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Coliphages as indicators of primary wastewater treatment efficiency by constructed wetlands

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ABSTRACT

Constructed wetlands are an efficient and cost-effective system for the treatment of wastewater that can be reused for diverse purposes, including irrigation; however, few studies have determined the efficiency of microbial removal by constructed wetlands in tropical regions. Therefore, the present study aimed to determine the microbial quality of the influent and effluent of a constructed wetland in Puerto Rico, using traditional bacterial indicators (i.e., thermotolerant coliforms and enterococci), as well as somatic and male-specific (F+) coliphages. Results showed that over 99.9 and 97.7% of thermotolerant coliforms and enterococci were removed after treatment by constructed wetlands, respectively. Notably, approximately 84.0% of male-specific (F+) coliphages were removed, while somatic and total coliphages exhibited differing removal percentages at different steps during treatment by constructed wetlands. The potential risk of the presence of enteric viruses in treated wastewater by constructed wetlands may increase when considering traditional bacterial indicators exclusively. The present study may aid in the efforts to determine public health concerns associated with the exposure of bioaerosols resulting from wastewater treatment by constructed wetlands.

Key words: coliphages, constructed wetlands, enterococci, thermotolerant coliforms

HIGHLIGHTS

- Microbial removal efficiency by constructed wetlands in tropical regions remains largely unexplored.
- Coliphages were tested as indicators of microbial removal efficiency by a constructed wetland in Puerto Rico.
- Approximately 84.0% of male-specific (F+) coliphages were removed.
- The present study aids to determine the effectiveness of coliphages as indicators of wastewater treatment by constructed wetlands.

INTRODUCTION

Point- and non-point sources of fecal contamination contribute to the introduction of enteric pathogens into water sources used for recreation and consumption, affecting water quality and consequently, public health (Sabwa & Githeko 1985; Krentz *et al.* 2013). Given the extent of the diversity of enteric microorganisms that may be introduced into water sources used for recreation and consumption, microbial indicators are used to infer their presence. While thermotolerant coliforms and enterococci are common indicators of fecal contamination (Rai *et al.* 2012), total, male-specific (F+) and somatic coliphages have also been used to monitor the microbial quality of water sources (Meloni *et al.* 2003). Coliphages have extensively been utilized as proxies of enteric pathogenic viruses and have been proposed as indicators of specific sources of fecal contamination (Simkova & Cervenka 1981; Muniesa *et al.* 2012).

Several methods have been employed to reduce the microbial load in wastewater, including septic tanks, intermittent sand filters and constructed wetlands (Mbuligwe 2005; Healy *et al.* 2007; Rodgers *et al.* 2011; Nasr & Mikhaeil 2013; Kauppinen *et al.* 2014). Constructed wetlands, particularly, have been used as a natural and effective system for wastewater treatment where the need for mechanical equipment, electrical power or monitoring by qualified operators is minimal, representing an alternative for wastewater treatment, particularly in developing countries (Zhang *et al.* 2014). These systems are designed to mimic natural wetland systems, utilizing wetland plants, soil and associated microorganisms to treat wastewater

(Kivaisi 2001). To this date, wastewater treatment efficiency by constructed wetlands has not been extensively examined in tropical regions.

The effluent of constructed wetlands may be reused for various purposes, including irrigation, which ultimately may lead to the dispersal of bioaerosols, whose microbial quality would also need to be determined (Koottatep *et al.* 2006; Gross *et al.* 2014). The microbial quality of wastewater effluent by constructed wetlands has been mainly determined using traditional bacterial indicators (e.g., thermotolerant coliforms and enterococci); yet, few data exist on coliphages as indicators of wastewater treatment efficiency by constructed wetlands in tropical regions (Avelar *et al.* 2014). Therefore, the present study aimed to monitor the microbial quality of the influent and effluent of a constructed wetland in Puerto Rico, along with the produced bioaerosols, using indicator bacteria (thermotolerant coliforms and enterococci) and coliphages.

