CHARACTERIZING DOMESTIC FECAL TRANSMISSION AND CHILD ENTERIC INFECTIONS DURING AN URBAN ONSITE SANITATION INTERVENTION

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ABSTRACT

David A. Holcomb: Characterizing Domestic Fecal Transmission and Child Enteric Infections during an Urban Onsite Sanitation Intervention
(Under the direction of Jill Stewart)

Several recent water, sanitation, and hygiene (WaSH) intervention studies to improve child health have highlighted the need for better understanding the environmental transmission and exposure patterns that drive fecal-oral disease. Most evaluations of sanitation interventions have been conducted in rural settings, even as an increasing proportion of the global population lives in crowded, informal urban settlements that lack basic services. We characterized fecal contamination in Mozambican households participating in the first rigorous evaluation of urban onsite sanitation (MapSan: the Maputo Sanitation Study), immediately before and one year after half the compounds replaced pit latrines in poor condition with pour-flush to septic systems. We measured general and host-associated fecal microbes at potential domestic exposure points, including household stored water, entrance soil, and food preparation surfaces, and compound source water and latrine entrance soil. Samples were analyzed using five locally validated microbial targets: culturable general fecal indicator *E. coli* (cEC), molecular *E. coli* marker EC23S, human-associated molecular markers HF183 and Mnif, and avian-associated molecular marker GFD. For each microbial target and sample type, we assessed pre-intervention associations with sociodemographic, meteorological, and physical sample characteristics, and employed a difference-in-differences (DID) approach to isolate intervention effects. We also

investigated associations between source-specific measures of domestic fecal contamination and objectively measured enteric infection in children. The domestic environment was heavily impacted by both general and human-source fecal contamination, and the majority of children were infected with multiple enteric pathogens. Associations with fecal contamination for both pre-intervention risk factors and child enteric infections were generally small and inconsistent in direction for different targets and sample types. The intervention also was not consistently associated with a change in *E. coli* concentrations or the odds of human target detection. Our results describe a setting impacted by pervasive domestic fecal contamination, including from human sources, that is largely disconnected from local variation in socioeconomic and sanitary conditions. This pattern suggests that in such heavily burdened settings, transformational changes to the community environment may be required before meaningful impacts on fecal contamination can be realized.

Melissa, you willed this one into existence.

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BLAST	Basic Local Alignment Search Tool
CBA	controlled before-and-after
cEC	cultured *Escherichia coli*
cfu	colony forming units
CI	confidence interval
Cq	quantification cycle/cycle threshold
DALY	disability adjusted life year
DID	difference-in-differences
DNA	deoxyribonucleic acid
FIB	fecal indicator bacteria
FIO	fecal indicator organism
FN	false negative
FP	false positive
gc	gene copies
GPP	Gastrointestinal Pathogen Panel
GPS	Global Positioning System
INS	National Institute of Health of Mozambique
IQR	interquartile range
JMP	Joint Monitoring Programme for Water Supply and Sanitation
LLoD	lower limit of detection
LLoQ	lower limit of quantification

LMIC	low- and middle-income countries
MapSan	Maputo Sanitation trial
MICE	multivariate imputation by chained equations
MISAU	Ministry of Health of Mozambique
MLE	maximum likelihood estimate
MST	microbial source tracking
mTEC	membrane thermotolerant *Escherichia coli*
NEC	negative extraction control
NFW	nuclease-free water
OR	odds ratio
PBS	phosphate buffered saline
PC	positive extraction control
PCR	polymerase chain reaction
POR	prevalence odds ratio
Q	target quantity (gene copies or colony forming units)
qPCR	(quantitative) real-time polymerase chain reaction
RFU	relative fluorescence units
ROC	receiver operator characteristic
rRNA	ribosomal ribonucleic acid
rxn	reaction
SD	standard deviation
SE	standard error
SPC	specimen processing control

tLLoD	theoretical lower limit of detection
TN	true negative
TNTC	too numerous to count
TP	true positive
ULoQ	upper limit of quantification
UNICEF	United Nations Children's Fund
USEPA	United States Environmental Protection Agency
VBNC	viable but non-culturable
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

Numerous and diverse pathogens can be shed in feces (Delahoy et al., 2018; Liu et al., 2016). Human feces have generally been viewed as presenting a greater hazard to human health, as any pathogens they contain necessarily infect humans (Field and Samadpour, 2007). However, human pathogens may also be shed in the feces of other animals, and exposure to domestic animals and livestock is associated with human illness (Conan et al., 2017; Delahoy et al., 2018; Penakalapati et al., 2017). Infection by fecal pathogens can result in diarrhea, malnutrition, growth faltering, cognitive delays, environmental enteric dysfunction, and death (Brown et al., 2013). Diarrhea, traditionally the enteric infection-related outcome most commonly assessed, accounts for a half-million annual deaths in children less than five years old—8.6% of all child mortality in 2015 (Schmidt et al., 2011, 2007; Wang et al., 2016). As the second-leading cause of death for children under five, it is estimated that nearly two-billion cases occur annually, most of which do not result in death, but are associated with stunting and malnutrition and account for 6.6% of disability adjusted life years (DALY) globally (Danaei et al., 2016; Fischer Walker et al., 2013; Stone et al., 2010). The diarrheal burden is greatest among infants 6-11 months old, with an average of 4.5 cases per year (Walker et al., 2012). Downstream implications include the 443 million days of school missed each year due to such illnesses (Moszynski, 2006).

While biologically diverse, fecal pathogens share the fecal-oral route of transmission, in which pathogens shed in the feces of an infected host are ingested by a new host, leading to further infection (Sobsey, 2015). Fecal-oral pathogen transmission is largely mediated through six principal environmental reservoirs: hands, water, soil, food, flies, and surfaces (Julian, 2016;

Wagner and Lanoix, 1958). Fecal transmission through the environment has long been conceptualized using the "F-diagram" (Figure 1.1), in which the path from infected feces to ingestion by the new host is presented unidirectionally through each reservoir; frequently, bars are placed across specific pathways representing interventions intended to interrupt transmission through the associated reservoirs (Mara et al., 2010; Wagner and Lanoix, 1958). The typical strategy for preventing fecal-oral infections is to interrupt environmental transmission through clean water, hand and food hygiene practices, and sanitation, the safe containment, removal, transport, treatment, and disposal or reuse of fecal waste, collectively known as "WaSH" (Bartram and Cairncross, 2010; Berendes et al., 2017; Blackett et al., 2014; Brown et al., 2013).

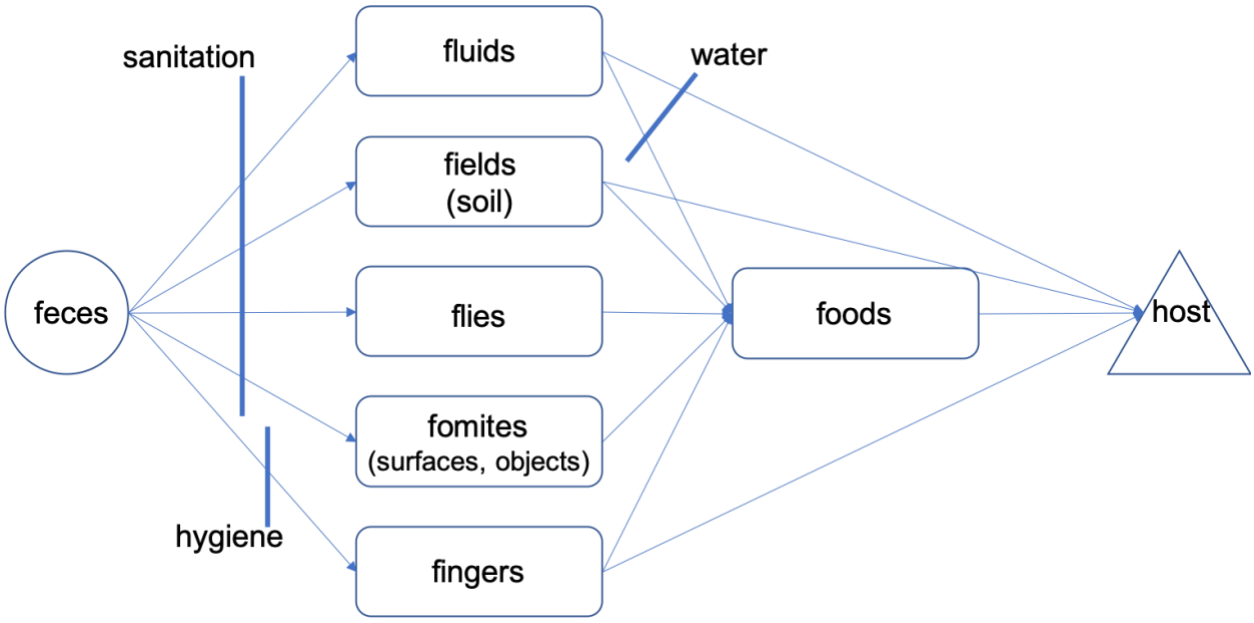

Figure 1.1: The F-diagram of fecal-oral disease transmission. Adapted from Wagner and Lanoix (1958) and Mara et al. (2010).

By separating pathogens from the environment at the point of origin, sanitation has the potential to interrupt the greatest number of pathways (Julian, 2016; Kawata, 1978; Mara et al., 2010). A recent meta-analysis found household sanitation reduced the risk of diarrheal disease by 16% on average, and an estimated 432,000 deaths and 26 million DALYs from diarrhea in 2016 were attributable to inadequate sanitation (Prüss-Ustün et al., 2019; Wolf et al., 2018). However,

a number of recent sanitation intervention trials have struggled to reduce both fecal-oral disease outcomes and measures of environmental fecal contamination, suggesting that the interventions did not fully interrupt the dominant transmission pathways in the specific context of each trial (Cameron et al., 2013; Clasen et al., 2014; Humphrey et al., 2019; Luby et al., 2018; Null et al., 2018; Patil et al., 2014; Pickering et al., 2015; Sclar et al., 2016; Sinharoy et al., 2017). The model presented in the F-diagram of unidirectional transmission separately through each reservoir, each pathway exposed to complete interruption by dropping in the appropriate intervention, is a dramatic oversimplification for many settings (Julian, 2016). In particular, the domestic environments in low- and middle-income countries (LMIC) are often characterized high levels of transmission through multiple interacting and cyclical pathways (Vujcic et al., 2014). Measures of fecal contamination along these pathways have typically been elevated and seldom, if ever, associated with household sanitary conditions other than direct interventions on specific pathways, such as household water treatment (Ercumen et al., 2018a, 2018b; Harris et al., 2016; Pickering et al., 2018; Sclar et al., 2016).

Fecal transmission has typically been assessed by testing samples of environmental reservoirs from different transmission and exposure pathways for fecal microbes, which serve as indicators of fecal contamination given the historic difficulty of direct pathogen detection in the environment (Field and Samadpour, 2007; Levy et al., 2012). Traditionally, easily culturable, non-specific fecal microbes have been targeted as fecal indicators. Recent advances in molecular detection has enabled molecular microbial source tracking (MST), in which the genes of microbes associated with the feces of a particular animal host are assayed to determine fecal source. Studies applying MST approaches to domestic settings have repeatedly identified domestic animals and livestock as key fecal sources, which generally are unaddressed by

household sanitation projects that focus on containing human excreta produced onsite (Boehm et al., 2016; Harris et al., 2016; Odagiri et al., 2016; Schriewer et al., 2015).

The purpose of this work is to evaluate the effects of sanitation on domestic fecal transmission in an urban setting. We apply microbial source tracking approaches to samples from multiple transmission pathways to characterize the sources and patterns of contamination, and relate these measures of fecal exposure to objectively measured health outcomes in children. Following this introduction, the dissertation is organized into three research chapters and a conclusion.

In Chapter 2: "Local Validation of Host-Associated Fecal Indicators and Risk Factors of Human Fecal Contamination in Urban Mozambican Households", we assess pre-intervention patterns of fecal contamination and their associations with socioeconomic, sanitary, and meteorological characteristics of the study sites. We provide detailed descriptions of the environmental sampling, laboratory, and data processing methods used throughout this work. We also describe the performance validation of a set of candidate microbial source tracking assays using fecal samples collected from the study area.

Chapter 3: "Impact of an Onsite Sanitation Intervention on Human Fecal Contamination of the Domestic Environment in Urban Maputo, Mozambique" describes the occurrence of several fecal microbes both before and after the intervention by treatment assignment. We present a difference-in-differences analysis of the intervention impact on traditional fecal indicators and host-associated microbial source tracking markers in multiple domestic transmission pathways.

An investigation of links between different measures of fecal contamination and objective measures of enteric infection in children is presented in Chapter 4: "Associations

Between Fecal Indicators and Child Health." We characterize enteric infection by testing child stool for 23 pathogens and conduct regression analysis to identify associations between both traditional and host-associated fecal microbes and infection by pathogen class for each domestic transmission pathway.

In Chapter 5: "Conclusion", we place our results and their limitations in the context of the broader literature. We explore the value of sanitation in the beyond enteric health impacts and suggest research avenues that may warrant greater focus.

CHAPTER 2: LOCAL VALIDATION OF HOST-ASSOCIATED FECAL INDICATORS AND RISK FACTORS OF HUMAN FECAL CONTAMINATION IN URBAN MOZAMBICAN HOUSEHOLDS

2.1 Introduction

Children who lack access to adequate sanitation suffer disproportionately from fecal-oral diseases, with symptoms including diarrhea, malnutrition, stunting, cognitive delay, and lasting damage to the gut (Brown et al., 2013; Prüss-Ustün et al., 2014). Diarrhea alone kills half a million children each year and is the second-leading cause of death globally for children under five (Wang et al., 2016). Strategies to prevent fecal-oral disease often focus on interrupting fecal pathogen transmission, which is mediated by six principle environmental reservoirs: hands, water, soil, food, flies, and surfaces (Julian, 2016; Wagner and Lanoix, 1958). However, a single reservoir may be contaminated by feces with different origins transmitted by multiple pathways with complex or cyclical interactions between them (Ercumen et al., 2017b; Harris et al., 2013; Schriewer et al., 2015; Vujcic et al., 2014). Several recent WaSH intervention studies to improve child health have highlighted the need for better understanding the environmental transmission and exposure patterns that drive fecal-oral disease (Cameron et al., 2013; Clasen et al., 2014; Luby et al., 2018; Null et al., 2018; Patil et al., 2014; Pickering et al., 2015; Sclar et al., 2016; Sinharoy et al., 2017). To date, such efforts have been focused largely in rural settings, even as an increasing proportion of the global population lives in crowded, informal urban settlements that lack basic services (Ercumen et al., 2018b, 2018a; Galli et al., 2014; Hawkins et al., 2013; Pickering et al., 2018).

Characterizing fecal origins is crucial to effectively intervening against pathogen transmission—domestic sanitation, for instance, only addresses human excreta produced onsite and would be ineffective where feces of exogenous origin or non-human source dominate. Fecal transmission has traditionally been evaluated by measuring fecal indicator organisms (FIO) like *Escherichia coli*, which are associated with disease outcomes but do not identify the pathways responsible—though clever sampling design can enable some transmission dynamics to be inferred (Ercumen et al., 2017b; Gruber et al., 2014). Sanitation interventions intended to interrupt domestic fecal transmission have generally not demonstrated an impact on measures of traditional FIO among multiple pathways, suggesting that the dominant transmission dynamics were not addressed (Sclar et al., 2016).

Recent technological advances enable molecular microbial source tracking (MST), in which samples are tested for the genetic material of bacteria specific to the gut of a particular host (e.g., human) to determine fecal source (Harwood et al., 2014). Several studies applying MST in developing countries have implicated livestock as a major source of domestic fecal contamination (Boehm et al., 2016; Harris et al., 2016; Odagiri et al., 2016; Schriewer et al., 2015). Because MST targets the gut microbiota, which varies among populations, it is necessary to validate MST assays in each new location to determine whether the selected microbial targets are both present and unique to the intended fecal source in the study area (Stewart et al., 2013). Most MST assays were developed for water quality monitoring purposes and some have often performed poorly when applied to domestic samples in highly-contaminated settings, highlighting not only the importance of diagnostic validation but also of understanding ecological, physical, and sociodemographic factors that may be driving local fecal transmission dynamics (Harris et al., 2016; Odagiri et al., 2015). We validated and applied a set of molecular

MST assays in households sharing poor-quality sanitation facilities in Maputo, Mozambique and assessed risk factors of general and human-source specific fecal contamination in multiple domestic transmission pathways.

2.2 Methods

2.2.1 Study setting

We conducted this study in the context of the Maputo Sanitation (MapSan) trial, a controlled, before-and-after study of urban sanitation and child health (Brown et al., 2015). The majority of households in Maputo (89%) use onsite sanitation (Blackett et al., 2014), much of which fails to meet the UNICEF/WHO Joint Monitoring Programme definition for "improved" sanitation, to which barely half of urban Mozambicans are estimated to have access (WHO/UNICEF, 2017). Frequent flooding, high population density, and inadequate management of three-quarters of the city's fecal waste contribute to a large burden of enteric infection and child mortality (Blackett et al., 2014; Knee et al., 2018; Sitoe et al., 2018; UN-HABITAT, 2014). The MapSan trial is evaluating a privately shared latrine intervention implemented in compounds—defined household clusters sharing an outdoor courtyard—with existing sanitation facilities in poor condition and shared by the households in the compound. Frequency-matched control compounds with similarly poor-quality shared sanitation were enrolled concurrently from the same unplanned, low-income neighborhoods of urban Maputo. We conducted a cross-sectional baseline assessment of domestic fecal contamination at an opportunistically selected subset of study compounds from both treatment arms as they were enrolled in the MapSan pre-intervention survey in May – August 2015.

2.2.2 Ethics Statement

This study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (IRB # 15-0963). The associated MapSan trial was pre-registered at ClinicalTrials.gov (NCT02362932) and was approved by the Comité Nacional de Bioética para a Saúde (CNBS), Ministério da Saúde, Republic of Mozambique (333/CNBS/14), the Ethics Committee of the London School of Hygiene & Tropical Medicine (reference # 8345), and the Institutional Review Board of the Georgia Institute of Technology (protocol # H15160). Environmental samples were collected only from households with children enrolled in the MapSan study, for whom a parent or guardian had provided written informed consent. Verbal assent was obtained from the head of each compound prior to initiating child enrollment and sampling activities.

2.2.3 Sample collection

We sampled three environmental reservoirs of fecal contamination—water, soil, and surfaces—at five nodes of potential transmission and exposure: source water, stored water, food preparation surfaces, latrine entrance soil, and household entrance soil. Source water and latrine soil samples were collected once from each compound, while stored water, food surfaces, and household soil samples were collected from each household with children enrolled in the MapSan trial. We also collected fecal material from study compounds to validate candidate MST assays. Due to the additional equipment and access required, we sampled latrine sludge during separate visits to certain compounds. Fresh feces were collected from animals observed defecating during any sampling visit. Sampling was conducted in the mornings, and all samples were immediately placed on ice for transport and maintained at 4 °C until processed, within 8

hours of collection for water samples and 30 hours for soil and surface samples. Fecal and sludge samples were immediately archived at -80 °C upon laboratory arrival.

Water samples were collected in approximately 1 L volumes in sterile plastic sample bags and immediately treated with approximately 20 mg of sodium thiosulfate (Brim Technologies, Eatontown, NJ, USA) to neutralize residual chlorine. Source water samples were collected directly. We asked a resident of each enrolled household to provide drinking water from a storage container as if they were giving water to a child to drink. We then asked the respondent to pour the water into a sterile sample bag and repeat the process until approximately 1 L was collected. The storage container material, mouth width, presence of a lid, and water extraction method were recorded. We also asked the household respondent to provide a surface regularly used to prepare foods in the condition in which it would typically be used. A 10 cm x 10 cm template was disinfected with 10% bleach followed by 70% ethanol and placed on the surface. A sterile, flocked nylon swab (Copan Diagnostics, Murrieta, CA, USA) was wetted in a centrifuge tube containing 12 mL sterile ¼-strength Ringer's solution (Oxoid, Hampshire, UK) and swabbed within the template in 3 directions to ensure complete coverage (Hedin et al., 2010; Moore and Griffith, 2007). The swab was clipped with disinfected scissors into the centrifuge tube, and the surface was again swabbed with a second, dry swab to collect any remaining wetting solution; the second swab was likewise clipped into the same tube. On surfaces with sufficient area, this procedure was repeated on a second 100 cm^2 area to produce a replicate swab sample. The surface type (e.g., table or bowl) and material were recorded.

Soil was collected 1 m in front of the compound latrine entrance and 1 m in front of the primary entrance to each household with enrolled children. A 10 cm x 10 cm square was drawn in the soil using a metal scoop disinfected with 10% bleach and 70% ethanol. We used the scoop

to gently homogenize the top 1 – 2 cm of soil and transfer it to a sterile sample bag (Pickering et al., 2012). Qualitative assessment of soil exposure to sunlight (full sun, partial sun, or shade) and any signs of visible surface wetness were recorded. Entrances fully covered in impervious surface were not sampled. We collected animal feces in a similar manner using a disinfected metal scoop to transfer individual stools into a sterile sample bag.

We sampled latrine sludge by attaching a sterile 50 mL centrifuge tube perpendicularly to a metal handle of sufficient length to lower through a latrine drophole to reach the sludge surface. This apparatus was used to scrape sludge into the tube from at least three locations on the sludge surface to collect a diverse sample.

