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Efficacy of locally-available cleaning methods in removing biofilms from taps and surfaces of household water storage containers

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Biofilms are aggregates of microorganisms attached to surfaces that can (re)contaminate water by releasing microorganisms. We grew *E. coli* biofilms on household taps and on storage container coupons, and cleaned them with locally-available agents (bleach, boiled water, soapy water, vinegar) and methods (flowing, scrubbing, or soaking taps; soaking and wiping container coupons). After cleaning, we enumerated surface *E. coli* and imaged biofilms using epifluorescence microscopy. Biofilms were removed from taps when: soaking assembled for five minutes (bleach, boiled water); and, scrubbing and soaking unassembled for five minutes (bleach, boiled water, vinegar). Only soaking methods removed *E. coli* from container coupons. To remove biofilms, we recommend soaking assembled taps in boiled water for five minutes. No recommendation for cleaning containers emerged as soaking storage containers is impractical. Results are consistent with biofilm research, and highlight the need for technical research to develop realistic cleaning recommendations for water storage containers in households.

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INTRODUCTION

Globally, 785 million people lack access to a basic drinking water source¹, and an estimated 1.8 billion depend on fecally contaminated sources². The aim of the Sustainable Development Goals (SDGs) is for all households to access safely-managed, on-plot water¹. In settings without access to safe water, waterborne diseases can cause high morbidity and mortality, particularly in children and susceptible populations³. Household water treatment and safe storage (HWTS) practices are promoted as interim solutions until the SDGs are achieved to reduce this health burden⁴. Household water treatment (HWT) has been shown to improve microbiological water quality and reduce diarrheal disease among users^{5,6}. Safe water storage consists of a storage container with physical barriers to contamination /recontamination of water (e.g., bucket with lid and tap, jerrican with small opening) that enable users to practice safe water access behaviors (e.g., pouring not dipping, dispensing from tap) and prevent fecal-oral transmission^{7–9}.

Research on safe water storage includes: (1) case-control studies on risks/benefits of un/safe storage¹⁰; (2) field evaluations of storage container cleaning methods^{11–13}; (3) impact assessments of safe storage^{11,14}; and (4) evaluations documenting recontamination during transport and storage^{15,16}. In a meta-analysis of case-control studies on cholera transmission, safe water transport and storage was significantly associated with lower odds of cholera (OR = 0.55, 95% CI = 0.39–0.80) and unsafe water transport and storage was significantly associated with higher odds of cholera (OR = 2.8; 95% CI = 2.1–3.7)¹⁰. This cholera data align with development context studies on risks/benefits of safe/unsafe water storage¹⁷. Given the known risks of unsafe storage, container cleaning activities are commonly promoted¹⁸. In emergency contexts, evaluations of three jerrican disinfection programs (using 2.5 mg/L to 5% chlorine solutions) documented

short-term increases in free chlorine residual and reductions in microbiological indicators, and regrowth of microbiological indicators in hours to days after cleaning^{11–13}. In an impact evaluation where control group participants received jerricans, diarrhea was significantly reduced from the prior week (OR = 0.84, 95% CI 0.82–0.86)¹⁴. Another impact evaluation found diarrhea reduction was not significant when improved buckets (with spout and lid) were compared with open buckets¹¹. However, a short-term 69% reduction in geometric mean fecal coliforms over 6 h of storage was documented. Lastly, when filter effluent and stored water samples were collected during biosand, ceramic, and Sawyer filters evaluations, geometric mean filter effluent results were lower than stored water sample results¹⁵. These results are consistent with a meta-analysis, which found water was more contaminated with microorganisms after transport and storage than at the source¹⁶. The above research highlights the public health risks of unsafe water storage. Of note is, while biofilms in storage containers were often mentioned as the cause of this risk, biofilms were not directly researched in the studies referenced above.

Biofilms are aggregates of microorganisms attached to a surface and coated within a self-created extracellular polymeric substance (EPS) matrix¹⁹. The EPS creates the microenvironment for biofilm cells, and is a complex matrix of biopolymers of microbial origin²⁰. The EPS provides structure for the biofilms and can protect microbial communities in a biofilm from unfavorable environmental conditions. Biofilms develop on all surfaces in contact with non-sterile water and, once established, 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 (DWDS), particularly in pipe and tubing distribution systems, and in taps.

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In particular, taps can be contaminated directly from the DWDS, or retrograde contamination as a result of backflow within the tap from an outlet contaminated via cleaning/contact in homes²¹ or cleaning cloths in hospitals²². Different parts of taps can be contaminated to different degrees, for example the solenoid valve of non-touch sensor taps²³ and flow straighteners²⁴ had higher biofilm concentrations than other parts of the tap. Polyvinyl chloride (PVC) taps were found to support a lower level of bacterial diversity and biofilm than stainless steel or cast iron taps, consistent with research on DWDS pipe materials finding plastic pipes with relatively less biofilm development²⁵. Biofilms in taps were remediated by removing contaminated parts²³, use of proprietary cleaners²⁶, or use of antimicrobial taps²². Biofilm contamination in taps can have severe health consequences; four neonates died in a neonatal care unit due to *P. aeruginosa* infection from contaminated taps in Ireland²⁴.

