# Quantification of human norovirus GII, human adenovirus, and fecal indicator organisms in wastewater used for irrigation in Accra, Ghana

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## ABSTRACT

Quantitative microbial risk assessment (QMRA) is frequently used to estimate health risks associated with wastewater irrigation and requires pathogen concentration estimates as inputs. However, human pathogens, such as viruses, are rarely quantified in water samples, and simple relationships between fecal indicator bacteria and pathogen concentrations are used instead. To provide data that can be used to refine QMRA models of wastewater-fed agriculture in Accra, stream, drain, and waste stabilization pond waters used for irrigation were sampled and analyzed for concentrations of fecal indicator microorganisms (human-specific *Bacteroidales*, *Escherichia coli*, enterococci, thermotolerant coliform, and somatic and F+ coliphages) and two human viruses (adenovirus and norovirus genogroup II). *E. coli* concentrations in all samples exceeded limits suggested by the World Health Organization, and human-specific *Bacteroidales* was found in all but one sample, suggesting human fecal contamination. Human viruses were detected in 16 out of 20 samples, were quantified in 12, and contained 2–3 orders of magnitude more norovirus than predicted by norovirus to *E. coli* concentration ratios assumed in recent publications employing indicator-based QMRA. As wastewater irrigation can be beneficial for farmers and municipalities, these results should not discourage water reuse in agriculture, but provide motivation and targets for wastewater treatment before use on farms.

**Key words** | enteric viruses, human-specific *Bacteroidales*, QMRA, wastewater irrigation, WHO Guidelines

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## INTRODUCTION

The use of untreated wastewater in urban and peri-urban irrigated agriculture is prevalent around the world. Increasing urban populations use more freshwater and produce a greater volume of wastewater that is, in many cases, discharged to the environment without treatment. Scarcity of (unpolluted) freshwater resources and year-round access to wastewater create conditions that encourage wastewater irrigation (Scott *et al.* 2004). Ghana's capital city, Accra, provides an example of wastewater irrigation practiced on a wide scale. Accra has an estimated 100 ha of land under irrigated vegetable production (Obuobie *et al.* 2006). Due to limited functional wastewater collection and treatment infrastructure (Murray & Drechsel 2011), as well as leaky septic systems and open defecation, untreated greywater and sewage (collectively 'wastewater') flow directly and indirectly into open drains and streams, which are the primary sources of water used by farmers in Accra to irrigate vegetables. The terms 'wastewater irrigation' and 'water reuse', therefore, encompass the use of water along the whole trajectory from raw wastewater to contaminated surface water. Alternative water sources are rare, making wastewater irrigation necessary for farmers who want to reap the benefits of urban or peri-urban agriculture. Year-round farming in proximity to Accra's markets offers a significant livelihood opportunity

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for small-scale farmers while supplying the city with most of its perishable vegetables (Raschid-Sally & Jayakody 2008). Additionally, municipalities and landowners benefit when farmers act as informal caretakers: farmers protect land from solid waste dumping and illegal land development, and farmed land can contribute to stormwater management (Lydecker & Drechsel 2010), an issue particularly important for Accra, which experiences annual flooding.

Despite the benefits, there are health risks associated with wastewater irrigation due to the potential presence of human pathogens. The connection between illness and agricultural use of human and animal waste has been demonstrated; epidemiological studies have found that the use of inadequately treated waste in agriculture can lead to an increased risk of viral, protozoan, intestinal helminth, and bacterial infection for farmers, their families, and consumers (Blumenthal & Peasey 2002; Ensink *et al*. 2006; Jiménez 2006). In an effort to reduce the health risks associated with water reuse, regulatory and health agencies, such as the United States Environmental Protection Agency and the World Health Organization (WHO), have developed water quality guidelines for the use of wastewater for agricultural purposes (Blumenthal *et al*. 2000; US EPA 2004; WHO 2006). The 2006 WHO Guidelines for the safe use of wastewater, excreta, and greywater in agriculture (hereafter referred to as the 'WHO Guidelines') stresses the development of location-specific, health-based water quality targets, and the use of water quality assessment and quantitative microbial risk assessment (QMRA) to determine health risks due to pathogens in wastewater. After estimating health risks, regulators can determine water treatment objectives to meet health-based targets.

QMRA models rely on estimates of pathogen exposure (e.g., estimates of pathogen concentrations in water and rates of ingestion) and appropriate dose–response models. Due to the complexity and expense of pathogen analyses, actual pathogen concentrations are often not measured. Instead, water quality is assessed using indicator organisms – typically fecal indicator bacteria (FIB) such as *Escherichia coli*, enterococci, or thermotolerant coliforms – and simple relationships between FIB and pathogens are used to predict the concentration of the target pathogen. However, relationships between indicator organism and pathogen concentrations are complex. For example, different organisms and classes of organisms (e.g., viruses, bacteria, protozoa, and helminths) are excreted in different ratios by varying proportions of the population depending on their health status, and have different fate and transport processes in the environment.

While bacterial pathogens can cause disease associated with wastewater irrigation, QMRA modeling has found viral pathogens, such as rotavirus and norovirus, to present greater health risks in wastewater irrigation than *Campylobacter*, which was used as an 'index' bacterial pathogen (WHO 2006). Viruses tend to be more persistent than bacteria in the environment and have been named some of the most important causes of food- and waterborne disease. Norovirus, for example, has been found to be *the* leading cause of foodborne illness in the USA (Widdowson *et al*. 2005), and a study conducted in the city of Tamale, in Northern Ghana, found rota-, adeno-, noro-, or astroviruses to be present in the stool of 73% of children aged 0–12 years suffering from diarrhea (Reither *et al*. 2007).

Previous researchers investigating irrigation water quality in Accra have quantified coliforms (Mensah *et al*. 2001; Keraita *et al*. 2003; Amoah *et al*. 2005), but not human viruses. To provide data that can be used to refine QMRA model inputs, irrigation waters from major farm sites around Accra were sampled and analyzed for concentrations of two types of human viral pathogen (human adenoviruses and norovirus genogroup II) and six fecal indicator organisms (human-specific *Bacteroidales*, *E. coli*, enterococci, thermotolerant coliform, and somatic and F+ coliphages). Norovirus and adenovirus are important waterborne viruses. Norovirus genogroup II was chosen over genogroup I given that genogroup II is a more prevalent etiology of disease in both Ghana (Armah *et al*. 2006; Silva *et al*. 2008) and around the world (Siebenga *et al*. 2009). Measured concentrations were used to determine relationships among fecal indicator microorganisms, and compute ratios between *E. coli* and human virus concentrations.

## METHODS

### Site selection and sample collection

Sampling sites were farming areas located within the Accra Metropolitan Assembly (Table 1). Water samples were

**Table 1** | Farm sites and sample locations

| Site | Water samples | Irrigation water used |
|---|---|---|
| Dzorwulu/Roman Ridge | Dzorwulu Drain, Roman Ridge Drain, Onyansa Stream | Piped water, open drains, and the Onyansa stream, which is contaminated by effluent from the non-functioning Roman Ridge wastewater treatment plant |
| La Fulani | La Drain, La WSP Influent, La WSP Effluent | Irrigation water sources include a drain containing sewage from the military base and WSP water |
| Marine Drive | Marine Drive Stream | Stream water |
| Korle Bu | Korle Bu Drain | Drain water, potentially from adjacent housing complexes |
| Nima Creek | Nima Creek | Nima Creek, which receives wastewater flows from houses along its course |
| Ghana Broadcasting Corporation (GBC) | GBC Drain | Main water source is an unlined drain that runs through the center of the site |
| Odaw River | Odaw River | The Odaw is the main river running through Accra and serves to drain an area of approximately 250 $km^2$, including the most urbanized areas of the city. While not a farm site per se, farmers located on the Odaw's banks use its water for irrigation |

collected in July 2010, from locations where farmers collect irrigation water and at a time when farmers were using the water (between 8:30 and 11:30 am). Each site was sampled on 2 separate days (except for La Drain and Odaw River which were each sampled once) for a total of 20 samples at 11 locations. Samples were collected using a bucket sterilized with 70% ethanol, wiped dry and rinsed with sample water before use. Water was poured from buckets into sterile, opaque cubitainers and transported to the laboratory on ice. All samples were analyzed within 6 h of collection.

