Distribution of human fecal marker GB-124 bacteriophages in urban sewage and reclaimed water of São Paulo city, Brazil

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ABSTRACT

Bacteriophages infecting *Bacteroides fragilis* GB-124 have been described as potential markers of human fecal contamination in water sources. The aim of this study was to evaluate the occurrence of GB-124 phages in raw sewage, secondary effluents and reclaimed water of the São Paulo city using a low-cost microbial source tracking method. Samples were collected monthly from April 2015 to March 2016 in four municipal wastewater treatment plants that operate with activated sludge processes followed by different tertiary treatments (sand-anthracite filtration, membrane bioreactor/reverse osmosis) and final chlorination. GB-124 phages were detected in 100% of the raw sewage samples, with viral loads varying from 7.5×10^3 to 1.32×10^6 PFU/L. Virus removal efficiency in activated sludge processes ranged from 1.89 to 2.31 $\log_{10}$. Frequencies of phage detection were lower in reclaimed water samples (0–22.2%). The results indicated that GB-124 phage could be a complementary low-cost viral marker for the detection of human fecal pollution in waters impacted with urban sewage in this region. However, the datasets of tertiary effluents resulted in several samples with concentrations below the detection limit (DL $\leq$1 PFU/mL) suggesting the need to obtain analytical methods with lower DL for greater accuracy of negative results.

Key words | bacteriophages, *Bacteroides fragilis* GB-124, human fecal indicator, microbial source tracking (MST), reclaimed water, urban sewage

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INTRODUCTION

The water demand associated with new patterns of economic development, consumption and increasing pollution levels are compromising the availability of this resource for various purposes, including drinking water supply. To prevent future environmental impacts and health public risks, new approaches to evaluate effluent sanitary quality are emerging (Diston *et al.* 2012; Rusiñol *et al.* 2014).

Traditional microbiological indicators (fecal indicators bacteria (FIB), e.g. thermotolerant and fecal coliforms, *Escherichia coli* and fecal enterococci) are not appropriate to evaluate the water contamination caused by other pathogens, such as protozoan and enteric viruses (Harwood *et al.* 2014). Moreover, they are not suitable to distinguish between human and animal sources of contamination (Harwood *et al.* 2014; Rusiñol *et al.* 2014). Determining sources of contamination is an important strategy in water management and this approach is often called microbial source tracking (MST) (Ahmed *et al.* 2010; Nnane *et al.* 2011; Araújo *et al.* 2014; Rusiñol *et al.* 2014).

Viruses are one of the major concerns in wastewater reclamation due to their high resistance to conventional wastewater treatment processes; their high rate of survival

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under adverse environmental conditions; and their serious implication in major outbreaks of waterborne diseases, especially those caused by enteric viruses, such as the norovirus, rotavirus, hepatitis A and E viruses, human enteroviruses, and adenoviruses (Bofill-Mas *et al.* 2013).

However, monitoring of multiple pathogens is not feasible and, therefore, researchers have focused on the study of viral indicators that could be used together with bacterial indicators for assessing human fecal contamination in the environment (Ahmed *et al.* 2010; Bofill-Mas *et al.* 2013; Rusiñol *et al.* 2014). Human adenovirus, polyomavirus and bacteriophages (F-specific, somatic coliphages and *Bacteroides* spp. phages) are considered potential candidates to assess human viral contamination in the environment (Payan *et al.* 2005; Ebdon *et al.* 2012; Bofill-Mas *et al.* 2013; Rusiñol *et al.* 2014; Purnell *et al.* 2015), and molecular methods (notably polymerase chain reaction and variants) are commonly used to track these microorganisms (Ahmed *et al.* 2010; Bofill-Mas *et al.* 2013; Rusiñol *et al.* 2014).

Bacteriophages infecting a specific host strain (*Bacteroides fragilis* GB-124) have been described as potential viral markers to evaluate human fecal pollution in different environmental matrices worldwide (Payan *et al.* 2005; Nnane *et al.* 2011; Diston *et al.* 2012; Ebdon *et al.* 2012; Jofre *et al.* 2014; McMinn *et al.* 2014). Most bacteriophages infecting different *Bacteroides* species have been reported to belong to the *Siphoviridae* family and are recognised to contain a dsDNA genome, a capsid composed of an icosahedral head, and a flexible tail (Jofre *et al.* 2014).

Advantages to the use of *Bacteroides* GB-124 phages to tracking human faecal pollution include their morphology, environmental survival, and persistence to treatment processes, characteristics similar to viruses of concern, such as enteric viruses (Diston *et al.* 2012; Ebdon *et al.* 2012; Jofre *et al.* 2014). Relatively high concentrations of GB-124 phages have been reported in municipal wastewaters worldwide and temporal and geographic stability were already observed (Ebdon *et al.* 2012; Jofre *et al.* 2014; McMinn *et al.* 2014). Additionally, assays using specific host strains have been developed, allowing the identification of human or animal sources of contamination (Payan *et al.* 2005; Ebdon *et al.* 2012).

