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Assessment of Recommendation for the Containment and Disinfection of Human Excreta in Cholera Treatment Centers

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Abstract: Containment, safe handling and disinfection of human excreta in cholera treatment centers (CTC) are key to preventing the onward spread of the disease. This study compared the efficacy of three chlorine-based approaches at concentrations of 0.5%, 1%, and 2% and one hydrated lime-based ($Ca(OH)_2$ at 30% w:v) approach. Experiments followed existing Médecins Sans Frontières (MSF) cholera guidelines. Three simulated human excreta matrices consisting of either raw municipal wastewater (4.5 liters), or raw municipal wastewater plus 1%, or 20% faecal sludge (w:v), were treated in 14 liter Oxfam® buckets containing 125 mL of chlorine solution or hydrated lime suspension. Bacterial indicators (faecal coliforms (FC) and intestinal enterococci (IE)) and viral indicator (somatic coliphages (SOMPH)) were used to determine treatment efficacy following contact times of 10, 30 and 60min. Results showed that efficacy improved as chlorine concentrations increased. No statistical differences were observed with respect to the various contact times. Overall median log removal for 0.5% chlorine were: FC (1.66), IE (1.41); SOMPH (1.28); for 1% chlorine: FC (1.98), IE (1.82); SOMPH (1.79); and for 2% chlorine: FC (2.88), IE (2.60), SOMPH (2.38). Hydrated lime (30%) provided the greatest overall log removal for bacterial indicators (FC (3.93) and IE (3.50), but not for the viral indicator, SOMPH (1.67)). These findings suggest that the use of 30% hydrated lime suspensions or 2% chlorine solutions may offer a simple public health protection measure for the containment, safe handling, and disinfection of human excreta during humanitarian emergencies.

Keywords: sanitation; wastewater; faecal sludge; excreta; pathogens; disinfection; chlorine; hydrated lime; WASH

1. Introduction

Cholera is a severe, diarrheal disease, which can lead to dehydration and even death. It is caused by the toxigenic bacterium, *Vibrio cholera*, serogroup O1 or O139 and is estimated to cause 2.9 million cases and 95,000 deaths each year around the world [1]. The cholerae bacterium is usually transmitted by faecally contaminated water or food, though it can also be spread by raw or undercooked shellfish [1]. Brackish rivers and coastal waters are environmental reservoirs for *Vibrio cholerae*. Optimum conditions for *Vibrio cholerae* growth include: Water temperature of approximately 37 °C (range 10–43 °C); pH 7.6 (range 5.0–9.6); and salinity 5–25 ppt [2]. Cholera outbreaks are most likely to occur in places with inadequate hygiene practices, poor water treatment, and/or poor sanitation.

During such outbreaks, patients are typically treated separately in central hospital-level facilities, such as cholera treatment centers (CTC) and/or at a series of medium-sized health facilities, such as cholera treatment units (CTU's) [3]. Symptomatic cholera patients present very acute diarrhea, with watery stools, greyish in colour, cloudy, and with flecks of mucus, appearing like "rice water" [4]. Patients may shed 10^7 to 10^8 *Vibrio cholerae* organisms per gram of stool [5] and can continue to shed organisms for 1 to 2 weeks [6]. Asymptomatic patients typically shed vibrios in their stool for only 1 day, at approximately 10^3 vibrios per gram [7]. *Vibrio cholerae* has a high infectious dose circa 10^8 cells, but this can vary greatly (from 10^3 to 10^{11} cells) depending on the bacterial strain and the health condition of the human host [8].

In CTC's and CTU's, the rapid provision of emergency sanitation may represent a key intervention to prevent the onward spread of disease. During the recent 2013–2016 West Africa Ebola outbreak, Water, Sanitation, and Hygiene (WASH) sector recommendations [9–11] suggested that human excreta (faeces, urine, vomit, and bodily fluids) produced in Ebola treatment centers (ETC's) should be collected and removed in buckets containing hyper-concentrated (0.5% or 5000 mg/L) chlorine solutions. Furthermore, Médecins Sans Frontières (MSF) cholera guidelines [12] recommend the use of higher chorine concentrations (2% or 20,000 mg/L) to disinfect patient's vomit and faeces. These approaches are mostly designed to contain pathogens and avoid the ongoing spread of disease until the patient's excreta can be disposed of safely in latrines, or further treated in a wastewater treatment plant. These approaches could also be regarded as the first stage towards the wastewater treatment process in an emergency setting sanitation chain, reducing the microbial load in patients' excreta. This is an important fact as it is well known that wastewater treatment plants are not able to reduce completely the microbiological contamination from their influent streams [13]. In reality, precarious sanitary installations mean that this might be the only form of treatment such excreta undergoes before being released into the environment. Furthermore, disposal of highly contaminated faecal sludge from CTC's and ETC's can contaminate groundwater aquifers and surface water sources.

Chlorine compounds commonly recommended for use in CTC's include: Powdered calcium hypochlorite (HTH); granular sodium dichloroisocyanurate (NaDCC); and liquid sodium hypochlorite (NaOCl) (domestic bleach). For each of these compounds, efficacy can vary, not only according to the concentration of the chlorine solution, but also with respect to contact time, temperature, pH level, and the proportion of organic matter present [14].

Chlorination is commonly used to treat drinking water in emergency settings [15]. However, it has been posited that the addition of chlorine compounds may be an ineffective way to disinfect waters containing large amounts of solids and dissolved organic matter (e.g., human excreta), because these compounds lose their bactericidal and virucidal properties when they react with organic matter, forming chloro-organic and chloramines with relatively low disinfecting power [16,17]. A recent study [18] highlighted the limitations of chlorine for disinfecting human excreta.