## Culture-based microbiological analyses

### Fecal indicator bacteria

Water samples were analyzed for *E. coli* and enterococci concentrations by membrane filtration using 47 mm diameter, 0.45 μm pore size, mixed cellulose ester HA filters (Millipore). *E. coli* were quantified using both mI (BD) and chromocult agars (Merck KGaA), with incubation at 37 °C for 24 h. Enterococci concentrations were quantified using mEI agar (BD), with incubation at 41 °C for 24 h. *E. coli* and enterococci were enumerated as colony forming units (CFU).

Thermotolerant coliform, also referred to as fecal coliform, were quantified by the multiple tube fermentation, most probable number (MPN) method with 1 mL sample inoculums, 5 mL sterile MacConkey broth (Merck KGaA), and three replicates of each dilution; tubes were incubated at 41 °C for 48 h. Replicates were marked as positive for thermotolerant coliform if color change or gas production was detected.

### Coliphage

Water samples were concentrated for coliphage enumeration by membrane filtration using HA filters. $MgCl_2$ (0.05 M final concentration) was added to water samples to facilitate virus adsorption to filters (Lukasik *et al.* 2000); samples were mixed and allowed to sit for 5 min before filtration. Between 15 and 150 mL of sample was subsequently filtered (at a rate approximately 100 mL/3 min), depending on water turbidity and filter clogging. Filters were preserved until elution by freezing at −20 °C on 300 μL of 50% glycerol (1:1 vol/vol with phosphate-buffered saline (PBS)).

Coliphage were eluted from filters by adding 3% beef extract (pH 9; 30 g/L beef extract, 30 mL/L Tween 80, 0.3 M NaCl) and swirling for 10 min. Filter eluent was assayed for coliphage using the double agar layer (DAL) method with 100 μL and 1 mL sample inoculums, modified tryptic soy agar (TSA) top and bottom agars, and appropriate hosts and antibiotics. F+ coliphage were assayed using *E. coli* $F_{amp}$ host bacteria with 1× ampicillin and streptomycin antibiotics (0.015 g/L of each); somatic coliphage were assayed using *E. coli* CN13 host with nalidixic acid (0.01 g/L). Modified

TSA consists of: BactoAgar (0.75% (top agar) or 1.5% (bottom agar) wt/vol; BD), 30 g/L tryptic soy broth (BD), and 0.002 M $CaCl_2$. Eluted filters were plated, face down, on top agar augmented with 0.3% Tween 80. Plates were incubated at 37 °C for 18–24 h and enumerated as plaque forming units (PFUs). Total phage concentrations were calculated by adding counts from the DAL and filter plates.

### Correlation between organisms

Correlation coefficients ($R^2$) between pairs of FIB and coliphage were determined using log-transformed concentrations.

## (RT-)qPCR analyses

Human-specific *Bacteroidales*, human adenovirus (HAdV; all 51 types), and norovirus genotype II (NV-GII) were concentrated, extracted, and then quantified by quantitative polymerase chain reaction (qPCR) or reverse transcription qPCR (RT-qPCR).

### Sample concentration

Bacteria and viruses were concentrated from water samples in the same manner as described above for coliphage, but with a $MgCl_2$ concentration of 0.025 M. For DNA/RNA preservation, filters were covered with 500 μL RNAlater® (Qiagen), allowed to sit for 1 min, and then excess liquid was removed by vacuum. Filters were aseptically rolled and placed in 5 mL DNA/RNA-free tubes, stored at 4 °C overnight to allow RNAlater® to further soak into the filter, then frozen at −20 °C until transported to University of California, Berkeley for analysis; once there, filters were stored at −80 °C until extracted.

### Preparation of recovery controls

MS2 (ssRNA bacteriophage) and *Pseudomonas syringae* pv. *phaseolicola* (pph6; Gram-negative bacteria) were used as control spikes to evaluate the combined efficiency of nucleic acid extraction and amplification for RNA and DNA, respectively.

MS2 was propagated by broth enrichment with *E. coli* $F_{amp}$ host and 1× ampicillin and streptomycin, then purified through chloroform extraction and polyethylene glycol precipitation, resuspended in PBS and frozen at −80 °C in single-use aliquots. The concentration of culturable MS2 in the control spike was determined by DAL with *E. coli* $F_{amp}$ host and 1× ampicillin and streptomycin.

Pph6 was cultured in modified King's B media (10 g/L tryptone, 10 g/L peptone, 10 mL/L glycerol, 8.3 mM $MgSO_4$, 5.7 mM $K_2HPO_4$) and incubated at 28 °C for 48 h. Freshly cultured cells were centrifuged for 10 min at 5,400 × *g* at 4 °C, the supernatant discarded, and then rinsed three times by resuspending with PBS, centrifuging and discarding the supernatant. After the final wash, the cells were resuspended in 15% glycerol–0.01 M $MgSO_4$. The cell suspension was frozen at −80 °C in single-use aliquots.

Fifty microliters of the $10^{-6}$ dilution of the MS2 spike (approximately 11 MS2 PFU) or the $10^{-1}$ dilution of the pph6 spike (approximately $1.5 \times 10^5$ gene copies) was spiked into each RNA or DNA sample tube, respectively, prior to nucleic acid extraction, but after sample concentration and tube transport. Identical control spikes were also added to extraction blanks, which did not contain filters. Extraction/amplification efficiency of each sample was calculated by comparing MS2 or pph6 concentration in that sample with the concentration in the extraction blank, and classified as poor (−; <25%), acceptable (+; 25–50%), or good (++ ; >50%); sample concentrations were not adjusted in relation to recovery efficiency. MS2 and pph6 recoveries do not include information on efficiency of the concentration step or potential loss during sample transport. Additionally, given variability in virus composition and structure, as well as interactions with sample matrices, MS2 may not behave exactly like the human viruses in extraction and amplification steps. Likewise, pph6 may not be a perfect proxy for extraction and amplification of human-specific *Bacteroidales* and HAdV in the irrigation water samples.

### Nucleic acid extraction

DNA and RNA were extracted from separate filters. As mentioned above, extraction/amplification control spikes (MS2 and pph6) were added to samples prior to extraction. DNA and RNA were extracted using the PowerWater DNA and RNA isolation kits (MoBio, Carlsbad, CA), respectively, following manufacturer protocols and one

modification: final DNA/RNA elution buffer was preheated to 95 °C before use to improve recovery (Viau *et al.* 2011). Extracted DNA and RNA were aliquoted in single-use aliquots and frozen at −80 °C.