Biofilm growth inside household water storage containers can act as microbial reservoirs, therefore resulting in recontamination of drinking water and deterioration of disinfectant residual. Biofilms have also been studied, via surrogate indicators, in water storage containers. In South Africa, Jagals et al. found statistically significant increases in turbidity, heterotrophic bacteria, and *C. perfringens* in undisturbed stored water versus source tap water, and in suspension water after scrubbing storage container walls versus undisturbed water²⁷. Using similar methods and qPCR sampling, *V. cholerae* and *V. parahaemolyticus* were found in both free volume and dislodged water samples from water storage containers²⁸. In the laboratory, using water sourced from a local river and standpipes, biofilms developed on polyethylene and galvanized steel household water storage containers after 48 h²⁹. Also in South Africa, Mellor used a sterilized swirl protocol (where sterilized water was added to empty containers, containers were shaken, and effluent collected) and determined a variable biofilm concentration across 40 samples of 1.85 ± 1.59 CFU/cm²³⁰. Biofilm concentration was not associated with container type, narrow versus wide neck, primary drinking water source, reported cleaning mechanism, or time since last washing. In a subsequent agent-based model developed using this data, biofilms had a significant impact on water quality, and subsequently, early childhood diarrhea³¹. In Cameroon, water from storage containers and cups, and swabs from emptied containers and cups were collected; qPCR results found higher copy numbers of *E. coli* and total coliform in containers and cups as compared to source water³². Additionally, fecal indicator bacteria presence was not associated with container type or opening, but was associated with storage time; no difference was found in water or biofilm gene copies after local cleaning ($n=2$). Lastly, Budeli et al. confirmed biofilm formation via scanning electron microscopy, in plastic storage container surfaces within 24 h of exposure to untreated water³³. After filtration with silver nanoparticle containing ceramic filters, biofilm formation was visually apparent at 14–21 days or 3–14 days, depending on filter. Budeli recommended washing plastic storage containers with water every 3–14 days. Overall, prior research has identified biofilms as a concern in water storage containers, but not directly measured biofilm growth using technical methods available or investigated the role of cleaning in removing biofilms.

We thus asked the research question: “What are the most efficacious locally-available cleaning agents and methods to remove biofilms from taps and storage container surfaces?” To answer this question we: (1) grew *E. coli* biofilms on tap and storage container surfaces; (2) applied locally-available cleaning agents (bleach, boiled water, soapy water, and vinegar) to those surfaces using locally-appropriate cleaning methods (soaking, scrubbing, etc.); (3) evaluated reductions in surface *E. coli* by culture and in biofilm structure by epifluorescence microscopy, stratified by cleaning agent and method; and (4) triangulated data,

including consideration of social factors, to develop cleaning recommendations for taps and storage containers.

RESULTS

Tap cleaning—confirmation of biofilm growth

E. coli and biofilm growth were confirmed via three mechanisms: daily *E. coli* enumeration during spiking; swabbing control taps immediately before destruction; and imaging control taps after destruction. Fifteen minutes after culture addition, culture *E. coli* concentrations were $1.00 \cdot 10^2$ – $8.67 \cdot 10^3$ CFU/mL. After 22.5 h, culture *E. coli* concentrations increased to $3.90 \cdot 10^5$ – $8.25 \cdot 10^8$ CFU/mL. Average *E. coli* concentration on control taps swabbed before destruction was 944–543,217 CFU/cm² (vertical sample) and 93.5–178,060 CFU/cm² (horizontal sample). Lower *E. coli* concentrations in horizontal samples is attributed to turbulence in the vertical section increasing *E. coli* deposition. Dense *E. coli* biofilm growth was confirmed via microscopy on all tested control taps (tap tube samples, Fig. 1; seat cup samples, Fig. 2). Please note circles are individual *E. coli* cells, and because the seat cup coupon is slightly convex, image stacks from these coupons contain a narrower slice of visible biofilm (Fig. 2). In image analysis, *E. coli* density (CFU/μm³) was calculated at 1.11 – $5.40 \cdot 10^{-3}$ in the tap tube and $1.18 \cdot 10^{-4}$ – $4.51 \cdot 10^{-3}$ in the seat cup. These consistent results (within one order of magnitude) confirm imaging as a more reliable method than swabbing (as inconsistencies in personnel technique and exact drying time can influence swabbing results) in this multi-day study context. Together, the three results sets confirm: *E. coli* was present in culture flowing through taps; *E. coli* was on control tap surfaces before tap destruction; and *E. coli* biofilms grew on control taps. These results confirm methods used to grow biofilms, and the validity of cleaning agent and method data.

Tap Cleaning—efficacy of cleaning agents and methods

The cleaning agents used were bleach (0.52%, pH = 12.4), recently boiled water at ~80 °C (no concentration, pH = 5.47), soapy water (20 g/L, pH = 11.5), and vinegar (5%, pH = 2.4). All cleaning agents were prepared and stored in sterile containers immediately prior to use. We evaluated efficacy of cleaning agents and methods by comparing surface swab *E. coli* results and biofilm imaging results to controls.

Results varied between cleaning agents and within cleaning agents indicating that the cleaning method also had an effect on the removal of biofilms. The bleach and vinegar cleaning agents removed all enumerable surface *E. coli* (≤ 1 CFU/cm²) from vertical and horizontal surfaces, for all cleaning methods (Table 1). Due to differences in *E. coli* surface concentrations in controls between runs, this is >2.3 log reduction value (LRV) for bleach, and >5.3 LRV for vinegar. While the cleaning agent of soapy water did reduce the amount of *E. coli* on surfaces compared to the control (1.05–6.04 LRV), soapy water did not remove all enumerable *E. coli* from horizontal and vertical surfaces except with the cleaning method of scrubbing and soaking disassembled for 5 min. While the cleaning agent of boiled water did reduce the amount of *E. coli* on surfaces from the control in most cases (–0.84–3.42 LRV), boiled water only removed enumerable *E. coli* from horizontal and vertical surfaces in cleaning methods incorporating a soaking step.

