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It's a trap! The development of a versatile drain biofilm model and its susceptibility to disinfection

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SUMMARY

Background: Pathogens in drain biofilms pose a significant risk for hospital-acquired infection. However, the evidence of product effectiveness in controlling drain biofilm and pathogen dissemination are scarce. A novel in-vitro biofilm model was developed to address the need for a robust, reproduceable and simple testing methodology for disinfection efficacy against a complex drain biofilm.

Methods: Identical complex drain biofilms were established simultaneously over 8 days, mimicking a sink trap. Reproducibility of their composition was confirmed by next-generation sequencing. The efficacy of sodium hypochlorite 1000 ppm (NaOCl), sodium dichloroisocyanurate 1000 ppm (NaDCC), non-ionic surfactant (NIS) and peracetic acid 4000 ppm (PAA) was explored, simulating normal sink usage conditions. Bacterial viability and recovery following a series of 15-min treatments were measured in three distinct parts of the drain.

Results: The drain biofilm consisted of 119 mixed species of Gram-positive and -negative bacteria. NaOCl produced a >4 $\log_{10}$ reduction in viability in the drain front section alone, while PAA achieved a >4 $\log_{10}$ reduction in viability in all of the drain sections following three 15-min doses and prevented biofilm regrowth for >4 days. NIS and NaDCC failed to control the biofilm in any drain sections.

Conclusions: Drains are one source of microbial pathogens in healthcare settings. Microbial biofilms are notoriously difficult to eradicate with conventional chemical biocidal products. The development of this reproducible in-vitro drain biofilm model enabled understanding of the impact of biocidal products on biofilm spatial composition and viability in different parts of the drain.

Introduction

The hospital water environment has been recognized as a reservoir of harmful pathogens. Sinks and taps can be a transmission source of dangerous bacteria, including carbapenem-resistant Enterobacteriaceae (CRE) [1], multi-drug-resistant Gram-negative bacilli [2] and extended-spectrum β-lactamase (ESBL)-producing Enterobacterales [3].

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Splashes from a contaminated sink can reach up to 1 m away from the sink [1], posing a threat to patients in close proximity. The main reservoir of pathogens resides in sink traps and U-bends of sinks that are constantly rich in nutrients and constantly hydrated. According to Kotay *et al.* [4], it takes only 7 days for *Escherichia coli* culture to travel from the contaminated P-trap to the strainer. An increase in hospital outbreaks linked to sinks is raising concerns [4].

Evidence of product effectiveness in controlling drain biofilms, pathogen dissemination and biofilm regrowth is limited. Interventions to tackle drain-associated nosocomial infections include covering the drains, replacing contaminated reservoirs and enhancing disinfection procedures (e.g. use of acetic acid). It is difficult to establish the most effective intervention as a combined approach is taken in most clinical cases. Gordon *et al.* [5] investigated 66 sink-related infection control interventions and found that only nine resulted in outbreak cessation and elimination of bacteria from the drain system. Twenty-two interventions only managed to discontinue the outbreak. This means that more than half of all infection control measures were not effective. Ineffective treatments included alcohol, bleach, chlorine, pressurized steam, hydrogen peroxide, silver nitrate and sodium hydroxide [5,6].

Controlling bacteria in drains is particularly challenging as they attach and grow as a biofilm, a complex microbial community that is highly resistant to disinfection [7]. Control of drain biofilm may thus be difficult to achieve. Efficacy of an intervention is measured as a decrease in bacterial viability following treatment. Biofilm regrowth and damage to the biofilm structure is rarely reported [1].

The aim of this study was to develop a robust, reproducible and reliable in-vitro model imitating the formation of a drain biofilm in a sink trap. Reproducibility of the drain biofilm allowed the performance of standardized disinfectant testing mimicking the use of a product in practice, giving valuable information about the bactericidal efficacy of various products against a mixed species drain biofilm, together with their impact on biofilm structure and biofilm regrowth after treatment.

Methods

Drain biofilm material

Drain biofilm material was collected from a sink U-bend from a common room at the School of Pharmacy and Pharmaceutical Sciences, Cardiff University. The room was used by members of staff for dishwashing, handwashing, meal preparation and beverage preparation.

