Dr Katarzyna Ledwoch

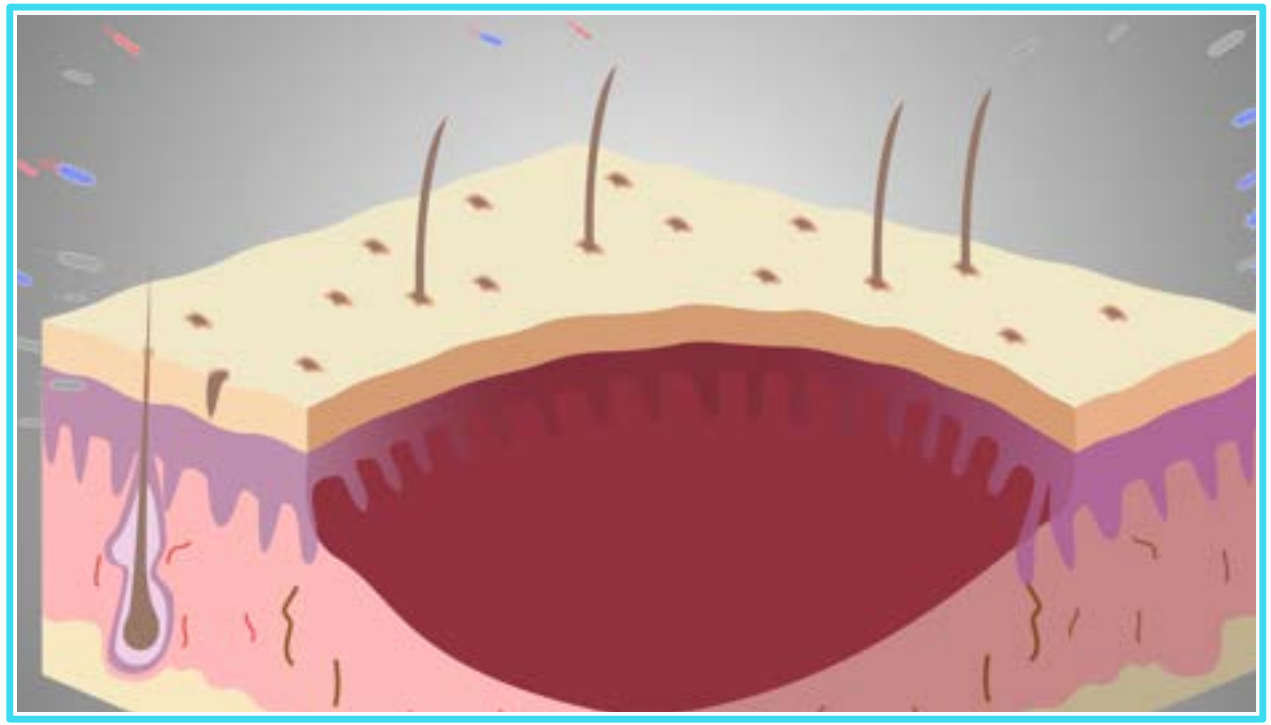
gama healthcare

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

What is biofilm?

- **Complex microbial communities**
- Microbial cells grow together attached to a surface/ forming aggregates
- Low metabolism
- Low motility
- Improved exchange of nutrients, information, DNA
- Phenotypic diversity
- **More resistant** than planktonic bacteria

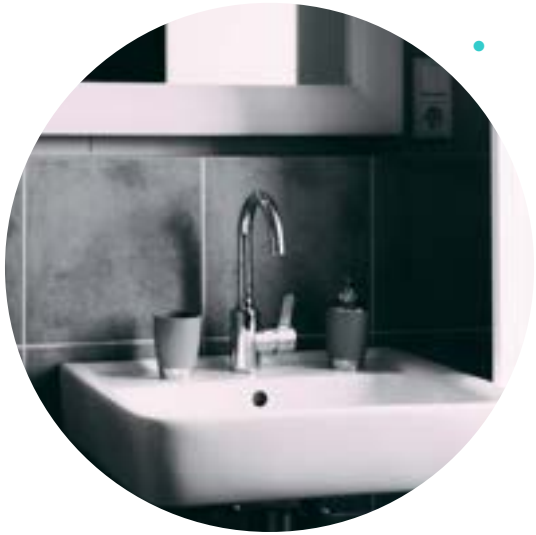
BIOFILM



Wound biofilm
Source: B. Braun International

Environmental biofilms in hospitals

- Dry biofilms on hospital surfaces (DSB)



- Wet/hydrated biofilms in drain systems

- Semi-dry biofilms on medical devices

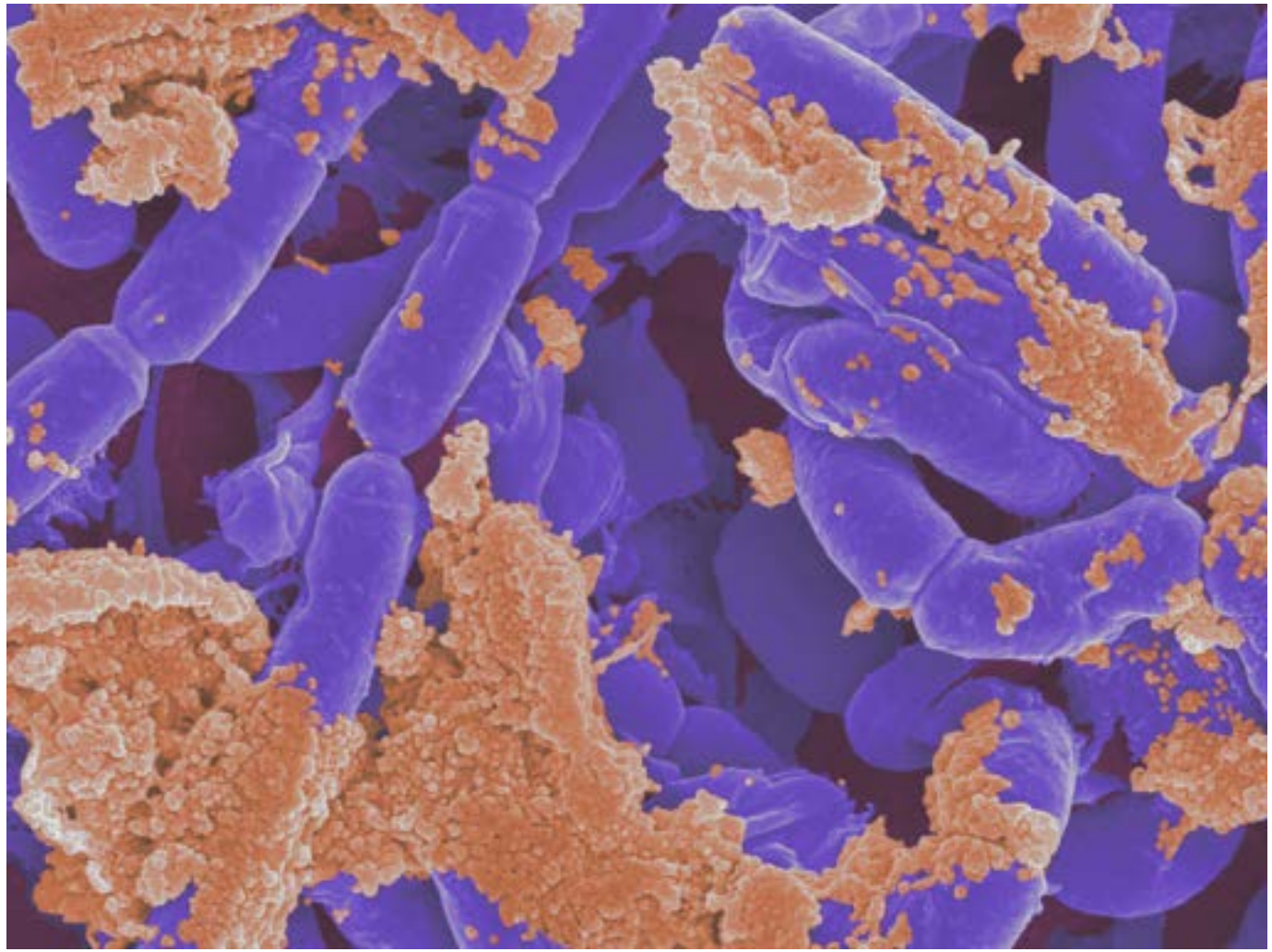
Environmental biofilms in hospitals

- Dry biofilms on hospital surfaces (DSB)

- Wet/hydrated biofilms in drain systems

- Semi-dry biofilms on medical devices

What is dry surface biofilm (DSB)?

