The effect of UV-C radiation (254 nm) on candidate microbial source tracking phages infecting a human-specific strain of *Bacteroides fragilis* (GB-124)

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ABSTRACT

The enumeration of phages infecting host-specific strains of *Bacteroides* has been widely recognised as an effective and low-cost method of microbial source tracking (MST). A recently described human-specific *Bacteroides* host strain (GB-124) has been shown to detect bacteriophages exclusively in human-impacted waters and is emerging as a useful MST tool. However, a better understanding of the morphology and ecological behaviour of the phages, especially in wastewater disinfection processes, is now required in order to validate their role as MST markers. Bacteriophages infecting *Bacteroides fragilis* GB-124 ($n = 21$) were isolated from wastewater effluent and irradiated using laboratory-based UV-C (254 nm) collimated beam experiments. Bacteriophages were found to be both a morphologically and ecologically homogeneous group, with all specimens showing highly similar first order log-linear inactivation profiles (mean fluence required to inactivate phages by 4-$\log_{10}$ was 36 mJ/cm^2). These findings present the first evidence that phages infecting GB-124 are inactivated by the levels of UV-C radiation routinely delivered during tertiary wastewater treatment processes. More importantly, comparison with previously published inactivation data suggests that their response to UV-C radiation makes GB-124 phages more suitable surrogates for selected enteric viruses in UV disinfection processes than traditional faecal indicator bacteria or human-specific molecular markers.

Key words | enteric viruses, faecal, point-source, wastewater

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INTRODUCTION

The correct identification of faecal sources delivering microbial pollution to waterbodies is key to enabling effective remediation efforts, reducing the health risk to both recreational and drinking water users from exposure to waterborne pathogens and delivering cost-effective water management strategies (Scott *et al*. 2002; Field & Samadpour 2007). Consequently, numerous microbial source tracking (MST) methods have been proposed in recent years that that can distinguish faecal pollution sources within waterbodies. Candidate methods include both library-dependent and independent approaches, those based on molecular methods and those based on more traditional culture techniques.

Among the culture-based methods currently in use, bacteriophages (phages) infecting *Bacteroides* host strains have shown promise (Payan *et al*. 2005; Ebdon *et al*. 2007; Wicki *et al*. 2011). The use of phages as an indicator of human faecal material is a relatively rapid and inexpensive way to determine the source of faecal material in aquatic systems. Moreover, the introduction of international standard methods for three phage groups (Anon 2001a, b, c) has provided clear protocols that allow comparison of data obtained from different studies, and this has greatly improved the quality and comparability of phage-based MST investigations. The US Environmental Protection Agency has recently identified research into phages infecting

doi: 10.2166/wh.2012.173

Bacteroides spp. as a 'high priority critical research need' (Ashbolt *et al.* 2007).

An effective MST marker, whether culture or molecular-based, should demonstrate similar ecological persistence to the pathogens of concern (Engelbrecht & Greening 1978; Cimenti *et al.* 2007), and in order to develop a useful model of enteric pathogen/indicator interaction, the survival characteristics of proposed indicators must first be described (Ballesté & Blanch 2010). Evidence suggests that UV irradiation is an important mechanism for the inactivation of both faecal indicator organisms (FIOs) and enteric pathogens (Sommer *et al.* 2001; Eischeid *et al.* 2009), whether it is delivered naturally through sunlight (UV-A and UV-B), or artificially as a tertiary wastewater disinfection process (UV-C). Whilst research into UV pathogen inactivation has been extensively reported (e.g. Gerba *et al.* 2002; Tree *et al.* 2005), the role of UV radiation in the inactivation of MST markers under controlled laboratory conditions has to date received less attention (Tartera *et al.* 1988; Sommer *et al.* 1989, 2001).

One host bacterium that has shown excellent human specificity is *Bacteroides fragilis* strain GB-124 (formerly considered to be most homologous with *Bacteroides ovatus* ATCC8483T; Payan *et al.* 2005; Ebdon *et al.* 2007). Phages infecting GB-124 have not previously been isolated and their persistence in the presence of UV radiation has not previously been explored. This study is the first to isolate and investigate members of this important group and to assess how their response to UV-C may influence their application in MST studies. The research sets out to determine the inactivation kinetics of GB-124 phages in response to 254 nm ultraviolet radiation (UV-C; representing the germicidal UV employed in wastewater treatment) under controlled laboratory conditions. Proposed standardised methods were used to irradiate phage in this study (Bolton & Linden 2003), allowing the data generated to be compared with those of other previously reported studies.

