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**Research Paper**

# Microbial hazards in real-world alternating dual-pit latrines treated with storage and lime in rural Cambodia

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## ABSTRACT

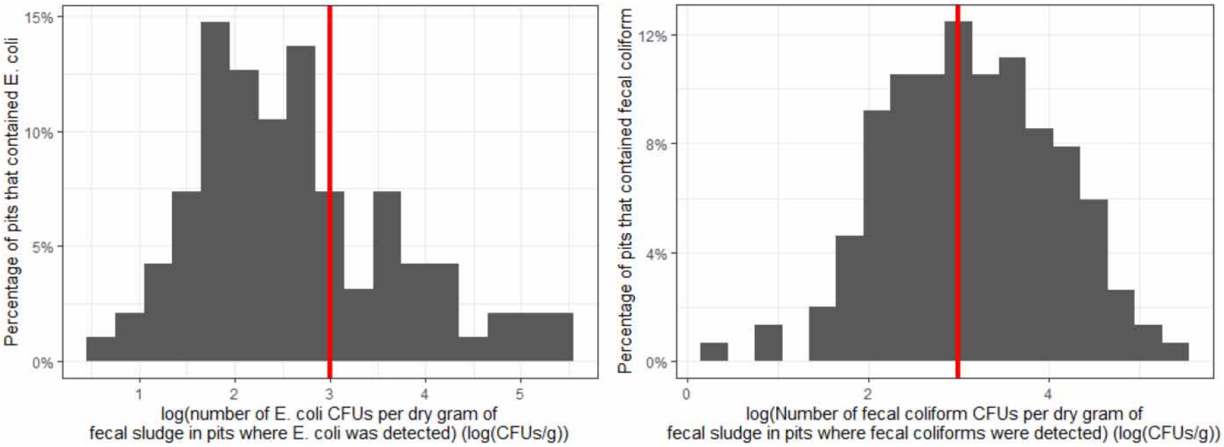
Achieving safely managed sanitation (SMS) in rural areas has spurred innovation in toilet designs that provide on-site treatment of fecal sludge (FS), including the development of International Development Enterprise (iDE)'s alternating dual-pit latrine upgrade (ADP). ADPs treat FS by inactivating pathogens using storage treatment with lime; however, ADPs' reduction in pathogenicity (and thus their associated public health benefit) has not yet been described in real-world pits at scale. We thus enumerate the fecal indicator bacteria *Escherichia coli* (*E. coli*) and fecal coliforms in 147 pits after two years of storage treatment with lime and compare detected concentrations to relevant standards. *E. coli* and fecal coliform concentrations indicated a risk to human health in 31 and 42% of sampled pits, respectively. Regression models described relationships between fecal indicator bacteria concentrations and measured factors (e.g., sludge pH, temperature) but did not reveal any meaningful associations. High rates of pit ineligibility also indicate that many ADPs are not operated as recommended. Results indicate a one-in-three chance that a household emptying their own pit would be exposed to health hazards and call into question the effectiveness of the standard two-year storage treatment in real-world applications. To improve rural SMS, various evidence-based recommendations are made.

**Key words:** fecal sludge management, on-site sanitation, pathogen inactivation, public health, safely managed sanitation, toilet

## HIGHLIGHTS

- Fecal sludge stored in real-world alternating dual-pit latrines after treatment were enumerated for fecal indicator bacteria.
- *E. coli* and fecal coliform concentrations indicated a risk to human health in 31 and 42% of sampled pits, respectively.
- Households emptying pits after treatment have a one-in-three chance of being exposed to health hazards.
- Many ADPs were not operated as recommended, reducing pathogen inactivation.

## GRAPHICAL ABSTRACT




## 1. INTRODUCTION

As defined by the Joint Monitoring Program (JMP), safely managed sanitation (SMS) requires people to use 'improved facilities' that are not shared with other households and where excreta are safely disposed of *in situ* or removed and treated offsite, thereby limiting contact with human feces and improving public health by preventing exposure to enteric pathogens (USEPA 1993; European Commission 2001; JMP 2019). While SMS could be achieved by simply containing fecal sludge (FS), leaving it underground untouched, and constructing new sanitation systems to contain more FS, many communities around the world opt to maintain sanitation systems by emptying, transporting, treating, and disposing of or reusing human feces and urine (Still & Foxon 2012). In rural areas, SMS has proven difficult to achieve because (1) sewer systems and wastewater treatment plants are not feasible due to high costs and dispersed populations and (2) challenges exist related to fecal sludge management (FSM), including treatment methods requiring specific environments (e.g., moisture levels, temperatures) and long durations; expensive transportation of FS; households' reluctance to pay for FSM services; a lack of safe home emptying practices; few safe disposal locations; undeveloped waste reuse markets; and/or a lack of desire to reuse human waste (Still & Foxon 2012; Hussain *et al.* 2017; Harper *et al.* 2018). As a result, rural sanitation in resource-limited contexts tends to be characterized by (1) few affordable services and related infrastructure and (2) rural households managing their own FS unsafely. A lack of regulations, or where they exist, a lack of enforcement, is also common in rural resource-limited contexts (Still & Foxon 2012). These challenges of rural sanitation have hindered progress toward achieving Sustainable Development Goal (SDG) 6 and must be addressed to improve public health in many rural areas globally (UN 2018).