Recently, it has been suggested that a physico-chemical approach using hydrated lime ($Ca(OH)_2$) suspensions might be a potential alternative to contain and treat human excreta in emergency settings [19]. Hydrated lime, also known as slaked lime or calcium hydroxide, is obtained when calcium oxide (CaO) is mixed with water to produce a highly alkaline suspension (pH 11–13). It has subsequently been observed that the effective treatment of wastewater may be achieved through exposure of excreta-borne pathogens to an alkaline environment, resulting in pathogen deactivation and destruction [20,21]. In addition to the chemical disinfection processes mediated by the high-pH environment, hydrated lime may also act as a coagulating agent, resulting in a coagulation-flocculation process in which pathogens adhere to solid flocs and are removed by a sedimentation stage, along with a significant portion of the organic component of the wastewater [22]. Consequently, hydrated lime has been proposed as an alternative means of treating municipal wastewater [23–25] and swine wastewater [26], and several studies have demonstrated the bactericidal and virucidal properties of lime in wastewater [20–22,27,28], faeces [29], and sewage sludge [30,31]. More recently, the successful application of hydrated lime to disinfect wastewaters from cholera

treatment centers (CTC) in Haiti following the 2010 cholera outbreak has been reported [32]. Also, a recent study in Malawi demonstrated that hydrated lime might also represent a promising faecal sludge sanitizer for emergency settings [33]. Although some authors have tested the use of hydrated lime in sanitary emergencies, this is an area that is still very little researched; furthermore, the effectiveness of some WASH sector recommendations (9–12) to contain and treat human excreta in emergency situations has not yet been tested.

Results of a previous research project undertaken at the University of Brighton (Applied Research on Disinfection to Prevent Ebola Transmission and funded by USAID) assessed the efficacy of various 0.5% (5000 mg/L) chlorine solutions (HTH, NaDCC, and household bleach) and hydrated lime suspensions (10%, 20%, and 30% (w:v)) at removing viruses and bacteria within excreta matrices. These recently published results [34] demonstrated that lime suspensions, particularly at 30% w/v, were considerably more effective than 0.5% chlorine solutions, especially when excreta matrices contained high concentrations of solids and organic matter. However, a chlorine solution of only 0.5% [9–11] was used for this previous study, which is considerably lower than the MSF cholera guideline [12], which recommends the use of 2% (20,000 mg/L) chlorine solutions for the containment and disinfection of human excreta in CTCs. Therefore, the following study sought to build upon the previous body of knowledge by assessing whether higher chlorine concentrations significantly increases their efficacy, compared to hydrated lime-based approaches, during the containment of human excreta in emergency settings.

2. Materials and Methods

Experiments followed methodologies previously described [34} with some modifications (as shown at the end of this section). Experiments were performed at the Environment and Public Health Research and Enterprise Group (EPHREG) laboratories of the University of Brighton (UoB). Excreta matrices (EM) were produced from dewatered faecal sludge and fresh untreated municipal wastewater. Samples of wastewater and sludge were collected weekly from a local municipal wastewater treatment plant (Hailsham North WWTP – with the permission of Southern Water Ltd (Worthing, U.K.)). All chlorine solutions (0.5% or 5000 mg/L; 1% or 10,000 mg/L; and 2% or 20,000 mg/L)) and hydrated lime ($Ca(OH)_2$) suspension (30% w:v) were prepared in the EPHReG laboratory (University of Brighton, Brighton, UK). Chlorine solutions were prepared from sodium dichloroisocyanurate ($C^3Cl^2N^3NaO^3$; NaDCC 65%; Minstral®). All experiments were undertaken within 14 liter high-density polyethylene (HDPE) plastic buckets provided by OXFAM®. Bacterial indicators (faecal coliforms (FC) and intestinal enterococci (IE)) and a viral indicator (somatic coliphages (SOMPH)) were used to evaluate approach efficacy.

2.1. Production of Excreta Matrices and Disinfectant Solutions

Three simulated excreta matrices (EM 0%, EM 10%, and EM 20%), containing varying amounts of suspended and dissolved organic matter, were produced. Their composition was intended to represent excreta from persons in different states of health. 'EM 20%' was composed of 80% raw wastewater (1200 mL) plus 20% faecal sludge (300 g) and represented excreta from healthy persons; 'EM 10%' was composed of 90% raw wastewater (1350 mL) plus 10% faecal sludge (150 g) and represented excreta from patients suffering from mild diarrhoea; 'EM 0%' was composed of 100% raw wastewater (1500 mL) and represented excreta from patients suffering from severe diarrhoea. EM 0% represented excreta with a consistency like 'rice water' similar to those described for symptomatic cholera patients [4]. First, the HACH® 8209 Iodometric titration method (HACH®, Loveland, CO, USA) was used in a series of tests to certify chlorine concentrations in NaDCC granules (65% available chlorine). Results showed a different percentage to that displayed on the product label, in that NaDCC granules recorded available chlorine levels of 53.7% (not 65%).

Prior to each disinfection experiment, one liter of each 0.5%, 1%, and 2% chlorine solution was prepared as follows. One liter of fresh deionized water was added to glass Schott bottles. Using an

OHAUS® Adventurer Pro digital scale (d = 0.01 g), 9.30 g, 18.60 g, and 37.20 g of NaDCC were weighed. The NaDCC chlorine compound was added to the bottles and stirred with a plastic-coated stirring rod. Each solution was labelled according to the chlorine concentration and allowed to stand for at least 30 min (to achieve total compound dissociation) prior to use. A total chlorine concentration test (HACH® 8209) was performed on each chlorine solution prior to experiments to ensure that solutions contained 0.5%, 1%, and 2% (±0.025%) of the available chlorine.

The hydrated lime suspension were prepared as follows: 300 g of hydrated lime (Rugby®, CEMEX U.K., Rugby, UK) were mixed with 700 mL of de-ionized water in glass Schott bottles (1 L), which were then used for experimental purposes within 24 h.