To create drain culture suspension, dense drain biofilm material (4.5 g) was diluted in phosphate buffered saline (PBS; Fisher Bioreagents, Fisher Scientific, Loughborough, UK) at 1:10 ratio and stored in a freezer at -20°C in 25% glycerol (Fisher BioReagents; Fisher Scientific).

Drain biofilm formation

Five millilitres of drain culture suspension (4.5 g of drain biofilm material mixed with 45 mL PBS) was further diluted in 45 mL 10% tryptone soya broth (TSB; Oxoid, Thermo Fisher Scientific, Newport, UK). To obtain inoculum of high cell density, the solution was grown slowly at ambient temperature (21°C) for 3 days. Following 3 days of growth, the high-density inoculum was placed in a sterile silicon rubber platinum-cured tube (outer diameter ø9 mm, inner diameter ø6 mm) (Fisherbrand; Fisher Scientific). To do so, 10 mL of high-density inoculum was pipetted into each 41-cm-long tube. Inoculum was left inside tubes for 2 days at 21°C to allow initial microbial attachment (inoculation phase).

Drain biofilm growth: trap model

Following the inoculation phase, the inoculum was drained out of the tubes. Each tube was cut into three sections (front, middle and back) and fitted into sterile 100-mL Duran clear glass laboratory bottles (Fisher Scientific) with GL45 screw cap twin hose connectors (Fisher Scientific) that imitated the construction of the sink trap (Figure S1, see online supplementary material). The inlet of each front section tube was connected to 10% TSB media via separate sterile media supply tubing, and the outlet of each back section tube was connected to waste via sterile waste collection tubing. The 10% TSB media was run through tubes via a FH100M multi-channel peristaltic pump (Fisher Scientific) at 30 rpm (equivalent of 30 mL/min) for 10 s every 2 h for a total duration of 6 days (media supply phase). The arrangement of the middle section tube allowed 56 mL of liquid (1:10 TSB unless otherwise stated) to be trapped inside the bottle. The schematic diagram of the whole drain biofilm model is shown in Figure S2 (see online supplementary material), with an accent on single tubing lines (the maximum pump capacity was six parallel lines). Overall, the complex drain biofilm was formed and grown for a total of 8 days (2 days inoculation phase and 6 days media supply phase).

Products tested

Four commercially available products were tested (Table I). Peracetic acid (PAA) and non-ionic surfactant (NIS) are specifically intended for drain cleaning in healthcare settings. All products were prepared according to the manufacturers' instructions. PAA came in granulated form and therefore was applied directly into the tubing; mixing PAA product with water induced foaming. The remaining products were in liquid form and were introduced directly through the pump. A 15-min contact time was applied for all products to maintain consistency in methods. Unformulated sodium hypochlorite (NaOCl) 1000 ppm (ACROS Organics; Fisher Scientific) was used as the reference.

Disinfectant testing: efficacy (log_{10} reduction) test — three 15-min doses

All volumes of product were reduced to reflect the smaller scale of the model. The drain biofilm model is a 5.3x in-vitro scale down of a sink with 32-mm-diameter tubing and a 300-mL capacity trap.

PAA was introduced into the ø9-mm tubing directly and wetted by 19 mL sterile water. Nineteen millilitres (100 mL equivalent for common sink) of NaOCl, NaDCC and NIS products were introduced with the peristaltic pump at 30 mL/min ensuring the solution reached the back section of the trap model (Figure S1, see online supplementary material). The

Table I
Characteristics of the products tested

Abbreviation	Form	Main active ingredient[a]	Excipients[a]	Concentration of main active ingredient (ppm)[b]	pH[c]	Application in the drain biofilm model
NaOCl 1000 ppm	Liquid solution	Sodium hypochlorite	n/a	1000	11.31	Through pump
NaDCC 1000 ppm	Liquid solution (tablet dissolved in water)	Sodium dichloroisocyanurate	Adipic acid, sodium toluene sulphonate, sodium n-lauroylsarcosinate	1000	5.93	Through pump
NIS <5%	Liquid solution	Non-ionic surfactants <5%	Perfumes	Not given	6.45	Through pump
PAA 4000 ppm	Granules mixed with water – foaming liquid	Peracetic acid	Sodium percarbonate, citric acid, sodium lauryl sulfate	4000[d]	8.12	Directly into the ø9-mm tube