Methods based on bacteriophage detection using culture assays provide a more reliable analysis of the infectivity of the virus strains detected. A simple and low-cost method to detect these viruses can be useful for laboratories, environmental sanitation companies or environmental regulatory agencies that need routine monitoring.

However, the occurrence of *Bacteroides fragilis* GB-124 seems to vary among distinct geographic regions because it depends on epidemiological and environmental factors (Payan *et al.* 2005). Therefore, it is necessary to identify distinct occurrence patterns of host strains under the local conditions (Payan *et al.* 2005; Ebdon *et al.* 2012; McMinn *et al.* 2014).

Knowledge about the occurrence of bacteriophages that infect *Bacteroides* host strain GB-124 in wastewaters, treated effluents and reclaimed water is limited in Brazil. Therefore, the aim of this study was to perform an environmental surveillance of these viruses in raw sewage and treated effluents (including reclaimed water) from four wastewater treatment plants (WWTPs) of São Paulo city and to evaluate bacteriophage removal at the different treatment processes. Physicochemical variables were also measured, and statistical analyses were performed to verify the influence of these parameters on virus detection.

MATERIAL AND METHODS

Location and characteristics of the WWTPs

The four WWTPs selected in this study are located in different regions of São Paulo metropolitan area, Brazil, and receive the sewage of approximately eight million people. São Paulo city is the capital of São Paulo state and is located in the southeast region of the country, being one of the most densely populated regions with approximately 21 million inhabitants.

These WWTPs receive mainly domestic sewage and comprise primary, secondary (activated sludge) and tertiary (filtration or ultrafiltration/reverse osmosis and chlorination) processes (Table 1). The WWTP-3 receives contributions from three collection networks, including two domestic sewage systems and one of a large chemical industry of nitrocellulose. The sewage collection network is separate from the urban drainage network.

Table 1 | Characteristics of the WWTPs selected for this study

WWTP	Treatment			Equiv. population	Inflow (m^3/s)	Reclaimed water uses
	Conventional	Tertiary or advanced	Disinfection			
1	AS	Sand-anthracite filter (20 μm)	Sodium hypochlorite (Ct = 30 min, 2–10 ppm (TRC))	4.4 million	9.7	Restricted urban use
2	AS	Coagulation (Aluminium polychloride – Al_n-$(OH)_m(Cl_3)_{n-m}$), flocculation/sedimentation, sand-anthracite/zeolites filter (20 μm)	Sodium hypochlorite (Ct = 30 min, 2–10 ppm (TRC))	1.2 million	2.5	Restricted urban use and industrial use
3	AS	Sand-anthracite filter (20 μm)	Sodium hypochlorite (Ct = 30 min, 2–10 ppm (TRC))	720 thousand	0.8	Restricted urban use
4	AS	Disk filter (ARKAL) (400 μm), anoxic chambre (N and P removal), MBR (pore size of 0.05 μm) – submerged membranes, reverse osmosis (<0.001 μm)	Chlorine dioxide (residual chlorine: 0.2 ppm)	1.4 million	1.9	Industrial use (boilers, cooling water), urban use (fire fighting system, street washing)

AS, activated sludge; Ct, contact time; TRC, total residual chlorine.

Sampling

Raw sewage, secondary effluent and reclaimed water were collected into sterile polypropylene bottles monthly during one year (April 2015–March 2016), totalling 144 samples (36 samples from each WWTP). Reclaimed water samples were dechlorinated with sodium thiosulphate (10%) after collection. The samples were maintained under refrigeration (4 °C) and transported to the laboratory for processing. The sample processing occurred up to 15 h after each collection.

For phage analysis, a raw sewage sample of WWTP-3 was composed in the laboratory considering the flow rate of each of the three individual influents discharging into this plant, because there is not a collect point resulting from the mixture of these influents before primary treatment in the respective WWTP.

Enumeration of phages

Bacteroides fragilis GB-124 phages were enumerated using a double-layer agar technique, according to ISO 10705-4 (ISO 2001) and as described elsewhere (Ebdon *et al.* 2012). In brief, 12 mL screw-topped tubes containing *Bacteroides* phage recovery medium (BPRM) broth were inoculated with 1 mL of host bacterium (GB-124) and incubated at 37 ± 0.2 °C for 18 h. One millilitre of bacteria was inoculated into BPRM broth and incubated at 37 ± 0.2 °C for approximately 3 h to achieve an optimal optical density for phage detection (approximately 0.33 at 620 nm).