MATERIALS AND METHODS

Isolation of phages infecting *B. fragilis* strain GB-124

One-litre grab samples of wastewater effluent were collected from a wastewater treatment works (WwTW) in South East England (population equivalent 37,327) on three separate occasions (autumn/winter 2008). Samples were collected in sterile polyethylene bottles (Fisher Scientific, UK), stored at 4 °C in the dark and transported to the Environment & Public Health Research Unit laboratory at the University of Brighton within 2 h. On arrival, all samples were filtered using sterile 10 ml plastic syringes (Becton Dickinson, USA) and sterile 0.22 μm polyvinylidene difluoride filters (Millipore, USA). Filtered samples were then stored in sterile glass tubes at 4 °C in the dark until assay (for no longer than 2 h).

After each final effluent sampling occasion, phages were isolated. During each round of phage isolation, filtered wastewater effluent samples were assayed in triplicate for phages infecting *B. fragilis* host strain GB-124 (Payan *et al.* 2005), using the double-agar protocol developed specifically for *Bacteroides* phages (Adams 1959; Anon 2001c). Agar plates exhibiting widely spaced plaques (zones of lysis) were selected for further isolation work, and 20 plaques were selected during each round of isolation; this is considered the minimum number required to estimate population diversity (Bianchi & Bianchi 1982). Agar plugs containing plaques were picked from plates using a sterile glass Pasteur pipette and were suspended in 400 μL of buffer (19.5 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 85.5 mM NaCl, 1.0 mM $MgSO_4$, 0.1 mM $CaCl_2$) in a sterile microcentrifuge tube. The suspension was incubated for 4 h at 4 °C to allow phage diffusion from the agar plug into the buffer. One hundred microlitres of phage-infected buffer (and dilutions thereof) were retested using the double-agar method mentioned above in order to confirm the presence of phages.

Plaques were picked for a second time from confirmatory plates with widely spaced zones of lysis and suspended in 400 μL of buffer for 4 h at 4 °C. This suspension was added to 27 ml of GB-124 in exponential growth phase (approximately 2×10^8 plaque forming units, pfu/ml) in a glass culture tube (filled to capacity to exclude air) and incubated anaerobically for 18 h at 36 °C, producing crude lysates. One millilitre of each crude lysate (and dilutions thereof) was assayed for phage viability, using the double-agar method described above. Lysates that produced plaques were selected for further work. Remaining volumes of lysates that produced plaques (seven in each round of isolation) were added to separate 620 ml suspensions of

GB-124 in exponential growth phase and incubated anaerobically for 18 h at 36 °C. Crude lysates containing no lytic phages (absence of plaques) were discarded.

Phage lysates were concentrated and purified by precipitation with polyethylene glycol in accordance with methods described elsewhere (Yamamoto *et al.* 1970). Briefly, following incubation, phage suspensions were kept at 4 °C and NaCl added to a final concentration of 1 M. After 1–2 h, suspensions were centrifuged at $1,800 \times g$ for 10 min to remove bacterial debris. Polyethylene glycol 8,000 was added to the supernatant to a final concentration of 10 % (w/v), mixed for 30 min using a magnetic stirrer and left for 12 h at 4 °C. Suspensions were centrifuged at $11,000 \times g$ for 10 min at 4 °C in order to produce a pellet of phage precipitate. The supernatant was discarded and 30 ml of buffer (prepared as above) added. Suspensions were stored at 4 °C for 18 h, allowing the pellet to soften. The pellet and overlying buffer were mixed by gentle pipetting and further debris removed by centrifugation at $1,500 \times g$ for 10 min. The observed titre of the resulting suspensions was between 2×10^8 and 3×10^{11} PFU/ml. Suspensions were stored in light-tight glass tubes in the dark at 4 °C. In total, 21 phages were isolated and named B124 phage.

Transmission electron microscopy

Ten microlitres of phage preparation (10^{-1} dilution of the original high titre lysate as detailed above) was deposited on 200 mesh Formvar/Carbon copper electron microscope grids (Agar Scientific, UK) and left to absorb for 2 min. The excess suspension was withdrawn using Whatman No. 1 filter paper (Whatman, UK) and 10 μL of 0.22 μm filtered 1% w/v uranyl acetate stain (pH 4–4.5) was added to the grid and left for 1 min. The excess was withdrawn using filter paper and grids were left to air dry before being viewed by transmission electron microscopy (Hitachi-7100 at 100 kV). Twenty-one phage suspensions were examined thus.