The cleaning method of scrubbing and soaking disassembled for 5 min removed all enumerable surface *E. coli* (≤ 1 *E. coli* CFU/cm²) from vertical and horizontal surfaces with all cleaning agents.

Quantitative image analysis found thin biofilms after: cleaning with boiling water (most methods) (0.0–41 μm); and bleach (all methods) (0.0–46 μm) (Fig. 3a). With vinegar, scrubbing methods and soaking disassembled resulted in the thinnest biofilms (0.0–26 μm). Cleaning with soapy water left thick biofilms (9.8–150 μm). Qualitative image analysis resulted in finding no

Fig. 1 Biofilms on Tap Tubes of control taps. The panel presents an example image slice from the control stacks for **a** bleach; **b** recently boiled water; **c** soapy water; and **d** vinegar. Scale bar is 10 µm.

biofilm or isolated *E. coli* cells with no clumping remaining on: (1) tap tube surfaces for flowing or all surfaces for soaking assembled taps in recently boiled water; (2) all surfaces for scrubbing assembled or scrubbing disassembled followed by soaking for five minutes in vinegar; and (3) tap tube surfaces for soaking assembled or all surfaces for soaking disassembled or scrubbing disassembled followed by soaking for five minutes with bleach (Fig. 3b). Small or large biofilm structures were identified on tap surfaces cleaned by flowing or soaking vinegar, scrubbing with bleach, and on most surfaces cleaned by soapy water.

Interesting results noticed in images were: scrubbing cleaning methods can align biofilm structures in the direction of scrubbing; soapy water cleaning methods can leave live *E. coli* cells surrounding the space where soap bubbles had been before bursting; and soaking cleaning methods with the cleaning agent bleach left elongated dead *E. coli* cells in biofilm structures (Fig. 4).

Storage container cleaning—confirmation of biofilm growth

E. coli and biofilm growth was confirmed via the same three mechanisms as in the spigot study. Fifteen minutes after culture addition, culture *E. coli* concentrations were $1.47 \cdot 10^3$ – $2.47 \cdot 10^4$ CFU/mL. After 22.5 h, culture *E. coli* concentrations increased to $2.67 \cdot 10^8$ – $7.27 \cdot 10^8$ CFU/mL. Average *E. coli* concentration, removed from control coupons via sonication, at the end of the 21-day growth period was $2.21 \cdot 10^7$ CFU/cm², confirming dense biofilm growth on the coupon surfaces. Contrary to the tap study, *E. coli* sonication results were more reliable than imaging results in the

coupon study, and could be attributed to error in preparation of imaging samples or sonication that improved removal of *E. coli* from surfaces as compared to the swabbing results from the taps. Together, the result sets confirm: *E. coli* was present in culture where coupons were soaked and *E. coli* was on control coupon surfaces before destruction. Imaging results confirmed the presence of biofilms on surfaces, but were not consistent and therefore not relied upon for further analysis. These results confirm methods used to grow biofilms, and the validity of cleaning agent and method *E. coli* surface data from sonication.

Storage container cleaning—efficacy of cleaning methods and agents

Across all cleaning agents, the cleaning method of soaking was efficacious in removing *E. coli* to non-detectable levels (<1 CFU/cm²) (Fig. 5). Compared to control surface *E. coli* values, this is an LRV of >7.95 for bleach and boiled water, and 6.78 for vinegar cleaning agents. Please note, the plates at all dilutions prepared for *E. coli* enumeration of wiping cleaning methods with all agents were too numerous to count. Thus, concentrations were all >12,500 CFU/cm², and we calculated conservative LRVs assuming the lowest dilution sample had 250 colonies, at <3.25 LRV.

Of the 10 coupons prepared for imaging, four yielded images with biofilms, and *E. coli* count, total area covered, average colony size, and percent area covered were unreliable from these data.

Fig. 2 Biofilms on Seat Cups of control taps. The panel presents an example image slice from the control stacks for **a** bleach; **b** recently boiled water; **c** soapy water; and **d** vinegar. Scale bar is 10 μm .

Table 1. Average *E. coli* concentrations (CFU/cm²) on tap surfaces from both horizontal and vertical swab locations for all cleaning agents (bleach, boiled water, soapy water, vinegar) and cleaning methods.

	Bleach		Boiled		Soap		Vinegar	
	H	V	H	V	H	V	H	V
Flowing agent through tap for 60 s	<1	1	1.6	<1	104	1	<1	1
Soaking assembled for 60 s	<1	<1	<1	<1	260	124	<1	<1
Soaking assembled for 5 min	<1	<1	<1	<1	92.2	<1	<1	<1
Soaking disassembled for 60 s	<1	<1	<1	<1	10.5	33	<1	<1
Scrubbing with a brush 5 times assembled	<1	<1	2670	3.15	7150	39,284	<1	<1
Scrubbing with a brush 5 times disassembled	<1	<1	80.2	25.6	35.8	15	<1	<1
Scrubbing and soaking disassembled for 5 min	<1	<1	<1	<1	<1	<1	<1	<1
Control—no cleaning	93.5	944	382	1319	81,081	543,217	178,060	117,504

H horizontal swab location, V vertical swab location.