The bacterial suspension was then used immediately. Samples were passed through a 0.22 μm syringe driven polyvinylidene difluoride filter (Millipore, USA) to remove background bacterial contamination. Filtered samples of raw sewage were also diluted (10-fold dilution) to avoid excessive lysis plaques on the agar. One millilitre of the samples was then combined with 1 mL of host (GB-124) in test tubes containing 2.5 mL of semisolid BPRM agar, poured into 90 mm Petri dishes with a monolayer of BPRM agar and allowed to solidify. The Petri dishes were inverted and incubated at 37 ± 0.2 °C for 18–24 h under anaerobic conditions using anaerobic jars and commercial anaerobic gas packs (BBL Gas Pack Plus, Maryland, USA).

The presence of phages resulted in the production of visible plaques (zones of lysis) in a confluent lawn of the host growth. A suspension of reference phage (B-14) known to infect the host strain was also included in all analyses as a

positive control. Negative controls (*B. fragilis* GB-124 culture) were also included in all experiments. All samples were analysed in duplicate, and the results were expressed as the mean number of plaque-forming units (PFU) per litre.

Physicochemical parameters

Water temperature, pH, conductivity and residual chlorine were measured in the field according to Standard Methods (methods 2550 B, 4500 H+ and 2510B, respectively) employing a pH metre (Mettler Toledo), conductivity metre (WTW) and colorimeter (Hach). The levels of total organic carbon, and turbidity were analysed at CETESB Chemistry Laboratory using methods 5310C, and 2130, respectively, recommended by *Standard Methods* (APHA/AWWA/WEF 2012).

Statistical analyses

The datasets of secondary and tertiary effluents resulted in the occurrence of several left-censored observation samples with concentrations below the theoretical detection limit (TDL ≤1 PFU/mL). In this case, for statistical analysis, the value considered was the TDL divided by the square root of 2 (Croghan & Egeghy 2003). The high concentration of points below the detection limit creates a non-standard distribution; however, it can be adjusted to a known distribution. The log (GB-124) was used as the response variable. It was noted that this variable follows the Zeros Adjusted Inverse Gaussian (ZAIG) distribution (Heller *et al.* 2006).

For achievement of the proposed objectives and considering the unique features of the data involved, the statistical modelling methodology Generalized Additive Models for Location, Scale and Shape (GAMLSS) was used (Stasinopoulos & Rigby 2007). A saturated model was started (with all the variables and factors present) and progressed by removing, one by one, those variables whose contribution was not significant in explaining variations in densities of GB-124 phages (Figure 1).

The final model was validated according to the hypotheses assumed: normal distribution and independence among residuals. The R packages used in this methodology were the GAMLSS family (Rigby & Stasinopoulos 2005). Statistical

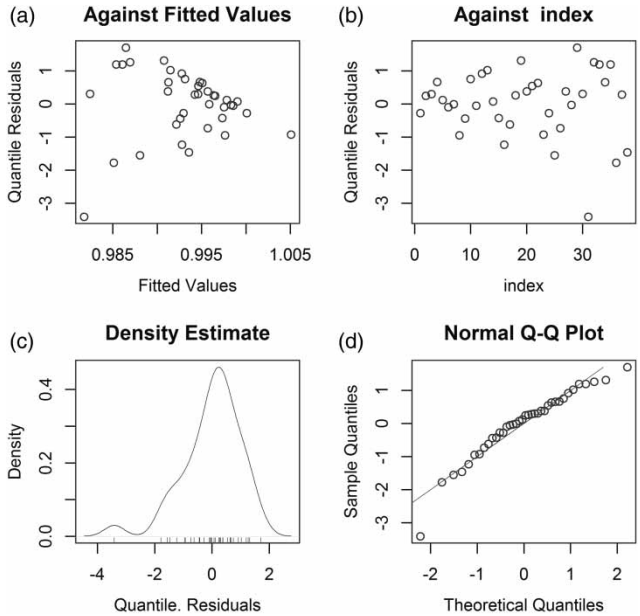

Figure 1 | Adjusted model (a)–(d) by the methodology GAMLSS to explain variations in densities of GB-124 phages in different WWTPs and treatment stages.

significance was obtained when the adjusted model produced $p \leq 0.001$ and all model coefficients showed $p < 0.10$.

RESULTS

Physicochemical parameters

The geometric means of the physicochemical parameters obtained during the sampling period are shown in Table 2. Water temperature did not vary greatly throughout the year in the WWTPs analysed, with values ranging from 24.1 to 25.5 °C for raw sewage samples, except for WWTP-3, which had a mean temperature of 35.1 °C for the chemical industry wastewater (Table 2). Higher values of water temperature were also observed in treated effluents and reclaimed water from WWTP-3 compared to the water temperature from the other WWTPs (Table 2).