NaOCl, sodium hypochlorite; NaDCC, sodium dichloroisocyanurate; NIS, non-ionic surfactants; PAA, peracetic acid.
[a] Main active ingredient and excipients mentioned in the material safety data sheet information of the commercial products used in this study.
[b] Concentration of available chlorine/peracetic acid concentration was measured with Pocket Colorimeter (HACH, Manchester, UK) (regardless of the product claim on label) via diethyl paraphenylene diamine method.
[c] pH was measured by FiveEasy Standard pH Meter (Mettler-Toledo Ltd, Leicester, UK).
[d] Initial concentration on application.

solution was left in the tubing for 15 min and then neutralized with Dey-Engley broth (DE broth; Neogen Corporation, Ayr, UK) for 5 min. Neutralization was performed by introducing the neutralizer with the peristaltic pump, ensuring the solution reached the whole length of the tubing. To reflect the use of products in practice, one 15-min dose of disinfectant was applied once per day for 3 days between each treatment (3 x 15 min), and the drain biofilm was supplied with sterile water (instead of 10% TSB) at 30 mL/min for 10 s every 2 h at 21°C. Following the last 15-min dose and neutralization step, the liquid was drained out from the system and tubing was disconnected. One-centimetre lengths of the silicone tubing were cut with sterile scissors from each section (i.e. front, middle and back) of the trap model.

Each tubing section (length 1 cm, outer diameter ø0.9 cm) was sliced open and placed in a sterile McCartney bottle containing 2 mL DE broth with 100 mg/mL proteinase K (Fisher Bioreagents; Fisher Scientific) and 1 g glass beads (Fisher Scientific), incubated for 1 h at 37° and then vortexed for 2 min. One hundred microlitres was serially diluted and 100 μL was plated on to tryptone soya agar (TSA; Oxoid) in two technical replicates. The viable count was read after overnight incubation of the TSA plates at 37°C, and $\log_{10}$ reduction was calculated in relation to untreated drain biofilm samples.

Disinfectant testing: 4-day regrowth test

Drain biofilm regrowth was tested 4 days after the three 15-min doses. The tubing was connected to sterile water between doses and during the regrowth period, with 10-s flushes at 30 rpm every 2 h at 21°C for up to 4 days. Four days after the last treatment, the liquid was drained out from the system and the tubing was disconnected. One-centimetre lengths of tubing were sampled from each section and processed as described above.

Next-generation sequencing

The DNA composition of the drain biofilm trap model was investigated using next-generation sequencing (NGS). Following the establishment of a complex drain biofilm for a total of 8 days, tubing sections (i.e. front, middle and back; Figure S1, see online supplementary material) were cut and biofilm was recovered as described above. DNA was extracted using Invitrogen PureLink Genomic DNA Mini Kit (Fisher Scientific) according to the manufacturer's instructions. Extracted DNA was quantified using the Quant-iT dsDNA Assay Kit, broad range (Fisher Scientific) according to the manufacturer's instructions.

NGS and quality analysis of FASTQ sequence reads were performed by BaseClear Group (Leiden, The Netherlands). To identify bacterial and archaeal isolates, the 16S rRNA gene (V3–V4) was polymerase chain reaction amplified before sequencing. The Illumina MiSeq (PE300) system was used to generate paired-end sequence reads, and blc2fastq2 2.18 software was used to produce FASTQ sequence files. Reads were filtered and clipped. Raw sequences were analysed with open source software Edge Bioinformatics (v.2.0.0).