2.2.4 Validation of microbial source tracking assays

2.2.4.1 DNA isolation from fecal samples

DNA was extracted from fecal samples in Maputo using the FastPrep SPIN Kit for Soils (MP Biomedicals, Santa Ana, CA, USA) and stabilized with DNAstable Plus (Biomatrica, San Diego, CA, USA) for ambient temperature transport to the US for further analysis. After lysing 500 mg thawed fecal sample in the supplied bead tubes by vortexing at maximum speed for 15 minutes, we completed the extractions according to the manufacturer protocol using a final elution volume of 70 µL. Eluted DNA was treated with 17.5 µL DNAstable Plus and maintained at room temperature for up to 14 days during transport to the United States, after which samples were stored at 4 °C and analyzed within 6 months. Latrine samples were extracted in duplicate, and an extraction blank was processed with each sample batch.

2.2.4.2 Identification of candidate MST assays

We considered open-source qPCR assays targeting general, human, and avian fecal microbes to assemble a panel of candidate MST markers (Table 2.1). Preference was given to

assays previously validated in multi-laboratory comparison studies, as well as ensuring a variety of organisms and gene targets were represented among the candidates (Johnston et al., 2013; Layton et al., 2013). We prioritized human source-associated assays in light of the concurrent intervention trial intended to reduce human fecal contamination, and considered avian-associated assays owing to the frequent observation of chickens and ducks in study compounds. While cats, dogs, goats, and pigs were also observed with varying frequency, we were unable to collect sufficient fecal samples from these sources to adequately validate any other animal-associated assays. We considered assays for non-host specific fecal microbes as a basis for relating molecular detection to culture-based detection of general fecal indicators.

Table 2.1: Candidate qPCR assays for microbial source tracking

target	host	organism/gene	class	assay	chemistry
BacUni	general	*Bacteroidales* 16S	bacterium	BacUni-UCD (Kildare et al., 2007)	TaqMan
EC23S	general	*E. coli* 23S	bacterium	EC23S857 (Chern et al., 2011)	TaqMan
BacHum	human	*Bacteroidales* 16S	bacterium	BacHum-UCD (Kildare et al., 2007)	TaqMan
HAdV	human	Adenovirus hexon gene	virus	HAdV (Jothikumar et al., 2005)	TaqMan
HF183	human	*B. dorei* 16S	bacterium	HF183/BacR287 (Green et al., 2014)	TaqMan
Mnif	human	*M. smithii nifH*	archaeon	Mnif (Johnston et al., 2010)	TaqMan
GFD	avian	*Helicobacter spp.*	bacterium	GFD (Green et al., 2012)	SYBR
LA35	avian	*Brevibacterium sp.* 16S	bacterium	LA35 (Weidhaas et al., 2010)	SYBR

2.2.4.3 Reference material for qPCR standard curves

Nucleotide Basic Local Alignment Search Tool (BLAST) searches were performed with the published primers and probe sequences for each candidate assay to ensure published sequence accuracy and to obtain the expected amplicon sequence (Agarwala et al., 2016). The matching amplicon sequence and ten additional bases on both ends were extracted from the

GenBank database to serve as reference sequences (Clark et al., 2016). Because all three assays targeting *Bacteroidales* 16S rRNA genes matched the same *B. dorei* gene sequence, a single reference sequence was extracted spanning the entire region targeted by these assays. The reference sequences obtained for the avian-associated assays were concatenated to construct a composite reference sequence for both assays. The reference sequences for the remaining (non-avian, non-16S) assays were likewise concatenated. These three composite reference sequences were commercially synthesized as artificial linear plasmids (Integrated DNA Technologies, Skokie, Il, USA) to serve as standard reference material for all candidate assays (Kodani and Winchell, 2012; Liu et al., 2013).

2.2.4.4 Validation qPCR

We validated the candidate assays against each fecal sample using singleplex qPCR. All validation reactions consisted of 12.5 µL TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA), 2.5 µL 10x primers and probe mix, and 10 µL of DNA template, for a total reaction volume of 25 µL (Odagiri et al., 2015). Reactions were performed on a CFX96 Touch thermocycler (Bio-Rad, Hercules, CA) with an initial 10-minute incubation at 95 °C, followed by cycles of denaturation and annealing for the durations, temperatures, and cycle numbers described in the original published protocol for each assay. Both ten-fold and hundred-fold sample dilutions were used as DNA template to account for potential PCR inhibition (Odagiri et al., 2015). We ran each sample dilution in duplicate, including duplicate ten-fold standard dilution series from $10^7 – 10^1$ copies (gc) of artificial plasmid standard and four non-template control (NTC) reactions on each instrument run. Raw qPCR output was processed using CFX Manager software (Bio-Rad) to calculate quantification cycle (Cq) values using the

baseline subtraction method with a 100 RFU florescence threshold (Cao et al., 2012; Layton et al., 2013).

2.2.4.5 Assay performance evaluation

We evaluated candidate assays primarily on the basis of binary diagnostic performance. The microbial target of a given assay was considered detected in reactions producing a Cq value lower than an assay-specific cutoff point. We considered a fecal sample positive for a given microbial target if the target was detected in any reactions containing DNA template from the sample. Latrine sludge samples were used as human fecal sources, duck and chicken samples represented avian fecal sources, and dog and pig fecal samples were non-target sources for all host-associated assays. General fecal assays were considered associated with all fecal samples for the purposes of performance evaluation. For each assay, we counted the true positive (TP) and false negative (FN) fecal samples from its associated animal host, as well as the true negative (TN) and false positive (FP) samples from non-associated fecal sources. We characterized diagnostic performance as the proportion of host samples correctly identified (sensitivity), the proportion of non-host samples in which the microbial target was not detected (specificity), and the proportion of all samples correctly identified (accuracy), as follows:

$$sensitivity = \frac{TP}{TP + FN} \quad \textbf{(2.1)}$$

$$specificity = \frac{TN}{TN + FP} \quad \textbf{(2.2)}$$

$$accurracy = \frac{TP + TN}{TP + FP + TN + FN} \quad \textbf{(2.3)}$$

2.2.4.6 Determination of optimal cycle cutoff points

To reduce the potential for false positives from amplification artifacts, we used receiver operator characteristic (ROC) analysis to obtain assay-specific cycle cutoff points for determining reaction detection status (Nutz et al., 2011). ROC curves were generated for cycle cutoffs in one Cq increments from 10 Cq to the maximum number of cycles described by the assay developers. Reactions with Cq values below the cutoff point were classified as positive and above the point as negative. Diagnostic sensitivity and specificity were calculated from all reactions (including extraction blanks) at each cutoff point. The highest whole Cq value that maximized the Youden index, computed as $J = sensitivity + specificity - 1$, was selected as the optimal cutoff point for each assay (Fluss et al., 2005; Nutz et al., 2011).

2.2.5 Microbial analysis of environmental samples

2.2.5.1 Filtering and culture-based analysis

Environmental samples were processed by membrane filtration prior to further microbial analysis. We filtered water samples without further processing and filtered eluate from surface swabs and soil samples. Food preparation surface swabs were eluted into the 12 mL quarter-strength Ringer's solution in the 15 mL centrifuge tubes into which they had been clipped in the field. Elution was accomplished through vigorous manual shaking of the tubes for 60 seconds (Pickering et al., 2012). We eluted soil samples by adding 1 g wet soil to 100 mL sterile, distilled water in a sterile sample bag and vigorously shaking by hand for 60 seconds. After settling for 15 minutes, 50 mL supernatant was extracted, deposited in a sterile sample bag, and used for filtrations (Boehm et al., 2009; Pickering et al., 2012). We determined soil moisture content by drying approximately five g wet soil by microwave oven until no additional changes in mass were detected.

Culture-based enumeration of *Escherichia coli* was performed following a modification of USEPA Method 1603 on 0.45 µm cellulose ester membranes (MilliporeSigma, Burlington, MA, USA) (USEPA, 2009). Filters were placed on sterile cellulose pads (Pall, Port Washington, NY, USA) saturated with modified mTEC broth (HiMedia, Mumbai, India) in sterile 50 mm metal plates and incubated at 44.5 ± 0.5 °C for 22 – 26 hours. We filtered 100 mL and 10 mL volumes of water samples and 1 ml and 0.1 mL of surface swab and soil eluate. Approximately 25 mL sterile PBS was added to the filter column before adding any sample volumes of 10 mL or less. If the lowest volume plate for a given sample was too numerous to count (TNTC), we filtered an additional 0.1 mL water sample and 0.01 mL for swab or soil eluate.

Following membrane filtration for culture-based *E. coli* enumeration, we filtered a larger volume of sample through the same column for molecular analysis. Up to 300 mL water, 12 ml swab eluate, and 30 mL soil eluate were filtered through 0.4 µm polycarbonate membranes (MilliporeSigma), which were folded into 2 mL cryovials and immediately archived at -80 °C. Filters were transported frozen on dry ice from Maputo to the United States and stored at -80 °C until DNA extraction, with the exception of eight surface swab filters that experienced room temperature conditions for approximately 24 hours before extraction.

2.2.5.2 DNA isolation from filtered samples

Anticipating heightened concentrations of both target DNA and PCR inhibitors in soil and surface swab samples relative to water samples, the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) was used to extract DNA from soil and swab sample filters, while the DNA-EZ ST01 (GeneRite, North Brunswick, NJ, USA) kit was chosen for water samples (Cox and Goodwin, 2013). To further address potential inhibition and provide a specimen processing control (SPC), 3 µg salmon testes DNA (MilliporeSigma) was added to all extraction bead tubes prior to

loading sample filters (Haugland et al., 2005, 2012). In each extraction batch, two tubes were filled with blank filters to serve as negative and positive extraction controls (NEC and PC, respectively), the PC spiked with 2×10^8 copies of each artificial plasmid standard. Samples were lysed with a Mini-Beadbeater (BioSpec, Bartlesville, OK, USA) at maximum speed for 120 seconds, after which we followed the PowerSoil manufacturer protocol or the Source Identification Protocol Project DNA-EZ ST01 protocol as appropriate (Boehm et al., 2013; Griffith et al., 2013). Purified DNA was eluted with 100 µL elution buffer, aliquoted in 25 µL volumes, and immediately stored at -80 °C, retaining one aliquot for further evaluation. The remaining aliquot was stored at 4 °C for up to 72 hours before measuring DNA concentration with a NanoDrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA) and testing for PCR inhibitors.

2.2.5.3 Molecular detection of microbial targets

We assessed microbial targets in environmental samples using four qPCR assays selected from the candidate set—EC23S, HF183, Mnif, and GFD—and assessed PCR inhibition with a fifth qPCR assay, Sketa22. Each reaction consisted of 12.5 µL TaqMan Environmental Master Mix 2.0, 2.5 µL 10x primers and probe mix, 5 µL nuclease free water (NFW), and 5 µL of DNA template, for a total reaction volume of 25 µL. Cycling conditions were identical to the validation analysis. Template DNA was used undiluted unless a specific sample was determined to be inhibited, in which case DNA was diluted five-fold (Haugland et al., 2012). We considered a sample to be inhibited if the Cq value for the Sketa22 assay was >3 Cq above the mean Cq of extraction controls (NEC and PC). Reactions were performed in duplicate for 10% of samples selected randomly within the set of samples of each type. Each 96-well reaction plate typically contained samples from three extraction batches, resulting in three NECs and three PCs per plate,

as well as three NTC reactions. We prepared five-point, ten-fold dilution series from each of the three PCs on a given plate, corresponding to triplicate reactions of 10^5 – 10^1 copies of each artificial plasmid standard before DNA extraction. All samples analyzed on the same plate were extracted using the same extraction method.

2.2.5.4 Calibration curve construction

We estimated microbial target abundance in environmental samples from observed Cq values using calibration curves fit to known concentrations of standard reference material. Two sets of standard dilution series were analyzed by qPCR for constructing calibration curves. The serial dilutions of extracted PCs, analyzed alongside environmental samples as described above, correspond to known concentrations before DNA isolation procedures to account for extraction loss. We also prepared three unextracted positive controls with 2×10^6 copies of each artificial plasmid standard and constructed dilution series corresponding to $10^7, 10^5, 10^4, 10^3, 10^2, 5 \times 10^1, 10^1$, and 5×10^0 copies per reaction. For each target, we analyzed the three dilution series in triplicate in three separate qPCR instrument runs, for 27 total reactions at each concentration—nine reactions from each dilution series across three plates. To relate these separate instrument runs to the analysis of environmental samples, each extracted PC was re-assayed alongside the unextracted dilution series in duplicate reactions corresponding to 10^5 pre-extraction copies.

Separate calibration curves were fit to the two sets of dilution series for each target using multilevel Bayesian regression to account for possible variation between reaction sets (Sivaganesan et al., 2010, 2008). We treated reaction Cq as the response, $\log_{10}$ copy number as a predictor, and allowed slopes and intercepts to vary by instrument run for both data sets and also by extraction batch for the curves fit to extracted PC data. We fit models with the **brms** package

in **R** version 3.5.1, using the default, improper flat priors on population-level coefficients and four chains with 2000 warmup iterations and 2000 sampling iterations (Bürkner, 2018, 2017; R Core Team, 2018).

2.2.5.5 Microbial target quantification

We quantified culturable *E. coli* (cEC) as colony forming units (cfu) on individual plates and molecular targets as gene copies (gc) in individual reactions. The sampling effort represented by each plate/reaction was used to compute target concentrations in environmental samples, normalized to 100 mL of water, 100 cm^2 of food preparation surface, or gram of dry soil. We considered each mL of sample eluate filtered to represent 0.01 g wet soil or 8.33 cm^2 surface area, and each reaction—containing 5 µL of the total 100 µL eluted from each filter—to represent $1/20^{th}$ of the filtered volume. Moisture content was used to normalize soil sampling efforts in terms of dry weight. We imputed missing moisture contents from observations of sun exposure, soil surface wetness, and precipitation, temperature, and wind conditions using multivariate imputation by chained equations (MICE) in the **R** package **mice** (Buuren and Groothuis-Oudshoorn, 2011). We calculated cEC abundance and sampling effort by summing the cfu counts and volumes filtered for all countable plates from a given sample (Levy et al., 2012).

We calculated molecular target concentration distributions from the calibration curve posterior draws to account for uncertainty in the concentration estimates (Gelman and Hill, 2007; McElreath, 2015). At each sampling iteration, we estimated target $\log_{10}$ gc in each reaction using the extraction batch- and instrument run-specific slope and intercept parameter values. We normalized the reaction $\log_{10}$ gc estimates by sampling effort and combined the transformed posterior draws from all replicate reactions to construct the posterior distribution of target

concentration in each environmental sample. Target concentration point estimates were obtained as the mean, standard deviation (SD), and 2.5% and 97.5% quantiles (95% confidence interval) of the $\log_{10}$ concentration posterior distributions.

2.2.5.6 Determining limits of detection

For cEC, we assumed a lower limit of detection (LLoD) of one cfu per plate and an upper limit of quantification (ULoQ) of 400 cfu per plate, as suggested by Levy et al. (2012) and supported by our samples. We obtained the limits in terms of cEC concentration using sample-specific sampling efforts, calculated using the largest volume filtered for samples with no growth on any plate and the smallest volume filtered for samples with all TNTC plates. We defined LLoDs for molecular targets as the $\log_{10}$ concentrations corresponding to the ROC-derived cutoff Cq value for each assay. Target concentration distributions were estimated from sample-specific sampling efforts and calibration curve posterior draws.

Theoretical detection limits (tLLoD) were also calculated using survival models to estimate the target concentration corresponding to a 95% probability of amplification, a common definition of LLoD for qPCR assays that requires substantial resources to establish empirically (Bustin et al., 2009; Stokdyk et al., 2016). Recognizing that not every copy of the target gene will successfully amplify but assuming each copy has an independent and identical probability of doing so, we estimated an exponential dose-response relationship between target concentration and detection in the serial dilution series reactions (Verbyla et al., 2016). For each reaction containing d_i $\log_{10}$ copies of the target, the detection status x_i follows a Bernoulli distribution with probability p_i given by

$$p_i = 1 - e^{-d_i r} \qquad (\mathbf{2.4})$$

where the survival coefficient r is the probability that each copy amplifies (Schmidt et al., 2013). We estimated r and solved for D, the $\log_{10}$ copy number for which $p_i = 0.95$, using Markov chain Monte Carlo (MCMC) implemented in JAGS with a uniform $\mathrm{Beta}(1,1)$ prior on r and three chains of 2000 warmup and 4000 sampling iterations each (Plummer, 2003). We characterized tLLoD for each assay as the mean and 95% confidence interval (CI) of the posterior distribution of D.

Separate tLLoD estimates were obtained for the serial dilution series of extracted and unextracted reference material for each target. Unextracted tLLoDs correspond to the minimum target concentration in individual reaction wells for reliable detection, while extracted tLLoDs reflect the required concentration in unprocessed sample to account for DNA loss during the extraction procedure. We estimated extraction efficiency, the proportion of DNA recovered following extraction, as the ratio of tLLoD posterior distributions from unextracted and extracted reactions.

2.2.5.7 Treatment of observations outside detection and quantification limits

When analyzing binary detection outcomes, we treated observations below the LLoD as negative and observations above the LLoD, including > ULoQ, as positive. For continuous concentration outcomes, we treated observations below the LLoD and above the ULoQ as left and right censored, respectively. We obtained maximum likelihood estimates (MLE) of the $\log_{10}$ concentration mean and SD assuming a censored normal distribution with the *fitdistcens* function in the **R** package **fitdistrplus** (Delignette-Muller and Dutang, 2015). We also imputed concentrations for censored observations as the expected value of a normal distribution truncated at the sample-specific LLoD or ULoQ using the *etruncnorm* function from the **R** package **truncnorm** and the MLE mean and SD (Mersmann et al., 2018; Messier et al., 2012).

2.2.6 Assessing risk factors of domestic fecal contamination

2.2.6.1 Risk factor data sources

We ascertained socioeconomic, demographic, sanitary, and health characteristics using surveys and direct observation. Questionnaires were administered to the head of each compound to assess compound characteristics and the mother or other guardian of each child for household and child characteristics. Characteristics identified as potential hazards include observed or reported feces, soiled diapers, standing wastewater, or domestic animals in the compound yard, previous compound flooding, and disposal of child feces elsewhere than the latrine. We noted amenities including household floor material and onsite access to latrines, source water points, and electricity, as well as physical characteristics of the latrine, if present. We calculated a household wealth index from survey responses using an asset-based scorecard developed for Mozambique, excluding sanitation-related assets (Knee et al., 2018; Schreiner et al., 2013). Other socio-demographic characteristics assessed include caregiver and household head educational attainment, household size and crowding (> three household members per room), and compound population and density. We represented population density as persons per latrine, per waterpoint, and per 100 m^2 of compound area. GPS-enabled tablets displaying orthorectified, geolocated satellite imagery were used to delineate compound boundaries, from which we calculated compound areas.

Daily meteorology records were obtained for the weather station at Maputo International Airport, located immediately adjacent to our study area, from the Global Surface Summary of Day dataset available through the National Oceanic and Atmospheric Administration's National Centers for Environmental Information (https://www7.ncdc.noaa.gov/CDO/cdoselect.cmd). We obtained daily records for mean, minimum, and maximum temperature, mean wind speed,

cumulative precipitation, and an indicator of whether any precipitation events occurred. In the case of insubstantial precipitation events, it was possible to both observe precipitation and report zero accumulation on the same day. Because meteorological variables were available only as daily summaries and sampling was conducted primarily in the mornings, we associated each environmental sample with meteorological values for the day prior to collection. We also calculated cumulative precipitation and the number of days with rain events in the week (seven days) and month (30 days) preceding sample collection.

Physical characteristics of each sample were observed during collection or determined during initial laboratory processing, in the case of soil moisture. Other sample characteristics investigated include source water point location, water storage container attributes, food preparation surface attributes, soil sun exposure, and soil surface wetness.

2.2.6.2 Statistical analysis

We used univariable analyses to test associations between each putative risk factor and occurrence of microbial targets. Separate analyses were performed for each sample type to allow for different patterns of fecal contamination. Normalized $\log_{10}$ target concentration was used as the response variable unless the target was detected in < 75% of samples of a given type, in which case the binary detection status served as the response. All continuous risk factors were mean centered and scaled, either by SD (i.e., standardized) or by a meaningful value for the particular variable (e.g., wealth index scaled such that each unit increase represented a 10-point increase on the original 0 – 100 index scale). We estimated associations for concentration responses with censored Bayesian regression to account for observations outside the limits of detection and quantification, which provides a measure of effect in terms of the change in target $\log_{10}$ concentration for a unit increase in the risk factor (Stan Development Team, 2019a).

Population-level parameters were assigned weakly regularizing normal priors with SD = 10 for the intercept and SD = 2 for predictors (McElreath, 2015). Bayesian logistic regression was used for binary responses with the odds ratio (OR) serving as the measure of effect. Weakly regularizing Student's *t* priors with 5 degrees of freedom were assigned to population-level parameters, using scale = 10 for the intercept and scale = 2.5 for predictors (Gelman et al., 2008; Stan Development Team, 2019b). When modeling responses in stored water, food surfaces, and household soil, which were collected from multiple households per compound, the intercept was allowed to vary by compound to account for clustering in outcomes. Models were fit in **brms** using four chains with 1500 warmup and 1000 sampling iterations each.

2.3 Results

2.3.1 Candidate assay diagnostic performance

Individual local fecal samples were collected from 10 chickens, 13 ducks, one dog, and two pigs, as well as a composite manure sample from 6 piglets. Surface sludge was obtained from 14 unimproved pit latrines, representing composite human-source fecal material. We analyzed each sample with eight qPCR assays to assess diagnostic performance, implementing ROC analysis to determine the optimal cutoff Cq value for each assay. Table 2.2 presents the ROC-derived optimal cutoff cycle for each assay as well as the sensitivity, specificity, and accuracy when assays were considered positive for a given sample if a Cq value below the cutoff was observed in any replicate reaction. Both general assays performed well, though EC23S was positive for 100% of samples while BacUni was negative for a single chicken sample. Host-associated assays were all reasonably specific, ranging from 71% (HF183) to 100% (GFD). All human assays cross-reacted with avian feces, though not to the extent seen in certain previous studies (Harris et al., 2016; Odagiri et al., 2015). HAdV was the most human-specific, cross-

reacting with only two duck fecal samples; BacHum and Mnif were both positive for certain chicken and duck samples, while HF183 cross-reacted with chickens, ducks, and a pig sample. All assays were negative for the dog sample with the exception of LA35, which was also positive for a single latrine sample.