2.2. Experimental Setup

Experiments were undertaken to simulate the containment and removal of human excreta in CTC's in accordance with the MSF cholera guideline [12]. Experiments were performed by applying 125 mL of chlorine (0.5%, 1%, and 2% NaDCC); or hydrated lime (30%) to excreta matrices within buckets filled to approximately one third of their capacity (4.5 L or 4500 g of excreta matrix). Therefore, the disinfectant volume used (125 mL) corresponds to 2.8% (v:v) of the excreta matrix volume to be treated (4.5 L). Proportional volumes of the previously described excreta matrices were used during the bucket experiments (EM 0% = 4500 mL of wastewater; EM 10% = 4050 mL of wastewater + 450 g of faecal sludge; and EM 20% = 3600 mL of wastewater + 900 g of faecal sludge) (Figure 1). The efficacy of each approach was then tested using each of the three excreta matrices and at three increasing contact times (Ct) of 10, 30, and 60 min. All tests were repeated six times for statistical relevance.

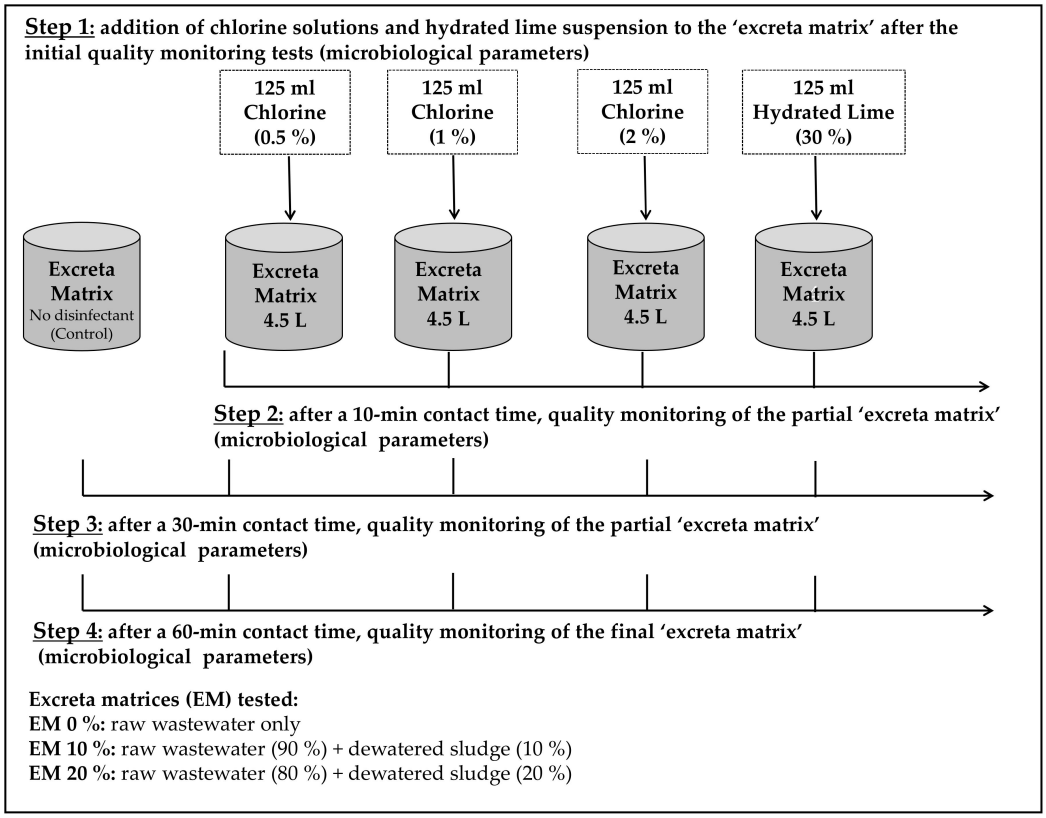

Figure 1. Flow chart of human excreta containment experiments.

Faecal sludge was weighed using an OHAUS® Ranger 3000 digital balance and volumes of wastewater were measured using measuring cylinders. Excreta matrices were then mixed and homogenized using an Arbeco® jar test machine (with a paddle speed of 200 rpm) for approximately 3 min. Excreta matrices were distributed to five buckets (four treatments plus one control).

All experiments were conducted at room temperature (approximately 21 °C). Before the addition of any disinfectant, and with the intention of quantifying the initial levels of all bacterial and viral indicators, a 5 mL sample was taken randomly from the buckets and poured into 50 mL self-standing centrifuge tubes (Corning®) containing 45 ml of quarter-strength Ringer's (QSR) solution. Producing an initial 10^{-1} 'control' dilution. Disinfectants were carefully poured into the four buckets simultaneously. Following contact times (Ct) of 10, 30, and 60 min, excreta matrices were rapidly stirred (3 secs) using a plastic coated stirring rod and 5 mL samples withdrawn for analysis from each bucket (including the control bucket). Samples were poured into 50 mL self-standing centrifuge tubes (Corning®) containing 45 mL of a solution (300 mg/L) of the dechlorinating agent sodium thiosulphate (BDH chemicals) (for chlorine-based disinfectants) or 45 mL of quarter-strength Ringer's (QSR) solution (for lime-based disinfection and control buckets). Producing 10^{-1} master dilutions. Dilution series (10^{-2}, 10^{-3}, and 10^{-4}) were produced from these master (10^{-1}) 50 mL dilutions and these were analyzed immediately.