UV irradiation and experimental design

It was not feasible to fully assay the inactivation kinetics of all 21 isolated phages for UV-C radiation. Therefore a representative subset of seven phages was selected for further in-depth UV inactivation experiments. This subset consisted of phages requiring the highest (B124-10, -12 and -21), the lowest (B124-1 and -29) and mean levels of UV-C radiation to inactivate them (B124-35 and 54) and was achieved using low-resolution, rapid screening tests (data not shown). The selected subset therefore encompassed the range of inactivation rates likely to be observed in phages infecting GB-124 present in wastewaters exposed to tertiary UV treatment.

Two monochromatic 254 nm (UV-C) low-pressure low-intensity Hg 15W UV bulbs (UVP, UK) were used during the experiments, and the fluence rate was measured using an IL1400A radiometer fitted with a SED005/U sensor and a WBS320 wide band UV-IR filter (both International Light; calibrated June 2009 by International Light). As radiometers provide only a reading of incident irradiation in the centre of the beam, average germicidal fluence rate delivered to test suspensions was calculated using corrections for the Petri factor, reflection factor, water factor and divergence factor, in accordance with previously described methods (Bolton & Linden 2003).

Phage suspensions for irradiation testing were prepared by diluting the previously prepared phage suspensions in the buffer (as detailed above) to a final concentration of 10^5 PFU/ml. Artificially seeded buffer solutions were used rather than sterile wastewater or impacted river water to reduce the chance of any phage inhibitors/photosensitisers being present in test suspensions, whilst also minimising particle shielding. A range of seven different fluences were selected that would allow bacteriophage inactivation to be observed: 0, 5, 10, 15, 20, 25 and 30 mJ/cm². Each exposure was repeated three times using new test suspensions, with samples being irradiated in a random order to minimise any error associated with UV bulb output fluctuation.

Upon completion of exposures, phage suspensions were stored in light-tight glass culture tubes, in the dark at 4 °C until all exposures for the phage suspension had been performed. A dilution series of each irradiated suspension was created immediately prior to analysis and assayed using the double-agar method described earlier. Two dilutions were selected for assay and each dilution was assayed in triplicate. In total, 882 double agar assays were performed during the study.

Statistical analysis

Pearson product-moment correlation was carried out to determine the significance of relationship using Microsoft Excel (v.12), whilst regression analysis and production of linear rate constants (k) was performed using Minitab (v.15). Identification of the phage inactivation kinetics was carried out using an inactivation model fitting tool [GInaFiT (v.1.5)] developed by Geeraerd *et al.* (2005).

RESULTS AND DISCUSSION

Phage morphology

All 21 phages isolated during the study displayed binary morphology, with an icosahedral capsid and helical tail, and on this morphological basis can be described as belonging to the *Siphoviridae* family (Van Regenmortel *et al.* 2000). Phage tail shape appeared to be homogenous, with little variation, and phages displayed straight or slightly curved tails, without visible tail fibres or other appendages (Figure 1). The predominance of phages exhibiting *Siphoviridae* morphology amongst the B124 phages isolated in this study is consistent with reports of other *Bacteroides* phages that have shown high 'within-family homogeneity' (i.e. siphoviruses with similar dimensions and morphology; Booth *et al.* 1979; Tartera & Jofre 1987; Lasobras *et al.* 1997; Queralt *et al.* 2003; Hawkins *et al.* 2008; Gómez-Doñate *et al.* 2011). It has been reported that different phage families display varying responses to environmental pressures, with siphoviruses showing greater resistance to inactivation (Dee & Fogleman 1992; Lasobras *et al.* 1997; Muniesa *et al.* 1999). In order to be an effective pathogen surrogate organism and MST tool, consistent ecological behaviour and response to environmental pressures should be demonstrated: this is most likely with a bacterial host detecting a homogeneous phage group (Queralt *et al.* 2003). The results of this study suggest that phage infecting GB-124 appear to be a morphologically homogeneous group, and this finding supports their use in MST studies.