MATERIALS AND METHODS

Sample site description and collection

The constructed wetlands in the present study are localized in Humacao, Puerto Rico (18° 05′ 30″ N and 065° 50′ 51″ W). The system consists of three connected constructed wetlands, where the influent originates from domestic wastewater from an adjacent school. Influent and effluent samples were collected from two of the constructed wetlands and effluent samples were collected from a third constructed wetland. All the generated, used water goes through a grease trap located in the school's kitchen. Once the water reaches the first wetland, it percolates through gravel and sand. The water flow is horizontal and it reaches a cement box that contains a flexible pipeline that controls the water level. From there, water moves to the second wetland and percolates in a similar manner as in the first wetland. The water then reaches the third and final wetland, which is superficial. The first two constructed wetlands can receive a wastewater volume of up to 69.6 cm^3, while the third constructed wetland can receive a volume of up to 673 m^3; thus, the total volume of all three constructed wetlands is 742.6 m^3. The first two wetlands contain plants from the genus *Scirpus* and the third wetland contains plants from the genus *Typha*. Supplementary Material, Figure 1 shows a diagram with a description of the constructed wetland. Samples were collected from April 2004 to December 2006 ($n = 47$). Samples were transported in the dark at 4–7 °C and processed within 24 h. Samples were analyzed in three different groups, depending on the time of collection: school period (August–May) ($n = 32$), summer period (June–July) ($n = 15$) and combined periods ($n = 47$).

Microbial analyses

The concentration of thermotolerant coliforms and enterococci was determined as previously described with several modifications (Britton & Greeson 1987; USEPA 2009). Briefly, several sample volumes (1–10 μL) were filtered through 0.45 μm membranes. Membranes were then placed on top of mFC or mEnterococcus media (Difco) for the detection of thermotolerant coliform or enterococci. Plates were then incubated at 44.5 or 35 °C for 24 or 48 h, respectively. Further confirmation of thermotolerant coliforms was performed by randomly choosing and inoculating five colonies (representing approximately 10% of the total colonies on the plate) in lauryl tryptose broth (Difco) containing an inverted Durham tube. Colonies were then incubated at 35 °C for 24 h and gas-producing isolates were considered positive. Similarly, for further confirmation of enterococci, five colonies were randomly picked and inoculated in azide dextrose broth (Difco) and plates were then incubated at 35 °C for 24 h. Positive samples would show growth after 24 h. Positive and blank controls were included in all the confirmation tests. Positive controls included *Escherichia coli* ATCC 15597 and *Enterococcus faecalis* strain ATCC 19433.

Total, somatic and male-specific (F+) coliphages were detected as previously described (USEPA 2001). Briefly, *E. coli* strains C3000 (ATCC 15597), CN-13 (ATCC 700609) and Famp (ATCC 700891) were used for the detection of total, somatic and male-specific (F+) coliphages, respectively. Briefly, 100 μL of 18–24 h cultures of the *E. coli* strains described and 1 mL of the sample were added to top agar media (0.75% agar w/v) containing trypticase soy broth (TSB) (Difco). For the detection of somatic coliphages, nalidixic acid was added to the media (final concentration of 10 mL/L) (Sigma). Streptomycin and ampicillin (final concentration of 10 mL/L) (Sigma) were added to the media used for the detection of male-specific (F+) coliphages. The mixture was poured onto petri dishes containing TSB and agar (1.5% w/v) and incubated at 35 °C for 24 h. Viral plaques were enumerated at 24 h and reported as plaque forming units (PFU) ($\log_{10}$)/100 mL.

Physicochemical parameters

Physicochemical parameters, including pH, temperature and total dissolved solids (TDS) or suspended solids were measured *in situ* (Patil *et al.* 2012). Phosphorus concentration was measured in the laboratory using a colorimetric test. Briefly, 50 mL

of the sample was filtered using sterilized coffee filters to eliminate large solids. Portable equipment (Hach Co) was used to determine the concentration of ortho-phosphates (PO_4). Values were divided by 50 to determine the concentration per liter.

Bioaerosol samples

The wastewater effluent is irrigated onto adjacent grass once it has reached the third and last constructed wetland. Bioaerosol samples originating from the effluent of the last constructed wetland were collected from three sample points, namely Point 2 ($n = 15$), Point 4 ($n = 15$) and Point 5 ($n = 15$) using an AGI-30 liquid impinger (Supplementary Material, Figure 2). Samples were collected in 40 mL of a sterile saline solution (0.85% NaCl) and the monitoring time was 10 min for each sample site. Samples were transported to the laboratory in the dark at 4–7 °C and processed within 24 h. Ten-mL of the sample was filtered in duplicate and tested for the bacterial indicators, as described above. For the viral indicators, 2 mL of the sample was processed as described previously.