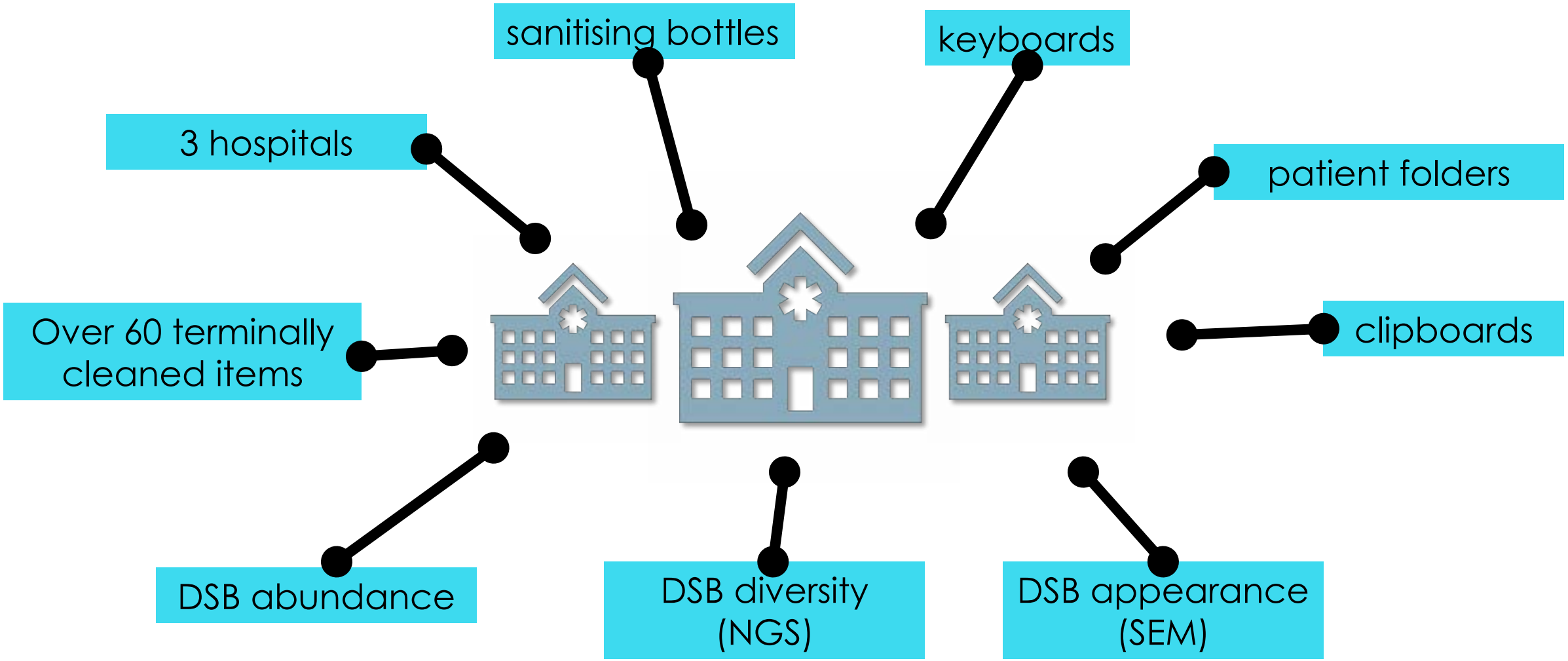


SEM image of DSB containing mixed hospital culture grown on stainless steel disc

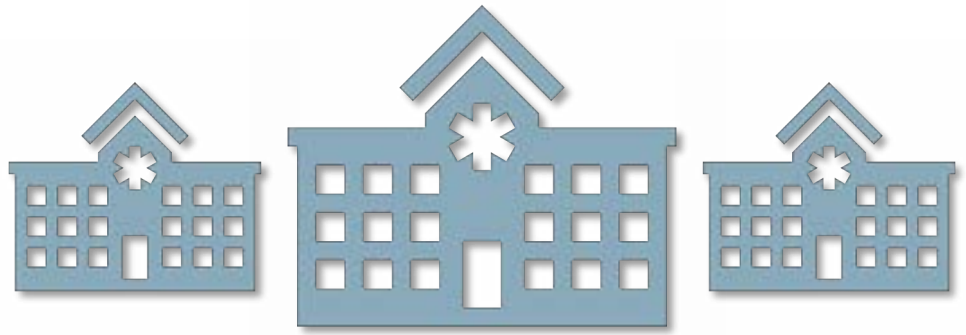
- Dry surface biofilms are **complex microbial communities** formed and grown in dry habitats.
- Much **less attention** has been paid to dry biofilms compared to most commonly researched wet/hydrated biofilms
- DSB colonize **various materials** from textile (chair), hard surfaces including plastic (PVC, PP), lacquered wood, wood, metal (stainless steel) to many others
- Dry biofilms have been isolated from diverse environmental conditions: **low moisture**, **varying temperature** and **nutrients levels**.