Due to the difficulties of achieving SMS in rural areas, safely managed on-site sanitation (SMOSS) systems that specifically decrease costs and complexities by not having to transport FS over long distances for treatment and disposal have become a focus of the rural sanitation sector in many countries (JMP 2021; Saxena & Den 2021). Such SMOSS systems can provide treatment where FS is contained and therefore allow households to manage their own FS with reduced risk of exposure to pathogens (JMP 2021). Examples of these SMOSS systems include alternating twin-pit latrines and composting toilets but can also include single-pit latrines whose pits are simply capped when full and a new pit constructed (Strande *et al.* 2014). Each type of SMOSS system has different advantages and disadvantages, with the selection of an appropriate SMOSS system depending on a variety of factors, including capital and operational costs; user preferences; user practices (e.g., wiping vs. washing); and system, material, and build-site availabilities (Chandana & Rao 2022).

Cambodia has recently experienced a marked expansion of improved sanitation coverage (i.e., sanitation systems that are used by only one household and hygienically separate human excreta from human contact; JMP 2019), primarily pit latrines (World Bank 2019). As most rural households in Cambodia use shallow-lined latrine pits that fill up within two years, and most pits are emptied using high-risk methods (USAID and PSI-PSK 2017; Harper 2019), Cambodia is faced with the urgent challenge of improving FSM in rural areas to ensure that health and other social benefits associated with improved sanitation standards are preserved through safer storage and treatment of FS (World Bank 2019). Currently, rural sanitation in Cambodia is characterized by few available and affordable pit-emptying service providers, and little to no infrastructure or enforced regulations that require FS treatment, safe disposal, and/or reuse (Harper 2019). Based on decades of experience in Cambodia and globally, the sanitation development sector currently prefers achieving SMS in rural Cambodia using on-site FS treatment and reusing FS as fertilizer (MRD 2020). More details about sanitation in Cambodia are available in Supporting Information 8.1.

To support achieving SMS in rural Cambodia, International Development Enterprises (iDE), a non-profit organization that works to improve rural sanitation via market development channels, introduced a new FSM product to rural Cambodia's sanitation market in 2017 – the alternating dual-pit latrine upgrade (ADP) with lime treatment – to provide accelerated on-site treatment of household FS. Building upon iDE's successful sanitation-marketing program, the ADP was inspired by the traditional Alternating Twin-Pit design with context-appropriate modifications including an upgrade installation process to existing latrines, lime treatment, and a pit gauge that signals when a household's pit is nearly full (Strande *et al.* 2014; iDE Cambodia 2020a, 2020b; Water for Women 2022). iDE ensures that ADPs are installed according to iDE's recommended practices, which include site selection and lime treatment protocols (see Supporting Information Figure S1 and ADP installation manual and technical guidelines are available in Supporting Information 8.2). More details about iDE's sanitation-marketing program are available in Supporting Information 8.3.

iDE's ADP is sold and constructed as an upgrade to an existing single-pit latrine, where an additional ('new') pit is added to an existing single-pit latrine. The new pit increases the household's capacity to store waste, effectively doubling their hygienic FS storage, and allows FS to be treated on-site while maintaining latrine functionality. When the pit connected to the pour-flush latrine in an ADP is more than two-thirds full, iDE recommends that the pits be 'switched', which requires (1) disconnecting the connected pit from the latrine by cutting and sealing either side of that pipe connection; treating its FS with lime; and leaving it to inactivate pathogens over time via storage treatment (two years recommended by the World Health Organization, or WHO; WHO 2006); and (2) emptying the disconnected pit, which has been undergoing storage treatment with lime and then connecting it to the toilet to maintain toilet functionality (see Supporting Information Figure S1). The intention is that by the time the new pit is full (an estimated two to five years for an average-sized rural Cambodian household), the treated FS in the original, lime-treated pit can be emptied using commonly available manual tools (e.g., shovels) without specific training or equipment. This method is designed to reduce the households', pit emptiers', and communities' risk of exposure to pathogens and increase the potential value of FS as agricultural fertilizer. Costing approximately $50 USD, an ADP upgrade is 16% less expensive than building a new single-pit latrine while still maintaining latrine functionality (iDE Cambodia 2020a, 2020b). In addition to a reduced cost, rural Cambodian households prefer the reduced space required by ADPs compared to multiple single-pit latrines (Harper 2019).

ADPs are designed to enable on-site treatment of household FS by storing FS underground in a capped pit for a specific amount of time (typically two years per the WHO) to reduce potential exposures to pathogens (WHO 2006; Still & Foxon 2012). Long-term storage of FS can inactivate pathogens via anaerobic digestion (Gantzer *et al.* 2001), reducing *Escherichia coli* (*E. coli*) and fecal coliform concentrations to levels unlikely to cause disease in humans after 6–12 months depending on the study (Musaazi *et al.* 2023). Storage treatment thus reduces the health risks associated with emptying on-site sanitation systems and the possibility for environmental fecal contamination via spills, leaks, and flooding (Saxena & Den 2021).