### (RT-)qPCR assays

Previously published primers, hydrolysis probes, and assays were used for (RT-)qPCR analyses (Table 2); probes were 5′ labeled with a 6′FAM reporter dye and 3′ labeled with a Black Hole 1 quencher. For qPCR assays (human-specific *Bacteroidales* (BacHum target), HAdV, and pph6), reactions included 1× TaqMan Fast Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems), the appropriate concentrations of primers and probe, 6 μL template, and nuclease-free water to obtain a final 20 μL volume. For (RT-)qPCR analyses (NV-GII and MS2), reactions included 1× TaqMan One-Step RT-PCR Master Mix with 1× RT Enzyme Mix (Applied Biosystems), the appropriate concentrations of primers and probe, 6 μL template, and nuclease-free water to obtain a final 20 μL volume. A 1:10 dilution of extracted DNA/RNA was used as template for all samples. Preliminary experiments found that 1:10 dilution of extracted DNA/RNA from pond water and primary sewage effluent reduced inhibition; further dilution did not provide an additional anti-inhibitory effect (data not shown). Reactions were run on an Applied Biosystems StepOnePlus Real-Time PCR system. Thermocycling conditions varied for each target based on previously published methods (Table 2). All (RT-)qPCR plates included a standard curve and a no-template control consisting of nuclease-free water.

### Generation of standards and calibration curves

Linearized plasmid standards were used for quantification of BacHum, HAdV, pph6, and NV-GII targets. *E. coli* containing the plasmid standard for the BacHum target was

**Table 2** | (RT-)qPCR targets, primers, probes, and thermocycling conditions. Hydrolysis probes were 5′ labeled with a 6′FAM reporter dye and 3′ labeled with a Black Hole 1 quencher. Primer and probe concentrations are presented in parentheses. Degenerate bases: N, any nucleotide; R, A or G; W, A or T

| Target organism (gene) | Primers/Probe (concentration in nM) | Oligonucleotide sequence (5′–3′) | Position | Thermocycling conditions |
|---|---|---|---|---|
| *Bacteria* | | | | |
| Human-specific *Bacteriodales*[a] (16 s gene) | BacHum-160f (400)<br>BacHum-241r (400)<br>BacHum-193p (80) | TGAGTTCACATGTCCGCATGA<br>CGTTACCCCGCCTACTATCTAATG<br>TCCGGTAGACGATGGGGATGCGTT | 160–180<br>241–218<br>193–217 | 95 °C, 10 min; 45 cycles: 95 °C, 15 s; 60 °C, 1 min |
| *Viruses* | | | | |
| Adenovirus[b] (hexon) | JTVXF (250)<br>JTVXR (250)<br>JTVXP (150) | GGACGCCTCGGAGTACCTGAG<br>ACIGTGGGGTTTCTGAACTTGTT<br>CTGGTGCAGTTCGCCCGTGCCA | 18895–18915<br>18990–18968<br>18923–18944 | 95 °C, 10 min; 45 cycles: 95 °C, 15 s; 55 °C, 1 min |
| Norovirus GII[c] (ORF1-ORF2 junction) | QNIF2d (200)<br>COG2R (200)<br>QNIFS (200) | ATGTTCAGRTGGATGAGRTTCTCWGA<br>TCGACGCCATCTTCATTCACA<br>AGCACGTGGGAGGGGATCG | 5012–5037<br>5100–5080<br>5042–5061 | 50 °C, 15 min; 95 °C, 10 min; 45 cycles: 95 °C, 15 s; 55 °C, 1 min |
| *Extraction controls* | | | | |
| MS2[d] (replicase β chain) | GJW-F (900)<br>GJW-R (900)<br>GJW-P (250) | CGGCTGCTCGCGGATA<br>ACTTGCGTTCTCGAGCGATAC<br>CCGTACCTCGGGTTTCCGTCTTGCT | 3165–3180<br>3228–3208<br>3182–3206 | 48 °C, 30 min; 95 °C, 10 min; 45 cycles: 95 °C, 15 s; 60 °C, 1 min |
| *Pseudomonas syringae*[e] (pph6) (Avr PphE) | Pph-409f (900)<br>Pph-465r (900)<br>Pph-428p (250) | CAGGCCCTAGCGTGGAAAC<br>GGTTCTGGGCCGATGATG<br>CACTGAAAAGGCTGTTCA | 409–427<br>465–448<br>428–445 | 95 °C, 10 min; 40 cycles: 92 °C, 15 s; 60°C, 1 min |

[a]Kildare *et al.* (2007).
[b]Jothikumar *et al.* (2005).
[c]Kageyama *et al.* (2003), Loisy *et al.* (2005), Viau *et al.* (2011).
[d]Williams (2009).
[e]Silkie & Nelson (2009).

obtained from Professor Stefan Wuertz at the University of California, Davis (Kildare *et al.* 2007). *E. coli* containing plasmid standards for HAdV and NV-GII were obtained from Professor Alexandria Boehm at Stanford University (Viau *et al.* 2011). *E. coli* containing the plasmid standard for pph6 was from Silkie & Nelson (2009). Plasmids were extracted using a QIAprep Spin MiniPrep kit (Qiagen). pph6, HAdV, and NV-GII plasmid standards were linearized using restriction enzyme *Bam*HI; the BacHum plasmid standard was linearized using restriction enzyme *Pst*I. Linearized plasmids containing the NV-GII target were *in vitro*-transcribed to RNA using MAXIscript (Ambion) and purified using MEGAclear (Ambion). A whole genome RNA standard was used for quantification of MS2. MS2 RNA was extracted from purified culture using guanidinium thiocyanide lysis buffer and a silica spin column (Qiagen), as described by Love *et al.* (2008).

DNA standard concentrations were quantified using the PicoGreen assay (Molecular Probes) for dsDNA on a NanoDrop ND-3300 fluorospectrophotometer (NanoDrop Technologies); RNA standard concentrations were quantified using the RiboGreen assay (Molecular Probes). Ten-fold serial dilutions of DNA and RNA standards in nuclease-free water were used to construct calibration curves to relate cycle thresholds ($C_t$) to concentrations (gene copies/μL). Calibration curves were pooled if an assay was run more than once.

### Data analysis

Nucleic acid extract from each sample was analyzed in triplicate reactions. If $C_t$ of all three reactions fell within the linear range of the calibration curve, $C_t$ values were used to calculate the average concentration and standard deviation of that sample's nucleic acid extract; to calculate the *sample* concentration, this value was multiplied by the volume of extracted DNA/RNA and normalized by the volume of water concentrated for that sample. If none of the replicates amplified within 45 cycles, the sample concentration was designated as 'less than the limit of detection (<LOD)'. If at least one replicate amplified, but it or other replicates had a $C_t$ value greater than the highest $C_t$ on the standard curve, that sample was designated as 'positive, but below the limit of quantification (+BLOQ)'.

## RESULTS

### Fecal indicator bacteria and coliphage

Average FIB and coliphage concentrations at each site are presented in Figure 1. Thermotolerant coliform concentrations ranged from 6.0 to 9.0 log MPN/100 mL. *E. coli* concentrations measured using mI and chromocult agars were similar: only 4 out of 20 samples had measured concentrations that differed by more than 0.1 log CFU/100 mL and the largest difference was 0.25 log CFU/100 mL. The correlation coefficient ($R^2$) between *E. coli* concentrations measured using the two media was 0.99. *E. coli* concentrations ranged from 4.2 to 7.5 log CFU/100 mL. Enterococci concentrations ranged from 3.3 to 7.1 log CFU/100 mL. Somatic coliphage concentrations ranged from 2.6 to 5.9 log PFU/100 mL, and F+ coliphage concentrations from 2.3 to 4.7 log PFU/100 mL. The ranking of indicator organisms from that with the highest concentration to least was in the following order for all samples, except the La waste stabilization pond (WSP) effluent: thermotolerant coliform concentration > *E. coli* > enterococci > somatic coliphage > F+ coliphage. In the La WSP effluent, the F+ coliphage concentrations were greater than somatic coliphage concentrations. No organisms were detected in negative controls.

