Environmental pathogen hazards reveal need for improved sanitation infrastructure in Alabama's Black Belt

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Abstract:

Many rural communities in Alabama's Black Belt region lack adequate sanitation resulting in wastewater discharges that may pose risks to residents. To understand the scope of the problem in one community, we conducted three cross-sectional surveys in a small town with limited sanitation in 2023. We measured a range of enteric pathogens in environmental samples by multi-parallel qPCR as well as fecal indicator bacteria *E. coli* and *Enterococcus* by culture and molecular methods. We examined soil samples (n = 55) from sites near failing septic systems or suspected direct surface discharges and comparison soil (n = 10) far from potential discharges to estimate sanitation-related pathogen hazards. We examined surface water samples from community (n = 8) and localized (n = 16) sites that may have been impacted by wastewater discharges. Comparing impacted and unimpacted soils samples revealed greater fecal contamination near known or suspected discharges, compared with control samples. The mean culturable *E. coli* count in impacted soils was 224 MPN/g (95% CI 0-510.5 MPN/g) and in unimpacted soils was 0.5 MPN/g (95% CI 0-1.5 MPN/g).We detected several pathogens via qPCR in impacted soil and surface water, including *Acanthamoeba* spp., *Balantidium coli*, *Blastocystis* spp., *Cryptosporidium* spp., and rotavirus. In community-level surface waters (n = 8), 88% of samples were positive for *E. coli* by culture (mean 3.04 x 10^5, 95% CI 0-8.96, x 10^5 MPN/100mL); 100% were positive for *Enterococcus* by culture (mean 1.10 x 10^4, 95% CI 0-2.55 x 10^4 MPN/100mL); and we detected *Acanthamoeba* spp., *Blastocystis* spp., *Cryptosporidium* spp., *Plesiomonas shigelloides*., rotavirus, and *Yersinia enterocolitica*, suggesting community-level wastewater discharges may degrade local surface water quality. Evidence suggests sanitation failures contribute to enteric pathogen hazards in this community, and culturable *E. coli* may provide a sensitive indicator of fecal contamination in prospective monitoring efforts.

Introduction:

Sanitary sewer connections are unavailable for many rural or disadvantaged communities within high-income countries (1,2). Residents without access to sewer connections typically rely instead on onsite sewage disposal systems (OSDS), though these systems are vulnerable to failure due to poor silting, poor draining, and climate-related disasters (3). The *Transforming Wastewater Infrastructure in the United States* project, supported by Columbia World Projects, aims to address these issues by presenting alternative wastewater technology solutions that are best suited for use in under-resourced settings.

As part of this project, a team will be piloting the installation of a decentralized wastewater system in a small town in the Black Belt region of Alabama. While most households in this town are connected to the county water supply, there is no existing municipal wastewater treatment system. Communities in the Black Belt region have received national recognition for failing or nonexistent wastewater infrastructure (4,5). Since shrink-swell clays, common to this area, lead to hydraulic failure of septic systems, a high proportion of residents rely instead on straight pipes and cesspools (3). This led to the Department of Justice's first Environmental Justice investigation under Title VI of the Civil Rights Act of 1964 (6). This investigation concluded with the citing of two areas of concern: the use of fines and law enforcement to punish people with inadequate wastewater treatment systems and inadequate action to assess and address health risks from raw sewage (4). The decentralized cluster system that will be piloted in this town is intended to model a novel system designed specifically to meet the needs of rural and resource-limited communities.

The primary objective of this study was to compare fecal indicator bacteria (*E. coli* and *Enterococcus*) between sites near failing septic systems or suspected direct surface discharges ("impacted") and sites far away from buildings, standing water, ditches, and other potential discharges ("unimpacted"). We aimed to establish baseline levels of fecal indicator bacteria (E. coli and Enterococcus) and human enteric pathogens in soil, surface water, and standing water prior to the installation of a decentralized wastewater system. Environmental samples were analyzed using culture-based methods and multiplex molecular techniques to detect and quantify microbial targets.

Materials and Methods:

We conducted three cross-sectional surveys in a small town in Alabama's Black Belt region during January, February, and May of 2023 to assess enteric pathogens and fecal indicator hazards in soil, surface water, and standing water samples. We collected samples across three different time points to capture potential microbial variations related to rainfall. Based on visual identification and local knowledge, we collected samples near failing septic systems or suspected direct surface discharges. We categorized these samples as "impacted." Additionally, we collected samples far away from known or identified wastewater discharge sites. We considered these "unimpacted."
We collected approximately 2 g of soil 2 inches below the soil surface and placed them into sterile WhirlPak bags. We collected 100-500 mL of surface and standing water and placed them into sterile WhirlPak bags. *We stored all soil samples at –20C until downstream processing*

Culture of Fecal Indicator Bacteria from Soil:

We cultured *E. coli* and *Enterococci* from soil samples using the Quanti-Tray 2000 System. Briefly, we diluted 3 g of soil into 30 mL of phosphate-buffered saline (PBS). After

homogenization by shaking, we diluted the solution 1:10 and 1:100 in PBS. . We prepared the 1:10 and 1:100 dilutions for culture using the IDEXX Colilert-18 and IDEXX Enterolert media and IDEXX QuantiTrays. We incubated the QuantiTrays at either 35°C (Colilert-18) or 41°C (Enterolert) for 18-24 hours. We scored the wells and determined the most probable number (MPN) concentration using on the IDEXX guidelines.