Table 2.2: Optimal cutoff cycle and diagnostic performance of candidate MST assays

assay	host	cutoff cycle	sensitivity	specificity	accuracy
BacUni	general	38	0.95	-	-
EC23S	general	39	1.00	-	-
BacHum	human	40	0.50	0.84	0.73
HF183	human	39	0.64	0.71	0.69
Mnif	human	41	0.71	0.74	0.73
HAdV	human	44	0.79	0.94	0.89
LA35	avian	45	0.43	0.91	0.67
GFD	avian	40	0.78	1.00	0.89

Sensitivity was lower than specificity for all host-associated assays. BacHum, the second-most specific human-associated target, was also the least sensitive. Mnif was the most balanced of the human markers with 71% sensitivity and 74% specificity. HAdV was the most sensitive human marker despite exceptionally low sensitivity in previous studie (Harwood et al., 2013). GFD was substantially more sensitive (78%) than LA35, which was positive in fewer than half the avian samples.

Given that environmental concentrations of microbial targets are generally much lower than in feces and sludge, we anticipated further reductions in assay sensitivity and improvements in specificity when applied to environmental samples. We therefore weighted sensitivity more highly when selecting assays for use in MST analysis of environmental samples. We selected EC23S as general fecal target, both for improved sensitivity relative to BacUni and to provide a molecular comparison to the cultured *E. coli* data. GFD was both more sensitive and specific than LA35 and was chosen for the avian target. Despite relatively strong specificity, we excluded

BacHum due to low sensitivity. We also did not proceed with HAdV owing to concerns about the potential for spatial and temporal discontinuity in its occurrence as well as previous reports of very low sensitivity. Accordingly, we selected Mnif and HF183 as human targets for further MST analysis.

2.3.2 Occurrence of fecal indicator organisms in the domestic environment

2.3.2.1 Characteristics of environmental samples

We collected 366 samples from 94 households in 58 compounds, home to 135 children enrolled in the MapSan trial. Samples were collected on 27 (noncontiguous) days. Source water was available for collection in only 44 compounds, with some compounds lacking water points and the municipal supply intermittently unavailable during the sampling visits. Soil was collected from 56 compound latrine entrances and 85 household entrances; soil could not be collected when the area around entrances was covered with impervious surfaces. We collected stored water and food preparation surface swabs from 91 and 90 households, respectively.

Soils were generally shaded to some extent and often had wet surfaces (Table 2.3), though latrine soils were somewhat more commonly exposed to full sun (27%) and wet (67%) than household soils (19% and 57%, respectively). Mean moisture content was nevertheless similar between soil samples from both locations. Plastic bowls comprised the large majority of food preparation surfaces sampled; nearly every water storage container was likewise constructed of plastic (92%). Storage containers typically had wide mouths (71%) with lids (70%), from which water was extracted by dipping a cup or pitcher inside the container. Conversely, water was typically poured out of narrow-mouthed containers, which were generally observed uncovered.

Table 2.3: Number (%) of samples observed with a given characteristic

type	characteristic	value	n	observations (%)
stored water	container material	plastic	91	84 (92)
		metal		4 (4)
		other		3 (3)
	container opening	covered	89	62 (70)
		uncovered		27 (30)
	container mouth	wide	89	63 (71)
		narrow		26 (29)
	extraction method	dip	89	62 (70)
		pour		27 (30)
food surface	type	bowl	90	83 (92)
		table		7 (8)
	material	plastic	90	79 (88)
		metal		8 (9)
		wood		3 (3)
latrine soil	sun exposure	full	49	13 (27)
		partial		34 (69)
		shaded		2 (4)
	surface wetness	dry	49	16 (33)
		wet		33 (67)
	moisture content	percent (IQR)	56	9.4 (8.4)
household soil	sun exposure	full	85	16 (19)
		partial		52 (61)
		shaded		17 (20)
	surface wetness	dry	83	36 (43)
		wet		47 (57)
	moisture content	percent (IQR)	85	8.4 (8.1)

2.3.2.2 Detection limits and extraction efficiency

The minimum concentration at which each target could be reliably detected was estimated for each sample at from the corresponding extracted PC calibration curve. Averaged across all batches and plates, the curves were relatively linear ($R^2 > 0.95$), although the amplification efficiency was somewhat poor for some targets, particularly HF183 (Table 2.4). Reduced linearity and amplification efficiency were both likely related to the use of reference materials that had been subjected to an extraction procedure, which helps account for target loss

during processing when quantifying unknown samples but introduces additional variability. Estimated target loss during DNA extraction, as implied by the ratio of extracted and unextracted tLLoDs (not shown), was 81% (95% CI: 77%, 90%) for EC23S, 79% (65%, 88%) for HF183, and 73% (51%, 85%) for Mnif; unextracted reactions were not run for GFD.

Table 2.4: Mean (95% CI) estimates of population-level intercept and slope, amplification efficiency, and R^2 for qPCR calibration curves fit to serial dilution series of extracted positive controls

target	intercept	slope	efficiency (%)	R^2
EC23S	47.91 (47.25, 48.64)	-3.50 (-3.64, -3.37)	93.11 (88.12, 98.14)	0.98 (0.97, 0.98)
HF183	47.45 (46.41, 48.58)	-3.85 (-4.07, -3.67)	81.83 (76.07, 87.36)	0.98 (0.97, 0.98)
Mnif	48.77 (47.66, 50.25)	-3.47 (-3.79, -3.23)	94.51 (83.57, 104.0)	0.95 (0.93, 0.95)
GFD	44.87 (43.70, 46.11)	-3.63 (-3.88, -3.40)	88.66 (81.06, 96.85)	0.98 (0.97, 0.98)

Sample-level LLoDs are a function of assay analytical sensitivity in individual reactions, variable target loss during processing, and the amount of sample processed. For culture-based detection, the analytical sensitivity was uniform (1 cfu per plate) but the amount of sample processed varied from sample to sample in order to obtain plates within the countable range. Conversely, the volume filtered for qPCR detection was consistent between samples of the same matrix (e.g., water) and the same filter extract was used for all qPCR assays, while analytical sensitivity varied not only between assays but also by extraction batch and instrument run. Normalizing concentrations by dry weight also contributed additional variability to soil sample LLoDs. Water samples were processed in the highest amounts relative to their normalization value, filtering 300 mL water for molecular analysis while expressing concentrations per 100 mL. By contrast, we filtered eluate equivalent to 0.3 g wet soil with concentrations expressed as 1 g dry soil. For a given target, average sample LLoDs were similar between samples of the same matrix (Table 2.5), although cEC LLoDs were notably higher in stored water than source water, which was generally processed in larger volumes. Of the molecular targets, GFD

consistently demonstrated the lowest detection limits while average LLoDs for EC23S, HF183, and Mnif were generally similar for a given sample type. This likely reflects higher analytical sensitivity on the part of GFD, as seen in the lower intercept values for the GFD calibration curves, which used non-specific SYBR chemistry for real-time detection in contrast with the probe-based chemistry of the other three assays. LLoD estimates indicate relatively high concentrations were required for reliable detection, with >1000 target copies per 100 mL water or 100 cm^2 surface and >10,000 per gram of soil generally needed for probe-based qPCR detection.

Table 2.5: Mean (SD) lower limits of detection as initial microbial target concentration in unprocessed sample

target	cEC	EC23S	HF183	Mnif	GFD
source water [log_{10}Q/100 mL]	0.04 (0.21)	3.38 (0.04)	2.77 (0.35)	3.14 (0.10)	2.41 (0.20)
stored water [log_{10}Q/100 mL]	0.35 (0.60)	3.36 (0.10)	3.04 (0.35)	3.11 (0.07)	2.30 (0.04)
food surface [log_{10}Q/100 cm^2]	1.59 (0.99)	3.91 (0.42)	3.50 (0.47)	3.52 (0.50)	2.83 (0.49)
latrine soil [log_{10}Q/dry g]	2.46 (0.52)	4.59 (0.11)	4.24 (0.19)	4.12 (0.16)	3.36 (0.17)
household soil [log_{10}Q/dry g]	2.40 (0.61)	4.50 (0.14)	4.45 (0.19)	4.34 (0.13)	3.31 (0.39)

2.3.2.3 Microbial target detection frequency

We detected at least one microbial target in 96% of samples (353/366). Highly credible *E. coli*, detected by both culture and qPCR, were present in the majority of samples (78%). EC23S and cEC were detected with similar frequency except in source water (Table 2.6), in which *E. coli* was detected twice as frequently by qPCR (66%) than culture (34%). Because source water was municipally treated and generally piped onto the compound premises, the elevated molecular signal may indicate the presence of DNA from organisms inactivated or rendered viable but non-culturable (VBNC) by treatment. The lowest detection frequencies for

all targets were observed in source water, the sample type furthest removed from domestic fecal transmission pathways.

Human targets were frequently detected in soils (59%) and occasionally in stored water (17%) but seldom in source water or on food surfaces. Mnif was more common than HF183 in both latrine and household soil, though HF183 was the only human marker detected with any frequency in samples other than soil. We observed the largest human fecal impact on latrine soil, with 68% positive for at least one human target and 27% positive for both, an indicator of highly credible human-source contamination. The avian target GFD was rarely detected in any sample type but was most common in soils (4%).

Table 2.6: Fraction (%) of samples positive for each target by sample type

target	source water	stored water	food surface	latrine soil	household soil
cEC	15/44 (34)	81/91 (89)	81/90 (90)	54/54 (100)	85/85 (100)
EC23S	29/44 (66)	79/91 (87)	75/89 (84)	53/56 (95)	84/84 (100)
any *E. coli*	34/44 (77)	90/91 (99)	89/90 (99)	55/55 (100)	85/85 (100)
both *E. coli*	10/44 (23)	70/91 (77)	67/89 (75)	52/55 (95)	84/84 (100)
HF183	1/44 (2)	15/91 (16)	1/89 (1)	21/56 (38)	21/84 (25)
Mnif	0/44 (0)	1/90 (1)	1/88 (1)	32/56 (57)	29/83 (35)
any human	1/44 (2)	15/90 (17)	2/88 (2)	38/56 (68)	45/84 (54)
both human	0/44 (0)	1/91 (1)	0/89 (0)	15/56 (27)	5/83 (6)
GFD	0/44 (0)	1/91 (1)	0/89 (0)	2/56 (4)	3/84 (4)
any target	34/44 (77)	90/91 (99)	89/90 (99)	55/55 (100)	85/85 (100)

2.3.2.4 Microbial target concentrations

Assuming normalized $\log_{10}$ concentrations follow a normal distribution with left- and right-censored observations, we obtained the MLE mean and SD concentration of each target detected in >10% of a given sample type (Table 2.7). Because we normalized according to the matrix sampled, concentrations may be directly compared between samples of the same matrix (e.g. source water and stored water) but not between matrices. Furthermore, while each cfu is assumed to correspond to a single organism present in the sample, organisms may carry different

numbers of each gene target, limiting the validity of comparisons between targets as well. We assessed cEC and EC23S concentrations in all sample types, HF183 concentration in stored water and soils, and Mnif concentration in soils only; GFD was detected too infrequently in any sample to characterize concentration.

E. coli was more abundant than human targets. Mean EC23S and HF183 concentrations were respectively 6.5 and 3.8 $\log_{10}$ gc/dry g of latrine soil and 4.3 and 1.4 $\log_{10}$ gc/100 ml of stored water. The censoring assumption implies that non-detected targets were not absent but rather present in concentrations too low for reliable detection, reflected in mean concentration estimates for human targets, and cEC in source water, below their estimated LLoDs (Table 2.5). By contrast, EC23S, which was detected in nearly every sample, has mean concentrations well above its LLoD for each sample type. In source water, the mean (SD) concentration among the 34% of samples positive for cEC was 8.5 (5.8) cfu/100 mL.

Table 2.7: Maximum likelihood estimate (SE) of target concentration mean and SD under a normal distribution with censored observations

	cEC		EC23S		HF183		Mnif	
type	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
source water [$\log_{10}$Q/100 mL]	-0.54 (0.33)	1.38 (0.29)	3.57 (0.14)	0.84 (0.12)				
stored water [$\log_{10}$Q/100 mL]	1.72 (0.16)	1.46 (0.12)	4.26 (0.09)	0.83 (0.07)	1.39 (0.48)	1.66 (0.36)		
food surface [$\log_{10}$Q/100 cm^2]	3.17 (0.21)	1.95 (0.18)	4.73 (0.10)	0.90 (0.08)				
latrine soil [$\log_{10}$Q/dry g]	3.95 (0.13)	0.97 (0.10)	6.48 (0.16)	1.17 (0.12)	3.79 (0.27)	1.39 (0.25)	4.31 (0.17)	1.11 (0.15)
household soil [$\log_{10}$Q/dry g]	4.14 (0.10)	0.90 (0.07)	6.72 (0.10)	0.88 (0.07)	3.31 (0.36)	1.65 (0.30)	4.11 (0.10)	0.57 (0.09)

2.3.3 Characteristics of study households, compounds, and sampling dates

All compounds had a latrine on premises and most covered the latrine drophole (86%), but otherwise latrine quality was poor: only 36% had a slab and 26% a permanent superstructure (Table 2.8). Most compounds (82%) had water sources on premises, though source water was

available in 70% at the time of sampling. Electricity was nearly universally available, and most households (97%) had impervious floors. Potential fecal hazards were present in many compounds, with standing wastewater observed in 58% of compounds, 56% owning domestic animals, and 49% reporting previous flooding. Additionally, disposal of feces outside the latrine was reported for at least one child in 86% of compounds.

The average compound had 17.3 members and 2.2 children enrolled in the study from 1.7 households, each of which had an average of 6.2 members and 1.3 enrolled children. About half of child caregivers (primarily mothers) reported completing primary school, though fewer household heads had done so (31%). The wealth of most households fell within the middle range of the 100-point asset-based index, with the typical household slightly below the index midpoint with a value of 46. Few households (12%) were crowded with more than three people per room. Compounds had a mean area of 280 m^2 and population density of 7.2 people/100 m^2. The weather during sampling was relatively dry and mild, with daily average temperatures of 20 °C. On the average sampling day it had rained a total of 4.2 cm on 2.5 days in the preceding month.

Table 2.8: Summary of household, compound, and sampling date characteristics; binary outcomes as positive observations (%) and continuous outcomes as mean (IQR)

		level	n	observations
hazards				
feces or soiled diapers observed		compound	57	21 (37)
standing wastewater observed		compound	57	33 (58)
prone to flooding		compound	57	28 (49)
animals present	any	compound	57	32 (56)
	poultry		57	8 (14)
	cat		57	27 (47)
	dog		57	6 (11)
	other		57	2 (4)
reported child diarrhea		household	90	8 (9)
	in any household	compound	57	6 (11)
unsafe child feces disposal		household	90	72 (80)
	in any household	compound	57	49 (86)
amenities				
latrine on premises		compound	57	57 (100)
	cabins (count)		57	1.0 (0.0)
	drophole cover		57	49 (86)
	slab or pedestal		57	22 (39)
	superstructure		57	15 (26)
	ventpipe		57	1 (2)
water on premises		compound	57	47 (82)
	water points (count)	compound	57	1.4 (1.0)
	household-reported access	household	91	79 (87)
	available during sampling	compound	57	40 (70)
electricity on premises		compound	57	55 (96)
covered floor		household	91	88 (97)
demographics				
completed primary education	head of household	household	91	28 (31)
	child caregiver	household	90	49 (54)
	child caregiver in any household	compound	57	41 (72)
wealth index (0 - 100)		household	91	45.8 (12.2)
household members (count)		household	91	6.2 (3.5)
	children enrolled		90	1.3 (1.0)
rooms in house (count)		household	91	2.8 (1.0)
persons per room (ratio)		household	91	2.3 (1.3)
	crowding (> 3)		91	12 (13)
compound population (count)		compound	57	17.3 (7.0)
	children enrolled		57	2.2 (2.0)
	households enrolled		57	1.7 (1.0)

compound area (m^2)		compound	52	279.5 (156.3)
compound population density (persons/100 m^2)		compound	52	7.2 (5.1)
persons per latrine (ratio)		compound	57	17.2 (7.0)
persons per water point (ratio)		compound	47	12.4 (7.8)
meteorology				
temperature previous day (°C)	mean	date	27	20.4 (1.7)
	minimum		27	14.1 (3.2)
	maximum		27	28.2 (4.4)
windspeed previous day (knots)		date	27	7.7 (3.2)
cumulative precipitation (mm)	previous day	date	26	2.8 (0.0)
	previous week	date	27	9.3 (3.0)
	previous month	date	27	41.6 (57.1)
days with any rain (count)	previous seven days	date	27	0.6 (1.5)
	previous 30 days	date	27	2.5 (4.0)

2.3.4 Associations with fecal indicator concentrations

We assessed risk factors of general fecal contamination as the expected linear change in normalized $\log_{10}$ concentration of cEC and EC23S given the presence of a binary predictor variable or a one unit increase in a scaled continuous variable. Predictors for which the 95% CI of the effect estimate included zero were not considered to be risk factors of contamination for the sample type tested, though we considered the sign of the point estimate across targets and sample types to evaluate the broader implications of each variable. There were few consistent trends in associations with target concentrations and most characteristics were not significantly associated with either target in most sample types. No compound, household, or meteorological characteristic was significant across all sample types; rather, the direction of the effect estimate often reversed between targets and sample types. cEC concentrations were elevated in all sample types with increased days of rain the previous week and attenuated with increased temperature, but most of these associations were not significant and did not hold for EC23S or for increased cumulative precipitation over the same period of time. Both cEC and EC23S concentrations declined when latrine dropholes were covered, but all the effects were relatively small and not

significant. The strongest effects were observed on EC23S concentration in latrine soil, with an expected increase of 1.2 (95% CI: 0.1 – 2.2) $\log_{10}$gc/dry g when at least one child in the compound had diarrhea in the previous week and 1.0 (0.3 – 1.7) $\log_{10}$gc/dry g when source water was available during sampling. EC23S was also elevated by 0.6 (0.2 – 1.0) $\log_{10}$gc/100 mL in stored water when feces were observed.

Among sample-level characteristics (Figure 2.3), wet soil surfaces were consistently associated with increased *E. coli* concentrations, significantly so for cEC in both latrine (0.7 (0.1 – 1.3) $\log_{10}$gc/dry g) and household (0.5 (0.1 – 0.9) $\log_{10}$gc/dry g) soils and also for EC23S in latrine soil (1.1 (0.5 – 1.7) $\log_{10}$gc/dry g). Food preparation surface characteristics were also associated with EC23S concentration, which was lower for plastic and bowl-type food preparation surfaces (the most common surfaces) than for metal or wooden and table-like surfaces.

2.3.5 Odds of detecting human fecal contamination

Risk factors of human source contamination were identified by the odds ratio for detection of human-associated targets given the presence of a binary predictor variable or a one unit increase in a scaled continuous variable. We used detection of HF183 and of any human target as response variables in soils and detection of HF183 in stored water, in which Mnif was rarely detected. Human targets were detected too infrequently to assess risk factors for food surface contamination. As with *E. coli* concentrations, we did not find significant associations between most compound, household, and meteorological characteristics and human-source contamination. However, several potential compound hazards were consistently associated with increased ORs for human target detection, including animal ownership, previous flooding, and observation of standing wastewater or feces. The associations were significant, as indicated by

95% CIs on the OR estimate that excluded one, for HF183 detection in latrine soil when domestic animals were present (OR: 4.3; 95% CI: 1.2 – 11.5) and for detecting any human target in household soil in the case of standing wastewater (OR: 8.9; 1.2 – 49). Completing primary school was associated with reduced odds of detecting any human target (but not HF183 specifically) in household soil (OR: 0.2; 0.03 – 0.6), while a 10-point increase in the household wealth index was associated with an increase in the odds of human target detection in household soils, significantly so for HF183 (OR: 7.4; 1.6 – 32.8). Human contamination was also significantly more common in the soils from latrines as the number of users increased. Increasing temperatures were generally associated with reduced odds of human target detection in soil. Rainy days in the past week and month also usually signaled increased human target detection, though the same association was not presence for cumulative precipitation over the same time periods. No variables were significantly associated with detecting HF183 in stored water.

The direction of associations between sample characteristics and human target detection were generally similar to those for *E. coli* concentrations (Figure 2.2). Soil surface wetness effects were less pronounced in household soils for human targets than for *E. coli*, though detection of any human target was significantly more likely in wet latrine soils (OR: 6.6; 1.5 – 20.2). Wet latrine soil was also significantly associated with HF183 detection: of the 18 of 33 wet latrine soils were positive for HF183 and only 1 of 16 dry soils were, which prevented stable estimation of the OR.