To evaluate the efficacy of each approach, bacterial and viral indicators were enumerated in initial, post-treatment, and control samples. Faecal coliforms (FC) and intestinal enterococci (IE) enumeration followed standard methods: Namely, ISO 9308/1:200035 [35] and ISO 7899/2:200036 [36], respectively. Duplicate samples were placed onto either m-Fecal Coliform (mFC) or m-Enterococcus (mEnt) agar (Difco®) in Ø 55mm Petri dishes. Results were expressed as colony-forming units (CFU) per mL. Somatic coliphages (SOMPH) were enumerated in accordance with ISO standard 10705-237 [37] and E. coli (WG5) was used as the host bacterium [38]. Enumeration was carried out in duplicate and results were expressed as plaque-forming units (PFU) per mL. For initial and control samples, in which bacterial and viral concentrations were higher, 10^{-3}, $10^{-4,}$ and 10^{-5} dilutions were used, while for post treatment samples, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were used. The limit of detection for all microorganisms was either 10 PFU mL^{-1} or 10 CFU mL^{-1} [35–37].

The main modifications from the previous study [34] are as follows:

- A smaller volume of disinfectant (125 mL) was used to treat the excreta matrices (4.5 L), in this case, 2.8% (v:v) instead of 10% (450 mL), the reason for this was to follow more closely the specific recommendations for cholera treatment centres [12].
- The chlorine approach was tested at three concentrations 0.5%, 1%, and 2% (not just 0.5% as recommended [9–11] for Ebola treatment centres)
- Contact time (Ct) was tested at 10, 30, and 60 min (not just 10 and 30 min).
- In this study, hydrated lime was only tested at the 30% concentration (not at 10% or 20%), this was because previous research [34] showed that it presented the highest efficacy to contain and treat excreta matrices.
- Only NADCC chlorine compound was used to produce chlorine solutions, the reason was that in the previous study [34], there was no statistical difference in treatment efficacy when compared to the other compounds (HTH and bleach (NaOCl)).
- Only somatic coliphages (not F-specific coliphages and/or phages of *Bacteroides fragilis* GB-124) were used as viral indicators when testing treatment efficacy, this was because somatic coliphages were the most resistant [34].

2.3. Statistical Analyses

Statistical analysis of data was accomplished with the aid of the 'IBM Statistical Package for the Social Sciences (SPSS) 24.0' and Minitab 18 statistical software. Non-parametric statistical tests were used and median values were chosen to express more accurately the average levels. The criterion of 95% confidence, or a 0.05 probability (p), were applied to test the significance of the various statistical tests.

The efficacy of various approaches was evaluated by recording the initial and final (surviving) concentrations of microorganisms and calculating the log reduction of each microorganism for each approach. Additionally, calculation of the log reduction was performed for each approach and for

the various contact times and excreta matrixes. To perform log reduction calculations, samples that displayed 'non-detect' values (i.e., values below the detection limit) were attributed values, which were half of the detection limit (i.e., 5 PFU mL^{-1} or 5 CFU mL^{-1}) in accordance with other studies [39]. The Kruskal-Wallis test was used to determine whether there were statistically significant differences between efficacies with respect to the four treatments, the three contact times (Ct), and in the three excreta matrices (EM). Furthermore, a Dunn-Bonferroni post hoc paired comparison test was performed to identify differences in disinfection efficacy between all four approaches. Furthermore, linear regression analysis was performed to determine how the chlorine concentration influenced the log reduction of microorganism in the various excreta matrices.

3. Results

Average (median) initial levels of indicator organisms (prior to treatment) were as follows: FC = 5.6 × 104 CFU mL^{-1}; IE = 4.4 × 104 CFU mL^{-1}; SOMPH = 2.6 × 104 PFU mL^{-1}. Average initial levels of indicator organisms for each excreta matrix are provided in Table S1. Below, Table 1 summarizes all median (range) log reductions with respect to approach, contact time, and excreta matrix.

Table 1. Summary of median (range) log reduction of all microorganism (faecal coliforms (FC), intestinal enterococci (IE) and somatic coliphages (SOMPH)) according to approach, contact time, and excreta matrix.

Approach	Contact Time	Excreta Matrix	FC	IE	SOMPH
Chlorine 0.5%	10 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.08)	1.52 (0.18–2.68)
		10%	1.57 (1.15–2.97)	1.38 (0.62–2.58)	1.09 (0.73–1.87)
		20%	1.37 (0.47–2.11)	.98 (0.59–1.76)	1.01 (0.28–2.18)
	30 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.08)	1.92 (0.58–3.38)
		10%	1.48 (0.98–2.34)	1.31 (0.83–1.65)	1.09 (0.71–1.84)
		20%	1.12 (0.38–1.97)	0.99 (0.32–1.83)	0.83 (0.06–1.98)
	60 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.01)	2.26 (1.87–3.98)
		10%	1.51 (0.65–2.32)	1.21 (0.60–1.73)	1.08 (0.75–1.80)
		20%	0.86 (0.45–4.28)	0.93 (0.39–1.67)	0.98 (0.02–3.98)
Chlorine 1%	10 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.01)	2.31 (0.83–3.38)
		10%	1.58 (1.49–2.56)	1.34 (0.57–2.28)	1.70 (1.02–2.37)
		20%	1.41 (0.78–2.29)	0.85 (0.54–1.76)	1.21 (0.61–2.19)
	30 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.01)	2.11 (1.33–3.98)
		10%	1.74 (1.31–3.10)	1.17 (.86–2.58)	1.53 (1.14–2.42)
		20%	1.33 (0.55–1.78)	1.17 (0.46–2.11)	1.27 (0.34–2.26)
	60 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.01)	2.47 (1.76–3.68)
		10%	1.69 (0.96–2.49)	1.44 (0.72–2.03)	1.35 (0.96–1.89)
		20%	1.17 (0.42–2.05)	0.96 (0.49–2.26)	1.40 (0.12–2.40)
Chlorine 2%	10 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.01)	2.83 (2.20 – 3.94)
		10%	1.99 (1.22–2.91)	1.59 (1.32–2.58)	1.80 (1.57–2.90)
		20%	1.77 (1.00- 3.26)	1.87 (1.08–2.61)	1.57 (1.04–2.80)
	30 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.01)	3.31 (2.12–3.94)
		10%	2.95 (1.56–4.01)	3.08 (1.38–3.88)	1.95 (1.36–3.05)
		20%	2.17 (0.46–4.34)	1.91 (0.97–4.01)	1.74 (0.53–3.10)
	60 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.01)	2.72 (2.40–3.34)
		10%	2.37 (1.48–3.70)	1.87 (1.27–3.88)	1.91 (1.33–3.65)
		20%	1.97 (0.69–4.34)	1.73 (0.85–2.82)	1.90 (0.42–3.40)