Scanning electron microscopy analysis

One-centimetre sections of tubing were cut in half lengthwise and incubated overnight in 2.5% glutaraldehyde solution

(Contain; Fisher Scientific), followed by immersion in successive concentrations of ethanol for 10 min each (10%, 25%, 50%, 70%, 90%, 100%). Prior to scanning electron microscopy scanning, samples were coated with 20 nm AuPd coating with sputter coater (SC500; Biorad, Hercules, CA, USA). Secondary electron images were acquired with a beam energy of 5 kV using an in-lens detector on a Sigma HD field emission gun scanning electron microscope (Carl Zeiss Ltd., Cambridge, UK) at 1000x magnification and 5–7-mm working distance with the help of the Earth and Ocean Sciences Department, Cardiff University, UK. The images were false-coloured using GNU Image manipulation program (GIMP 2.8) software. The images were not otherwise altered.

Statistical analysis

Statistical significance of data sets was evaluated with GraphPad PRISM (v. 7.04) using single-way analysis of variance (ANOVA). The statistical analysis was performed at a 0.05 confidence level ($P<0.05$), comparing the effect of different disinfectant treatments on drain biofilm viability. The standard deviation of environmental and technical replicates was evaluated with Bassel's correction. All measurements, if not stated otherwise, were performed in triplicate. Each triplicate was plated in two environmental replicates.

Results

Drain biofilm viability

There was no significant difference (single-way ANOVA, $P=0.8295$) in viable aerobic counts between the front, middle and back tubing sections in the trap model from seven independent drain biofilm batches. The average total bacterial numbers recovered from the front, middle and back sections were 8.3 ± 0.6, 8.5 ± 0.7 and 8.3 ± 0.7 $\log_{10}$ colony-forming units (cfu)/cm^2, respectively.

Drain biofilm development and appearance

During the 2-day inoculation phase, the drain biofilm culture adhered to the silicone tubing. Under no-flow conditions, bacteria grow in evenly scattered firm clusters (Figure 1). The biofilm was then subjected to periodical flushes when 10% TSB was supplied at 30 mL/min for 10 s every 2 h. Bacteria started to establish biofilm communities, which can be observed as a dense matrix with distinctive extracellular polymeric substances (EPS) (Figure 1). After 2 days of periodically flushing the media through drain biofilm, bacteria covered a larger surface area. At the end of the 8th day, the drain biofilm established a rigid thick layer of tightly embedded cells (Figure 1).

Drain biofilm composition

The composition of the drain biofilm in the front, middle and back sections from three independent batches is given in Table S1 (see online supplementary material). Overall, 119 different species were identified, with 76 species detected in two or more samples. The most prevalent species were *Klebsiella oxytoca* (12–33%, mean 22%), *Escherichia coli* (3–47%, mean 20), *Klebsiella pneumoniae* (10–27%, mean 19%), *Serratia marcescens* (0.001–33%, mean 10%), *Enterobacter cloacae* (4–10%, mean 8%), *Salmonella bongori* (2–16%, mean 5%), *Erwinia pyrifoliae* (0.4–9%, mean 4%) and *Klebsiella aerogenes* (2–6%, mean 4%).

