

## Article

# The Effect of Protozoa Indigenous to Lakewater and Wastewater on Decay of Fecal Indicator Bacteria and Coliphage

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**Abstract:** Fecal indicator bacteria (FIB: *Escherichia coli* and enterococci) are used to assess recreational water quality. Viral indicators (i.e., somatic and F+ coliphage), could improve the prediction of viral pathogens in recreational waters, however, the impact of environmental factors, including the effect of predatory protozoa source, on their survival in water is poorly understood. We investigated the effect of lakewater or wastewater protozoa, on the decay (decreasing concentrations over time) of culturable FIB and coliphages under sunlight and shaded conditions. FIB decay was generally greater than the coliphages and was more rapid when indicators were exposed to lake vs. wastewater protozoa. F+ coliphage decay was the least affected by experimental variables. Somatic coliphage decayed fastest in the presence of wastewater protozoa and sunlight, though their decay under shaded conditions was 10-fold less than F+ after 14 days. The protozoa source consistently contributed significantly to the decay of FIB, and somatic, though not the F+ coliphage. Sunlight generally accelerated decay, and shade reduced somatic coliphage decay to the lowest level among all the indicators. Differential responses of FIB, somatic, and F+ coliphages to environmental factors support the need for studies that address the relationship between the decay of coliphages and viral pathogens under environmentally relevant conditions.

**Keywords:** predation; fecal indicator bacteria; bacteriophage; ambient water; wastewater



**Citation:** Korajkic, A.; McMinn, B.R.; Harwood, V.J. The Effect of Protozoa Indigenous to Lakewater and Wastewater on Decay of Fecal Indicator Bacteria and Coliphage. *Pathogens* **2023**, *12*, 378. <https://doi.org/10.3390/pathogens12030378>

Academic Editor: Sara Bonetta

Received: 11 January 2023

Revised: 20 February 2023

Accepted: 23 February 2023

Published: 25 February 2023



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## 1. Introduction

Enteric viruses have been identified as the main etiological agents of waterborne illness in recreational settings [1–4]. However, the sanitary quality of recreational waters is routinely and most commonly assessed by enumeration of culturable fecal indicator bacteria (FIB), such as *Escherichia coli* and enterococci, and more recently quantitative polymerase chain reaction (qPCR) in the United States [5]. Despite a long history of use as an indicator of fecal contamination, there are many criticisms of the FIB approach, at least partially due to the different fate and transport characteristics of FIB and viral pathogens (recently reviewed in [6–8]). For example, the decay of FIB in ambient waters is generally faster than that of viral pathogens due to greater susceptibility to a range of biotic and abiotic environmental factors. If indicators of fecal contamination are to accurately predict human health risk, the decay rate of indicators and pathogens in aquatic environments should be similar, or at least predictably related [8].

In recent years, there has been renewed interest in bacteriophages, such as *E. coli*-infecting coliphages, for many different applications [9–12], including as viral indicators of fecal pollution in recreational waters [13] due to their many similarities to enteric viral pathogens [14]. Coliphages infect *E. coli*, a commensal human gastrointestinal tract species, and are subsequently shed in feces by hosts, following routes of dissemination into the environment that are similar to those of enteric viral pathogens. The utilization of coliphages in this context is further supported by epidemiological studies demonstrating the association between coliphage levels and gastrointestinal illness in recreational

bathers [15–18]. However, additional information regarding the effect of various biotic and abiotic environmental factors on FIB and coliphage decay [19–22] in aquatic habitats is needed to assist with future recreational water quality criteria (RWQC) development. While the effect of some parameters (e.g., temperature) is well characterized, others (e.g., microbial interactions including viral lysis, intra- and interspecies competition, and protozoan predation) are less well understood [8].

Protozoan grazers, which consume bacteria and viruses, are a vital part of microbial food webs in aquatic habitats [23], and these top-down processes are especially important in oligotrophic environments [24–26], such as William H. Harsha Lake, the subject of this study [27,28]. The effect of protozoan grazing on FIB has been documented in several studies, mainly utilizing singular, laboratory-cultured strains of FIB [29–33]. A limited number of studies relied on more realistic sources of FIB (e.g., human and animal feces and wastewater), however, FIB sources in these studies were not manipulated and therefore contained protozoan predators that are ubiquitous in such environments (e.g., *Blastocystis*, *Entamoeba*, *Bodo*, *Colpidium* spp.) [34–36]. These studies consistently found a greater decay of FIB in the presence of aquatic protozoa populations, however, the magnitude of the predator effect was reduced compared to studies that used FIB cultured in the laboratory [20–22,37]. The presence of protozoan communities indigenous to feces and/or wastewater in the inoculum may have contributed to the difference in magnitude of the protozoan effect on FIB decay in these experiments that was not explored in the studies.

The role of protozoan predation on the decay of viruses, including coliphage, is not as clear and has been studied less frequently [8]. However, some controlled laboratory feeding studies indicated the ingestion of T4 and MS2 coliphages by *Tetrahymena*, *Thaumatomonas*, and *Salpingoeca* spp. [38,39]. Earlier studies generally indicated faster decay of enterophage (bacteriophage infecting enterococci), F+, and somatic coliphage in the presence of autochthonous aquatic protozoan communities compared to autoclaved and filtered river, lake, and marine waters [19,40,41]. However, similar to some FIB studies, these observations were recorded for either singular bacteriophage strains or bacteriophage cultivated from wastewater, thereby eliminating any possible effect of predatory protozoa from wastewater or feces. The only field study utilizing wastewater and human feces as a source of bacteriophages (somatic and F+ coliphages, GB-124 bacteriophage infecting *Bacteroides fragilis*) noted minimal effects of marine protozoan communities on decay as compared to filtered marine water controls [20]. Furthermore, this was the only study that contained wastewater protozoan communities contributed by the inoculum, making it unclear whether the effect of protozoa autochthonous to ambient waters observed in earlier studies was confounded by the presence of wastewater protists, or whether the source of the bacteriophage (i.e., laboratory propagated strains vs. wastewater/feces) influenced the results.

Unlike the effect of the source of predators, the contribution of ambient sunlight to the decay of FIB and coliphages has been more extensively documented and is reviewed in [8]. In general, culturable FIB and infectious coliphage exhibit greater decay when exposed to ambient sunlight, compared to dark or shaded controls [8], and this effect is attributed to either direct damage to nucleic acids in the form of pyrimidine dimers caused by UVB radiation or endogenous and/or exogenous photo-oxidative damage caused by UVA radiation [42] although the precise mechanism of UV-induced damage is likely to differ among different species and taxonomic groups.

The slower decay of FIB and coliphages in the absence of any protozoan predators and in dark or shaded conditions compared to sunlight exposure has been documented extensively and reviewed in [8,42–45]. However, the effect of protozoa source has not been studied before. Therefore, we opted to focus on the effect of protozoan predators from lakewater vs. wastewater and potential interactions with ambient sunlight instead. Through selective removal of protozoan communities from either the lakewater or the wastewater, we were able to investigate the effects of different grazer sources on the decay of the diverse communities of FIB and coliphage contained in lakewater and wastewater.

Incubation under sunlight and shaded conditions enabled characterization of the relative influence of and interactions between predator source and ambient sunlight. Finally, a direct comparison of FIB and coliphage decay characteristics allowed us to document differential and temporal responses of these two fecal indicator groups to biotic and abiotic environmental stressors.

## 2. Materials and Methods

### 2.1. Experimental Design

The goal of the study was to compare the effect of protozoa from lakewater (treatments: A and B) vs. wastewater (treatments: C and D) on decay ( $\log_{10}$  reduction) of bacterial and viral indicators under conditions of high (treatments A and C) or low (treatments: B and D) light intensity (Table 1). The experimental treatments were as follows: (A) exposure to lake protozoan predators and ambient sunlight (lake protozoa/sun), (B) exposure to lake protozoan predators only (lake protozoa/shade), (C) exposure to wastewater protozoan predators and ambient sunlight (wastewater protozoa/sun) and (D) exposure to wastewater protozoan predators only (wastewater protozoa/shade) (Table 1). A submersible aquatic mesocosm (SAM) device, constructed as previously described [8,19,22,46,47] was used to conduct the experiment in situ. Even though a singular SAM device was deployed for this experiment, each treatment and time point consisted of three independent dialysis bag replicates, prepared as described below, as is common practice for similar field studies, e.g., [20,46,48–50]. Fifty percent of bags with each inoculum type (i.e., lake protozoa or wastewater protozoa) were placed at the upper level of the SAM for the sunlight-exposed treatment (approximately 2–5 cm below the water surface), while the remaining half was placed at the lower level (approximately 25–30 cm below the water surface) underneath the heavy-duty black plastic tarp covering to simulate shaded conditions. Independent triplicate dialysis bags for each treatment were collected for enumeration of FIB and coliphages (as described below) immediately after the inoculum preparation ( $T_0$ ) and after 24h ( $T_1$ ), 72h ( $T_3$ ), 120h ( $T_5$ ), 192h ( $T_8$ ), and 336h ( $T_{14}$ ) of in situ incubation.