Statistical analyses

Linear regression analyses were performed to determine correlations between bacterial and viral indicators and physicochemical parameters. A one-way analysis of variance was performed to determine possible differences in the prevalence of the microbial indicators, stations and sample dates. All analyses were performed using Minitab (Osman 1997) (Minitab Release 12, Minitab Inc., 1997). The data were separated into class and summer periods to determine if season may influence the prevalence of the microbial indicators. Data were also combined and analyzed as described.

RESULTS

Physicochemical parameters and prevalence of microbial indicators

The physicochemical parameters measured in the present study are shown in Table 1. No significant differences in the physicochemical parameters were noted across the sampling sites and collection periods.

Thermotolerant coliforms and enterococci

The prevalence of thermotolerant coliforms and enterococci is shown in Figure 1. During the school period, thermotolerant coliform concentrations were 10^6 and 10^5 CFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^5 and 10^4 CFU ($\log_{10}$)/100 mL in Sites 2A and 2B, respectively; and 10^3 CFU ($\log_{10}$)/100 mL in Site 3 (Figure 1(a)). Enterococci concentrations were 10^5 and 10^4 CFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; and 10^3 CFU ($\log_{10}$)/100 mL in Sites 2A, 2B and 3. During the summer period, thermotolerant coliforms concentrations were 10^7 and 10^5 CFU ($\log_{10}$)/100 mL in

Table 1 | Physicochemical parameters considered in the present study

	Sample[a]	pH	Temp (°C)	TDS (ppt)	PO_4 (mg/L)
School period	1A	6.7 ± 0.2 (6.1–6.9)	27.3 ± 1.3 (25.0–30.0)	0.40 ± 0.10 (0.19–0.58)	0.27 ± 0.11 (0.02–0.42)
	1B	6.6 ± 0.3 (5.9–6.9)	27.2 ± 2.0 (23.0–31.5)	0.32 ± 0.08 (0.16–0.46)	0.30 ± 0.07 (0.14–0.42)
	2A	6.7 ± 0.3 (6.0–7.1)	27.4 ± 2.2 (23.0–32.0)	0.32 ± 0.08 (0.16–0.45)	0.33 ± 0.10 (0.10–0.50)
	2B	6.5 ± 0.2 (5.9–6.9)	26.7 ± 1.9 (23.0–29.5)	0.29 ± 0.08 (0.12–0.44)	0.30 ± 0.11 (0.04–0.48)
	3	7.2 ± 0.3 (6.7–8.3)	26.2 ± 2.2 (22.0–30.5)	0.24 ± 0.08 (0.09–0.41)	0.30 ± 0.11 (0.06–0.48)
Summer period	1A	6.6 ± 0.1 (6.4–6.8)	28.9 ± 0.5 (28.0–29.5)	0.34 ± 0.14 (0.21–0.63)	0.30 ± 0.12 (0.06–0.44)
	1B	6.6 ± 0.2 (6.3–6.8)	29.1 ± 1.0 (28.0–31.0)	0.28 ± 0.10 (0.17–0.49)	0.23 ± 0.10 (0.04–0.38)
	2A	6.8 ± 0.2 (6.4–7.4)	29.3 ± 1.0 (28.0–31.0)	0.28 ± 0.10 (0.17–0.50)	0.23 ± 0.14 (0.04–0.46)
	2B	6.4 ± 0.1 (6.3–6.6)	28.6 ± 1.1 (27.0–31.0)	0.23 ± 0.09 (0.14–0.43)	0.27 ± 0.11 (0.10–0.38)
	3	7.4 ± 0.5 (6.8–8.4)	29.4 ± 1.0 (27.5–31.0)	0.21 ± 0.10 (0.11–0.43)	0.22 ± 0.13 (0.06–0.42)
Combined periods	1A	6.7 ± 0.2 (6.1–6.9)	27.8 ± 1.4 (25.0–30.0)	0.38 ± 0.12 (0.19–0.63)	0.28 ± 0.11 (0.02–0.44)
	1B	6.6 ± 0.2 (5.9–6.9)	27.8 ± 2.0 (23.0–31.5)	0.31 ± 0.09 (0.16–0.49)	0.29 ± 0.09 (0.04–0.42)
	2A	6.8 ± 0.3 (6.0–7.4)	28.0 ± 2.1 (23.0–32.0)	0.31 ± 0.09 (0.16–0.50)	0.31 ± 0.11 (0.03–0.50)
	2B	6.5 ± 0.2 (5.9–6.9)	27.3 ± 1.9 (23.0–31.0)	0.28 ± 0.09 (0.12–0.44)	0.29 ± 0.11 (0.04–0.48)
	3	7.3 ± 0.4 (6.7–8.4)	27.2 ± 2.4 (22.0–31.0)	0.23 ± 0.09 (0.09–0.43)	0.28 ± 0.12 (0.06–0.48)