### Virus and human-specific *Bacteroidales* concentrations

Samples positive for NV-GII, HAdV, and human-specific *Bacteroidales* are indicated in Figure 2; the locations of these samples are presented in Figure 3. Measured virus and human-specific *Bacteroidales* concentrations, as well as extraction/amplification recoveries, are presented in Table 3.

Recovery efficiencies of pph6, the DNA extraction/amplification control, ranged from 0 to 115% (mean = 46%), with 1 and 7 out of 20 samples categorized as having acceptable and good efficiency, respectively. Recovery efficiencies of MS2, the RNA extraction/amplification control, ranged from 0 to 145% (mean = 62%), with 6 and 9 out of 20 samples categorized as having acceptable and good efficiency, respectively. Poor extraction/amplification control recovery in some samples could be due to poor nucleic acid extraction and/or (RT-)qPCR inhibition.

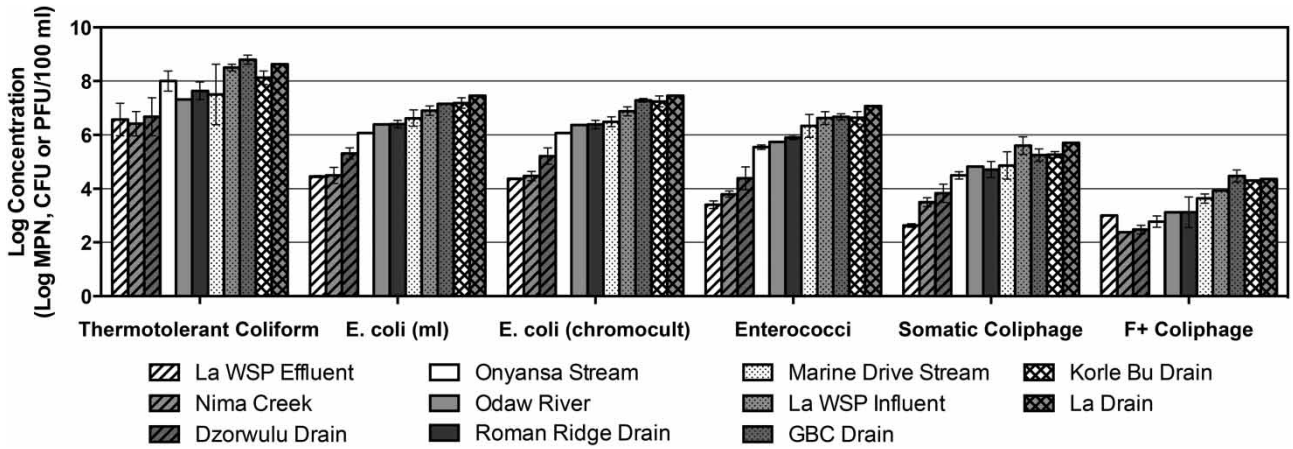


**Figure 1** | Average FIB and coliphage concentrations. $n = 2$ for all samples, except for all organisms in La Drain and Odaw Stream, and F+ coliphage in Nima Creek, La WSP Influent, and Korle Bu Drain, where $n = 1$. Error bars designate the range of values.

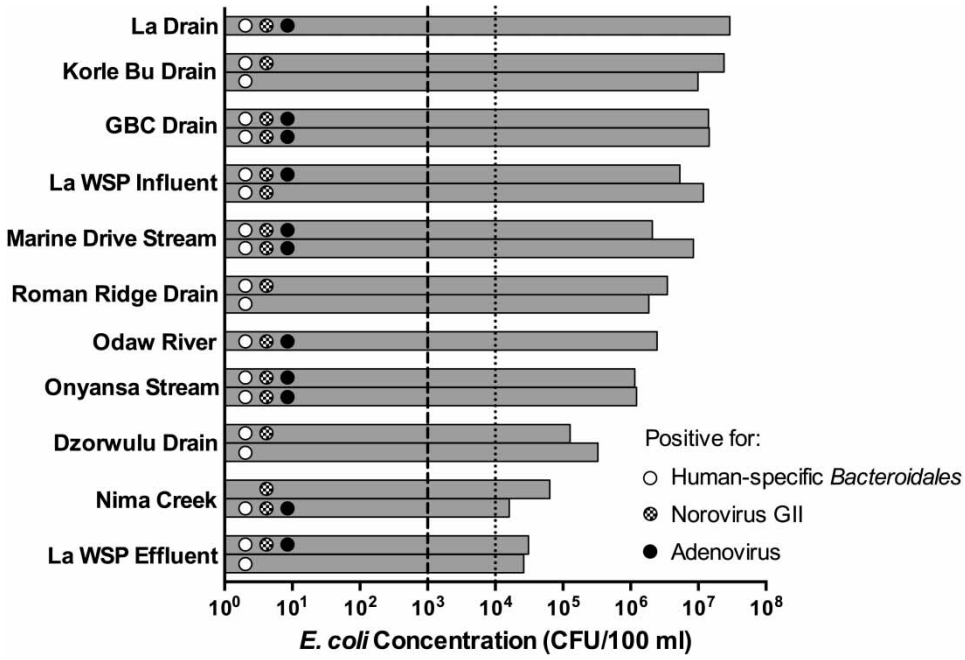


**Figure 2** | *E. coli* concentrations; each bar represents water sampled from the denoted site on 1 day. White circles indicate samples positive for human-specific *Bacteroidales*, black and white check circles indicate samples positive for norovirus GII, and black circles indicate samples positive for human adenoviruses. The dashed line ($10^3$ *E. coli* CFU/100 mL) designates water quality criteria suggested by 2006 WHO Guidelines for irrigation of root crops while the dotted line ($10^4$ *E. coli* CFU/100 mL) designates that for irrigation of leaf crops.

Microorganism concentrations measured by (RT-)qPCR, therefore, may not reflect actual virus concentrations, but conservative estimates. Additionally, because recovery efficiencies varied from sample to sample, the relative concentrations between samples may not be accurate.