Culture of Fecal Indicator Bacteria from Water:
We cultured *E. coli* and *Enterococci* from surface water and standing water samples using the Quanti-Tray 2000 System. We prepared 1:10 and 1:100 dilutions of each water sample in sterile PBS. We prepared the samples with either the IDEXX Colilert-18 media or the IDEXX Enterolert (*Enterococcus*) media in the Quanti-Tray 2000 trays. Once we sealed and labeled the trays, we incubated them at 41°C for 24 hours for Enterolert or 35°C for 18 hours for Colilert-18.

Soil and Surface Water Processing:
For surface water samples transported to UNC , we thawed them at 4°C for 72 hours before processing. From each sample, we filtered 25 mL to 200 mL onto a 47-mm uM Millipore HA membrane, as shown in Appendix 1. For soil samples we weighed out 0.189 grams to 0.485 grams and placed them into a 2 mL tube. Exact processing volumes and weights are included in Appendix 2.

Isolation of Total Nucleic Acids and Analysis:
From the soil and water samples, we isolated total nucleic acids using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research). Immediately prior to extraction, We spiked all samples with 10 uL of Bovine herpesvirus (BHV) and Bovine respiratory syncytial virus (BRSV), carried in the Zoetis Inforce-3 vaccine, which served as the extraction control. For each batch of extractions, we included a negative extraction control (PCR-grade water), and a positive extraction control (Inforce-3). From the extraction eluates, we measured the concentration of double-stranded DNA (dsDNA) and RNA using the Qubit High Sensitivity dsDNA and Qubit RNA High Sensitivity, Broad Range Assay Kits (Invitrogen, Carlsbad, CA, USA) respectively.

We analyzed the isolated total nucleic acids from soil and surface water samples using a custom TaqMan Array Card (TAC) (ThermoFisher Scientific, Waltham, MA) targeting 39 enteric pathogens and fecal markers including: 20 types of bacteria (*Acanthamoeba* spp., *Campylobacter jejuni/coli*, *Clostridium difficile*, *E. coli* O157:H7, *E. coli* ybbW, Enteroaggregative *E. coli* aaiC, Enteroaggregative *E. coli* aatA, *Enterococcus* spp., Enteropathogenic *E. coli* bfpA+, Enteropathogenic *E. coli* eae+, Enterotoxigenic *E. coli* heat-labile, Enterotoxigenic *E. coli* STh, Enterotoxigenic *E. coli* STp, *Helicobacter pylori, Plesiomonas shigelloides, Salmonella enterica,* Shiga toxin producing *E. coli* Stx1, Shiga toxin producing *E. coli* Stx2, *Shigella* spp. and enteroinvasive *E. coli*, and *Yersinia enterocolitica*), 9 viruses (Adenovirus 40/41, *Astroviridae*, Hepatitis A, Norovirus GI, Norovirus GII, Rotavirus, Sapovirus I/II/IV/V, SARS-CoV-2, Zika Virus), 6 helminths (*Ancylostoma duodenale*, *Ascaris lumbricoides*, *Enterobius vermicularis*, *Necator americanus*, *Strongyloides stercoralis*, and *Trichuris trichiura*), 6 protozoans (*Balantidium coli*, *Blastocystis* spp., *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Entamoeba histolytica*, and *Giardia* spp.), and 2 MST Makers (Human Mitochondrial DNA and Human-specific HF183 Bacteroides 16S rRNA genetic marker). More details on the targets included are in Appendix 4. We added 33 uL of nucleic acid extraction and 67 uL of mastermix

(Hepatitis G, Exo IPC Mix (10X), Exo IPC DNA (50X), AgPath Buffer (2X), and AgPath Enzyme) into one port in each TAC. We spiked in Hepatitis G to the master mix as the qPCR control.

Once we filled each TAC port, we performed one-step reverse transcription qPCR using a QuantStudio 7 (Thermo Fisher Scientific, Waltham, MA) at the following thermocycling conditions: 45°C for 20 minutes and 94°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute, with a ramp rate of 1°C/second between each step. Once complete, we compared the curves and multicomponent plots with the control plots to ensure only positive amplifications were included by either flagging them or omitting unreliable results(7).

Standard Curves:

We generated standard curves by running a 7-fold dilution series of positive control material to determine assay efficiency. Next, we compiled the raw TAC data into a single data frame. We then implemented quality control steps and flagged failed assays where the IPC did not amplify. We set all Ct values above the y-intercept of the standard curve for that particular target to NA to remove suspicious data points (8). We performed quality assurance checks, including evaluating amplification of the different targets across different TAC cards in the positive PCR control and identifying failed targets based on Ct thresholds ($10 < Ct > 40$). We also assessed the amplification of the Hepatitis G target, which was included in the PCR master mix, across all samples and excluded any sample that did not meet the Ct thresholds ($10 < Ct > 40$). We calculated recovery efficiency for BHV and BRSV by comparing sample Ct values against the average value of each target in the positive extraction controls samples.

Quality Control:

The targets *Enterococcus* spp. lsrRNA and *E. coli ybbw* appeared in some of the NEC (negative extraction control) and PEC (process extraction control) (Appendix 5). We identified the matching environmental samples with the same extraction group for each target detected in NEC or PEC controls then subtracted the corresponding copies per uL. If both controls showed detection, the higher of the two was subtracted to account for background or contamination, improving the accuracy of quantification. This final data frame is included in Appendix 6.

Data Analysis:

We analyzed all data using RStudio (R Foundation for Statistical Computing, Vienna, Austria). To compare sites that were assumed to be impacted by failing sanitation to sites that were assumed to be unimpacted by failing sanitation, we used the Wilcoxon Rank Sum Test.