However, while FS storage can adequately mitigate hazards from viruses and bacteria, controlling hazards from protozoa and helminths typically requires longer storage times and/or alkaline treatment (e.g., lime treatment; Musaazi *et al.* 2023). Lime treatment (i.e., lime stabilization) is thus also included in iDE's ADP upgrade product because it has been described as a 'simple and cost-effective chemical treatment process' for FS and is commonly used in fecal sludge treatment plants to inactivate pathogens and stabilize biosolids for safe disposal or reuse (Farzadkia & Bazrafshan 2014; Odey *et al.* 2017; Zewde *et al.* 2021). The ADP upgrade adds 6 kg of hydrated lime (calcium hydroxide, $Ca(OH)_2$) into an existing 0.8-m-wide 1.5-m-deep cylindrical latrine pit of a single-pit latrine that is full of FS. This lime and FS are then mixed thoroughly to inactivate pathogens by creating a highly alkaline environment (target $pH = 12$), which exceeds the pH of 9 at which *E. coli* and other pathogens can survive (de Jonge 2003; iDE Cambodia 2015; Emersan 2022). Additional details about lime treatment are available in Supporting Information 8.4.

In addition to a well-designed latrine, the proper operation of ADPs by households is also critical to inactivate pathogens within FS. How rural Cambodian households operate and maintain their ADPs is explored in detail in a companion article published concurrently with this article (Harper *et al.* 2023). Post-installation messages and steps required to properly operate an ADP are described in a leaflet distributed to households by iDE at the time of purchase (Supporting Information Figure S2).

Although lime treatment has been shown to inactivate pathogens in FS in laboratories and fecal sludge treatment plants (Strande & Brdjanovic 2014), few studies have investigated how enteric pathogens inactivate in real-world pits via storage treatment with or without lime treatment (Chakraborty *et al.* 2014; Anderson *et al.* 2015; iDE Cambodia 2015). Testing for enteric pathogens is often expensive, and instead fecal indicator bacteria (FIB), such as *E. coli* and fecal coliforms, are typically measured due to the low cost and availability of necessary materials in low- and middle-income countries (European Commission 2001; USEPA 2021; Velkushanova *et al.* 2021). Although fecal coliforms are not pathogenic themselves, they are indicative of other disease-causing bacteria (e.g., *Salmonella enterica* serovar typhi, which causes typhoid in humans; *Vibrio cholerae*, which causes cholera in humans; USEPA 2022). While Cambodia does not have FS regulations, European Union and the United States regulations require <1,000 colony forming units (CFUs) of *E. coli* and fecal coliform bacteria per gram of dry sludge for land application or surface disposal (European Commission 2012; USEPA 2020). While these regulations have many more stipulations that apply to other aspects of waste management (e.g., metal and other organic pollutants), we consider the regulations related to *E. coli* and fecal coliform to be reasonable references to describe the risk of infection due to exposure to FS. Moreover, research has shown that FS in rural Cambodia is likely to be used for land application as agricultural fertilizer (USAID & PSI 2017), making these regulations applicable to this study.

With over 22,000 ADPs in use by rural households in Cambodia since 2017, the reduction in pathogenicity provided by storage treatment with lime in pit latrines can now be evaluated at scale under real-world conditions. Using biological testing data that describes the presence of FIB in real-world FS samples from rural Cambodian households, this study investigates the research question 'Can lime-treated pits be emptied safely without specific techniques or equipment after the WHO-recommended two years of storage treatment?' The above-mentioned advantages of incubating the FIB *E. coli* and fecal coliform allow us to characterize FIB in FS to assess if two years of storage treatment with lime consistently reduces FIB concentrations below established guidelines (1,000 CFUs; European Commission 2001; USEPA 2020). We then compare the detected concentrations of FIB to relevant standards from the European Union and United States to comment on the potential risk to public health posed by FS emptied from ADPs in rural Cambodia (European Commission 2001; USEPA 2020).

This study was performed concurrently with another study that described how households operate and maintain their ADPs in relation to recommended practices (Harper *et al.* 2023). That study's results provide relevant context and should be considered together with the results of this study to provide a more holistic description of household ADPs in rural Cambodia.

## 2. METHODS

### 2.1. Study design, sampling frame, inclusion criteria, and sample size

Samples were collected from pits that were lined with concrete rings; 0.8–1.0 m in diameter; 2 m deep; were unsealed at the bottom; had undergone lime mixing 22–28 months prior to sampling; and had subsequently been sealed for two years of storage treatment to determine if two years of storage treatment with lime was sufficient to reduce FIB concentrations to safer levels that might be acceptable for manual emptying by household members. This time frame was selected to ensure that enough pits that had undergone approximately two years of storage treatment after lime treatment were available to sample.

The sampling frame included households in Prey Veng province that both (1) had an ADP upgrade installed for their existing single-pit pour-flush latrine 22–28 months prior to sampling and (2) were present in iDE's sales database. These households would likely need FSM services due to a high likelihood of their pit filling, which typically occurs after two to five years in rural Cambodia (MRD 2020). All sampled pits met the inclusion criteria described in Supporting Information 8.2.

Prey Veng province was selected for study due to the many ADPs in use and its proximity to laboratory testing facilities to reduce transport time and ensure the quality of the samples. Considering result generalizability, there are no known or expected differences between pits in Prey Veng and those in other provinces, with the exception of small-scale (e.g., between villages) variations in groundwater conditions that may affect pits' microbiological conditions (Stenström 2011). Thus, the results of this study are generalizable to all pits that have undergone lime treatment and two years of storage treatment in rural Cambodia and other nearby countries that share similar socioeconomic and culture characteristics including Thailand, Lao PDR, and Vietnam (Mensah & Chen 2013).