Data triangulation and recommendation development

An ideal cleaning method would: (1) remove live *E. coli* on surfaces; (2) remove the biofilm structure (EPS and dead *E. coli* cells); and (3) be simple to complete. This is because risk would be reduced from live organisms in the biofilm, and reattachment of new organisms would be delayed until the biofilm structure was regenerated³⁴. Furthermore, research has shown that simpler

actions require less training and are more likely to occur at the household level³⁵.

In taps, three options reduced both *E. coli* on surfaces and biofilm structure: bleach (all cleaning methods), boiled water (soaking methods only), and vinegar (scrubbing methods only) (Table 2). Given these three technically efficacious methods, we consider ease-of-use, ease-of-preparation, and availability of

Fig. 3 Biofilm image analysis. Biofilm image analysis data is stratified by cleaning method and agent from the Tap Tube (TT) and the Seat Cup (SC), including **a** average biofilm thickness (μm) and **b** qualitative analysis.

materials in the household. Based on experience working with these methods, we found it: challenging to fully disassemble the taps, including removing the seat cup as recommended, without damaging or contaminating it; easier to prepare cleaning agents that did not need dilutions; difficult to purchase and keep sterilized a scrub brush of the correct size to reach within the entire tap (we purchased and sterilized laboratory bottle brushes); and, that boiled water can present a burning hazard. As such, we recommend cleaning methods that do not involve tap disassembly, solution preparation, or purchase of specialized equipment. Based on the above, considering both technological efficacy and social factors, we recommend soaking an assembled tap in recently boiled water ($<76.7^\circ\text{C}$, the temperature rating of the tap) in a container (such as a pan water was boiled in) for five minutes. However, other options could be used, including bleach with all cleaning methods, recently boiled water with soaking methods only; and, vinegar with scrubbing methods only. Please note a benefit of boiled water is that it would be more effective against complex biofilm structures containing protozoa than chlorine-based agents³⁶. Furthermore, it is likely that cleaning agent choice

will be a household level decision based upon familiarity with the cleaning agent, ease of use, access and availability, and cost.

In storage container coupons, the only option that reduced surface *E. coli* was soaking for 5 min, with all three tested cleaning agents. Although soaking samples was technically efficacious, it is impractical for households to clean their storage container by soaking with large quantities of a cleaning solution. Thus, current results do not lead to a recommendation for efficacious container cleaning.

DISCUSSION

Using methods developed and adapted from a related study “Efficacy of locally-available cleaning methods and household chlorination at preventing biofilm development, removing *E. coli*, and maintaining free chlorine residual in jerricans used to store household drinking water”, we successfully grew *E. coli* biofilms on taps and storage container surfaces. We then tested four cleaning agents and seven cleaning methods on taps, and three cleaning agents and three cleaning methods on storage containers. After

Fig. 4 **Interesting imaging results.** The panel presents an example image slice from representative image stacks cleaned via: **a** scrubbing; **b** soapy water; and **c** bleach. Scale bar is 10 µm.

Fig. 5 **Efficacy of cleaning methods/agents at removing surface *E. coli* from container coupons.** Graphs present the **a** concentrations and **b** LRV.

Table 2. Summary of *E. coli* and biofilm structure results for both tap and storage container cleaning for all cleaning agents and cleaning methods.

	Tap cleaning		Storage container cleaning	
	<i>E. coli</i>	Biofilm structure	<i>E. coli</i>	Biofilm Structure
Bleach	Reduced to ND All methods	Removed structures Most methods	Reduced to ND Soaking only	Inconclusive
Boiled water	Reduced to ND Soaking methods only	Removed structures Soaking assembled methods	Reduced to ND Soaking only	Inconclusive
Soapy water	Did not reduce to ND All methods	Did not remove structures All methods	Not tested	Not tested
Vinegar	Reduced to ND All methods	Removed structures Most scrubbing methods	Reduced to ND Soaking only	Inconclusive

ND non-detectable.

triangulating technically efficacious results with user considerations, we developed a recommendation to remove biofilms from taps by soaking for 5 min in recently boiled water at 80 °C. We were not able to develop recommendations for container cleaning. Below we discuss reasons for differences seen across cleaning agents and methods, and recommendations for further research.

The efficacy of sodium hypochlorite in removing biofilms is influenced by contact time and concentration. In dental biofilms, 2% sodium hypochlorite at 10 min contact time was found most efficacious at removing fungal biofilms from acrylic³⁷ and soaking dentures 3 min a day in 0.5% sodium hypochlorite reduced

microorganisms and fungal biofilms³⁸. In medical applications, soaking PVC tracheostomy tubes 5 min in 0.3% sodium hypochlorite reduced *Staphylococcus aureus* and *Pseudomonas aeruginosa*³⁹. In the food industry, 100 parts-per-million aerosolized sodium hypochlorite reduced foodborne pathogens on PVC with 50 min contact time⁴⁰. Thus, while it would be expected *E. coli* would be rapidly inactivated by sodium hypochlorite due to low CT factor³⁶, removing the EPS structure with sodium hypochlorite requires contact time, consistent with our results.