It is interesting to note that although a total of 60 phages were isolated, only 21 phages consistently exhibited lytic life cycles during concentration/purification. Recent work (Ogilvie pers comm) has shown that the GB-124 phage genome contains phage anti-repressors and recombination proteins that may facilitate a lysogenic life cycle. It is possible that the phage infecting GB-124 isolated during this study switched from a lytic cycle when isolated to a lysogenic cycle during concentration/purification. The ability of siphoviruses to switch between lytic and lysogenic life cycles has been well documented for lambda phage (Cheng *et al.* 1988; Oppenheim *et al.* 2005).
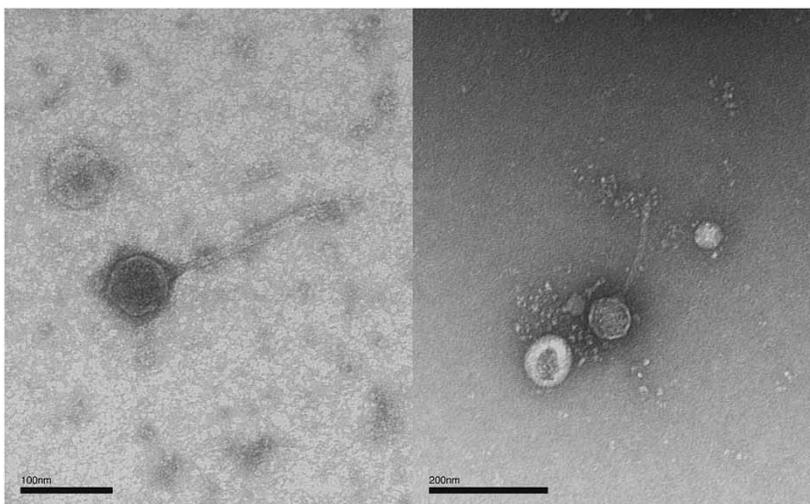

Figure 1 | Typical B124 siphoviruses [B124-1 (left) and B124-12 (right)].

UV-C inactivation kinetics

The dose-response curves for UV-C irradiation of all seven phage specimens are shown in Figure 2 (data are presented as $\log_{10}$ phage inactivation (N_o/N) versus fluence (mJ/cm^2)). All irradiated phage specimens showed a significant ($p = 0.000$) negative relationship between $\log_{10}$ PFU/100 μL and fluence, with minimal range: Pearson product-moment correlations ranged from -0.98% (B124-10, -12, -29 and -35) to -0.99% (B124-1, -21 and -54), with a mean value of -0.98% and $0.006 \, mJ/cm^2$ standard deviation (SD). Using the GInaFiT modelling software, the relationship between $\log_{10}$ inactivation and fluence was linear positive for all phage specimens examined. All phage displayed a similar relationship to increasing fluence, and the inactivation rate constants (k) derived from linear regression analysis are shown in Table 1. The k values varied from $0.099 \, mJ/cm^2$ (B124-54) to $0.139 \, mJ/cm^2$ (B124-10), with a mean value of $0.114 \, mJ/cm^2$ (SD = $0.014 \, mJ/cm^2$, range = $0.04 \, mJ/cm^2$). Fluences needed to achieve a 4-$\log_{10}$ inactivation of the phage ranged from $29 \, mJ/cm^2$ (B124-10) to $41 \, mJ/cm^2$ (B124-54; Table 1). The mean value was 36 mJ/cm^2 (range = $12 \, mJ/cm^2$; SD = $4.07 \, mJ/cm^2$). Statistical analysis showed that the inactivation curves of the seven

phages were not significantly different to each other (t test; $p = 0.874$). The fact that all phage suspensions irradiated in this study had similar inactivation rate constants is consistent with their similar morphology, and, as consistent environmental behaviour is more likely, again supports their candidacy as an effective MST tool.