Table 1. *Cont.*

Approach	Contact Time	Excreta Matrix	FC	IE	SOMPH
Hydrated lime 30%	10 min	0%	3.62 (3.26–4.28)	3.68 (3.00–4.01)	3.11 (2.12–3.94)
		10%	3.96 (3.70–4.32)	2.86 (2.07–3.88)	1.40 (0.78–1.60)
		20%	4.27 (3.36–4.82)	3.39 (1.29–3.72)	1.45 (0.74–2.49)
	30 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.01)	3.34 (2.90–3.94)
		10%	3.81 (2.23–4.32)	2.92 (1.82–4.03)	1.22 (0.85–2.45)
		20%	4.30 (3.82–4.82)	3.37 (1.78–3.72)	1.18 (0.64–3.94)
	60 min	0%	3.62 (3.26–4.28)	3.69 (3.26–4.01)	3.48 (2.20–3.98)
		10%	3.81 (2.53–4.32)	3.21 (1.19–3.88)	1.16 (0.81–1.97)
		20%	4.08 (3.68–4.82)	3.04 (1.40–3.96)	1.51 (0.47–2.10)

3.1. Overall Efficacy with Respect to Each Approach

Overall log reduction levels for each bacterial and viral indicator and each treatment are displayed in Figure 2. These values are based on median values from pooled samples of all excreta matrices (EM 0%, 10%, and 20%) and all contact times (Ct = 10, 30, and 60 min).

For FC, 'Lime 30%' provided the greatest overall efficacy (M = 3.93), followed by 'Chlorine 2%' (M = 2.88), 'Chlorine 1%' (M = 1.98), and 'Chlorine 0.5%' (M = 1.66). For FC, 'Chlorine 2%' and 'Lime 30%' demonstrated significantly ($p < 0.05$) greater log reductions than 'Chlorine 0.5%' solutions (File S1). For IE, 'Lime 30%' demonstrated the greatest overall efficacy (M = 3.50), followed by 'Chlorine 2%' (M = 2.60), 'Chlorine 1%' (M = 1.82), and 'Chlorine 0.5%' (M = 1.41). Only 'Lime 30%' demonstrated a significantly ($p < 0.05$) greater log reduction than other treatments (File S1). For SOMPH, 'Chlorine 2%' demonstrated the greatest overall disinfection efficacy (M = 2.38), followed by 'Chlorine 1%' (M = 1.79), 'Lime 30%' (M = 1.67), and 'Chlorine 0.5%' (M = 1.28). For SOMPH, 'Chlorine 2%' and 'Lime 30%' demonstrated significantly ($p < 0.05$) greater log reductions than 'Chlorine 0.5%' solutions (File S1).

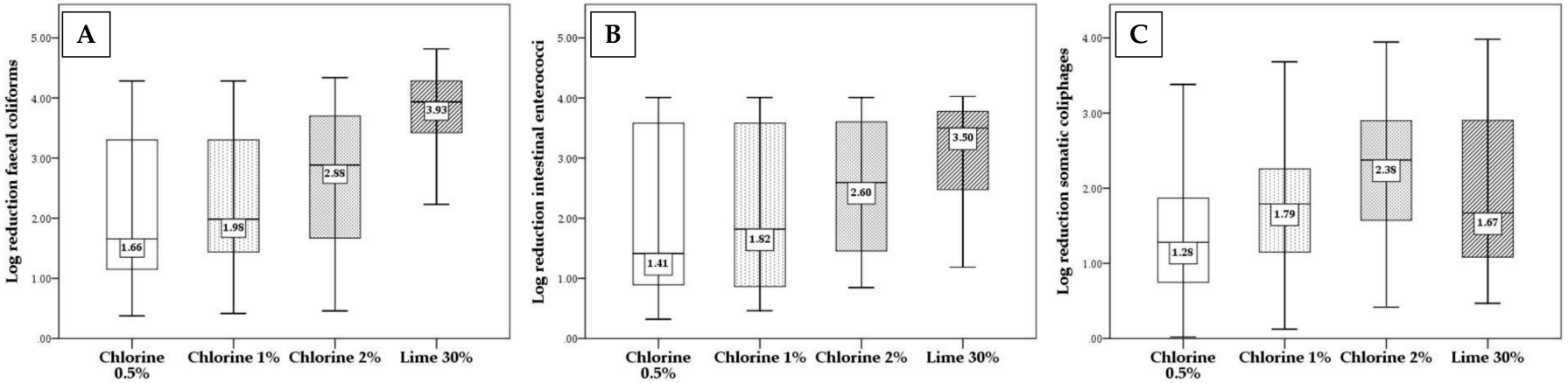

Figure 2. Box-plots displaying overall log reduction levels for faecal coliforms (**A**), intestinal enterococci (**B**), and somatic coliphages (**C**) following chlorine-based and hydrated lime-based approaches for the containment of human excreta matrices.