Acetic acid has been investigated to prevent biofilms in the dental⁴¹, food⁴², and wound health⁴³ contexts. However, contact times are measured in hours, and there is less existing research

relevant to cleaning storage containers. Soaking in vinegar was not as effective as soaking in bleach or recently boiled water, supporting previous research that found acidic solutions to be less effective than alkaline solutions at penetrating into the acidic biofilm structure⁴⁴. However, scrubbing was effective, as once the acidic vinegar penetrated the biofilm EPS matrix, it came into contact with *E. coli* cells.

Overall, we found scrubbing the tap and wiping the container to be less effective than soaking. For taps, the difficulty of scrubbing with a bottle brush was it streaked the biofilm around. For containers, after a 21-day growth period, it is possible that the relatively thick biofilm prevented an effective diffusion-penetration interaction between the *E. coli* and the cleaning agents present on the sponge and cloth⁴⁵. In one study investigating cleaning cobalt-chrome implantables with autoclaving followed by mechanical scrubbing, scrubbing only increased efficacy when used with 4% chlorhexidine and not when used with a saline solution⁴⁶. It is possible that scrubbing needs to be both quite vigorous and include a disinfectant to be effective.

To our knowledge, boiled water and soapy water have not previously been evaluated in this context for biofilm removal. A contact time of 5 min with boiled water could 'melt' EPS components, as soaking in boiling water has removed biofilms from baby pacifiers⁴⁷. However, scrubbing with boiled water would not have sufficient temperature/contact time to impact the live *E. coli* on the surface or biofilm structure (as seen in results). Please note EPS components vary based on type and concentration of microorganisms and environment; it is thus possible EPS vary in resistance to boiled water²⁰. The surfactant soap would not be expected to be effective against biofilms because the lipophilic end of soap that attaches to remove microorganisms, dirt, and grease would not be able to 'extract' these particles from the biofilm structure (and soapy water was not tested on surface containers after poor results from taps). Similarly, room-temperature water alone would not be effective as a cleaning agent to remove biofilms with cleaning methods tested herein.

Limitations to our work include: (1) biofilms were grown from high-concentration cultures, which will form a biofilm faster than would be seen in field circumstances; (2) biofilms were grown from *E. coli* ATCC 11229, which produces biofilms of moderate adherence⁴⁸; in real-world settings biofilms would be a mix of organisms; (3) we used only one concentration of each cleaning solution per test; (4) bleach and vinegar were toxic to *E. coli* only biofilms, but they may not be effective against biofilms comprised of different organisms; (5) imaging of the storage container surface was not successful; (6) we used new, clean taps and containers in our study; (7) even though sonication is more effective at removing *E. coli* from surfaces than swabbing⁴⁹, we were not able to sonicate taps due to size and wanting to obtain multiple samples; thus surface *E. coli* results are more reliable for container coupons than taps; and, (8) we did not stain separately for dead cells during image processing to determine live and dead cell biofilm composition. Despite these limitations, we feel study results are valid. A high-concentration *E. coli* only biofilm is a conservative challenge case, and boiled water inactivates the protozoa, bacteria, and viruses causing diarrheal disease. Additionally, our research did not address how often taps should be cleaned. A reasonable cleaning recommendation, based on the fact biofilms can grow on PVC in less than 1 week given sub-optimal water quality⁵⁰, could be one time per week. Lastly, an important consideration is that in this research we focused on removing existing biofilms from these surfaces. We did not research how to prevent the development of biofilms on these surfaces; please see "Efficacy of locally-available cleaning methods and household chlorination at preventing biofilm development, removing *E. coli*, and maintaining free chlorine residual in jerricans used to store household drinking water" for information on prevention of biofilm development in jerricans.

We recommend further research, including: field research on acceptability of tap cleaning methods, including burden to household, and impact of cleaning solution residue on taste/acceptability of water; laboratory research to determine cleaning frequency recommendations, including regrowth period after a cleaning event, and cleaning methods to prevent biofilm growth on taps and storage containers; laboratory and field research of efficacy and effectiveness of taps and water storage containers containing antimicrobial compounds at inhibiting biofilm growth; research on more realistic complex biofilms; research on methods for container cleaning that have sufficient contact time but do not require soaking (e.g., using a spray bottle to spray cleaning solution on a container (shown effective in cleaning cutting boards⁵¹), scrubbing a container vigorously with an abrasive); and development of storage containers embedded with antimicrobial compounds that inhibit biofilm development able to be produced locally at low-cost. Additionally, in literature review we noted the lack of a comprehensive systematic review on the outcomes and impacts of safe water storage; it is recommended this be completed.

Overall, in the laboratory we cleaned *E. coli* biofilms from 96 taps and 30 container surfaces using locally-available cleaning agents and methods that we hypothesized households could employ. After triangulating cleaning results, we recommend that households clean taps by soaking them in recently boiled water (above 80 °C) for 5 min. Unfortunately, a recommendation of soaking water storage containers is impractical and we thus have no recommendation for cleaning storage containers at this time. This research is a successful example of using technical laboratory methods to answer field-relevant questions; further research such as this to close evidence gaps in WASH is recommended.

METHODS

Tap cleaning

To assess tap cleaning agent and method efficacy: (1) a protocol was developed; (2) a system was built to group biofilms in taps and one trial run was completed; (3) four data collection runs, one for each of the four cleaning agents, were completed; and (4) data were analyzed.