Ledwoch et al (2018) J Hosp Infect 100:3:e47-e56

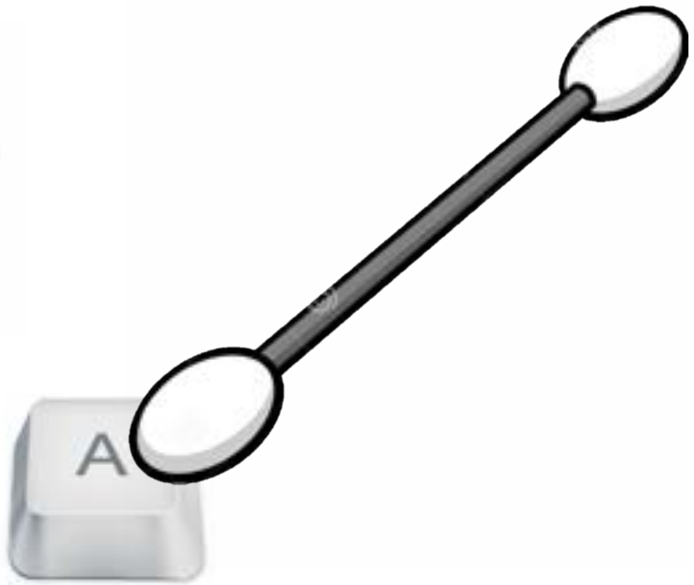
Multicentre study



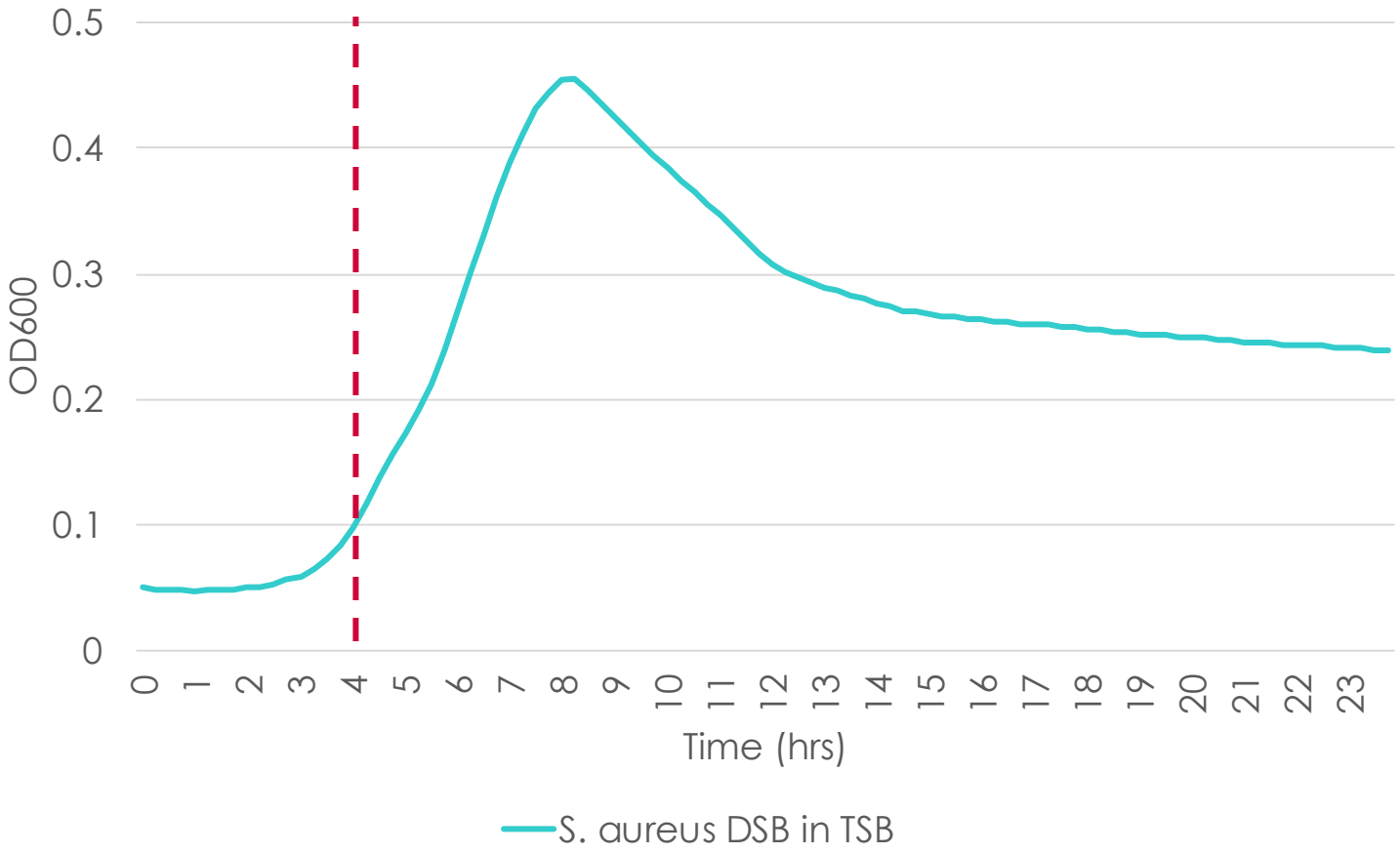
DSB abundance - swabbing



0%



DSB abundance – metabolic activity of DSB



Isabella Centeleghe
PhD student
Cardiff University

DSB abundance – recovery in broth

95%

DSB appearance: SEM

Image source: https://panolam.com

DSB appearance: SEM

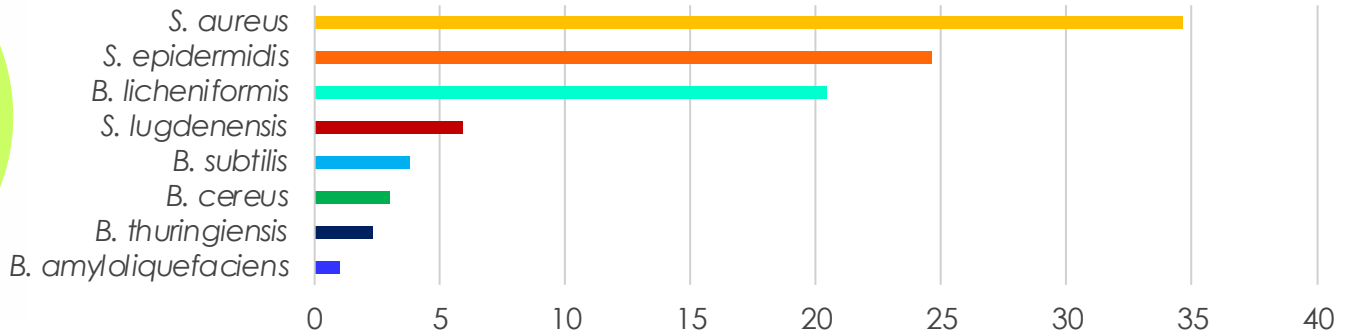
Image source: https://www.medaptus.com

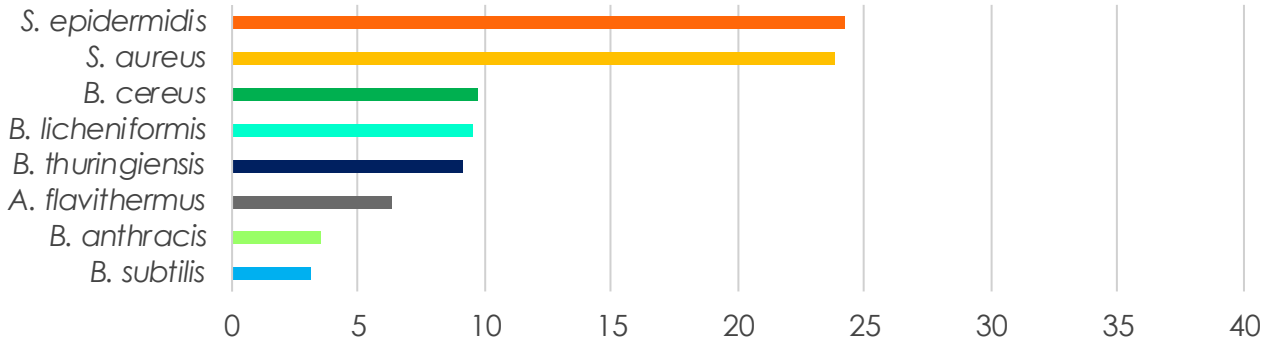
DSB composition: NGS

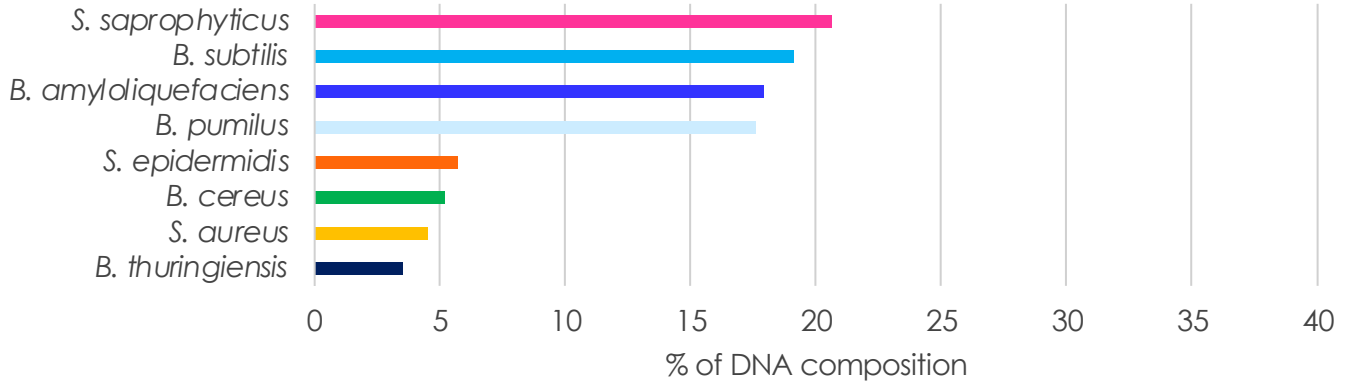
A

B

C







DSB in healthcare settings

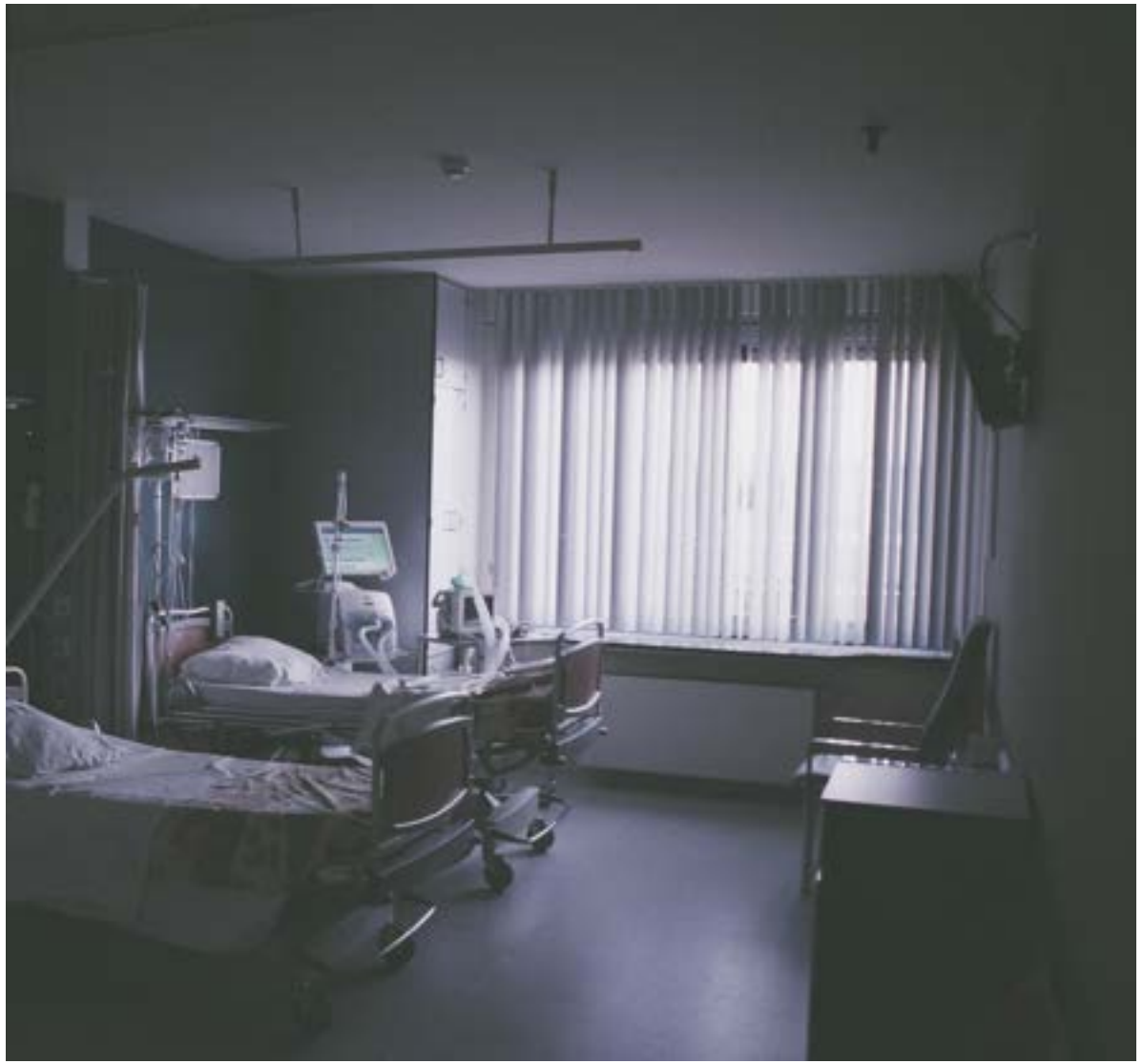
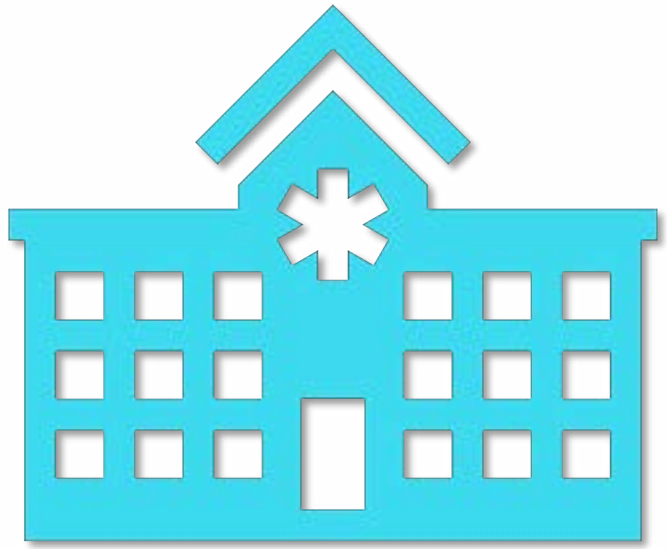


Image source: Unsplash

- Dry surface biofilms are **widespread** on surfaces in hospitals
- DSB contribute to pathogens survival and HAIs **despite cleaning and disinfection**
- DSB **cannot be detected** by swabbing or contact plates
- All biofilms harboured gram-positive bacteria including pathogens associated with HCAIs
- Dry surface biofilms **regrow within one day** when provided with nutrients

Why DSB can be a problem?



- Patient safety
- Staff safety
- Increased mortality
- Hospital credibility
- Can not be detected from dry surface
- Increased hospitalisation costs
- Less responsive to biocidal products

Why DSB can be a problem?

"(…) hand-touch sites are habitually contaminated by hospital pathogens, which are then delivered to patients on hands"
- Dancer (2009) J Hosp Infect 73: 378-385

"Contamination of the inanimate environment around patients constitutes an important reservoir of MRO with the risk of HAI (…)"
- Vickery et al. (2012) J Hosp Infect 80: 52-55

"In 2002, the estimated number of HAIs in U.S. hospitals, (…) was approximately 1.7 million (..). The estimated deaths (…) were 98,987."
-Klevens R, et al. (2007) Public Health Rep 122:160–16

"(…) current cleaning practices are inadequate to control biofilm development."
- Vickery et al. (2012) J Hosp Infect 80: 52-55

"(…) evaluating the clinical effectiveness of cleaning and disinfecting methods is challenging"
- Han et al. (2015) Ann Intern Med 163(8):598-607

Why DSB can be a problem?

TECH EXPLORIST TECHNOLOGY SCIENCE HEALTH SPACE INVENTION INSPIRATION

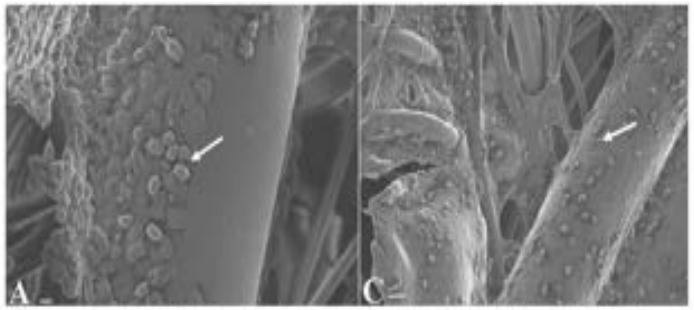
Home › Health › Hospital gowns retain superbugs even after being treated with disinfectant

Health

Hospital gowns retain superbugs even after being treated with disinfectant

The study showed that C difficile - a bacteria that can cause severe diarrhea, bowel complications and even death - is surviving on surgical gowns.

By Pranjal Mehar - July 13th, 2019



Microscope image showing C difficile on hospital gown fibres before (left) and after (right) disinfectant treatment

Surgical gowns and other apparel masks, footwear, gloves) fill two needs: 1. protecting patients from microorganisms conveyed by the surgical team or patients themselves, and 2. protecting healthcare services from contact with irresistible microorganisms harbored by the patient.

However, a new study by the University of Plymouth suggests that surgical gowns used in hospitals retain superbugs. Even after single-use, the gowns had been infected with superbug Clostridium difficile (C difficile), even after being treated with the recommended amount of disinfectant.

Study links hospital Candida auris outbreak to reusable thermometers

Filed Under: Antimicrobial Stewardship; Fungal Infection

Chris Dall | News Reporter | CIDRAP News | Oct 03, 2018

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allanswart / iStock

A large *Candida auris* outbreak at a hospital in England appears to be linked to reusable patient-monitoring equipment, a team of researchers reports today in the *New England Journal of Medicine*.