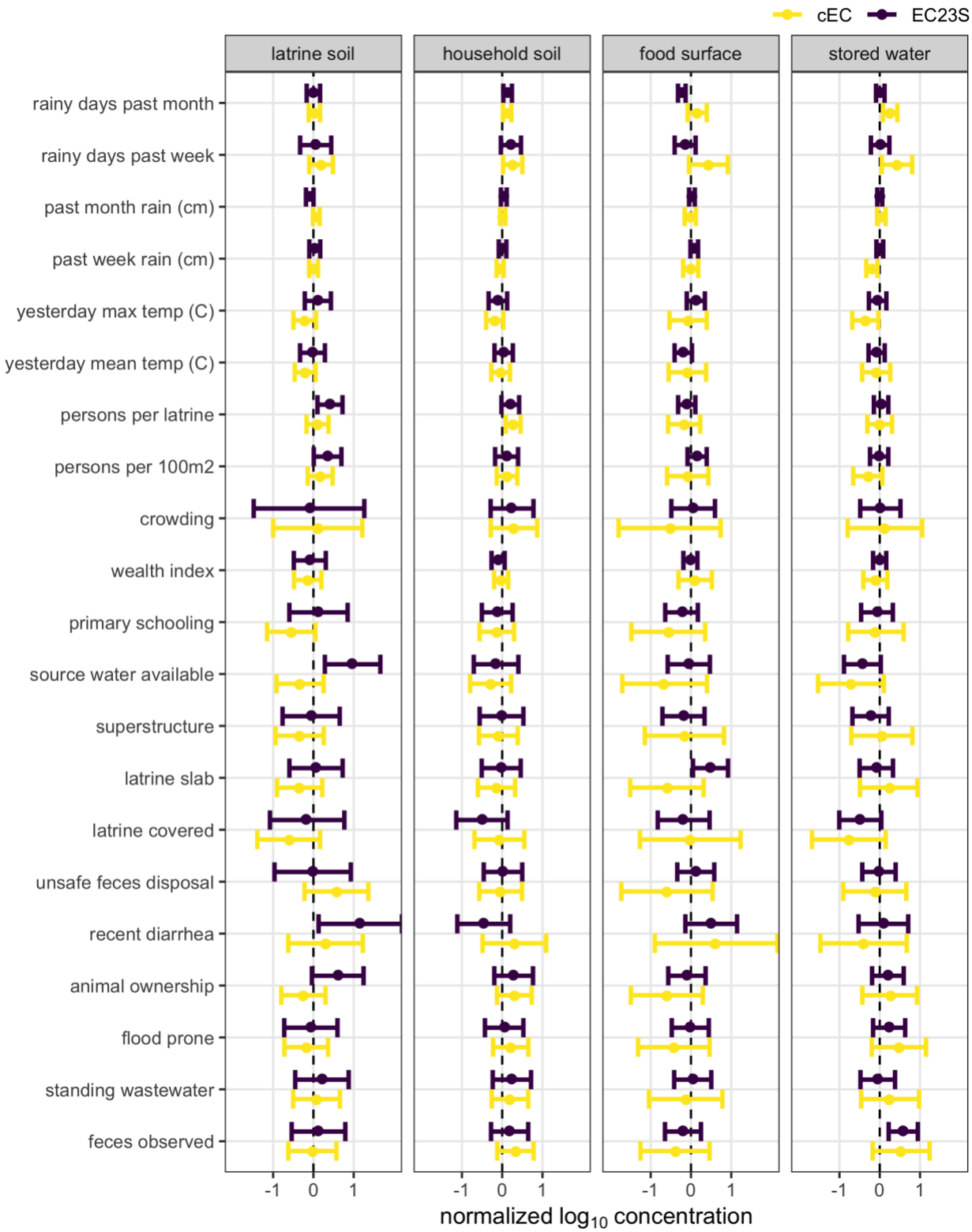

Figure 2.1: Estimated mean (points) and 95% CI (bars) change in *E. coli* target concentrations (horizontal axis) associated with sociodemographic, sanitary, and meteorological risk factors (vertical axis) by target (colors) and sample type (columns).

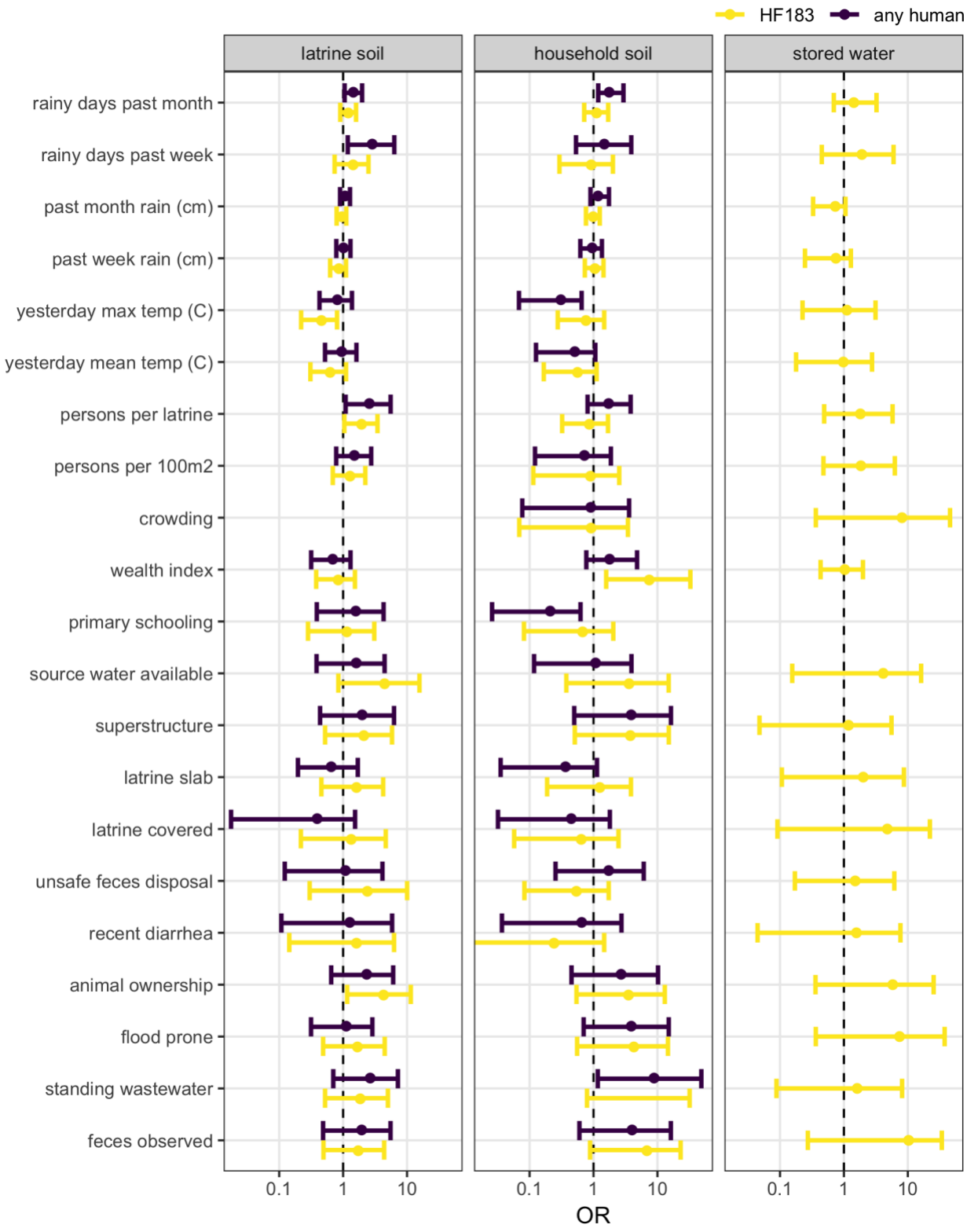

Figure 2.2: Mean (points) and 95% CI (bars) estimates of odds ratio for human target detection (horizontal axis) associated with sociodemographic, sanitary, and meteorological risk factors (vertical axis) by target (colors) and sample type (columns).

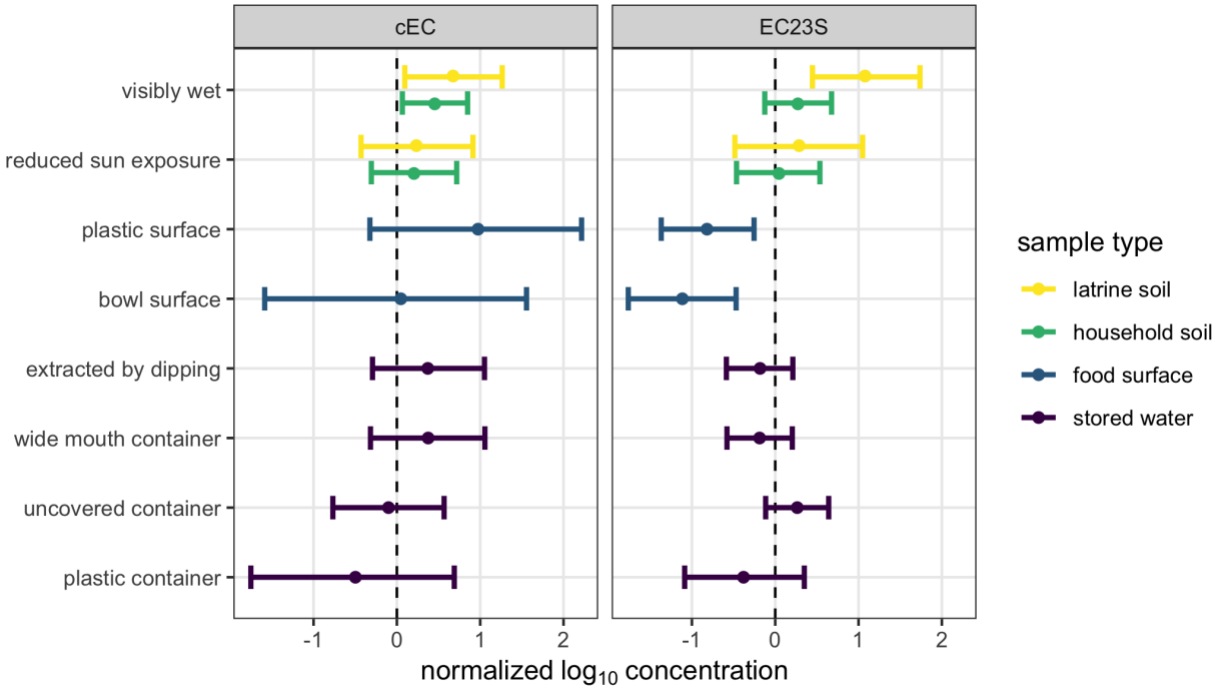

Figure 2.3: Estimated mean (points) and 95% CI (bars) change in target concentrations (horizontal axis) associated with sample characteristics (vertical axis) by target (columns) and sample type (colors).

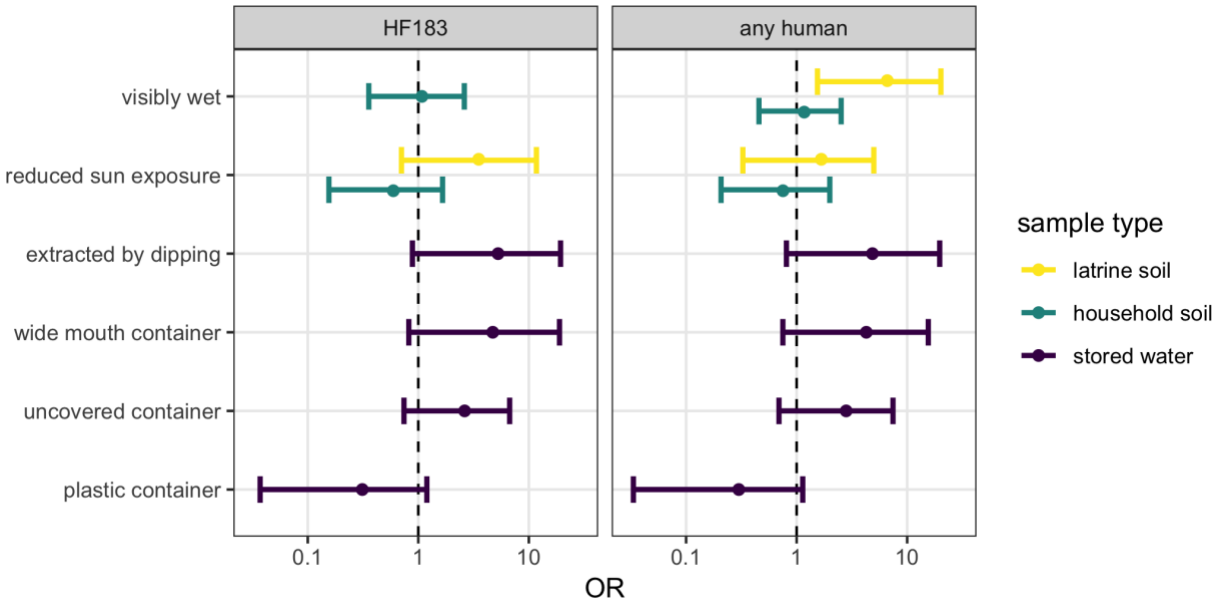

Figure 2.4: Mean (points) and 95% CI (bars) estimates of odds ratio for human target detection (horizontal axis) associated with sample characteristics (vertical axis) by target (columns) and sample type (colors).

2.4 Discussion

We found evidence of widespread fecal contamination, including from human sources, throughout the domestic environment. However, municipal source water was relatively high quality: two thirds of samples were free from culturable *E. coli*, and the typical concentration in *E. coli*-contaminated samples was less than 10 cfu/100 mL, considered "low risk" under previous WHO drinking water guidelines—though not a distinction with strong support in the literature (Gruber et al., 2014; WHO, 1997). Human targets were detected in the majority of soil samples and a meaningful percentage of stored water samples despite relatively low diagnostic sensitivity and high detection limits, suggesting that human-source contamination may have been even more pervasive than observed. The three sample types with the highest occurrence of human targets—stored water, household soil, and latrine soil—were also the most subject to onsite fecal transmission dynamics. Unlike source water, which was presumably removed from onsite transmission, food preparation surfaces were subject to both endogenous contamination, such as contact with household soil, and exogenous contamination, as may be introduced on food purchased outside the home. Food surfaces were rarely observed with human contamination. By contrast, stored water was primarily subject to onsite transmission, particularly in the case of human contamination, as containers were generally filled within the compound with human target-free source water. The frequent occurrence of human-associated microbes in samples dominated by onsite transmission suggests onsite origins driving the human-source fecal contamination we observed.

The diagnostic performance of all host-associated assays was relatively poor, especially in comparison to their performance in previous multi-site, multi-laboratory studies (Boehm et al., 2013; Layton et al., 2013; Reischer et al., 2013). The exception was human adenovirus assay

HAdV, which demonstrated substantially higher sensitivity (with slightly reduced specificity) than previous studies (Harwood et al., 2013). Because all latrine samples were collected over a two-week period, it is possible that we captured a period of elevated adenovirus shedding in the study population and that HAdV sensitivity could be much lower at other times. Avian marker GFD also performed relatively well in comparison with the human markers and has been successfully applied in both urban and rural Bangladesh previously (Boehm et al., 2016; Harris et al., 2016). However, we rarely detected GFD in environmental samples despite reported ownership of domestic poultry and frequent observations of poultry and poultry defecation in compound yards while conducting study activities, raising questions about the suitability of GFD in our study area in practice. The lower performance of other human MST assays aligns with several other studies that attempted human fecal source tracking in developing contexts to evaluate domestic sanitary conditions (Boehm et al., 2016; Harris et al., 2016; Jenkins et al., 2009; Odagiri et al., 2015). Presuming lower abundance of both targets and cross-reacting non-targets in environmental samples than in feces, we expect diagnostic sensitivity to decline and specificity to increase in practice. That we nevertheless frequently detected human targets while failing to detect avian feces, a known source of cross-reaction, suggests a heavy burden of human-source fecal contamination in the domestic environment of our study sites.

Despite analyzing more than 350 samples, by collecting five samples types and considering each separately the number of observations involved in any particular comparison was necessarily limited. Coupled with primarily binary predictor variables, our power to identify risk factors among the covariates considered was severely restricted for all but the largest effects. Furthermore, where large effects were observed the particular combination of target, sample type, and risk factor often resulted in (nearly) empty cells in the 2×2 of exposure and outcome,

for which stable estimates were difficult to obtain. Accordingly, great care must be taken both in dismissing covariates as potential risk factors when no associations were found and in identifying a potential covariate as a risk factor on the apparent strength of its association, as the association is likely to be fragile and highly dependent on the particular set of data observed. With such limitations in mind, both non-specific and human-source fecal contamination were largely disconnected from local variation in socioeconomic and sanitary conditions. While the variables considered may be related to fecal contamination in the absolute sense, the range of conditions present in the study setting may be too narrow to observe meaningful differences in contamination. This pattern suggests that in such heavily burdened settings, transformational changes to the community environment may be required before meaningful impacts on fecal contamination can be realized (Eisenberg et al., 2012; Husseini et al., 2018).

CHAPTER 3: IMPACT OF AN ONSITE SANITATION INTERVENTION ON HUMAN FECAL CONTAMINATION OF THE DOMESTIC ENVIRONMENT IN URBAN MAPUTO, MOZAMBIQUE

3.1 Introduction

Public health engineers have long recognized several generalized pathways of fecal-oral disease transmission, mediated by six principal environmental reservoirs: hands, water, soil, food, flies, and surfaces/fomites (Wagner and Lanoix, 1958). Many strategies to reduce the burden of fecal-oral disease center on preventing oral exposure to fecal pathogens by interrupting these transmission pathways (Julian, 2016). Improving sanitation infrastructure is one such strategy, which has the potential to impact all transmission pathways by separating pathogens from the environment at the point of origin (Julian, 2016; Kawata, 1978; Mara et al., 2010). While meta-analyses have found a general association between sanitation and health (Cairncross et al., 2010; Norman et al., 2010; Waddington et al., 2009; Wolf et al., 2018, 2014), a number of sanitation intervention trials have struggled to reduce both fecal-oral disease outcomes and measures of environmental fecal contamination, suggesting that the interventions did not fully interrupt the dominant transmission pathways in the specific context of each trial (Cameron et al., 2013; Clasen et al., 2014; Patil et al., 2014; Pickering et al., 2015; Sclar et al., 2016; Sinharoy et al., 2017). Recently, more comprehensive interventions succeeded in reducing fecal contamination in pathways directly intervened upon, namely household water and food, but did not meaningfully affect indirect pathways and ambient environmental contamination (Ercumen et al., 2018b, 2018a). Better design and targeting of interventions to prevent fecal-oral exposure

requires, in part, an improved understanding of context-specific transmission dynamics (Ercumen et al., 2017b; Schriewer et al., 2015).

Field evaluation of fecal transmission is generally conducted by measuring fecal indicator organisms (FIO) in point samples of the environmental reservoirs that mediate fecal transmission pathways and serve as exposure routes (Sclar et al., 2016). Such measurements are indicative of fecal contamination generally within the sampling context (e.g., a household), and can be related to potential fecal dose presented by the exposure route (Julian, 2016). Less readily apparent is the transmission history leading to fecal contamination detected in a point sample of environmental reservoir, as a single reservoir may be contaminated by feces of different origins transmitted by multiple pathways (Ercumen et al., 2017b; Harris et al., 2013; Schriewer et al., 2015). Recently, microbial source tracking (MST), in which molecular detection of host-associated FIO is used to determine fecal source, has been deployed to investigate transmission in various settings (Boehm et al., 2016; Harris et al., 2016; Odagiri et al., 2016; Schriewer et al., 2015), though the correlations between various MST targets and fecal pathogens and disease are not as well established as for generic FIO (Gruber et al., 2014; Harwood et al., 2014; Hodge et al., 2016; Wu et al., 2011). Sampling the same reservoir (e.g., water) at multiple nodes of potential fecal transmission (e.g., source, storage) provides additional information to help identify the contribution of various transmission pathways to contamination of a given exposure route (e.g., drinking water) (Ercumen et al., 2017b).

We characterized the sources and extent of fecal contamination in urban Mozambican households participating in the first rigorous evaluation of urban onsite sanitation to elucidate the dominant pathways of transmission and assess the degree to which each was interrupted by the intervention.

3.2 Methods

3.2.1 Study setting and description of intervention

We characterized fecal transmission in households with children participating in the Maputo Sanitation (MapSan) study (clinicaltrials.gov NCT02362932), a prospective, controlled health impact trial of an urban, onsite sanitation intervention (Brown et al., 2015). The non-randomized intervention was delivered to compounds, self-defined clusters of households sharing outdoor space, in low-income neighborhoods of Maputo, Mozambique, areas with high burdens of enteric disease and predominantly onsite sanitation infrastructure (Devamani et al., 2014; Knee et al., 2018). Similar compounds in the same neighborhoods not selected to receive the intervention were recruited to serve as control sites. At baseline, both intervention and control compounds shared sanitation facilities in poor-condition. The existing shared latrines in intervention compounds were replaced with pour-flush latrines on infiltration pits with sturdy, private superstructures. Intervention latrines were constructed in 2015 – 2016 by Water and Sanitation for the Urban Poor (WSUP), which selected intervention sites primarily according to engineering and demand criteria (Brown et al., 2015).

3.2.2 Study design

The intervention impact on fecal contamination of the domestic environment was evaluated using a controlled before-and-after study design (Schmidt, 2017). Children in each intervention compound were enrolled immediately before the new latrine was opened for use by compound residents, with ongoing enrollment of control compounds conducted at a similar frequency. Follow-up visits to each compound were conducted approximately 12 months following baseline enrollment. We administered compound-, household-, and child-level surveys and collected child stool during both baseline and follow-up visits. Concurrent with child stool

collection in both survey rounds, we collected environmental samples from each household with children participating in the health study and from the shared outdoor space in an opportunistically selected subset of MapSan study compounds. During the 12-month follow-up visit we also collected environmental samples from new households with children in previously sampled compounds, as well as from additional compounds not sampled at baseline as time permitted.