Possible implications for pathogen surrogacy

If the fluences required to inactivate an indicator are more closely aligned to pathogens of concern (such as culturable, viable enteric viruses), then a more accurate assessment of the health risk and a more effective plan for pollution remediation may be possible. One of the difficulties with using proposed molecular MST markers is the amplification of non-infectious strands of nucleic acid using polymerase chain reaction (PCR) (Jofre & Blanch 2010). These positive signals may overestimate the contribution of human faecal load (and hence level of health risk) and may result in expensive and unnecessary remediation efforts. For example, it has been demonstrated that human-specific 16S rRNA Bacteroidales markers show minimal attenuation after UV-C tertiary treatment (Stapleton *et al.* 2009; Wyer *et al.* 2010) and may persist longer in tertiary treated
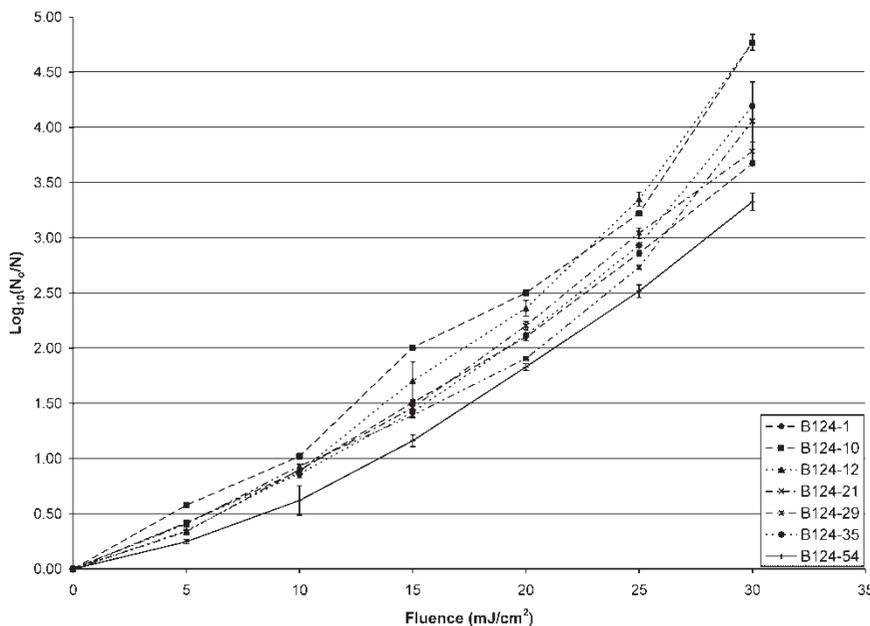

Figure 2 │ Dose-response curves of phages B124-1, B124-10, B124-12, B124-21, B124-29, B124-35 and B124-54 during UV-C inactivation experiments (data points show the mean of nine values. Error bars represent one standard deviation above and below the mean).

Table 1 | Inactivation rate coefficients (*k*) and fluences required for log$_{10}$ reductions of B124 phage specimens during UV-C irradiation

Phage specimen ID (B124-)	*k* (mJ/cm^2)	−1 log$_{10}$ reduction (mJ/cm^2)	−2 log$_{10}$ reduction (mJ/cm^2)	−3 log$_{10}$ reduction (mJ/cm^2)	−4 log$_{10}$ reduction (mJ/cm^2)
1	0.113	9	18	27	35
10	0.139	7	14	22	29
12	0.121	8	17	25	33
21	0.117	9	17	26	34
29	0.101	10	20	30	40
35	0.107	9	19	28	37
54	0.099	10	20	30	41

wastewater effluents than traditional faecal indicator bacteria (Anon 2008). Therefore, caution is required when using such methods to quantitatively apportion and forecast faecal inputs from wastewaters subjected to UV treatment processes.

The fluence requirements necessary to inactivate B124 phages were shown to be closely aligned with those of other phages and viruses reported in the literature (Table 2). It is interesting to note that siphoviruses *B. fragilis* phage B40-8 and *Staphylococcus aureus* phage A994

Table 2 | UV-C inactivation requirements of viruses and phages similar to B-124 phages

Organism	4 log$_{10}$ reduction (mJ/cm^2)	Reference
B40-8 phage	28–29	Sommer *et al.* (1998, 2001)
PRD-1 phage	30.1	Meng & Gerba (1996)
Staphylococcus aureus phage A994	36	Sommer *et al.* (1989)
Calicivirus canine	30	De Roda Husman *et al.* (2004)
Calicivirus feline	36	Thurston-Enriquez *et al.* (2003)
Coxsackievirus B3	32	Gerba *et al.* (2002)
Coxsackievirus B5	36	Gerba *et al.* (2002)
Echovirus I	33	Gerba *et al.* (2002)
Echovirus II	28	Gerba *et al.* (2002)
Hepatitis A HM175	29.6	Wilson *et al.* (1992)
Poliovirus type 1	27	Tree *et al.* (2005)
Rotavirus SA 11	38	Sommer *et al.* (1989)

required similar levels of UV-C to achieve a 4-log$_{10}$ inactivation as B124 phages, indicating the influence of phage morphology on inactivation requirements. Recent work (Ogilvie pers comm.) suggests that the genomes of B124 phages are highly homologous to *B. fragilis* phage B40-8 (Puig & Gironés 1999). Such limited diversity, and the aforementioned predominance of siphoviruses within the *Bacteroides* phage population, indicates that it is highly likely that phages capable of infecting the same *Bacteroides* host may be highly similar to one another in terms of response to UV radiation. Unfortunately, further data on phage family and inactivation requirements were not available for comparison in this study.