**Table 1.** Schematic of the experimental design.

Designation	Light Intensity (Sun/Shade)	Protozoa Removed by Filtration	Overall Effects Studied
A	High (Sun)	Wastewater protozoa removed	Effect of lake protozoa on decay rates of indicators under high light intensity
B	Low (Shade)	Wastewater protozoa removed	Effect of lake protozoa on decay rates of indicators under low light intensity
C	High (Sun)	Lakewater protozoa removed	Effect of wastewater protozoa on decay rates of indicators under high light intensity
D	Low (Shade)	Lakewater protozoa removed	Effect of wastewater protozoa on decay rates of indicators under low light intensity

Hourly light intensity (lux) and temperature ( $^{\circ}\text{C}$ ) readings were recorded at both the upper and lower levels of the SAM using HOBO<sup>®</sup> UA 002-08 data loggers (Onset Computer Corporation, Bourne, MA USA). The mean and standard deviation for the water temperature readings were  $16.7 \pm 1.2$   $^{\circ}\text{C}$  and  $16.5 \pm 0.90$   $^{\circ}\text{C}$  for upper (sunlight) and lower (shade) levels of SAM, respectively. The mean and standard deviation for light intensity measurements were  $585.0 \pm 3490.0$  lux for the upper and  $111.2 \pm 429.0$  lux for the lower level of the SAM. Mean light intensity was significantly higher at the upper level of the

SAM compared to the lower level ( $p < 0.0001$ ) indicating that our experimental design adequately created shaded conditions.

## 2.2. Mesocosm Preparation

Primary wastewater effluent and ambient water samples (~15 L each) were collected from a local wastewater treatment plant (Little Miami Wastewater Treatment Plant, Cincinnati, OH: 39.1038889° N, -84.4330556° W) and William H. Harsha Lake (Batavia, OH: 39.0252°N, -84.1303° W), respectively. Immediately after collection,  $\frac{1}{2}$  of each sample type was passed through a (0.80  $\mu\text{m}$  pore size, 47 mm diameter) nitrocellulose membrane filter (Pall Corporation, Port Washington, NY USA) to remove protozoan predators and other particulate matter. Removal of protozoa via filtration is a common procedure and less detrimental to the integrity of the water sample compared to other techniques (e.g., heat and chemical treatments) [51–55]. Both filtered and unfiltered samples were held at 4 °C overnight to minimize any changes in microbial populations.

The following day (<24 h after sample collection) the mesocosm inoculum was prepared by mixing a 1:1 ratio of either unfiltered lakewater with filtered primary wastewater effluent (treatments A and B) or filtered lakewater with unfiltered primary wastewater effluent (treatments C and D) (Table 1). Given that we used wastewater as the source of FIB and coliphage, and therefore could not modify the starting concentrations, and accounting for a  $\sim 2 \log_{10}$  difference in concentrations between the two indicator types, this particular ratio was chosen to ensure that quantifiable densities could be obtained for a maximum number of sampling time points. Two hundred milliliters of each inoculum type were used to fill regenerated cellulose dialysis bags (75 mm flat width, 13–14 kD pore size MWCO, Spectrum Labs, Rancho Dominguez, CA USA) that were rehydrated for 24 h in sterile diH<sub>2</sub>O prior to the start of the experiment. Potential attenuation of ambient sunlight by the regenerated cellulose dialysis bag material has been tested previously and found to be minimal (<10%) [21]. Prepared dialysis bags were placed in Ziplock™ bags containing approximately 50 mL of ambient water to prevent desiccation and transported to the field site (William H. Harsha Lake) on ice.

## 2.3. FIB and Bacteriophage Enumeration

The FIB and coliphage concentrations were measured using the standard membrane filtration technique [56,57] and double agar layer (DAL) assays [58], respectively. When necessary, decimal dilution series were prepared using a sterile 1X phosphate-buffered saline (PBS) solution (0.0425 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> and 0.4055 g L<sup>-1</sup> of MgCl<sub>2</sub>: pH 7.2 Sigma Aldrich, St. Louis, MO). For FIB enumeration, samples were filtered through 0.45  $\mu\text{m}$  (47 mm diameter) nitrocellulose filters and incubated on either mEI for 16–18 h at 41 °C (enterococci) or modified mTEC agar for 2 h at 35 °C, followed by 14–16 h at 44.5 °C (*E. coli*). For the somatic and F+ coliphages, 1 mL of sample was added to 5 mL of the molten top (0.7% agar) tryptic soy agar (TSA) overlay containing 0.1% of appropriate antibiotic stock solution (100  $\mu\text{g mL}^{-1}$  nalidixic acid for somatic or 15  $\mu\text{g mL}^{-1}$  streptomycin/ampicillin for F+ coliphage [Fisher Scientific, Waltham, MA]) followed by the addition of 200  $\mu\text{L}$  of appropriate *E. coli* host (CN-13 ATCC#700609 [somatic] or F<sub>amp</sub> ATCC#700891 [F+], American Type Culture Collection, Manassas, VA USA) in the midlog growth phase. The top agar overlay mixture was poured on the bottom agar TSA plates (1.5% agar and containing 0.1% of appropriate antibiotic stock solution) and then incubated at 37 °C for 16–18 h. The following day, characteristic colony-forming units (CFU) and plaque-forming units (PFU) were enumerated. During each sampling event, for both FIB and coliphages, method blank (sample substituted with 1X PBS) and media sterility negative controls were performed. For the duration of the study, no CFUs or PFUs were observed indicating the absence of contamination.

#### 2.4. Data Analyses

FIB and coliphage concentrations were  $\log_{10}$  transformed prior to data analyses. The decay of FIB and coliphages was calculated as cumulative  $\log_{10}$  reduction ( $\log_{10} C_0 - \log_{10} C_T$ ) where  $C_T$  represents the concentration at different sampling time points ( $T_1$ ,  $T_3$ ,  $T_5$ ,  $T_8$ , and  $T_{14}$ ) and  $C_0$  represents the starting concentrations measured at  $T_0$ . To facilitate comparisons with other studies, daily decay rates for each organism and treatment are also provided in Table S1. Out of 72 samples collected during the study, the proportion of samples containing no detectable FIB or coliphage was low (i.e., 12.5% for enterococci, 0% for *E. coli*, 11.1% for F+ coliphage, 1.38% for somatic coliphage) and it occurred in the later stages of the experiment ( $T_5$ – $T_{14}$ ). Table S2 contains details on observations below the detection limits. GraphPad Prism version 8.1.2 (GraphPad Software, La Jolla, CA USA) was used to conduct a two-way analysis of variance (ANOVA) with Tukey's multiple comparison test to evaluate the effects of the two factors (source of protozoan predators and exposure to ambient sunlight) on decay (Table 2). The same software was used to conduct the one-way ANOVA to compare decay across different indicators within the same treatment and Wilcoxon matched pairs signed rank test to assess differences in light temperature readings between the upper and lower levels of the SAM. The pairing was effective as indicated by a high Spearman correlation coefficient ( $r = 0.9252$ ) and a low corresponding  $p$  value ( $<0.0001$ ).

**Table 2.** Two-way ANOVA summary of the effect of treatment variables (predator source and sunlight) on indicator decay at each time point. Statistically significant factors are bolded.