Sample size calculations were performed to select a reasonable statistical power while balancing budgetary and operational complexity. Additional details are available in Supporting Information 8.3. This study was approved by Solutions IRB on 26 October 2021 under protocol number 2021/10/2 (Solutions IRB 2022).

### 2.2. Sample collection

Cross-sectional field sampling of FS samples from 150 latrine pits that had undergone lime mixing followed by two years of storage treatment was performed in Prey Veng province between March and May 2022. Three pits were later identified to be ineligible or were unable to be matched to laboratory data and were ultimately excluded from the study. The final sample for analysis included a total of 147 pits. All samples were collected by Rural Water and Sanitation (RWST), a local data collection firm experienced with this type of sampling. RWST sample collectors were accompanied by an iDE-trained laborer familiar with how to safely open and reseal ADP pit lids to allow for sampling (Capone *et al.* 2020; Velkushanova *et al.* 2021). Fecal sludge samples were taken using sterile sample containers mounted on a custom-built sampling arm that is similar to a Nasco Sludge Nabber (see pictures in Supporting Information 8.6; Nasco 2021). For pits containing FS that did not have surface water deeper than 5 mm across its surface, each sample container was lowered into each pit along its center line until the top of the container just became submerged below the surface of the fecal sludge. For pits containing fecal sludge with surface water deeper than 5 mm across its surface, a large shovel was used to scoop sludge from the center of the pit to ensure that sufficient sludge was collected and not just surface water; then, samples were collected from the large shovel. Samples were transported on ice to the laboratory. The pH and temperature of each sample were also measured in the field using an Extech PH90 meter during sampling. Additional details about sample collection are provided in Supporting Information 8.6 and are validated by existing literature (Capone *et al.* 2020; Velkushanova *et al.* 2021).

### 2.3. Laboratory testing

Fecal sludge samples were tested in a biological laboratory at the Institute of Technology of Cambodia (ITC). In each sample, *E. coli* and fecal coliform were enumerated by growing cultures with Compact Dry EC plates (Hardy Diagnostics 2021). All samples remained on ice until testing and were tested within 24 h of collection. A laboratory technician took 1.0 g of fecal sludge; diluted it with sterile deionized (DI) water between $1{:}10^1$ and $1{:}10^6$; applied 1 ml of each solution to each plate; incubated the plates at 35 °C for 24 h; counted the number of *E. coli* and fecal coliform colonies (blue and purple in color, respectively); and adjusted for dilution to determine the number of CFUs in 1 g of FS. Additional details of the testing protocols are shown in Supporting Information 8.4 and 8.5.

The solid contents of each sample were also measured in the laboratory via oven baking using the gravimetric method (Velkushanova *et al.* 2021). An approximately 5.0-g sample was baked in an aluminum foil weight boat or similar container at 105 °C overnight. The moisture content was then calculated from the loss in mass due to drying with an error of no more than $\pm 2\%$.

To verify laboratory testing accuracy, 10% of pits ($n = 15$) were sampled twice as replicate samples, and 15 negative and 23 positive control samples were created using sterile DI water and animal feces, respectively, and were also tested.

### 2.4. Data analysis

To characterize the risk to public health posed by emptying the FS from the tested pits, we first calculated various frequency metrics to describe the characteristics of the FS samples. Then, to identify and quantify the factors affecting this risk to public health, we created four regression models (two logistic with marginal effects and two multiple linear). These models were designed to describe the presence and number of *E. coli* and fecal coliform CFUs, respectively, as functions of sludge pH; temperature; solid content; number of pit users; and the presence of animals near the pit. These independent variables were identified in the literature to affect biological processes within pit latrines and were therefore included in these models (Capone 2020; Velkushanova *et al.* 2021).

Two logistic regression models with marginal effects were used to identify the relationships between key pit characteristics and the presence of *E. coli* and fecal coliform in each pit:

$$\log_{10}\left(\frac{P(N_{\text{bacteria}})}{1 - P(N_{\text{bacteria}})}\right) = \beta_0 + \beta_1 \text{pH} + \beta_2 \text{Temp} + \beta_3 \text{SolidCon} + \beta_4 N_{\text{users}} + \beta_5 \text{SurfWat} + \beta_6 \text{Cat} + \beta_7 \text{Chicken} + \beta_8 \text{Cow} + \beta_9 \text{Dog} + \beta_{10} \text{Other Animal}$$

where, for *E. coli* and fecal coliform bacteria separately, $\log_{10}(P(N_{\text{bacteria}})/(1 - P(N_{\text{bacteria}})))$ is the base-10 log odds that a pit contains any *E. coli* or fecal coliform bacteria; $\beta_i$ are regression coefficients; pH is the sludge's pH at sampling; Temp is the sludge's temperature in °C at sampling; SolidCon is the sludge's solid content in dry grams of FS per total grams of FS as measured in the laboratory; $N_{users}$ is the number of people defecating in a pit daily; SurfWat describes the observed (binary) presence or absence of surface water on top of the FS stored in a pit; and the remaining variables describe the observed presence or absence of the specified animals' feces near the pit. Although seasonal flooding is common in Cambodia and likely affects results due to the effects of moisture on pathogen inactivation, data describing the seasonal flooding of each sampled pit from households is not available. We attempted to consider these effects by observing the presence or absence of surface water as well as by measuring the solid content of each sample. The results of this model are presented as an odds ratio. For independent variables that showed significant effects on the log odds that a pit contained any *E. coli* or fecal coliform, marginal effects were then used to calculate the predicted probability of a pit containing *E. coli* or fecal coliform as a function of the independent variable of interest, holding all other variables constant.