3.2. Efficacy of Each Approach with Respect to Contact Time (Ct)

A graphical comparison of the efficacy of each treatment approach with respect to contact time (Ct) for the three excreta matrices can be found in Figure S1. Interestingly, there was no significant difference (p-value > 0.05) between the efficacy of each approach with respect to contact times (Ct 10, 30, and 60 min) (File S2).

3.3. Efficacy of Each Disinfectant with Respect to Excreta Matrix (EM)

Figure 3 compares the efficacy of each treatment approach with respect to the excreta matrix for the various contact times. There was no significant difference (p-value > 0.05) in the log reduction of any bacterial or viral indicators when EM 10% and EM 20% outcomes were statistically compared. Although, there was always a significant difference (p-value < 0.05) in the log reduction of all bacterial and viral indicators when EM 10% and EM 20% outcomes were statistically compared to those of EM 0% (File S2).

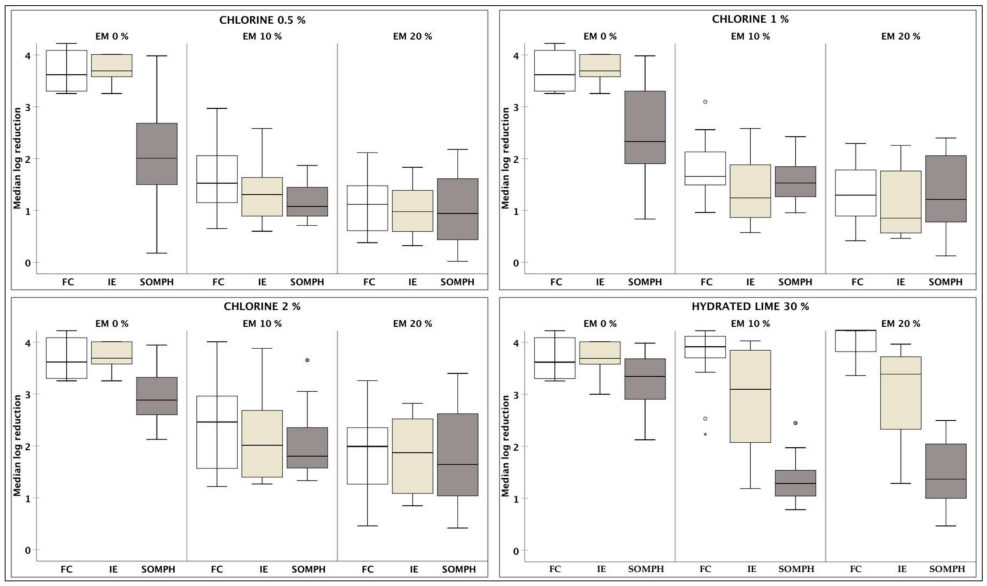

Figure 3. Box-plots displaying median log reduction of all microorganism (faecal coliforms (FC), intestinal enterococci (IE) and somatic coliphages (SOMPH)) levels, with respect to treatment approach and each excreta matrix (EM).

It is important to note that some log reduction levels of FC, IE, and SOMPH for 'Lime 30%' were higher in excreta matrices containing higher levels of dewatered sludge (EM 10% and EM 20%). This was the result of higher initial levels of indicator organisms in these matrices when compared to the initial levels in EM 0%

3.4. Fitted Line Plots with Regression Equations, Representing Log Reduction of Microorganisms, according to Chlorine Concentrations

As noted from previous boxplots results (Figure 2 - Overall efficacy with respect to each treatment approach), increasing the concentration of the chlorine solution increases its ability to reduce the levels of indicators within the excreta matrices. Linear regression analysis was performed to determine how the chlorine concentrations influence the log reduction of the indicator organism in the excreta matrices. Figure 4 below displays fitted line plots and regression equations in respect to the overall log reduction of indicator organisms according to chlorine concentrations. Note that the r-squared values for all equations were low (20.1% to 35.1%); this can be chiefly explained by the large variations in log reduction data with respect to the different excreta matrices and contact times. Outputs for the linear regression analysis can be found in File S3.

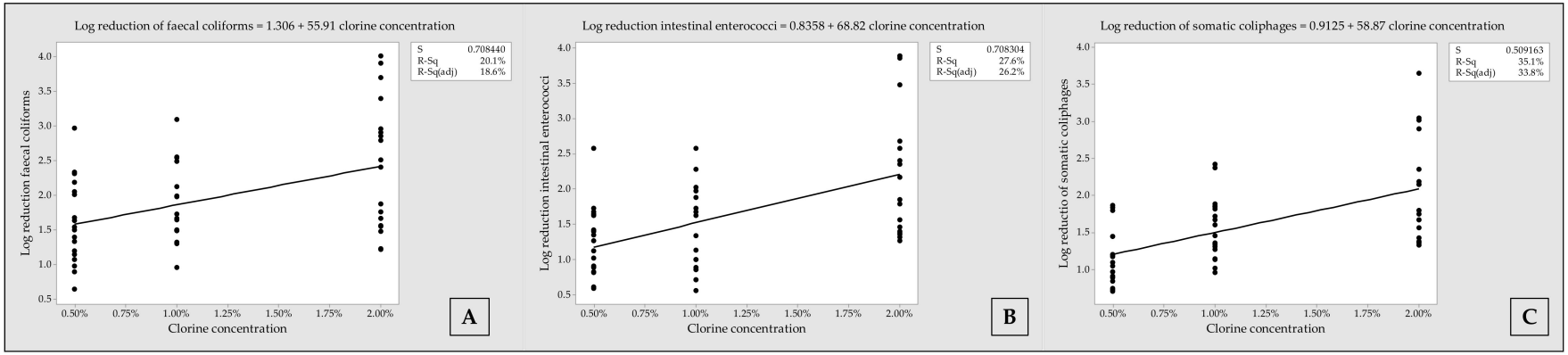

Figure 4. Fitted line plots with regression equations, representing the log reduction of faecal coliforms (**A**), intestinal enterococci (**B**), and somatic coliphages (**C**), according to chlorine concentrations.