The similarity of B124 phage UV-C inactivation requirements to coxsackievirus, echovirus, hepatitis A virus, poliovirus and rotavirus (Table 2), indicates that B124 phages may have a role as a surrogate for these viruses during wastewater treatment. It would therefore be useful to examine further the behaviour of these pathogens and B124 phages under a range of UV-influenced conditions. Comparison of the results of this study with those in the literature show that phages infecting GB-124 may demonstrate greater resistance to UV-C radiation than the traditional faecal indicator bacteria used extensively in the control and management of faecal pollution tools in river catchments; *Escherichia coli* strains typically require between 5.6 (Otaki *et al.* 2003) and 7.5 mJ/cm^2 (Eischeid & Linden 2007) to achieve 4-log$_{10}$ inactivation, whereas 5 mJ/cm^2 has been reported for *Enterococcus faecalis* (Metzger *et al.* 2006). B124 phages also show greater resistance to UV-C radiation than many non-viral pathogens; *Campylobacter jejuni* (4.6 mJ/cm^2; Wilson *et al.* 1992), *Salmonella typhi* (7.1 mJ/cm^2; Chang *et al.* 1985), *Giardia lamblia* (2.0 mJ/cm^2; Shin *et al.* 2000) and *Cryptosporidium parvum* (2.2 mJ/cm^2; Morita *et al.* 2002). Therefore, it is plausible to suggest that B124 phages may offer a better indication of viral, but not bacterial or protozoan, risk to the health of downstream users than traditional FIOs in waters receiving UV-C treated wastewater.

UV-C fluences commonly used in tertiary wastewater treatment vary, according to site, local requirements and established operator practice. For instance, in the UK, Southern Water plc (the main water supply and sewerage company in Southeast England and operator of the WwTW

from which both GB-124 and B124 phage specimens were isolated) conforms to fluence consents (i.e. minimum requirements) of 32 and 35 mJ/cm^2, but it is common practice to deliver fluences of 50 mJ/cm^2 (I Mayor-Smith pers comm.). Delivery of these fluences may inactivate both B124 phages and the pathogens/indicators present in municipal wastewater (Table 2). These findings suggest that GB-124 phages may have limitations in their capacity to identify human faecal contamination downstream of WwTW employing tertiary UV-C disinfection. However, the fact that phages infecting GB-124 appear to be efficiently inactivated by UV-C treatment in conjunction with a range of important viral pathogens, may offer a distinct advantage over other approaches. Those that rely on the detection of molecular markers may demonstrate increased persistence and traditional FIOs demonstrate lower persistence, and therefore phages infecting GB-124 may be more useful surrogates for viral pathogens in waters receiving UV-C disinfected final effluents.

CONCLUSIONS

The findings from this study may be summarised as follows:

1. Phages infecting GB-124 appear to be a homogeneous group demonstrating morphology typical of the *Siphoviridae* family. Work is currently being undertaken to sequence B124 phages to support efforts to understand the mobile genome of the human gut microbiome and to aid family classification.
2. The UV-C inactivation kinetics of phages infecting *Bacteroides* strain GB-124 are highly homogeneous and similar to other siphoviruses reported in the literature.
3. B124 phages are likely to be inactivated by the UV-C fluences delivered in WwTW though the rate of inactivation will be highly dependent on a number of external factors, including composition of the wastewater matrix and the efficiency of the UV plant.
4. B124 phages may not be suitable MST markers of human faecal pollution in waters receiving UV-C disinfected wastewater effluents.
5. Similarities in UV-C inactivation rates suggest that B124 phages may have a role as an easily assayed indicator of selected enteric viruses during tertiary UV-C disinfection, supporting more accurate assessments of human viral health risks than commonly used FIOs or more recently proposed molecular markers.

This study represents an important and essential first step in fully elucidating the ecological inactivation kinetics of B124 phages currently used in MST studies. *In situ* inactivation experiments within a variety of environmental matrices to investigate stressors other than UV-C radiation, would demonstrate whether selected waterborne pathogens and these candidate indicators demonstrate similar ecological behaviour. This future work would further improve waterborne disease risk prediction models.

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