For pits in which *E. coli* or fecal coliform was detected, two unweighted multiple linear regression models were used to describe the relationships between key pit characteristics and the number of *E. coli* or fecal coliform CFUs in each pit:

$$\log_{10}(N_{bacteria}) = \beta_{11} + \beta_{12}\text{pH} + \beta_{13}\text{Temp} + \beta_{14}\text{SolidCon} + \beta_{15}N_{users} + \beta_{16}\text{SurfWat} + \beta_{17}\text{Cat} + \beta_{18}\text{Chicken} + \beta_{19}\text{Cow} + \beta_{20}\text{Dog} + \beta_{21}\text{Other Animal}$$

where, for *E. coli* and fecal coliform bacteria separately, $\log_{10}(N_{bacteria})$ is the base-10 log of the number of *E. coli* CFUs per dry gram of FS; and the remaining variables are as described above.

## 3. RESULTS

The characteristics of sampled pits and FS, including pH, temperature, and solid content, are available in the Supporting Information (Figures S3–S5). Important results about these characteristics are discussed below in relation to FIB concentrations.

### 3.1. Pit eligibility

Pit eligibility for this study was low across the sampling frame. Of the 682 pits visited by the iDE and RWST teams, only 23% (157) were eligible to sample. Most commonly, pits were disqualified from the study because the household had either reconnected them to a toilet (34%) or to another pit (22%) since the ADP was installed, contrary to iDE recommended protocols.

### 3.2. *E. coli*

*E. coli* was detected in nearly half of pits ($n = 68$ of 147 pits, 46%), and 31% of all pits exceeded 1,000 *E. coli* CFUs of per dry gram of fecal sludge (mean = 5,100 CFUs; standard deviation = 22,500 CFUs; Figure 1). Thus, approximately one-in-three pits

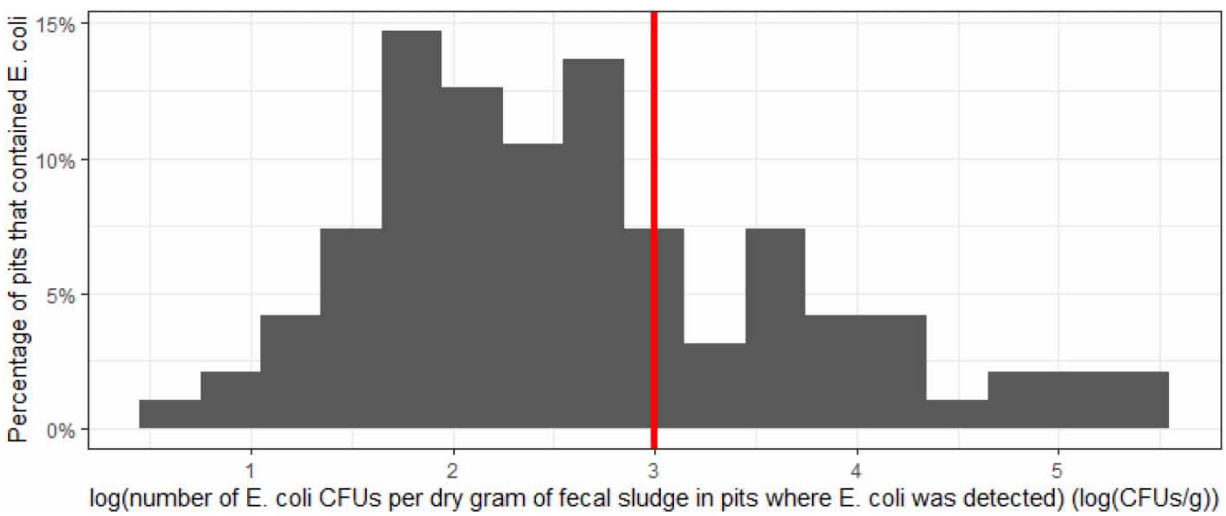



**Figure 1** | Logarithm of the number of *E. coli* CFUs per dry gram of fecal sludge in pits that contained *E. coli* ($n = 83$ of 147). The red line indicates the threshold above which risks to public health exist.[3,25] Please refer to the online version of this paper to see this figure in colour: https://dx.doi.org/10.2166/washdev.2023.016.

after two years of storage treatment with lime posed a risk to public health due to *E. coli* infection if not emptied using specific equipment or techniques.

The presence of *E. coli* was not found to be associated with any of the fecal sludge or household characteristics included in the models (Supporting Information Table S1). Fecal sludge pH, temperature, solid content (Supporting Information Figures S3–S5); or the number of pit users or the presence of animals near the pit were associated with the likelihood of a pit containing *E. coli*. Although the pH increased to near 12 when lime was added, per the lime treatment process protocols (Supporting Information 8.1), most of the sampled pH decreased to near neutral (Supporting Information Figure S3) after two years of storage treatment due to natural biological and physical processes (e.g., metabolization, diffusion, dilution from groundwater). Thus, the pH measured at sampling was not found to be associated with *E. coli* presence. Also, although many pits sampled contained surface water covering the entire pit, this was also not found to be associated with the likelihood that a pit contained *E. coli*.