Mean values of pH in all stages of the WWTPs were near neutrality (pH = 7.0), with low standard deviation (Table 2). The water temperature and pH results in the treated effluents of all WWTPs were in compliance with standards established by the national legislation for effluent discharges in receiving waters (water temperature below 40 °C and pH of 6.0–9.0) (CONAMA, Resolution n°. 430 2011).

Table 2 | Geometric mean values of physicochemical parameters obtained for the different WWTPs during April 2015 to March 2016

	Physicochemical parameters (±SD)					
Stage	**Water temperature (°C)**	**pH**	**Conductivity (μS/cm)**	**Turbidity (NTU)**	**TOC (mg/L)**	**Residual chlorine (mg/L)**
Raw sewage ($n = 12$)						
WWTP-1	24.1 (± 1.78)	6.96 (± 0.31)	646.6 (± 97.7)	109.7 (± 62.0)	106.04 (± 121.01)	
WWTP-2	25.4 (± 1.72)	7.49 (± 0.19)	1,145.02 (± 154.7)	144.5 (± 115.3)	155.1 (± 57.08)	–
WWTP-3[a]						
Chemical industry	35.1 (± 2.58)	7.07 (± 0.27)	3.74 (± 0.61)	49.5 (± 96.8)	296.7 (± 118.8)	
Domestic sewage	25.5 (± 2.8)	7.09 (± 0.22)	481.5 (± 489.7)	127.4 (± 124.0)	110.3 (± 58.4)	–
Domestic sewage	25.5 (± 1.59)	7.25 (± 0.28)	38.9 (± 707.8)	56.1 (± 48.3)	69.7 (± 55.6)	–
WWTP-4	25.2 (± 1.79)	7.02 (± 0.25)	802.5 (± 140.4)	131.6 (± 84.8)	125.5 (± 86.11)	–
Treated effluents ($n = 12$)						
WWTP-1	24.4 (± 1.84)	7.32 (± 0.16)	768.53 (± 140.8)	7.56 (± 11.9)	20.5 (± 7.9)	–
WWTP-2	26.6 (± 1.67)	7.18 (± ±0.15)	745.4 (± 207.6)	21.6 (± 48.4)	28.2 (± 17.5)	–
WWTP-3	27.4 (± 1.72)	6.89 (± 0.15)	97.9 (± 791.14)	4.37 (± 4.79)	10.19 (± 3.39)	–
WWTP-4	25.6 (± 2.15)	6.4 (± 0.34)	563.4 (± 106.2)	5.6 (± 7.27)	10.6 (± 4.4)	–
Reclaimed water ($n = 12$)						
WWTP-1 ($n = 9$)	22.9 (± 2.79)	7.14 (± 0.37)	792.2 (± 140.1)	4.8 (± 2.73)	16.4 (± 5.92)	1.35 (±2.01)
WWTP-2	26.5 (± 2.27)	7.06 (± 0.33)	901 (± 198.6)	2.84 (± 7.45)	14.8 (± 4.64)	1.55 (±2.4)[b]
WWTP-3	26.8 (± 1.69)	6.86 (± 0.09)	57.85 (± 793.13)	3.92 (± 6.66)	10.8 (± 3.0)	0.84 (±0.6)[b]
WWTP-4	25.8 (± 2.0)	6.69 (± 0.2)	484.09 (± 49.2)	1.38 (± 0.28)	4.64 (± 1.25)	0.36 (±0.15)[c]

SD, standard deviation; NTU, nephelometric turbidity unit; TOC, total organic carbon.
[a]WWTP-3 receives three sewage collection networks, including wastewaters from a large chemical industry.
[b]Residual chlorine measured in WWTP-2 and 3 ($n = 11$).
[c]Residual chlorine measured in WWTP-4 ($n = 8$).

The conductivity results varied widely, but were in accordance with values commonly found in wastewaters and effluents from WWTPs that receive domestic and industrial sewage.

Higher levels of total organic carbon (TOC) were observed in raw sewage from the industrial wastewaters from WWTP-3, while in the treated effluents, higher mean concentrations were found in WWTP-2 (Table 2).

In general, lower levels of turbidity and TOC were observed in reclaimed water samples from WWTP-4, which operates with membrane bioreactor (MBR) and reverse osmosis (Table 2), indicating a better performance of this system for pollutant removal.

Residual mean chlorine values were also within the range of standards commonly found for the disinfection of effluents in tertiary treatment, and lower concentrations were found in reclaimed water samples from WWTP-4 which uses chlorine dioxide as a disinfecting agent (Table 2).