We assessed fecal contamination at five nodes of fecal transmission to represent three fecal-oral exposure routes: drinking water, food ingestion, and soil ingestion (Julian, 2016). Two transmission nodes, piped water source and latrine entrance soil, were sampled once at each compound. Household water storage, food preparation surfaces, and entrance soil were sampled at each household with children enrolled in the health impacts trial, with the potential for multiple samples per compound. Piped water source was chosen as a transmission node to indicate fecal contamination of exogenous origin, to be compared with household water storage, which presents the opportunity to accumulate contamination domestically. Conversely, latrine entrance soil was selected to represent endogenous fecal origin, while household entrance soil suggests contamination accumulated from sources in addition to human excreta produced on site. Finally, food preparation surfaces were swabbed, representing contamination of the food exposure route that could be reliably measured in every household visited. Hands were not sampled; while they serve as an important mechanism of both transmission and exposure, pervasive hand contamination detected in a pilot study of the same neighborhoods suggests little specific transmission information to be gained measuring hand contamination directly (Devamani et al., 2014). Each sample type was collected as described previously (Chapter 2),

with stored water and food surface samples in particular collected in such a manner as to capture fecal contamination and exposure under typical usage conditions.

Verbal assent was obtained from the head of each compound before commencing study activities and a parent or guardian provided written, informed consent for each child to participate in the study. Environmental samples were only collected from households with enrolled children for whom parental consent had been given. This study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (IRB # 15-0963) and the associated health study was approved by the Comité Nacional de Bioética para a Saúde (CNBS), Ministério da Saúde, Republic of Mozambique (333/CNBS/14), the Ethics Committee of the London School of Hygiene and Tropical Medicine (reference # 8345), and the Institutional Review Board of the Georgia Institute of Technology (protocol # H15160).

3.2.3 Microbial detection and quantification

Environmental samples were processed by membrane filtration, preceded by manual elution for soil and swab samples, as described elsewhere (Chapter 2). We analyzed sample filters for five locally-validated microbial targets using both culture- and molecular-based detection (Table 3.1). We enumerated culturable *Escherichia coli* (cEC) from filters on modified mTEC broth (Hi-Media, Mumbai, India) and immediately archived additional filters at -80°C for molecular analysis.

DNA was isolated from soil and surface sample filters using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) and from water sample filters with the DNA-EZ ST01 Kit (GeneRite, North Brunswick, NJ, USA), with a positive control (PC) and negative extraction control (NEC) included in each batch of up to 22 sample filters. Filters were treated with 3 µg salmon testes DNA (MilliporeSigma, Burlington, MA, USA) immediately prior to extraction as a

specimen processing control (SPC), used in part to assess PCR inhibition in the sample (Haugland et al., 2005, 2012). We tested each extract using five separate qPCR assays, four targeting fecal microbes and the final assay, Sketa22, targeting the salmon DNA SPC (Haugland et al., 2010), with 10% of each sample type analyzed in duplicate for all microbial targets. Each reaction consisted of 12.5 µL TaqMan Environmental Master Mix 2.0, 2.5 µL 10x primers and probe mix, 5 µL nuclease free water (NFW), and 5 µL of DNA template, for a total reaction volume of 25 µL. After an initial 10-minute incubation period at 95 °C for all assays, the cycling conditions specified by the original assay developers were followed using a CFX96 Touch thermocycler (Bio-Rad, Hercules, CA). Samples with Sketa22 cycle quantification (Cq) values > 3 above the mean Cq of extraction controls (NEC and PC) were diluted 1:5 for further analysis. Each plate included three no-template controls (NTCs) as well as five-point, ten-fold dilution series of three extracted PCs, corresponding to triplicate reactions with $10^5 – 10^1$ or $10^6 – 10^2$ target gene copies (gc). Target concentrations were estimated from calibration curves fit to the standard dilution series reactions using multilevel Bayesian regression in which the slopes and intercepts were permitted to vary by extraction batch and instrument run (Sivaganesan et al., 2010).

Table 3.1: Microbial targets used to assess fecal contamination

target	host	organism/gene	class	assay	detection
cEC	general	culturable *E. coli*	bacterium	modified mTEC (USEPA, 2009)	culture
EC23S	general	*E. coli* 23S	bacterium	EC23S857 (Chern et al., 2011)	qPCR
HF183	human	*B. dorei* 16S	bacterium	HF183/BacR287 (Green et al., 2014)	qPCR
Mnif	human	*M. smithii nifH*	archaeon	Mnif (Johnston et al., 2010)	qPCR
GFD	avian	*Helicobacter spp.*	bacterium	GFD (Green et al., 2012)	qPCR

3.2.4 Characterizing patterns of fecal transmission

We calculated the detection frequency and concentration mean and standard deviation (SD) of each microbial target at each transmission node, as well as the proportion of samples from a given compound during each study phase (baseline and 12-month follow-up) positive for each target. Target concentrations were $\log_{10}$ transformed and normalized according to sample matrix, expressed as $\log_{10}$ target quantity ($\log_{10}$Q) per 100 mL water, 100 cm^2 surface, and 1 dry gram soil. Maximum likelihood estimates (MLE) of concentration mean and SD were obtained assuming the $\log_{10}$-transformed microbial target concentrations followed a normal distribution with left- and right-censored observations.

We determined fecal source by considering detection of host-associated microbial targets and regarded co-detection of targets with the same host (e.g., Mnif and HF183) or organism (e.g., cEC and EC23S) indicative of highly credible contamination by the associated host/organism. Within-sample associations between different targets were investigated using multilevel logistic regression with varying intercepts and slopes by sample type to estimate the odds of detecting each target given the detection of another target. We also tested within-sample associations between untransformed concentrations of different targets using Spearman rank correlation, without distinguishing between sample type and treating non-detect (ND) observations as zero concentration (Schriewer et al., 2015).

Fecal origins and transmission pathways were inferred from the patterns of contamination between different sample types collected from the same household and compound. We compared target occurrence between source and stored water; between latrine soil and stored water, household soil, and food surfaces; between stored water and household soil and food surfaces; and between household soil and stored water and food surfaces. Between-sample associations in

detection of each target were estimated using logistic regression with compound varying intercepts.

All models were implemented in the **R** package **brms** using four chains with 1500 warmup iterations and 1000 or 1500 sampling iterations each (Bürkner, 2018, 2017; R Core Team, 2018). Weakly regularizing Student's *t* priors with 5 degrees of freedom were assigned to population-level parameters, using scale = 10 for the intercept and scale = 2.5 for predictors (Gelman et al., 2008; Stan Development Team, 2019b). As each model included a single, binary predictor variable, we estimated the probability of detection for the response variable given the detection status of the predictor variable by applying the inverse-logit transformation to the appropriate combinations of population- and group-level parameters. Risk ratios (RR) were estimated by dividing the estimated probability of response variable detection given detection of the predictor variable by the probability of detecting the response variable when the response variable is not detected. All transformations and calculations were performed on model posterior draws and point estimates were obtained as the mean, 2.5^{th} percentile, and 97.5^{th} percentile (95% confidence interval, CI) of the posterior distributions of the parameters and their derived values.

3.2.5 Evaluating intervention effects on fecal contamination

We used a difference-in-differences (DID) approach to estimate the effect of the intervention on non-specific and human-source fecal contamination. DID enables unbiased estimation of the treatment effect in the absence of randomization, even when different individuals (locations, in the context of this study) are measured pre- and post-treatment (Schmidt, 2017). This approach requires the twin assumptions that all unmeasured covariates related to the outcome that vary in time are constant across treatment groups, and that unmeasured covariates that vary between treatment groups are constant through time (Abadie,

2005; Wing et al., 2018). These assumptions imply that the outcome would have followed parallel trends in both the intervention and control groups were it not for the intervention. To increase the likelihood these assumptions are satisfied, treatment and control arms should be balanced in both the outcome and measured characteristics related to the outcome pre-treatment (Schmidt, 2017).

We conducted regression analysis using the standard DID estimator, in which the predictor set includes indicator variables for study phase (baseline and 12-month follow-up) and treatment assignment (control and intervention), as well as their interaction (Gelman and Hill, 2007; Wing et al., 2018). The intervention effect estimate is provided by the coefficient for the interaction term and was estimated separately for each combination of microbial target and sample type using Bayesian multilevel models. We did not analyze intervention effects on any target in source water due to the lack of a direct causal path between household sanitation infrastructure and contamination of piped municipal water supply. For targets detected in >75% of a given sample type we used $\log_{10}$ concentration as the response variable and binary detection status for targets detected less frequently. We also assessed the intervention effect on the overall rate of contamination as the number of samples from a given compound positive for each target. Models for all responses allowed the intercept to vary by compound to account for repeated measures at the compound level.

Censored linear regression was used to estimate the intervention impact in terms of the change in target $\log_{10}$ concentration with weakly regularizing normal priors with SD = 10 on the population-level intercept and SD = 2 on predictor variables, including the DID terms (Bürkner, 2017; McElreath, 2015; Stan Development Team, 2019a). We estimated the effect of the intervention on target prevalence with logistic regression and the prevalence odds ratio (POR) as

the measure of effect, using the same weakly regularizing Student's *t* priors as in the target co-detection models. Compound-level detection counts were analyzed with negative-binomial regression to estimate the incidence rate ratio (IRR) using the total number of samples collected in each compound as an offset and the same regularizing priors as for continuous outcomes. All models were fit in **brms** with 1500 warmup iterations and 1000 sampling iterations on four chains, and estimates of the intervention effect summarized by the mean and 95% CI of the resulting 4000 posterior draws.

We fit unadjusted models with only DID estimator terms as predictors as well as adjusted models with additional predictor variables for potential confounders of the intervention effect on fecal contamination. Only time-varying covariates that were not anticipated to be affected by the treatment were considered for the adjustment set. We controlled for compound population density, presence of domestic animals, and asset-based household wealth scores, all derived from household and compound surveys administered during each study phase. Previous day mean temperature and cumulative rainfall in the preceding seven days were drawn from daily summary records for a local weather station. The sources and processing of compound, household, and meteorological covariates have been described elsewhere (Chapter 2). For stored water samples, we considered whether the storage container was covered and if the mouth was wide enough to admit hands. The surface material was considered for food surface swabs, and for soil samples we accounted for sun exposure and visibly wet soil surfaces. All continuous variables were mean centered and scaled by SD or a physically meaningful value. Covariate data sources and treatment are described in greater detail elsewhere (Chapter 2).

3.3 Results

3.3.1 Sample characteristics

We collected environmental samples from 142 households in 72 compounds with 305 children enrolled in the associated health study (Table 3.2). In total, 785 samples were collected from 518 unique locations, 267 (52%) of which were sampled during both study phases for 534 paired pre-/post-intervention samples. We visited additional households and compounds in the follow-up study phase, collecting more samples than at baseline. However, fewer source water samples were collected at follow-up because the municipal water supply was available less often.

Table 3.2: Sampling units and environmental sample counts in each study phase and treatment arm, and number of unique units sampled in both phases and total

	before		after		paired	total
unit	control	intervention	control	intervention		
compounds	31	27	32	34	52	72
households	50	44	59	58	69	142
children	72	63	86	84	112	305
source water	21	23	18	24	29	86
stored water	47	44	55	56	64	202
food surface	47	43	56	52	64	198
latrine soil	31	25	32	30	49	118
household soil	46	39	50	46	61	181

Characteristics suspected as possible confounders of the relationship between sanitation and fecal contamination were generally balanced between treatment arms during each study phase (Table 3.3). However, cumulative precipitation was higher on average in intervention compounds at baseline and in control compounds at follow-up. Water storage containers were also more frequently covered in intervention (78%) than control households (62%) as baseline, though the majority of containers were covered in all strata. Soils were more often wet from control households (48%) than intervention (38%) at follow-up, both of which were lower than at baseline (56% – 59%). The majority of all food surfaces were made of plastic, though more

often so in control households during both study phases. A higher percentage of compounds from both treatment arms reported owning domestic animals at follow-up (81% – 88%) than baseline (53 – 59%), which may be related to differences in the questionnaire between survey phases. The wealth of the average household in all strata was in the low-mid 40s on a 100-point scale, slightly below the midpoint. Mean population density ranged from 5.4 – 7.5 people/100 m^2 compound area.

Table 3.3: Summary statistics and unique observations of compound, household, sampling date, and sample-specific characteristics by treatment arm and study phase. Continuous variables are summarized as median (IQR) and categorical variables as number (%) of observations with a given realization

		before		after	
variable	**level**	**con***	**int#**	**con**	**int**
animals present	compound	16 (53%), n=30	16 (59%), n=27	26 (81%), n=32	30 (88%), n=34
population density (persons/100 m^2)	compound	5.4 (3.7), n=28	7.5 (6.1), n=24	5.6 (4.9), n=30	6.3 (4.6), n=33
wealth index (0 - 100)	household	43.0 (11.9), n=48	43.0 (12.2), n=43	44.2 (17.4), n=57	45.3 (14.0), n=53
previous day mean temperature (°C)	date	20.5 (1.5), n=17	20.1 (2.0), n=15	20.1 (1.2), n=19	20.9 (3.0), n=18
seven-day cumulative precipitation (mm)	date	6.1 (3.0), n=17	14.6 (3.0), n=15	11.6 (19.6), n=19	6.7 (0.0), n=18
water container covered	sample	29 (62%), n=47	32 (78%), n=41	34 (62%), n=55	37 (66%), n=56
narrow-mouth water container	sample	13 (28%), n=47	13 (32%), n=41	16 (29%), n=55	15 (27%), n=56
plastic food surface material	sample	43 (91%), n=47	35 (83%), n=42	53 (95%), n=56	43 (83%), n=52
latrine soil shaded	sample	23 (74%), n=31	13 (72%), n=18	26 (81%), n=32	23 (77%), n=30
latrine soil wet surface	sample	21 (68%), n=31	12 (67%), n=18	19 (59%), n=32	20 (67%), n=30
household soil shaded	sample	36 (78%), n=46	32 (84%), n=38	47 (94%), n=50	37 (82%), n=45
household soil wet surface	sample	27 (59%), n=46	20 (56%), n=36	24 (48%), n=50	17 (38%), n=45

*control treatment assignment; #intervention treatment assignment

3.3.2 Microbial target detection

3.3.2.1 Detection frequencies

We detected at least one microbial target in 93% of samples (733/785), with both *E. coli* targets present in the majority of samples (72%) (Table 3.4). Human targets were frequently detected in soils (60%) and occasionally in stored water (15%) but seldom in source water or on food surfaces. The avian target GFD was rarely detected in any sample type but was most common in soils (4%). cEC was present in every soil sample >85% of stored water and food surface samples collected in the pre-intervention phase but was detected far less often at follow-up, particularly in latrine soils. A similar broad decrease in EC23S was not observed. Water sources in control compounds were much more frequently contaminated with cEC (48%) than in intervention compounds (22%) at baseline but the opposite pattern was observed for EC23S (48% control, 83% intervention). Human targets were somewhat less common in stored water from control households (10% - 13%) than intervention (20%) at both time points. The frequency of HF183 detection increased and detection of Mnif decreased in all soils at follow-up, such that human markers were present in similar proportions of soils at both time points. However, co-detection of both human markers, which suggests highly-credible human-source contamination, increased sharply in control compounds and decreased in intervention compounds for latrine soils collected at follow-up.

Table 3.4: Fraction (%) of samples positive for each target by sample type, study phase, and treatment assignment

	source water		stored water		food surface		latrine soil		household soil	
	con*	int#	con	int	con	int	con	int	con	int
cEC										
before	10/21 (48)	5/23 (22)	44/47 (94)	37/44 (84)	43/47 (91)	38/43 (88)	31/31 (100)	23/23 (100)	46/46 (100)	39/39 (100)
after	5/18 (28)	6/22 (27)	45/55 (82)	44/52 (85)	38/56 (68)	31/47 (66)	28/32 (88)	20/26 (77)	48/50 (96)	37/41 (90)
EC23S										
before	10/21 (48)	19/23 (83)	39/47 (83)	40/44 (91)	35/47 (74)	40/42 (95)	29/31 (94)	24/25 (96)	45/45 (100)	39/39 (100)
after	10/18 (56)	15/24 (62)	48/52 (92)	47/54 (87)	42/56 (75)	32/52 (62)	32/32 (100)	28/30 (93)	48/50 (96)	46/46 (100)
both *E. coli*										
before	6/21 (29)	4/23 (17)	36/47 (77)	34/44 (77)	32/47 (68)	35/42 (83)	29/31 (94)	23/24 (96)	45/45 (100)	39/39 (100)
after	3/18 (17)	5/22 (23)	41/53 (77)	39/50 (78)	31/56 (55)	23/49 (47)	28/32 (88)	19/27 (70)	46/50 (92)	37/41 (90)
HF183										
before	0/21 (0)	1/23 (4)	6/47 (13)	9/44 (20)	1/47 (2)	0/42 (0)	12/31 (39)	9/25 (36)	9/45 (20)	12/39 (31)
after	0/18 (0)	1/24 (4)	5/52 (10)	11/54 (20)	5/56 (9)	1/52 (2)	18/32 (56)	13/30 (43)	25/50 (50)	15/46 (33)
Mnif										
before	0/21 (0)	0/23 (0)	0/46 (0)	1/44 (2)	0/46 (0)	1/42 (2)	16/31 (52)	16/25 (64)	21/45 (47)	8/38 (21)
after	0/18 (0)	0/24 (0)	0/52 (0)	0/54 (0)	1/54 (2)	0/52 (0)	16/32 (50)	11/30 (37)	13/50 (26)	11/46 (24)
either human										
before	0/21 (0)	1/23 (4)	6/46 (13)	9/44 (20)	1/46 (2)	1/42 (2)	22/31 (71)	16/25 (64)	28/45 (62)	17/39 (44)
after	0/18 (0)	1/24 (4)	5/52 (10)	11/54 (20)	6/55 (11)	1/52 (2)	22/32 (69)	22/30 (73)	31/50 (62)	21/46 (46)
both human										
before	0/21 (0)	0/23 (0)	0/47 (0)	1/44 (2)	0/47 (0)	0/42 (0)	6/31 (19)	9/25 (36)	2/45 (4)	3/38 (8)
after	0/18 (0)	0/24 (0)	0/52 (0)	0/54 (0)	0/55 (0)	0/52 (0)	12/32 (38)	2/30 (7)	7/50 (14)	5/46 (11)
GFD										
before	0/21 (0)	0/23 (0)	0/47 (0)	1/44 (2)	0/47 (0)	0/42 (0)	1/31 (3)	1/25 (4)	1/45 (2)	2/39 (5)
after	0/18 (0)	0/24 (0)	1/52 (2)	0/54 (0)	0/56 (0)	0/52 (0)	0/32 (0)	2/30 (7)	2/50 (4)	2/46 (4)
any target										
before	14/21 (67)	20/23 (87)	47/47 (100)	43/44 (98)	46/47 (98)	43/43 (100)	31/31 (100)	24/24 (100)	46/46 (100)	39/39 (100)
after	12/18 (67)	16/24 (67)	53/54 (98)	52/56 (93)	49/56 (88)	41/50 (82)	32/32 (100)	29/29 (100)	50/50 (100)	46/46 (100)

*control treatment assignment; #intervention treatment assignment

3.3.3 Microbial target concentrations

Target concentrations were similar, on average, between treatment arms for all sample types with the exception of cEC in source water, which was more common and at higher concentration in control compounds. Concentrations of cEC decreased at follow-up relative to baseline for all sample types across both treatment arms. With the exception of cEC, no distinct trends in average concentration were observed between study phases for any target. Of the molecular targets, EC23S concentrations were higher on average than HF183 or Mnif, which accords with the less frequent detection of the two human targets.

Table 3.5: Maximum likelihood estimate of target concentration mean (SD) by sample type under a normal distribution with censored observations, stratified by study phase and treatment assignment

		cEC		EC23S		HF183		Mnif	
		con*	int#	con	int	con	int	con	int
source water [log_{10}Q/100 mL]	before	-0.1 (1.2)	-1.2 (1.5)	3.3 (0.8)	3.8 (0.8)				
	after	-0.3 (0.5)	-0.8 (1.2)	3.2 (0.4)	3.5 (0.7)				
stored water [log_{10}Q/100 mL]	before	2.0 (1.3)	1.4 (1.6)	4.1 (0.9)	4.5 (0.8)	1.1 (1.6)	2.0 (1.5)		
	after	1.1 (1.3)	1.1 (1.2)	4.1 (0.8)	4.0 (0.7)	1.3 (1.0)	2.1 (0.8)		
food surface [log_{10}Q/100 cm^2]	before	3.4 (1.8)	2.9 (2.0)	4.4 (0.8)	5.1 (0.9)				
	after	2.0 (1.9)	1.9 (1.9)	4.7 (1.2)	4.4 (1.3)				
latrine soil [log_{10}Q/dry g]	before	4.0 (1.0)	3.9 (0.9)	6.2 (1.1)	6.9 (1.1)	3.8 (1.3)	3.7 (1.5)	4.2 (1.4)	4.4 (0.7)
	after	3.3 (1.2)	2.9 (1.2)	6.8 (1.2)	6.5 (1.2)	4.2 (1.5)	3.9 (0.8)	4.3 (0.9)	4.0 (0.7)
household soil [log_{10}Q/dry g]	before	4.1 (0.9)	4.1 (0.9)	6.7 (0.8)	6.7 (0.9)	3.4 (1.3)	3.4 (1.8)	4.3 (0.4)	3.7 (0.7)
	after	3.7 (0.9)	3.4 (1.2)	6.7 (1.0)	6.6 (0.8)	4.0 (0.6)	3.6 (1.6)	3.4 (1.0)	3.5 (0.7)

*control treatment assignment; #intervention treatment assignment

3.3.4 Co-occurrence of microbial targets

3.3.4.1 Within-sample co-detection of microbial targets

Among all samples, we found significant ($p < 0.05$) correlation between untransformed concentrations of nearly all target pairs in the same sample by non-parametric Spearman rank correlation when non-detect observations were assigned zero concentration (Figure 3.1). Only Mnif and GFD, the least frequently detected targets, were not correlated. The strongest correlation was between *E. coli* targets (ρ: 0.58; 95% CI: 0.53 – 0.62). Molecular *E. coli* target EC23S was more highly correlated with molecularly-detected human targets HF183 (ρ: 0.41; 0.35 – 0.47) and Mnif (ρ: 0.36; 0.44 – 0.54) than culture-based cEC. Though significant, the correlation between the two human targets was relatively weak at $\rho = 0.25$ (0.19 – 0.32).