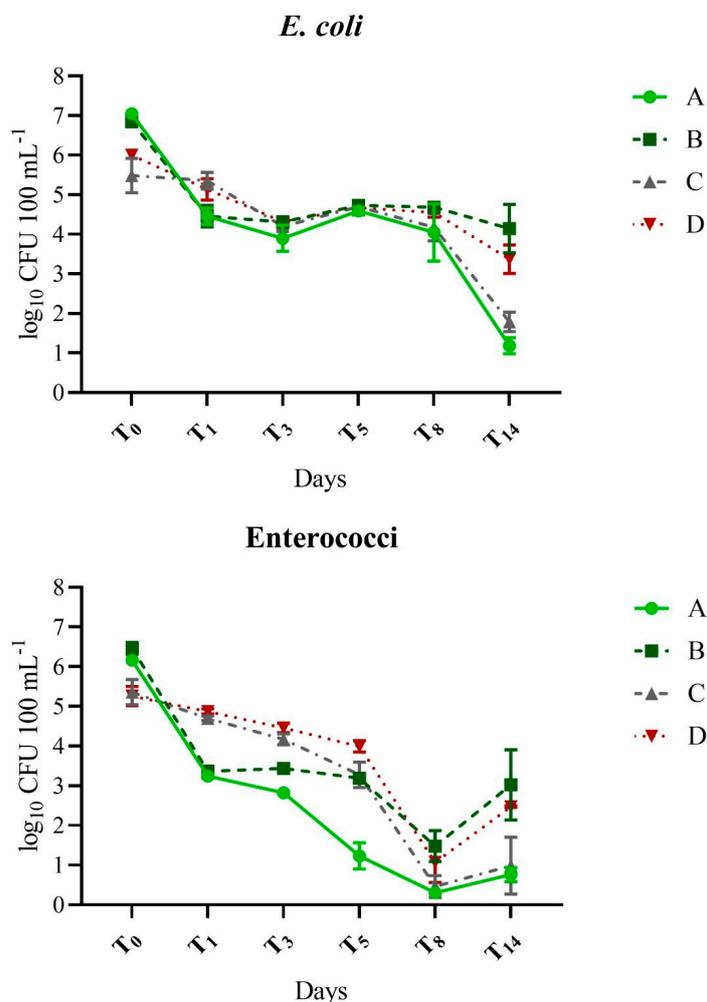
Indicator	Time Point (days)	Factor					
		Source of Predators <sup>a</sup>		Sunlight		Interaction <sup>b</sup>	
		%	<i>p</i> Value	%	<i>p</i> Value	%	<i>p</i> Value
<i>E. coli</i>	T <sub>1</sub>	86.37	<0.0001	1.513	0.2398	4.895	0.0542
	T <sub>3</sub>	85.43	0.0004	0.126	0.7657	13.57	0.0224
	T <sub>5</sub>	81.43	0.0001	0.519	0.5561	10.65	0.0265
	T <sub>8</sub>	64.70	0.0008	5.350	0.1719	10.93	0.0643
	T <sub>14</sub>	20.45	0.0118	57.11	0.0013	18.16	0.0148
Enterococci	T <sub>1</sub>	96.72	<0.0001	0.033	0.7506	0.806	0.1424
	T <sub>3</sub>	98.46	<0.0001	0.309	0.1821	0.309	0.1821
	T <sub>5</sub>	75.05	<0.0001	19.53	0.0001	2.270	0.0431
	T <sub>8</sub>	42.59	0.0043	35.12	0.0071	0.420	0.7054
	T <sub>14</sub>	13.84	0.1770	59.67	0.0224	1.806	0.5950
F+ coliphage	T <sub>1</sub>	13.06	0.2147	13.80	0.2034	15.63	0.1787
	T <sub>3</sub>	1.713	0.6984	5.678	0.4854	7.708	0.4189
	T <sub>5</sub>	5.885	0.2175	45.59	0.0057	22.48	0.0303
	T <sub>8</sub>	13.81	0.1422	33.97	0.0340	10.51	0.1933
	T <sub>14</sub>	0.753	0.6851	19.17	0.0821	66.04	0.0100
Somatic coliphage	T <sub>1</sub>	54.89	0.0019	8.966	0.1028	15.00	0.0444
	T <sub>3</sub>	86.57	<0.0001	0.493	0.5945	0.111	0.7989
	T <sub>5</sub>	47.29	0.0059	25.23	0.0266	0.003	0.9775
	T <sub>8</sub>	10.72	0.0174	77.91	<0.0001	1.762	0.2605
	T <sub>14</sub>	0.775	0.6236	82.75	0.0029	0.426	0.7143

<sup>a</sup> Percent contribution of each treatment variable (predator source and sunlight) to the observed variability in the dataset. <sup>b</sup> Interaction between treatment variables (predator source and sunlight).

### 3. Results

#### 3.1. Effect of Predator Source and Sunlight on FIB and Coliphage Decay

The source of protozoan predators had a profound and significant effect on the decay of *E. coli* and enterococci in the first 24 h, from T<sub>0</sub> to T<sub>1</sub> (Figure 1, Tables 2 and 3), accounting for >86% of the observed variability in log<sub>10</sub> reduction (Table 2). FIB exposed to lake protozoa (A and B) decreased by 2.39–3.10 log<sub>10</sub> by T<sub>1</sub>, while those exposed to wastewater protozoa (C and D) decreased only 0.13–0.87 log<sub>10</sub> in the first 24 h of the experiment (Table 3). The influence of protozoan source remained high at T<sub>8</sub>, accounting for 64.7% and 42.6% of variability for *E. coli* and enterococci, respectively (Table 2). At T<sub>14</sub>, the protozoa source was a significant factor in *E. coli* decay, accounting for 20.5% of variability, although it was not significant in enterococci decay. As the influence of protozoan source on decay decreased over time, sunlight became a significant factor, accounting for 57.1% of the variability in *E. coli* decay at T<sub>14</sub>. The interaction of variables was also a significant factor at T<sub>14</sub>, contributing 18.2% of the variability and indicating that the influence of sunlight was dependent on the protozoa source (Table 2). Sunlight became a significant factor in the decay of the enterococci at T<sub>5</sub> and it remained so until T<sub>14</sub> when it accounted for 59.7% of the variability (Table 2).



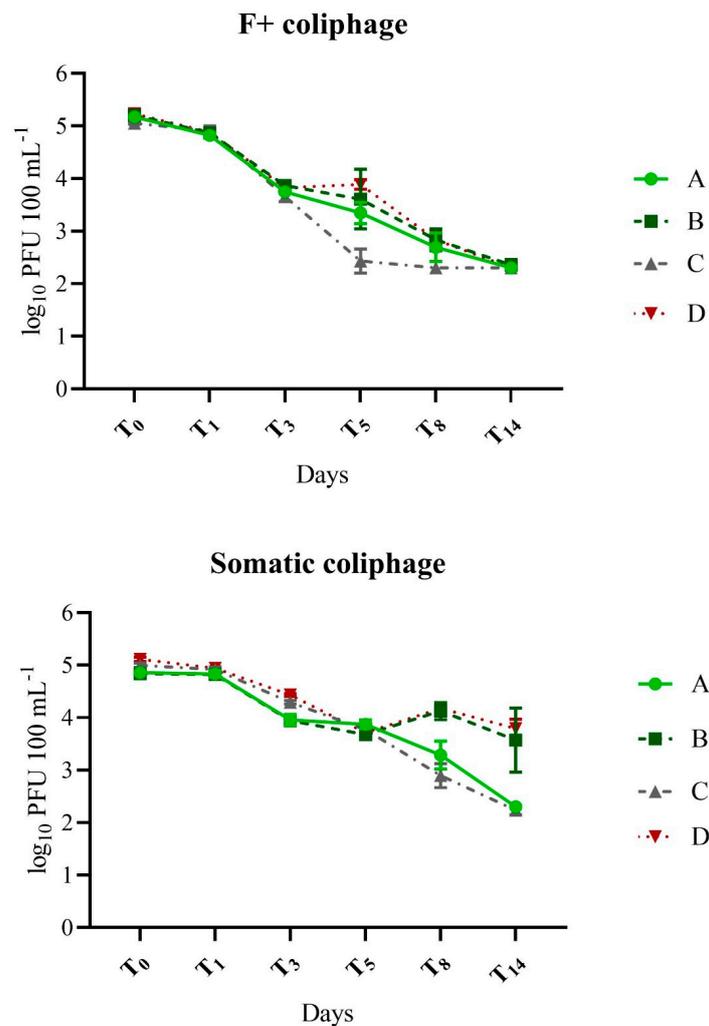
**Figure 1.** Change in concentrations of *E. coli* and enterococci over time in response to the source of protozoan predators and exposure to ambient sunlight. Treatments: A (lake protozoa/sun), B (lake protozoa/shade), C (wastewater protozoa/sun), D (wastewater protozoa/shade). Error bars represent the standard deviation between independent dialysis bag replicates. All data shown including samples where LOD was used.