Results:

Across the three sampling campaigns, we collected and processed 123 samples (soils weighed and membrane filtration of water samples). Of the samples collected, 35% (43 of 123) were cultured for *E. coli* via IDEXX, 25 % (31 of 123) were cultured for *Enterococcus* via IDEXX, 88% (108 of 123) underwent nucleic acid extraction, and 74% (91 of 123) were analyzed using multi-parallel qPCR. These data are summarized in Table 1 below.

198 *Table 1: Summary of how the Samples were Processed*

Month Collected	Impact Status	Type of Sample	# Collected	# Cultured for *E. coli*	# Cultured for *Enterococcus*	# Extracted	# Run on TAC
January	Impacted	Surface Water	4	0	0	4	2
		Soil	17	0	0	17	13
	Unimpacted	Soil	5	0	0	5	3
February	Impacted	Surface Water	13	10	0	12	12
		Soil	27	0	0	24	21
	Unimpacted	Soil	6	0	0	6	4
May	Impacted	Surface Water	12	12	12	12	12
		Soil	34	15	15	24	21
	Unimpacted	Soil	5	4	4	3	3
ALL	Impacted	Surface Water	29	24	12	29	26
		Soil	78	15	15	65	55
	Unimpacted	Soil	16	4	4	14	10

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200
201 *Detection and Quantification of Enteric Pathogens and Fecal Indicator Bacteria of Soil Samples.*
202 To assess patterns of microbial contamination across different environmental contexts, we
203 evaluated soil from sites identified as impacted or unimpacted by wastewater. Figure 1 summarizes
204 the comparison of cultured fecal indicator bacteria (*E. coli*, *Enterococcus*, and total coliform) and
205 molecular screening via multi-parallel qPCR of enteric pathogens and human-associated markers
206 in impacted and unimpacted soil samples.
207
208 **Figure 1. Comparison of Human Pathogens in Impacted VS. Unimpacted Soil Samples by Culture and**
209 **Molecular Methods.** Human pathogens found in detectable concentrations from impacted and unimpacted soil.
210 Wilcoxon rank sum p-values are shown for pairwise comparisons between impacted and unimpacted soil samples
211 analyzed via culture by IDEXX and molecular methods by multi-parallel qPCR ($p < 0.05$).
212
213 Of the 19 soil samples examined by culture, total coliforms were detected in 19 samples (100%)
214 at a range of 1,120 to > 2,419.6 MPN/g, *E. coli* was detected in 12 samples (63%) at a range of 0
215 to 2,247 MPN/g, *Enterococcus* was detected in 19 samples (100%), at a range of 41 to > 2,419.6
216 MPN/g (Table 2).
217
218 **Table 2: Detection and Quantification of Total Coliform, *E. coli,* and *Enterococcus* in Soil by IDEXX.**

	Total Coliform		*E. coli*		*Enterococcus*	
Sample Description	Prevalence (%)	Average Concentration (MPN/g)	Prevalence (%)	Average Concentration (MPN/g)	Prevalence (%)	Average Concentration (MPN/g)
All	19/19 (100)	107,000± 118,00	12/19 (63)	177 ± 509	19/19 (100)	3,080 ± 5580
Impacted	15/15 (100)	116,000±	11/15 (73)	224 ±	15/15 (100)	2,250 ±

		122,000		567		2,390
Unimpacted	4/4 (100)	73,700 ± 113,00	1/4 (25)	0.5 ± 1	4/4 (100)	6210 ± 12,000

Neither the prevalence nor the concentration of total coliform or *Enterococcus* were significantly different between the sites assumed to be impacted and the sites assumed to be unimpacted by failing or non-existent wastewater treatment systems (Figure 1). However, the prevalence and concentration of *E. coli* was significantly higher for samples assumed to be impacted by failing or non-existent wastewater treatment systems than for samples assumed to be unimpacted.

From the 49 soil samples analyzed by multi-parallel qPCR that met all the quality control requirements, 11 samples (22%) were found to contain detectable levels of the *ybbW* gene target for *E. coli*. Of the 51 soil samples that met all the quality control requirements, 31 samples (61%) were found to contain detectable levels of the gene targets specific to *Enterococcus* spp. lsrRNA (Table 3). The concentration of *E. coli* ranged from not detectable to 8.32 x 10^5 copies per gram. The concentration of *Enterococcus* ranged from not detectable to 1.12 x 10^6 copies per gram.

Table 3: Detection and Quantification of *E. coli* and *Enterococcus* in Soil by Multi-parallel qPCR

	E. coli		*Enterococcus*	
Sample Description	Prevalence (%)	Average Concentration Log_{10} (copies per g)	Prevalence (%)	Average Concentration Log_{10} (copies per g)
All	11/49 (22)	4.72 ± 0.82	31/51 (61)	3.92 ± 0.97
Impacted	9/42 (21)	4.92 ± 0.68	27/44 (61)	4.04 ± 0.96
Unimpacted	2/7 (29)	3.79 ± 0.98	4/7 (57)	3.15 ± 0.72

There was no significant difference in prevalence or concentration of these indicators, *E. coli. ybbW* and *Enterococcus* spp. lsrRNA, between the sites assumed to be impacted and the sites assumed to be unimpacted by failing or non-existent wastewater treatment systems as seen in Figure 1.

The only pathogen detected in both impacted and unimpacted soil samples besides *E. coli* ybbW and *Enterococcus* spp. lsrRNA was *Acanthamoeba* spp. Of the 38 impacted soil samples analyzed that met all the quality control requirements, 37 samples (97%) were found to contain detectable levels of *Acanthamoeba* spp. with concentrations ranging from 1.22 x 10^3 copies per g to 6.06 x 10^5 copies per g. Of the 7 unimpacted soil samples analyzed that met all the quality control requirements, 7 samples (100%) were found to contain detectable levels of *Acanthamoeba* spp. with concentrations ranging from 2.61 x 10^5 copies per g to 2.86x 10^4 copies per g. There was no significant difference in prevalence or concentration of *Acanthamoeba* spp. between the sites assumed to be impacted and the sites assumed to be unimpacted by failing or non-existent wastewater treatment systems as seen in Figure 1.

