Environmental Science
Water Research & Technology

Efficacy of locally-available cleaning methods and household chlorination at inhibiting biofilm development in jerricans used to store household drinking water

Journal:	*Environmental Science: Water Research & Technology*
Manuscript ID	EW-ART-08-2020-000748.R1
Article Type:	Paper

Based on our results, we emphasize that biofilm formation on jerrican surfaces in contact with contaminated water is not completely preventable using field-available cleaning methods. An unknown number of households may store water in biofilm contaminated jerricans, representing a crucial gap in ensuring access to safely managed drinking water for all as outlined under Sustainable Development Goal 6.

Efficacy of locally-available cleaning methods and household chlorination at inhibiting biofilm development in jerricans used to store household drinking water

Gabrielle M. String[*1], *Marta Domini*[1], *Hanaa Badr*[1,2], *Hannah Brodsky*[1,3], *Yarmina Kamal*[1], *Tyler Tatro*[1], *Miranda Johnston*[1,4], *Anthonia Ogudipe*[1], *Trang Nha Vu*[1], *Marlene K. Wolfe*[1,5], *Daniele S. Lantagne*[1]

1. Tufts University School of Engineering, Medford, MA
2. Tanta University, Faculty of Science, Egypt
3. Cornell University College of Engineering, Ithaca, NY, USA
4. Arlington High School, Arlington, MA, USA
5. Stanford University, Stanford, CA, USA

*Corresponding author: gabrielle.string@tufts.edu

ABSTRACT

Jerricans are commonly used to store household drinking water. However, biofilms can grow on jerrican surfaces and contaminate water. To investigate mechanisms to inhibit biofilm growth, 72 5-L jerricans containing *E. coli*-spiked water were incubated for 10 weeks in the laboratory. Jerricans were stratified by: water treatment with chlorine; turbidity; and, regular cleaning with NaOCl, rocks, sand, NaOCl/rocks, and NaOCl/sand. At study end, surface *E. coli* was enumerated and biofilms imaged using epifluorescence microscopy; free chlorine residual was tested regularly. We found biofilms grew rapidly in jerricans (presence confirmed in 3 weeks), and chlorine demand within jerricans increased over time as FCR decreased from 1.16 to 0.59 mg/L over the course of the study in 5 NTU treated waters. Biofilm growth was significantly inhibited by water treatment with chlorine ($p=0.03$), and was inhibited by cleaning with NaOCl or NaOCl/rocks. Cleaning with rocks increased surface roughness, which promotes biofilm growth. These results highlight the need for research on locally-appropriate cleaning methods and/or alternatives (e.g. replacement, biofilm-resistant surfaces) to ensure jerricans provide safe storage of drinking water.

Keywords

Biofilm, chlorine, cleaning, disinfection, jerrican, water storage container

Abbreviations

CFU	Colony forming units
cm	Centimeter
DAPI	4',6-diamidino-2-phenylindole
E. coli	*Escherichia coli*
FCR	Free chlorine residual
g	Gram
HDPE	High-density polyethelene
Hz	Hertz
L	Liter
LB	Lauria-Bertani medium
LMIC	Low- and middle-income countries
mg	Milligram
nm	Nanometer
NTU	Nephelometric turbidity units
PBS	Phosphate buffered saline
RMS	Root mean square
rpm	Rotations per minute
WASH	Water, sanitation, and hygiene
μm	Micrometer

1 INTRODUCTION

In 2017, 5.3 billion people (71%) had access to safely managed water supplies; the remaining 2.2 billion, mainly in low- and middle-income countries (LMIC) relied on basic, limited, unimproved, or surface water sources [1]. Additionally, a recent systematic review found a higher odds (OR = 1.09, 95% CI = 1.04-1.13) of diarrhea in children under five years with 1-$\log_{10}$ higher fecal indicator bacteria concentrations in drinking water[2]. Evidence-based strategies for providing safe water, sanitation, and hygiene (WASH) to populations in LMIC and emergency-affected areas are necessary to uphold dignity and control communicable diseases [3-5]. Recent systematic reviews have found WASH interventions consistently reduce the risk of disease transmission, and the risk of disease, in humanitarian contexts [6, 7].

Previous research has highlighted the importance of safe water storage: a case-control study identified safe storage as a protective factor (OR = 0.55, 95% CI = 0.39–0.80), and unsafe storage a risk factor (OR = 2.8; 95% CI = 2.1–3.7), for cholera transmission; evaluations identified a clean household water storage container is associated with reduced risk of water contamination [8, 9]; and, one impact evaluation showed safe storage reduced diarrhea (OR = 0.84, 95% CI 0.82–0.86) while another found no diarrheal reduction but 69% reduction in fecal coliforms over six hours of storage[10, 11]. Evaluations have also documented risks of unsafe water storage practices, including recontamination of household stored water from filter effluent to storage [12-14], and increased contamination after transport and storage compared to source water [15].

Two commonly implemented safe water storage interventions in LMIC and humanitarian response are distributions of chlorine tablets to treat household drinking water and jerricans to safely store household drinking water [6]. Chlorine tablets are widely distributed because chlorine

effectively inactivates most bacterial and viral pathogens; leads to residual protection; is low cost; and, is easy to use [16]. Users add a tablet(s) in an appropriately sized storage container, wait 30 minutes, and drink. Recommendations for tablet dosage depend on water turbidity, volume, and whether the tablets are deployed for outbreak or household use; commonly recommended dosages are 1.9 mg/L for clear water and 3.8 mg/L for turbid water[17, 18]. Reported and confirmed use of chlorine tablets varies widely; more successful programs deliver tablets of appropriate dosage with a storage container and training to households already familiar with their use [6, 19]. Chlorine efficacy against common water pathogens has been widely documented; for instance the Ct factor for *E. coli* is <0.25 mg•min/L [18].

Safe water storage consists of a container, such as a jerrican, with physical barriers to contamination/recontamination of water (e.g. small opening) that enable users to practice safe water access behaviors (e.g. pouring) and reduces fecal-oral transmission [20-22]. One review found that safe water storage reduced the risk of diarrhea more than water filtration alone (45% compared to 33%)[23]. Prevalent in LMIC and emergency contexts, jerricans are made of opaque high density polyethylene (HDPE) and manufactured with a small, screw-cap opening that can protect water from external contaminants during storage, but inhibits cleaning the inside surface. As cleaning narrow-mouth containers can be difficult [24], users have developed cleaning methods including adding disinfectants (e.g. chlorine powder) and/or locally-available abrasives (e.g. sand/rocks) into the jerrican, shaking, and rinsing to clean [11].

Evaluations of three short-term jerrican disinfection programs in emergency contexts (using 2.5-50,000 mg/L chlorine solutions at 1-8 time points, and/or abrasives) documented short-term increases in free chlorine residual (FCR) with reductions in microbiological indicators, and regrowth of microbiological indicators in hours-to-days [11, 25, 26]. Of note is biofilms were

mentioned as the fundamental cause of contamination of water, but were not directly researched in these studies.

Biofilms are aggregates of microorganisms attached to a surface and coated within a self-created extracellular polymeric substance matrix [27]. Biofilms develop on all surfaces in contact with non-sterile water and are self-sustaining. Due to their ability to harbor, and shed, infectious pathogens, biofilms have been extensively researched in hospital settings and drinking water distribution systems; limited research exists for water storage containers. In South Africa and Cameroon, samples taken after scrubbing, shaking, or swabbing storage container surfaces had more contamination than stored water [28-31]. One small sample size study found biofilm coliform concentration ranged 1.85±1.59 CFU/cm^2 and was not associated with container shape, primary drinking water source, reported cleaning mechanism, or 'time since last washing' [30]. In subsequent agent based modeling, biofilms were modeled as contributing between 0-5000 coliform CFU/100mL to stored water and were found to have a significant impact on coliform concentration in stored water ($p<0.001$) and to be the most important factor in high stored water coliform concentrations[32]. On plastic container surfaces in a laboratory, biofilms formed within 24 hours storing raw water, and 3-21 days storing filtered water [33]. Overall, while not focusing specifically on jerricans nor investigating the relationship between chlorine tablet use and the development of biofilms, prior research has indirectly measured biofilm formation in household water storage containers and found local cleaning methods ineffective at removing biofilms. Furthermore, an unknown number of households may store water in biofilm contaminated jerricans, representing a crucial gap in ensuring access to safely managed drinking water for all as outlined under Sustainable Development Goal 6 [34].

We hypothesized *E. coli* biofilms would grow in jerricans, and when biofilms grow, chlorine demand and aqueous *E. coli* would increase in stored water. Furthermore, biofilm growth would be conditioned on water treatment, water turbidity, cleaning methods used, and frequency of cleaning. To test our hypothesis, we first completed pre-testing to develop methods to grow and measure *E. coli* biofilm formation in jerricans. A non-pathogenic strain of *E. coli* (ATCC 11229) was chosen for this study as it is known to form biofilms, is easy to culture and quantify, has a short growth period [35]. Furthermore, *E. coli* is a standard indicator bacteria for water quality and >1000 *E. coli* CFU/100mL is considered very high risk water [36]. We conducted a 10-week laboratory study divided into three phases (based on cleaning and treatment frequency) with 72 jerricans stratified by chlorine tablet treatment (yes/no), turbidity (5/50 NTU), six cleaning methods, and daily-to-weekly cleaning. Outcomes included testing end-of-study surface roughness; end-of-phase epifluorescence imaging and surface *E. coli;* weekly aqueous *E. coli*; and, daily-to-weekly free chlorine residual (FCR) (Figure 1). Lastly, we conducted follow-on testing of the relationship between household use and cleaning characteristics, surface roughness, and biofilm growth in previously used jerricans.

2 METHODS

This study consisted of three stages: 1) pre-testing to develop methods to grow and confirm biofilm presence using microbiological testing and imaging; 2) full-scale study to test the efficacy of household chlorination and cleaning methods at preventing biofilm growth; and, 3) follow-on study on surface roughness and biofilm growth in field-collected jerricans.

2.1 Pre-testing

Coupons (1cm^2), a standard method for growing biofilms on a sub-stratum [37], were cut from smooth, flat sidewalls of new 5 L HDPE jerricans (Air Sea Containers Inc, Miami, FL, USA). In total, 288 coupons were cut from the containers and stratified by different test parameters: 1) concentration of *E. coli* spiking solution (10^4, 10^5, 10^6 CFU/100mL); 2) the liquid environment the *E. coli* were developing in (growth media or buffered water; 3) biofilm growth period (10 or 21 days); 4) *E. coli* removal method for surface enumeration (swabbing or sonicating); and 5) manufactured surface roughness of coupon (virgin or 120, 240, 400 grit sandpaper). To create surface scratches, coupons were ground on a polishing table for 1 minute using either 120, 240, or 400 grit sandpaper; coupons with no scratches were left as control. Surface roughness of coupons was measured using a Dektak XT-S Profilometer with *Vision64* software (Bruker, Billerica, MA, USA). Surface profiles were collected using a 12.5 μm tip stylus with 29.4 μN contact force at 166.7 μm/s scan speed by scanning each coupon for 5000 μm in three directions (x, y, and xy). Root-mean squared (RMS) surface roughness was calculated after leveling the profile using two points, and entered into Microsoft Excel. Coupons were then sterilized with 70% ethanol, individually placed in 50 mL Falcon tubes, and immersed in 25 mL of either Luria-Bertani (LB) broth or Type-1 laboratory grade water (Milli-Q® Reference, MilliporeSigma, Burlington, MA, USA) filtered through a 0.22 μm filter, hereafter termed "Milli-Q", that was buffered with phosphate buffered saline (PBS) (pH=7.4). *E. coli* (ATCC 11229) stock was streaked onto LB agar plates, incubated at 35ºC, and stored at 4ºC. The night before each use of *E. coli,* a streak plate colony was used to inoculate 20 mL of LB broth, and incubated at 35ºC for 12-18 hours with shaking at 70 rpm. The culture was then diluted (1:20) in sterile LB broth and incubated at 35ºC for 3 hours with shaking, or until a concentration of ~10^{10} cells/mL was reached, as estimated using a spectrophotometer (OD=600 nm). *E. coli* was spiked into each tube

at a concentration of either 10^4, 10^5, or 10^6 CFU/100mL. Falcon tubes were incubated at 35°C on an orbital shaker at 70 rpm for two days. Coupons were aseptically transferred to new 50 mL Falcon tubes with 25mL of fresh LB Broth or buffered Milli-Q and spiked with fresh *E. coli* culture at the appropriate concentration, every 48 hours. This cycle was repeated for either 10 or 21 days.