The outbreak in the neurosciences intensive care unit (ICU) at Oxford University Hospitals involved 70 patients who were infected or colonized with *C auris*, a fungus that has become increasingly resistant to azoles, echinocandins, and polyenes—the three classes of antifungals used to treat infections caused by *Candida* and other fungal species.

An epidemiologic investigation and case-control study by investigators from the University of Oxford, Public Health England, and elsewhere found that the most compelling explanation for the prolonged outbreak was the persistence of the organism on reusable skin-surface axillary probes, a device placed in a patient's armpit for continuous temperature monitoring.

"Our results indicate that reusable patient equipment may serve as a source of healthcare-associated outbreaks of infection with *C. auris*," the authors of the study write.

C auris was first identified in Japan in 2009 and since then has emerged as a significant healthcare-associated pathogen in several countries, causing hospital outbreaks on five continents. The multidrug-resistant fungus can cause severe infections in immunocompromised patients and has been associated with high mortality.

Medscape Wednesday, July 17, 2019

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The Latest Dirt on Hospital Cleanliness

Sonali D. Advani, MD, MBBS, MPH

DISCLOSURES | January 15, 2019

Read Comments

EDITORIAL COLLABORATION Medscape & SHEA

How 'Clean' Is the Hospital Environment?

A recent multistate survey of 183 acute care hospitals in the United States revealed that approximately 1 of every 25 inpatients developed at least one healthcare–associated infection per day.[1] *Clostridioides difficile* was reported as the most common healthcare–associated pathogen, leading to an increased focus on prevention strategies.

A growing body of evidence suggests that hospital surfaces, floors, sheets, sinks, curtains and equipment are contaminated with pathogens that can serve as sources of infection. Rigorous environmental cleaning is required to ensure that hospital surfaces, equipment, and linens are safe for patient use and to prevent transmission of such pathogens as C difficile

Why DSB can be a problem?

Dry surface biofilms are a major contributor of pathogens persistence on surfaces

Many treatments fail to eradicate dry surface biofilms highlighting their high resistance and transferability

We hypothesize that dry surface biofilms play a significant role in Healthcare associated infections

How to solve the problem?

Improved hand hygiene

Image source: Unsplash

Improved cleaning

Image source: http://resistancecontrol.info

Improved monitoring of contamination level

Image source: Unsplash

Disinfectants targeting DSB

Image source: Unsplash

Testing disinfectants

Log reduction

gives the number of bacteria that were removed after treatment

Disinfectants targeting DSB

Image source: Unsplash

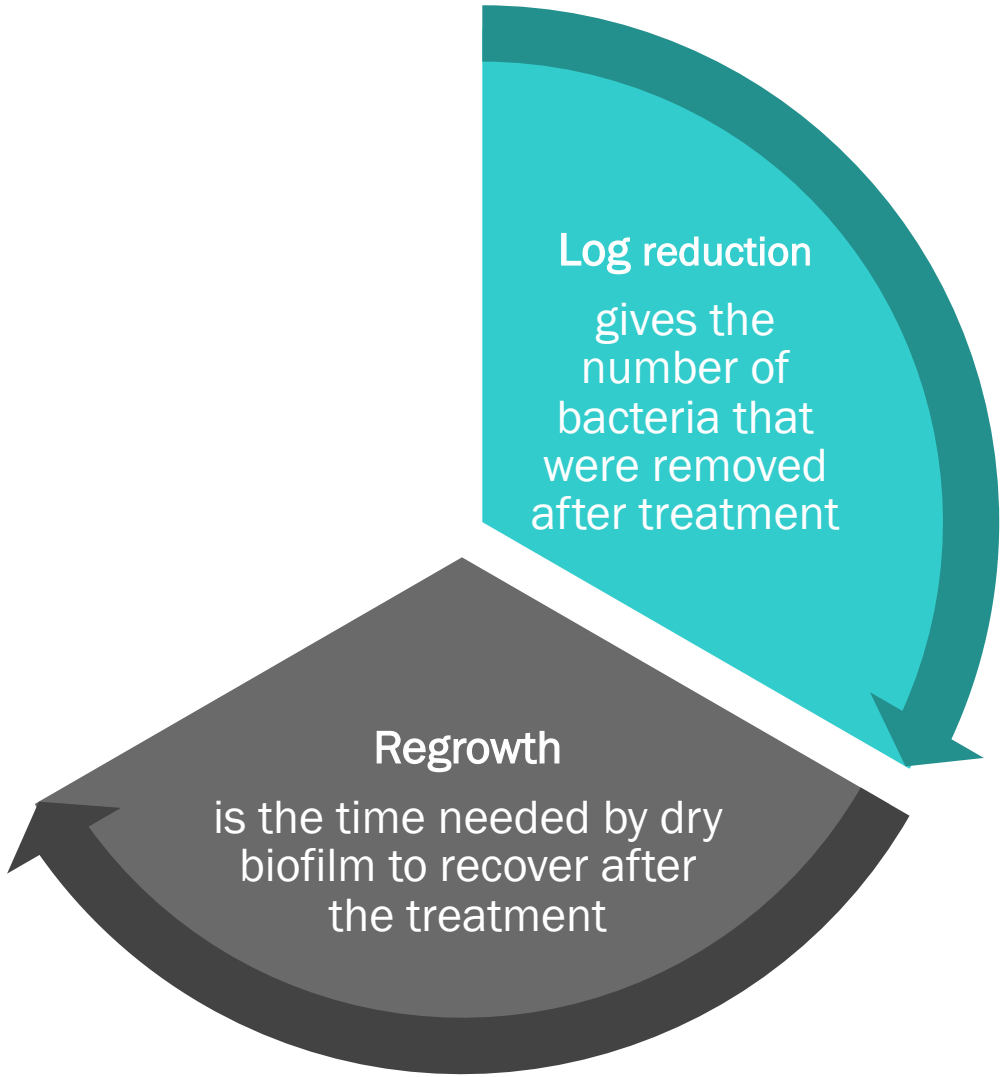
Regrowth

Image source: Unsplash

"Biofilms can reform rapidly; repeated debridement alone is unlikely to prevent biofilm regrowth (…)"
- Schultz et al. (2017) Wound Rep Reg 25:744–757

"(…) any bacteria in the biofilms that survives the sanitizer treatment may initiate biofilm regrowth."
- Han et al. (2017) Front. Microbiol. 8: 988

Testing disinfectants



Disinfectants targeting DSB

Image source: Unsplash

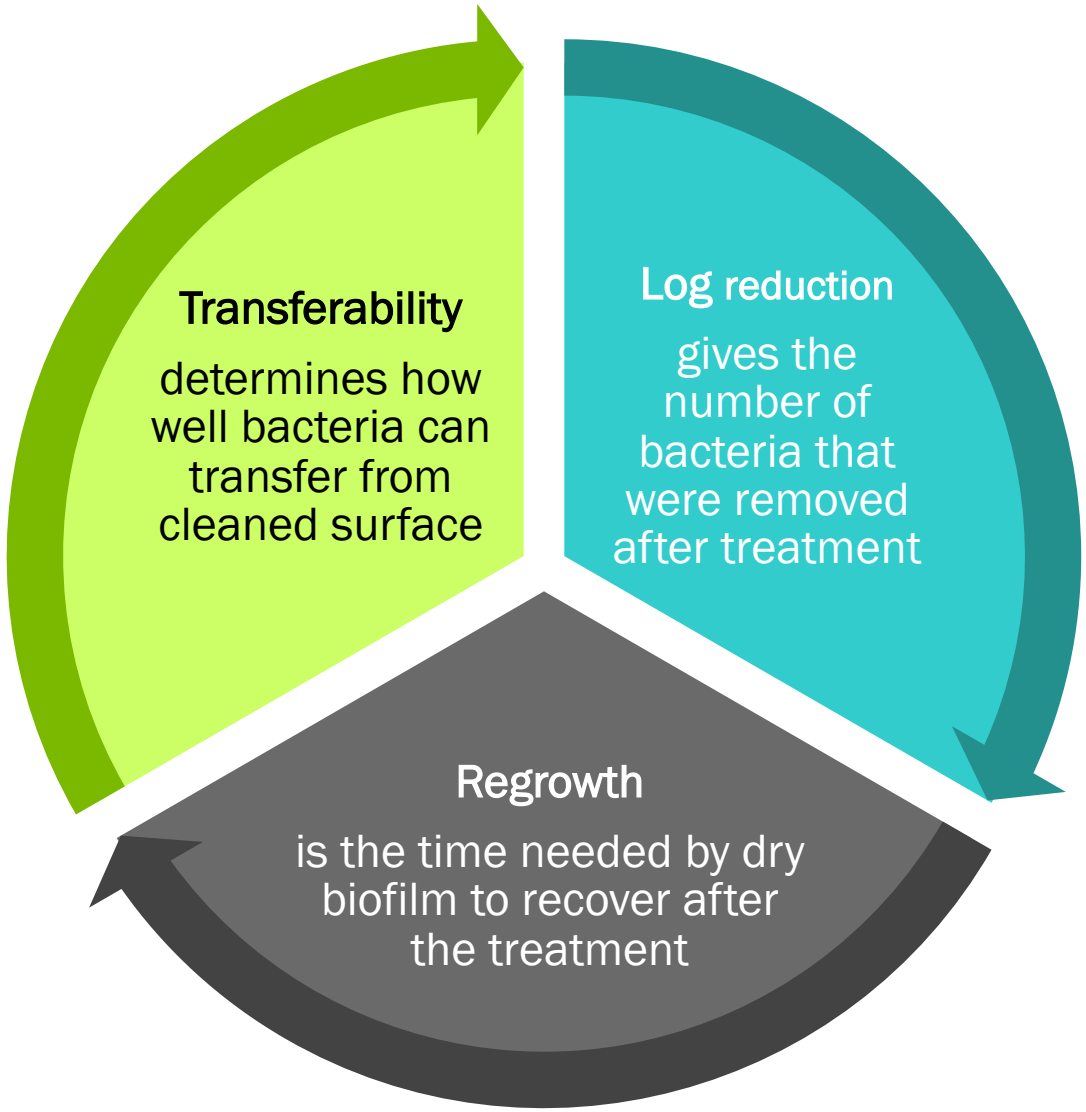
Transferability

"DSB bacteria are transferred by hands from one fomite to multiple fomites, suggesting that DSB may serve as a persistent environmental source of pathogens."
- Chowdhury et al. (2018) J Hosp Infect 100:e85-e90