Blastocystis spp, *Cryptosporidium* spp., and *Balantidium coli* were only detected in impacted soil samples. *Blastocystis* spp. were detected in 3 of 33 impacted soil samples that met all the quality control requirements (9%), with concentrations ranging from 2.12 x 10^3 copies per g to 4.08 x 10^3 copies per g. *Cryptosporidium* spp. were detected in 1 of 35 impacted soil samples that met all the quality control requirements (3%) at 7.33 x 10^2 copies per g. *Balantidium coli* were detected in 1 of 43 impacted soil samples that met all the quality control requirements (2%) at 2.87 x 10^4 copies

per g. There was no significant difference in prevalence or concentration of *Blastocystis* spp., *Cryptosporidium* spp., or *Balantidium coli* between the sites assumed to be impacted and the sites assumed to be unimpacted by failing or non-existent wastewater treatment systems as seen in Figure 1.

Rotavirus and *Astroviridae* were only detected in unimpacted soil samples. Rotavirus were detected in 1 of 7 unimpacted soil samples that met all the quality control requirements (14%) at 2.45×10^3 copies per g. *Astroviridae* were detected in 1 of 7 unimpacted soil samples that met all the quality control requirements (14%) at 5.57×10^3 copies per g. There was a significantly greater difference in prevalence and concentration of Rotavirus and *Astroviridae* at the sites assumed to be unimpacted and the sites assumed to be impacted by failing or non-existent wastewater treatment systems as seen in Figure 1.

All soil samples were also screened for host-associated genetic markers (MST markers). Two soil samples were found to have a detectable amount of human mitochondrial DNA. Human mitochondrial DNA is a genetic marker that is found in all human cells and can be used to indicate the presence of human biological material and fecal presence (9). Of the 40 impacted soil samples analyzed that met all the quality control requirements, 1 sample (3%) was found to contain detectable levels of human mitochondrial DNA at 7.41×10^2 copies per g. Of the 6 unimpacted soil samples analyzed that met all the quality control requirements, 1 sample (17%) was found to contain detectable levels of human mitochondrial DNA at 8.71×10^2 copies per g. There was no significant difference in prevalence or concentration of human mitochondrial DNA between the sites assumed to be impacted and the sites assumed to be unimpacted by failing or non-existent wastewater treatment systems as seen in Figure 1.

Detection and Quantification of Enteric Pathogens and Fecal Indicator Bacteria of Surface Water Samples.

While all the surface water samples are from “impacted” sources, the sampling locations represent two distinct spatial contexts: "localized" (collected near known or suspected discharge points) or "community" (collected from bodies of water such as ponds and streams in the community representative of broader environmental conditions). Figure 2 compares fecal markers, analyzed by culture and molecular methods, and pathogen concentrations in surface water by proximity to suspected discharges, comparing samples classified as either community or localized.

Figure 2. Comparison of Human Pathogens in Surface Water Samples Collected at Community VS. Localized Discharges Surface Water Samples by Culture and Molecular Methods. Wilcoxon rank sum p-values are shown for pairwise comparisons between surface water samples collected in the community and localized sites via culture by IDEXX and molecular methods by multi-parallel qPCR ($p < 0.05$).

Of the 24 surface water samples examined by culture, total coliforms were detected in 24 samples (100%) at a range of 109 to > 2,419.6 MPN/100 mL. Of the 24 surface water samples examined by culture for *E. coli, E. coli* was detected in 23 samples (95.8%) at a range of 0 to > 2,419.6 MPN/100 mL. Of the 12 samples examined by culture for *Enterococcus*, *Enterococcus* was detected in 12 samples (100%), at a range of 74 to > 2,419.6 MPN/100 mL (Table 2). All surface water samples collected and analyzed by culture were assumed to be in areas impacted by failing or non-existent wastewater treatment systems. The EPA criteria for recreational water quality is 33 MPN/100 mL of *Enterococcus* and 126 MPN/100 mL of *E. coli* (10). All of the surface water

samples are above the guideline for *Enterococcus* (100%) and all but six (75%) are above the guidelines for *E. coli.*

Table 4: Detection and Quantification of Total Coliform, *E. coli,* and Enterococcus in Surface Water by IDEXX.

	Total Coliform		*E. coli*			Enterococcus		
Sample Description	Prevalence (%)	Average Concentration (MPN/100 mL)	Prevalence (%)	Average Concentration Measured (MPN/100 mL)	EPA Maximum Concentration (MPN/100 mL)	Prevalence (%)	Average Concentration Measured (MPN/100 mL)	EPA Maximum Concentration (MPN/100 mL)
All	24/24 (100)	464,000± 898,000	23/24 (95.8)	305,000 ± 817,000	126	12/12 (100)	220,000 ± 694,000	33
Community	8/8 (100)	56,400 ± 101,817	7/8 (88)	304,000 ± 855,000		8/8 (100)	11,000 ± 14,800	
Localized	16/16 (100)	667,000 ± 1,050,000	16/16 (100)	305,000 ± 8226,000		16/16 (100)	324,000 ± 848,000	

Neither the prevalence nor the concentration of *E. coli* or *Enterococcus* were significantly different between the community and localized sites (Figure 2). However, the prevalence and concentration of was total coliform significantly were significantly greater at between the community sites and localized sites.