Through partner discussions, a literature review, and author experience, four locally-available cleaning agents (bleach (sodium hypochlorite), boiled water, soapy water, and vinegar), and seven cleaning methods (flowing agent through tap for 60 s, soaking assembled for 60 s, soaking assembled for 5 min, soaking disassembled for 60 s, scrubbing with a bottle brush five times assembled, scrubbing with a brush five times disassembled, and scrubbing with a bottle brush five times and soaking disassembled for 5 min) were selected. A control of no cleaning was included. Test taps were manufactured by Tomlinson Industries (HFSLT Faucet 3/4"-16 UNF 1" Long, Cleveland, Ohio, US) and consist of a PPE body and jam nut with two washers.

A system was built to continuously flow 18 L of *E. coli*-spiked Luria-Bertani (LB) broth through taps to grow biofilms (Fig. 6). The system consisted of three plastic 10 L buckets, with eight taps attached to each bucket (24 taps total). Taps were latched open and drained by gravity into an enclosed funnel and 20 L collection bucket. The three 20 L buckets drained via 3/4" braided hose into a centrifugal pump (Grundfos CM 3-4, Bjerringbro, Denmark), and liquid from all buckets mixed together. A pressure-reducing valve on the pump was used to hold pressure in the 1" discharge hose at 20 psi. From the discharge hose, the fluid was pumped to the top of the system and distributed via a manifold into each of the three 10 L buckets with taps. Manual ball valves on the manifold were tuned to ensure that flow rate into each 10 L bucket maintained a 1" fluid head above the taps. This head produced a laminar flow regime (Reynolds number = 1899) and a draining flow rate of 1.5 L/min through each tap. The system was run once for optimization, after which cooling controls were added, including a: chiller pumping a mixture of 80% ethylene glycol and 20% water at 0 °C through copper tubing wrapped around the left bucket in the system; a cooler filled with ice packs on the return line; and a fan to cool the pump.

Four experimental runs were completed, each testing the efficacy of one of the cleaning agents using each of the cleaning methods. A run

* Left bucket is shown open to show the flow through the taps; center bucket shows the funnel that collected flow into 20 L bucket; right bucket shows the taps fully enclosed as they were in operation.

Fig. 6 Pumping system. Schematic of the pumping system constructed in the laboratory for growing biofilm in taps*.

consisted of: sterilizing the pumping system; daily culture preparation; operation and monitoring of the system; cleaning agent preparation; cleaning and destruction of taps; microbiological sampling; and imaging.

Before starting an experimental run, the system was cleaned by removing buckets, taps, and funnels, and washing pieces with 0.5% sodium hypochlorite. After reassembly, the system was sterilized by pumping 5% sodium hypochlorite through all lines for 1 h, followed by one rinse cycle with 18 L sterile LB Broth, and rinse cycles with Type 1 laboratory-grade water (Milli-Q® Reference, MilliporeSigma, Burlington, MA, USA) filtered through a 0.22 µm filter (Millipak®, MilliporeSigma, Burlington, MA, USA), hereafter termed “Milli-Q”, to remove any residual chlorine. Confirmation that chlorine had been fully rinsed from the system was completed using a Lamotte 1200 colorimeter and DPD-1 instrument grade tablets (Lamotte, Chestertown, MD, USA) to ensure a non-detect chlorine residual before proceeding.

E. coli (ATCC 11229) stock was streaked onto LB agar plates, incubated at 35 °C, and stored at 4 °C. The night before each system refill, a streak plate colony was used to inoculate 20 mL of LB broth, and incubated at 35 °C on a rotating plate for 12–18 h with shaking. The culture was then diluted (1:20) in sterile LB broth and incubated at 35 °C on a rotating plate for 3 h, or until a concentration of $\sim 10^{10}$ cells/mL was reached, as estimated using a spectrophotometer (OD = 600 nm). The volume of culture used to spike 10^2 – 10^3 CFU/mL *E. coli* into the sterile LB broth added to the pumping system was estimated from the spectrophotometer reading.

The system was operated by adding 6 L of *E. coli*-spiked LB broth to each of the 3 buckets (20 L each), pumping the broth through the system for 24 h, and draining the system of the broth. This process was repeated for 5 days to grow biofilms on surfaces in contact with spiked broth. To monitor *E. coli* concentrations in the LB broth, an LB broth sample was aseptically collected at two time points: 15 min after adding fresh *E. coli*-spiked LB broth to the system; and, after 22.5 h of operation. Appropriate dilutions of these samples were prepared in phosphate buffered saline (PBS) (pH = 7.4), filtered through a 0.45 µm membrane, plated on mColiBlue24® (Hach, Loveland, CO) media, inverted and incubated at 35 °C for 24 h following standard methods. *E. coli* colonies were then enumerated and recorded. Please note we refer to this “*E. coli*-spiked LB broth” as “culture” herein. Culture temperature and volume were monitored twice daily during system operation, and 4 liters of Milli-Q water were added to the system every evening.