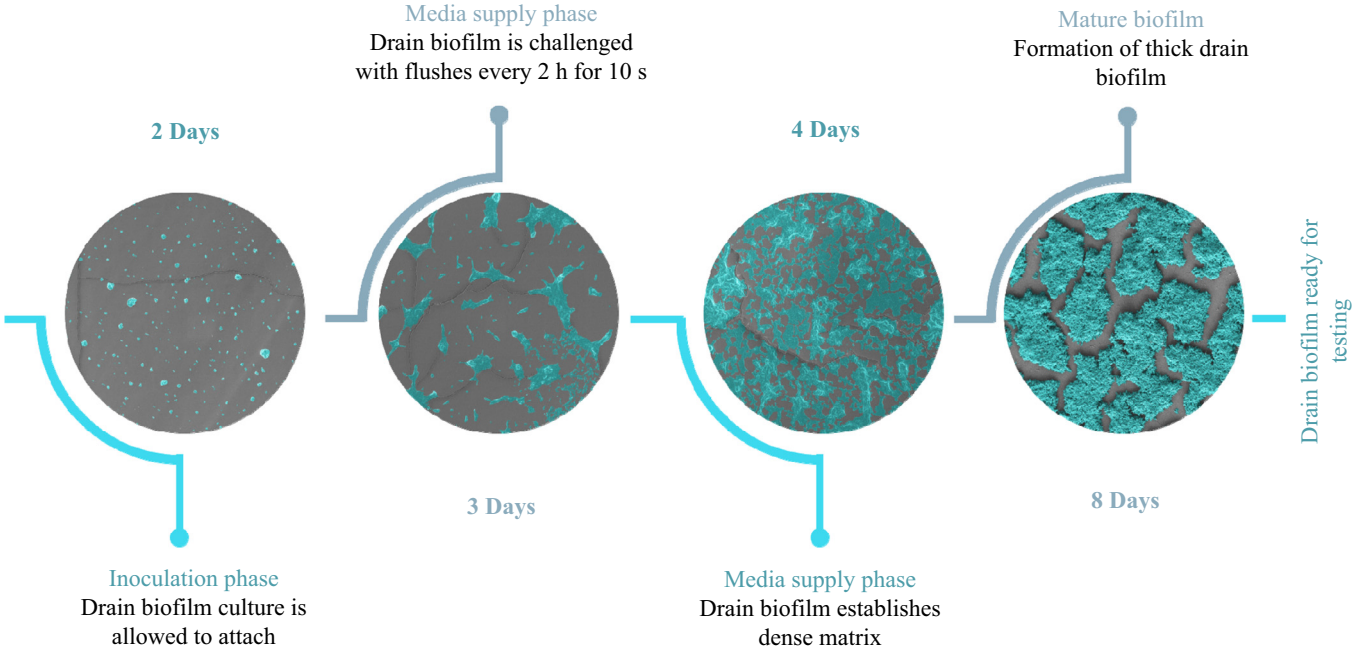

Figure 1. Drain biofilm development over time. Images are representative observations from three independent drain biofilm batches.

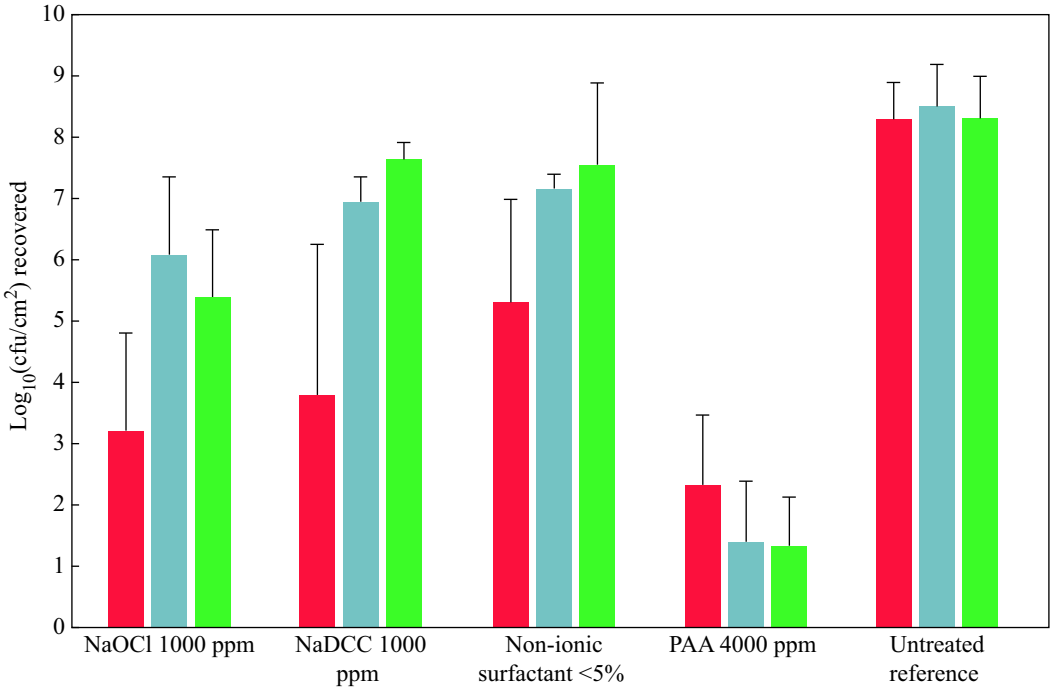

Figure 2. Total viable bacteria recovered (log_{10}) from different sections of the model following product treatment (three 15-min doses). Red bars, front section; blue bars, middle section; green bars, back section. cfu, colony-forming unit.