Average and standard deviations for pH, temperature (°C), total dissolved solids (TDS) (ppt) and PO_4 (mg/L) are shown. Summer period included $n = 15$ samples for which pH, temperature and TDS were measured and nine samples for which PO_4 was measured for all five sample types. Minimum and maximum values for each measurement are shown in parenthesis.

[a]1, 2 or 3, constructed wetland; A, influent; B, effluent.

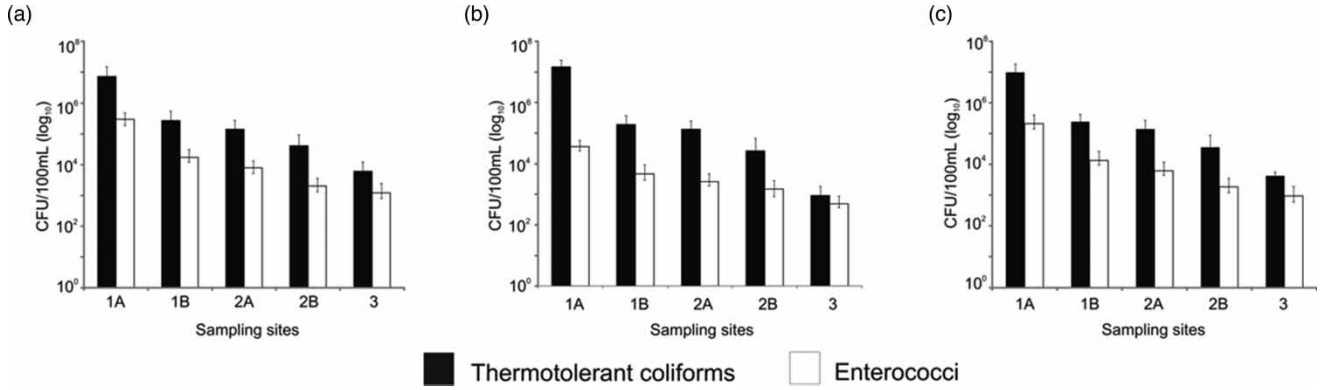

Figure 1 | Bacterial indicators in constructed wetland. Arithmetic means for thermotolerant coliforms and enterococci are represented by black and white bars, respectively. Figure shows indicator bacteria concentrations (CFU ($\log_{10}$)/100 mL) during the school (a), summer (b) and combined (c) periods. Standard deviations are shown with error bars.

Sites 1A and 1B, respectively; 10^5 and 10^4 CFU ($\log_{10}$)/100 mL in Sites 2A and 2B, respectively; and 10^2 CFU ($\log_{10}$)/100 mL in Site 3. Enterococci concentrations were 10^4 and 10^3 CFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^3 CFU ($\log_{10}$)/100 mL in Sites 2A and 2B; and 10^2 CFU ($\log_{10}$)/100 mL in Site 3 (Figure 1(b)). For the combined periods, thermotolerant coliform concentrations were 10^6 and 10^5 CFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^5 and 10^4 CFU ($\log_{10}$)/100 mL in Sites 2A and 2B, respectively; and 10^3 CFU ($\log_{10}$)/100 mL in Site 3. Finally, enterococci concentrations were 10^5 and 10^4 CFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^3 CFU ($\log_{10}$)/100 mL in Sites 2A and 2B; and 10^2 CFU ($\log_{10}$)/100 mL in Site 3 (Figure 1(c)). Arithmetic mean values and standard deviations used to generate Figure 1 can be found in Supplementary Material, File 1.