Among pits in which *E. coli* was found, the number of *E. coli* CFUs per dry gram of fecal sludge was found to be associated with the presence of chickens near the ADP pit ($p = 0.04$, respectively; Supporting Information Table S2). Sludge pH and temperature, the number of pit users, and the presence of water in the sludge were not associated with the number of *E. coli* CFUs found.

*E. coli* concentrations between replicate samples matched (median difference in $\log_{10}$ concentration between replicates = $0.21 \pm 0.07$), positive and negative control samples matched expected values, and no other problems with laboratory testing were found.

### 3.3. Fecal coliform

Fecal coliform was detected in three-quarters of all pits ($n = 110$ of 147 pits, 75%), and 42% exceed 1,000 fecal coliform CFUs per gram (mean = 7,900 CFUs; standard deviation = 24,300 CFUs; Figure 2). Thus, approximately two-in-five pits posed a risk to public health due to infections from enteric pathogens.

Few variables were found to be associated with the presence of fecal coliform (Supporting Information Table S3). Sludge pH was found to be associated with the likelihood that fecal coliform was present in sludge. Holding all other variables constant, a one unit increase in pH decreased the likelihood that a sample contained fecal coliform by 6% ($p = 0.07$). Also, the presence of cow manure near the pit during sample collection was associated with a 20% decrease in the probability that a pit contained fecal coliform ($p = 0.005$). While the presence of cows alone is unlikely to reduce fecal coliform concentrations in pits, the ownership of cows is likely associated with one or multiple other unmeasured variables (e.g., household diet, socioeconomic status, hygiene practices, pit-specific variations in temperature, humidity, and bacterial regrowth) that may be

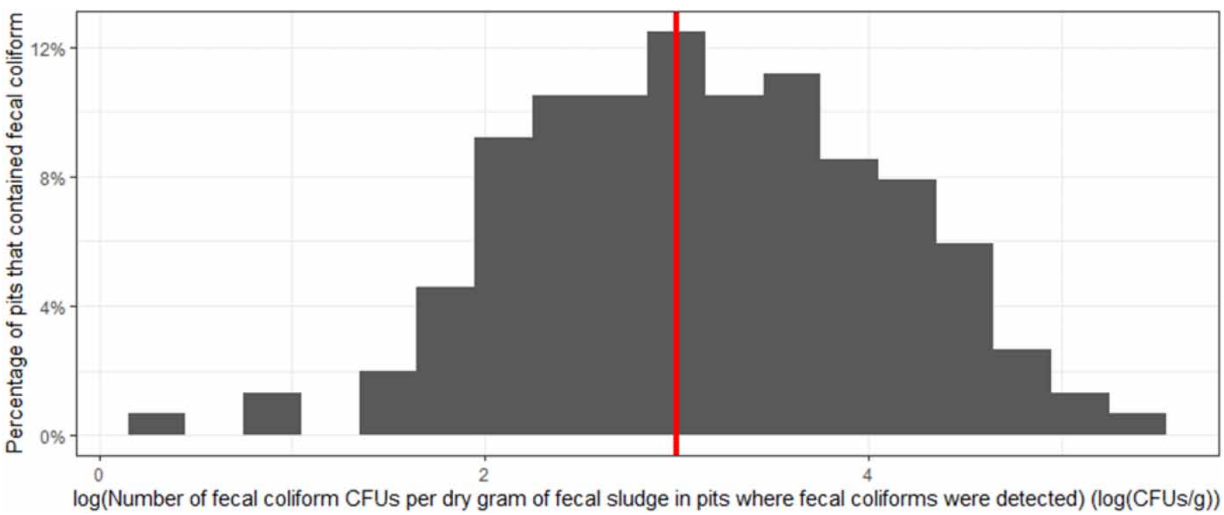



**Figure 2** | Logarithm of the concentration of fecal coliform (CFU per dry gram of fecal sludge in pits that contained fecal coliform ($n = 110$ of 147). The red line indicates the threshold above which risks to public health exist.[3,25] Please refer to the online version of this paper to see this figure in colour: https://dx.doi.org/10.2166/washdev.2023.016.

associated with the reduced presence of fecal coliform in a household's pit. Other variables, including sludge temperature and solid content; the number of pit users; and the presence of surface water in the pit, showed no significant effects on the presence of fecal coliform.

In pits where fecal coliform was detected, the concentration of fecal coliform was only found to be associated with the presence of dog feces and other unknown animals near the pit during sampling (Supporting Information Table S4). Pits with dogs in the vicinity at the time of sampling were found to contain fewer fecal bacterial colonies, a result which was significant at the 3% level of confidence. Conversely, pits for which sample collectors reported observing signs of other, unknown animals nearby were found to contain a greater number of fecal coliform bacterial colonies, a result which was significant at <1% level of confidence. It is important to note that signs of other animals were only observed near 16 of the 110 pits (15%), meaning that this result is likely at least in part due to a small sample size. Additionally, similar to the results described above, it is unlikely that the presence of these animals directly affects the number of bacterial coliform present (e.g., via groundwater infiltration, rainfall, or flooding). Most plausibly, the presence of various animals near the pit is also correlated with household diet and hygiene habits, which subsequently plays a role in the bacterial content of the pit. Sludge pH, temperature, and solid content; the number of pit users; and the presence of surface water in a pit were not found to be associated with the concentration of fecal coliform.