Detection of GB-124 phages

GB-124 phages were recovered in 100% of the raw sewage samples from all the WWTPs (Table 3). Viral loads varied from 3×10^4 (min) to 2.7×10^6 (max) PFU/L in WWTP-1, from 2.3×10^4 (min) to 1.32×10^6 (max) PFU/L in WWTP-2, from 7.5×10^3 (min) to 1.01×10^6 (max) PFU/L in WWTP-3 and from 4.7×10^4 (min) to 1.3×10^6 (max) PFU/L in WWTP-4, with geometric mean viral loads (PFU/L) from 2.09×10^5 (±SD 3.42) (WWTP-1), 2.91×10^5 (±SD 3.45) (WWTP-2), 8.1×10^4 (±SD 3.49) (WWTP-3) and 2.18×10^5 (±SD 3.0) (WWTP-4) (Table 3).

Table 3 | Densities and annual frequency of bacteriophages of host strain *B. fragilis* GB-124 (PFU/L) in the different treatment stages of WWTPs

	Stages of treatment process											
	Raw sewage				Treated effluents				Reclaimed water			
Sampling date	WWTP-1	WWTP-2	WWTP-3	WWTP-4	WWTP-1	WWTP-2	WWTP-3	WWTP-4	WWTP-1	WWTP-2	WWTP-3	WWTP-4
April	1.9×10^5	3.6×10^5	2.5×10^5	1.05×10^5	4.0×10^4	ND	ND	ND	NP	ND	ND	ND
May	3.0×10^4	7.7×10^5	4.5×10^4	NP*	ND	6.0×10^3	ND	2.0×10^3	NP	ND	ND	ND
June	8.0×10^4	3.7×10^5	1.8×10^4	6.9×10^4	1.7×10^4	ND	ND	ND	ND	ND	ND	ND
July	3.7×10^5	3.7×10^5	1.4×10^5	3.4×10^5	1.5×10^4	1.6×10^4	ND	1.5×10^3	ND	ND	ND	ND
August	2.7×10^6	7.6×10^5	1.1×10^5	3.95×10^5	1.6×10^4	3.5×10^3	ND	3.0×10^3	9.5×10^3	ND	ND	ND
September	6.8×10^5	7.5×10^5	1.5×10^5	4.95×10^5	ND	1.0×10^3	ND	ND	ND	ND	ND	ND
October	7.0×10^5	4.0×10^5	1.0×10^5	3.45×10^5	ND	ND	ND	5.0×10^2	1.0×10^3	ND	ND	ND
November	2.3×10^5	2.3×10^4	6.3×10^4	1.3×10^6	ND	ND	ND	ND	ND	5.0×10^2	ND	ND
December	6.95×10^4	7.9×10^4	7.7×10^4	1.3×10^5	5.0×10^3	ND	1.0×10^3	ND	NP	ND	2.0×10^3	ND
January	1.48×10^5	4.7×10^4	7.5×10^3	6.76×10^5	7.0×10^3	ND	ND	1.5×10^4	ND	ND	ND	ND
February	1.68×10^5	1.3×10^6	1.0×10^6	4.7×10^4	ND	2.5×10^3	4.7×10^4	ND	ND	8.5×10^3	4.0×10^3	ND
March	8.4×10^4	3.8×10^5	4.5×10^4	6.0×10^4	ND	ND	ND	ND	ND	ND	ND	ND
Frequency	100%	100%	100%	100%	50%	41.6%	16.6%	41.6%	22.2%	16.6%	16.6%	0

NP, not provided: operational problems in WWTP-1; ND, not detected (below detection threshold (≤1 PFU/mL); PFU, plaque forming units; NP*, not performed.

Lower frequencies of detection were observed for secondary effluent and reclaimed water samples (Table 3). In secondary effluents, GB-124 phages were detected in 50% of WWTP-1, in 41.6% of WWTPs 2 and 4 samples and only in 16.6% of WWTP-3 samples (Table 3). Discharge of chemical sewage entering directly in the system could contribute to viral inactivation. Geometric mean viral loads obtained in treated effluents were as follows: 2.53×10^3 (±SD 4.68), 1.43×10^3 (±SD 2.92), 1.03×10^3 (±SD 3.34) and 1.16×10^3 (±SD 2.65) (PFU/L) for WWTPs 1, 2, 3 and 4, respectively (Table 3).

The highest percentage of phage detection in reclaimed water was observed in the system composed of sand-anthracite and chlorination treatment from WWTP-1 (Table 3). Only two samples were positive (16.6%) for WWTP-2 and 3 (Table 3). No GB-124 phages were recovered from reclaimed water samples from WWTP-4, which operates with MBR, reverse osmosis and disinfection with chlorine dioxide (Table 3), suggesting a better virus-removal efficiency by this system. Geometric mean viral loads obtained after adjustment of left-censored data set in reclaimed water samples were 9.81×10^2 (±SD 2.36), 8.45×10^2 (±SD 2.08), 8.91×10^2 (±SD 1.75) and 7.07×10^2 (±SD 1.0) for WWTPs 1, 2, 3 and 4, respectively (Table 3).