From the 25 surface water samples analyzed by multi-parallel qPCR that met all the quality control requirements, 24 samples (96%) were found to contain detectable levels of the *ybbW* gene target for *E. coli.* Of the 24 surface water samples that met all the quality control requirements, 24 samples (100%) were found to contain detectable levels of the gene targets specific to *Enterococcus* spp. lsrRNA (Table 5). The concentration of *E. coli* ranged from not detected to 7.41×10^7 copies per 100 mL. The concentration of *Enterococcus* spp. lsrRNA ranged from 2.57×10^2 to 9.12×10^6 copies per 100 mL.

Table 5: Detection and Quantification of *E. coli* and *Enterococcus* in Surface Water by Multi-parallel qPCR

	E. coli		*Enterococcus* spp. lsrRNA	
Sample Description	Prevalence (%)	Average Concentration Log_{10} (copies per 100 mL)	Prevalence (%)	Average Concentration Log_{10} (copies per 100 mL)
All	24/25 (96)	5.99 ± 1.31	24/24 (100)	4.82 ± 0.95
Community	6/6 (100)	6.49 ± 0.88	6/6 (100)	5.02 ± 0.52
Localized	18/19 (95)	5.82 ± 1.41	18/18 (100)	4.76 ± 1.06

There was no significant difference in prevalence or concentration of these indicators, *E. coli. ybbW* or *Enterococcus* spp. lsrRNA, between the community and localized sites as seen in Figure 2.

The enteric pathogens that were detected in both surface water classified as community and localized sites, besides *E. coli* ybbW and *Enterococcus* spp. lsrRNA, are *Acanthamoeba* spp., *Blastocystis* spp., *Cryptosporidium* spp., and *Plesiomonas shigelloides*. Of the 20 surface water samples classified as localized that met all the quality control requirements, 18 samples (90%) were found to contain detectable levels of *Acanthamoeba* spp. with concentrations ranging from 1.95×10^2 to 3.31×10^4 copies per 100 mL. Of the 6 community surface water samples analyzed that met all the quality control requirements, 4 samples (67%) were found to contain detectable levels of *Acanthamoeba* spp. with concentrations ranging from 1.23×10^3 to 2.75×10^3 copies per 100 mL. Of the 16 surface water samples classified as localized that met all the quality control requirements, 11 samples (69%) were found to contain detectable levels of *Blastocystis* spp. with concentrations ranging from 2.04×10^2 to 3.09×10^5 copies per 100 mL. Of the 4 community surface water samples analyzed that met all the quality control requirements, 3 samples (75%) were found to contain detectable levels of *Blastocystis* spp. with concentrations ranging from 1×10^3 to 8.91×10^4 copies per 100 mL. Of the 20 surface water samples classified as localized that met all the quality control requirements, 15 samples (75%) were found to contain detectable levels of *Cryptosporidium* spp. with concentrations ranging from 4.37×10^2 to 8.32×10^4 copies per 100 mL. Of the 6 community surface water samples analyzed that met all the quality control requirements, 6 samples (100%) were found to contain detectable levels of *Cryptosporidium* spp. with concentrations ranging from 1.51×10^2 to 1.17×10^4 copies per 100 mL. Of the 20 surface water samples classified as localized that met all the quality control requirements, 1 sample (5%) were found to contain detectable levels of *Plesiomonas shigelloides.* with concentrations at 3.8×10^2 copies per 100 mL. Of the 6 community surface water samples analyzed that met all the quality control requirements, 2 samples (33%) were found to contain detectable levels of *Plesiomonas shigelloides*. with concentrations ranging from 1.1×10^2 to 3.09×10^3 copies per 100 mL. There was no significant difference in prevalence or concentration of *Acanthamoeba* spp*., Blastocystis* spp., *Cryptosporidium* spp., or *Plesiomonas shigelloides*. between the community and localized sites as seen in Figure 2.

Rotavirus and *Yersinia enterocolitica* were only detected in community surface water samples. Rotavirus and *Yersinia enterocolitica* was found in 1 of 6 surface water samples at community sites that met all the quality control requirements (17%) at 7.94×10^2 and 2.95×10^2 copies per 100 mL respectively. There was no significant difference in prevalence or concentration in Rotavirus or *Yersinia enterocolitica* between the community and localized sites as seen in Figure 2.

Adenovirus 40/41 and *Balantidium coli* were only detected in surface water samples at localized sites. Adenovirus 40/41 was found in 1 of 19 localized surface water samples that met all the quality control requirements (5%) at 1.95×10^2 copies per 100 mL. *Balantidium coli* was found in 2 of 14 localized surface water samples that met all the quality control requirements (14%) ranging from 1.91×10^6 to 2.34×10^6 copies per 100 mL. There was no significant difference in prevalence or concentration in Adenovirus 40/41 or *Balantidium coli* between the community and localized sites as seen in Figure 2.