"Contamination of high-touch environmental surfaces plays an important role in transmission of pathogens in the acute care hospital setting"
- Han et al. (2015) Ann Intern Med 163(8):598-607

"(…) hand-touch sites are habitually contaminated by hospital pathogens, which are then delivered to patients on hands."
- Dancer (2009) J Hosp Infect 73:378-385

Testing disinfectants



Disinfectants targeting DSB

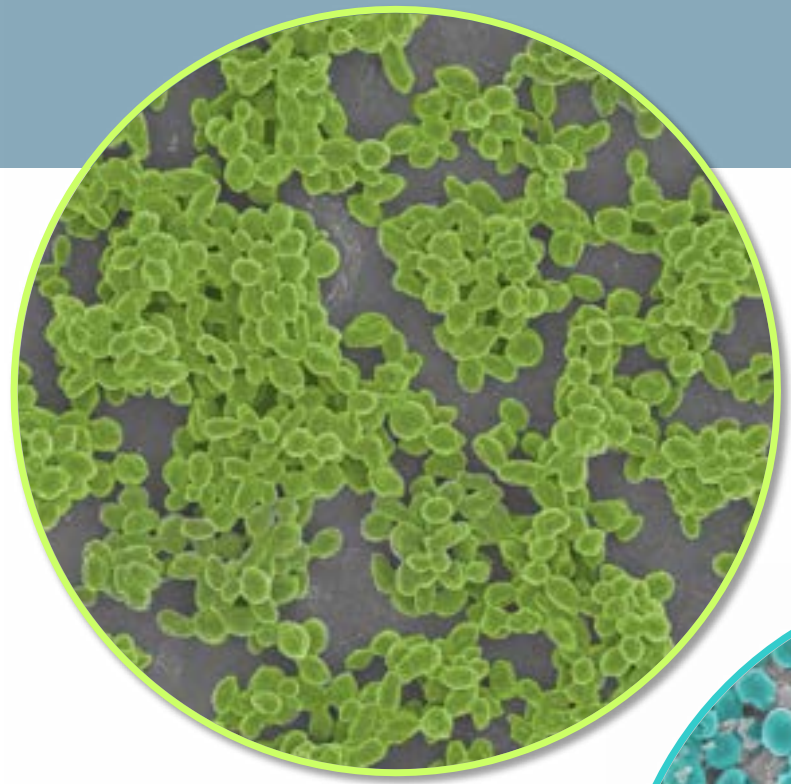
Image source: Unsplash

DSB models

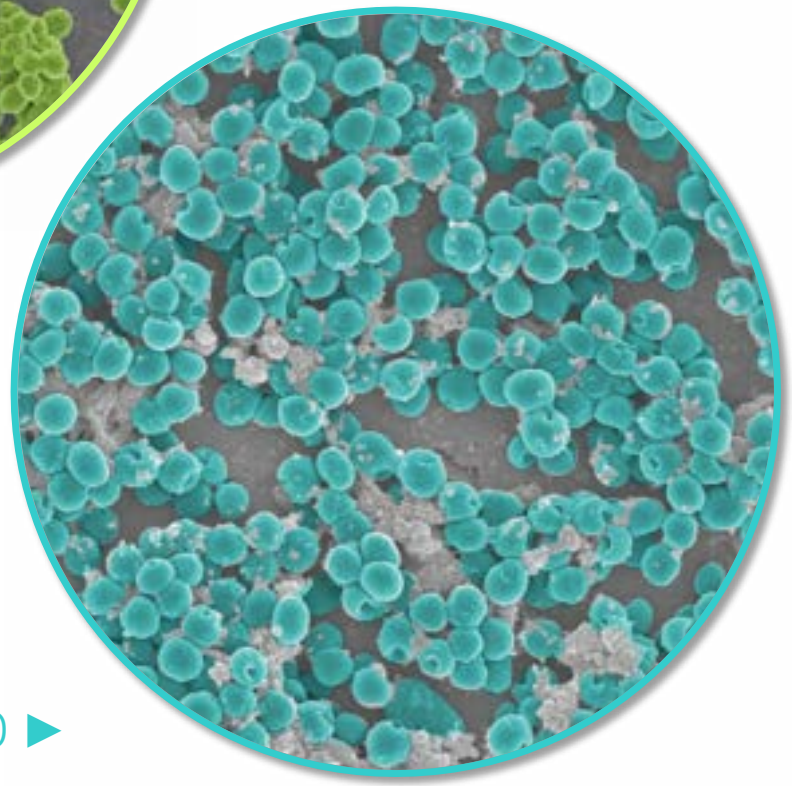
- AISI 430 stainless steel sterile discs
- 10^6 CFU/ml *S. aureus* NCTC 10788
- 10^6 CFU/ml *C. auris* DSM 21092
- 1ml of inoculum per disc
- TSB/MEB + 3 g/L BSA

- 48h wet/dry cycles for of 12 days
- High uniformity ensuring testing repeatability

- More resistant to disinfection treatment than dried bacterial suspension



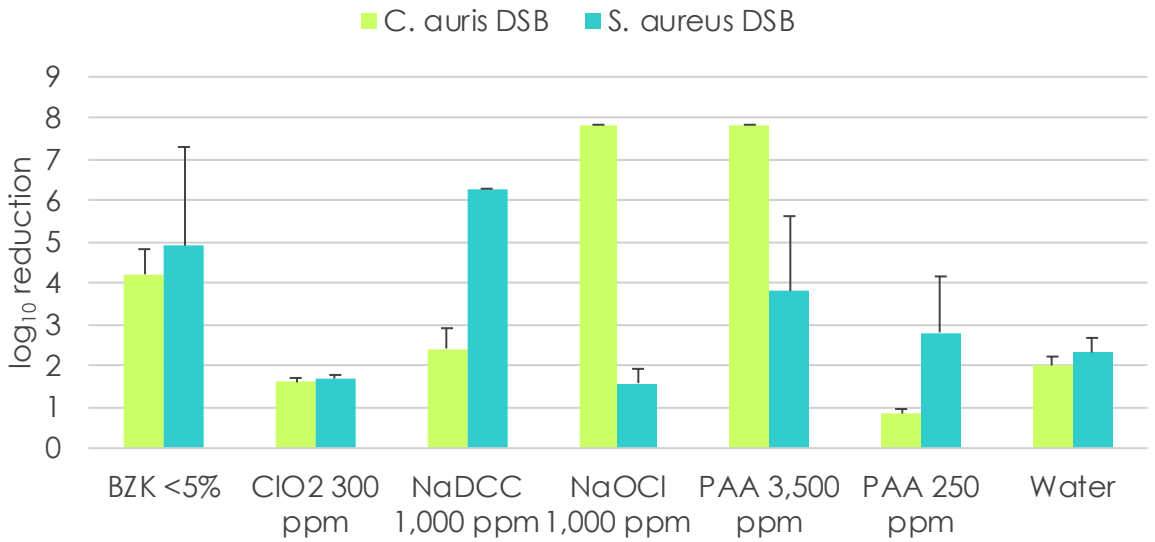
◀*C. auris* DSB x2,000

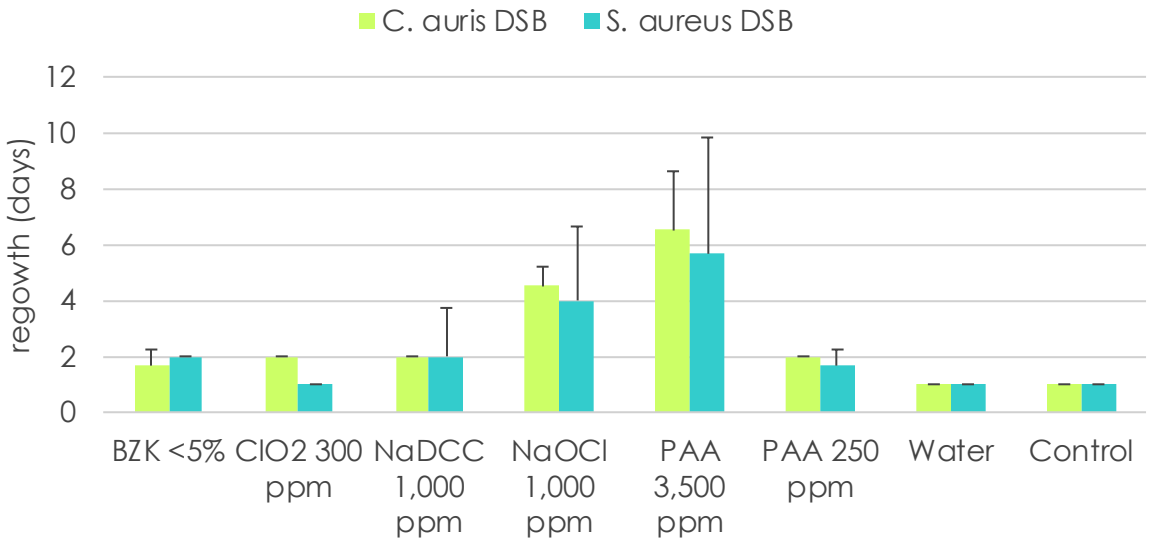


S. aureus DSB x10,000 ▶

Ledwoch and Maillard (2019) Materials (Basel) 12(1):18 ; Ledwoch et al (2019) Lett Appl Microbiol 68(4): 329-336

Disinfectants testing





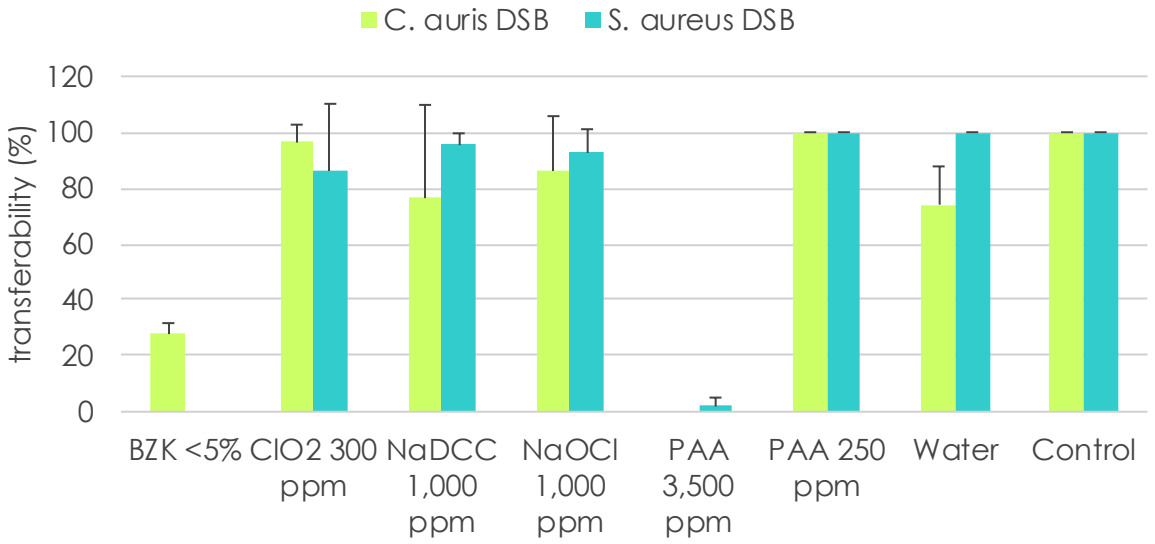


Image source: Pxhere

Disinfectants testing

- Transferability and recovery of DSB post treatment are essential to describe biocide efficacy