Figure 2 shows the median and interquartile range of log (GB-124) phages values achieved in each stage of WWTP. Statistical analysis showed that the stage of treatment has a significant impact on the concentrations of GB-124 ($p < 0.001$), while there was no significant difference in concentrations of phages among the WWTPs.

Mean virus removal efficiency achieved by activated sludge processes varied from 1.89 $\log_{10}$ (min) (WWTP-3) to 2.31 $\log_{10}$ (max) (WWTP-2) (Figure 2). Tertiary

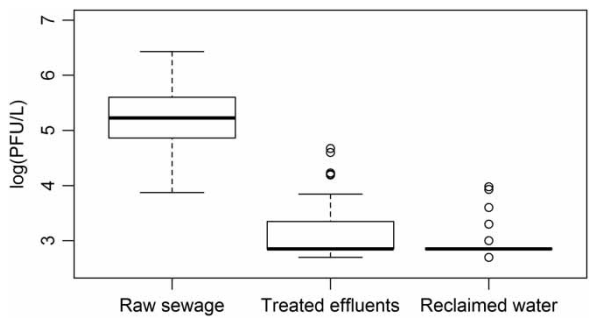

Figure 2 | Box-plot of median densities of GB-124 phages (log units) at each treatment stage considering all the WWTPs.

treatments were able to increase the virus removal efficiency up to 0.41 $\log_{10}$ (WWTP-1) (Figure 2).

Water temperature, TOC and conductivity were statistically significant for GB-124 phage occurrence in the different treatment stages of WWTPs (Figure 3), while turbidity and pH did not present significant contributions. GB-124 phage occurrence decreased with increased water temperature in the raw sewage and treated effluents of the WWTPs ($p \leq 0.0021$), while at higher values of conductivity, the densities of GB-124 phages increased in raw sewage and in the secondary treatment stage in all WWTPs ($p \leq 0.091$) (Figure 3). In raw sewage samples, the detection rates of phages decreased with higher levels of TOC ($p \leq 0.0046$), while the opposite occurred in treated effluent samples from WWTPs with higher levels of TOC ($p \leq 0.100$) (Figure 3), although TOC concentrations below 100 mg/L were observed in these samples.

DISCUSSION

Analysis of GB-124 phages in environmental samples has been considered a suitable approach in MST studies since they present a narrow host range and are highly specific to identify human fecal contamination (Nnane *et al.* 2011; Ebdon *et al.* 2012; McMinn *et al.* 2014).

Similar to data from other studies, environmental surveillance of GB-124 phages demonstrated that these viruses are widely disseminated in municipal wastewaters from São Paulo city, being detected throughout the year. Moreover, no significant difference was obtained among viral concentrations present in raw sewage of different WWTPs located in different regions and receiving different inflows of sewage.

The viral loads obtained are within the range of those of other studies where the presence of GB-124 phages was demonstrated in municipal wastewaters in Europe (Ebdon *et al.* 2012; Jofre *et al.* 2014; Purnell *et al.* 2015) and the USA (McMinn *et al.* 2014), suggesting that these microorganisms could also be potential indicators of human fecal pollution in water impacted with urban sewage in São Paulo city.

Although correlations among physicochemical parameters and concentrations of phages observed in different stages of the WWTPs should be interpreted with caution, results have suggested that the density of phages can be dependent on wastewater temperature.