Product efficacy: decrease in bacterial viability in the trap model after three 15-min doses

In practice, drain treatments are often repeated on different dates or are applied more frequently when the product is being used for the first time to eradicate the heavy biofilm that accumulated in untreated drains for a prolonged period of time. After three consecutive 15-min daily doses, NaOCl (1000 ppm), NaDCC (1000 ppm) and PAA (4000 ppm) treatments were effective at killing bacteria in the drain biofilm in the front section of the trap model (5.1, 4.5 and 6.0 log_{10} reduction in

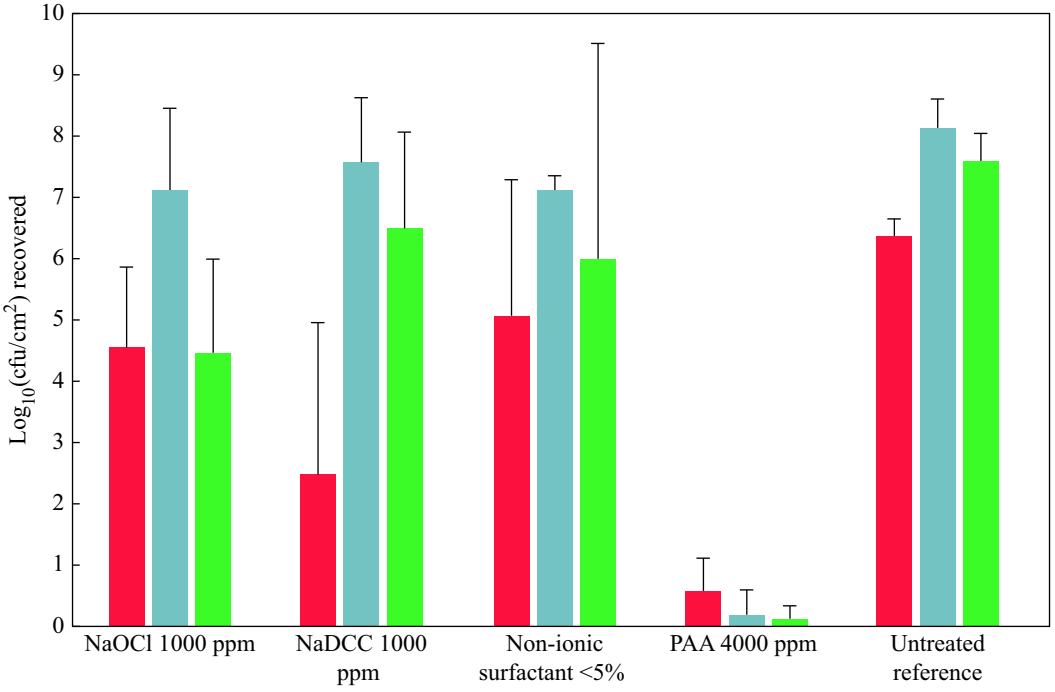

Figure 3. Log_{10} colony-forming units (cfu)/cm^2 of bacteria recovered from biofilm 4 days after a series of three 15-min treatments. Red bars, front section; blue bars, middle section; green bars, back section. cfu, colony-forming unit.

bacterial viability, respectively). NIS <5% treatment decreased viability by 3.0 $\log_{10}$ in the front section alone (Figure 2).