4. Discussion

The findings revealed significant differences between the removal efficacies achieved with respect to the various treatment approaches tested. All chlorine and lime-based approaches demonstrated a significant log reduction for both IE (3.69) and FC (3.62) for EM 0%. This is an important finding as it is this matrix that most closely resembles cholera patients' 'rice water' stools, in terms of consistency. However, when all excreta matrices were considered, hydrated lime suspensions achieved greater IE and FC reductions, compared with the chlorine-based approaches. The results also demonstrated that increasing the concentration of the chlorine solution unsurprisingly increases its ability to disinfect the excreta matrices; with 2% chlorine solutions demonstrating higher log reductions compared with the weaker (0.5% and 1%) chlorine solutions in all excreta matrices (Table 1, Figures 2 and 3). Despite the improvement of efficacy when chlorine concentrations were increased, the proposed method (i.e., a 30% suspension of hydrated lime) demonstrated superior log reductions when compared with all the chlorine concentrations for two (FC of (+1.05) and IE (+1.10)) of the three indicator organisms investigated (Figure 2). However, a lower log reduction of somatic coliphages (−0.71) was achieved using hydrated lime compared to the 2% chlorine solution.

Both the 0.5% and 1% chlorine solutions only performed well in the EM 0% (Table 1 and Figure 3). The fact that watery feces from symptomatic cholera patients have a 'rice water'-like appearance [4] and is most similar to the EM 0% may give the false impression that concentrations of chlorine at 0.5% and 1% would be sufficient to treat and contain excreta from CTC's. However, the appearance and consistency of cholera patients' diarrhea has been shown to vary widely, and even asymptomatic patients may shed *Vibrio cholerae* in their normal stools [6,7]. Therefore, as a precautionary measure, the authors think that each approach should be effective regardless of stool consistency (hence the testing of three distinct excreta matrices. Chlorine 0.5% and 1% solutions' efficacy was significantly lower than that of the 30% lime solution, in the 10% and 2% excreta matrices (Figure 3). This was perhaps unsurprising given that chlorine-based disinfectants have been shown to lose their bactericidal and virucidal properties rapidly when in contact with high levels of organic matter [14].

It is interesting to note that the 30% lime solution did not lose its efficacy even as the load of organic matter and suspended solids increased in the excreta matrices from 0% to 10% and 20%. As mentioned previously, this study evolved from (and built upon findings of) earlier research [34]. However, the study differed (as outlined in the materials and methods section) in that it focused on trying to understand the performance of higher chlorine concentrations (1% and 2%), and different volumes (125 mL) of the chlorine solutions and lime suspension to treat and contain human excreta. This was hoped to more closely mirror those recommended in MSF's guidelines for use within CTC's. Therefore, while the previous study used approximately 10% (v:v), that is 450 mL of chlorine solution or hydrated lime suspensions in a 4.5 L of excreta matrix, this study used only 2.8% (v:v), that is 125 mL. Despite this study involving lesser volumes of disinfectants (when compared to our previous study [34]), the results showed some similarities. For example, hydrated lime 30% demonstrated a higher efficacy than chlorine 0.5% in reducing FC, IE, and SOMPH. However, the efficacy of both chlorine and lime-based treatments was reduced when a lesser volume (125 mL) of solution or suspension were added to the excreta matrices (Table 2).

Table 2 above demonstrates that the log reduction of indicator organisms generally decreased because of the addition of a lower volume of chlorine solution and/or hydrated lime suspension to the excreta matrices. This decrease was more evident in the case of the chlorine-based treatments. This is an important finding and the authors recommend the use of a 10% ratio (disinfectant: excreta) during the containment and safe handling of excreta matrices.

Table 2. Comparison of treatment efficacy (log reduction of indicator organisms (faecal coliforms (FC), intestinal enterococci (IE) and somatic coliphages (SOMPH)) with respect to the volume of chlorine or lime suspension used during current and previous studies [34].

Indicator Organism	Chlorine 0.5%			Hydrated Lime 30%		
	Previous Study *	This Study **	Difference	Previous Study *	This Study **	Difference
FC	2.78	1.66	**−1.12**	4.41	3.93	**−0.48**
IE	1.92	1.41	**−0.51**	3.94	3.50	**−0.44**
SOMPH	3.24	1.28	**−1.96**	1.80	1.67	**−0.13**

* 10%(v:v) = ratio chlorine solution or lime suspension used to excreta matrix (450 mL:4.5 L). ** 2.8%(v:v) = ratio chlorine solution or lime suspension used to excreta matrix (125 mL:4.5 L).

Current WASH recommendations [9–12] suggest excreta/disinfection contact times of 10, 15, and 30 min. However, the findings of this study, which looked at contact times of 10, 30, and 60 min, showed that the difference in log reductions between these times was not statistically significant, suggesting that after 10 min, most of the reduction in organisms had already occurred. Despite not being within the original scope of this project, the authors also explored the efficacy of each approach following a contact time of 24 h. The results (see File S4), especially with regards to 0.5% chlorine solutions, displayed decreased reduction levels of faecal coliforms and intestinal enterococci compared to contact times of 10, 30, and 60 min, suggesting possible bacterial regrowth [40]. This could be explained by the fact that levels of available free chlorine are likely to be low (following the high chlorine demand exerted by organic matter), though further research is needed to confirm this. However, this observation suggests that the timing of disposal, or further treatment of the human excreta, may be an important factor to consider to effectively protect health workers tasked with handling and emptying such buckets.