Differences in detection frequency between targets and sample types present challenges for estimating the predictive value of detecting a particular target. In stored water, for instance, Mnif was very rarely detected and only once co-detected with HF183, suggesting no association. However, HF183 and Mnif were co-detected in 30% of soil samples (primarily latrine soils) and 41% of soil samples positive for one target were also positive for the other, indicating a possible weak association. Among all samples, the odds ratio for HF183 detection given Mnif detection was significant (OR: 3.7; 2.4 – 5.6), but this apparent relationship can be attributed to the nearly exclusive detection of Mnif in soil samples. In essence, Mnif detection serves as an indicator of soil sample type, in which HF183 occurred much more frequently than other sample types. The apparent association between HF183 and Mnif disappears when the analysis is restricted to soil samples. Furthermore, *E. coli* targets were nearly universally detected in soils, such that *E. coli* was necessarily present when human targets were detected. Accordingly, detecting *E. coli* in soil provides little addition information about the likelihood of human target detection; while

detecting human targets in soil without also detecting *E. coli* is unlikely, it is difficult to determine to what extent this is due simply to the low probability of *E. coli* absence generally.

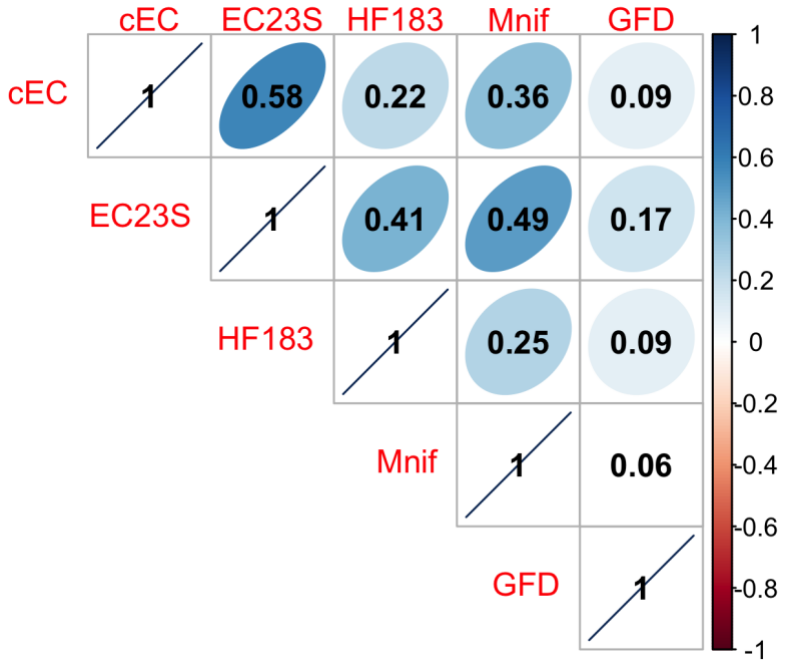

Figure 3.1: Spearman rank correlations of target concentrations within samples, non-detects treated as zero concentration. Positive correlations are shaded blue, negative correlations red, and shading is omitted for non-significant (p > 0.05) correlations. Shading ovals narrow with increasing strength of the association.

3.3.4.2 Within-compound sample contamination

In the median compound, 100% of samples collected during a given study phase were positive for at least one *E. coli* target, regardless of treatment assignment (Table 3.6). Between 20 – 30% of samples were positive for human targets in the median compound, though the proportion positive for Mnif fell to zero in the median intervention compound at follow-up. The median proportion of samples positive for HF183 increased slightly at follow-up for both treatment arms, while the proportion positive for Mnif fell.

We estimated odds ratios for detecting the same microbial target in sample types from the same household or compound that potentially share fecal transmission pathways. Presence of either *E. coli* target in a compound's source water was not associated with its detection in water

stored in the compound's households. HF183 was more likely to be detected in stored water when also detected in the household entrance soil (OR: 3.3; 95% CI: 1.3 – 7.3) but not when detected in the latrine entrance soil. The odds of detecting cEC on food surfaces increased significantly when detected in latrine soils (OR: 5.3; 1.4 – 15) and were also weakly associated with detection of cEC in stored water (OR: 2.5; 0.9 – 5.4). Detection of human targets in latrine soil was corresponded with human target detection in household soil, with a significant association for Mnif detection (OR: 3.7; 1.4 – 8.6) and a weak association for HF183 detection (OR: 2.0; 1.0 – 3.8). As for within-sample co-detection, the near ubiquity of soil *E. coli* prevented estimating associations in most cases.

Table 3.6: Median (IQR) proportion of samples collected from a given compound positive for each target

	before		after	
target	**control**	**intervention**	**control**	**intervention**
cEC	1.00 (0.19)	0.80 (0.24)	0.79 (0.21)	0.80 (0.28)
EC23S	0.80 (0.25)	1.00 (0.12)	0.89 (0.20)	0.81 (0.35)
any EC	1.00 (0.00)	1.00 (0.00)	1.00 (0.10)	1.00 (0.19)
both EC	0.80 (0.07)	0.80 (0.14)	0.75 (0.24)	0.60 (0.30)
HF183	0.18 (0.25)	0.14 (0.25)	0.24 (0.23)	0.19 (0.28)
Mnif	0.20 (0.34)	0.14 (0.25)	0.12 (0.24)	0.00 (0.20)
any human	0.27 (0.18)	0.20 (0.24)	0.33 (0.27)	0.23 (0.26)
both human	0.00 (0.00)	0.00 (0.16)	0.05 (0.14)	0.00 (0.00)
GFD	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
any target	1.00 (0.00)	1.00 (0.00)	1.00 (0.09)	1.00 (0.19)

3.3.5 Difference-in-differences estimates of intervention effect

3.3.5.1 Fecal contamination of individual samples

The intervention was associated with a significant decrease in latrine soil EC23S concentration of -0.9 (95% CI: -1.7 – -0.1) $\log_{10}$gc/dry g in an unadjusted model and -1.2 (-2.1 – -0.4) $\log_{10}$gc/dry g when adjusted for confounding (Figure 3.2). EC23S concentrations also decreased by -0.9 (-1.5 – -0.3) $\log_{10}$gc/100 cm^2 on food surfaces and -0.5 (-1.5 – -0.3)

$\log_{10}$gc/100 mL in stored water, though the strength of both effects was diminished in adjusted models. Intervention effects were not observed for EC23S concentrations in household soil and cEC concentrations in all sample types showed no association with the intervention. We found weak evidence for a reduction in human target HF183 with the intervention in household soils, with an adjusted OR of 0.28 (95% CI: 0.03 – 1.05). Detection of any human target was elevated under the intervention in stored water, household soil, and latrine soil, though the 95% CIs included the null for both unadjusted and adjusted models. Effect estimates in adjusted models generally had wider intervals than in unadjusted models, indicating that consideration of potential confounders increased uncertainty about the intervention effect, though there was little consistency in the direction of change in point estimates towards or away from the null for adjusted models.

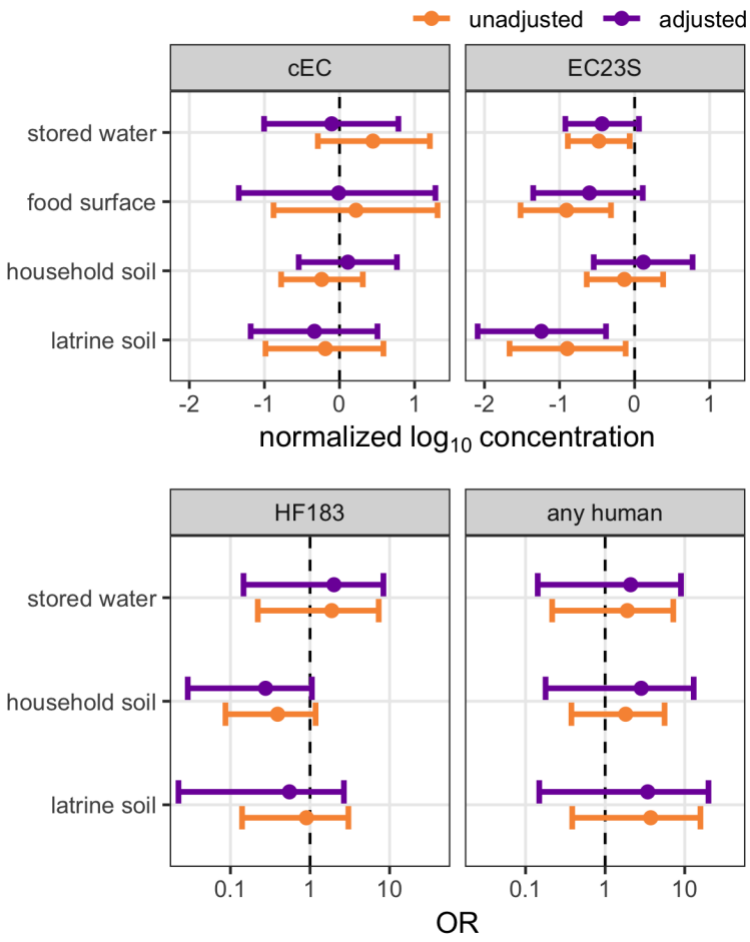

Figure 3.2: Difference-in-differences mean (point) and 95% CI (bar) estimates of change in normalized log_{10} concentration of general fecal indictors (top) and odds ratios for detection of human-associated fecal microbes (bottom). Adjusted models (purple) included precipitation, temperature, compound population density, domestic animals, household wealth, and sample-specific characteristics, including water storage container cover and mouth width, food surface material, and soil sun exposure and surface wetness.

3.3.5.2 Rate of sample contamination within-compounds

We did not find a significant effect of the intervention on the compound-level sample contamination rates for any microbial target (Figure 3.3). In keeping with patterns observed among individual samples, estimated contamination rates for EC23S and HF183 are lower under the intervention but not reliably so, as indicated by 95% CIs spanning the null.

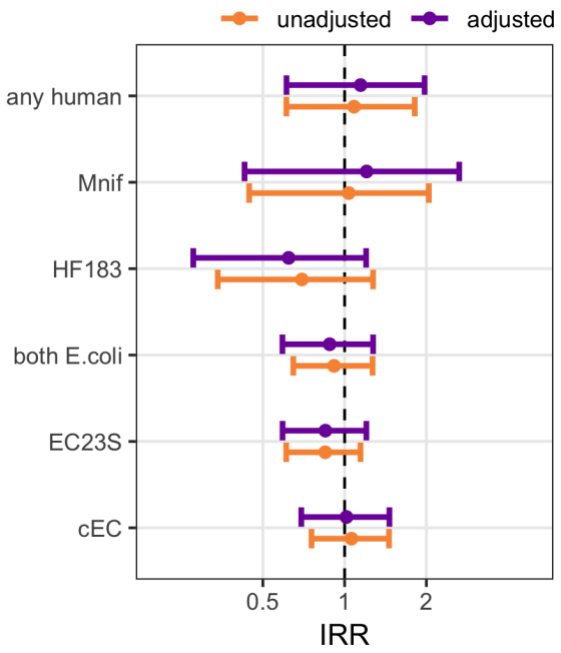

Figure 3.3: Difference-in-differences mean (point) and 95% CI (bar) estimates of incidence rate ratios (horizontal axis) for sample contamination by microbial targets (vertical axis). Adjusted models (purple) included precipitation, temperature, compound population density, domestic animals, and household wealth.

3.3.5.3 Sensitivity of effect estimates to individual observations

A single compound with one enrolled household, visited only during the follow-up study phase, was initially incorrectly recorded as a control compound. Reidentifying each sample from this compound as controls created a natural experiment to explore how sensitive the effect estimates were to the values of individual observations (Figure 3.4). While the direction of each estimated intervention effect was unchanged by the treatment reassignment, the magnitude of many estimates changed substantially and the significance of the effect, as indicated by the 95% CI excluding the null, reversed in two cases. EC23S concentrations in latrine soil were substantially lower when the treatment assignment was corrected, while the OR for HF183 detection in household soil shifted slightly but definitively towards the null.

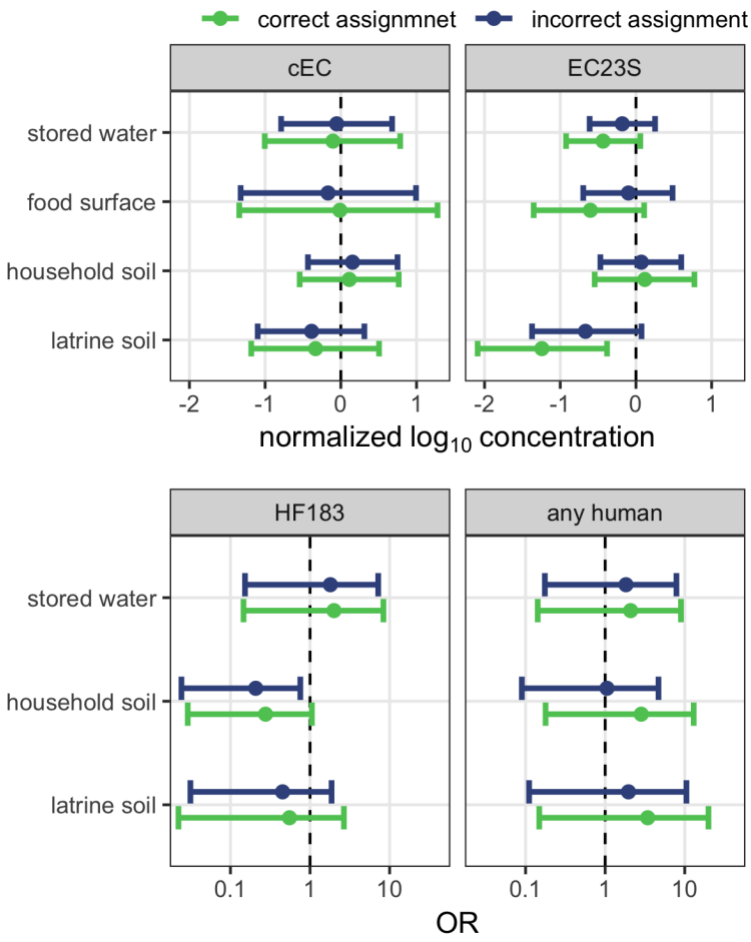

Figure 3.4: Effect of changing treatment assignment for a single observation on adjusted DID intervention effect estimates.

3.4 Discussion

We observed pervasive non-specific fecal contamination in both treatment arms both before and after the intervention. There was a decline in cEC detection frequency and concentration during the follow-up across all sample types and treatment groups. Although we conducted the study during the winter season and there was relatively little rain during each sampling period, Maputo experienced a drought in the intervening months between concluding the baseline sampling activities and beginning the follow-up samples. *E. coli* has been known to reside in tropical and sub-tropical soils, where soil moisture content is closely associated with regrowth (Rivera et al., 1988; Solo-Gabriele et al., 2000). Persistent drought conditions between

study phases may have reduced the population of naturally occurring *E. coli*, thereby reducing the overall levels of *E. coli* contamination observed in samples of the domestic environment. Occurrence of *E. coli* gene targets, however, did not notably change between phases. Human-source contamination was also evident, though there was generally poor correspondence between the two human-associated microbial targets, particularly in stored water. While neither target was wholly specific and some proportion of observations may have been false positives, the low diagnostic sensitivity and relatively high detection limits suggest that further human source contamination may have been overlooked, particularly in soils.

A key limitation of this study was a lack of statistical power, particularly as our sample set was divided between five different sample types. An estimated 345 observations from each treatment arm (690 observations total) were required to detect a 16% reduction in a binary outcome with 70% baseline prevalence at 80% power for the health impact trial in which this study was nested (Brown et al., 2015). By comparison, our largest sample set, stored water, contained about 55 post-intervention observations in each arm. We therefore would not expect to detect any but the largest of intervention effects on fecal contamination of a scale that has not been reported previously (Sclar et al., 2016).

With only a single set of observations pre- and post-intervention, it was not possible to validate the parallel trend assumption inherent in the DID approach (Wing et al., 2018). However, the patterns of human target detection may call into question the suitability of DID analysis in this case (Table 3.4). The detection frequency of HF183 was well matched at baseline between treatment arms in latrine soils but notably lower in the control arm for household soils. Mnif was somewhat more common in intervention latrine soils than control but more than twice as prevalent in soils from control households than intervention households. At 12-month follow-

up, HF183 detection increased in latrine soils for both treatment arms, though the increase was much greater in the control group. The same trend was amplified in household soils, where baseline HF183 detection was relatively rare. Mnif, on the other hand, decreased in all soil samples at follow-up, particularly in control household soils—precisely the group for which HF183 showed the largest increase. For both targets the detection frequency in intervention household soils remained fairly stable between phases, but due to large and opposite changes in detection at control households, the intervention appeared to substantially reduce HF183 in household soils while increasing Mnif in the same set of samples. One would draw opposite conclusions about the impact of the intervention on human-source contamination were only one or the other of these targets used. Both conclusions are likely incorrect and possibly attributable to imperfect assays operating at the edge of analytical sensitivity randomly succeeding and failing to detect underlying contamination; indeed, characterizing human contamination as detection of either target results in relatively steady prevalence across time within each treatment arm for both soil types. By contrast, identifying human contamination as the co-detection of both human targets gives the impression of an exceptionally large intervention effect in latrine soils, as detection was substantially higher in interventions than controls at baseline, with a precipitous decline in intervention detection and a sharp increase in control detection at follow up.

Further evidence potential challenges with the DID estimates for this dataset can be seen in an unplanned sensitivity analysis that arose from the re-categorization of a single compound from intervention to control due to a coding error (Figure 3.4). Sampled only at follow-up and containing a single enrolled household, this compound represented at most one observation in each model yet reversed the interpretation of the intervention effect for two cases and substantially changed the point estimate in several others. While perhaps a useful reminder of the

hazard in interpreting associations on the basis of whether the 95% CI of the estimate excludes the null, the magnitude of the change in multiple effect estimates due to re-categorization of a single observation raises concern about the interpretation of such estimates based on this dataset.

Sanitation conditions have rarely been associated with measures of domestic fecal contamination (Sclar et al., 2016). This may be due in part to the typical approach of measuring fecal microbes, which have long served as useful fecal indicators in settings where fecal impacts are unexpected, such as natural waters, but appear nearly ubiquitous in environments where humans and animals spend their time. The noise inherent in fecal microbe measurements likely obscures any sanitary signals that may be present. Furthermore, the existence of multiple transmission pathways can prevent even substantial interruptions in transmission along a dominant pathway from producing measurable effects (Briscoe, 1984). A threshold-level of disruption to both primary and secondary transmission pathways may need to be achieved before improvements to fecal contamination and health can be observed. This threshold remains poorly defined, but likely represents a transformative change living conditions for both households and neighborhoods (Husseini et al., 2018; Otaki et al., 2007).

CHAPTER 4: ASSOCIATIONS BETWEEN FECAL INDICATORS AND CHILD HEALTH

4.1 Introduction

Many different microorganisms cause human disease through fecal-oral infection, in which pathogens shed in the feces of an infected host are ingested by a new host, leading to further infection (Sobsey, 2015). A diverse set of pathways, many environmentally mediated, serve to transmit fecal pathogens between individuals (Feachem et al., 1983; Wagner and Lanoix, 1958). Due to typically low environmental abundance, difficulty to culture, and the variety of organisms potentially present, the risk of environmental exposure to fecal pathogens has traditionally been evaluated using fecal indicator organisms (FIO) like *Escherichia coli* (Field and Samadpour, 2007; Harwood et al., 2014). The assumption underlying this approach is that detection of FIO, which are abundant in the feces of most animals and easily quantified from environmental media, represents recent fecal contamination and the probable presence of fecal pathogens. It is also assumed that FIO abundance is proportional to fecal pathogen abundance, such that higher FIO concentration indicates high risk of fecal pathogen exposure. However, many fecal pathogens, including viruses responsible for the greatest burden of enteric disease globally (Kotloff et al., 2013; Platts-Mills et al., 2015), are highly host-specific. Non-human source fecal contamination therefore likely carries a different risk to human health than human fecal contamination (Field and Samadpour, 2007), which traditional FIO are unable to differentiate. Furthermore, detection of *E. coli* and other coliform bacteria may not be indicative of fecal sources at all, as they have been repeatedly shown to naturally occur in the environment

(Rivera et al., 1988; Solo-Gabriele et al., 2000). However, traditional FIO have been repeatedly associated with the risk of enteric disease, particularly under strong study designs that minimize bias (Ercumen et al., 2017a; Gruber et al., 2014).

Recently, microbial source tracking (MST), in which molecular detection of host-associated FIO is used to determine fecal source, has been deployed to investigate transmission in various settings (Boehm et al., 2016; Harris et al., 2016; Odagiri et al., 2016; Schriewer et al., 2015). Unlike traditional FIO, the extent to which MST markers are indicative of pathogens and disease has yet to be fully investigated (Harwood et al., 2014). A study in rural India found an increased risk of child diarrhea in the six weeks following the detection of MST markers in the home, but no study has yet explored the relationship between MST marker abundance in multiple domestic exposure routes and health using objective and accurate health outcomes (Odagiri et al., 2016).

Although diarrhea is the second-leading cause of death for children globally, the majority of infections with enteric pathogens are asymptomatic (Kotloff et al., 2013; Taniuchi et al., 2013; Wang et al., 2016). These infections are increasingly thought to be associated with growth faltering, environmental enteric dysfunction, and other distal health outcomes, but are not captured by caregiver reported diarrhea commonly used as the health outcome in evaluations of water, sanitation, and hygiene interventions (George et al., 2017; Kosek et al., 2017). Accordingly, we assessed associations between objective measures of enteric infection in child stool and exposure to fecal microbes, including traditional FIO, molecularly-detected FIO, and human-associated MST markers, in multiple domestic fecal transmission pathways.