The performance of most disinfectants in treating the complex biofilm in the middle and back sections of the trap model was lower than their performance in the front section. NaOCl (1000 ppm), NaDCC (1000 ppm) and NIS (<5%) products decreased biofilm viability by 2.4, 1.6 and 1.4 $\log_{10}$ reduction in the middle section, and 2.9, 0.7 and 0.8 $\log_{10}$ reduction in the back section, respectively. PAA (4000 ppm) with three 15-min doses performed very well in the middle and back sections: 7.1 $\log_{10}$ reduction in the middle section and 7.0 $\log_{10}$ reduction in the back section, respectively (Figure 2).

Product efficacy: 4-day regrowth test

Biofilm recovered slowly from the front section (Figure 3), while biofilm recovery was rapid in the middle and back sections, notably for NaOCl (1000 ppm) with a *c.* 2 $\log_{10}$ increase in number. Not surprisingly, the bacterial number in all three drain sections remained high 4 days after NaDCC (1000 ppm) or NIS treatment (Figure 3). Four days after treatment with PAA (4000 ppm; three 15-min doses), bacterial viability within the drain biofilm remained low (0.6 ± 0.5, 0.2 ± 0.4 and 0.1 ± 0.2 $\log_{10}$ recovered from front, middle and back sections, respectively) and was significantly lower compared with the other treatments (single-way ANOVA, $P<0.05$ for all treatments and trap sections, with the exception of NaDCC for the front section with $P=0.1878$).

Discussion

Drain biofilm model

In this in-vitro model, a mature complex biofilm was grown for 8 days. This biofilm formation period is a compromise between allowing the development of mature biofilm and enabling the rapid testing of disinfectants. It is acknowledged that this does not represent a complex drain biofilm formed over years in hospital water lines [4].

Looking at biomass, initial attachment and EPS production, Andersson *et al.* showed that the strongest biofilm is formed by a mixture of 13 various species, as opposed to single or dual species biofilms of the same strains [8]. Bacteria thrive in a highly diverse community, and such varied complex biofilms will be found in hospital drain traps [9]. The drain biofilm formed in the model is a multi-species community, mainly composed of Gram-negative bacteria that belong to the Enterobacterales family. Few of the species present in the drain biofilm culture were reported as linked to drain-associated hospital infections, including: *K. pneumoniae* [10], *K. oxytoca* [11], *E. cloacae*, *K. aerogenes*, *Enterobacter asburiae* [12], *Raoultella ornithinolytica* [3] and *S. marcescens* [13]. Some of the species reported by McBain *et al.* [14] in their study on domestic drains were also isolated from the sink U-bend in the present study. It needs to be stressed, however, that the 16S rRNA gene sequencing used in this study is limited to the detection of bacterial and archaeal isolates. Therefore, the role of fungal species in the drain biofilm model composition remains unknown.

No distinctive difference in drain biofilm composition was observed by NGS between the front, middle and back sections of the trap model.

Drain biofilm susceptibility to disinfection

Elimination of bacteria from all sections of the drain biofilm model is essential to prevent the spread of pathogens, as bacteria from drain biofilm can migrate to the entire drain [1,4,15]. Successful disinfection treatment should therefore be able to control all parts of the drainage system effectively. It is important to conduct tests with drain system models that imitate those parts, just like the model developed in this study. This study investigated the effect of the disinfectant treatments on the viability of drain biofilm following three 15-min doses; however, it needs to be stressed that the amount of biomass detached by the action of disinfectant was not investigated. Therefore, the efficacy of the disinfectants presented here might result from their ability to inactivate cells, but also from their effect on the extracellular matrix, contributing to drain biofilm detachment.

This study showed that biofilms in the trap and back section were not controlled by NaOCl (1000 ppm), following three 15-min daily doses. In addition, NaOCl (1000 ppm) disinfection wore off quickly, with drain biofilm recovering within days post treatment. Poor performance of chlorine was shown in another study, where only 2 $\log_{10}$ reduction against drain biofilm was achieved with 6% NaOCl treatment [16]. During a drain-associated *Acinetobacter baumannii* outbreak, disinfection with NaOCl five times per day was not effective and the outbreak was only controlled when sinks were replaced [17]. Sodium hypochlorite solution was also not effective in stopping a drain-related *A. baumannii* outbreak in France [18] or a *K. pneumoniae* outbreak in Spain [19].