**Table 3.** Decay (cumulative log<sub>10</sub> reduction ± standard deviation for independent dialysis bag replicates) values for FIB and coliphage for each treatment and sampling day.

Days	<i>E. coli</i>				Enterococci				F+ Coliphage				Somatic Coliphage			
	Treatment <sup>1</sup>															
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
T <sub>1</sub>	2.60 ± 0.19	2.39 ± 0.16	0.13 ± 0.53	0.87 ± 0.27	2.92 ± 0.10	3.10 ± 0.22	0.66 ± 0.33	0.39 ± 0.26	0.35 ± 0.11	0.34 ± 0.15	0.15 ± 0.08	0.35 ± 0.14	0.03 ± 0.04	0.02 ± 0.06	0.08 ± 0.02	0.17 ± 0.02
T <sub>3</sub>	2.97 ± 0.09	2.49 ± 0.07	1.10 ± 0.52	1.69 ± 0.06	3.34 ± 0.09	3.03 ± 0.18	1.19 ± 0.23	0.93 ± 0.01	1.42 ± 0.05	1.34 ± 0.14	1.40 ± 0.07	1.40 ± 0.02	0.90 ± 0.05	0.90 ± 0.05	0.70 ± 0.03	0.68 ± 0.06
T <sub>5</sub>	2.46 ± 0.12	2.12 ± 0.08	0.79 ± 0.47	1.33 ± 0.04	4.93 ± 0.35	3.27 ± 0.23	2.08 ± 0.21	1.27 ± 0.39	1.82 ± 0.12	1.60 ± 0.63	2.62 ± 0.21	1.34 ± 0.17	0.99 ± 0.14	1.16 ± 0.04	1.23 ± 0.15	1.40 ± 0.06
T <sub>8</sub>	2.99 ± 0.74	2.16 ± 0.08	1.30 ± 0.24	1.45 ± 0.11	5.86 ± 0.06	4.98 ± 0.53	4.90 ± 0.35	4.19 ± 0.43	2.48 ± 0.22	2.37 ± 0.20	2.75 ± 0.02	2.39 ± 0.10	1.57 ± 0.29	0.71 ± 0.16	2.10 ± 0.26	0.94 ± 0.10
T <sub>14</sub>	5.87 ± 0.18	2.70 ± 0.66	3.52 ± 0.18	2.63 ± 0.36	5.43 ± 0.12	3.44 ± 0.90	4.32 ± 1.14	2.92 ± 0.11	2.91 ± 0.06	2.85 ± 0.05	2.76 ± 0.03	2.98 ± 0.06	2.54 ± 0.06	1.27 ± 0.57	2.77 ± 0.04	1.30 ± 0.21

<sup>1</sup> A (lake protozoa/sun), B (lake protozoa/shade), C (wastewater protozoa/sun), and D (wastewater protozoa/shade).

The effect of the source of protozoan predators on the coliphages varied between somatic and F+ coliphages (Figure 2, Tables 2 and 3). Predator source was not a significant factor in the  $\log_{10}$  reduction of F+ coliphage, at any of the time points (Table 2). In contrast, the predator source significantly influenced somatic coliphage decay at T1–T8, accounting for the maximum variability of 86.6% at T3, though diminishing to 10.7% at T8 and becoming a negligible factor at T14 (Table 2). Somatic coliphage decay was generally greater in the presence of wastewater protozoa compared to lake protozoa (T1, T5, and T8) except at T3 (Table 3), although the magnitude of the difference was not as pronounced as it was for FIB.



**Figure 2.** Change in concentrations of somatic and F+ coliphage over time in response to source of protozoan predators and exposure to ambient sunlight. Treatments: A (lake protozoa/sun), B (lake protozoa/shade), C (wastewater protozoa/sun), D (wastewater protozoa/shade). Error bars represent standard deviation-independent dialysis bag replicates. All data shown including samples where LOD was used.

The effect of sunlight on bacteriophage decay was different for F+ vs. somatic coliphage, particularly toward the end of the experiment (T8 and T14). While sunlight was not a significant factor in the decay of either coliphage group until T5. At T8, sunlight contributed nearly 80% to variability in somatic coliphage decay, though less than half that to F+ coliphage decay (i.e., ~34%) (Table 3). The difference was even more apparent at T14 when the effect of sunlight on somatic coliphage increased further to 83%, and  $\log_{10}$  reduction values in the sun were double the  $\log_{10}$  reduction values in the shade (Figure 2, Table 3). Sunlight was not a significant contributor to F+ coliphage decay at T14, further

highlighting disparities in the sunlight effect on the two coliphage groups. In fact, the magnitude difference in the decay of the F+ coliphage in the sun vs. the shade at T<sub>8</sub> and T<sub>14</sub> was only a small fraction of that observed for the somatic coliphage. (log<sub>10</sub> reduction: 0.06–0.22) (Figure 2, Table 3). For example, at T<sub>8</sub>, when sunlight was a significant factor in decay for somatic and F+ coliphages, decay of the somatic coliphage in sunlight was twice that of the shaded conditions (difference of 0.86 and 1.16 log<sub>10</sub> between sun and shade in the presence of lake and wastewater protozoa, respectively) (Figure 2, Table 3). In contrast, F+ coliphage decay in sun vs. shade at T<sub>8</sub> differed by only 0.11 and 0.36 log<sub>10</sub> in the presence of lake and wastewater protozoa, respectively.

### 3.2. Decay of FIB Compared to Coliphage

*E. coli* and enterococci typically decayed more rapidly than coliphages throughout the study (Figures 1 and 2, Table 3,) and this trend was particularly evident in treatments containing lake protozoa (A: lake protozoa/sun and B: lake protozoa/shade). For example, at the last time point, T<sub>14</sub>, log<sub>10</sub> reduction of FIB in treatment A (lake protozoa/sun), was nearly double that of the coliphages ( $\geq 5.4$  vs. 2.9). This difference was statistically significant ( $p \leq 0.0001$ ) when either FIB group was compared to either coliphage group. In treatment B (lake protozoa/shade) at T<sub>14</sub>, enterococci decayed faster compared to somatic coliphage ( $p = 0.0121$ ), though there were no other statistically significant comparisons. While there was a trend for the faster decay of FIB compared to coliphages for treatment C (wastewater protozoa/sun) (Table 2), there were no significant differences in decay among all microorganisms ( $p \geq 0.1659$ ) at the last time point. Finally, all microorganisms decayed significantly faster ( $p \leq 0.0126$ ) than the somatic coliphage at T<sub>14</sub> in treatment D (wastewater protozoa/shade).

## 4. Discussion

Measurement of viable and culturable FIB used historically to assess water quality may not be sufficient indicators of the sanitary quality of recreational waters, considering that the majority of recreational water disease outbreaks are caused by viral pathogens [1,2,59]. Therefore, viral indicators, specifically somatic and F+ coliphage, have been suggested as additional monitoring tools for recreational waters [13]. In order to improve our understanding of the utility of both FIB and coliphage as fecal indicators, more information is needed about their fate in ambient waters.

While the direct effect of protozoan grazing on the decay of FIB and coliphage has been well documented [8,23,44], the effect of protozoa source on indicator decay remains unexplored. To the best of our knowledge, this is the first field study where the effect of predator source on the decay of fecal microorganisms was investigated through systematic removal of protists from either the wastewater inoculum or lakewater medium.

The source of predators had a significant impact on the decay rates of *E. coli* and enterococci, as FIB decay in the presence of lake predators was frequently  $\geq 1$  log greater than in the presence of wastewater protozoa. The protozoan communities autochthonous to lakewater and wastewater are fundamentally different [35,36,60–68]. Ciliates are typically the dominant group in wastewater (by biomass and the number of species) while small flagellates are the most abundant form in lakewater [69]. Direct comparisons of protist diversity between human feces and both marine and freshwater aquatic environments indicated lower diversity in feces [35]. While the diversity of protists in wastewater is higher than in fecal specimens, it is still lower compared to environmental waters [47,70]. The increased diversity and richness of predatory species have been linked to elevated predator production and higher grazing rates [71] and offer a plausible explanation for our observations regarding the greater decay of FIB in the presence of protozoa indigenous to lakewater compared to those from the wastewater inoculum.