Somatic, male-specific (F+) and total coliphages

The prevalence of coliphages is shown in Figure 2. During the school period, somatic coliphage concentrations were 10^4 and 10^3 PFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^4 and 10^3 PFU ($\log_{10}$)/100 mL in Sites 2A and 2B, respectively; and 10^3 PFU ($\log_{10}$)/100 mL in Site 3. Male-specific (F+) coliphages concentrations were 10^4 and 10^3 PFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^3 and 10^2 PFU ($\log_{10}$)/100 mL in Sites 2A and 2B, respectively; and 10^2 PFU ($\log_{10}$)/100 mL in Site 3. Total coliphage concentrations were 10^4 and 10^3 PFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^3 and 10^2 PFU ($\log_{10}$)/100 mL in Sites 2A and 2B, respectively; and 10^2 PFU ($\log_{10}$)/100 mL in Site 3 (Figure 2(a)). During the

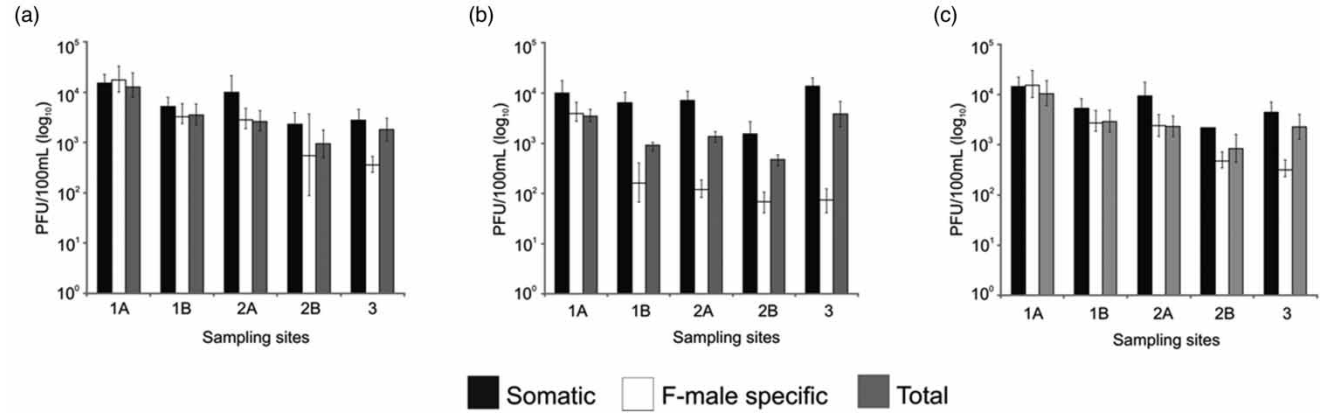

Figure 2 | Coliphages in constructed wetland: (a) shows coliphages concentrations during the school period; (b) shows coliphages concentrations during summer period; (c) shows coliphages concentrations when both class and summer periods are combined. Data show arithmetic means and are represented as PFU ($\log_{10}$)/100 mL. Somatic, male-specific (F+) and total coliphages are represented by black, white and gray bars, respectively. Standard deviations are shown with the error bars.

summer period, somatic coliphages concentrations were 10^3 PFU ($\log_{10}$)/100 mL in Sites 1A, 1B, 2A and 2B; and 10^4 PFU ($\log_{10}$)/100 mL in Site 3. Male-specific (F+) coliphage concentrations were 10^3 and 10^2 PFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^2 and 10^1 PFU ($\log_{10}$)/100 mL in Sites 2A and 2B, respectively; and 10^1 PFU ($\log_{10}$)/100 mL in Site 3. Total coliphage concentrations were 10^3 and 10^2 PFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^2 and 10^1 PFU ($\log_{10}$)/100 mL in Sites 2A and 2B, respectively; and 10^3 PFU ($\log_{10}$)/100 mL in Site 3 (Figure 2(b)). When combining both periods, somatic coliphage concentrations were 10^4 and 10^3 PFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; and 10^3 PFU ($\log_{10}$)/100 mL in Sites 2A, 2B and 3. Male-specific (F+) coliphage concentrations were 10^4 and 10^3 PFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^3 and 10^2 PFU ($\log_{10}$)/100 mL in Sites 2A and 2B, respectively; and 10^2 PFU ($\log_{10}$)/100 mL in Site 3. Total coliphage concentrations were 10^4 and 10^3 PFU ($\log_{10}$)/100 mL in Sites 1A and 1B, respectively; 10^3 and 10^2 PFU ($\log_{10}$)/100 mL in Sites 2A and 2B; and 10^3 PFU ($\log_{10}$)/100 mL in Site 3 (Figure 2(c)). Arithmetic mean values and standard deviations used to generate Figure 2 can be found in Supplementary Material, File 2.