All surface water samples were also screened for host-associated genetic markers (MST markers). Seven surface water samples were found to have a detectable amount of human mitochondrial DNA. Of the 20 surface water samples classified as localized that met all the quality control

requirements, 6 samples (30%) were found to contain detectable levels of human mitochondrial DNA with concentrations ranging from 2.69 x 10^2 to 5.5 x 10^2 copies per 100 mL. Of the 5 community surface water samples analyzed that met all the quality control requirements, 1 sample (20%) were found to contain detectable levels of human mitochondrial DNA with concentrations at 1.05 x 10^2 copies per 100 mL. There was no significant difference in prevalence or concentration of human mitochondrial DNA between the community and localized sites as seen in Figure 2. Six surface water samples were found to have a detectable amount of Human-specific HF183 *Bacteroides.* Human-specific HF183 *Bacteroides* is a molecular marker from the human gut microbiome that is used to detect human fecal contamination (11). Of the 19 surface water samples classified as localized that met all the quality control requirements, 2 samples (11%) were found to contain detectable levels of Human-specific HF183 *Bacteroides* with concentrations ranging from 2.04 x 10^3 to 5.37 x 10^3 copies per 100 mL. Of the 6 community surface water samples analyzed that met all the quality control requirements, 4 sample (68%) were found to contain detectable levels of Human-specific HF183 *Bacteroides* with concentrations ranging from 1.62 x 10^2 to 1.17 x 10^3 copies per 100 mL. The prevalence and concentration of Human-specific HF183 *Bacteroides* were significantly greater at the community sites when compared to localized sites as seennin Figure 2.

Effect of Rainfall on the Detection and Quantification of Fecal Indicator Bacteria Analyzed Multi-parallel qPCR in Impacted Soil and Surface Water

We collected samples across three different time points, January, February, and May of 2023, to capture potential microbial variations related to rainfall. We collected samples on January 23rd, after heavy rainfall (38.6 mm in the past 48 hours), February 25th after minimal rainfall (1.01 mm in the past 48 hours), and May 13th after moderate rainfall (12.7 mm in the past 48 hours)(12). The American Meteorological Society (AMS) classifies rain in three categories: light (0-6 mm/day), moderate (6-18 mm/day), and heavy (more than 18 mm/day)(13). Therefore, according to the AMS we sampled during all three rainfall categories: heavy (January), light (February), and moderate (May). As seen in Appendix 7, rainfall categories did not significantly impact *Enterococcus* spp. lsrRNA in either sample type or *E. coli* ybbW concentrations in impacted soil samples. However, *E. coli* ybbW concentrations in impacted surface water were significantly greater during moderate when compared to light rainfall conditions. Due to the fact there was only one sample type where there was a significant difference between rainfall categories the rest of this paper will be combining the time points for analysis.

Discussion and Limitations:

The presence and extent of *E. coli* and *Enterococcus* in both the culture and molecular data suggests widespread fecal contamination in the study setting. Fecal indicator bacteria were found in high prevalence and concentration in soil and surface water by both culture and molecular methods. While the prevalence of the enteric pathogens examined were low, several targets important to public health were identified, such as *Cryptosporidium* spp., and *Blastocystis* spp. *Cryptosporidium* spp. is a protozoan parasite that can infect a wide range of animal hosts, including birds, reptiles, and mammals. The assay for *Cryptosporidium* spp. targeted the 18s rRNA region of all human-pathogenic crypto species, including *C. hominis*, *C. parvum*, *C. meleagridis*, *C. canis*, *C. felis*, *C. muris and C. suis* (14). Future analyses will include species-specific assays to interrogate the likely source (human or animal) of *Cryptosporidium* spp. within these environmental samples. *Blastocystis* spp. are enteric parasites, known to infect a range of human and animal hosts (15). Infections of *Blastocystis* spp. can cause gastrointestinal distress; carriers

may also be asymptomatic. *Blastocystis* spp. are considered to be the cause of emerging infectious disease (15), and its prevalence is thought to be higher than *Giardia* spp. and *Cryptosporidium* spp. in the United States and globally(15). In a study that examined enteric pathogens in children in the Black Belt, the prevalence of *Blastocystis* spp. in stool was found to be 3.7% (18/488) in a cohort of children from this study region (16). This gives more evidence that this target is likely to be valuable to measure the effect of the sanitation intervention.

Other pathogens of interest were detected besides *Cryptosporidium* spp. and *Blastocystis* spp. *Acanthamoeba* spp. are amoebae found in soil and water. These amoebae are pathogens that have been implicated in cases of amebic encephalitis, an infection of the central nervous system, and amebic keratitis, an infection of the eye. However, since these amoebae are free-living, and commonly found in the environment, we cannot conclusively relate their detection to sanitation infrastructure in the region. Instead, we may continue to monitor this pathogen in future work as a control. *Plesiomonas shigelloides* has been implicated in cases of childhood diarrhea and it is commonly found in surface water samples (17). *Balantidium coli,* while mostly presenting as asymptomatic, can lead to persistent diarrhea and occasionally dysentery. It presents most commonly in reservoirs where animals are kept and in areas where sanitation is poor (18). Astrovirus is also very common globally, it has been shown that 90% of children will be infected with this virus, through the fecal-oral route, by the time they are 9 years old(19). Rotavirus is another virus that is transmitted through the fecal-oral route and commonly linked to limited sanitation service (20). All of these pathogens were also found in the stool of children living in the Black Belt in a previous study (16).

Our study had several limitations. First, we determined assay lower limits of detection (LLOD) using criteria outlined in Sahoo et. al, which defines the y-intercept of the standard curve as "the theoretical limit of detection of the assay" (8) and may differ from other methods of empirically deriving detection limits. We therefore excluded some samples with Ct values near the LLOD as a conservative approach to pathogen detection via molecular methods. We necessarily omitted some presumptive detections at the LLOD as potentially spurious (e.g., one detection of *Giardia* spp in surface water) as a conservative approach. Details on samples can be found in Appendix 8. Second, the typology of impacted and unimpacted sampling sites were limited by the potential subjectivity of researcher observations in the field. Since all environmental samples were collected in public spaces around the community, we were not certain to what degree each site was impacted by the sanitation status in the community, beyond directly observable criteria such as proximity to a failing septic system or direct surface discharge. Sites may have been categorized incorrectly, for example if unobserved discharges were present. Third, limiting our sampling to publicly accessible sites may have constrained our analysis by excluding potentially important (but inaccessible) sites on private property. For example, households with failing septic systems may have sewage pooling in the yard around the tank or drain fields. In this case, the "impacted" site would be on private property and therefore unavailable given the restraints of this study. In future sampling, researchers will be sampling from sites at households that have enrolled into a prospective impact study. Finally, the sample size for culture data was relatively small, limiting statistical power. Out of the 123 samples that were collected across three timepoints, only 43 samples (35%) were cultured at all, and out of the samples that were cultured, not all of them were cultured for the same organism. This more than likely affected the analysis of the culture data and

limited the conclusions we could draw about what these samples tell us about the potential exposure relevance of sanitation infrastructure in this area.