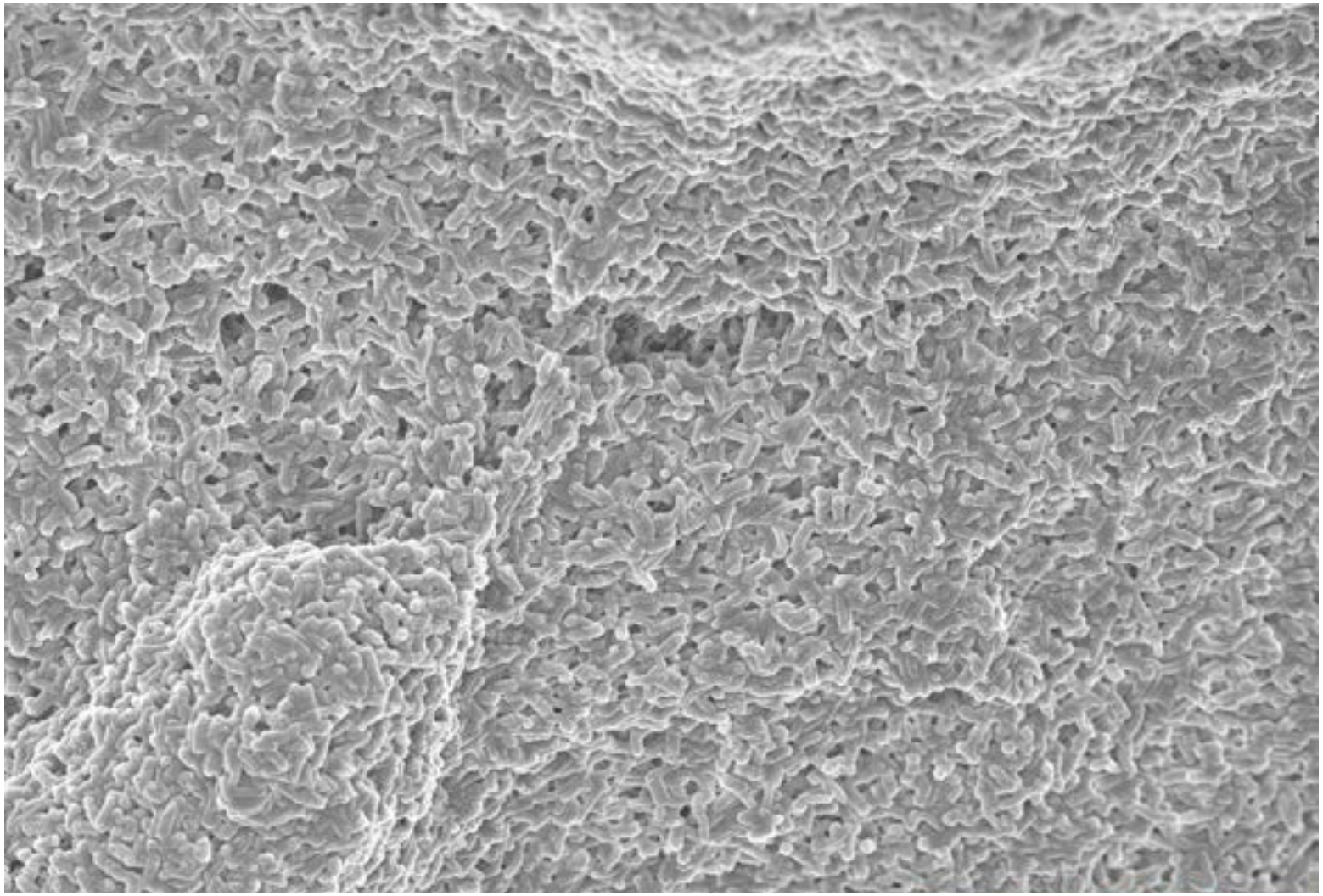
- Most disinfectants tested cannot prevent DSB transferability and regrowth

- High log_{10} reduction does not correlate to better overall performance of the product

Disinfectants targeting DSB

Image source: Unsplash

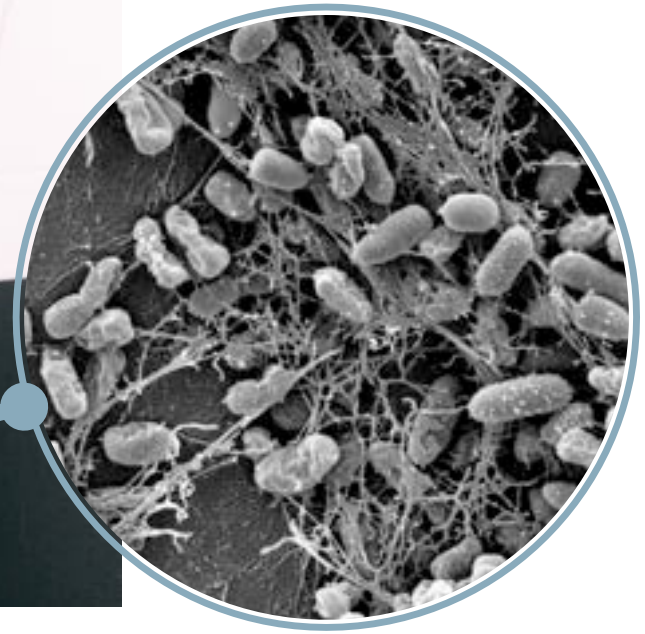
What is drain biofilm?



SEM image of mixed species drain biofilm formed on silicone tubing (dominating specie *Ps. aeruginosa*)

- **Wet/hydrated** biofilm in sink u-bent or trap
- But also, partially dry biofilm at the front section of drainage system; occasionally wetted
- Pathogens in drain biofilms pose a significant **HCA risk**
- The evidence of product effectiveness in controlling drain biofilms, pathogen dissemination and biofilm regrowth **are scarce**

Drain biofilm appearance: SEM



◀Mulamattathil et al. (2014) Afr J Sci. 110(11/12);

▼Aspelund et al. (2016) J Hosp Infect 94:13-20

Image source: Pexel

Why drain biofilm is a problem?

- Widespread
- Persistent
- Staff safety
- Recoverable
- Hospital credibility
- Patient safety
- Increased hospitalisation costs
- Increased mortality
- Less responsive to biocidal products

Why drain biofilm is a problem?

"For many years, patient-area wastewater drains (ie, sink and shower drains) have been considered a potential source of bacterial pathogens that can be transmitted to patients."
- Carling (2018) Infect Control Hosp Epidemiol 39:972–979

"(…) hospital sink drains can accumulate strains with resistance genes and become a potential source of CPE."
- De Geyter et al. (2017) Antimicrob Resist In 6:24

"Many recent reports demonstrate that sink drain pipes become colonized with highly consequential multidrug-resistant bacteria, which then results in hospital-acquired infections."
- Kotay et al. (2017) Appl Environ Microbiol 83(8)

Why drain biofilm is a problem?

A to Z Guides > News >

Dangerous Bacteria May Lurk in Hospital Sinks

By Robert Preidt

HealthDay Reporter

THURSDAY, Feb. 7, 2019 (HealthDay News) -- Hospital germ detectives say the sinks next to toilets in patient rooms may harbor potentially dangerous bacteria.

Researchers at the Medical College of Wisconsin checked a large Wisconsin hospital for *Klebsiella pneumoniae carbapenemase*. KPC is a type of bacteria that can cause health care-associated infections such as pneumonia, bloodstream infections, wound infections or surgical site infections.

Working in the medical intensive care unit, the researchers tested drains in sinks next to patient toilets, and sinks closer to the entrance of patient rooms.

KPC was found in 87 percent of patient sinks next to toilets, compared with about 22 percent of sinks near room entry doors.

In 4 out of 5 rooms where KPC was found in the entry door sinks, it was also found in the sink near the toilet, suggesting a possible source of cross-contamination.

ScienceDaily

Your source for the latest research news

A reservoir of bacteria: Sink drains next to toilets in patient rooms may harbor dangerous organisms

Date: February 5, 2019

Source: Elsevier

Summary: Sinks situated next to patient toilets in hospital rooms may be reservoirs for Klebsiella pneumoniae carbapenemase (KPC), increasing the risk of dangerous germ transmission, according to new research.

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FULL STORY

Sinks situated next to patient toilets in hospital rooms may be reservoirs for *Klebsiella pneumoniae* carbapenemase (KPC), increasing the risk of dangerous germ transmission, according to new research published in the *American Journal of Infection Control* (AJIC), the journal of the Association for Professionals in Infection Control and Epidemiology.

How to solve the problem?