The growth of *E. coli* biofilms on coupon surfaces was confirmed using enumeration by *E. coli* culture and imaging. Two methods were trialed to remove *E. coli* from coupon surfaces for enumeration by culture: swabbing and sonicating [38, 39]. Swabbing was conducted by passing a Sanicult Hygiene Monitoring swab (Starplex Scientific, Etobicoke, Ontario, Canada) over the coupon surface five times, returning swabs to their peptone broth, vortexing, and storing on ice. Sonication was conducted by wiping the exterior coupon surface with 70% ethanol to sterilize, then aseptically placing coupons in sterile Falcon tubes with 25 mL of PBS. Tubes were vortexed for 30 seconds at 1200 rpm, then sonicated for 5 minutes at 40,000 Hz in ice water. Appropriate dilutions from each swab or sonicated sample were prepared, filtered through a membrane, plated on mColiBlue24® media (Hach, Loveland, CO), and incubated at 35°C for 24 hours following Standard Methods 9222B [40]. *E. coli* colonies were enumerated and recorded.

For imaging, each coupon was rinsed in a sterile field by gently pipetting 2 mL of PBS across the surface to remove planktonic cells [41], air dried, mounted onto a glass microscope slide. In the dark, 50 µL of a 600 µM solution of 4',6 diamidino-2-phenylindole dihydrochloride (DAPI) stain (MilliporeSigma, Burlington, MA, USA) were pipetted onto each coupon and incubated at room temperature for 30 minutes. A drop of fluorescent mounting media (MilliporeSigma, Burlington, MA, USA) was applied as an anti-fading agent to each coupon, and a glass coverslip placed on top. Slides were wrapped in aluminum foil and stored at 4°C until imaging. Coupons

were imaged by epifluorescence microscopy, using a Leica SPE confocal microscope (Leica, Wetzlar, Germany) under 63x objective in immersion oil. Images were acquired by exciting the DAPI using a 405 nm visible laser diode. The DAPI stain had a peak excitation wavelength of 350 nm and a peak emission wavelength of 470 nm. Three randomly selected fields of view were imaged for each coupon by scanning at 400 Hz from the surface of the coupon up through to the biofilm surface. Image slices were recorded at predefined z-step sizes (ranging 0.3-2.0 μm), and an image stack for each field of view was exported for analysis.

2.2 Full-scale

2.2.1 Study design

The full-scale study was designed based on pre-testing results, and occurred over a 10-week time period with new 5 L HDPE jerricans. Jerricans were stratified by different test parameters: 1) turbidity (5 or 50 NTU); 2) chlorine treatment (treated with Aquatabs® (Medentech, Wexford, Ireland) or non-treated); and, 3) six cleaning methods (125 mL 5% NaOCl (chlorine); 220 g pebbles (rocks); 150 g sand (sand); 125 mL 5% NaOCl and 220 g pebbles (chlorine/rocks); 125 mL 5% NaOCl and 150 g sand (chlorine/sand); and, no cleaning (control)). Each combination was tested in triplicate (Figure 1) for a total of 72 jerricans at study beginning. Jerricans were maintained at 35°C except when being emptied/cleaned/refilled (~3 hours per day) when they were at 21°C.

2.2.2 Empty/clean/refill cycle

Jerricans were emptied, cleaned, and refilled with 4.5 L of *E. coli* spiked water daily (Weeks 1-4), 2x/week (Weeks 5-8), and 1x/week (Weeks 9-11). Please note during weeks 5-11, *E. coli* was also spiked into every jerrican each non-cleaning day.

Each morning, jerricans were emptied then cleaned using the assigned cleaning material(s). The chlorine cleaning solution was prepared by diluting 5.25% NaOCl (Austin's A-1 Bleach, Pittsburg, PA, USA) to ~0.5% by volume. Rocks ("Pea Pebbles", Vigoro, Lake Forest, IL, USA) and sand ("All Purpose Play Sand", Pavestone, Atlanta, GA, USA) were washed with tap water to remove excess dust, dried in an oven at 100°C for 24 hours, and stored in sterile containers until use. After adding the cleaning materials, jerricans were shaken 5 times in each of three directions: "up -down", "front-back" and "left-right". Jerricans were then emptied and rinsed three times with 300-400 mL of Milli-Q to remove excess cleaning material.

After cleaning, jerricans were refilled with test water freshly prepared in four batches in sterilized tubs by buffering 90 L of Milli-Q with 54 mL 5M NaOCl, 52.2 mL 1M K_2HPO_4, and 34.8 mL 1M KH_2PO_4. To create 5 and 50 NTU turbidity waters, creek-bed sediments were manually mixed into the tubs for 15 minutes using sterile metal stirrers at concentrations of 0.3 g/L sediment for 5 NTU and 4.5 g/L for 50 NTU. Sediments were collected by removing the top 5 cm of material from the Mystic River creek-bed (Medford, MA, USA), and collecting the 10 cm layer beneath. In the laboratory, sediments were sieved through a 18x14 mesh, rinsed, and allowed to settle for 24 hours. Supernatant was poured off, and remaining solids were dried at 100°C for 72 hours in an oven and stored in sterile containers. After preparation, turbidity was confirmed to be within 10% of the target (4.5-5.5 NTU; 45-55 NTU) in each tub using a turbidimeter calibrated daily (Lamotte 2020we, Chestertown, MD, USA). If needed, turbidity was adjusted/retested.

177 *E. coli* cultures (ATCC 11229) were prepared as in 2.1. After spiking 10^3 CFU/mL into each tub,
178 the water was manually mixed for 15 minutes, and 4.5 L was dispensed into appropriate
179 jerricans. The target *E. coli* concentration from one sample of spiking solution and water samples
180 from 2-4 randomly selected jerricans was verified using membrane filtration as in 2.1.

181 Lastly, for chlorine treated jerricans, a 17 mg Aquatabs® tablet was added to 5 NTU jerricans
182 (dose 2mg/L) or a 32 mg tablet was added to 50 NTU jerricans (dose 4mg/L) daily (Weeks 1-8),
183 or when free chlorine residual (FCR) values were equal or lower than the Week 1-8 average
184 (Week 9-11).

185 **2.2.3 Testing**

186 Each empty/clean/refill day, FCR was measured in triplicate and an average recorded at 1, 4, and
187 22 hours after treatment using a calibrated colorimeter and DPD-1 instrument grade tablets
188 (Lamotte 1200, Chestertown, MD, USA), which were expected to be accurate and return
189 readings with low measurement error [42]. Samples were collected by pouring an aliquot from the
190 container into a rinsed sample collection beaker. Sample from the beaker was poured into the
191 colorimeter tube, the DPD-1 tablet was added, the vial was wiped with a Kimwipe, and color
192 was allowed to develop for at least 30 seconds and no more than 2 minutes before being read in
193 the colorimeter. Weekly, *E. coli* was enumerated in samples collected 1 and 22 hours after test
194 water addition, via membrane filtration, as in 2.1. Timings for FCR and *E. coli* samplings were
195 chosen to ensure completion of experimental procedures were consistent from day to day. At
196 end-of-phase, one jerrican per triplicate was destructed and 2 cm^2 coupons (two from treated
197 jerricans, four from non-treated) were cut from the bottom, side, and front surfaces of jerricans
198 using tinsnips, for a total of six coupons for treated, and 12 coupons for non-treated, jerricans.

One coupon from treated jerricans and two from non-treated jerricans, from each location, were prepared using sonication and *E. coli* in supernatant was enumerated as in 2.1. The remaining one coupon from treated and two from non-treated jerricans were prepared and imaged as in 2.1. After imaging, coverslips were discarded, coupons were gently washed with a sponge, soap, and tap water to remove the biofilm layer, sprayed with 70% ethanol, and surface roughness measured as in 2.1.

2.2.4 Analysis

Data were entered into Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA). The geometric mean of plates in countable range (10-200 colonies) was calculated for each cultured samples and reported in CFU/100mL for liquid samples and CFU/cm^2 for surface samples. Samples below detection limit were replaced with half the detection limit, zero values were replaced with 0.5, and samples above detection limit were counted as at detection limit. Data was analyzed in Excel and Stata, with differences in surface *E. coli*, biofilm thickness, and roughness between side and bottom coupons assessed using a paired t-test, and differences by turbidity and water treatment assessed using independent sample t-tests. Differences in FCR at study beginning and end, and between *E. coli* at 1 and 22 hours at study end, were assessed using paired t-tests. Both parametric and non-parametric tests were checked, with no resulting difference; thus t-tests are presented.

Images were imported and visualized for qualitative analysis in FIJI/ImageJ 1.52i. Image stacks were trimmed so that the top of the stack was the first slice with cells in focus and the bottom of the stack was the last slice with cells in focus for that field of view. Stacks were then converted to 8-bit black and white, thresheld using the Otsu algorithm, downsampled using a 3D-Gaussian

Blur algorithm (alpha=0.5 in x, y, z), then dilated once, eroded once, and inverted to remove background noise. Each image stack was then analyzed using the Particle Analyzer algorithm, *E. coli* counts and biofilm thickness recorded, and a density (CFU/μm^3) calculated for each image stack. In denser biofilms, *E. coli* cells clumped together to form colonies, which the algorithm falsely counted as one, resulting in calculated density value sometimes being artificially low. Thus, processed images were also qualitatively evaluated. A rating system was developed to qualitatively categorize image stacks: no growth; a few disconnected *E. coli* cells and no clumping; a small amount of clumping and visually moderate biofilm structure; and, large clumping and visually dense structure. All images were qualitatively classified separately by two trained individuals.

2.3 Follow-on

Jerricans were collected from households during surveys as part of field evaluations for other studies (Tufts SBER IRB #1712003 and #1712004, and appropriate local approvals). Enrolled households were surveyed about their household water storage and handling practices; those that stored water in a jerrican and reported to clean their jerrican were eligible for inclusion in this follow-on study. Jerricans were collected from consenting households and replaced with new, equivalent containers. In the field, jerricans were washed and filled with locally available bleach to sterilize the containers, before transport to Tufts in checked baggage, and stored at room temperature for 6-11 months. Prior to study start, jerricans were cleaned with 5.25% bleach and sets of three 4 cm^2 coupons were cut from the center of one side and from the bottom of jerricans. Coupons were gently hand-washed with soap and tap water, cleaned with 0.5% bleach, and sterilized with 70% ethanol. A surface profile was collected from a similar location on each coupon as described in 2.1.

Coupons were then sterilized with 70% ethanol solution and aseptically transferred into individual, sterile 50 mL Falcon tubes containing 25 mL of LB Broth spiked with *E. coli* at a concentration of 10^3 CFU/mL prepared as described in 2.1 and were maintained for 21 days. *E. coli* concentration in LB Broth was tested 1 and 48 hours after spiking to confirm growth using membrane filtration methods as described in 2.1.

After 21-days, each coupon exterior surface was wiped with 70% ethanol, rinsed by gently pipetting 2 mL of PBS across the surface, and air dried in a sterile field. Two coupons from each triplicate were then aseptically transferred into Falcon tubes containing 20 mL of PBS (pH=7.4), sonicated, and processed via membrane filtration as in 2.1. Data was entered and *E. coli* CFU/cm^2 concentrations were calculated as in 2.2.4. Data was analyzed in Excel and Stata, with differences between side and bottom coupons assessed using a paired t-test, use of abrasive agents assessed using an independent sample t-test, and differences between countries assessed using a one-way ANOVA and a post-hoc Tukey's HSD test to identify significant differences.

The remaining coupon from each triplicate was processed for imaging via epifluorescence microscopy and analysis as described in 2.1 and 2.2.4.

3 RESULTS

Results from the study include pre-testing results; full-study results including end-of-phase surface *E. coli* testing, end-of-study biofilm imaging and surface roughness results, weekly aqueous *E. coli*, and daily-to-weekly FCR; and, follow-on study results.

3.1 Pre-testing

E. coli biofilms were grown on HDPE coupon surfaces of varying roughness using two media and three *E. coli* concentration spikes changed every 48 hours for two time periods. Additionally, two removal methods (swabbing and sonication) were tested for enumerating live *E. coli* attached to coupon surfaces, and coupons were imaged using epifluorescence microscopy.