Sixteen samples (80%) were positive for NV-GII: 11 were quantifiable and 5 classified as +BLOQ. Of the samples with quantifiable concentrations of NV-GII, concentrations ranged between $(4.75 \pm 2.20) \times 10^2$ and $(1.58 \pm 0.28) \times 10^4$ gene copies/100 mL: eleven samples (55%)

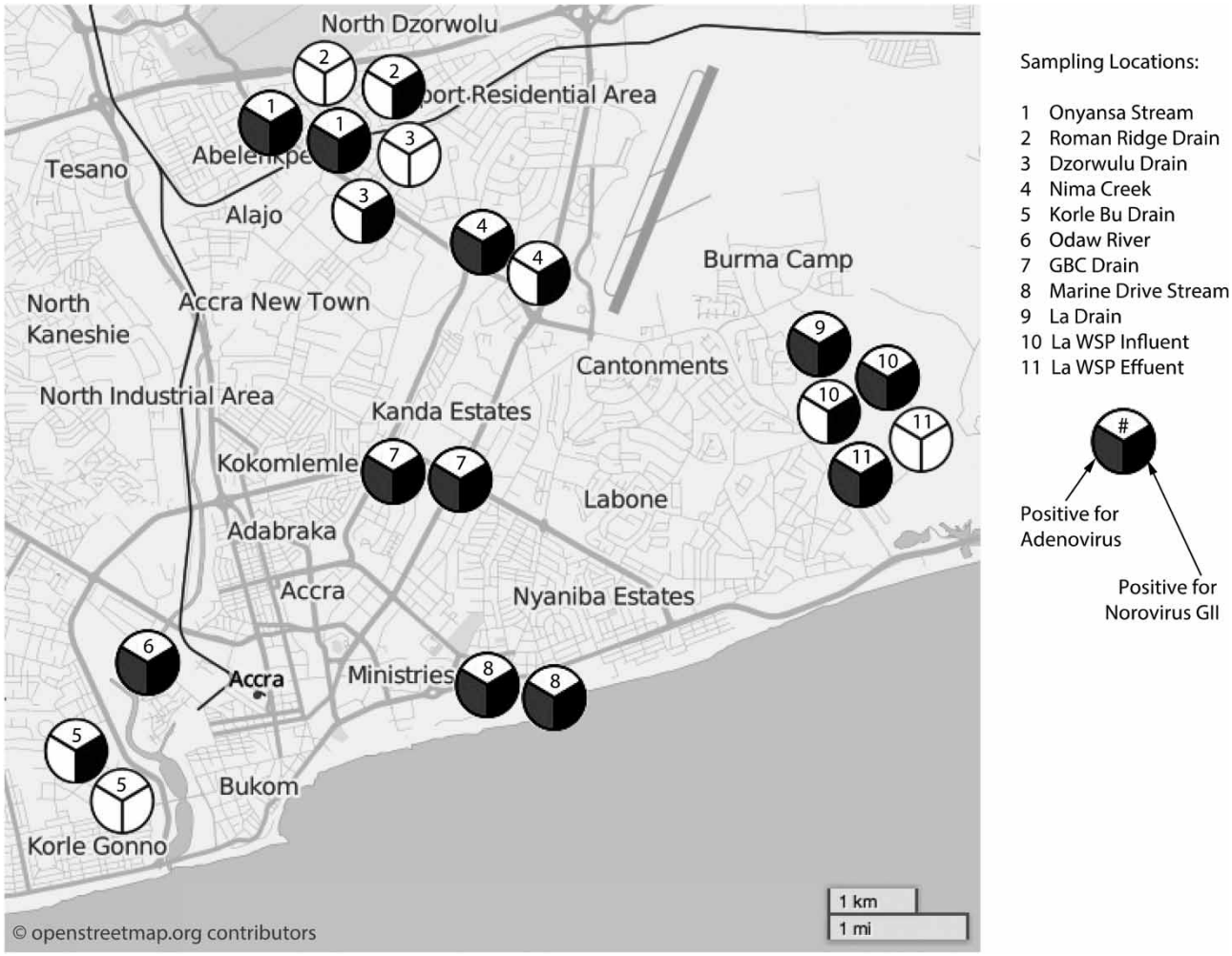


**Figure 3** | Location of samples positive for human adenoviruses and norovirus GII.

were positive for HAdV: seven were quantifiable and four classified as +BLOQ. Of the samples with quantifiable concentrations of HAdV, concentrations ranged between $(2.80 \pm 0.92) \times 10^2$ and $(6.50 \pm 0.60) \times 10^4$ gene copies/100 mL. NV-GII was detected more frequently than HAdV: 11 samples contained both viruses, 5 samples contained NV-GII only, and 4 samples contained neither virus (Figure 2). All sites contained either NV-GII or HAdV in at least one sample.

Human-specific *Bacteroidales* was quantified in 15 out of 20 samples and classified as <LOD in only 1 sample (from Nima Creek); the samples classified as +BLOQ and <LOD for human-specific *Bacteroidales* were also classified as having poor DNA extraction/amplification efficiency. Measured human-specific *Bacteroidales* concentrations ranged from $(9.7 \pm 5.0) \times 10^2$ gene copies/100 mL in La WSP effluent to $(4.9 \pm 0.02) \times 10^6$ gene copies/100 mL in La drain water.

Negative controls that captured the entire analysis chain (from sample concentration to amplification), and no-template (RT-)qPCR amplification controls were negative.

## Limitations

While (RT-)qPCR is a sensitive tool for the quantification of microorganisms, its limitations could result in an under- or overestimation of pathogen concentrations and health risks. (RT-)qPCR does not differentiate between infectious and non-infectious pathogens, and could overestimate the risk of infection presented by a particular organism. On

**Table 3** | Human-specific *Bacteroidales*, human adenovirus, and norovirus GII concentrations. Samples are listed in order of decreasing *E. coli* concentration within each category (e.g., streams, drains, and WSP samples). Concentrations listed as 'below the limit of detection (<LOD)' or 'positive, but below the limit of quantification (+BLOQ)' are followed, in parentheses, by the LOQ of that particular sample for that particular assay. Concentrations are listed as gene copies/100 mL. Recovery efficiency categories are as follows: – poor (<25%), + acceptable (25–50%), and ++ good (>50%)

| | | DNA target concentrations (gene copies/100 mL) | | | RNA target concentration (gene copies/100 mL) | |
|---|---|---|---|---|---|---|
| **Sample** | **Date** | **Human-specific *Bacteroidales* (Mean ± SD)** | **Human adenovirus (Mean ± SD)** | **Pph6 recovery efficiency** | **Human norovirus GII (Mean ± SD)** | **MS2 recovery efficiency** |
| *Streams* | | | | | | |
| Marine Drive Stream | 07/21/10 | $3.58 \pm 0.36 \times 10^5$ | $3.97 \pm 1.01 \times 10^2$ | – | $4.48 \pm 2.35 \times 10^3$ | ++ |
| | 07/27/10 | $5.86 \pm 0.11 \times 10^5$ | $2.80 \pm 0.92 \times 10^2$ | ++ | +BLOQ ($<6.26 \times 10^2$) | – |
| Odaw River | 07/22/10 | $3.64 \pm 0.17 \times 10^5$ | $1.70 \pm 0.44 \times 10^4$ | ++ | $2.62 \pm 0.14 \times 10^3$ | ++ |
| Onyansa Stream | 07/26/10 | $1.50 \pm 0.06 \times 10^6$ | $3.77 \pm 1.26 \times 10^2$ | ++ | $1.04 \pm 0.47 \times 10^3$ | ++ |
| | 07/28/10 | $1.27 \pm 0.04 \times 10^6$ | $3.71 \pm 0.34 \times 10^2$ | ++ | $2.18 \pm 0.58 \times 10^3$ | ++ |
| Nima Creek | 07/19/10 | $2.28 \pm 0.26 \times 10^3$ | +BLOQ (<27.2) | ++ | +BLOQ ($<3.13 \times 10^2$) | + |
| | 08/02/10 | < LOD ($<2.05 \times 10^2$) | <LOD (<35.3) | – | $4.75 \pm 2.20 \times 10^2$ | ++ |
| *Drains* | | | | | | |
| La Drain | 07/21/10 | $4.86 \pm 0.02 \times 10^6$ | $2.06 \pm 0.48 \times 10^4$ | + | $8.04 \pm 2.22 \times 10^3$ | ++ |
| Korle Bu Drain | 07/22/10 | + BLOQ ($<1.02 \times 10^3$) | <LOD ($<1.77 \times 10^2$) | – | <LOD ($<1.88 \times 10^3$) | – |
| | 07/28/10 | $6.08 \pm 3.79 \times 10^3$ | <LOD ($<1.77 \times 10^2$) | – | $2.01 \pm 1.46 \times 10^3$ | + |
| GBC Drain | 07/21/10 | $2.82 \pm 0.61 \times 10^5$ | +BLOQ ($<2.35 \times 10^2$) | – | $1.58 \pm 0.28 \times 10^4$ | ++ |
| | 07/28/10 | $1.23 \pm 0.14 \times 10^5$ | +BLOQ ($<1.18 \times 10^2$) | – | +BLOQ ($<1.25 \times 10^3$) | ++ |
| Roman Ridge Drain | 07/20/10 | + BLOQ ($<4.10 \times 10^2$) | <LOD (<70.6) | – | <LOD ($<7.51 \times 10^2$) | – |
| | 07/26/10 | $1.46 \pm 0.22 \times 10^5$ | <LOD (<58.8) | – | $9.89 \pm 3.64 \times 10^2$ | ++ |
| Dzorwulu Drain | 07/20/10 | + BLOQ ($<3.41 \times 10^2$) | <LOD (<58.8) | – | <LOD ($<6.26 \times 10^2$) | – |
| | 07/26/10 | + BLOQ ($<2.56 \times 10^2$) | <LOD (<44.1) | – | +BLOQ ($<4.69 \times 10^2$) | + |
| *WSP* | | | | | | |
| La WSP Influent | 07/21/10 | $6.63 \pm 4.86 \times 10^3$ | <LOD ($<1.18 \times 10^2$) | – | $4.39 \pm 0.63 \times 10^3$ | + |
| | 07/27/10 | $2.75 \pm 0.11 \times 10^6$ | $6.50 \pm 0.60 \times 10^4$ | ++ | $1.26 \pm 0.56 \times 10^3$ | + |
| La WSP Effluent | 07/21/10 | $6.29 \pm 1.27 \times 10^3$ | <LOD (<39.2) | ++ | <LOD ($<4.17 \times 10^2$) | – |
| | 07/27/10 | $9.66 \pm 5.02 \times 10^2$ | +BLOQ (<39.2) | – | +BLOQ ($<4.17 \times 10^2$) | + |