Preparation of cleaning agents is as follows. Bleach (0.5% sodium hypochlorite (NaOCl)) was prepared by diluting 6% laboratory-grade commercial bleach (Pure Bright, KIK International, Houston, TX, USA);

concentration was confirmed using iodimetric titration (Hach Method 8209, Loveland, CO, USA). A 0.5% concentration was selected because: a 10:1 dilution of 5% commercial bleach is simple for a user to prepare; and 0.5% is recommended during Ebola outbreaks for disinfection of non-living surfaces, and has shown to efficaciously remove *E. coli* and viral surrogates from surfaces⁵². To prepare boiled water, Milli-Q water was dispensed into a 5 L metal cooking pot, placed on a hot plate, and brought to a rolling boiling. Before cleaning, water was poured into a sterilized 5 L graduated cylinder, from which water was dispensed as needed during cleaning. Please note water was not continuously boiling during cleaning but was checked to ensure it was ~ 80 °C. To prepare soapy water, commercially-available unbranded local bar soap was purchased in Dhaka, Bangladesh and shipped to Tufts University. Soap was grated with a kitchen cheese grater; 400 g of grated soap was then stirred into 20 L of Milli-Q water until mostly dissolved. This concentration was selected based on previous results finding this soapy water concentration was more effective at removing thermotolerant coliforms and *C. perfringens* from hands than water alone⁵³. Lastly, commercially-available household vinegar (5% acetic acid) was purchased from a Boston-area grocery store and used without modification (Heinz, Pittsburgh, PA, USA).

At the conclusion of each experimental run, the system was drained and the 24 taps were unmounted. Three taps were cleaned using each of the seven cleaning methods; three control taps were not cleaned. Of the triplicate tap samples, two were swabbed for *E. coli* and one was destructed for imaging. To clean taps by flowing agents through them, taps were individually mounted on sterilized 10 L buckets, 4 L of agent was added to buckets and allowed to completely drain through open spigots (approximately 60 s), then taps were aseptically unmounted. To soak taps, 200 mL of cleaning agent were added to sterile 300 mL Whirl-Pak® bags (Nasco, Fort Atkinson, WI, USA), a tap was added to the bag, bags were closed so taps were fully submerged, and after soaking, bags were drained and taps aseptically removed. To scrub taps, the sterile bottle brush was: (1) dipped into cleaning agent and the horizontal part was scrubbed in-and-out five times; and (2) dipped into cleaning agent and moved circularly around the inside surface of the horizontal part five times (Fig. 7). This scrubbing method was repeated for the vertical portion. To disassemble taps, the nut, two washers, and flow lever were unscrewed from the main body of the tap.

Swabbing was conducted using a Sanicult Hygiene Monitoring swab (Starplex Scientific, Etobicoke, Ontario, Canada). Each tap was swabbed separately in the horizontal and vertical directions by moving the swab ten times around the inner surface of the corresponding tap area (Fig. 7). The

Fig. 7 Tap sample collection locations. Tap scrubbing, swabbing, and coupon cutting locations, including **a** horizontal and vertical scrubbing and swabbing locations and **b** locations of the Tap Tube (TT) and the Seat Cup (SC) coupons removed for imaging⁵⁴.

surface areas swabbed were $\sim 31.45 \text{ cm}^2$ and 4.59 cm^2 for the horizontal and vertical surfaces, respectively. After swabbing, the swab was returned to the Sanicult broth, vortexed, and stored on ice. Appropriate dilutions of swab samples were prepared via membrane filtration, as described above.

One representative surface area ('coupon') was cut from each of two locations on each tap, the seat cup on the flow lever (SC) and the tap tube at the base of the tap's horizontal section near the internal corner (TT) (Fig. 7). Each coupon was carefully cut from the tap using a sterile utility knife, rinsed with PBS, and allowed to air dry in a sterile field. Coupons were then mounted onto a glass microscope slide with nail polish and moved to a biosafety cabinet in a darkroom. In the dark, $50 \mu\text{L}$ of a $600 \mu\text{M}$ solution of 4',6' diamidino-2-phenylindole dihydrochloride (DAPI) stain (Millipore-Sigma, Burlington, MA, USA) were pipetted onto each coupon and incubated at room temperature for 30 min. Lastly, 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 (Wetzlar, Germany) under $\times 63$ objective in immersion oil. Images were acquired using a 405 nm visible laser diode. After locating surface *E. coli*, gain and offset were adjusted for each image to bring cells into focus. Three randomly selected fields of view were acquired for each coupon by scanning at 400 Hz from the top of the biofilm to the coupon's surface. Image slices were recorded at predefined z-step sizes (ranging $0.3\text{--}2.0 \mu\text{m}$). A resulting image stack ($116.52 \mu\text{m}^2 \times$ depth of the sample) for each field of view was exported for analysis.

All collected microbiological data were entered into Microsoft Excel (Redmond, WA, USA). The geometric mean of plates in the countable range ($10\text{--}200$ colonies) was calculated for each swab after accounting for dilution, divided by the swabbed surface area, and reported as the number of colony forming units (CFU) per swabbed surface area (cm^2). Results were averaged for each location for replicate taps.

Images were imported and visualized for qualitative analysis in the open-source software FIJI/ImageJ 2.0.0-rc-69/1.52n. They were converted to 8-bit black and white images, thresholded using the Otsu algorithm, downsampled using a 3D Gaussian Blur algorithm ($\alpha = 0.5$ all directions), then dilated once, eroded once, and inverted to remove background noise. Each image stack was then processed using the Particle Analyzer algorithm, *E. coli* counts and biofilm thickness recorded, and a density ($\text{CFU}/\mu\text{m}^3$) calculated for each image stack. In denser biofilms *E. coli* cells clumped together to form colonies, which the algorithm counted as one, thus the calculated density value was sometimes artificially low. Thus, processed images were also qualitatively evaluated. A rating system of 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 by two trained individuals separately.