Fecal coliform concentrations between replicate samples matched (median difference in $\log_{10}$ concentration between replicates $= 0.24 \pm 0.11$), positive and negative control samples matched expected values, and no other problems with laboratory testing were found.

## 4. DISCUSSION AND CONCLUSIONS

Two years of storage treatment with lime reduced FIB concentrations below established guidelines in approximately three in five latrine pits in rural Cambodia. Indicating that ADPs have enabled SMS at scale in rural Cambodia, these results also highlight that even after the WHO-recommended two years of storage treatment, there is a two-in-five chance that a household emptying their own pit would be exposed to health hazards.

*E. coli* likely survived the lime treatment process due to multiple factors. Incomplete mixing likely produced certain areas within each pit that did not have sufficient lime to increase the FS' pH to a level that could destroy all *E. coli* bacteria. Also, the correct ratios of lime to FS, quantities of lime applied, and the final pH level reached in the FS could not be verified; these issues are noted as limitations of this study below.

With high FIB concentrations found in real-world pits after the WHO-recommended two years of storage treatment, even with lime treatment, the effectiveness of storage treatment in real-world applications is called into question. Although incomplete lime mixing may have led to the reported *E. coli* detections, none should have been detected after two years of storage treatment. Thus, the timing of storage treatment should be re-evaluated as a practical and effective on-site FS treatment method in relatively shallow-lined latrine pits due to a high prevalence of pour-flush toilets, and in regions like rural Cambodia that have relatively high groundwater and experience regular flooding.

The high rates of pit ineligibility encountered in this study also clearly show that many ADPs are not operated per recommended protocols. Most households reconnected the disconnected pit to a toilet or to another pit, which is likely to introduce fresh FS and additional live pathogenic organisms into the sludge undergoing limed storage treatment in a capped pit. Also, inadequate lime mixing may have hindered pathogen inactivation. Thus, these practices can hinder or even negate the FS treatment achieved by lime treatment and storage treatment. Additionally, households typically waited until their pit was full or nearly full before considering switching their pits, putting them at risk of latrine dysfunction and their pit overflowing. Few households followed recommended practices while most actively engaged in practices that increased their risk of exposure to contaminants in FS, including piercing their pit, early emptying, and dumping untreated waste near to living areas. With most households not following recommended ADP practices, this study highlights the challenges of ensuring recommended practices are followed in rural contexts, particularly when multiple barriers limit compliance. This issue of ADP household practices is explored in more detail in a companion study (Harper *et al.* 2023).

### 4.1. Limitations

The results of this study are subject to some important limitations. The sampling frame limited our description of ADP-owning households to only those that had their ADP installed 22–28 months prior to sampling by an iDE-trained LBO; all other ADPs in rural Cambodia are not well described by the results of this study. Relatedly, we do not compare ADPs that had been treated with lime to ADPs that had *not* been treated with lime (i.e., only treated with storage), which were either not present or not easily identifiable in the same study locations.

FS samples were also not taken from studied pits prior to or immediately after treatment began due to limited funding and the logistics of coordinating ADP construction and sampling FS; we thus cannot describe how FIB were inactivated over time. The amount of lime added to each pit and the completeness of lime mixing in each pit were also not verifiable because LBOs perform this process independently (see Supporting Information 8.2). These variables can markedly affect pathogen inactivation in FS (Chakraborty *et al.* 2014; Anderson *et al.* 2015; Emersan 2022; Harper *et al.* 2023). However, we remain confident that the pH of each pit increased as expected after lime treatment based on research conducted during the development of the lime treatment protocol and associated published literature (e.g., Chakraborty *et al.* 2014), and the high-quality training of latrine installers provided by iDE.

During laboratory testing, control and replicate samples were used to detect errors in laboratory analysis; while no errors were detected, the results of this study are dependent on the quality of the laboratory analysis performed by ITC. While this study originally intended to enumerate Ascaris ova in each FS sample (see Supporting Information 8.9), confidence in the reported results regarding *E. coli* and fecal coliform concentrations remains high due to the technicians' strong familiarity and experience with enumerating those organisms, and the consistent results with replicate samples.

Additional discussion of limitations is available in Supporting Information 8.12.

## 5. SECTOR RECOMMENDATIONS

The results of this study provide a detailed picture of the microbiology within ADPs at rural Cambodian households. From these results, we make the following recommendations to improve ADPs, rural FSM, and the rural sanitation sector in general.

### 5.1. Monitor and evaluate existing on-site FSM solutions, and use findings to improve SMS interventions

This study evaluated two proven methods of FS treatment at scale in real-world latrine pits (two-year storage treatment and lime treatment), and unfortunately, reductions in FIB were lower than expected. Extra caution by the sector must be taken when recommending a two-year storage treatment period to rural households for safe emptying and FS reuse as fertilizer. In rural Cambodia, designing new safe emptying and burial methods must be considered where a two-year storage treatment period is not possible, especially in regions like rural Cambodia that have relatively high groundwater and experience regular flooding.