Coupon RMS surface roughness ranged from 1.43 µm (virgin) to 1.60 µm (400 grit, fine scratches) to 5.20 µm (120 grit, coarse scratches). A range of geomean *E. coli* concentrations were observed at 10 days (ranges across the four surface roughnesses presented for each spike concentration): $2.2 \bullet 10^{9}$-$1.6 \bullet 10^{11}$ CFU/100mL (10^{4} CFU/mL spike) to $8.9 \bullet 10^{12}$ -$9.6 \bullet 10^{12}$ CFU/100mL (10^{2} CFU/mL spike) in spiked LB broth, and $2.7 \bullet 10^{8}$-$9.1 \bullet 10^{7}$ CFU/100mL (10^{2} CFU/mL spike) to $6.8 \bullet 10^{8}$-$4.7 \bullet 10^{9}$ CFU/100mL (10^{4} CFU/mL spike) buffered Milli-Q. At 21 days, concentrations for a 10^{4} CFU/mL spike (across the four surface roughnesses) increased to range $3.2 \bullet 10^{9}$- $3.8 \bullet 10^{11}$ CFU/100mL (LB broth) and $2.1 \bullet 10^{9}$ -$5.3 \bullet 10^{10}$ CFU/100mL (buffered Milli-Q). Thus, surface roughness varied by grit and biofilms grew in all conditions (as seen by increases in media concentrations over time). Biofilms grew at increased rates with LB broth (compared to buffered Milli-Q) and with longer growth periods (21 compared to 10 days).

When sampled by swabbing, geomean *E. coli* surface concentrations in 10^{4} CFU/mL spiked Milli-Q varied from 13-2,600 CFU/cm^2 (virgin, 240 grit) at 10 days of growth; at 21 days the concentration increased to 240-63,000 CFU/cm^2 (400 grit, 240 grit). When sampled by sonication, geomean *E. coli* surface concentrations in 10^{4} CFU/mL spiked Milli-Q varied from 48,000-80,000 CFU/cm^2 (240, 400 grit) at 10 days of growth; at 21 days the concentration increased to 720,000-2,400,000 CFU/cm^2 (virgin, 400 grit). Overall, sonication had higher recovery rates and more consistent results.

Surface *E. coli* and imaging results found, after 21 days of growth, *E. coli* surface concentrations were an order of magnitude higher in scratched as compared to virgin samples, despite similar spiking conditions. These results were consistent in imaging, where *E. coli* cells aligned in scratches on abraded coupons as compared to virgin surfaces (Figure 2).

3.2 Full-study

3.2.1 End-of-phase *E. coli* surface results

A total of 432 *E. coli* surface samples were analyzed during the study. For treated jerricans, across three phases and three samples, none of 54 samples (0%) in 5 NTU treated jerricans had >1 CFU/cm^2. In the 50 NTU treated jerricans, 13 of 54 locations (24%) had >1 CFU/cm^2. Concentrations >1 CFU/cm^2 were seen in control samples, and chlorine, sand, and chlorine/sand cleaning methods. No sample in rocks or chlorine/rocks cleaning methods was >1 CFU/cm^2. Of the 13 positive samples, six (46%) were in control jerricans (as compared to cleaned jerricans), and six (46%) were in the third (last) sampling phase (as compared to Phase 1 and 2) (Figure 3).

For non-treated jerricans across three phases, the geometric mean *E. coli* concentration in 5 NTU non-treated jerricans across three surface locations was 42 CFU/cm^2 (control=77; min=13 (sand); max=137 (rocks)). In 50 NTU non-treated jerricans, geometric mean *E. coli* concentrations across three phases and three surface locations was 1,167 CFU/cm^2 (min=694 (control); max=1,943 (chlorine/rocks)).

E. coli from surfaces was significantly lower in treated jerricans in all phases (all phases, <0.01). *E. coli* from surfaces did not differ significantly by turbidity for any phase (p=0.16, p=0.15, p=0.14 for Phase 1-3, respectively).

3.2.2 End-of-study biofilm imaging and surface roughness results

Across all three phases, 324 coupons were stained and imaged. At study end, in treated jerricans, biofilms were visually detected on at least 1/3 coupons (bottom, side, or front) in 5/6 (5 NTU) and 6/6 (50 NTU) cleaning methods. In non-treated jerricans, biofilms were detected in all cleaning methods. No biofilms were detected: in 5 NTU treated jerricans on all surfaces when cleaning with chlorine, and on bottom and side surfaces when cleaning with chlorine/rocks (Figure 4); and, in 50 NTU treated jerricans on bottom and side surfaces when cleaning with chlorine, and on bottom and front surfaces when cleaning with chlorine/rocks.

Treated jerrican biofilm thickness ranged from 0.0-21 μm (5 NTU) and 0.0-42 μm (50 NTU); non-treated jerrican biofilm thickness ranged from 1.5-43 μm (5 NTU) and 0.0-40 μm (50 NTU) (Figure 4). Treated jerrican density ranged from 0.0-690 CFU/μm^3 (5 NTU) and 0.0-449 CFU/μm^3 (50 NTU); non-treated jerrican biofilm density ranged from 30.4-316 CFU/μm^3 (5 NTU) and 0.0-284 CFU/μm^3 (50 NTU). Biofilm thickness was significantly less in treated as compared to non-treated jerricans ($p=0.03$) and did not differ significantly by turbidity ($p=0.28$).

The surface roughness of the 108 imaged coupons from Phase 3 varied by cleaning method. Across the three surfaces per jerrican, RMS surface roughness was 1.31-3.27μm in control jerricans, 2.09-5.95μm in jerricans cleaned with chlorine, 2.73-12.1 μm in rocks, 1.34-5.49 μm in sand, 2.45-10.4 μm in chlorine/rocks, and 1.00-3.99 μm in chlorine/sand (Figure 5). Water turbidity and treatment were not associated with surface roughness ($p=0.70$ and $p=0.71$,

respectively). Please note there was insufficient sample size to complete formal statistical analysis by cleaning method.

3.2.3 Weekly aqueous *E. coli* results

Overall, there were 480 weekly aqueous *E. coli* results. In 5 NTU treated jerricans, the geomean *E. coli* concentration 1 hour after spiking was <1 CFU/100mL (range <1-600); at 22 hours it remained <1 (range <1-40), with 3% of samples >1 and 2% >10 CFU/100mL. In 50 NTU treated jerricans, the geomean *E. coli* concentration 1 hour after spiking was 108 CFU/100mL (range <10-25,000); at 22 hours it decreased to 12 (range <10-2,906), with 100% of samples >1 and 23% >10 CFU/100mL.

Across the 10 weekly samples in non-treated jerricans, all samples were >1 and >10 CFU/100mL (Figure 6). At 1 hour after spiking in 5 NTU non-treated jerricans, the geomean *E. coli* concentration was $1.7 \bullet 10^5$ CFU/100mL (range across cleaning methods $1.1 \bullet 10^5$-$2.7 \bullet 10^5$), at 22 hours it increased to $1.2 \bullet 10^6$ CFU/100mL (range $7.2 \bullet 10^5$-$2.1 \bullet 10^6$). In 50 NTU non-treated jerricans, the geomean *E. coli* concentration was $3.1 \bullet 10^5$ CFU/100mL (range $2.1 \bullet 10^5$-$3.9 \bullet 10^5$) 1 hour after spiking, at 22 hours it increased to $1.2 \bullet 10^7$ CFU/100mL (range $2.8 \bullet 10^6$-$3.0 \bullet 10^7$) (Table 1).

Please note 14/480 samples (2.9%) were above detection limit (10/14 in the first two sampling weeks) and in Week 5 there was a potential error with disinfection residue remaining on challenge water preparation buckets and/or mixing paddles after sterilization that led to low *E. coli* spiking concentrations.

353 At study end, non-treated jerricans experienced a significant increase in *E. coli* concentration
354 from 1 to 22 hours ($p<0.001$) and treated jerricans experienced a significant decrease ($p<0.001$).
355 There were no significant differences in *E. coli* concentration at the two time points when
356 stratified by turbidity (5 NTU $p=0.23$; 50 NTU $p=0.62$). Final *E. coli* concentration (22-hour
357 measurement at 10-weeks) was significantly higher in non-treated jerricans ($p<0.001$) and did
358 not differ significantly by turbidity ($p=0.31$).

359 **3.2.4 Daily-to-Weekly Free Chlorine Residual Results**

360 In total, 8,132 FCR measurements were conducted. Please note, results from study days 1-9
361 (1,296 samples) were discarded as some non-Lamotte DPD-1 tablets were accidentally used
362 during this time.

363 In non-treated jerricans across all three time points from day 10-64, average FCR was 0.03 mg/L
364 (min=0.00, max=0.14) (Figure 7). Five of 900 averaged samples (0.005%) were ≥0.10 mg/L.
365 Thus, non-treated samples, including samples from cleaning methods that included chlorine
366 (chlorine, chlorine/rocks, chlorine/sand), did not have FCR in jerrican water on any study day.

367 In 5 NTU treated jerricans (Table 2) 1 hour after spiking, average FCR declined from 1.16 mg/L
368 to 0.80 to 0.59 over the three study phases; 149/150 (99%) samples met recommended minimum
369 FCR criteria of ≥0.2 mg/L [36, 43]. At 4 hours after spiking, average FCR declined from 0.83 mg/L
370 to 0.34 over three study phases; 131/150 (87%) samples met criteria. At 22 hours after spiking,
371 average FCR declined from 0.34 mg/L to 0.17 to 0.04 over three study phases and was
372 significantly lower on the last day of the study compared to the first ($p=0.01$); 89/150 (59%) met
373 criteria.

In 50 NTU treated jerricans (Table 2), average FCR declined from 0.38 mg/L to 0.07 over the three study phases; 49/150 (33%) samples met criteria of ≥0.2 mg/L, all in the first phase. At 4 and 22 hours after spiking, all averages were at or below realistic detection limit of 0.10 mg/L; 10/150 (7.0%) samples at 4 hours and 2/150 (1.3%) samples at 22 hours met criteria of ≥0.2 mg/L. FCR at 22 hours was significantly lower on the last day of the study compared to the first (p=0.03).

3.3 Follow-on

Jerricans were collected from four different contexts (Mbuji-Mayi, Democratic Republic of Congo (5 jerricans); Cox's Bazar, Bangladesh (4); Port-au-Prince, Haiti (5); and, Goma, Democratic Republic of Congo (5)). All households (100%) reported cleaning their jerricans; most reported cleaning daily (57%), or 1-2 times per week (31%). Households reported cleaning jerricans with water with soap (52%), or water and an abrasive (42%). Within each context, households all obtained jerricans at similar times (either all containers were obtained before or after emergency onset) and water sources were similar (e.g. all households collected river water in Mbuji-Mayi; open well in Cox's Bazar).

Biofilms were grown on 114 coupons cut from the 19 HDPE field-collected jerricans. The *E. coli* concentration in biofilms grown on side coupons varied from $2.49 \bullet 10^6$ CFU/cm^2 (Goma) to $1.48 \bullet 10^8$ (Mbuji-Mayi) and on bottom coupons from $2.00 \bullet 10^6$ CFU/cm^2 (Goma) to $4.18 \bullet 10^8$ (Port-au-Prince). No statistically significant differences were identified between *E. coli* concentration in biofilms grown on the side and bottom of the same container (paired t-test, p=0.41), or between surface *E. coli* from containers cleaned with and without abrasive agents

(p=0.57). *E. coli* differed significantly by country of origin (p=0.01), with jerricans from Port-au-Prince having significantly more *E. coli* than from Goma and Cox's Bazar.

The thickness of biofilms grown on surfaces were 13.7-65.3 μm (Port-au-Prince), 1.0-69.5 μm (Mbuji-Mayi), 4.5-35.8μm (Goma), and 2.5-46.6 μm (Cox's Bazar). No trends were observed in biofilm thickness by location or by country.

RMS surface roughness varied on side coupons from 1.76 μm (Port-au-Prince) to 9.54 μm (Cox's Bazar), and on bottom coupons from 1.52-11.48 μm (both Mbuji-Mayi). The jerrican averaged RMS surface roughness varied from 1.92 μm (Port-au-Prince) to 10.51 μm (Mbuji-Mayi). The RMS surface roughness of bottom coupons was significantly greater than that of side coupons (paired t-test, p=0.04). Side coupons did not differ significantly by household reported use of an abrasive agent (p=0.13), and bottom coupon roughness was greater in jerricans in which abrasive agents were reported (p=0.01). Side coupon roughness did not differ by country of origin (ANOVA, p=0.22), and bottom coupon roughness did differ significantly (p=0.02) with samples from Mbuji-Mayi significantly rougher than Cox's Bazar.