Other studies have reported that bleach was effective to combat hospital-drain-related infections, although complex interventions were implemented. Disinfection with 0.1% sodium hypochlorite was part of an intervention undertaken to successfully tackle an *A. baumannii* outbreak at the National Taiwan University Hospital [20]. Similar results were obtained by La Forgia *et al.*, where a weekly cleansing protocol of the whole drainage system with diluted sodium hypochlorite reduced *A. baumannii* infection rates significantly [21].

NaDCC has been considered safer than NaOCl when used in tablet form [22]. Some manufacturers recommend using their NaDCC tablets for drain cleaning, and their advised concentration of available chlorine ranges from 250 to 500 ppm. Peer-reviewed information on the efficacy of NaDCC on the eradication of drain biofilm is scarce. This study showed that 1000 ppm NaDCC was not effective against the biofilm in all of the drain sections, even with three 15-min daily doses.

PAA (4000 ppm) was very effective on the model in all drain sections. Other studies have reported the efficacy of PAA against biofilm formed on PVC piping [23] and against *E. coli* biofilm from a drinking water pipeline system [24]. The formation of foam following mixing the PAA product with water may have contributed to enhanced efficacy. Foam products have been found to be more effective in drain decontamination compared with their liquid equivalents [25].

As shown in this study, biofilms in drains regrow quickly even after treatment that effectively reduces biofilm viability. Similar findings were shown in the study by Jones *et al.*, where drain biofilm regrew to its initial concentration within 7 days of treatment with four different foaming products [25]. Buchan *et al.* showed that biofilm recovered fully within 7 days of bleach or H_2O_2 treatment [16]. Rapid recovery of drain biofilm is an unavoidable fact that underlines the importance of frequent disinfection.

Modelling complex drain biofilm in sink trap

The drain biofilm model developed in this study is a cheaper alternative to performing in-vivo studies, when products are tested in full-scale sinks. The model allows six lines of identical drain biofilms to form and grow simultaneously, allowing for many testing possibilities, notably with realistic sink usage scenarios with the introduction of liquids commonly poured down hospital sinks (e.g. intravenous fluid, coffee with sugar, urine, etc.). The trap model generates much less pathogenic waste and is less prone to cross-contamination due to its contained structure compared with an actual sink (Table S2, see online supplementary material). It also occupies significantly less space, thanks to its compact design (an alternative of six identical full-scale sinks could take up a whole room).

However, the model does have limitations. In a standard sink design, the trap outlet pipe is located at a lower level than the trap inlet pipe. Intensively foaming product would therefore escape entirely through the output pipe, and would not reach the strainer. The inlet tube in the trap model is located 3 cm above the outlet tube; however, some of the PAA product still foamed up to the front part. Moreover, the front section does not reflect the complex structure of a sink strainer. The material used as the biofilm attachment substrate was chemically different to PVC, PP and other plastics commonly used as drainage pipes. As there are limited studies on the effectiveness of disinfection against drain biofilms, the model would also need to be validated against full-scale sinks.

In conclusion, the in-vitro drain biofilm model presented in this study allowed for reproducible testing against a complex biofilm, including measuring viability, composition, regrowth after treatment, and biofilm structure in different parts of the drain. The disinfection susceptibility test showed that bleach, in widespread use, is only partially effective against drain biofilm, with good efficacy in the front section following three consecutive doses but no substantial reduction in bacterial viability in the middle and back sections of the model. Moreover, biofilm had recovered steadily 4 days after the last dose of sodium hypochlorite 1000 ppm. On the contrary, three consecutive 15-min doses of peracetic acid 4000 ppm were highly successful at eradicating and preventing biofilm regrowth in every part of the drain model.

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Conflict of interest statement
K Ledwoch is employed part-time by GAMA Healthcare Ltd, and P. Norville is an employee of GAMA Healthcare Ltd. GAMA Healthcare Ltd, the funder of this project, is also the manufacturer of the tested PAA drain product.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2020.08.010.

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