The influence of the protozoa source was not as clear for coliphages, as the predator source did not have a significant effect on the decay of F+ coliphage. Although the protozoan source significantly affected the decay of somatic coliphage, the predator source with

more impact changed over time during the study, and the magnitude of difference in decay rates between predator sources was always much less than 1 log. Nonetheless, the starting concentrations of both coliphages and the decay observed in this study were comparable to an earlier field study utilizing wastewater as the source of coliphages [20], however, it was considerably lower than the studies utilizing laboratory-propagated coliphages under similar conditions [19,41]. While the preferential protozoan grazing on some viral species over others has been previously documented [38,72,73], the potential influence of coliphage source (i.e., wastewater versus laboratory cultivated strains) is novel and merits further consideration. Furthermore, while lower starting concentrations of coliphage provided a more realistic representation of levels expected in ambient waters following a wastewater pollution event, this difference could influence the observed effect (or the lack thereof) of the predatory protozoa [74–76]. Finally, another important distinction between our study and earlier works [20,40,41], is that we filtered lakewater and wastewater through a 0.80 µm filter, which would have retained the bacterial population from both sources, while others utilized either autoclaving or filtration through 0.22 µm, which likely resulted in their removal. The presence of the autochthonous bacterial community provided an additional abundance of potential prey for protozoan grazers, and it could have affected the time-dependent influence of protozoan source on somatic coliphage, as well as lack of predator source on F+ coliphage decay rates.

The contribution of ambient sunlight to the decay of fecal microbiota is arguably one of the best-studied environmental parameters [8,42,44]. As expected, we observed that the decay of FIB and somatic coliphages is faster under sunlight-exposed conditions compared to the shaded controls. The exception to this observation was the F+ coliphage, which decayed similarly under all treatment conditions. While F+ coliphage has been reported to be more resilient than the somatic subgroup to wastewater UV disinfection [77,78], which relies on germicidal action of UV-C spectrum [79], extrapolation to ambient sunlight conditions (mainly consisting of UV-A and UV-B spectra) [42] is not appropriate given the great differences in these two irradiation sources and intensities. Furthermore, while somatic and F+ coliphage decay rates were similar under sunlight-exposed conditions, somatic coliphage decay rates under shaded conditions were considerably lower compared to F+ ( $\leq 1.5 \log_{10}$ ). This implies that consistently higher concentrations of somatic coliphage (as compared to F+) frequently detected in ambient waters [78,80–83] may be at least in part due to greater persistence of the somatic subgroup under shaded conditions (i.e., no direct exposure to ambient sunlight). This finding is novel and warrants further study, particularly as a comparison with viral pathogens.

A limited number of studies comparing decay characteristics of coliphages and viral pathogens (i.e., poliovirus-1-Sabin, norovirus GI-1, human adenovirus 2) suggest that viral indicators are more appropriate proxies for viral pathogen persistence in environmental waters than FIB [19,84,85]. We generally observed accelerated decay of both FIB compared to somatic coliphages. However, FIB decay rates under shaded conditions were similar to those of the F+ coliphages. Some viral indicators may be more resilient to environmental stressors compared to their bacterial counterparts. However, this study found that environmental conditions and the type of coliphage influence the relative rapidity of their decay rates. Previous reports also noted the extended persistence of coliphages compared to FIB in freshwaters [86–88] and marine environments [87–89]. A trend of faster decay of culturable FIB compared to viral pathogens (i.e., adenovirus 40/41 and coxsackie A9) [90–93] further supports the need for viral indicator(s).

In summary, we demonstrated that the decay of FIB in the presence of protozoa is generally more rapid than that of coliphages, further supporting the need for viral indicators of fecal pollution to better reflect the decay characteristics of pathogenic viruses in recreational waters. We have also shown that somatic coliphage decay rates under shaded conditions are considerably slower than those of F+ coliphage or FIB. This difference has important implications for the selection of coliphage groups as viral indicators for recreational water quality and highlights the need for studies that compared the decay

of pathogenic viruses to that of coliphages under environmentally relevant conditions. Furthermore, we established that the lake protozoa were more influential in the decay of FIB compared to wastewater predators, though the effect of predator source on the decay of somatic coliphages, and the lack of effect of predatory source on F+ coliphage, was less clear and merits further research. While our study adds to the growing body of knowledge regarding the effect of biotic and abiotic parameters on decay, the findings should not be directly extrapolated to other geographic regions or seasons due to the inherent variability of factors such as autochthonous microbial communities, intensity, duration of UV radiation, and water temperature.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pathogens12030378/s1>, Table S1. Cumulative decay rate per day values for FIB and coliphage for each treatment and sampling day. Table S2. Analyses with observations below the assay limit of detection.

**Author Contributions:** Conceptualization, A.K.; Methodology, A.K. and B.R.M.; Formal Analysis, A.K.; Investigation, A.K. and B.R.M.; Writing Original Draft Preparation, A.K. and V.J.H.; Writing Review & Editing, A.K., B.R.M. and V.J.H. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the United States Environmental Protection Agency.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data available in a publicly accessible repository (<https://catalog.data.gov/dataset/>), accessed on 10 January 2023).

**Conflicts of Interest:** The authors declare no conflict of interest.

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

## References

1. Sinclair, R.G.; Jones, E.L.; Gerba, C.P. Viruses in recreational water-borne disease outbreaks: A review. *J. Appl. Microbiol.* **2009**, *107*, 1769–1780. [[CrossRef](#)] [[PubMed](#)]
2. Graciaa, D.S.; Cope, J.R.; Roberts, V.A.; Cikesh, B.L.; Kahler, A.M.; Vigar, M.; Hilborn, E.D.; Wade, T.J.; Backer, L.C.; Montgomery, S.P.; et al. Outbreaks Associated with Untreated Recreational Water—United States, 2000–2014. *Am. J. Transplant.* **2018**, *18*, 2083–2087. [[CrossRef](#)]
3. Hlavsa, M.C.; Roberts, V.A.; Kahler, A.M.; Hilborn, E.D.; Mecher, T.R.; Beach, M.J.; Wade, T.J.; Yoder, J.S. Outbreaks of Illness Associated with Recreational Water—United States, 2011–2012. *MMWR Morb. Mortal. Wkly. Rep.* **2015**, *64*, 668–672. [[CrossRef](#)] [[PubMed](#)]
4. Zlot, A.; Simckes, M.; Vines, J.; Reynolds, L.; Sullivan, A.; Scott, M.K.; McLuckie, J.M.; Kromer, D.; Hill, V.R.; Yoder, J.S.; et al. Norovirus outbreak associated with a natural lake used for recreation—Oregon, 2014. *MMWR Morb. Mortal. Wkly. Rep.* **2015**, *64*, 485–490. [[CrossRef](#)]
5. EPA-821-R-10-004; Method A: Enterococci in Water by TaqMan Quantitative Polymerase Chain Reaction (qPCR) Assay. United States Environmental Protection Agency: Washington, DC, USA, 2010.
6. Korajkic, A.; McMinn, B.R.; Harwood, V.J. Relationships between Microbial Indicators and Pathogens in Recreational Water Settings. *Int. J. Environ. Res. Public Health* **2018**, *15*, 2842. [[CrossRef](#)]
7. Fout, G.S.; Borchardt, M.A.; Kieke, B.A., Jr.; Karim, M.R. Human virus and microbial indicator occurrence in public-supply groundwater systems: Meta-analysis of 12 international studies. *Hydrogeol. J.* **2017**, *25*, 903–919. [[CrossRef](#)]
8. Korajkic, A.; Wanjugi, P.; Brooks, L.; Cao, Y.; Harwood, V.J. Persistence and Decay of Fecal Microbiota in Aquatic Habitats. *Microbiol. Mol. Biol. Rev. MMBR* **2019**, *83*, e00005–e00019. [[CrossRef](#)]
9. Wang, M.; Liu, P.; Zhou, Q.; Tao, W.; Sun, Y.; Zeng, Z. Estimating the contribution of bacteriophage to the dissemination of antibiotic resistance genes in pig feces. *Environ. Pollut.* **2018**, *238*, 291–298. [[CrossRef](#)]
10. Wang, M.; Xiong, W.; Liu, P.; Xie, X.; Zeng, J.; Sun, Y.; Zeng, Z. Metagenomic Insights Into the Contribution of Phages to Antibiotic Resistance in Water Samples Related to Swine Feedlot Wastewater Treatment. *Front. Microbiol.* **2018**, *9*, 2474. [[CrossRef](#)]