The effectiveness of storage treatment with lime compared to storage treatment without lime should also be investigated to describe any changes in pathogen inactivation provided by lime compared to only storage treatment.

Additionally, no clear link was found between measured characteristics and the presence or number of *E. coli* concentrations at households that followed recommended ADP practices. Thus, unknown factors strongly affected results, hindering pathogen inactivation. One possible factor is incomplete lime mixing or inadequate lime addition, which could not be verified in this study. While training sanitation service providers is necessary, it does not guarantee practices will be followed. Thus, compliance with recommended lime-mixing practices must be improved by improving monitoring and evaluation processes to ensure that lime is thoroughly mixed in each pit every time, and that enough lime is used. One potential approach to improve treatment effectiveness with ADPs is to increase the dosage of lime while improving lime mixing. High-dose lime treatment would also avoid the problem of storage treatment, which require longer treatment times with lower levels of solid contents in the pit. If high-dose lime treatment effectively reduces FIB concentrations, a full pit may be treated much more quickly (e.g., within days or weeks) and then emptied at the household's convenience, making it available to switch to when the connected pit fills. The effectiveness of this process and simple storage treatment should be evaluated using a longitudinal study design that, for example, samples lime-treated pits every month for one year to describe how pathogen inactivation proceeds over time in real-world pits.

### 5.2. When designing FSM solutions, both technical (treatment and disposal) and behavioral (household practices around technology/service) aspects must be considered

Safely managed rural sanitation is affected by many factors, including household practices. Even if two years of storage treatment with lime had reduced FIB concentrations sufficiently across all tested pits, SMS may not be achieved in rural Cambodia due to the critical problems related to household ADP practices. Although training and education efforts around these issues did occur with this sample, most studied households did not follow recommended practices, increasing the risk of infection to themselves and their communities. Improved and expanded interventions are needed to reduce costs, improve convenience, and support households in gaining a better understanding of the pathogens contained within FS, how these pathogens affect human health, how these pathogens are transmitted, and how to keep families safe. These interventions also need to support households' understanding of how an ADP operates and how each of the recommended practices serve to keep people safe. In addition to education, practices could also be improved by clear government standards, enforced regulations, and more consideration of household practices in the design of FSM products and services. These issues must be considered to achieve SMS in rural Cambodia and SDG 6 globally, and therefore must be the primary focus of future sanitation development. Based on findings from this study, iDE updated the household leaflet instructions to emphasize the need to empty the old pit before switching pits and to be cautious about using the pit's content as fertilizer before three years of storage treatment (Supporting Information Figure S2).

### 5.3. Develop and institutionally implement more practical tools that easily, accurately, and affordably monitor progress toward access to safely managed sanitation in rural areas

The costs associated with collecting, testing, and analyzing biological samples for this study were $13,067 USD ($89 USD per sample) and may not be sustainable as a long-term monitoring process for local government and communities. US EPA and EU standards allow simply specifying FS treatment conditions and monitoring of the treatment process without testing FS for organism survival. Some such monitoring tools have been developed (e.g., the Sanitation Safety Planning toolbox, related WHO materials; WHO 2022). However, these tools are not widely applied by governing authorities, highlighting the need for higher level institutional changes, which include clarification of responsibilities and accountability. Also, developing individual tools and implementing processes that are not integrated will likely be insufficient to sustainably improve the sanitation sector or achieve SMS in rural Cambodia. Therefore, more affordable and practical SMS monitoring tools that can enable regular evaluation of FSM solutions under real-world conditions and at scale must be developed and integrated into relevant institutions.

### 5.4. Conduct follow-up research designed to better understand the relationship between initial fecal indicator bacteria concentrations, lime treatment, and storage treatment

This study did not include a baseline (samples collected prior to treating and sealing each pit) and was not designed to be comparative. As a result, it is not possible to definitively state that storage treatment with lime was directly responsible for the low or undetected FIB concentrations observed in nearly 6 0% of pits, especially considering the fact that high concentrations of FIB were observed in four of ten pits that were treated in the same manner. To better understand the direct impact of storage treatment, additional research comparing baseline FIB concentrations to those detected after two years is necessary. Preferably, this research would include samples collected at multiple points in time, allowing for the comparison of FIB concentrations over time. This knowledge would provide more insight into the precise impact of storage treatment on pathogen inactivation, including identifying the optimal time to empty the pit after treatment.

All sampled pits were treated with lime prior to being sealed. Although not strictly required according to WHO guidelines, treatment with lime is reported to accelerate pathogen inactivation by elevating the pH in each pit. Treatment with lime, however, requires households to either purchase lime and mix it themselves or hire a service provider to do so. Results from the companion article show that the majority of households in this rural Cambodian sample did not mix their pit with lime due to cost or service availability. Thus, storage treatment without lime is a more accessible FSM treatment method, but additional research is required to describe how pathogens inactivate in real-world pits without lime. Such research is on-going, and some has been recently published (Musa *et al.* 2022).

For public health to continue to be improved through SMS, pathogen inactivation must be understood and monitored when designing and implementing on-site sanitation systems. The results of this study provide evidence that current SMS interventions should continue to be evaluated and improved to ensure that JMP indicators and SDG6 goals are achieved.

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## DATA AVAILABILITY STATEMENT

All relevant data are available from an online repository or repositories.

## CONFLICT OF INTEREST

The authors declare there is no conflict.

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