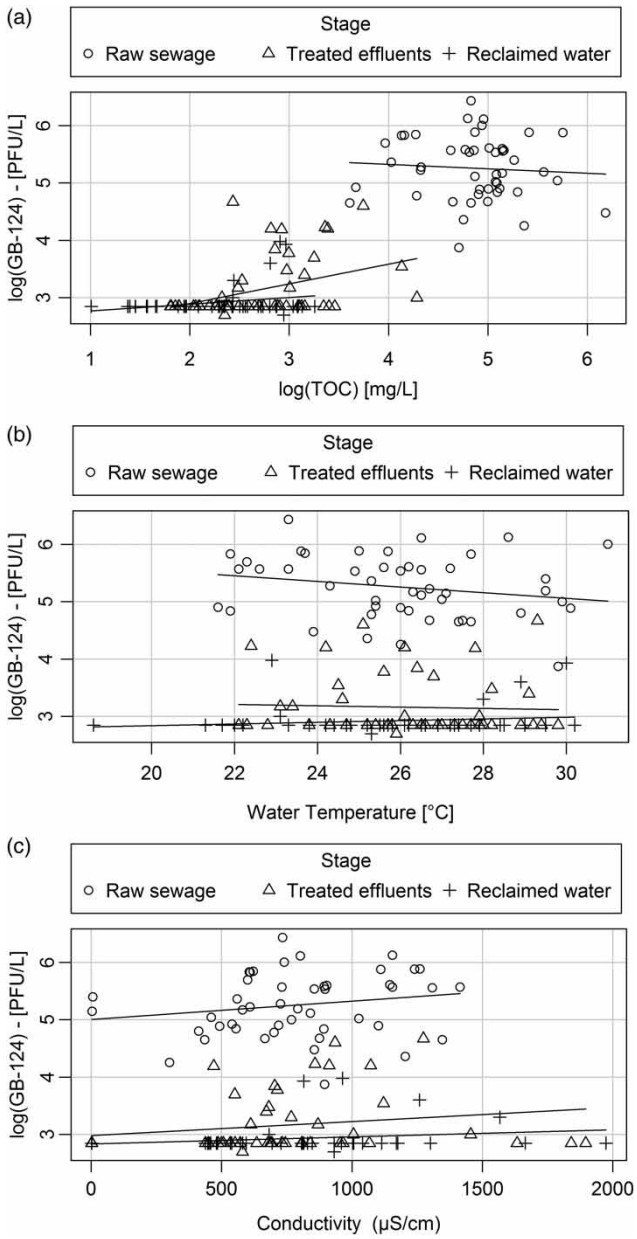

Figure 3 | Influence of physicochemical parameters in GB-124 phages occurrence in different treatment stages of WWTPs: TOC (a), water temperature (b), and conductivity (c).

The stability of GB-124 phages in sewage at different temperatures has been evaluated (McMinn *et al.* 2014). In general, phages persist longer at lower temperature (5 °C) than at higher temperature (i.e. 20 and 35 °C). After 21

days at 20 °C, no GB-124 phages could be detected by McMinn *et al.* (2014) in sewage samples, and inactivation was achieved after 7 days at 35 °C. Therefore, although GB-124 phages may be a potential viral marker to assessment of human fecal pollution of receiving waters, their applicability to evaluate the contamination of water downstream from a wastewater treatment plant in hot climate regions should be confirmed.

GB-124 phages were also observed after secondary treatment by activated sludge processes. Virus removal efficiency in activated sludge processes is recognized to achieve 0.7–2.9 log (Zhang *et al.* 2016). In this study, virus removal efficiencies ranged from 1.89 to 2.31 $\log_{10}$ and were similar to other studies that reported virus removal efficiencies achieved by activated sludge processes (Zhang & Farahbakhsh 2007; Carducci *et al.* 2008). Carducci *et al.* (2008) found an average reduction of 2.2 log of somatic coliphages in activated sludge processes, and Zhang & Farahbakhsh (2007) observed a range of 1.5–2.3 log removal for the same types of viruses.

Although phage densities still remain in effluents from activated sludge processes, the importance of this treatment in virus removal cannot be disregarded. However, the presence of cultivable viruses in these effluents suggests a potential risk of dissemination of other enteric viruses into the receiving waters.

A fraction of these effluents is further treated by tertiary treatments in WWTPs to generate reclaimed water that is used by City Hall for urban and industrial purposes, such as washing streets and monuments, cooling boilers, and other non-potable reuses.

Mixed-media (sand, gravel, anthracite, coal) filtration is commonly used as a tertiary treatment to enhance the reduction of pathogens. In this study, sand-anthracite filters followed by chlorination are used as tertiary treatment in three WWTPs. As few reclaimed water samples were positive for GB-124 phages, results were adjusted to a known distribution using a statistical model, providing a more realistic set of data. GB-124 phage removal achieved by tertiary treatments varied from 0.06 $\log_{10}$ in WWTP-3 up to 0.41 $\log_{10}$ for WWTP-1. These results are in accordance with other studies that reported typical removals from sand filtration to be 0- to 1-$\log_{10}$ for enteric viruses and ~0.14- to 2-$\log_{10}$ for coliphages (USEPA 2015).

Interestingly, in some samples from WWTP-2, reductions in GB-124 phages were not observed: the number present in effluent from tertiary treatment were greater than the number found in secondary treatment effluent. Ebdon *et al.* (2012) also observed that phage concentrations in treated effluent samples from WWTPs were not significantly different from concentrations detected in raw sewage samples, suggesting that the behaviour of these phages under different environmental conditions needs to be more understood.

In Brazil, chlorination is the most common disinfection process used and, in general, the cheaper technology. Higher resistance of viruses to disinfection by chlorination is recognised (Durán *et al.* 2003; Jofre *et al.* 2014), and the presence of interfering substances, such as organic matter and suspended solids in treated effluents can reduce the disinfection efficacy of chlorine (Carducci *et al.* 2008). Durán *et al.* (2003) demonstrated that bacteriophages infecting *Bacteroides fragilis* and selected somatic coliphages are more resistant to chlorination than bacterial indicators and other enteric viruses in sewage effluents.