the other hand, the method employed to concentrate samples before extraction did not allow for the concentration of large volumes of water. This resulted in LOQs that were large compared with the infectious doses of the human virus targets: 18 virus particles for NV (Teunis *et al.* 2008), and unknown but assumed to be low for waterborne HAdV. Samples found to be <LOD could potentially contain virus concentrations capable of causing infection.

There are additional uncertainties in virus and human-specific Bacteroidales concentrations measured by (RT-) qPCR. The extraction/amplification controls used in this study (MS2 and pph6) may not perfectly model extraction and amplification of the (RT-)qPCR targets, due to physical and chemical properties that can differ between microorganisms. These properties may be particularly different between pph6 and HAdV given that bacteria and viruses have different sizes and structures. Also, we were unable to measure the efficiency of sample concentration in the field. Inefficient sample concentration would lead to measured virus concentrations that are less than actual concentrations in source waters; the use of measured virus concentrations in risk assessment could underestimate health risks associated with using these waters for irrigation.

Pathogen concentrations in wastewater and the environment can vary both spatially and temporally depending on local conditions, that include climate, weather, the health

status of the population, the availability and condition of wastewater infrastructure, and the environmental fate and transport of a particular microorganism. This limitation may make generalization and extrapolation of study findings to wider contexts difficult.

## DISCUSSION

### Water quality and the WHO Guidelines

The 2006 WHO Guidelines call for the use of QMRA to develop location-specific, health-based water quality standards for irrigation water. The Guidelines used an illustrative example, with rotavirus as an 'index' viral pathogen, to determine that an *E. coli* concentration of no more than $10^3$/100 mL (for root crops) or $10^4$/100 mL (for leaf crops) would meet the health-based target of no more than $10^{-6}$ disability-adjusted life years (DALYs) lost per person per year for farmers practicing wastewater irrigation. This recommendation was also deemed appropriate to protect the health of consumers of raw vegetables as long as post-water treatment control measures (such as the use of drip irrigation, pathogen die-off in the field, and vegetable washing and disinfection) are also employed. While Seidu *et al.* (2008) employed QMRA to determine health risks associated with wastewater irrigation in Accra (using measured fecal coliform concentrations and an assumed ratio between concentrations of fecal coliform and rotavirus), QMRA has not yet been conducted with actual virus concentrations measured in Accra, nor has it been used to establish Accra- or Ghana-specific health-based water quality standards.

None of the irrigation water samples analyzed in this study met the water quality standards suggested by the 2006 WHO Guidelines (Figure 2): all samples contained *E. coli* concentrations greater than $10^4$/100 mL, and some samples contained *E. coli* concentrations over three orders of magnitude larger than this limit. Additionally, thermotolerant coliform concentrations in all samples greatly exceeded the limit of $10^3$ thermotolerant coliforms/100 mL set by the 1989 WHO Guidelines for irrigation of vegetables that could be eaten raw (WHO 1989). Similar results were found by Mensah *et al.* (2001), Keraita *et al.* (2003), and Amoah *et al.* (2005). Amoah *et al.* (2005) analyzed irrigation water samples collected from streams and drains in Accra and Kumasi, Ghana over the course of a year and found all but two samples to have fecal coliform concentrations that exceeded the 1989 WHO Guidelines, despite considerable variation over time.

A challenge with the use of FIB like *E. coli*, enterococci, and thermotolerant coliforms is that they can have animal fecal sources, as well as environmental sources and reservoirs. While FIB have been observed to multiply in tropical environments (Field & Samadpour (2007) and references within), bacteria in the order *Bacteroidales* are strict anaerobes, and are less likely to grow outside human and animal hosts (Kreader 1995; Kildare *et al.* 2007; Walters & Field 2009). The molecular marker BacHum was designed to detect human-specific *Bacteroidales* (Kildare *et al.* 2007), however some cross-reactivity with animal feces has been observed (Kildare *et al.* 2007; Silkie & Nelson 2009). All but one sample contained detectable levels of human-specific *Bacteroidales*, an indication that the water sources sampled were contaminated with human feces. While one of the Nima Creek samples had a human-specific *Bacteroidales* concentration <LOD, this sample had poor DNA extraction/amplification efficiency and contained $(4.75 \pm 2.20) \times 10^2$ NV-GII gene copies/100 mL, indicating that this water source also had human fecal contamination. Overall, the BacHum qPCR target was a sensitive marker for sewage in Accra, similar to studies conducted in California (Kildare *et al.* 2007; Silkie & Nelson 2009), but different from that of Jenkins *et al.* (2009) who found limited sensitivity of the BacHum qPCR target to detect human-specific *Bacteroidales* in human feces and sewage in Kenya. The levels of BacHum in Accra samples ranged from $<10^{2.3}$ to $10^{6.7}$ gene copies/100 mL, which is lower than the concentrations reported for sewage samples in California: $10^{8.0}$–$10^{9.8}$ gene copies/100 mL (Silkie & Nelson 2009) and $10^{7.8}$–$10^{9.9}$ gene copies/100 mL (Van De Werfhorst *et al.* 2011). A potential explanation why lower BacHum concentrations were found in the Accra samples could be that most consisted of diluted sewage; this was also seen with *E. coli* concentrations, which were lower in the Accra samples ($10^{4.2}$–$10^{7.5}$ CFU/100 mL) than in California sewage ($10^{6.1}$–$10^{6.8}$ MPN/100 mL; Silkie & Nelson 2009). Lower BacHum concentrations in Accra could potentially also be attributed to a lower concentration of the BacHum target in human feces in Accra than in California, or to DNA extraction/amplification efficiencies that were less than 100%.