For the school period, significant correlations were noted between thermotolerant coliforms and pH ($p < 0.001$) and TDS ($p < 0.001$). Enterococci were correlated with pH ($p = 0.004$), temperature ($p = 0.008$) and TDS ($p < 0.001$). There were also significant correlations between total ($p = 0.040$) and somatic coliphages with TDS ($p < 0.001$) and male-specific (F+) coliphages with temperature ($p = 0.038$). No other correlations were noted. For the summer period, there were significant correlations between coliforms and pH ($p = 0.001$) and TDS ($p < 0.001$). Enterococci concentrations were also significantly correlated with pH ($p = 0.001$) and TDS ($p < 0.001$). Significant correlations were found between total coliphages and TDS ($p = 0.020$) and somatic coliphages with temperature ($p = 0.044$). No other correlations were noted. For the combined periods, there was a significant correlation between thermotolerant coliforms and enterococci with pH ($p < 0.001$) and TDS ($p < 0.001$). Similarly, there was a significant correlation between total ($p = 0.005$), somatic ($p = 0.001$) and male-specific (F+) coliphages ($p = 0.035$) with TDS. No other correlations were noted.

Removal percentages

Thermotolerant coliforms, enterococci and coliphages removal percentages (± standard deviation) for school, summer and combined periods are shown in Table 2. PO_4 removal percentages (± standard deviation) for all periods are shown as well.

Bioaerosol microbial analyses

A total of 15 samples per sampling point were obtained for the bioaerosol arm of the study. CFU ($\log_{10}$)/100 mL values (average ± standard deviation) for thermotolerant coliforms and enterococci are shown in Figure 3(a). Thermotolerant coliforms were not detected in the first sampling point, while they exhibited a decrease of $10^{0.2}$ in the second sampling point and were detected at the third sampling point in concentrations of approximately $10^{2.5}$. Enterococci, on the other hand, were detected in all sampled points in concentrations ranging from $10^{0.5}$ to $10^{3.5}$, depending on the sampling point. Coliphages were

Table 2 | Removal percentages of bacterial and viral indicators in this study during school, summer and combined periods

					Coliphages		
	Site[a]	Enterococci	Thermotolerant coliforms	Somatic	Male-specific (F+)	Total	PO_4
School period	1A–1B	94.85 ± 6.85	90.27 ± 12.70	36.65 ± 74.42	28.05 ± 164.73	44.85 ± 57.00	−108.79 ± 369.24
	2A–2B	−97.83 ± 558.68	−120.51 ± 575.39	47.73 ± 63.78	28.40 ± 132.99	1.40 ± 718.82	−5.50 ± 35.34
	1A–2B	98.76 ± 3.44	96.43 ± 12.40	80.10 ± 26.15	42.10 ± 164.07	51.30 ± 176.94	−102.20 ± 366.14
	1A-3	99.83 ± 0.45	98.02 ± 3.32	−64.77 ± 614.75	83.75 ± 30.49	−14.00 ± 403.69	−72.13 ± 268.87
Summer period	1A–1B	93.75 ± 11.67	79.73 ± 23.76	−48.04 ± 260.55	84.16 ± 20.47	45.98 ± 55.09	15.98 ± 46.66
	2A–2B	74.61 ± 36.67	11.54 ± 166.58	21.15 ± 69.36	43.59 ± 51.22	57.33 ± 27.15	−144.47 ± 308.27
	1A–2B	98.38 ± 6.23	94.67 ± 6.47	9.37 ± 162.07	66.54 ± 46.75	67.70 ± 42.75	−26.50 ± 133.48
	1A-3	99.94 ± 0.13	96.49 ± 4.57	−506.08 ± 692.20	64.29 ± 47.38	−69.21 ± 259.24	15.41 ± 55.13
Combined periods	1A–1B	93.62 ± 11.67	85.02 ± 19.80	24.06 ± 121.22	35.37 ± 154.43	45.34 ± 55.24	−79.24 ± 326.40
	2A–2B	−18.91 ± 426.60	−51.27 ± 437.61	43.47 ± 63.95	30.30 ± 125.03	14.30 ± 221.94	−30.03 ± 160.21
	1A–2B	98.75 ± 4.29	95.83 ± 10.17	65.58 ± 68.60	48.18 ± 153.41	55.09 ± 155.97	−84.27 ± 326.14
	1A-3	99.89 ± 0.34	97.65 ± 3.70	−132.79 ± 622.01	84.01 ± 29.31	−26.74 ± 372.92	−51.40 ± 238.30

Sample sites included constructed Wetlands 1, 2 and 3. For constructed Wetlands 1 and 2, influent and effluent samples were collected, while only effluent samples were collected for constructed Wetland 3. Microbial indicators included thermotolerant coliforms, enterococci, total, somatic and male-specific (F+) coliphages. The removal percentage of PO_4 is shown as well. Results show the average and standard deviations.