4.2 Methods

4.2.1 Study setting

The capital of Mozambique, Maputo is a rapidly growing city with an expected population of 1.8 million by 2025 (UN-HABITAT, 2014). An estimated 70% of residents live in unplanned slum settlements with poor services and low-quality infrastructure. These areas are densely populated, with mean population densities greater than 15,000 people/km^2, compared with 4086 people/km^2 for the city as a whole (Barros et al., 2014; UN-HABITAT, 2010). The risk of exposure to fecal pathogens in these neighborhoods is of particular concern given that onsite sanitation systems are used by 89% of Maputo residents, the majority of urban Mozambicans lack access to improved sanitation as defined by UNICEF/WHO Joint Monitoring Programme, and three-quarters of Maputo's fecal waste is not safely managed (Blackett et al., 2014; WHO/UNICEF, 2017). High burdens of fecal-oral disease, a leading cause of child mortality in Mozambique and globally, have been reported in urban Maputo and southern Mozambique. (Devamani et al., 2014; Fonseca et al., 2014; Knee et al., 2018; Kotloff et al., 2013; Nhampossa et al., 2013; Sitoe et al., 2018).

4.2.2 Study design

The Maputo Sanitation (MapSan) study (clinicaltrials.gov NCT02362932) was conducted in low-income neighborhoods in urban Maputo to evaluate an onsite sanitation intervention to reduce child enteric infections (Brown et al., 2015). The intervention, a pour-flush latrine with infiltration pit, was implemented in compounds (household clusters) in 2015 – 2016 to replace existing shared sanitation in poor condition. Additional compounds with similarly poor-quality shared sanitation served as control sites. We conducted two cross-sectional assessments of child health and domestic fecal contamination in MapSan compounds. Baseline child enrollment was

conducted shortly before latrines were opened for use by residents of intervention compounds, with ongoing frequency-matched enrollment of control compounds. All children living in study compounds between 29 days and 48 months old at baseline were enrolled after a parent or guardian provided written, informed consent. We conducted follow-up visits approximately 12-months after baseline enrollment, resurveying previously enrolled children and enrolling any new children and households not present at baseline. Deworming treatment was provided to all compound members by the Ministry of Health after each study visit. This study was approved by the Comité Nacional de Bioética para a Saúde (CNBS), Ministério da Saúde, Republic of Mozambique (333/CNBS/14), the Ethics Committee of the London School of Hygiene & Tropical Medicine (reference # 8345), the Institutional Review Board of the University of North Carolina at Chapel Hill (IRB # 15-0963), and the Institutional Review Board of the Georgia Institute of Technology (protocol # H15160).

4.2.3 Data collection

4.2.3.1 Child characteristics

Health, socioeconomic, demographic, and sanitary characteristics were assessed by surveys administered during each study phase, as described previously (Knee et al., 2018). Child- and household-level questionnaires were administered to the parent or other caregiver of each enrolled child and compound-level questionnaires to the head of each compound. Child characteristics collected include age, sex, breastfeeding status, and caregiver-reported diarrhea, defined as three or more loose or watery stools or any bloody stool in a 24-hour period in the previous seven days. Household assets were ascertained to compute a household wealth index using a scorecard developed for Mozambique, excluding sanitation-related assets (Schreiner et al., 2013). We calculated population density from reported compound population and estimated

compound area, which was calculated by delineated the boundaries of each compound on high-resolution aerial photographs.

4.2.3.2 Child stool

After completing enrollment and administering surveys, the child's caregiver was provided a disposable diaper and a large, plastic, sterile sampling bag and was requested to place the diaper containing the child's next bowel movement in the bag, to be retrieved by the study team the following morning. If the stool sample was not available the next day, a team member would return on subsequent days to obtain the sample, coordinating with the caregiver and providing additional sampling supplies as necessary. Stool samples were placed in a cooler and transported to the medical parasitology laboratory at the National Institute of Health (INS) in the Ministry of Health of Mozambique (MISAU) in central Maputo, typically by early afternoon. Upon arrival at the lab, a portion of each sample was transferred to up to three 2 mL cryovials and immediately archived at -80 °C. The remaining sample was processed the same afternoon for soil transmitted helminth analysis by the Kato Katz microscopy method, with results read within 30 minutes for hookworm and the remaining results read the following day (WHO, 2004). We prioritized sample archives over helminth analysis if the volume of stool was limited. If the sample was sufficiently liquid that stool could not be transferred to a cryovial, we instead archived a section cut from the saturated diaper. Archived stool samples were transported on dry ice to Atlanta, GA, USA, where they were stored at -80 °C awaiting further analysis.

4.2.3.3 Environmental samples

During the return visit to collect stool from children enrolled the previous day, we sampled multiple locations in domestic environment representing potential routes of fecal-oral exposure through drinking water, food ingestion, and soil ingestion (Julian, 2016). At each

household with enrolled children, we sampled stored drinking water, soil from the entrance to the residential structure, and swabs of surface used in food preparation, following sampling procedures described elsewhere (Chapter 2). We also collected a single sample from a piped water source in each compound, when available, and a soil sample from the entrance to the compound latrine. Respondents were asked to provide stored water and food surfaces in the manner and condition under which they would typically be used in caring for a child to account for realistic exposure conditions. Samples were immediately placed on ice and transported to MISAU with the child stool samples for same-day processing of water samples and same- or next-day processing of soil and surface samples by membrane filtration, preceded by manual elution for soil and swab samples by a procedure described previously (Chapter 2). We adapted USEPA Method 1603 to enumerate culturable *Escherichia coli* (cEC) from filters on modified mTEC broth (Hi-Media, Mumbai, India) and immediately archived additional filters at -80 °C for molecular analysis (USEPA, 2009). Archived filters were transported to Atlanta, GA, USA with the stool samples, transferred to Chapel Hill, NC on dry ice, and returned to storage at -80 °C awaiting further analysis.

4.2.4 Enteric pathogen detection in stool

Stool samples were analyzed for the presence of 15 enteric pathogens using the Luminex MagPix xTAG Gastrointestinal Pathogen Panel (GPP, Luminex Corp, Austin, TX). The GPP uses multiplex RT-PCR with bead-based fluorescent reporting to directly molecular signatures from nine bacteria, three viruses, and three protozoa in stool (Claas et al., 2013). Bacterial targets include *Campylobacter* spp. (*C. jejuni, C. coli, C. lari*), *Clostridium difficile* Toxin A/B, *Salmonella* spp., *Yersinia enterocolitica, Vibrio cholerae*, *Shigella* spp. (*S. boydii, S. sonnei, S. flexneri, S. dysenteriae*), and three pathogenic *E. coli* strains: enterotoxigenic *E. coli* (ETEC)

LT/ST, Shiga-like toxin-producing *E. coli* (STEC) stx1/stx2, and *E. coli* O157. Adenovirus 40/41, norovirus GI/GII, and rotavirus A comprise the viral targets and *Cryptosporidium* spp. (*C. parvum, C. hominis*), *Entamoeba histolytica*, and *Giardia lamblia* the protozoan parasites.

We pretreated bulk stool samples with 1 mL of ASL stool lysis buffer (Qiagen, Hilden, Germany) and eluted diaper samples in 2.5 mL of ASL buffer using a sterile 10 mL syringe to forcefully rinse the sample five times. Nucleic acids were isolated from 1 mL of pretreated stool or eluate using the QIAamp 96 Virus QIAcube HT Kit on the QIAcube HT platform (Qiagen) and analyzed by GPP within 24 hours.

4.2.5 Fecal microbe detection and quantification in environmental samples

We analyzed archived filter membranes for three fecal microbe gene targets, two corresponding to organisms associated specifically with human feces (HF183 and Mnif) and one corresponding to the general fecal indicator *E. coli* (EC23S857, "EC23S") to complement the culture-based assessment of *E. coli* (Chern et al., 2011; Green et al., 2014; Johnston et al., 2010). Molecular targets were detected with qPCR assays that were previously validated on fecal samples from study sites using a CFX96 Touch thermocycler (Bio-Rad, Hercules, CA) and cycling conditions specified by the assay developers (Chapter 2). We used salmon testes DNA as a specimen processing control to assess PCR inhibition, treating each filter with 3 µg before extracting DNA (Haugland et al., 2005, 2012). The DNeasy PowerSoil Kit was used to extract DNA from soil and sample filters and the DNA-EZ ST01 Kit (GeneRite, North Brunswick, NJ, USA) was used for water samples (Cox and Goodwin, 2013). We used 5 µL of purified DNA as template in each 25 µL reaction, which contained 12.5 µL TaqMan Environmental Master Mix 2.0 and 2.5 µL 10x primers and probe mix, with nuclease free water making up the remaining volume. Triplicate no-template control (NTC) reactions and triplicate reactions at each point of

serial dilution series of extracted reference DNA corresponding to $10^5 – 10^1$ or $10^6 – 10^2$ target gene copies (gc) were included on each instrument run.

The concentration of each target in microbial samples was estimated from calibration curves fit to all reactions containing the standard serial dilution series. Processing variation was accounted for by allowing the calibration curve slopes and intercepts to vary by extraction batch and instrument run using multilevel Bayesian regression fit in **R** version 3.5.1 with the **brms** package (Bürkner, 2017; Sivaganesan et al., 2010). Target concentrations were calculated on the $\log_{10}$ scale and were normalized according to sample type: per 100 mL water, 100 cm^2 surface, or dry gram of soil. We imputed concentrations for samples in which the target was not detected as the expected value of the sampling distribution of concentrations truncated at the sample-specific LLoD (Messier et al., 2012). We assumed the $\log_{10}$ concentrations followed a normal distribution subject to censoring, for which we obtained maximum likelihood estimates of the mean and standard deviation (SD) using the *fitdistcens* function in the **R** package **fitdistrplus** and expected values of the truncated distribution with the package **truncnorm** (Delignette-Muller and Dutang, 2015; Mersmann et al., 2018)**.**

4.2.6 Statistical analysis

We investigated associations between child enteric health outcomes and domestic fecal contamination, as indicated by both non-specific and human-associated fecal microbes, in multiple exposure pathways. Child enteric health was characterized as infections by pathogen class (bacterial, viral, protozoan, and STH), the number of concurrent infections (out of 23 pathogens tested), the number of classes represented among concurrent infections, and reported diarrheal illness. Occurrence of individual microbial targets was represented by $\log_{10}$-transformed, mean-centered, and standard deviation-scaled concentration. The combined

influence of multiple microbial targets was characterized by a binary indicator of joint detection status, representing whether both *E. coli* targets or either human target were detected in each sample.

Associations with microbial target occurrence were estimated by multilevel logistic regression for binary response variables, including infection status for each pathogen class and reported diarrhea, and multilevel negative binomial regression for counts of concurrent infections and pathogen classes. We fit single exposure models to test associations with microbial target occurrence in each sample type individually and multiple exposure models that included a predictor variable for target occurrence in each sample type. All models were adjusted for child age, sex, and breastfeeding status, caregiver education, and household wealth. The adjustment set was selected *a priori*, comprising known confounders of the relationship between other exposures and child enteric infection (Knee et al., 2018). A binary indicator of whether the child was living in a compound that had received the intervention was also included as a model predictor to account for the possibility of intervention effects on child health not mediated by the reduction in environmental fecal contamination. The intercept was allowed to vary by compound in all models to account for clustering in responses by compound. While repeated measures were also collected at the household and child level, the inclusion of varying intercepts for these more deeply nested groups produced unstable estimates of population-level parameters.

4.3 Results

4.3.1 Enrollment and child characteristics

We collected environmental samples associated with 193 unique children from 135 households in 71 compounds (Table 4.1). Paired baseline and 12-month follow-up responses were obtained for 122 children. Child health outcomes were drawn from caregiver surveys and

two separate analyses of child stool: GPP analysis to diagnose bacterial, viral, and protozoan infections and Kato Katz analysis to detect STH infections. All three health outcome data sources were available for 89 children at baseline and 87 at follow-up. We sampled from 11 additional compounds and 22 additional households during the 12-month study phase, with a corresponding increase in environmental samples over baseline. Source water and latrine soil were sampled once per compound for fewer total samples compared to stored water, food surfaces, and household soil, which were collected at each household. Soil could not be collected from household entrances surrounded by impervious surface, resulting in fewer household soil samples than other household-level samples.

Table 4.1: Number of sampling units by study phase

sampling units	count	
total child records	305	
unique children	193	
paired responses	112	
	baseline	**follow-up**
caregiver surveys	121	133
GPP stools	93	108
Kato Katz stools	90	89
complete responses	89	87
compounds	55	66
households	91	113
source water	43	41
stored water	89	100
food surface	88	98
latrine soil	53	59
household soil	81	85

The median child was 1.7 years old at baseline and 2.2 years old at 12-month follow-up, reflecting the enrollment of new children at follow-up, particularly infants born since baseline (Table 4.2). Although more child age observations were missing than other survey-derived child characteristics, age was available for nearly every stool sample analyzed by GPP and Kato Katz. Males were slightly overrepresented among stool samples despite a slightly high proportion of

female children among all survey responses. Approximately a third of children were breastfed at baseline, dropping to a quarter of children at 12 months, reflecting aging of the study population; exclusive breastfeeding was rare. The caregivers for about half the children had completed primary school. Median household wealth fell somewhat below the midpoint of the asset-based wealth index (43 on a 100-point scale) across study phases and response subgroups. Just over half of children (51 – 52%) at 12-months lived in compounds that had received the latrine intervention.

Table 4.2: Summary of child characteristics by health outcome source, as count (%) for binary variables and median (IQR) for continuous variables

	surveys				GPP				Kato Katz			
	baseline		12-months		baseline		12-months		baseline		12-months	
	n	value	n	value	n	value	n	value	n	value	n	value
child age (years)	95	1.7 (1.9)	104	2.2 (2.2)	92	1.7 (1.9)	106	2.2 (2.2)	89	1.7 (1.9)	87	2.1 (2.0)
child sex, female	117	60 (51)	133	69 (52)	90	42 (47)	108	53 (49)	86	41 (48)	89	42 (47)
any breastfeeding	121	42 (35)	133	31 (23)	93	33 (35)	107	26 (24)	90	31 (34)	88	21 (24)
exclusive breastfeeding	121	10 (8)	133	4 (3)	93	7 (8)	107	3 (3)	90	7 (8)	88	2 (2)
caregiver primary educated	121	60 (50)	133	70 (53)	93	46 (49)	107	55 (51)	90	44 (49)	88	45 (51)
wealth index (0 - 100)	121	43 (12)	129	43 (12)	93	43 (11)	106	43 (12)	90	43 (9)	87	43 (15)
received intervention	121	0 (0)	133	69 (52)	93	0 (0)	108	56 (52)	90	0 (0)	89	45 (51)

4.3.2 Child enteric health outcome prevalence

Enteric infections were very common among the study population. At baseline, 86% of all children were infected with at least one pathogen (of 23 assayed), which increased to 92% during follow-up. Infections were even more common among children with paired observations, with 92% infected with at least one pathogen at baseline and 97% infected during follow-up. Two-thirds of children at baseline were co-infected with a median of two pathogens from two different classes, rising to 90% at follow-up for children with paired observations. The majority

of infections were asymptomatic, however, with only 8 – 11% of children experiencing diarrhea in the previous seven days. In contrast with enteric infections, children with paired observations at baseline were least affected by diarrhea. Bacteria were the most common pathogen class, present in about three-quarters of children, followed by protozoa and helminths, which each affected about half the study population. Viral infections affected about 15% of children.

Table 4.3: Fraction (%) of observations positive for each health outcome among all children and among only children with paired observations at baseline and follow-up, stratified by study phase

	all observations		paired observations	
outcome	**baseline**	**follow-up**	**baseline**	**follow-up**
bacterial infection	66/93 (71)	84/108 (78)	30/39 (77)	34/39 (87)
viral infection	14/93 (15)	17/108 (16)	5/39 (13)	6/39 (15)
protozoan infection	48/93 (52)	60/108 (56)	20/39 (51)	27/39 (69)
STH infection	39/90 (43)	49/89 (55)	15/30 (50)	19/30 (63)
any infection	81/94 (86)	99/108 (92)	36/39 (92)	38/39 (97)
> 1 infection	63/94 (67)	81/108 (75)	28/39 (72)	35/39 (90)
median (IQR) infections	2 (2)	2 (2.25)	3 (2)	3 (2)
> 1 pathogen class	59/94 (63)	76/108 (70)	27/39 (69)	34/39 (87)
median (IQR) classes	2 (2)	2 (2)	2 (2)	2 (1)
diarrhea	10/121 (8)	14/133 (11)	6/64 (9)	2/64 (3)

4.3.3 Abundance and sources of environmental fecal contamination

The domestic environment was heavily impacted by both general and human-source fecal contamination. We detected the general fecal indicator *E. coli* by both culture and qPCR approaches in > 75% of all samples other than source water, which was municipally treated and piped on premises. However, 31% of source water samples were positive for cEC and 22% were positive for both cEC and EC23S, which suggests highly credible *E. coli* contamination. Soils were most frequently contaminated, with EC23S detected in 96% of latrine entrance and 99% of household entrance soil samples. Human source contamination was also most common in soils and was particularly frequent in latrine entrance soils. Human-associated Mnif and HF183 were each present in about half of latrine soils and a third of household soils but were co-detected in

only 24% and 9% of latrine and household soils, respectively. Mean EC23S concentrations were as much as 1000 gc/dry gram of soil higher than the mean concentration of either human target.

Mnif was rarely detected outside soil, while HF183 was also present in 16% of source water samples and on 7% of food preparation surfaces. Food preparation surfaces also had the lowest frequency of *E. coli* contamination among samples subject to domestic fecal transmission with detection frequencies of 76% and 78% for EC23S and cEC, respectively. As in soils, mean EC23S concentrations were substantially higher (2.6 $\log_{10}$gc/100 mL) than HF183 in stored water samples.

Table 4.4: Number of samples positive (%) for each microbial target and corresponding mean (SD) normalized log_{10} concentration maximum likelihood estimates under a censored normal distribution, by sample type

	source water			stored water			food surface			latrine soil			household soil		
target	**n**	**detect**	**conc.***	**n**	**detect**	**conc.**	**n**	**detect**	**conc.**	**n**	**detect**	**conc.**	**n**	**detect**	**conc.**
cEC	83	26 (31)	-0.6 (1.2)	193	165 (85)	1.3 (1.4)	188	146 (78)	2.5 (2.0)	109	99 (91)	3.5 (1.2)	171	166 (97)	3.9 (1.0)
EC23S	85	53 (62)	3.4 (0.8)	192	169 (88)	4.1 (0.8)	192	145 (76)	4.6 (1.1)	115	110 (96)	6.6 (1.2)	175	173 (99)	6.7 (0.9)
HF183	85	2 (2)		192	30 (16)	1.5 (1.3)	192	7 (4)		115	50 (43)	3.9 (1.3)	175	58 (33)	3.7 (1.3)
Mnif	85	0 (0)		191	1 (1)		190	2 (1)		115	58 (50)	4.2 (1.0)	174	52 (30)	3.7 (0.8)
both *E. coli*	83	18 (22)		189	145 (77)		189	117 (62)		111	96 (86)		170	163 (96)	
any human	85	2 (2)		191	30 (16)		191	9 (5)		115	80 (70)		175	94 (54)	
both human	85	0 (0)		192	1 (1)		191	0 (0)		115	28 (24)		174	16 (9)	

*target normalized log_{10} concentration

4.3.4 Associations between fecal microbe targets and child enteric health

We did not observe any particular trends between different microbial indicators of fecal contamination and infection with enteric pathogens (Figure 4.1). Associations between a particular pathogen class and microbial target from a specific location in the domestic environment were occasionally significant. A one-SD increase in cEC concentration in source water, for example, was strongly associated with increased odds of viral infection (aOR: 12.6; 95% CI: 1.4 – 67.5), though only in a multiple exposure model that included standardized cEC concentrations for each sample type as predictor variables. The odds of viral infection were still elevated with increasing source water cEC concentration in a single exposure model but the strength of the association was greatly diminished (OR: 1.9; 1.0 – 3.6). A similar scenario was observed for STH infection with stored water cEC concentration (multiple exposure OR: 5.2; 1.2 – 19.2). By contrast, bacterial infection was weakly associated with source water concentrations of both cEC (OR: 2.2; 1.0 – 4.4) and EC23S (OR: 2.0; 1.0 – 3.9) in single exposure models only. Intriguingly, protozoan infections were reduced with higher HF183 concentrations, though the relationships were not significant except in latrine soil, where it was weakly so (single exposure OR: 0.7; 0.4 – 1.0). We were unable to estimate associations with human targets in source water and on food surfaces, where they were rarely detected.

No microbial target and sample type were associated with the number of pathogens or pathogens classes coinfecting a child and only latrine soil EC23S concentration was strongly associated with diarrhea (OR: 20; 1.8, 100), an exceptionally imprecise estimate that demands particular caution to interpret. The number of concurrent pathogens was marginally diminished with increased HF183 concentration in stored water while rising slightly with the detection of any human target in household soils, neither of which are readily differentiated from the null.