Overall, field-collected jerricans differed across countries in terms of surface roughness and *E. coli* concentration. Additionally, there were differences between side and bottom samples, and data indicates abrasive cleaning methods may increase the bottom surface roughness of real-world jerricans.

4 DISCUSSION

In this large exploratory laboratory study, we adapted and developed methods to grow and test *E. coli* biofilms in jerricans to further understanding of contamination risk in unsafe water storage. We found: 1) biofilms grew on jerricans rapidly; 2) biofilm growth and aqueous *E. coli* concentration were inhibited by chlorine treatment, regardless of turbidity; 3) over the study time, chlorine demand increased and FCR decreased; 4) there were qualitative indications that, in particular, abrasive cleaning methods reduced biofilm thickness and increased jerrican surface roughness; and, 5) in field jerricans, when abrasive cleaning was reported, bottom surface roughness increased.

We found biofilms grew on jerrican surfaces when exposed to *E. coli* spiked water within days, as shown in pre-testing results, end-of-phase surface *E. coli,* and imaging results. These are consistent with existing research on biofilm development in drinking water systems with chlorine residual [27, 44], and confirm biofilm growth is a concern in jericans currently distributed and used in LMIC and humanitarian contexts. Please note we used a high *E. coli*-only spike concentration, which is both a worst-case (high concentration) and conservative (single organism) biofilm growth scenario (String et al. 2020).

As hypothesized, regular water treatment with chlorine significantly inhibited *E. coli* biofilm growth and reduced geomean aqueous *E. coli* concentrations over 10 weeks in 5 NTU stored water at 22 hours from $1.2 \bullet 10^6$ CFU/100mL in non-treated jerricans to <1 CFU/100mL in treated jerricans. However, over 10-weeks, there was consistent and statistically significant FCR decline, to the point where only chlorine treatment in 5 NTU waters maintained FCR ≥0.2 mg/L at 22 hours. Although not directly linked, attributing FCR decay to biofilm growth is consistent

436 with previous research [45]. Other possible explanations for increased chlorine demand include
437 sediment accumulation and increased surface area from scratches. Furthermore, this result
438 potentially explains an inconsistency in household chlorination literature, where some studies
439 have found consistent FCR and other studies have noted no FCR and slight *E. coli* growth in
440 stored water after treatment [46-48]. These results emphasize the importance for practitioners and
441 researchers to monitor the effectiveness of chlorine treatment, as it may change with container
442 condition over time. That is also consistent with previous literature, which has documented, even
443 in absence of FCR, little *E. coli* in chlorine treated household stored water [49]. These results
444 highlight the benefits of filtering water before chlorination, reducing the number of
445 microorganisms available to form biofilms. We note, consistent with previous studies, no water
446 safety benefit was observed by cleaning jerricans with chlorine in the absence of water treatment
447 [6].

448 There were qualitative indications of differences within treatment and cleaning methods:
449 biofilms were not observed in low turbidity water treated with chlorine and chlorine/rocks
450 reduced biofilm thickness while increasing jerrican surface roughness. While these results are
451 not statistically significant due to low sample size, they are consistent across study outcomes,
452 including: 1) in treated jerricans, no sample cleaned with rocks or chlorine/rocks had enumerable
453 surface *E. coli*, however in non-treated jerricans, samples cleaned with rocks or chlorine/rocks
454 had the highest surface *E. coli* (which could be attributed to *E. coli* growing in scratches as seen
455 in Figure 2); 2) in treated jerricans, no biofilms were detected in images in 5/6 surfaces cleaned
456 with chlorine and 4/6 surfaces cleaned with chlorine/rocks; and, 3) in non-treated jerricans,
457 chlorine/rocks had the lowest number of surfaces with visible biofilm (4/6 surfaces had only a
458 “few cells, no structure”). Additionally, surface roughness was highest in rocks and

chlorine/rocks cleaning methods; and, bottom coupon roughness was statistically greater in field jerricans where abrasives were reported used for cleaning. Overall, these results indicate chlorine and chlorine/rocks are potentially more efficacious than other cleaning methods tested in this study. However, rock cleaning methods can increase surface roughness, which can present opportunities for microorganism attachment to the surface and biofilm growth [50].

Although surface *E. coli* did not vary significantly between bottom and side surfaces on field-collected jerricans, surface roughness did vary by location. As surface roughness can impact biofilm growth, it is important for researchers and practitioners to consider collecting microbiological samples from various surfaces within the same container when collecting household stored water samples to ensure contamination is not under, or over, estimated.

The limitations to our work include: 1) biofilms in the full study were grown in jerricans storing water with *E. coli* concentrations of 10^5 CFU/100mL, which is very high risk water[36]; 2) biofilms in the follow-on study were grown by placing coupons in culture, which forms a denser biofilm faster than might be seen in field circumstances [37]; 3) biofilms were *E. coli*-only biofilms, in a real-world setting biofilms would contain mixtures of organisms; 4) the 50 NTU turbidity might have been too high, especially because large volumes of settled sediment impacted some of the imaging; 5) we did not stain for live as compared to dead *E. coli* cells in imaging, which would have provided a better indication of recontamination potential of cells being released from the biofilm, and we recommend future studies stain for viable/non-viable cells; 6) we did not test the chlorine cleaning solution concentration daily in the full-scale study; and, 7) we had limited variability in surface roughness in field-collected jerricans. While we acknowledge these limitations, we highlight that many of these limitations bias the study towards conservative results.

Based on our results, we emphasize that biofilm formation on jerrican surfaces in contact with contaminated water does not appear to be completely preventable. Since biofilms can function as a reservoir for pathogens [27], water storage containers must be considered as a contamination pathway. We recommend several actions to inhibit biofilm growth in jerricans used for safe water storage: regular chlorination of water, noting that chlorination also has other water quality and health benefits [16], biofilm growth does occur even with daily chlorination (as seen in control samples), and some biofilms are resistant to chlorination [51, 52]; regular cleaning of interior jerrican surfaces (as all cleaning methods were better than control); replacement of severely scratched or damaged jerricans, and the possible development of coatings for jerrican surfaces to prevent biofilm development. Unfortunately, our results do not lead to a simple, universal recommendation for jerrican cleaning, particularly as cleaning material availability and acceptance is highly context dependent. However, our results highlight cleaning is necessary, and suggest, of the methods tested herein, 5% chlorine and 5% chlorine/clean rocks inhibited biofilm growth better than other tested methods. We note that these would not work against protozoal biofilms, the rocks used in this study were sterilized and rocks in the field will be contaminated, and over time abrasives degraded jerrican surfaces. Therefore, there is an unknown balance between using rocks to remove biofilms, and not damaging the jerrican surface which can promote biofilm growth.

Overall, our results stress the difficulty of cleaning, which is not surprising to those in the water utility sector trying to remove biofilms from pipelines [53-55], but may be surprising to many in the WASH in humanitarian response sector, where household cleaning of water storage containers is often recommended to reduce risk [4]. We recommend further laboratory based efficacy research: 1) the inhibition of biofilm development using other disinfectants and dosages; 2) the impact of

different cleaning methods on biofilms grown in lower turbidity water (1 NTU) and water of varied organic content; 3) the prevention of biofilm development with different cleaning frequencies; 4) the impact of disinfectant cleaning methods against biofilms comprised of varied microorganisms (including protozoa); 5) the impact of different water storage container materials on biofilm growth and cleaning methods; and, 6) the impact of other, locally-appropriate cleaning methods such as boiling water, vinegar and the use of a scrub brush (String et al. 2020). Additionally, we recommend researching alternative methods to prevent/inhibit biofilm growth in jerricans, such as the addition of antimicrobial additives to jerrican surfaces or the replacement of jerricans on a regular basis. Furthermore, a targeted longitudinal study varying only cleaning frequency is needed to clarify these results. We note the methods herein could be applied to researching cleaning other household water storage containers, such as 1,000 L rooftop tanks. Moreover, we recommend further field research on actual cleaning methods practiced by households, the acceptability of cleaning methods, and the surface roughness of local jerricans. Lastly, we highlight this work was about prevention/inhibition of biofilm growth in jerricans, and further work on how to effectively clean already-established biofilms from jerricans is recommended.

5 CONCLUSIONS

The benefits of this complex study design were it allowed testing of multiple variables and hypotheses to answer field-relevant questions, and provided a basis to define a future research agenda on safe water storage. We found biofilms will develop on water storage container surfaces, cleaning is complex and nuanced, and field relevant recommendations for inhibiting the development of biofilms are needed. We hope this study is a platform from which future

technical research on biofilm formation in water storage containers used in LMIC and humanitarian response can be investigated to ensure safely managed drinking water for all.

ACKNOWLEDGEMENTS

We thank the Office of US Foreign Disaster Assistance for funding, Medentech for donating Aquatabs, and Emily Decker, Kelly Donohue, Sejal Dua, John Fraser, Himamshu Ghimire, Nabila Khandakar, Molly Lie, Tharina Messeroux, Magnifique Mukundwa, Katie Painter, Faith Patrick, Tom Shimkus, Derrick Sosa, Katherine Sweetser, and Jolie Wasserman for laboratory, data entry, and data analysis assistance. We thank Camille Heylen for laboratory training, Alenka Lovy and the microscopy facilities at the Imaging Core at the Tufts Center for Neuroscience Research, sponsored by P30 NS047243 (Jackson), Brandon Stafford at Tufts University NOLOP/FAST facility for follow-on study coupon cutting, Jim Vlahakis at Tufts University Micro and Nano Fabrication Facility for training and profilometer access.

FUNDING

This work was funded by the USAID Office of US Foreign Disaster Assistance

humanitarian response can be investigated to ensure safely managed drinking water for all.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

DL developed the project idea, obtained funding, reviewed all protocols, wrote drafts of the manuscripts, and supervised the project. GS developed or reviewed all protocols, completed or

supervised all laboratory work, analyzed and wrote up data, and wrote and edited drafts of the manuscript. MD led data collection on the full-scale jerrican project for four months, analyzed full-scale data, and completed literature review and writing. HBadr assisted with data collection on the full-scale jerrican project and completed literature review. HB, YK, TT, and MJ developed follow-up study protocol, completed follow-up laboratory work and analysis, and wrote up follow-up data. TNV drafted the protocol for, and led data collection on, the full-scale jerrican study for one month. AO assisted with image analysis. MW completed statistical analysis, assisted with data collection, and contributed to framing and writing the manuscript. All authors reviewed and approved the final manuscript.

REFERENCES

1. W. U. JMP, *Progress on household drinking water, sanitation, and hygiene 2000-2017: Special focus on inequalities*, Joint Monitoring Programme, New York, 2019.

2. F. G. B. Goddard, A. J. Pickering, A. Ercumen, J. Brown, H. H. Chang and T. Clasen, Faecal contamination of the environment and child health: a systematic review and individual participant data meta-analysis, *The Lancet Planetary Health*, 2020, **4**, e405-e415.

3. J. Watson, M. Gayer and M. Connolly, Epidemics after Natural Disasters, *Emerging Infectious Diseases*, 2007, **13**, 1-5.

4. SPHERE, *Humanitarian Charter and Minimum Standards in Disaster Response, 2nd Edition*, The Sphere Project, Geneva, Switzerland, 2011.

5. M. Toole, Vulnerability in emergency situations, *Lancet*, 1996, **348**, 840.

6. T. Yates, J. Vujcic, M. Leandre Joseph, K. Gallandat and D. Lantagne, Water, sanitation, and hygiene interventions in outbreak response: a synthesis of evidence, *Waterlines*, 2018, **38**, 5-30.

7. T. Yates, J. Vujcic, M. Leandre Joseph, K. Gallandat and D. Lantagne, Efficacy and effectiveness of water, sanitaiton, and hygiene interventions in emergencies in low- and middle-income countries: a systematic review, *Waterlines*, 2018, **37**.

8. M. Wolfe, M. Kaur, T. Yates, M. Woodin and D. Lantagne, A Systematic Review and Meta-Analysis of the Association between Water, Sanitation, and Hygiene Exposures and Cholera in Case-Control Studies, *Am J Trop Med Hyg*, 2018, **99**, 534-545.