Another useful point to bear in mind when using chlorine-based approaches is that the 2% concentration seems to be more adequate (than other concentrations tested both here and in previous studies) for containing human excreta and wastewater. This also reinforces the importance of checking the shelf-life of chlorine solutions [41] and also of the need for adequate methods with which to test the concentration of such solutions in situ [42]. Although this research demonstrates that hyperchlorination (chlorine 2%) appears to be a potentially effective way to treat and contain human wastewaters, chlorine-based products are considerably less effective when it comes in more concentrated forms of human excreta, which seem to be better suited to lime-based treatment. Although our results demonstrate that increasing the chlorine concentration improved treatment efficacy (Figure 4), caution should be exercised when deciding whether to further increase chlorine concentrations (i.e., 3%) as there are other safety concerns surrounding the routine use of chlorine products, such as the possible production of toxic by-products or volatile gases [43,44] and the potential re-growth of resistant pathogenic microorganisms [40]. Despite this, chlorine remains a very important and widely-used disinfectant, which clearly will continue to have a pivotal role to play in the treatment of drinking water [9] and in the cleaning of surfaces during infectious disease outbreaks.

The price of chlorine and hydrated lime compounds may vary by country. In this study, the price to prepare hydrate lime suspension was U$ 0.23/L; compared to the price to prepare chlorine solution of 0.5%, 1%, and 2%, which was, respectively, U$ 0.18/L; U$ 0.36/L, and U$ 0.54/L. However, the production of lime suspensions requires larger volumes of hydrated lime compared with the equivalent volume of chlorine compounds required to produce the solutions used in this study. Transportation of large quantities of hydrated lime powder may therefore be considered a logistical problem in cholera treatment centers. Nevertheless, hydrated lime powder is a very common building material, which is available in most parts of the world and resources of limestone suitable for its manufacture are very large [45].

Whilst *Vibrio cholerae* is known to be resistant to environmental stresses [46] and has been shown to tolerate pH levels of up to 9.6 [2], the pH levels achieved in the hydrated lime approach from this study were extremely high (ranging from 12 to 13). One study, which used a far lower concentration (0.15%) of hydrated lime, demonstrated a considerable bactericidal effect on *Vibrio cholerae* [47]. Furthermore, studies have also suggested that *Vibrio cholerae* virulence expression is strongly repressed at high pH [48] (e.g., 8.5). The findings of this research (along with that from previous studies [33], including by this group [34]) continue to support the use of hydrated lime as a preferred emergency sanitation option, due to operational advantages that include: Relatively low cost, accessibility and reliance of supplies, operational stability across wide temperature ranges, and relatively short treatment times. Therefore, the international WASH sector should consider the use of hydrated lime as part of the basket of approaches needed to safely and effectively contain, handle, and treat human excreta in future emergency scenarios (e.g., cholera and Ebola treatment centers).

Whilst this study did not involve the use *Vibrio cholerae* (as its primary focus was on developing protocols for the use of conventional fecal indicators in field testing), previous research using biocides has demonstrated that *Vibrio cholerae* levels were reduced to <1 CFU/mL (6 log decrease) after exposure to just 800 ppm chlorine, following a contact time of 20 min [49]. This is important information given that we used chlorine concentrations ranging from 5,000 to 20,000 ppm, followed by a contact times of between 10 to 60 min. Given that symptomatic patients have been shown to shed *Vibrio cholerae* at levels of $10^{7–8}$ per gram [5] and that the infective dose has been estimated to be in the region of 10^{8} [8], log reductions of approximately 3.6 should ensure that any risk to operators or those responsible for handling cholera waste in CTC's is likely to be greatly reduced. These levels can be further decreased if additional wastewater/sludge treatments are subsequently employed in the sanitation chain.

Alternative treatment technologies to sanitize faecal sludge, such as the use of wood ash [50], have previously proven to be less effective than hydrated lime. Another potential alternative is the use of peracetic acid [51], which, when added to wastewaters, was shown not to generate significant amounts of toxic or mutagenic disinfection by-products or chemical residues in effluents. Also, there are some emerging technologies, such as the use of ultrasound for the treatment of municipal wastewaters [52] and the use of microwaves to treat faecal sludge [53], that still need to be further tested. Furthermore, a compendium that has been recently published summarizes sanitation technologies to be used in emergencies [54]. However, there is little research into the containment, safe handling, treatment, and disinfection of human excreta and wastewater in emergency settings.

Results from this study are very encouraging, but further research is still required in this overlooked area by the research community (i.e., production of toxic gases by the mixture of chlorine solutions and human excreta; assessment of the regrowth of bacteria after the chlorine demand exerted by the organic matter during these treatments; establishment of optimum ratios and contact times between solutions/suspensions and excreta matrices; repeating of experiments with vibrio strains, which may better resemble *Vibrio cholerae*; and, more importantly, the use of hydrated lime to treat/disinfect larger quantities of faecal sludge in emergency settings).

To conclude, the authors recommend either the use of 30% hydrated lime suspensions (at a ratio of 10% solution: excreta) or 2% chlorine solutions (at a ratio of 10% solution: excreta) as the preferred options for the containment and safe handling of human excreta in emergency settings, such as cholera treatment centers. As such, it is important that hydrated lime (as well as chlorine-based products) are included in emergency WASH response protocols and in inventory lists for emergency settings.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/11/2/188/s1, Figure S1: Bar charts displaying median log reduction levels for all microorganisms, following each approach, after three contact times, Table S1: Initial levels of microorganisms prior to experiments, File S1: Statistical analysis for overall log reduction of microorganisms according to each approach, File S2: Statistical analysis for log reduction of microorganisms, according to contact time and excreta matrix, for each approach, File S3. Outputs of linear regression analysis of log reduction microorganisms vs. chlorine concentration. File S4. Log reduction of each microorganism, according to each approach, after 24 hours of contact time.

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