Despite limitations, initial results show that widespread sanitation deficits in the study setting present pathogen hazards in the community. These hazards are most commonly associated with uncontained fecal waste discharges. Planned sanitation infrastructure expansion may be effective in limiting the potential for exposures to residents which in turn could improve overall health and well-being.

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Supporting Information:
S1 Appendix. Example of the Membrane Filtration Set Up.
S2 Appendix. General Descriptive Information for Environmental Samples.
S3 Appendix. Raw Culture Data for Environmental Samples Analyzed via IDEXX.
S4 Appendix. qPCR primer and probe sequences for TaqMan Array Card.
S5 Appendix. Quality Control Findings: Detection of Targets in NEC and PEC Samples.
S6 Appendix. Raw qPCR Data for Environmental Samples Analyzed via TaqMan Array Card.
S7 Appendix. Quantification of Fecal Indicator Bacteria (*E. coli* ybbW and *Enterococcus* spp. lsrRNA) from impacted Environmental Samples in January, February, and May 2023.
S8. Excluded Samples with Ct Values Near the Limit of Detection.

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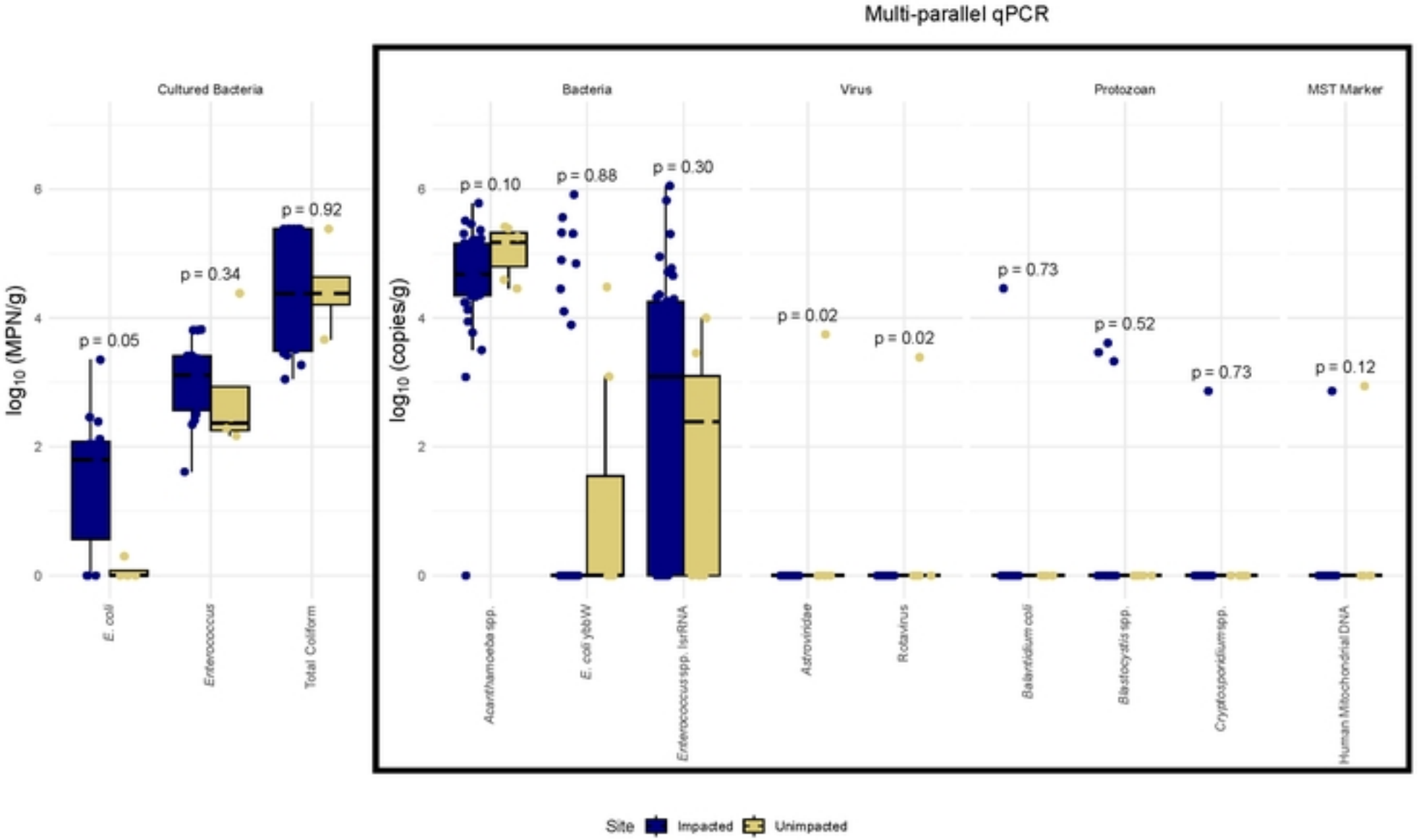
Multi-parallel qPCR
Cultured Bacteria
Bacteria
Virus
Protozoan
MST Marker
log10 (MPN/g)
log10 (copies/g)
p = 0.05
p = 0.34
p = 0.92
p = 0.10
p = 0.88
p = 0.30
p = 0.02
p = 0.02
p = 0.73
p = 0.52
p = 0.73
p = 0.12
E. coli
Enterococcus
Total Coliform
Acanthamoeba spp.
E. coli yebW
Enterococcus spp. IsrRNA
Astroviridae
Rotavirus
Balantidium coli
Blastocystis spp.
Cryptosporidium spp.
Human Mitochondrial DNA
Site
Impacted
Unimpacted