Surface container cleaning

To assess surface container cleaning efficacy: (1) a protocol was developed and coupons cut from containers; (2) one 21-day data collection run was completed, followed by a cleaning, *E. coli* testing, and image preparation; and (3) data were analyzed.

Polypropylene water storage containers were provided to Tufts University by partners. Using a scroll saw, 45 cm^2 coupons were cut from the flat sides and bases of the containers. Using a Dektak XT-S Profilometer with Vision64 software (Bruker, Billerica, MA, USA), a surface

profile was collected using a $12.5 \mu\text{m}$ tip stylus with $29.4 \mu\text{N}$ contact force at $166.7 \mu\text{m/s}$ scan speed by scanning each coupon for $5000 \mu\text{m}$ in three directions (x , y , and xy). Average roughness (Pa) and root-mean squared (Pka) values were calculated after leveling the profile using two points, and entered into Microsoft Excel. Outlier coupons were identified using the IQR and a trendline and removed from the sample set. The final sample size was 30 coupons of comparable surface roughness values ($0.462\text{--}1.098 \mu\text{m}$).

Coupons were sterilized with 0.5% bleach solution and 70% ethanol, and aseptically submerged in individual 50 mL Falcon tubes containing 25 mL of LB broth spiked with *E. coli* at a target concentration of 10^3 CFU/mL . *E. coli* culture was prepared as described in the tap study. Falcon tubes were incubated for 21 days at 35°C on a ThermoFisher MaxQ 2000 orbital shaker (Fisher Scientific, Hampton, NH, USA) at 70 rpm . Coupons were aseptically transferred to new 50 mL Falcon tubes with 25 mL of fresh culture every 48 h, a total of 11 times throughout the 21-day experiment. Each spiking day, *E. coli* concentrations in culture were confirmed as in the tap study.

At the end of the 21-day growth period, the exterior surface of each coupon was sterilized with ethanol, rinsed by gently pipetting 2 mL of PBS across the interior surface to remove planktonic cells settled on the surface, and air dried in a sterile field. This ensured live *E. coli* in biofilm remained only on the interior PPE surface. Coupons were cleaned by applying three cleaning agents (0.53% bleach solution, 6% acetic acid vinegar, and boiled tap water) using three cleaning methods (soaking for five minutes, wiping with a sterile Whirl-Pak[®] hydrated PolySponge[™] representative of a cleaning sponge (Nasco, Fort Atkinson, WI, USA), and wiping with a cloth (100 cm^2 , 60% cotton, 40% polyester). These methods were selected based on experience and results from the tap cleaning study. Before cleaning, cloth pieces were sterilized by soaking in bleach for 15 min, soaking in ethanol for 15 min, and drying in a biosafety cabinet for 18 h. A new, sterile sponge or cloth was used to clean each coupon. To apply the soaking cleaning method, a coupon was placed in a sterile 50 mL Falcon tube with 25 mL of cleaning agent, allowed to soak for 5 min, and removed from the Falcon tube. To apply the wiping cleaning methods, a coupon was placed interior facing upwards on a sterile surface, the sponge or cloth was soaked in the cleaning agent, and the coupon was wiped five times back and forth in each of four directions (xx , yy , xy , and yx). Please note, the same person wiped all coupons to maintain wiping consistency. Three replicate coupons were cleaned per agent/method combination, and three control coupons were left uncleaned (30 coupons total; three agents by three methods in triplicate, plus three controls).

After cleaning, two coupons from each triplicate were aseptically transferred into Falcon tubes containing 20 mL of PBS and if necessary a dissolved neutralizing agent (sodium thiosulfate for bleach, sodium bicarbonate for vinegar). Falcon tubes were vortexed on a Fisherbrand[™] Analog Vortex Mixer for 30 s at 1200 rpm (Fisher Scientific, Hampton, NH, USA), then sonicated in a Fisherbrand[™] FS20 Sonicator for 5 min at 40 KHz (Fisher Scientific, Hampton, NH, USA) in water at 20°C . Falcon tubes samples were prepared as described in the above tap study to enumerate *E. coli* removed from surfaces via sonication. Additionally, one coupon from each triplicate was stained and imaged, as described above, with the exception the z-step size was maintained at $0.5 \mu\text{m}$. Data were entered into Microsoft Excel and analyzed in Excel and R (RStudio, the R Foundation for Statistical Computing, Vienna, Austria).

DATA AVAILABILITY

We confirm all data and images are available from the corresponding author upon request.

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AUTHOR CONTRIBUTIONS

G.S. developed methods for testing, designed and built the testing systems, developed the tap cleaning method and destruction protocols, assisted with analysis of tap cleaning data, supervised storage container laboratory work, and completed literature review. D.L. supervised protocol development, wrote/collated manuscript drafts, completed literature review, and obtained funding for research. HBadr completed literature review. H.B., Y.K., T.T., and M.J. developed storage container cleaning protocol, completed storage container laboratory work and analysis,

completed literature review, and wrote up storage container data. P.M. contributed to tap cleaning protocol and completed four laboratory runs for the tap cleaning protocol and assisted with tap cleaning analysis. M.D. ran/optimized the first tap cleaning run, modified protocols, supervised all laboratory work for tap cleaning, assisted with tap cleaning analysis, and completed literature review. All authors reviewed and edited manuscript drafts.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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