9. K. Heitzinger, C. A. Rocha, R. E. Quick, S. M. Montano, D. H. Tilley, Jr., C. N. Mock, A. J. Carrasco, R. M. Cabrera and S. E. Hawes, "Improved" But Not Necessarily Safe: An Assessment of Fecal Contamination of Household Drinking Water in Rural Peru, *Am J Trop Med Hyg*, 2015, **93**, 501-508.

10. S. Doocy and G. Burnham, Point-of-use water treatment and diarrhoea reduction in the emergency context: an effectiveness trial in Liberia, *Trop Med Int Health*, 2006, **11**, 1542-1552.

11. L. Roberts, Y. Chartier, O. Chartier, G. Malenga, M. Toole and H. Rodka, Keeping clean water clean in a Malawi refugee camp: a randomized intervention trial, *Bull World Health Organ*, 2001, **79**, 280-287.

12. J. Rayner, A. Murray, M. Joseph, A. J. Branz and D. Lantagne, Evaluation of household drinking water filter distributions in Haiti., *Journal of Water, Sanitation and Hygiene for Development*, 2016, **6**, 42-54.

13. J. Kohlitz, T. Hasan, K. Khatri, A. Sokota, S. Iddings, U. Bera and R. Psutka, Assessing reported use and microbiological performance of a point-of-use household water filter in rural Fiji. , *Journal of Water, Sanitation and Hygiene for Development*, 2013, **3**, 207-215.

14. A. Murray, B. Stewart, C. Hopper, E. Tobin, R. J, H. Mut-Tracy, B. Stewart, C. Stewart, C. Tobin, M. Goeb, C. Mueb and D. Lantagne, Laboratory efficacy and field effectiveness of hollow fiber membrane microfilters used for household water treatment in Honduras, *Journal of Water, Sanitation and Hygiene for Development*, 2017, **7**, 74-84.

15. J. Wright, S. Gundry and R. Conroy, Household drinking water in developing countries: a systematic review of microbiological contamination between source and point-of-use, *Trop Med Int Health*, 2004, **9**, 106-117.

16. A. Branz, M. Levine, L. Lehmann, A. Bastable, S. Ali, K. Kadir, T. Yates, D. Bloom and D. Lantagne, Chlorination of Drinking Water in Emergencies: A Review of Knowledge, Recommendations for Implementation, and Research Needed, *Waterlines*, 2017, **36**, 4-39.

17. Medentech, Aquatabs: The world's most trusted water purification tablet, http://www.aquatabs.com/index.php?id=774, (accessed May 2, 2019).

18. CDC, Effect of Chlorination on Inactivating Selected Pathogen, https://www.cdc.gov/safewater/effectiveness-on-pathogens.html, (accessed August 8, 2019).

19. T. Yates, J. Vujcic, M. Leandre Joseph, K. Gallandat and D. Lantagne, Efficacy and effectiveness of water, sanitaiton, and hygiene interventions in emergencies in low- and middle-income countries: a systematic review, *Waterlines*, 2018, **37**.

20. E. D. Mintz, F. M. Reiff and R. V. Tauxe, Safe water treatment and storage in the home. A practical new strategy to prevent waterborne disease, *JAMA*, 1995, **273**, 948-953.

21. D. Lantagne, R. E. Quick and E. Mintz, *Household water treatment and safe storage options in developing countries: a review of current implementation practices*, Woodrow Wilson International Center for Scholars, Environmental Change and Security Program, 2006.

22. CDC, *Safe Water for the Community: A Guide for Establishing a Community-based Safe Water System Program*, Centers for Disease Control and Prevention, Atlanta, GA, USA, 2008.

23. J. Wolf, A. Prüss-Ustün, O. Cumming, J. Bartram, S. Bonjour, S. Cairncross, T. Clasen, J. M. Colford, Jr., V. Curtis, J. De France, L. Fewtrell, M. C. Freeman, B. Gordon, P. R. Hunter, A. Jeandron, R. B. Johnston, D. Mäusezahl, C. Mathers, M. Neira and J. P. Higgins, Assessing the impact of drinking water and sanitation on diarrhoeal disease in low- and middle-income settings: systematic review and meta-regression, *Trop Med Int Health*, 2014, **19**, 928-942.

24. P. K. Jensen, J. H. Ensink, G. Jayasinghe, W. van der Hoek, S. Cairncross and A. Dalsgaard, Domestic transmission routes of pathogens: the problem of in-house contamination of drinking water during storage in developing countries, *Trop Med Int Health*, 2002, **7**, 604-609.

25. V. M. Walden, E. A. Lamond and S. A. Field, Container contamination as a possible source of a diarrhoea outbreak in Abou Shouk camp, Darfur province, Sudan, *Disasters*, 2005, **29**, 213-221.

26. A. Steele, B. Clarke and O. Watkins, Impact of jerry can disinfection in a camp environment - experiences in an IDP camp in Northern Uganda, *J Water Health*, 2008, **6**, 559-564.

27. J. Wingender and H. C. Flemming, Biofilms in drinking water and their role as reservoir for pathogens, *Int J Hyg Environ Health*, 2011, **214**, 417-423.

28. P. Jagals, C. Jagals and T. C. Bokako, The effect of container-biofilm on the microbiological quality of water used from plastic household containers, *J Water Health*, 2003, **1**, 101-108.

29. V. M. Ntema, N. Potgieter and T. G. Barnard, Detection of Vibrio cholerae and Vibrio parahaemolyticus by molecular and culture based methods from source water to household container-stored water at the point-of-use in South African rural communities, *Water Sci Technol*, 2010, **61**, 3091-3101.

30. J. E. Mellor, J. A. Smith, A. Samie and R. A. Dillingham, Coliform Sources and Mechanisms for Regrowth in Household Drinking Water in Limpopo, South Africa, *J Environ Eng (New York)*, 2013, **139**, 1152-1161.

31. S. Bae, C. Lyons and N. Onstad, A culture-dependent and metagenomic approach of household drinking water from the source to point of use in a developing country, *Water Res X*, 2019, **2**, 100026.

32. J. E. Mellor, J. A. Smith, G. P. Learmonth, V. O. Netshandama and R. A. Dillingham, Modeling the complexities of water, hygiene, and health in Limpopo Province, South Africa, *Environ Sci Technol*, 2012, **46**, 13512-13520.

33. P. Budeli, R. C. Moropeng, L. Mpenyana-Monyatsi and M. N. B. Momba, Inhibition of biofilm formation on the surface of water storage containers using biosand zeolite silver-impregnated clay granular and silver impregnated porous pot filtration systems, *PLoS One*, 2018, **13**, e0194715.

34. WHO UNICEF JMP, *Progress on household drinking water, sanitation and hygiene I 2000-2017: Special Focus on Inequalities*, Joint Monitoring Programme 2019.

35. L. C. Skillman, I. W. Sutherland, M. V. Jones and A. Goulsbra, Green fluorescent protein as a novel species-specific marker in enteric dual-species biofilms, *Microbiology*, 1998, **144**, 2095-2101.

36. WHO, Guidelines for Drinking-water Quality: fourth edition incorporating the first addendum.*Journal*, 2017.

37. J. Azeredo, N. F. Azevedo, R. Briandet, N. Cerca, T. Coenye, A. R. Costa, M. Desvaux, G. Di Bonaventura, M. Hebraud, Z. Jaglic, M. Kacaniova, S. Knochel, A. Lourenco, F. Mergulhao, R. L. Meyer, G. Nychas, M. Simoes, O. Tresse and C. Sternberg, Critical review on biofilm methods, *Crit Rev Microbiol*, 2017, **43**, 313-351.

38. T. A. Branck, M. J. Hurley, G. N. Prata, C. A. Crivello and P. J. Marek, Efficacy of a Sonicating Swab for Removal and Capture of Listeria monocytogenes in Biofilms on Stainless Steel, *Appl Environ Microbiol*, 2017, **83**.

39. C. Larimer, E. Winder, R. Jeters, M. Prowant, I. Nettleship, R. S. Addleman and G. T. Bonheyo, A method for rapid quantitative assessment of biofilms with biomolecular staining and image analysis, *Anal Bioanal Chem*, 2016, **408**, 999-1008.

40. APHA/AWWA/WEF, *Standard Methods for the Examination of Water and Wastewater, 20th Edition*, Washington, DC, 1998.

41. C. Hannig, M. Follo, E. Hellwig and A. Al-Ahmad, Visualization of adherent micro-organisms using different techniques, *J Med Microbiol*, 2010, **59**, 1-7.

42. A. Murray and D. Lantagne, Accuracy, precision, usability, and cost of free chlorine residual testing methods, *J Water Health*, 2015, **13**, 79-90.

43. WHO, *Guidelines for drinking-water quality, 4th Edition.*, World Health Organization, Geneva, Switzerland, 2011.

44. K. E. Fish and J. B. Boxall, Biofilm Microbiome (Re)Growth Dynamics in Drinking Water Distribution Systems Are Impacted by Chlorine Concentration, *Front Microbiol*, 2018, **9**, 2519.

45. J. Xu, C. Huang, X. Shi, S. Dong, B. Yuan and T. H. Nguyen, Role of drinking water biofilms on residual chlorine decay and trihalomethane formation: An experimental and modeling study, *Sci Total Environ*, 2018, **642**, 516-525.

46. D. Lantagne, Sodium hypochlorite dosage for household and emergency water treatment, *Journal of the American Water Works Association*, 2008, **100**, 106-119.

47. K. Levy, L. Anderson, K. A. Robb, W. Cevallos, G. Trueba and J. N. Eisenberg, Household effectiveness vs. laboratory efficacy of point-of-use chlorination, *Water Res*, 2014, **54**, 69-77.

48. N. Wilhelm, A. Kaufmann, E. Blanton and D. Lantagne, Sodium hypochlorite dosage for household and emergency water treatment: updated recommendations, *J Water Health*, 2018, **16**, 112-125.

49. C. Null and D. Lantagne, Microbiological quality of chlorinated water after storage in ceramic pots, *Journal of Water, Sanitation, and Hygiene for Development*, 2012, **2**, 250-253.

50. M. van Loosdrecht, D. Eikelboom, A. Gjaltema, A. Mulder, L. Tijhuis and J. Heijnen, Biofilm structures, *Water Sci Technol*, 1995, **32**, 35-43.

51. Z. Xue, V. R. Sendamangalam, C. L. Gruden and Y. Seo, Multiple roles of extracellular polymeric substances on resistance of biofilm and detached clusters, *Environ Sci Technol*, 2012, **46**, 13212-13219.

52. A. Bridier, R. Briandet, V. Thomas and F. Dubois-Brissonnet, Resistance of bacterial biofilms to disinfectants: a review, *Biofouling*, 2011, **27**, 1017-1032.