[a]1, 2 and 3, constructed wetland; A, influent, B, effluent.

detected in all the sampled points in concentrations ranging from approximately $10^{0.5}$ to $10^{2.5}$, depending on the coliphage type (Figure 3(b)). Values used to generate Figure 3 can be found in Supplementary Material, File 3.

DISCUSSION

Similarities between periods considered

Thermotolerant coliforms, enterococci, somatic, male-specific (F+) and total coliphages were evaluated as indicators of wastewater treatment efficiency by a constructed wetland in Puerto Rico. While the efficiency of wastewater treatment by constructed wetlands has revealed varying results in previous studies, they still could represent one of the most cost-efficient systems for wastewater treatment (Hill & Sobsey 2001). The potential of wetland technology is increasing, especially in developing countries. Many developing countries are in subtropical and tropical climates, similar to that in Puerto Rico, which may promote higher microbial and biological activity and possibly, a more efficient wastewater treatment by constructed wetlands (Kivaisi 2001).

The constructed wetland in the present study had a processing capacity of 14.6 m^3/per day and a retention capacity of 52 days, indicating that it operated in a relatively efficient manner. While most of the physicochemical parameters did not fluctuate, there was a reduction in TDS throughout the system and an unclear pattern in terms of PO_4 that merits further investigation. While this study focused on specific parameters, future and similar studies can include other parameters such as Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). Correlations between the indicators tested and several of the physicochemical parameters considered may not be surprising; however, these correlations open the opportunity to understand how the system operates in real-time by measuring the parameters online. TDS measurements could represent an alternative to rapidly (and perhaps online and real-time) determine the efficiency of the system in locations where it could be difficult to process samples using traditional and alternative microbial and viral indicators.

The reduction of approximately 2–4 logs for enterococci and thermotolerant coliforms, respectively (representing 96.5 to >99.0%, depending on the period and sampling site), is comparable with the removal efficiency of bacterial indicators by constructed wetlands in other countries (Verhoevena & Meulemanb 1999; Thurston *et al.* 2001; Vymazal 2007). However, in the present study, CFU (log_{10})/100 mL in some of the samples did not follow US Environmental Protection Agency (USEPA) guidelines (USEPA 2000). This suggests that an additional step, such as chlorination, might be necessary to ensure the removal of the bacterial indicators according to USEPA guidelines.

There were no clear removal patterns for somatic coliphages during any of the periods considered. Male-specific (F+) coliphages exhibited a removal of 84.0%, which is below that previously reported (Thurston *et al.* 2001; Hench *et al.* 2003). However, it has been demonstrated that the removal percentages of coliphages tend to decrease from 1 year to another. Notably, the constructed wetland in the present study was approximately 12 years-old by the time samples were collected. Interestingly, total coliphages exhibited a reduction of 0.5 logs from Points 1A to 1B and from Points 2A to 2B, but exhibited an increase of almost 1 log at Point 3 during the summer period. While no further testing was performed, it could be speculated that fecal pellets originating from animals could be a possible source of coliphages in the third swamp, which is