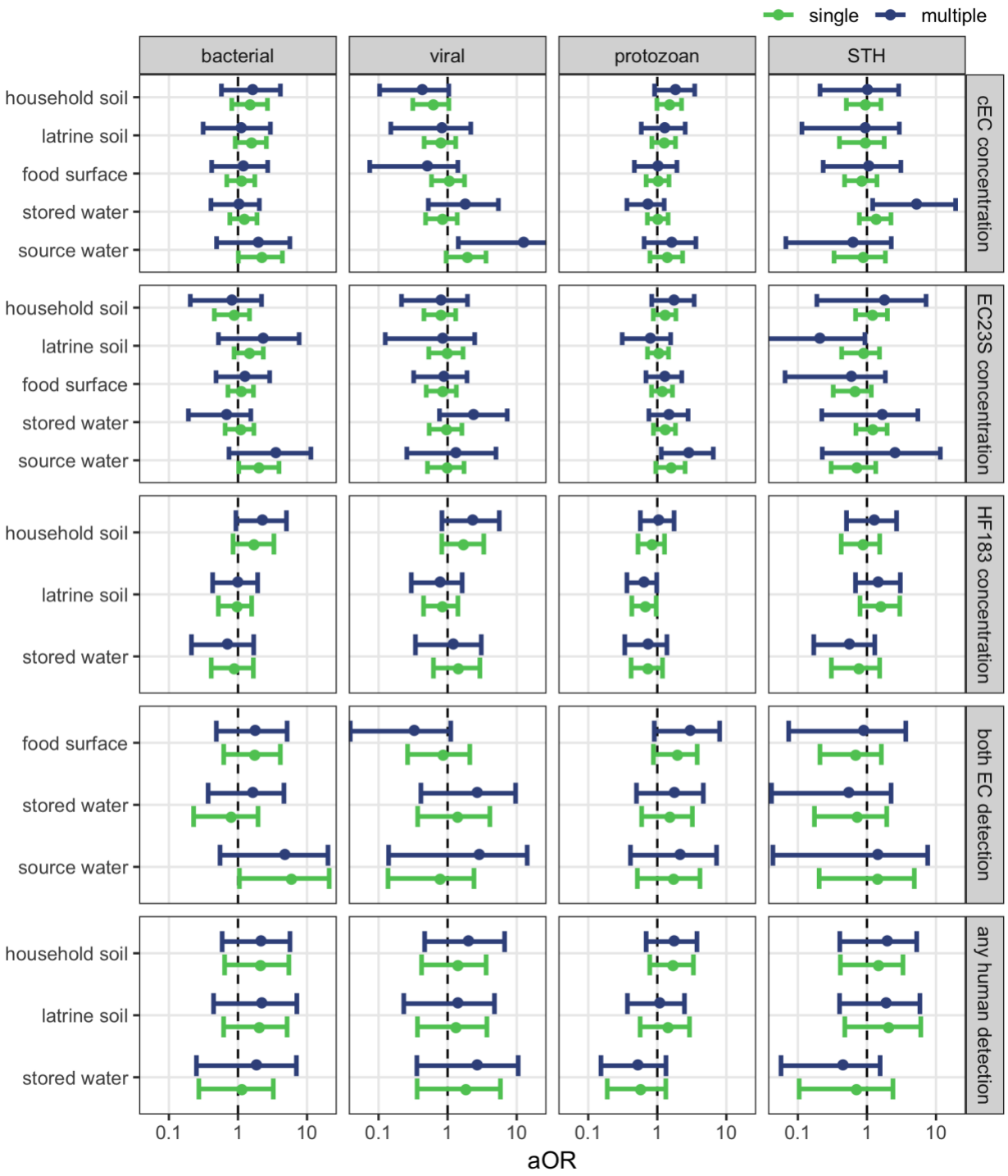

Figure 4.1: Adjusted odds ratio of infection by pathogen class (columns) given contamination of environmental samples (vertical axis) with fecal microbes (rows) from single-exposure (green) and multiple-exposure (blue) models.

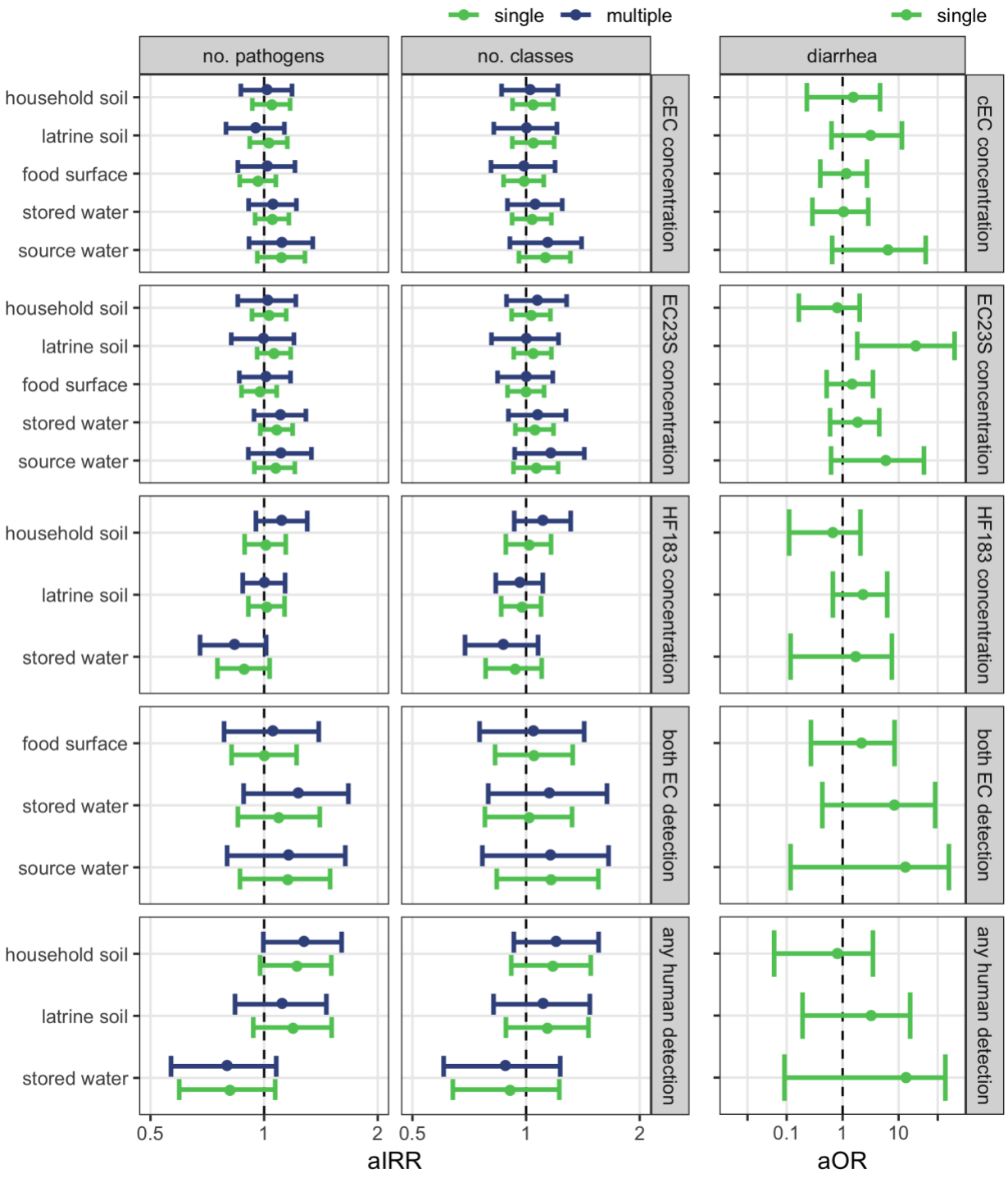

Figure 4.2: Adjusted incidence rate ratio (right) and odds ratio (left) of pathogens, classes, and diarrhea (columns) given contamination of environmental samples (vertical axis) with fecal microbes (rows) from single-exposure (green) and multiple-exposure (blue) models.

4.4 Discussion

Enteric infections and domestic fecal contamination were both exceptionally common in our study setting. Between two-thirds and three-quarters of child were infected with multiple pathogens from different classes. *E. coli* was present in at least three-quarters of all domestic locations sampled (excluding municipally treated source water) and was nearly ubiquitous in soils. Although most children were asymptomatic, the high prevalence and diversity of pathogens shed confirm that children were exposed to multiple fecal pathogens, and the high correspondence between infections assessed twelve months apart suggest that children were exposed persistently. The presence of human-associated fecal microbes in household stored water and domestic soils suggests that fecal pathogen transmission and exposure may occur in the domestic environment. However, we did not find consistent evidence that the occurrence of fecal microbes, including human-associated targets, on pathways of fecal-oral exposure was associated with the enteric health of children.

Despite high prevalence of most outcomes, the statistical power of our study was limited by a relatively small sample size, which was constrained by the number of compounds from which it was feasible to collect extensive environmental samples. Furthermore, by collecting child stool and environmental samples concurrently we effectively assessed exposures after the outcome, with the implied assumption that exposures were relatively stable through time such that post-outcome exposures are representative of pre-outcome exposures. However, fecal microbes can be highly variable over short time scales and prospective microbial exposure assessment has shown better association with fecal-oral disease than cross-sectional assessment (Ercumen et al., 2017a; Holcomb et al., 2018; Levy et al., 2009, 2008; Luby et al., 2015). Human-associated fecal microbes in the domestic environment were found to be associated with

diarrhea in a large study with prospective exposure assessment conducted in rural India, but to our knowledge a similar analysis has not been conducted in a crowded, urban setting (Odagiri et al., 2016).

Given the low power, it is not surprising that estimated associations between most microbial targets and health outcomes were weak or absent. However, for a number of comparisons the bounds of the 95% CI fell curiously close to the null value. Perhaps the most consistent associations were with source water, contamination of which generally corresponded to increased odds of individual infections and rate of coinfections. Many of the estimates that nearly or barely excluded the null were for source water exposures. The distinct fecal transmission dynamics governing source water, which was generally municipally treated, piped to compound premises, free from human fecal contamination, and lightly impacted by non-specific fecal contamination, may support cautious interpretation of a genuine signal between source water contamination and risk of enteric infection. Because contaminated water would have been delivered concurrently to all compounds with water points, multiple children participating in the study could have been exposed during each episode of source water contamination, potentially resulting in clusters of infection observations. Non-specific fecal contamination of source water has previously been associated with risk of diarrheal illness, though not necessarily to a greater degree than household water (Ercumen et al., 2015; Gruber et al., 2014; Levy et al., 2012; Luby et al., 2015; Moe et al., 1991).

CHAPTER 5: CONCLUSION

In keeping with the majority of studies reported previously, we did not find consistent evidence of a relationship between ambient fecal contamination of the domestic environment and sanitation conditions (Sclar et al., 2016). Where impacts on fecal contamination have been observed previously, the pathways affected were directly intervened upon by a specific intervention, such as household water treatment, with no concurrent reduction along indirect pathways, even in the context of combined water, sanitation, and hygiene interventions (Ercumen et al., 2018a, 2018b). Most previous studies of sanitation and domestic fecal contamination were conducted in rural settings, where transmission patterns likely differ from those in the dense, informal urban communities we investigated. Nevertheless, livestock have been identified as major fecal sources in both settings (Boehm et al., 2016; Ercumen et al., 2017b; Harris et al., 2016; Schriewer et al., 2015).

Fecal contamination has primarily been measured using fecal indicator organisms, both non-specific and host-associated. These tools were largely developed for monitoring surface water quality and were later applied to drinking water. While associations between fecal indicators and fecal pathogens are weak and variable (Korajkic et al., 2018; Wu et al., 2011), both recreational and drinking water exposure to fecal indicators, particularly culturable *E. coli*, are associated with increased risk of diarrhea and other adverse health outcomes under certain conditions and using appropriate study designs (Ercumen et al., 2017a; Gruber et al., 2014; Wade et al., 2003). However, many organisms used as fecal indicators are known to persist and replicate in the environment, such that their presence is not necessarily indicative of recent fecal

contamination (Rivera et al., 1988; Solo-Gabriele et al., 2000). In settings where one would not expect terrestrial animal-associated microbes, such as natural waters, their presence appears to serve as a reliable indicator of animal impacts. When impacts are entirely expected, as is the case with domestic environments in which humans live in close proximity and conduct much of their daily activity, we seem to find their associated microbes everywhere we choose to look. Regardless of whether fecal material is notably present in the environment, fecal microbes appear to be shed into the ambient environment wherever humans and animals spend their time.

Host-associated fecal microbes, as obligate anaerobes with constrained source distributions, may be expected to provide a clearer fecal signal in settings with ubiquitous non-specific fecal microbes. Indeed, livestock-associated microbial source tracking markers have effectively demonstrated the contribution of domestic animals to the fecal contamination of many households (Boehm et al., 2016; Harris et al., 2016). However, these were again cases of microbial indicators revealing impacts from an unexpected (if unsurprising) source given the human-dominated settings. Human-associated targets have proven further limited in domestic contexts due to poor diagnostic performance (Boehm et al., 2016; Harris et al., 2016; Odagiri et al., 2015). The pervasive exposure to fecal contamination in these settings may have contributed to horizontal transfer of microbes between hosts, such that there are not endemic distributions of fecal bacteria specific to humans (Harris et al., 2016). In any event, occurrence of non-specific, human-associated, and livestock associated fecal microbes in the domestic environment have generally shown little relationship to sanitary conditions, providing little additional information beyond indicating that the environment is indeed contaminated—an outcome also strongly suggested by the incredibly high prevalence of enteric infections, a definitive indicator fecal pathogen exposure (Knee et al., 2018; Kotloff et al., 2013). Until more sensitive and specific

microbial source tracking methods are developed, perhaps including improved community-based profiling and source apportionment techniques, assessing ambient fecal microbe occurrence in domestic environment is unlikely to provide actionable information with which to improve interventions that could not be more readily be obtained through observation (Cao et al., 2013; Knights et al., 2011; Roguet et al., 2018; Wang et al., 2013). Continued microbial monitoring of municipal source water quality is warranted, as one would not expected human and animal impacts on treated water and their detection would indicate failure of the treatment or distribution process, while information on fecal source could aid in identifying the point of contamination. Measuring fecal microbes in stored drinking water may be similarly informative, particularly when the source water is relatively uncontaminated, by implicating patterns of recontamination in the home. However, one would not necessarily expect stored water quality to respond to indirect interventions, including household sanitation, while the ambient environment from which the re-contaminating microbes presumably originate remains heavily impacted (Harris et al., 2019).

As noted, nearly every child in our study had at least one enteric infection and a large majority had multiple co-infections. With such ubiquitous transmission and exposure to fecal pathogens, it may be that even a highly-effective intervention to contain household excreta would not produce a measurable effect on environmental contamination and enteric infections. Mathematical modeling has shown that substantial reductions in pathogen exposure through a particular pathway translates into small, potentially undetectable reductions in disease with multiple complementary pathways and high transmission burdens (Briscoe, 1984). While combined interventions targeting multiple pathways have generally demonstrated no greater impact than individual interventions (Wolf et al., 2018), there is likely a threshold effect in which

both dominant and secondary transmission pathways must be sufficiently interrupted to reduce risk. Interrupting only secondary pathways is insignificant while the dominant pathway remains, while only interrupting the dominant pathway may result in negligible health gains if even small amounts of secondary transmission persist (Briscoe, 1984). All transmission, minor and major, may need to be prevented to improve health, which in turn requires transformational changes to the environment to overcome the transmission threshold (Husseini et al., 2018). Furthermore, sanitation and hygiene both require adequate water access and quality, such that all interventions are to some degree combined, whether or not explicitly described as such; the lack of improvement from combined interventions more likely reflects the general inadequacy of household WaSH generally to interrupt all fecal transmission pathways in heavily impacted settings (Carter, 2017).

Just as fecal contamination is ubiquitous in many domestic settings, sanitation likely must achieve complete community coverage in order to prevent fecal transmission (Cairncross et al., 1996; Shuval et al., 1981). Modeling exercises provide theoretical support for a herd protection effect due to sanitation, though the threshold coverage level has not been adequately characterized and indeed may not exist (Carter, 2017; Fuller et al., 2016; Fuller and Eisenberg, 2016). Latrine coverage has also been associated with diarrhea risk in individual studies where household sanitation has not (Berendes et al., 2018; Harris et al., 2017). In the neighborhoods of Maputo in which we conducted our study, however, basic sanitation access was essentially universal, although the majority of residents used traditional latrines that did not meet the JMP definition for improved sanitation (Blackett et al., 2014; WHO/UNICEF, 2017). The participants in our study were drawn from the minority of households using shared latrines, likely among the poorest residents in the community. In addition to poor latrine quality, the lack of adequate fecal

sludge management services contributed to an environment in which fecal waste was far from adequately contained, high coverage of basic sanitation notwithstanding (Blackett et al., 2014; Shiras et al., 2018).

A recent meta-analysis of sanitation impacts on diarrhea risk stratified their analysis by studies achieving > 75% community coverage and those with coverage < 75%, finding a much larger impact among high-coverage studies (Wolf et al., 2018). Notably, however, only four studies achieved the 75% coverage threshold, of which three were sewer interventions. By nature, sewers are a community-level intervention that combine fecal waste management, in the sense that once flushed the feces are no longer the responsibility of the individual households and are immediately removed, at least initially, from the community. Sewers are by no means a panacea—if improperly treated, as is very often the case, sewage is simply and rather efficiently transported elsewhere in the broader environment (Mills et al., 2018; Mitchell et al., 2016; Williams and Overbo, 2015). Early sewers that discharged directly to surface water likely increased typhoid incidence in European and North American cities, which local governments mostly remedied by filtration and chlorination of source water (Abellan, 2017; Cutler and Miller, 2005; Tarr, 2016). That drinking water treatment nevertheless substantially reduced fecal-oral disease suggests that the introduction of basic sewerage successfully removed fecal wastes from the immediate domestic and community environments (Cutler and Miller, 2005).

The relatively strong performance of sewer interventions in recent health evaluations may be attributable to the prerequisite socioeconomic and structural conditions in the community to support the installation of sewers, rather than any particular strength of the technology itself. A flush toilet in the home suggests that reliable piped water is also available, in a structure of sufficient quality to support and warrant the installation of water and sewer hardware. In-home

piped water and sewer connections also likely indicate a sufficiently formal recognition of occupancy and title rights, which could also encourage further investment in the housing quality and provide collateral for accessing capital for further productive use (De Soto, 2000; Tidwell et al., 2019a). In essence, the introduction of sewers may serve as an indicator of a community having achieved the threshold-level of housing and environmental quality necessary to interrupt fecal transmission, perhaps providing a final push in the form of excreta removal to clear the threshold (Husseini et al., 2018).

Flush toilets with sewer connections do demonstrate at least one innate quality: near universal desirability, even in societies often regarded as holding cultural or religious reservations about adopting sanitation infrastructure (Coffey et al., 2017; Jain and Subramanian, 2018; Subramanian et al., 2016). Flush toilets absolve all individual responsibility to further manage fecal waste, a stressful task most would prefer (or are societally obligated) to avoid (Jain and Subramanian, 2018; Shiras et al., 2018). The obvious and global preference for flush toilets, when financially attainable, illustrates the intrinsic value of sanitation facilities beyond any specific health and environmental quality improvements they may offer (Jain and Subramanian, 2018; Schmidt, 2014). As human beings, we desire secure, private, and dignified spaces—not only for urination and defecation, but also to manage menstruation and simply to take a bath. Such desires in turn highlight an instrumental role sanitation plays in well-being, as perceived lack of privacy or safety in the sanitation options available have repeatedly been associated with risk to mental health, including increased psychosocial stress (Sclar et al., 2018). Lack of private and secure facilities in schools presents particular barriers to attendance and engagement by girls who are menstruating (Mason et al., 2013; Sommer, 2010). The risks of gender-based violence are aggravated by inadequate sanitation infrastructure, so much so that open defecation has been

much more strongly associated with non-partner sexual violence than child diarrhea (Jadhav et al., 2016; Winter and Barchi, 2016).

Unlike efforts to interrupt fecal transmission, even small improvements to the quality of sanitation can provide meaningful benefits to safety and well-being. Girls attending schools in Bangladesh that made gender-segregated toilets more accessible by not keeping them locked from the outside had better attendance records (Alam et al., 2017). Interviews and focus group discussions conducted in the same neighborhoods of Maputo as our research revealed widespread safety, privacy, and embarrassment concerns related to sanitation, but participants who had received the MapSan latrine intervention reported far fewer sanitation-related stressors than those using traditional latrines (Shiras et al., 2018). A large majority of participants reported the intervention latrines reduced stress in comparison to their old latrines, most frequently in reference to mitigating disgust at unhygienic conditions and embarrassment over presenting the latrine to visitors, but also commonly by improving perceptions of privacy and security. Door locks were frequently identified as a key determinant of safety and privacy; of the two intervention designs implemented, the latrine type with more door locks remaining several months after the intervention was more frequently considered private than the other design, despite being shared with more people.

Given the intrinsic value of sanitation and the benefits to well-being of even minor improvements, greater focus should be placed on improving the strategies used to promote acceptable sanitation infrastructure that more effectively supports privacy, security, and dignity (Carter, 2013). Sanitation interventions have not historically demonstrated noteworthy success at increasing latrine coverage and use, and there are ethical as well as practical concerns about many demand-oriented intervention strategies (Bartram et al., 2012; Garn et al., 2017; Jain and

Subramanian, 2018). However, in dense, urban settings where basic sanitation is nearly universal, it may be possible in the short term to spur improvements to sanitation quality at scale, while laying the foundation for the transformative improvements to the city's physical infrastructure. For example, a carefully designed information campaign caused landlords in peri-urban Lusaka, Zambia to voluntarily implement latrine improvements for their tenants similar to those identified by MapSan participants as important for perceptions of safety and reduced disgust (Shiras et al., 2018; Tidwell et al., 2019a, 2019b). While far from sufficient to meet the sanitation needs of a rapidly urbanizing world, such approaches may help improve the immediate well-being of urban residents during the decades of concerted and sustained development of city-wide infrastructure that the recent history of Singapore demonstrates can successfully address urban fecal transmission and child health (Otaki et al., 2007).

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