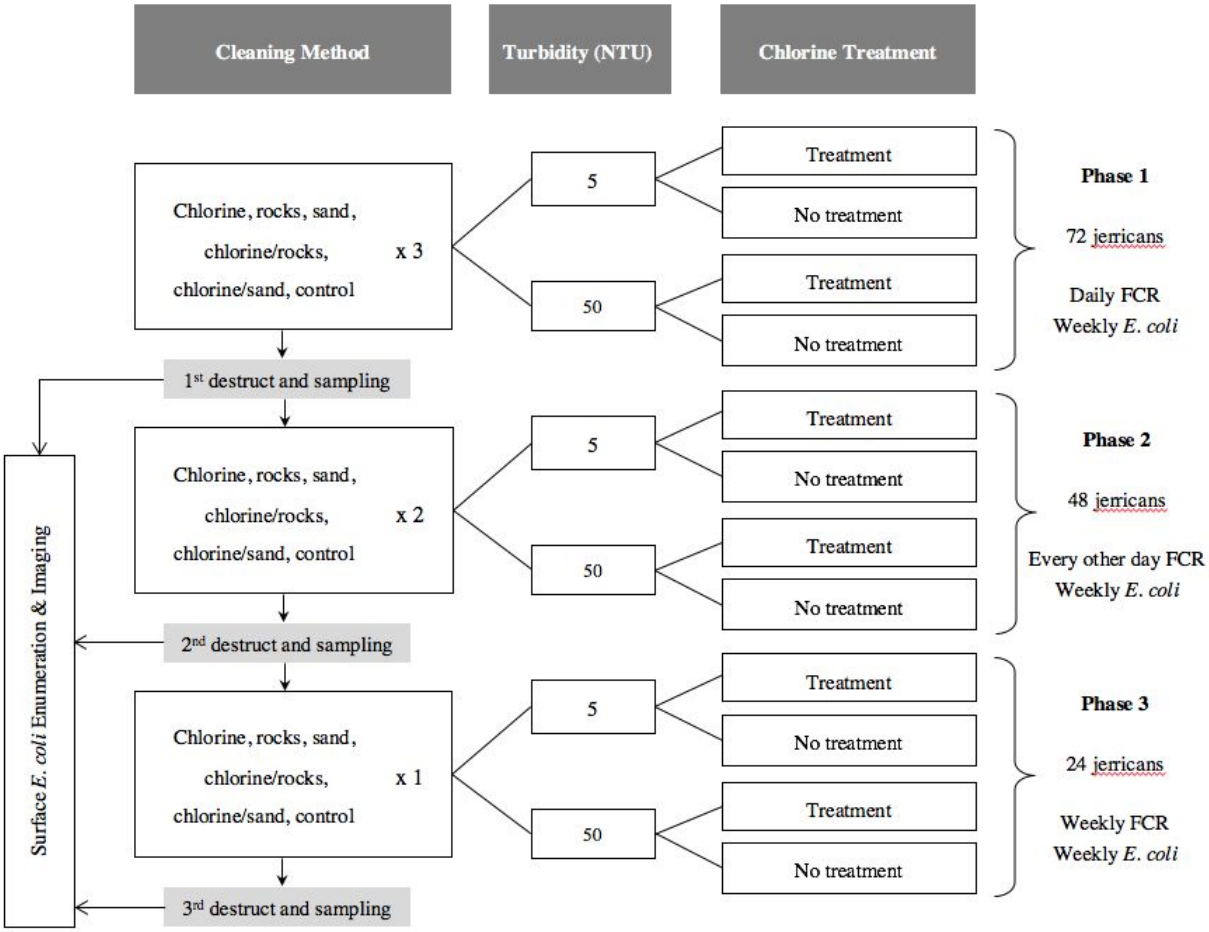
53. E. Kumpel and K. Nelson, Mechanisms Affecting Water Quality in an Intermittent Piped Water Supply, *Environmental Science & Technology* 2014, **48**, 2766-2775, DOI: 2710.1021/es405054u.

54. A. F. Barton, M. R. Wallis, J. E. Sargison, A. Buia and G. J. Walker, Hydraulic Roughness of Biofouled Pipes, Biofilm Character, and Measured Improvements from Cleaning, *Journal of Hydraulic Engineering*, 2008, **135**.

55. M. W. LeChevallier, C. D. Lowry and R. G. Lee, Disinfection Biofilms in a Model Distribution System, *Journal - American Water Works Association*, 1990, **82**, 87-99, doi:10.1002/j.1551-8833.1990.tb06996.x.

700 **FIGURES**

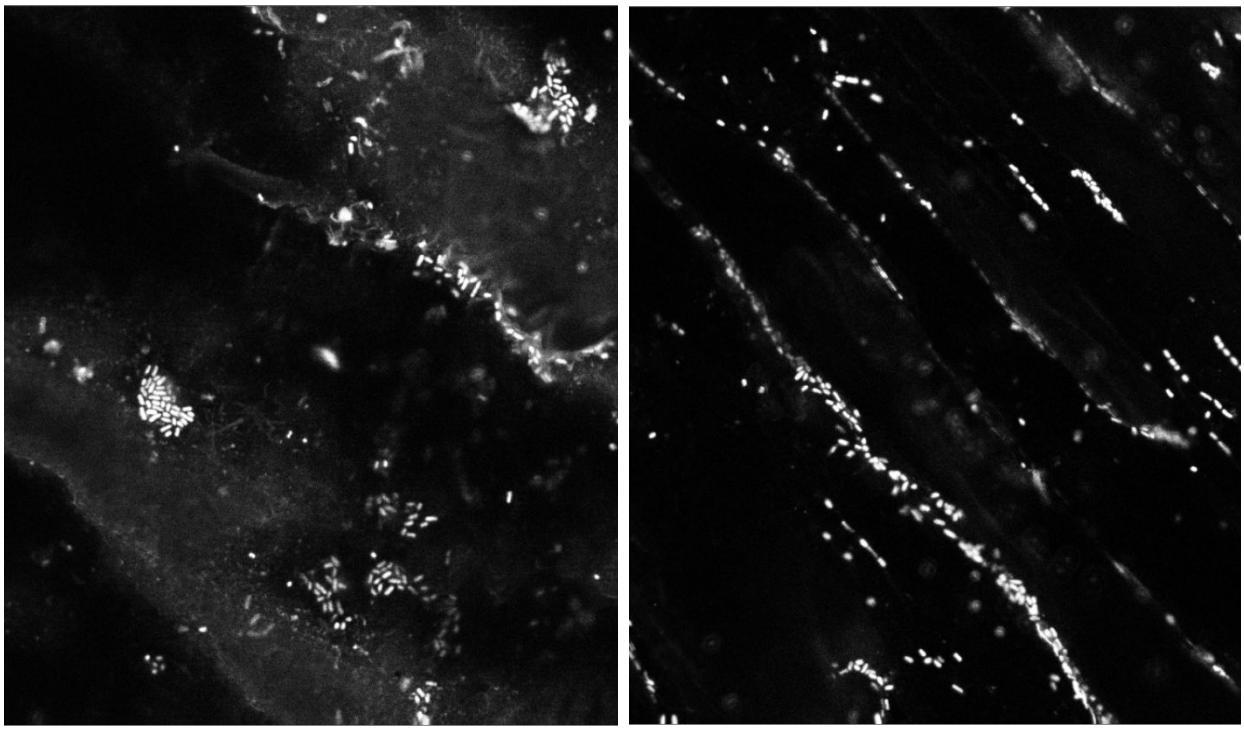
701 Figure 1. Full-scale study design.



702

703

704 Figure 2. Images of *E. coli* growth on coupons from pre-testing trials on (a) a virgin plastic
705 coupon surface and (b) a 120-grit scratched plastic coupon surface.
706



(a) (b)

707
708

Figure 3. Surface *E. coli* removed from coupons during destruction at the end-of-phases 1-3. Data stratified in plots by phase, coupon location, and cleaning method. Plots for each stored water condition: (a) 5 NTU treated; (b) 5 NTU non-treated; (c) 50 NTU treated; and, (d) 50 NTU non-treated.

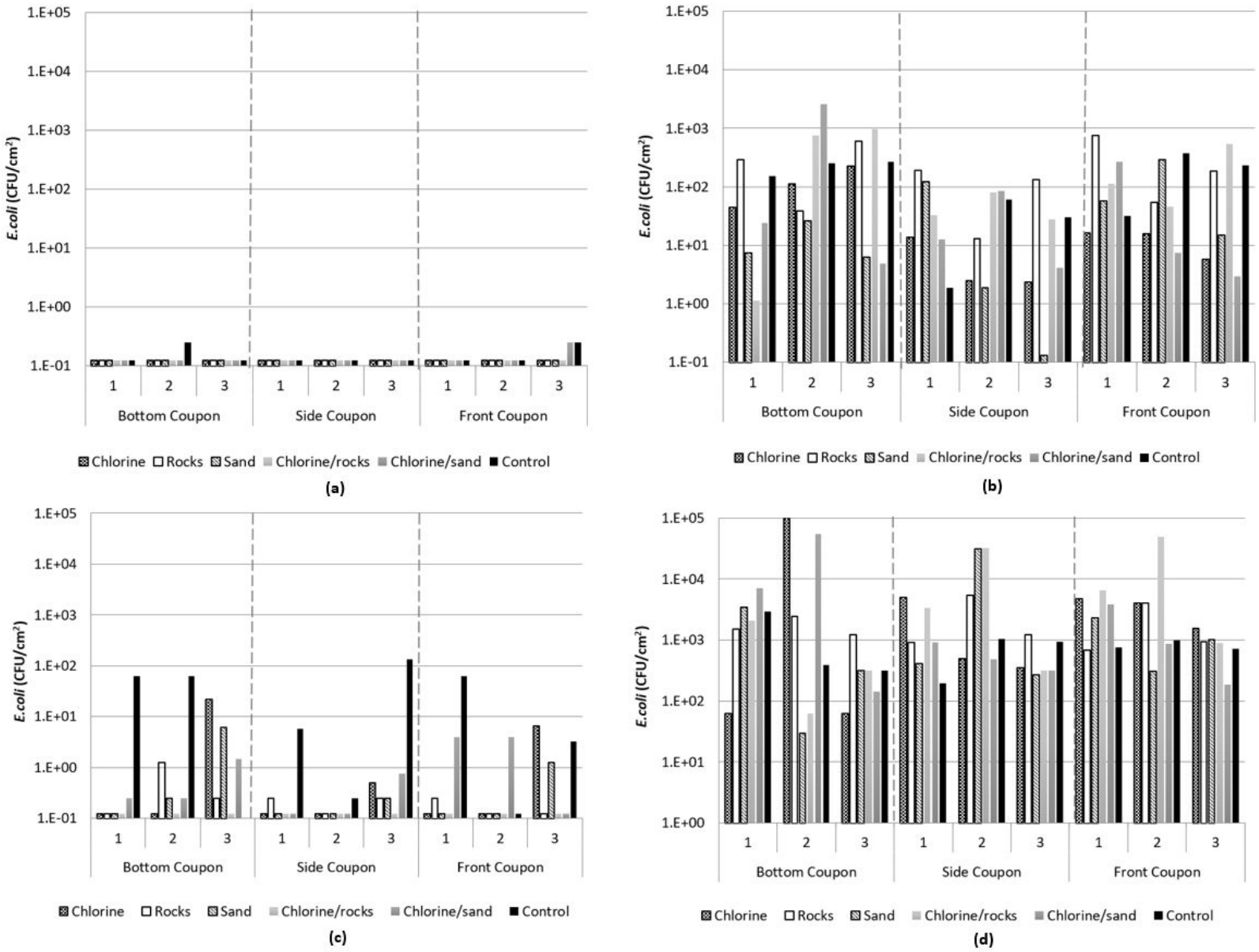


Figure 4. Geomean biofilm thickness on coupons at the end-of-study. Plots for each stored water condition: (a) 5 NTU treated; (b) 5 NTU non-treated; (c) 50 NTU treated; (d) 50 NTU non-treated; and, (e) qualitative imaging results.