The largest indicator organism concentrations were observed in the La, Korle Bu, and GBC drains. Some drains, like the Roman Ridge and Dzorwulu drains, were likely built to channel greywater and stormwater, not untreated sewage; however, given the presence of human-specific *Bacteroidales* and NV-GII, it is likely that untreated human waste was discharged into these drains as well. The streams sampled as part of this study also contained human-specific *Bacteroidales* and human viruses, highlighting the fact that, in Accra, many streams act as de facto drains.

Samples were collected from the inlet and outlet of a WSP on the La Fulani farm site. WSPs remove microorganisms from wastewater through sedimentation, predation, competition, and sunlight inactivation, with sunlight inactivation playing an important role (Curtis *et al.* 1992; Davies-Colley *et al.* 1999, 2000; Sinton *et al.* 2002; Shilton 2005). Although we had a limited sample size for evaluating the La WSP ($n = 2$), microorganism concentrations were observed to decrease between the WSP influent and effluent – measured *E. coli*, enterococci, thermotolerant coliform, somatic and F+ coliphage concentrations decreased by 2.5, 3.3, 1.6, 3.1, and <1 log, respectively – and water samples collected at the outlet of the La WSP had lower concentrations of *E. coli*, enterococci, thermotolerant coliform, and somatic coliphage than other water sources. However, La WSP effluent still did not meet the WHO recommended limit for *E. coli* in irrigation water. Additionally, we observed little reduction in F+ coliphage concentrations between the WSP inlet and outlet, possibly due to slower rates of F + RNA coliphage inactivation than the other organisms in sunlit WSP water (Sinton *et al.* 2002). Viruses have different rates of sunlight inactivation depending on their composition and structure (Love *et al.* 2010; Silverman *et al.* 2013) and, generally, have slower inactivation rates than bacteria. The WHO Guidelines include WSPs on the list of health protection measures that can be used to achieve water quality targets. While bacteria, and some virus, removal occurred in the WSP, additional treatment would be required to meet WHO recommended bacteria levels and remove or inactivate persistent viruses.

## Correlation between indicator organisms

Log concentrations of indicator organisms were compared to determine correlations between them. *E. coli* was generally correlated with enterococci and somatic coliphage ($n = 20$; $R^2 = 0.97$ and 0.89, respectively), and less so with thermotolerant coliform and F+ coliphage ($n = 20$ and 17; $R^2 = 0.68$ and 0.62, respectively). $R^2$ between enterococci and somatic coliphage log concentrations was 0.91 ($n = 20$). Thermotolerant coliform and F+ coliphage were the least correlated with other fecal indicators (Table 4).

When looking at correlation charts between F+ coliphage and *E. coli*, enterococci, and somatic coliphage, the most apparent outlying points are those sampled from the La WSP effluent (circled in Figure 4). The outlying points could be caused by the longer persistence of F+ coliphage in WSP (as compared with FIB and somatic coliphage), which would result in higher relative concentrations of F+ coliphage in WSP effluent than in fresher wastewater. When the La WSP effluent samples are removed from analysis, the correlation coefficients between F+ coliphage and *E. coli*, enterococci, and somatic coliphage increase to 0.82, 0.74, and 0.66, respectively. These results serve as a reminder that when making comparisons between microorganism concentrations, it is important to consider the environmental conditions and processes the samples have been exposed to, as different organisms have different fates and transport in the environment.

## QMRA assumptions: The ratio between norovirus and *E. coli*

The 2006 WHO Guidelines employed rotavirus as an index organism for QMRA used to make recommendations for health-based water quality standards. However, with the generation of dose–response data for norovirus (Teunis *et al.* 2008), some (including Mara & Sleigh (2010) and Mara *et al.* (2010)) have called for the use of norovirus in these models. While norovirus and rotavirus are both highly infectious, norovirus is frequently associated with food and waterborne outbreaks and is capable of infecting people of all ages (Widdowson *et al.* 2005), whereas rotavirus predominantly infects children under the age of five.

As viruses are not regularly quantified in water samples, QMRA models frequently employ assumptions of virus concentration based on the concentration of FIB. Shuval *et al.* (1997), Seidu *et al.* (2008), and the 2006 WHO Guidelines used the assumption that wastewater contains 0.1–1

**Table 4** | $R^2$ between microorganism log concentrations ($n = 20$ for all except $n = 17$ for those with F+ coliphage). Values in parentheses in F+ coliphage column are $R^2$ values determined with the exclusion of La WSP effluent samples

| | ***E. coli* (chromocult agar)** | **Enterococci** | **Somatic coliphage** | **Thermotolerant coliform** | **F+ coliphage** |
|---|---|---|---|---|---|
| *E. coli* (mI agar) | 0.99 | 0.97 | 0.89 | 0.68 | 0.62 (0.82) |
| *E. coli* (chromocult agar) | | 0.96 | 0.88 | 0.68 | 0.64 (0.83) |
| Enterococci | | | 0.91 | 0.67 | 0.58 (0.74) |
| Somatic coliphage | | | | 0.69 | 0.43 (0.66) |
| Thermotolerant coliform | | | | | 0.46 (0.49) |