Covering the drains

Image source: https://healthybuilds.com

Sink replacement

Image source: Pxhere

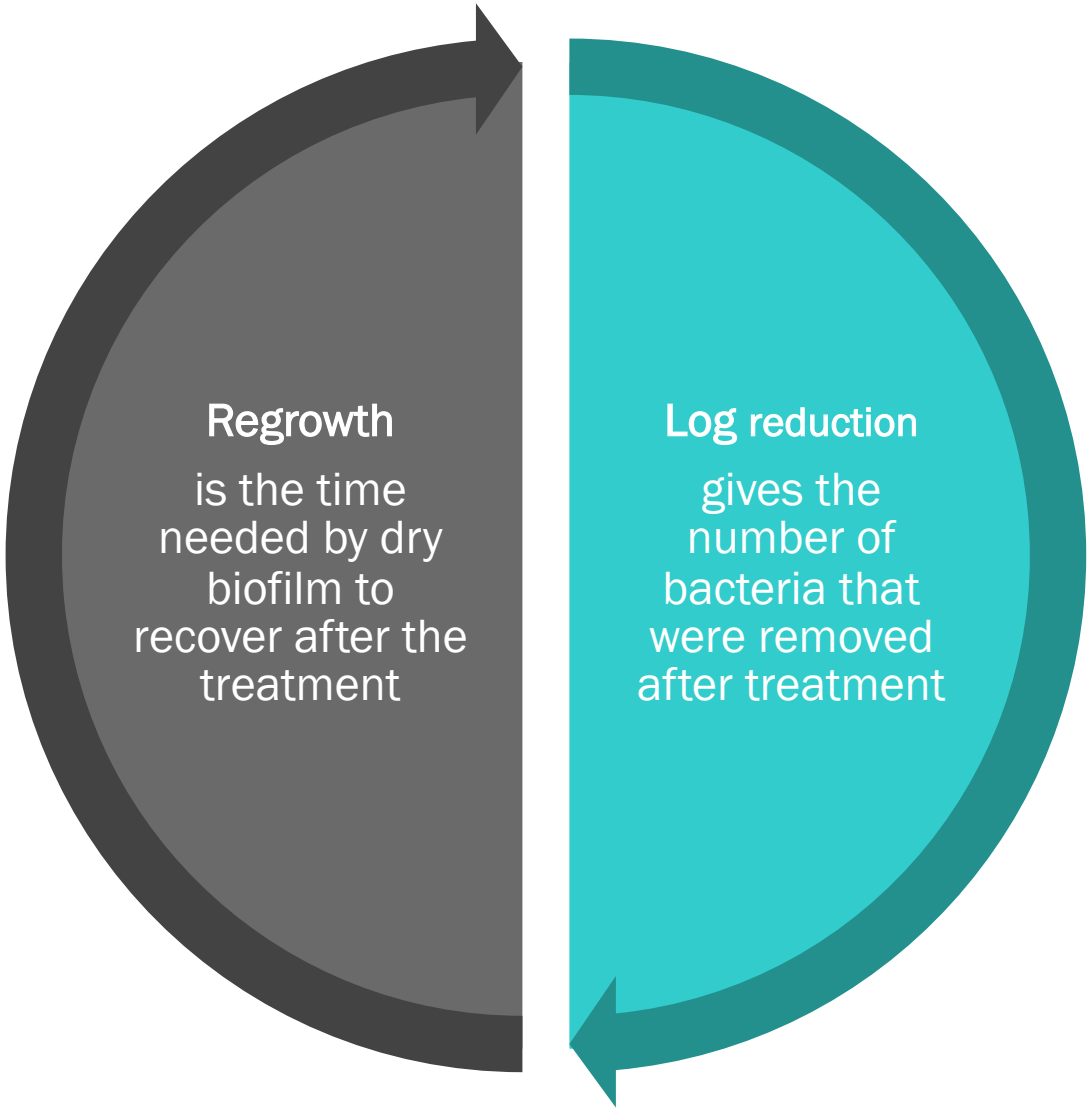
Image source: Unsplash

Improved monitoring of contamination level

Enhancing disinfection procedures

Image source: Unsplash

Disinfectants testing against drain biofilm



Enhancing disinfection procedures

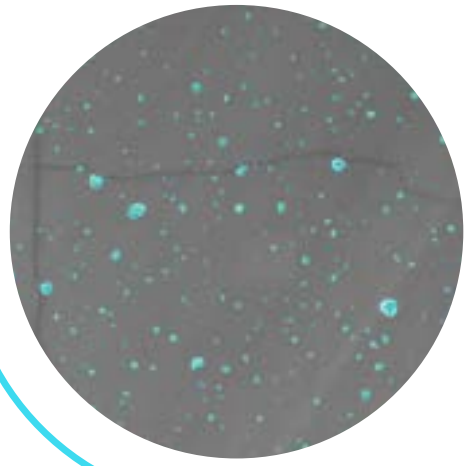
Image source: Unsplash

Drain biofilm models

- Sterile silicone tubing
- Mixed species drain culture collected from a communal sink
- A peristaltic pump perfuse diluent, diluted growth medium, or biocidal products through the tube
- 1:10 TSB for growth promotion

Drain biofilm development

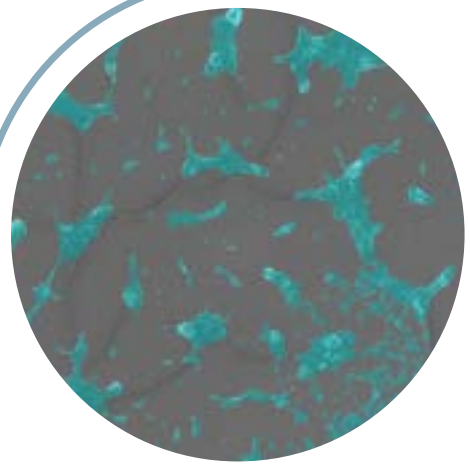
2 DAYS



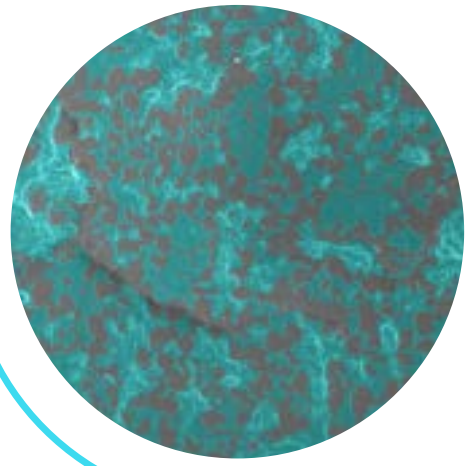
Inoculation phase
Drain biofilm culture is allowed to attach