The Environmental Company of the São Paulo State (CETESB), which is a regulatory agency, perform periodic monitoring (twice a year) in WWTPs using FIB (thermotolerant coliforms (TC) and *E. coli*) to evaluate reuse water quality produced by sanitation companies. Data on the presence of fecal indicators (i.e. TC and *E. coli*) performed in 2015 and 2016 demonstrated that these microorganisms were not detected in effluents from tertiary treatments of the respective WWTPs analysed in this study.

MBRs and reverse osmosis have been described as more effective technologies in virus removal compared to other tertiary treatments (Chaudhry *et al.* 2015; Pype *et al.* 2016). In MBRs, several factors could contribute in virus removal, including differences in morphology, attachments to biomass in the mixed liquor, inactivation by enzymes or predation, retention by the fouling cake layer and backwashing of membranes (Chaudhry *et al.* 2015; Purnell *et al.* 2015).

Zhang & Farahbakhsh (2007) found that MBR achieved 5.8-$\log_{10}$ removal of coliphages, and more than 4.0 $\log_{10}$ was achieved for adenoviruses and noroviruses removal in a study conducted by Chaudhry *et al.* (2015). In a full-scale MBR study, Purnell *et al.* (2015) reported a 5.3 $\log_{10}$ reduction in indigenous somatic coliphages and a 3.8 log removal for GB-124 phages.

Although there are few studies about virus removal by reverse osmosis treatment, this system can present high virus-removal efficiency, achieving more than 5.0 $\log_{10}$ for enteric viruses (Pype *et al.* 2016). However, operational problems or damage to the membranes could allow the passage of viruses, and therefore, continuous monitoring is recommended. In this study, no GB-124 phages were detected in reclaimed water treated by MBR coupled with reverse osmosis and disinfection by chlorine dioxide. Studies have demonstrated that chlorine dioxide concentrations of 0.1–1.0 mg/L are more effective in inactivating bacteria and viruses in water than chlorine (USEPA 2015).

Although a low frequency of GB-124 phage detection was observed in reclaimed water samples, the data obtained in this study indicate that these viruses could survive even after tertiary treatment by filtration and disinfection processes. In this way, although non-potable reuse is practiced, some applications such as street washing and irrigation of public gardens could pose some risk either by direct or indirect contact or by inhalation of aerosols containing considerable numbers of viable viral particles because a positive correlation between the detection rates of the studied phages and other pathogenic viruses in effluents from WWTPs have been observed (Ebdon *et al.* 2012).

It is also important to emphasise that the method used here to recover GB-124 phages uses 1 mL of samples to detect these viruses, which could be unsuitable to detect bacteriophages from reclaimed water samples or cleaner water (Harwood *et al.* 2013).

The absence of positivity of GB-124 phages in MBR effluents was already reported by other authors, although other enteric viruses and coliphages had been detected in the same samples (Purnell *et al.* 2016). The same authors also discuss that negative results for GB-124 phages in effluents from MBR systems could be associated with the detection limit of the used method, but different behaviour characteristics, such as attachment properties onto biomass in the mixed liquor, could also explain the absence of GB-124 phages in treated effluents (Purnell *et al.* 2015, 2016). Nevertheless, in the present study, the MBR system is coupled with reverse osmosis and final disinfection by chlorine dioxide, which could provide better virus removal efficiency. In this way, negative results obtained in effluents from the MBR/reverse osmosis system can be more associated with the performance of this system in virus removal than the limitations of the method used to detect phages. The same hypothesis would not be suitable for effluents from sand-anthracite filters, as the performance for virus removal is lower than MBR/reverse osmosis systems.

While the behaviour of GB-124 phages is not completely understood during the treatment stages in WWTPs (Ebdon *et al.* 2012; Purnell *et al.* 2016), it is more reasonable to consider that these bacteriophages could not be a good viral marker to assess human fecal pollution in these environmental matrices using the present methodology. Larger sample volumes or the use of methods to concentrate viruses could be applied to increase the probability of bacteriophage detection in these environmental matrices (Harwood *et al.* 2013). Further studies should be carried out using different methods to detect GB-124 phages and comparisons with other viral markers to evaluate their potential to tracking human fecal pollution in reclaimed or reuse water.

CONCLUSIONS

Although GB-124 phages were less abundant in reclaimed water samples, the test is a simple performance analysis, and then these bacteriophages could be considered complementary low-cost viral markers for tracking sources of human fecal pollution in municipal wastewaters and impacted receiving waters, as described previously (Nnane *et al.* 2011; Ebdon *et al.* 2012; McMinn *et al.* 2014). Therefore, the use of this marker should be encouraged, and other studies involving GB-124 phages detection or bacteriophages that infect other host strains of *Bacteroides* spp. in several environmental matrices, under different local conditions, could provide important information to policy makers for orientation or implementation of future water reuse and wastewater regulation programmes.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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