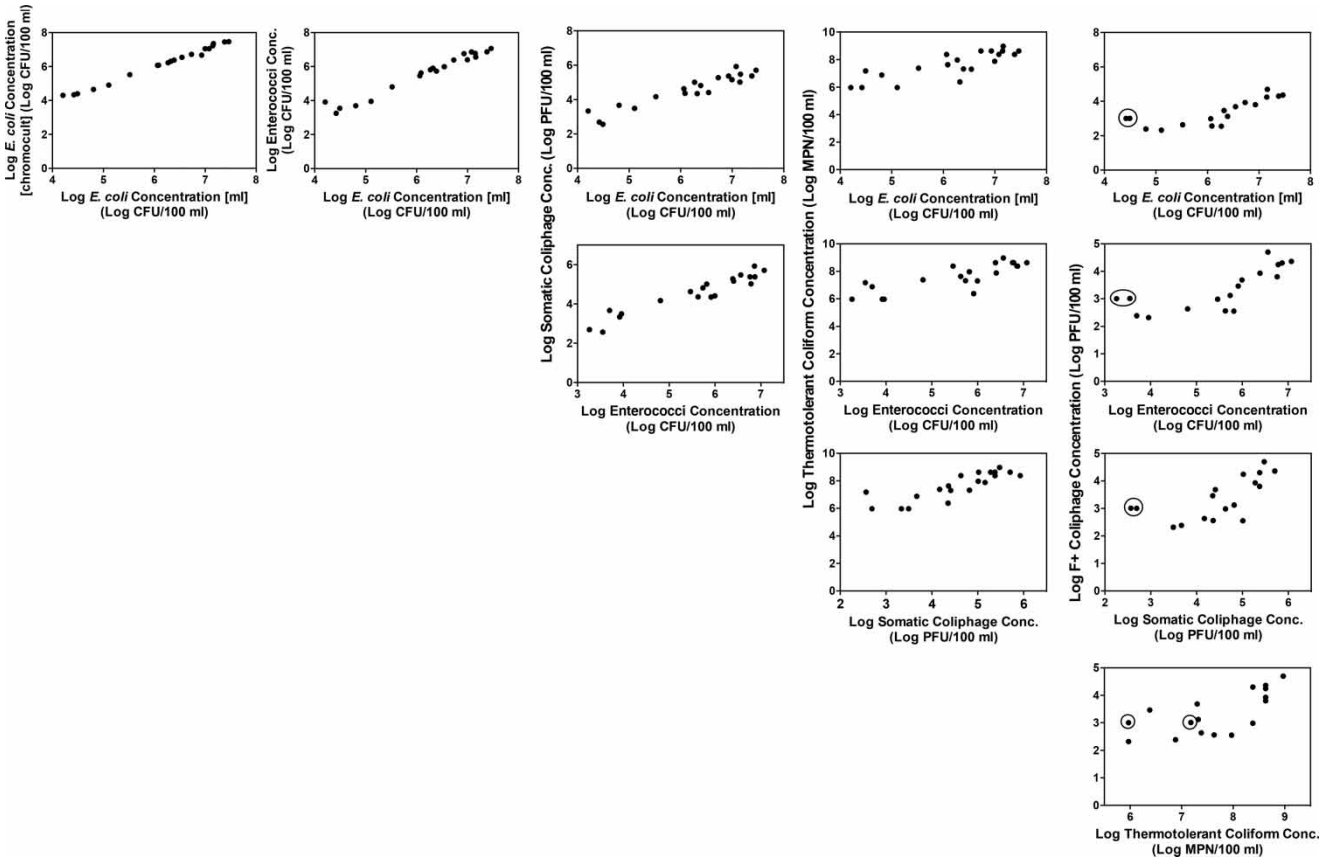


**Figure 4** | Correlations between microorganism log concentrations. Data points circled in the rightmost column correspond to La WSP effluent samples.

rotavirus particles per $10^5$ fecal coliforms based on work by Schwartzbrod (1995). Schwartzbrod employed a literature review to determine that enteric viruses exist in untreated wastewater at concentrations ranging from $10^2$ to $10^3$ PFU/L; the majority of studies cited were conducted in the United States and Europe. Mara *et al.* (2007) used an assumption of 0.1–1 rotavirus particles per $10^5$ *E. coli* with the reasoning that *E. coli* is a better indicator of fecal contamination; Mara *et al.* (2007) also cited work by Oragui *et al.* (1987) in northeastern Brazil, although this publication measured concentrations of fecal coliform – not *E. coli* – and found a ratio of four rotaviruses per $10^5$ fecal coliforms in raw wastewater. In recent QMRA modeling, Mara & Sleigh (2010) and Mara *et al.* (2010) assumed a ratio of

0.1–1 noroviruses per $10^5$ *E. coli*, a ratio likely carried over from the rotavirus assumption and a lack of information about norovirus concentrations in irrigation waters.

In the present study, in samples with quantifiable concentrations of NV-GII, we found an average of one NV-GII per $10^{3.2\pm1.1}$ *E. coli* or $10^{4.8\pm1.0}$ thermotolerant coliform ($10^{\text{mean}\pm95\%\text{ confidence interval}}$; $n = 11$). Samples that did not have quantifiable NV-GII concentrations include five that had poor RNA extraction/amplification efficiency and three that had low concentrations of *E. coli*, which would correspond to NV-GII concentrations below the LOQ; one sample (GBC drain) had good extraction/amplification efficiency and a sufficiently high concentration of *E. coli*, but did not contain a quantifiable NV-GII concentration. The NV-GII to thermotolerant coliform ratio found here is similar to the rotavirus to fecal coliform ratio used previously (Shuval *et al.* 1997; WHO 2006; Seidu *et al.* 2008); however, our NV-GII to *E. coli* ratio is larger than the norovirus to *E. coli* ratio assumed in recent publications (Mara & Sleigh 2010; Mara *et al.* 2010). The larger NV-GII to *E. coli* ratio could be attributed to: (1) the use of *E. coli* instead of thermotolerant coliform in the ratio, since thermotolerant coliform concentrations are usually greater than those for *E. coli*; (2) a difference in the number of norovirus infections at the time of sample collection; or (3) the virus quantification method employed. Virus concentrations determined through (RT-)qPCR are greater than those determined by culture-based methods, because total particle to PFU ratios can range from 100–1,000 to 1 (Flint *et al.* 2004). Given that no fully permissive cell culture system for norovirus exists at present, norovirus must be measured by (RT-)qPCR or direct particle count. The use of (RT-)qPCR to quantify norovirus and membrane filtration to quantify *E. coli* could be preferred in this context given that (RT-)qPCR was used to quantify norovirus used in dose–response work (Teunis *et al.* 2008), and membrane filtration is regularly used to quantify *E. coli* in environmental samples. Although there are uncertainties surrounding the use of (RT-)qPCR in norovirus quantification – such as the exclusion of norovirus genogroup I from analysis, and imperfect sample concentration, RNA extraction, and amplification – correction of these limitations would act to further increase the norovirus to *E. coli* ratio.

As discussed above, pathogen concentrations, and the ratios between pathogens and fecal indicator organisms, can change spatially and temporally depending on local conditions. While the ratio of NV-GII to *E. coli* or thermotolerant coliform is likely to differ over place and time, it is an important finding that the current assumption of 0.1–1 norovirus particles per $10^5$ *E. coli* would underestimate virus dose with exposure to the wastewater and surface waters sampled in Accra by approximately 2–3 orders of magnitude. The use of the NV-GII to *E. coli* ratio determined in the present study would result in a higher estimate of health risk.

The ratios of HAdV to fecal indicator bacteria were similar to those of NV-GII, but more variable. The ratio of HAdV to *E. coli* was one to $10^{3.2\pm1.7}$ and the ratio of HAdV to thermotolerant coliform was one to $10^{4.6\pm2.0}$ ($10^{\text{mean}\pm95\%\text{ confidence interval}}$; $n = 7$).

## CONCLUSION

No water sources sampled as part of this study met water quality criteria recommended by either the 1989 or 2006 WHO Guidelines, and all but one contained detectable concentrations of human-specific *Bacteroidales*, suggesting human fecal contamination. NV-GII was detected in 16/20 samples and HAdV in 11/20 samples. Given that wastewater irrigation can be beneficial to farmers and municipalities, it is unlikely, and potentially inadvisable, for water reuse in agriculture to cease. Instead, health risks should be addressed by treating wastewater before use on farms. The 2006 WHO Guidelines suggest the use of the 'multi-barrier approach' to reduce microbial contamination from 'farm to fork'; this approach can utilize measures such as WSPs, on-farm wastewater treatment ponds (Keraita *et al.* 2008; Amoah *et al.* 2011) and produce disinfection (Amoah *et al.* 2011). While the La WSP reduced concentrations of indicator microorganisms, it did not do so to levels that met the WHO recommended limits, highlighting the importance of multiple barriers. Wastewater collection and treatment before release to the environment should be promoted in Accra in general, given that some of the contaminated surface waters we studied flow through densely populated parts of the city, and can provide the

general population with a source of exposure to waterborne pathogens.

As the WHO Guidelines and QMRA models are implemented and updated, it is important to have reasonable estimates of virus concentrations to use as inputs to these models. In the present study, we found an average of one NV-GII per $10^{3.2\pm1.1}$ *E. coli*. While this ratio is expected to vary depending on local conditions, including the health status of the contributing population, it could be used to calculate an informed estimate of virus concentration for modeling health risks associated with wastewater irrigation in Accra.

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