11. Lekunberri, I.; Subirats, J.; Borrego, C.M.; Balcazar, J.L. Exploring the contribution of bacteriophages to antibiotic resistance. *Environ. Pollut.* **2017**, *220*, 981–984. [[CrossRef](#)]
12. Ross, J.; Topp, E. Abundance of Antibiotic Resistance Genes in Bacteriophage following Soil Fertilization with Dairy Manure or Municipal Biosolids, and Evidence for Potential Transduction. *Appl. Environ. Microbiol.* **2015**, *81*, 7905–7913. [[CrossRef](#)]
13. 820-R-15-098; Review of Coliphages as Possible Indicators of Fecal Contamination for Ambient Water Quality. United States Environmental Protection Agency: Washington, DC, USA, 2015.
14. King, A.M.Q.; Adams, M.J.; Carstens, E.B.; Lefkowitz, W.J. *Virus Taxonomy: Classification and Nomenclature of Viruses*; Ninth Report of the International Committee on Taxonomy of Viruses; International Committee on Taxonomy of Viruses: London, UK, 2011.
15. Griffith, J.F.; Weisberg, S.B.; Arnold, B.F.; Cao, Y.; Schiff, K.C.; Colford, J.M., Jr. Epidemiologic evaluation of multiple alternate microbial water quality monitoring indicators at three California beaches. *Water Res.* **2016**, *94*, 371–381. [[CrossRef](#)]
16. Wade, T.J.; Sams, E.; Brenner, K.P.; Haugland, R.; Chern, E.; Beach, M.; Wymer, L.; Rankin, C.C.; Love, D.; Li, Q.; et al. Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: A prospective cohort study. *Environ. Health A Glob. Access Sci. Source* **2010**, *9*, 66. [[CrossRef](#)] [[PubMed](#)]
17. Wiedenmann, A.; Kruger, P.; Dietz, K.; Lopez-Pila, J.M.; Szewzyk, R.; Botzenhart, K. A randomized controlled trial assessing infectious disease risks from bathing in fresh recreational waters in relation to the concentration of *Escherichia coli*, intestinal enterococci, *Clostridium perfringens*, and somatic coliphages. *Environ. Health Perspect.* **2006**, *114*, 228–236. [[CrossRef](#)] [[PubMed](#)]
18. Colford, J.M.; Wade, T.J.; Schiff, K.C.; Wright, C.C.; Griffith, J.F.; Sandhu, S.K.; Burns, S.; Sobsey, M.; Lovelace, G.; Weisberg, S.B. Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. *Epidemiology* **2007**, *18*, 27–35. [[CrossRef](#)] [[PubMed](#)]
19. McMinn, B.R.; Rhodes, E.R.; Huff, E.M.; Korajkic, A. Decay of infectious adenovirus and coliphages in freshwater habitats is differentially affected by ambient sunlight and the presence of indigenous protozoa communities. *Virol. J.* **2020**, *17*, 1. [[CrossRef](#)]
20. Wanjugi, P.; Sivaganesan, M.; Korajkic, A.; Kelty, C.A.; McMinn, B.; Ulrich, R.; Harwood, V.J.; Shanks, O.C. Differential decomposition of bacterial and viral fecal indicators in common human pollution types. *Water Res.* **2016**, *105*, 591–601. [[CrossRef](#)] [[PubMed](#)]
21. Korajkic, A.; McMinn, B.R.; Shanks, O.C.; Sivaganesan, M.; Fout, G.S.; Ashbolt, N.J. Biotic interactions and sunlight affect persistence of fecal indicator bacteria and microbial source tracking genetic markers in the upper Mississippi river. *Appl. Environ. Microbiol.* **2014**, *80*, 3952–3961. [[CrossRef](#)] [[PubMed](#)]
22. Korajkic, A.; McMinn, B.R.; Harwood, V.J.; Shanks, O.C.; Fout, G.S.; Ashbolt, N.J. Differential decay of enterococci and *Escherichia coli* originating from two fecal pollution sources. *Appl. Environ. Microbiol.* **2013**, *79*, 2488–2492. [[CrossRef](#)]
23. Pernthaler, J. Predation on prokaryotes in the water column and its ecological implications. *Nat. Rev. Microbiol.* **2005**, *3*, 537–546. [[CrossRef](#)]
24. Pace, M.L.; Cole, J.J. Comparative and experimental approaches to top-down and bottom-up regulation of bacteria. *Microb. Ecol.* **1994**, *28*, 181–193. [[CrossRef](#)] [[PubMed](#)]
25. Feichtmayer, J.; Deng, L.; Griebler, C. Antagonistic Microbial Interactions: Contributions and Potential Applications for Controlling Pathogens in the Aquatic Systems. *Front. Microbiol.* **2017**, *8*, 2192. [[CrossRef](#)] [[PubMed](#)]
26. Baltar, F.; Aristegui, J.; Gasol, J.M.; Herndl, G.J. Microbial functioning and community structure variability in the mesopelagic and epipelagic waters of the subtropical northeast atlantic ocean. *Appl. Environ. Microbiol.* **2012**, *78*, 3309–3316. [[CrossRef](#)] [[PubMed](#)]
27. Muller, H. The relative importance of different ciliate taxa in the pelagic food web of lake constance. *Microb. Ecol.* **1989**, *18*, 261–273. [[CrossRef](#)]
28. Boenigk, J.; Arndt, H. Bacterivory by heterotrophic flagellates: Community structure and feeding strategies. *Antonie Van Leeuwenhoek* **2002**, *81*, 465–480. [[CrossRef](#)]
29. Korajkic, A.; Wanjugi, P.; Harwood, V.J. Indigenous microbiota and habitat influence *Escherichia coli* survival more than sunlight in simulated aquatic environments. *Appl. Environ. Microbiol.* **2013**, *79*, 5329–5337. [[CrossRef](#)]
30. Wanjugi, P.; Fox, G.A.; Harwood, V.J. The Interplay Between Predation, Competition, and Nutrient Levels Influences the Survival of *Escherichia coli* in Aquatic Environments. *Microb. Ecol.* **2016**, *72*, 526–537. [[CrossRef](#)]
31. Wanjugi, P.; Harwood, V.J. The influence of predation and competition on the survival of commensal and pathogenic fecal bacteria in aquatic habitats. *Environ. Microbiol.* **2013**, *15*, 517–526. [[CrossRef](#)]
32. Pirlot, S.; Unrein, F.; Descy, J.P.; Servais, P. Fate of heterotrophic bacteria in Lake Tanganyika (East Africa). *FEMS Microbiol. Ecol.* **2007**, *62*, 354–364. [[CrossRef](#)]
33. Menon, P.; Billen, G.; Servais, P. Mortality rates of autochthonous and fecal bacteria in natural aquatic ecosystems. *Water Res.* **2003**, *37*, 4151–4158. [[CrossRef](#)]
34. Burian, A.; Pinn, D.; Peralta-Maraver, I.; Sweet, M.; Mauvisseau, Q.; Eyice, O.; Bulling, M.; Rothig, T.; Kratina, P. Predation increases multiple components of microbial diversity in activated sludge communities. *ISME J.* **2022**, *16*, 1086–1094. [[CrossRef](#)] [[PubMed](#)]
35. Parfrey, L.W.; Walters, W.A.; Lauber, C.L.; Clemente, J.C.; Berg-Lyons, D.; Teiling, C.; Kodira, C.; Mohiuddin, M.; Brunelle, J.; Driscoll, M.; et al. Communities of microbial eukaryotes in the mammalian gut within the context of environmental eukaryotic diversity. *Front. Microbiol.* **2014**, *5*, 298. [[CrossRef](#)] [[PubMed](#)]
36. Scanlan, P.D.; Marchesi, J.R. Micro-eukaryotic diversity of the human distal gut microbiota: Qualitative assessment using culture-dependent and -independent analysis of faeces. *ISME J.* **2008**, *2*, 1183–1193. [[CrossRef](#)] [[PubMed](#)]