Figure1

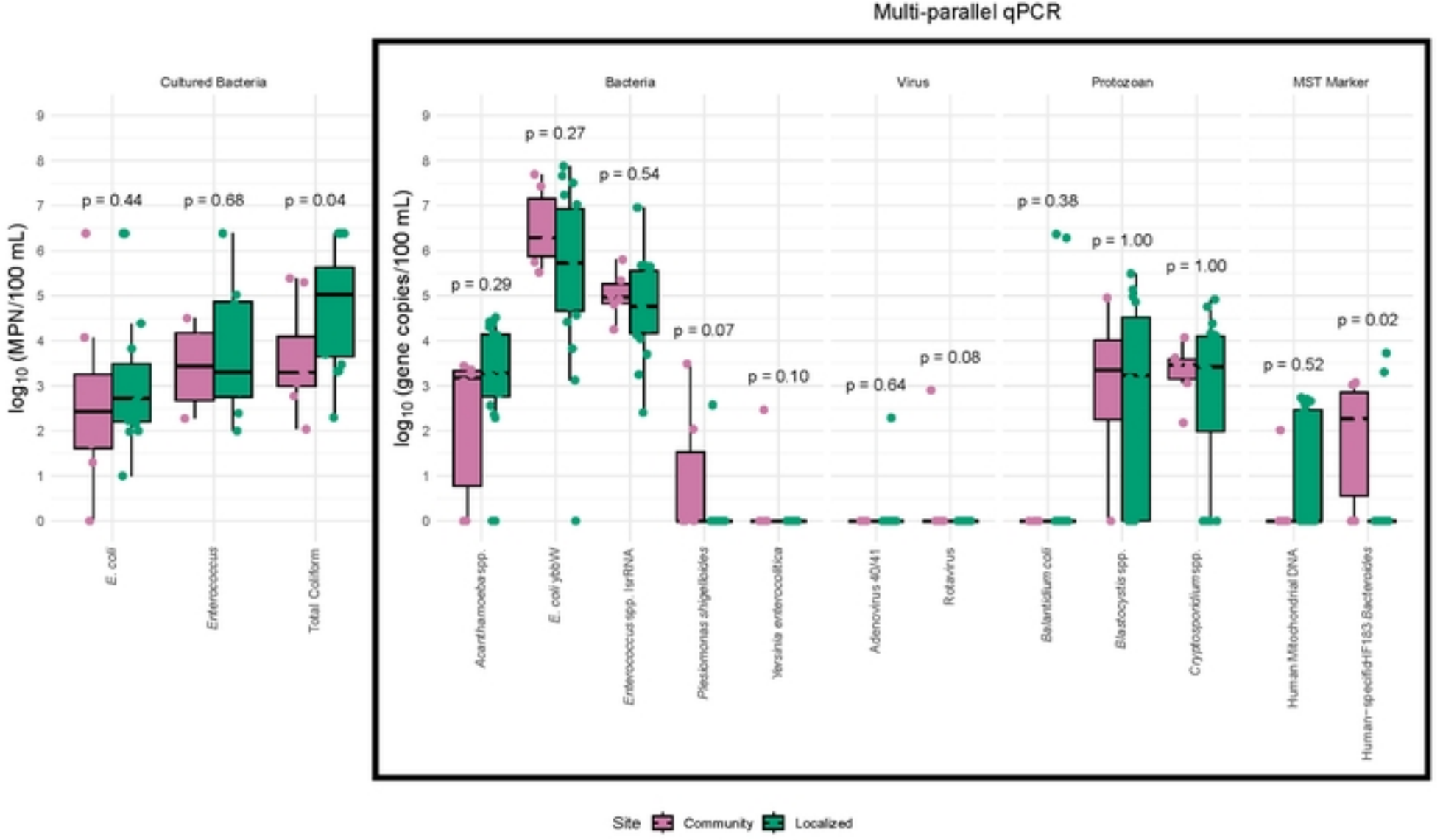
Multi-parallel qPCR
Cultured Bacteria
Bacteria
Virus
Protozoan
MST Marker
log10 (MPN/100 mL)
log10 (gene copies/100 mL)
p = 0.44
p = 0.68
p = 0.04
p = 0.29
p = 0.27
p = 0.54
p = 0.07
p = 0.10
p = 0.64
p = 0.08
p = 0.38
p = 1.00
p = 1.00
p = 0.52
p = 0.02
E. coli
Enterococcus
Total Coliform
Acanthamoeba spp.
E. coli ybbW
Enterococcus spp. IsrRNA
Pleisomonas shigelloides
Yersinia enterocolitica
Adenovirus 40/41
Rotavirus
Balantidium coli
Blastocystis spp.
Cryptosporidium spp.
Human Mitochondrial DNA
Human-specific HF183 Bacteroides
Site
Community
Localized

Figure2