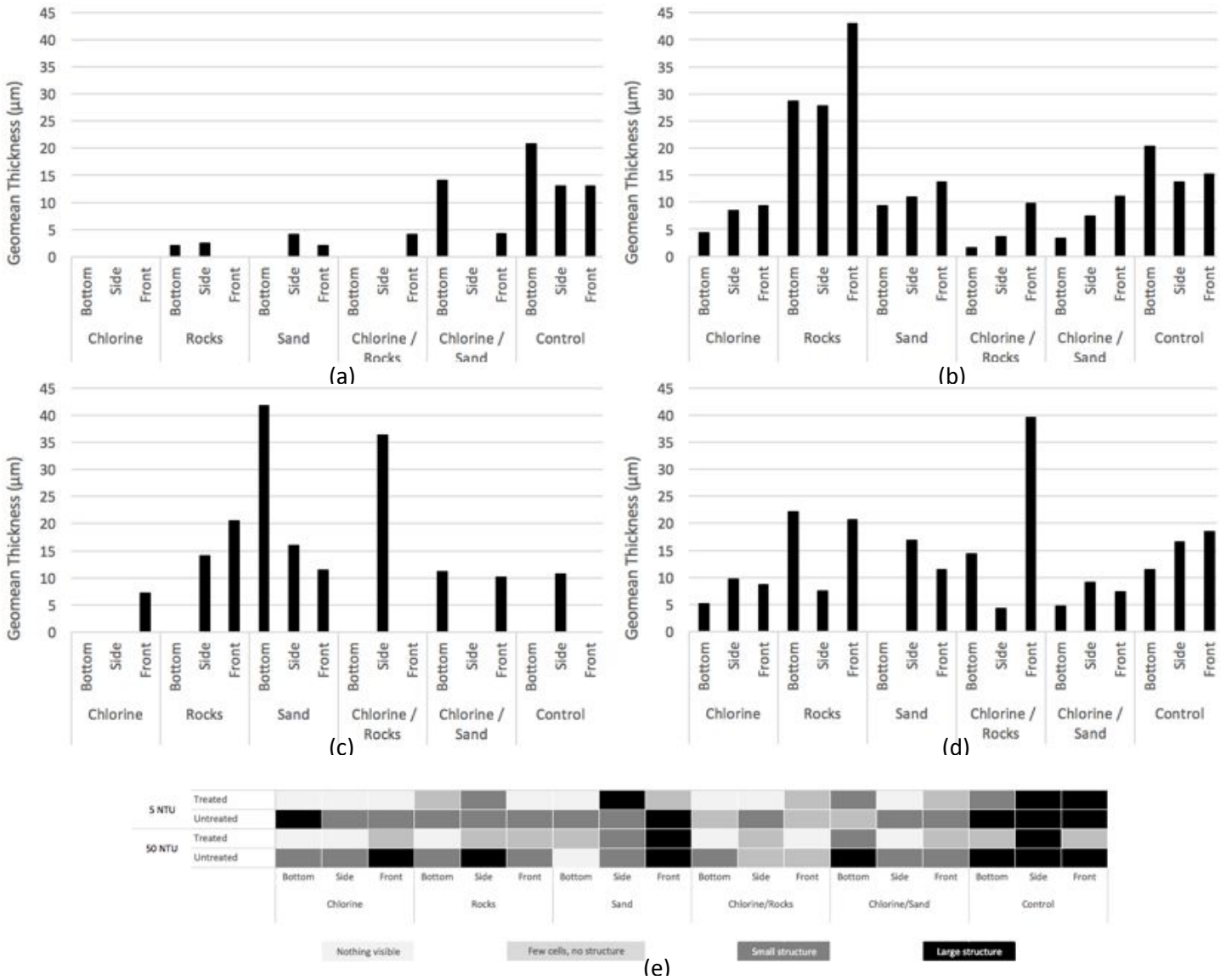
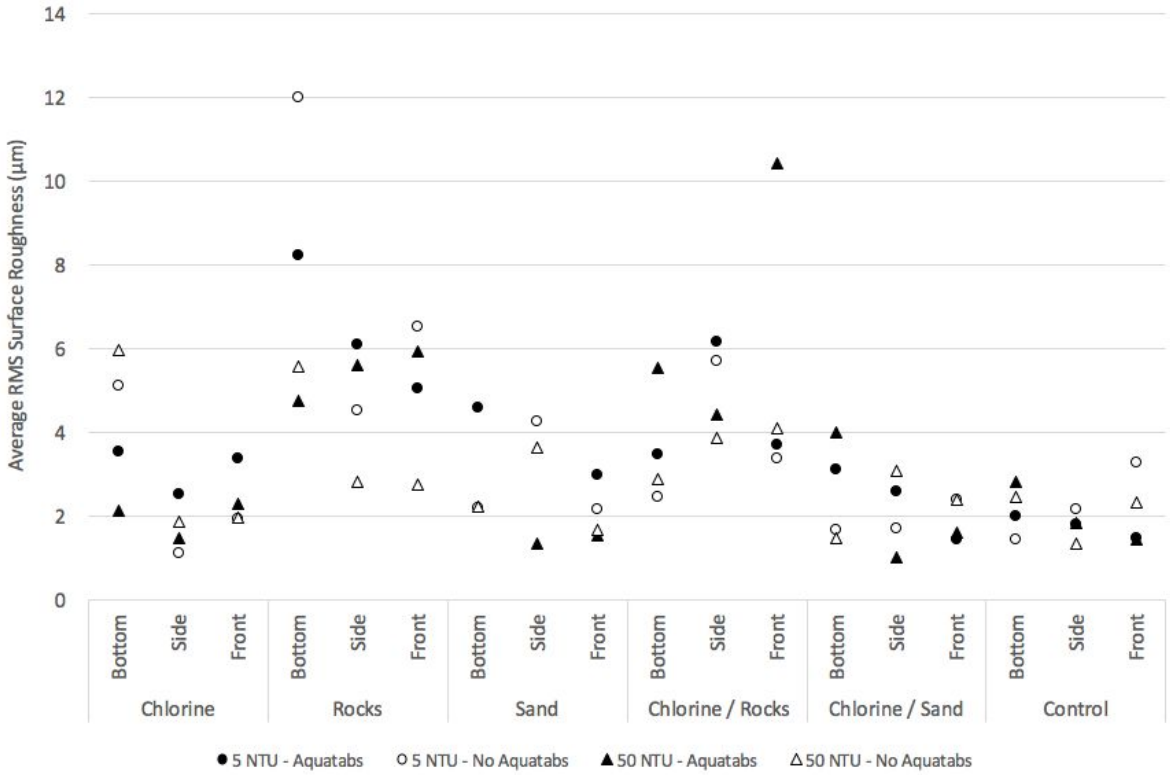


Figure 5. Average root mean squared surface roughness measured on coupons at end-of-study. Data stratified by cleaning method, coupon location, and stored water condition (turbidity and treatment).



763 Figure 6. Weekly aqueous *E. coli* (CFU/100mL) concentrations measured at 1 and 22 hours. Data stratified in plots by cleaning
764 method and presented for each water storage condition: (a) 5 NTU treated; (b) 5 NTU non-treated; (c) 50 NTU treated; and,
765 d) 50 NTU non-treated jerricans
766

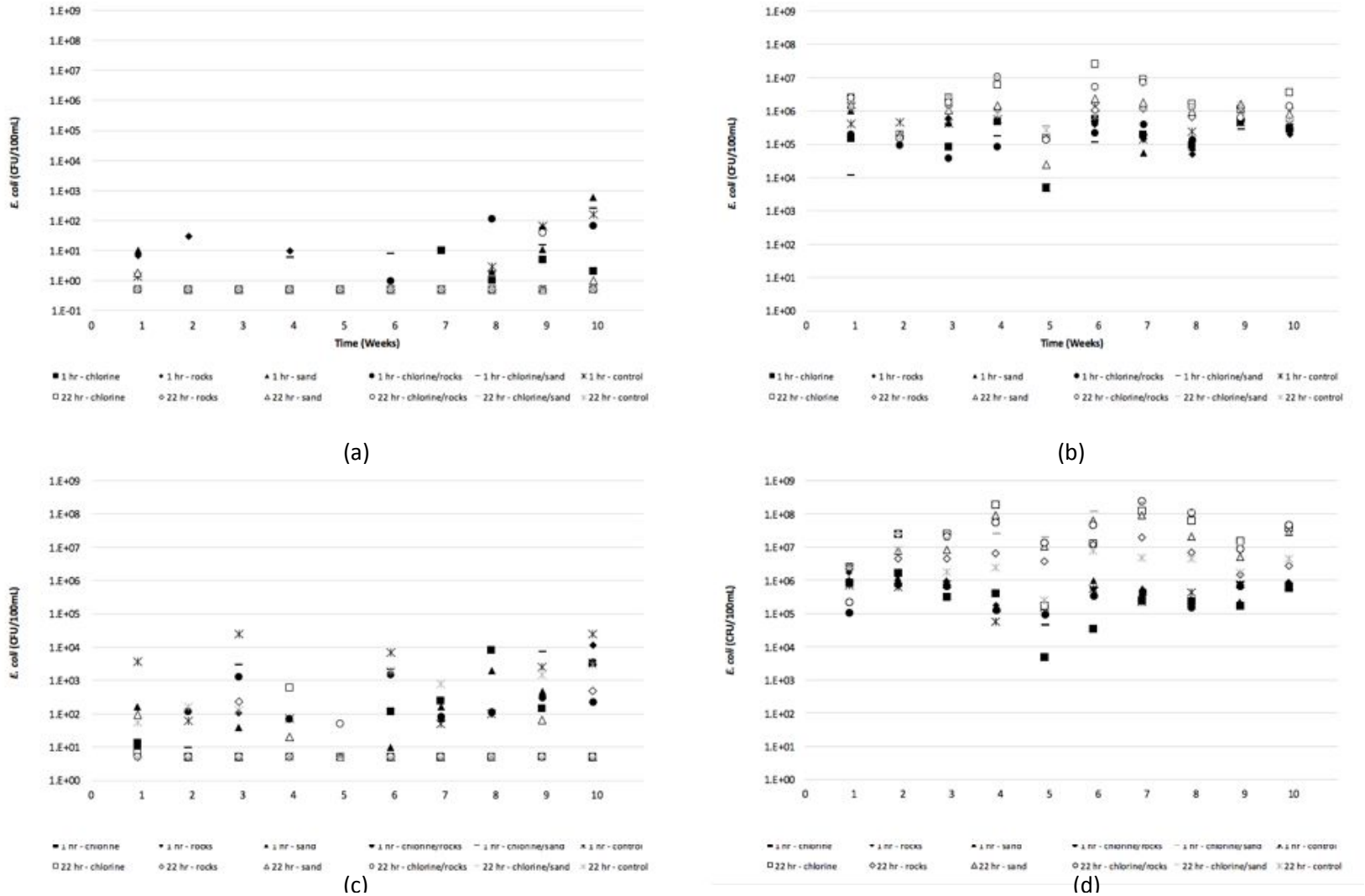


Figure 7. Free chlorine residual measured over all three study phases stratified by water treatment (AT=Aquatabs, N-AT=not treated), turbidity (5 or 50 NTU), and cleaning method. Measurements plotted here at a) 1 hour; b) 4 hours; and, c) 22 hours after treatment.