37. Dick, L.K.; Stelzer, E.A.; Bertke, E.E.; Fong, D.L.; Stoeckel, D.M. Relative decay of Bacteroidales microbial source tracking markers and cultivated *Escherichia coli* in freshwater microcosms. *Appl. Environ. Microbiol.* **2010**, *76*, 3255–3262. [[CrossRef](#)] [[PubMed](#)]
38. Deng, L.; Krauss, S.; Feichtmayer, J.; Hofmann, R.; Arndt, H.; Griebler, C. Grazing of heterotrophic flagellates on viruses is driven by feeding behaviour. *Environ. Microbiol Rep* **2014**, *6*, 325–330. [[CrossRef](#)] [[PubMed](#)]
39. Pinheiro, M.D.; Power, M.E.; Butler, B.J.; Dayeh, V.R.; Slawson, R.; Lee, L.E.; Lynn, D.H.; Bols, N.C. Use of *Tetrahymena thermophila* to study the role of protozoa in inactivation of viruses in water. *Appl. Environ. Microbiol.* **2007**, *73*, 643–649. [[CrossRef](#)] [[PubMed](#)]
40. Booncharoen, N.; Mongkolsuk, S.; Sirikanchana, K. Comparative persistence of human sewage-specific enterococcal bacteriophages in freshwater and seawater. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 6235–6246. [[CrossRef](#)]
41. Yang, Y.; Griffiths, M.W. Comparative persistence of subgroups of F-specific RNA phages in river water. *Appl. Environ. Microbiol.* **2013**, *79*, 4564–4567. [[CrossRef](#)]
42. Nelson, K.L.; Boehm, A.B.; Davies-Colley, R.J.; Dodd, M.C.; Kohn, T.; Linden, K.G.; Liu, Y.; Maraccini, P.A.; McNeill, K.; Mitch, W.A.; et al. Sunlight-mediated inactivation of health-relevant microorganisms in water: A review of mechanisms and modeling approaches. *Environ. Sci. Process. Impacts* **2018**, *20*, 1089–1122. [[CrossRef](#)]
43. Boehm, A.B.; Silverman, A.I.; Schriewer, A.; Goodwin, K. Systematic review and meta-analysis of decay rates of waterborne mammalian viruses and coliphages in surface waters. *Water Res.* **2019**, *164*, 114898. [[CrossRef](#)]
44. Dean, K.; Mitchell, J. Identifying water quality and environmental factors that influence indicator and pathogen decay in natural surface waters. *Water Res.* **2022**, *211*, 118051. [[CrossRef](#)]
45. McMinn, B.R.; Ashbolt, N.J.; Korajkic, A. Bacteriophages as indicators of faecal pollution and enteric virus removal. *Letts. Appl. Microbiol.* **2017**, *65*, 11–26. [[CrossRef](#)]
46. Korajkic, A.; McMinn, B.R.; Ashbolt, N.J.; Sivaganesan, M.; Harwood, V.J.; Shanks, O.C. Extended persistence of general and cattle-associated fecal indicators in marine and freshwater environment. *Sci. Total Environ.* **2019**, *650*, 1292–1302. [[CrossRef](#)] [[PubMed](#)]
47. Korajkic, A.; Parfrey, L.W.; McMinn, B.R.; Baeza, Y.V.; VanTeuren, W.; Knight, R.; Shanks, O.C. Changes in bacterial and eukaryotic communities during sewage decomposition in Mississippi river water. *Water Res.* **2015**, *69*, 30–39. [[CrossRef](#)]
48. Mattioli, M.C.; Sassoubre, L.M.; Russell, T.L.; Boehm, A.B. Decay of sewage-sourced microbial source tracking markers and fecal indicator bacteria in marine waters. *Water Res.* **2017**, *108*, 106–114. [[CrossRef](#)] [[PubMed](#)]
49. Balleste, E.; Garcia-Aljaro, C.; Blanch, A.R. Assessment of the decay rates of microbial source tracking molecular markers and faecal indicator bacteria from different sources. *J. Appl. Microbiol.* **2018**, *125*, 1938–1949. [[CrossRef](#)]
50. Bae, S.; Wuertz, S. Rapid decay of host-specific fecal Bacteroidales cells in seawater as measured by quantitative PCR with propidium monoazide. *Water Res.* **2009**, *43*, 4850–4859. [[CrossRef](#)] [[PubMed](#)]
51. Batani, G.; Perez, G.; de la Escalera, G.M.; Piccini, C.; Fazi, S. Competition and protist predation are important regulators of riverine bacterial community composition and size distribution. *J. Freshw. Ecol* **2016**, *31*, 609–623. [[CrossRef](#)]
52. Doblin, M.A.; Dobbs, F.C. Setting a size-exclusion limit to remove toxic dinoflagellate cysts from ships' ballast water. *Mar Pollut Bull* **2006**, *52*, 259–263. [[CrossRef](#)] [[PubMed](#)]
53. Oemcke, D.J.; Hans van Leeuwen, J. Ozonation of the marine dinoflagellate alga *Amphidinium* sp.—Implications for ballast water disinfection. *Water Res.* **2005**, *39*, 5119–5125. [[CrossRef](#)] [[PubMed](#)]
54. Suttle, C.A.; Chen, F. Mechanisms and rates of decay of marine viruses in seawater. *Appl. Environ. Microbiol.* **1992**, *58*, 3721–3729. [[CrossRef](#)] [[PubMed](#)]
55. Worsfold, N.T.; Warren, P.H.; Petchey, O.L. Context-dependent effects of predator removal from experimental microcosm communities. *Oikos* **2009**, *118*, 1319–1326. [[CrossRef](#)]
56. EPA-821-R-14-010; Method 1603: *Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using Modified Membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC). United States Environmental Protection Agency: Washington, DC, USA, 2014.
57. EPA-821-R-06-009; Method 1600: Enterococci in Water by Membrane Filtration Using Membrane-Enterococcus Indoxyl- $\beta$ -D Glucoside Agar (mEI). United States Environmental Protection Agency: Washington, DC, USA, 2006.
58. Adams, M.H. *Bacteriophages*; Interscience Publishers: New York, NY, USA, 1959.
59. Guzman-Herrador, B.; Carlander, A.; Ethelberg, S.; Freiesleben de Blasio, B.; Kuusi, M.; Lund, V.; Lofdahl, M.; MacDonald, E.; Nichols, G.; Schonning, C.; et al. Waterborne outbreaks in the Nordic countries, 1998 to 2012. *Eurosurveillance* **2015**, *20*, 21160. [[CrossRef](#)] [[PubMed](#)]
60. Hamad, I.; Sokhna, C.; Raoult, D.; Bittar, F. Molecular detection of eukaryotes in a single human stool sample from Senegal. *PLoS ONE* **2012**, *7*, e40888. [[CrossRef](#)]
61. Nam, Y.D.; Chang, H.W.; Kim, K.H.; Roh, S.W.; Kim, M.S.; Jung, M.J.; Lee, S.W.; Kim, J.Y.; Yoon, J.H.; Bae, J.W. Bacterial, archaeal, and eukaryal diversity in the intestines of Korean people. *J. Microbiol.* **2008**, *46*, 491–501. [[CrossRef](#)]
62. Comtet-Marre, S.; Parisot, N.; Lepercq, P.; Chaucheyras-Durand, F.; Mosoni, P.; Peyretailade, E.; Bayat, A.R.; Shingfield, K.J.; Peyret, P.; Forano, E. Metatranscriptomics Reveals the Active Bacterial and Eukaryotic Fibrolytic Communities in the Rumen of Dairy Cow Fed a Mixed Diet. *Front. Microbiol.* **2017**, *8*, 67. [[CrossRef](#)]
63. Russell, J.B.; Rychlik, J.L. Factors that alter rumen microbial ecology. *Science* **2001**, *292*, 1119–1122. [[CrossRef](#)] [[PubMed](#)]
64. Weimer, P.J. Redundancy, resilience, and host specificity of the ruminal microbiota: Implications for engineering improved ruminal fermentations. *Front. Microbiol.* **2015**, *6*, 296. [[CrossRef](#)]