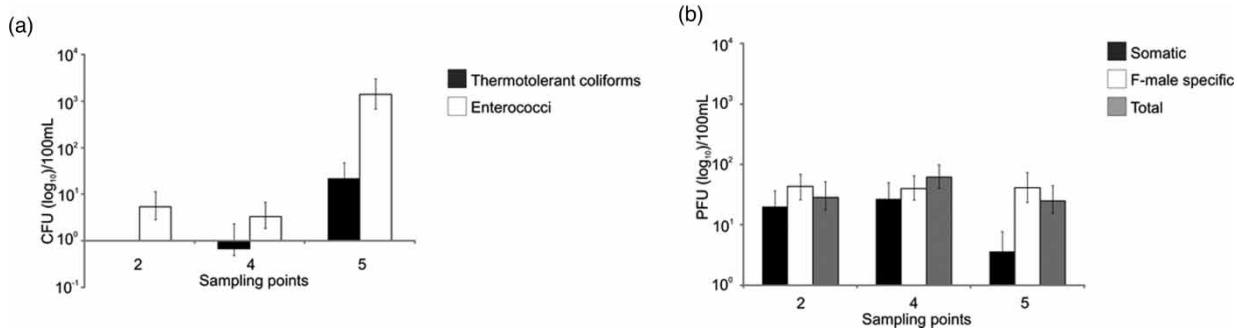

Figure 3 | Bacterial and viral indicators arithmetic means in the bioaerosols samples: (a) shows the concentration of thermotolerant coliforms (black bars) and enterococci (white bars) in bioaerosols (CFU (log_{10})/100 mL); (b) shows somatic (black bars), male-specific (F+) (white bars) and total coliphages (gray bars). Thermotolerant coliforms concentration a sampling point 2 was 0 prior log_{10} normalization. Standard deviations are shown with error bars.

characterized by surface flow and easy access to animals and/or lysogenic coliphages could be entering the lytic phase due to environmental factors that could promote induction (Hernandez-Delgado & Toranzos 1995).

In terms of PO_4, no reduction throughout the constructed wetland was noted. Previous studies of PO_4 removal in sub-superficial constructed wetlands have reported similar results. Of the studies reported by the USEPA, only one study reported a 95% PO_4 removal. This could be due to the use of fine river gravel, which contained iron and aluminum oxides that mediated the removal of PO_4. In Europe, sand is used instead of gravel, which helps in PO_4 removal, but requires a greater treatment surface due to the reduced hydraulic capacity. It should be noted that PO_4 represents 5–20% of total phosphates and its removal will be due to the absorption and precipitation reactions with calcium. The type of gravel utilized in the constructed wetland in our study does not contain iron or aluminum oxides, neither any other component necessary for the removal of PO_4. Notably, there were very similar trends in terms of removal percentages and correlations of the microbial indicators with the physicochemical parameters during the school, summer or combined periods. A possible explanation could be that weather conditions in Puerto Rico, remain relatively constant throughout the year.

Given that the treated wastewater in the present study is used for irrigation purposes, the prevalence of thermotolerant coliforms, enterococci and coliphages in the bioaerosols was investigated. Notably, the distance between one of the bioaerosol sampling sites and the playground area is approximately 65.5 m. Previous studies have suggested that bioaerosols may not represent a threat to public health if they are set at a distance of >100 m (Darvodelsky & Fien 2005). The same study suggested that microorganisms in bioaerosols are rapidly inactivated once they reach the surface due to radiation, desiccation and high temperatures (Darvodelsky & Fien 2005). It is feasible that the detection of bacterial and viral indicators in the present study is due to the relatively shorter set distance (i.e., 65.5 m).

Interestingly, CFU (log_{10}) and PFU (log_{10}) per 100 mL were low in some of the samples (<1.0 log) and in some cases, none of the microbial indicators were detected. Several reasons could explain these low numbers. For instance, some microbes remain attached to particulate matter; thus, the number of these microorganisms could be underestimated (Peccia 2007). In addition, it remains feasible that the microbial indicators may be in the viable but non-cultivable state. If this is the case, future studies would need to determine the prevalence of bacterial and viral indicators in bioaerosols using both culture- and molecular-based techniques. While the number of bacterial indicators in the present study may not represent a health concern, this may not be the case for enteric viruses. One to 10 viral particles may cause infection in the host and the low coliphage numbers (being used as indicators of enteric viruses) may also indicate the possible presence of enteric viruses. Coliphages, particularly male-specific (F+), may serve as surrogates of enteric pathogenic viruses, and may represent one of the most reliable indicators of wastewater treatment efficiency by constructed wetlands (Hernandez-Delgado & Toranzos 1995). While more studies are still needed, the data in the present study aid to better understand the public health concerns associated with the exposure to bioaerosols resulting from wastewater treatment when using constructed wetlands.

CONCLUSIONS

The microbial quality of treated wastewater by constructed wetlands in subtropical and tropical regions may not be influenced by the time of year samples are collected. This is mostly due to the temperature not drastically changing; thus, results are expected to be relatively similar throughout the year. Measurement of several physicochemical parameters online may provide an insight of how the system operates in real-time. This represents an advantage given that many biological measurements and parameters may usually take 24 h for completion. Coliphages, particularly male-specific (F+), may be the most appropriate indicator of wastewater treatment by constructed wetlands in regions with similar climates as Puerto Rico.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

Tasha M. Santiago-Rodriguez is a current employee of Diversigen, a microbiome services company.

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