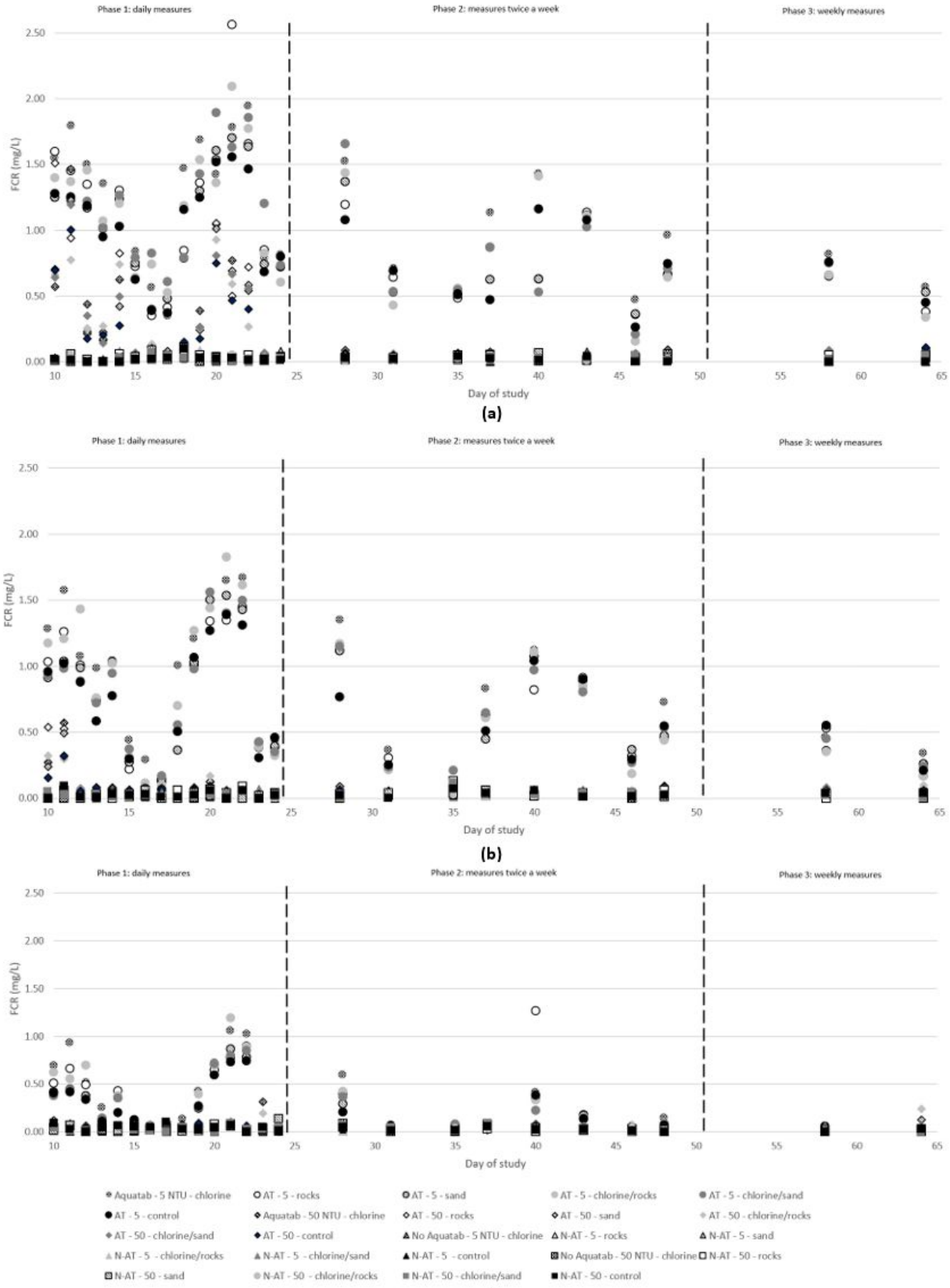
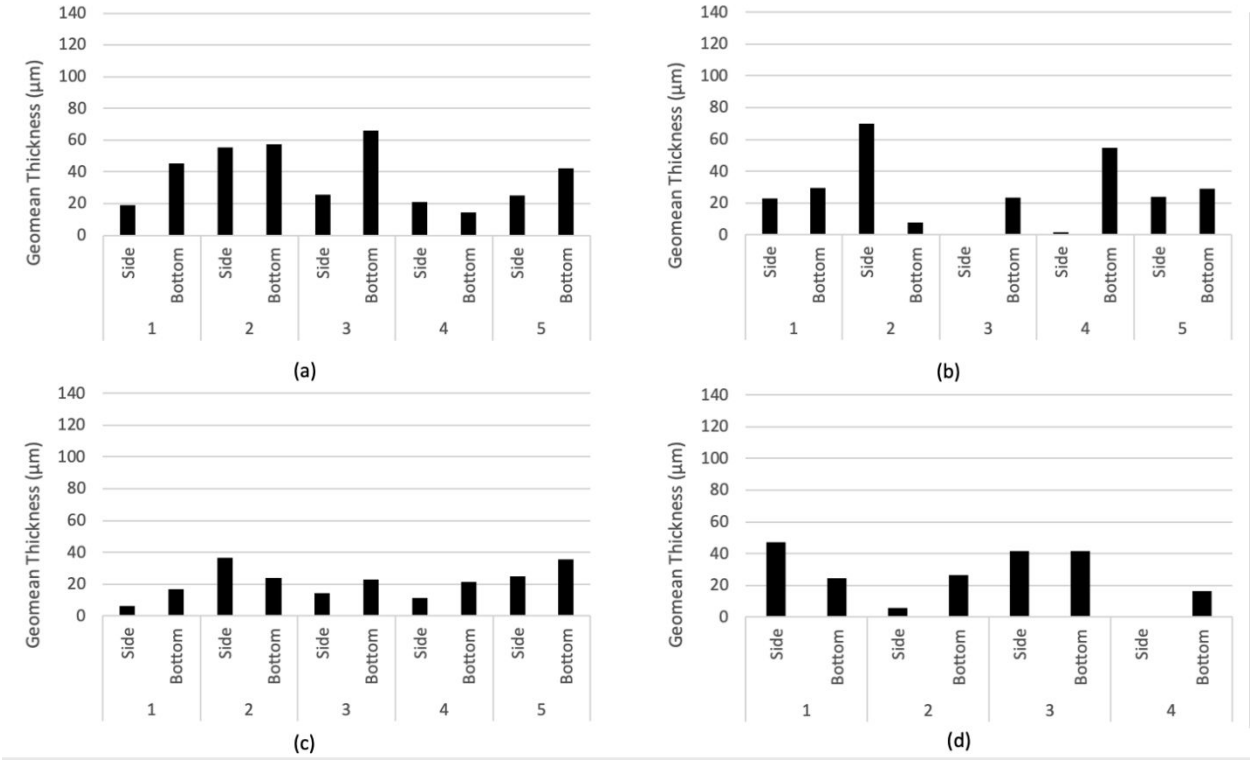


Figure 8. Geomean biofilm thickness grown on field collected jerrican coupons, measured at the end of the experiment. Data stratified by jerrican country of origin: (a) Port-au-Prince, Haiti (n=5); (b) Mbuji-Mayi, DRC (n=5); (c) Goma, DRC (n=5); (d) Cox's Bazar, Bangladesh (n=4).



781 Table 1: Weekly aqueous *E. coli* concentration (CFU/100mL) for jerricans. Stratified here by
782 treatment, turbidity, and cleaning method.
783

	Non-Treated Jerricans			
	5 NTU		**50 NTU**	
	1 hour E. coli (CFU/100 mL)		**1 hour E. coli (CFU/100 mL)**	
	Geomean	**% >1, % >10**	**Geomean**	**% >1, % >10**
Chlorine*	$1.5•10^5$	100, 100	$2.1•10^5$	100, 100
Rocks	$2.2•10^5$	100, 100	$3.2•10^5$	100, 100
Sand	$2.1•10^5$	100, 100	$3.4•10^5$	100, 100
Rocks/chlorine	$1.2•10^5$	100, 100	$3.1•10^5$	100, 100
Sand/chlorine	$1.1•10^5$	100, 100	$3.2•10^5$	100, 100
Control	$2.7•10^5$	100, 100	$3.9•10^5$	100, 100
ALL	*$1.7•10^5$*	*100, 100*	*$3.1•10^5$*	*100, 100*
	22 hour E. coli (CFU/100 mL)		**22 hour E. coli (CFU/100 mL)**	
	Geomean	**% >1, % >10**	**Geomean**	**% >1, % >10**
Chlorine	$2.1•10^6$	100, 100	$1.8•10^7$	100, 100
Rocks	$7.9•10^5$	100, 100	$4.9•10^6$	100, 100
Sand	$7.6•10^5$	100, 100	$1.7•10^7$	100, 100
Rocks/chlorine	$1.5•10^6$	100, 100	$2.3•10^7$	100, 100
Sand/chlorine	$1.8•10^6$	100, 100	$3.0•10^7$	100, 100
Control	$7.2•10^5$	100, 100	$2.8•10^6$	100, 100
ALL	*$1.2•10^6$*	*100, 100*	*$1.2•10^7$*	*100, 100*
	Treated Jerricans			
	5 NTU		**50 NTU**	
	1 hour E. coli (CFU/100 mL)		**1 hour E. coli (CFU/100 mL)**	
	Geomean (min, max)	**% >1, % >10**	**Geomean (min, max)**	**% >1, % >10**
Chlorine	1.1 (<1, 10)	30, 0	62 (<10, 8,200)	100, 60
Rocks	2.5 (<1, 56)	40, 20	41 (<10, 11,100)	100, 50
Sand	2.2 (<1, 600)	40, 30	84 (<10, 3,700)	100, 70
Rocks/chlorine	1.5 (<1, 117)	20, 20	118 (<10, 1,500)	100, 80
Sand/chlorine	3.5 (<1, 270)	60, 30	107 (<10, 7,600)	100, 70
Control	1.9 (<1, 160)	40, 20	577 (<10, 25,000)	100, 90
ALL	*<1 (<1,600)*	*38, 20*	*108 (<10, 25,000)*	*100, 70*
	22 hour E. coli (CFU/100 mL)		**22 hour E. coli (CFU/100 mL)**	
	Geomean	**% >1, % >10**	**Geomean**	**% >1, % >10**
Chlorine	<1 (<1, <1)	0, 0	8.3 (<10, 600)	100, 10
Rocks	<1 (<1, <1)	0, 0	12 (<10, 474)	100, 20
Sand	<1 (<1, 1.8)	10, 0	10 (<10, 93)	100, 30
Rocks/chlorine	<1 (<1, 40)	10, 10	6.2 (<10, 50)	100, 10
Sand/chlorine	<1 (<1, <1)	0, 0	<10 (<10, <10)	100, 0
Control	<1 (<1, <1)	0, 0	125 (<10, 2,906)	100, 70
ALL	*<1 (<1, 40)*	*3, 2*	*12 (<10-2,906)*	*100, 23*

784 *n=10 for each cleaning method, N=60

785

Table 2: Free chlorine residual measured at 1, 4, and 22 after Aquatabs treatment in jerricans storing water of both 5 and 50 NTU turbidity. Note: data on non-treated jerricans is not presented in this table.

	5 NTU – 1 hour FCR (mg/L) Average, % ≥0.2 mg/L			**50 NTU – 1 hour FCR (mg/L) Average, % ≥0.2 mg/L**		
Day Samples	**10-24 (n=15)**	**25-48 (n=8)**	**49-64 (n=2)**	**10-24 (n=15)**	**25-48 (n=8)**	**49-64 (n=2)**
Chlorine	1.28 (100)	0.98 (100)	0.70 (100)	0.43 (60)	0.07 (0)	0.09 (0)
Rocks	1.18 (100)	0.75 (100)	0.52 (100)	0.44 (53)	0.05 (0)	0.08 (0)
Sand	1.07 (100)	0.73 (100)	0.60 (100)	0.38 (53)	0.05 (0)	0.07 (0)
Rocks/chlorine	1.19 (100)	0.83 (88)	0.50 (100)	0.35 (60)	0.05 (0)	0.06 (0)
Sand/chlorine	1.18 (100)	0.76 (100)	0.60 (100)	0.37 (53)	0.06 (0)	0.09 (0)
Control	1.04 (100)	0.75 (100)	0.61 (100)	0.30 (47)	0.05 (0)	0.06 (0)
*ALL**	*1.16 (100)*	*0.80 (98)*	*0.59 (100)*	*0.38 (54)*	*0.05 (0)*	*0.07 (0)*
	5 NTU – 4 hour FCR (mg/L) Average, % ≥0.2 mg/L			**50 NTU – 4 hour FCR (mg/L) Average, % ≥0.2 mg/L**		
Day Samples	**10-24 (n=15)**	**25-48 (n=8)**	**49-64 (n=2)**	**10-24 (n=15)**	**25-48 (n=8)**	**49-64 (n=2)**
Chlorine	0.98 (93)	0.70 (88)	0.40 (100)	0.10 (13)	0.07 (0)	0.05 (0)
Rocks	0.79 (87)	0.58 (88)	0.35 (50)	0.12 (13)	0.04 (0)	0.07 (0)
Sand	0.79 (87)	0.58 (88)	0.31 (100)	0.10 (13)	0.05 (0)	0.06 (0)
Rocks/chlorine	0.91 (87)	0.59 (75)	0.26 (50)	0.09 (13)	0.04 (0)	0.07 (0)
Sand/chlorine	0.79 (87)	0.60 (100)	0.35 (100)	0.08 (6.7)	0.04 (0)	0.07 (0)
Control	0.73 (87)	0.55 (88)	0.38 (100)	0.08 (6.7)	0.04 (0)	0.06 (0)
*ALL**	*0.83 (88)*	*0.60 (88)*	*0.34 (83)*	*0.10 (11)*	*0.05 (0)*	*0.06 (0)*
	5 NTU – 22 hour FCR (mg/L) Average, % ≥0.2 mg/L			**50 NTU – 22 hour FCR (mg/L) Average, % ≥0.2 mg/L**		
Day Samples	**10-24 (n=15)**	**25-48 (n=8)**	**49-64 (n=2)**	**10-24 (n=15)**	**25-48 (n=8)**	**49-64 (n=2)**
Chlorine	0.43 (60)	0.20 (25)	0.05 (0)	0.07 (6.7)	0.04 (0)	0.04 (0)
Rocks	0.34 (53)	0.27 (25)	0.04 (0)	0.05 (0)	0.04 (0)	0.04 (0)
Sand	0.31 (53)	0.14 (25)	0.03 (0)	0.06 (0)	0.05 (0)	0.09 (0)
Rocks/chlorine	0.39 (53)	0.15 (25)	0.03 (0)	0.06 (0)	0.05 (0)	0.14 (50)
Sand/chlorine	0.31 (53)	0.13 (25)	0.04 (0)	0.06 (0)	0.05 (0)	0.03 (0)
Control	0.28 (53)	0.12 (25)	0.05 (0)	0.05 (0)	0.04 (0)	0.00 (0)
*ALL**	*0.34 (54)*	*0.17 (24)*	*0.04 (0)*	*0.06 (1.1)*	*0.04 (0)*	*0.05 (8)*

* N=90 for day 10-24, 48 for day 25-48, and 12 for day 49-64; 150 samples total