65. Berdjeb, L.; Pollet, T.; Domaizon, I.; Jacquet, S. Effect of grazers and viruses on bacterial community structure and production in two contrasting trophic lakes. *Bmc Microbiol* **2011**, *11*, 88. [[CrossRef](#)]
66. Comte, J.; Jacquet, S.; Viboud, S.; Fontvieille, D.; Millery, A.; Paolini, G.; Domaizon, I. Microbial community structure and dynamics in the largest natural French lake (Lake Bourget). *Microb. Ecol.* **2006**, *52*, 72–89. [[CrossRef](#)]
67. Graham, J.M.; Kent, A.D.; Lauster, G.H.; Yannarell, A.C.; Graham, L.E.; Triplett, E.W. Seasonal dynamics of phytoplankton and planktonic protozoan communities in a northern temperate humic lake: Diversity in a dinoflagellate dominated system. *Microb. Ecol.* **2004**, *48*, 528–540. [[CrossRef](#)] [[PubMed](#)]
68. Khomich, M.; Kausrud, H.; Logares, R.; Rasconi, S.; Andersen, T. Planktonic protistan communities in lakes along a large-scale environmental gradient. *FEMS Microbiol. Ecol.* **2017**, *93*, fiw231. [[CrossRef](#)] [[PubMed](#)]
69. Pauli, W.; Jax, K.; Berger, S. Chapter 3: Protozoa in Wastewater Treatment: Function and Importance. In *The Handbook of Environmental Chemistry Vol. 2 Part K Biodegradation and Persistence*; Beek, B., Ed.; Springer: Berlin/Heidelberg, Germany, 2001.
70. Maritz, J.M.; Ten Eyck, T.A.; Elizabeth Alter, S.; Carlton, J.M. Patterns of protist diversity associated with raw sewage in New York City. *ISME J.* **2019**, *13*, 2750–2763. [[CrossRef](#)]
71. Saleem, M.; Fetzter, I.; Harms, H.; Chatzinotas, A. Diversity of protists and bacteria determines predation performance and stability. *ISME J.* **2013**, *7*, 1912–1921. [[CrossRef](#)] [[PubMed](#)]
72. Olive, M.; Gan, C.; Carratala, A.; Kohn, T. Control of Waterborne Human Viruses by Indigenous Bacteria and Protists Is Influenced by Temperature, Virus Type, and Microbial Species. *Appl. Environ. Microbiol.* **2020**, *86*, e01992-19. [[CrossRef](#)]
73. Olive, M.; Moerman, F.; Fernandez-Cassi, X.; Altermatt, F.; Kohn, T. Removal of Waterborne Viruses by Tetrahymena pyriformis Is Virus-Specific and Coincides with Changes in Protist Swimming Speed. *Environ. Sci. Technol.* **2022**, *56*, 4062–4070. [[CrossRef](#)] [[PubMed](#)]
74. Thomas, R.; Berdjeb, L.; Sime-Ngando, T.; Jacquet, S. Viral abundance, production, decay rates and life strategies (lysogeny versus lysis) in Lake Bourget (France). *Environ. Microbiol.* **2011**, *13*, 616–630. [[CrossRef](#)]
75. Surbeck, C.Q.; Jiang, S.C.; Grant, S.B. Ecological control of fecal indicator bacteria in an urban stream. *Environ. Sci. Technol.* **2010**, *44*, 631–637. [[CrossRef](#)]
76. Parikka, K.J.; Le Romancer, M.; Wauters, N.; Jacquet, S. Deciphering the virus-to-prokaryote ratio (VPR): Insights into virus-host relationships in a variety of ecosystems. *Biol. Rev.* **2017**, *92*, 1081–1100. [[CrossRef](#)]
77. Havelaar, A.H.; Pot-Hogbeem, W.M.; Kooti, W.; Pot, R. F-Specific Bacteriophages as Indicators of the Disinfection Efficiency of Secondary Effluent with Ultraviolet Radiation. *Ozone Sci. Eng.* **1987**, *9*, 353–367. [[CrossRef](#)]
78. Korajkic, A.; McMinn, B.R.; Herrmann, M.P.; Pemberton, A.C.; Kelleher, J.; Oshima, K.; Villegas, E.N. Performance evaluation of a dead-end hollowfiber ultrafiltration method for enumeration of somatic and F+ coliphage from recreational waters. *J. Virol. Methods* **2021**, *296*, 114245. [[CrossRef](#)] [[PubMed](#)]
79. Collivignarelli, M.C.; Abba, A.; Miino, M.C.; Caccamo, F.M.; Torretta, V.; Rada, E.C.; Sorlini, S. Disinfection of Wastewater by UV-Based Treatment for Reuse in a Circular Economy Perspective. Where Are We at? *Int. J. Environ. Res. Public Health* **2020**, *18*, 77. [[CrossRef](#)] [[PubMed](#)]
80. Wanjugi, P.; Sivaganesan, M.; Korajkic, A.; McMinn, B.; Kelty, C.A.; Rhodes, E.; Cyterski, M.; Zepp, R.; Oshima, K.; Stachler, E.; et al. Incidence of somatic and F+ coliphage in Great Lake Basin recreational waters. *Water Res.* **2018**, *140*, 200–210. [[CrossRef](#)] [[PubMed](#)]
81. McMinn, B.R.; Rhodes, E.R.; Huff, E.M.; Wanjugi, P.; Ware, M.M.; Nappier, S.P.; Cyterski, M.; Shanks, O.C.; Oshima, K.; Korajkic, A. Comparison of somatic and F+ coliphage enumeration methods with large volume surface water samples. *J. Virol. Methods* **2018**, *261*, 63–66. [[CrossRef](#)] [[PubMed](#)]
82. McMinn, B.R.; Huff, E.M.; Rhodes, E.R.; Korajkic, A. Concentration and quantification of somatic and F+ coliphages from recreational waters. *J. Virol. Methods* **2017**, *249*, 58–65. [[CrossRef](#)] [[PubMed](#)]
83. Zimmer-Faust, A.G.; Griffith, J.F.; Steele, J.A.; Asato, L.; Chiem, T.; Choi, S.; Diaz, A.; Guzman, J.; Padilla, M.; Quach-Cu, J.; et al. Assessing cross-laboratory performance for quantifying coliphage using EPA Method 1642. *J. Appl. Microbiol.* **2022**, *133*, 340–348. [[CrossRef](#)] [[PubMed](#)]
84. Skraber, S.; Gassilloud, B.; Schwartzbrod, L.; Gantzer, C. Survival of infectious Poliovirus-1 in river water compared to the persistence of somatic coliphages, thermotolerant coliforms and Poliovirus-1 genome. *Water Res.* **2004**, *38*, 2927–2933. [[CrossRef](#)]
85. Bae, J.; Schwab, K.J. Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. *Appl. Environ. Microbiol.* **2008**, *74*, 477–484. [[CrossRef](#)] [[PubMed](#)]
86. Duran, A.E.; Muniesa, M.; Mendez, X.; Valero, F.; Lucena, F.; Jofre, J. Removal and inactivation of indicator bacteriophages in fresh waters. *J. Appl. Microbiol.* **2002**, *92*, 338–347. [[CrossRef](#)]
87. Noble, R.T.; Lee, I.M.; Schiff, K.C. Inactivation of indicator micro-organisms from various sources of faecal contamination in seawater and freshwater. *J. Appl. Microbiol.* **2004**, *96*, 464–472. [[CrossRef](#)]
88. Sinton, L.W.; Hall, C.H.; Lynch, P.A.; Davies-Colley, R.J. Sunlight inactivation of fecal indicator bacteria and bacteriophages from waste stabilization pond effluent in fresh and saline waters. *Appl. Environ. Microbiol.* **2002**, *68*, 1122–1131. [[CrossRef](#)] [[PubMed](#)]
89. Sinton, L.W.; Finlay, R.K.; Lynch, P.A. Sunlight inactivation of fecal bacteriophages and bacteria in sewage-polluted seawater. *Appl. Environ. Microbiol.* **1999**, *65*, 3605–3613. [[CrossRef](#)]
90. Ahmed, W.; Toze, S.; Veal, C.; Fisher, P.; Zhang, Q.; Zhu, Z.G.; Staley, C.; Sadowsky, M.J. 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.* **2021**, *751*, 141475. [[CrossRef](#)] [[PubMed](#)]

91. Nasser, A.M.; Zaruk, N.; Tenenbaum, L.; Netzan, Y. Comparative survival of *Cryptosporidium*, coxsackievirus A9 and *Escherichia coli* in stream, brackish-and sea waters. *Water Sci. Technol.* **2003**, *47*, 91–96. [[CrossRef](#)] [[PubMed](#)]
92. Ahmed, W.; Zhang, Q.; Kozak, S.; Beale, D.; Gyawali, P.; Sadowsky, M.J.; Simpson, S. Comparative decay of sewage-associated marker genes in beach water and sediment in a subtropical region. *Water Res.* **2019**, *149*, 511–521. [[CrossRef](#)] [[PubMed](#)]
93. Ahmed, W.; Gyawali, P.; Sidhu, J.P.; Toze, S. Relative inactivation of faecal indicator bacteria and sewage markers in freshwater and seawater microcosms. *Lett. Appl. Microbiol.* **2014**, *59*, 348–354. [[CrossRef](#)] [[PubMed](#)]

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