3 DAYS

Media supply phase
Drain biofilm is challenged with flushes every 2h for 10s



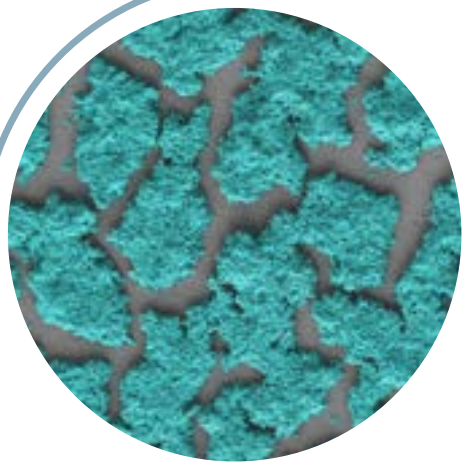
4 DAYS



Media supply phase
Drain biofilm establishes dense matrix

8 DAYS

Mature biofilm
Formation of thick drain biofilm

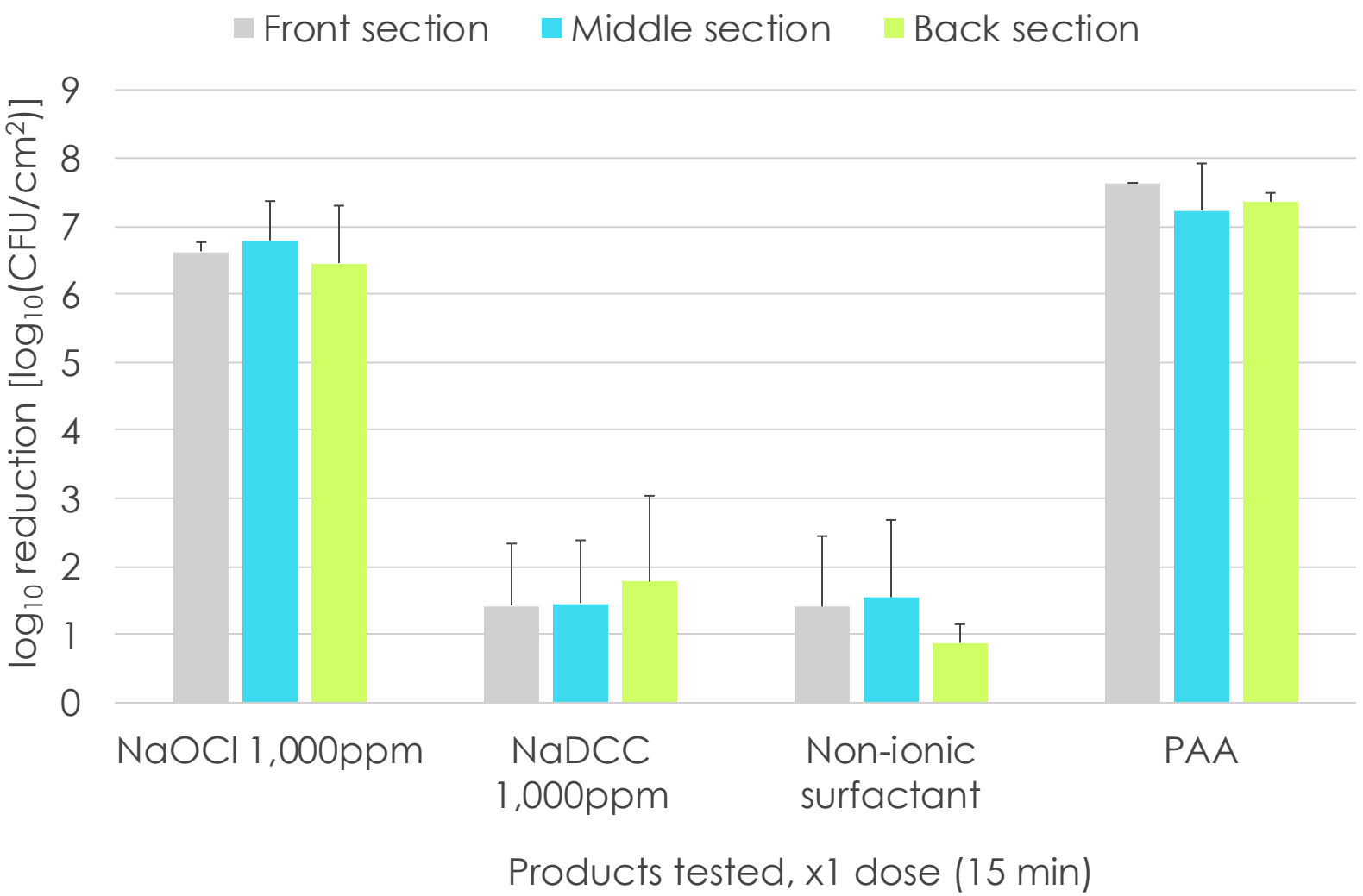


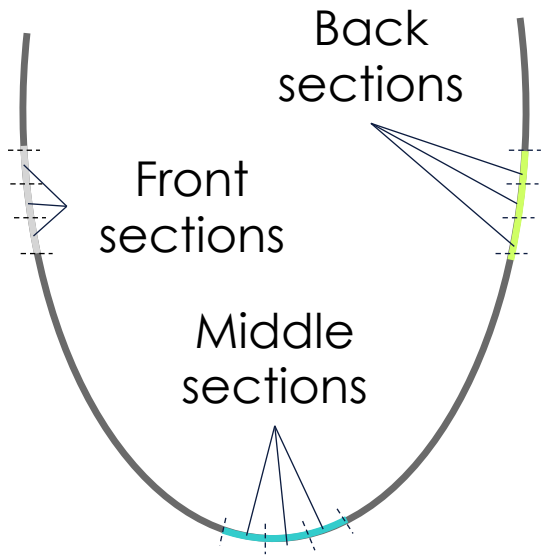
DRAIN BIOFILM READY FOR TESTING

Drain biofilm models

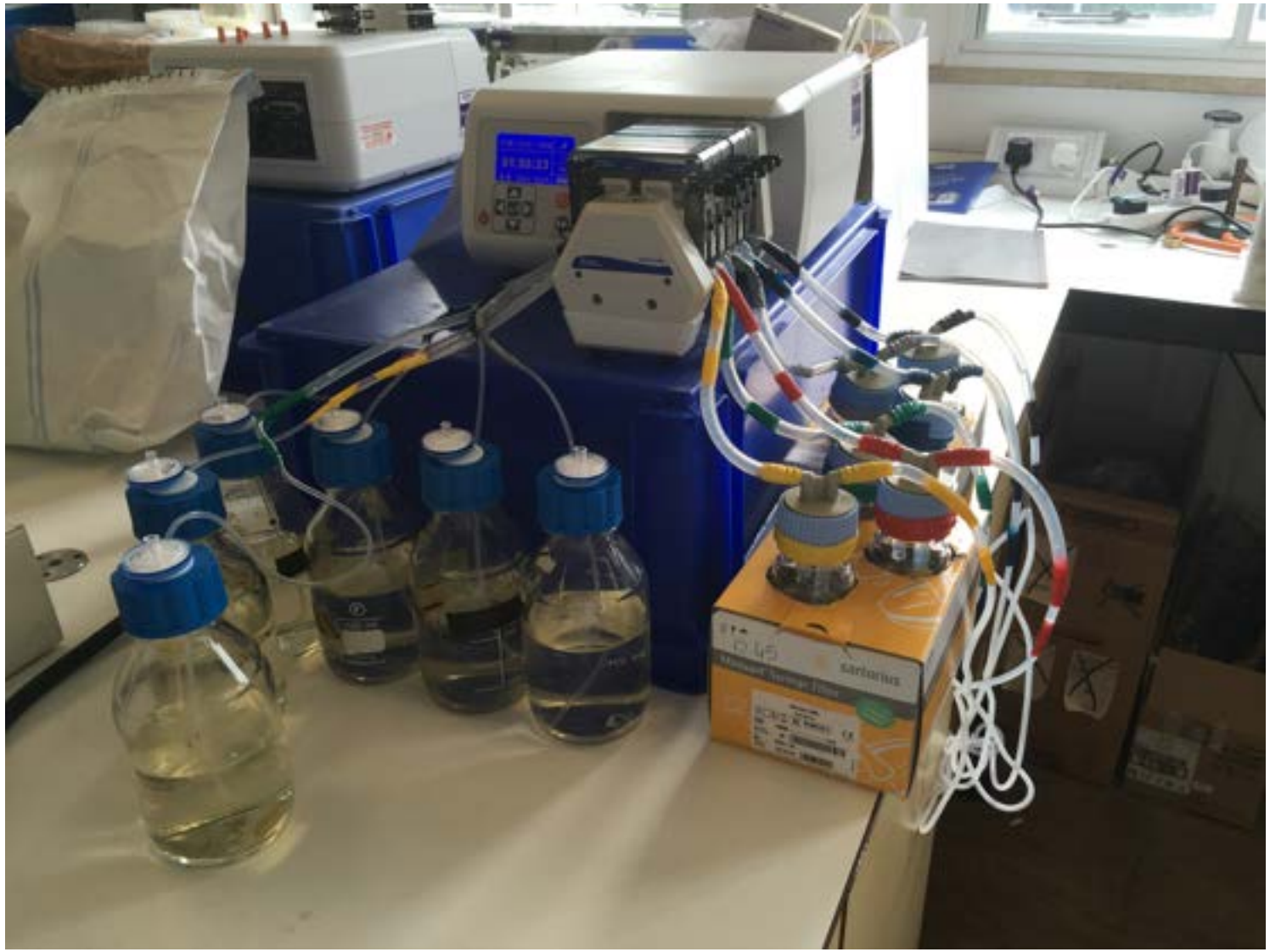
U-bent model

Disinfection tests: u-bent model

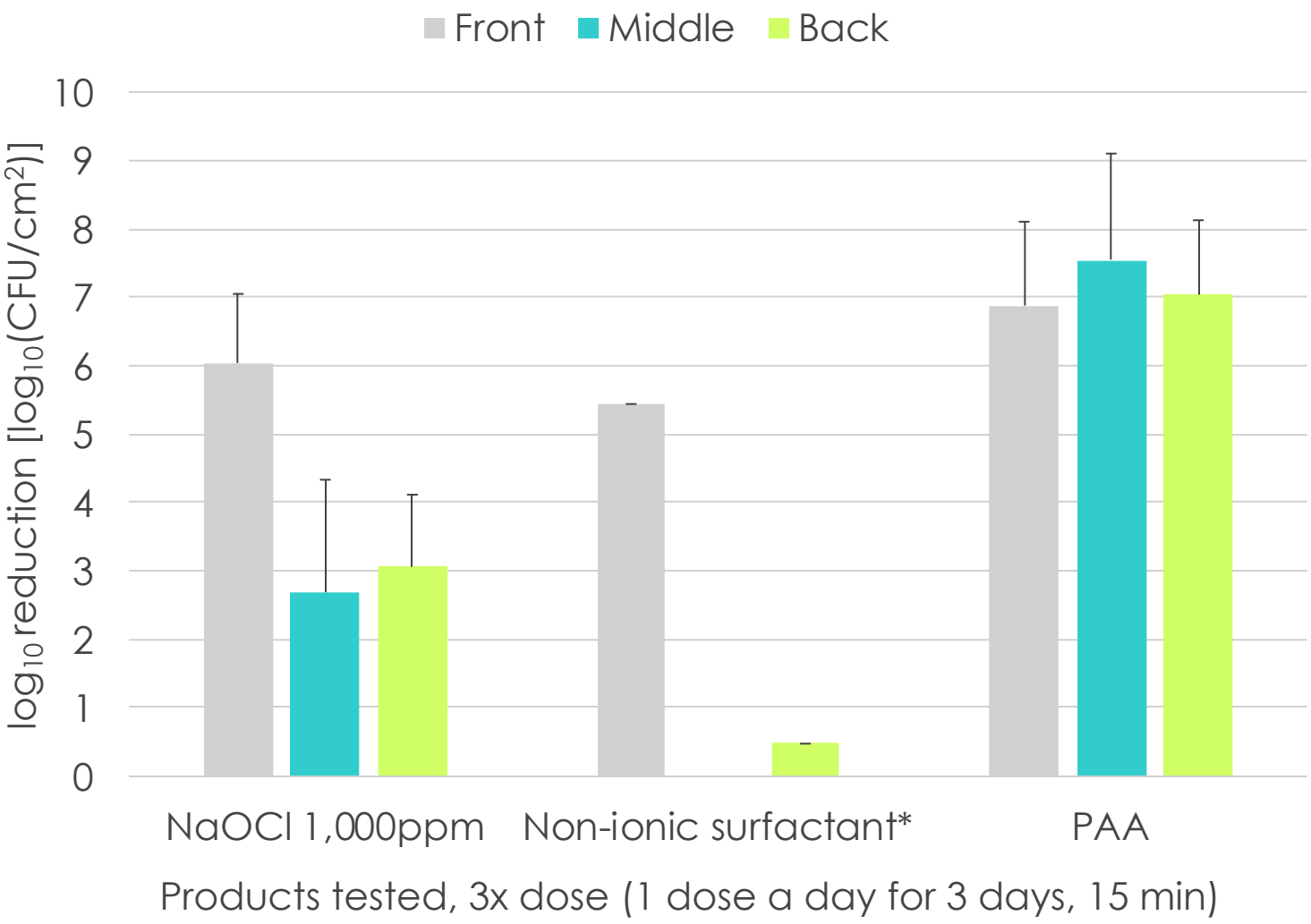


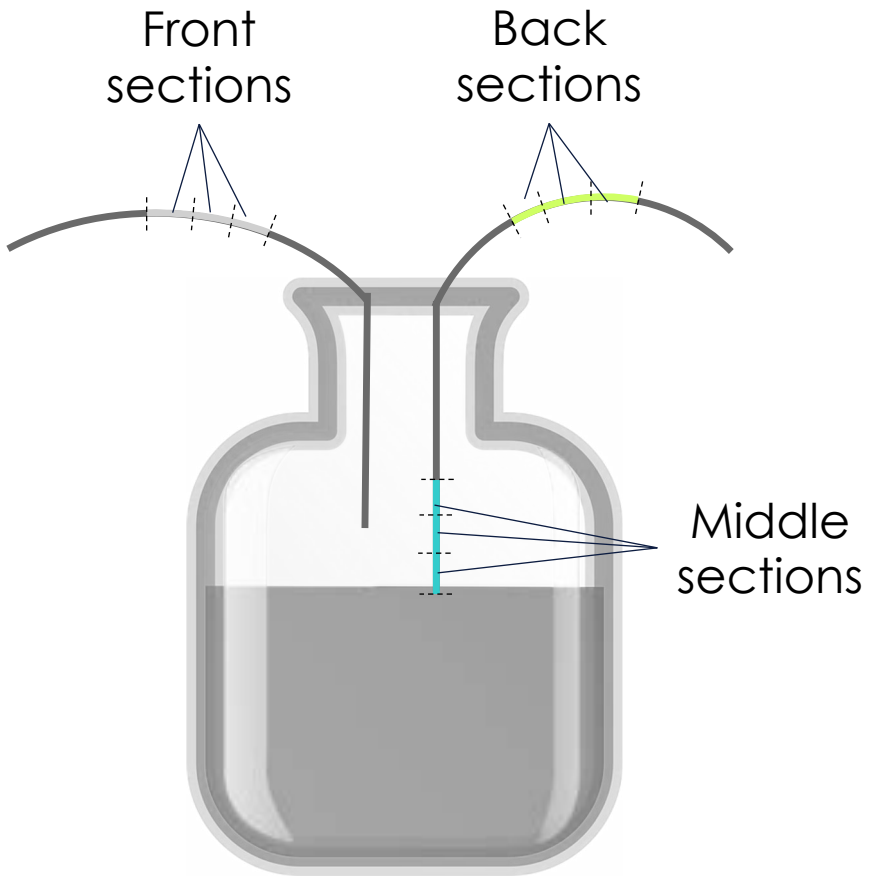


Trap model



Disinfection tests: trap model

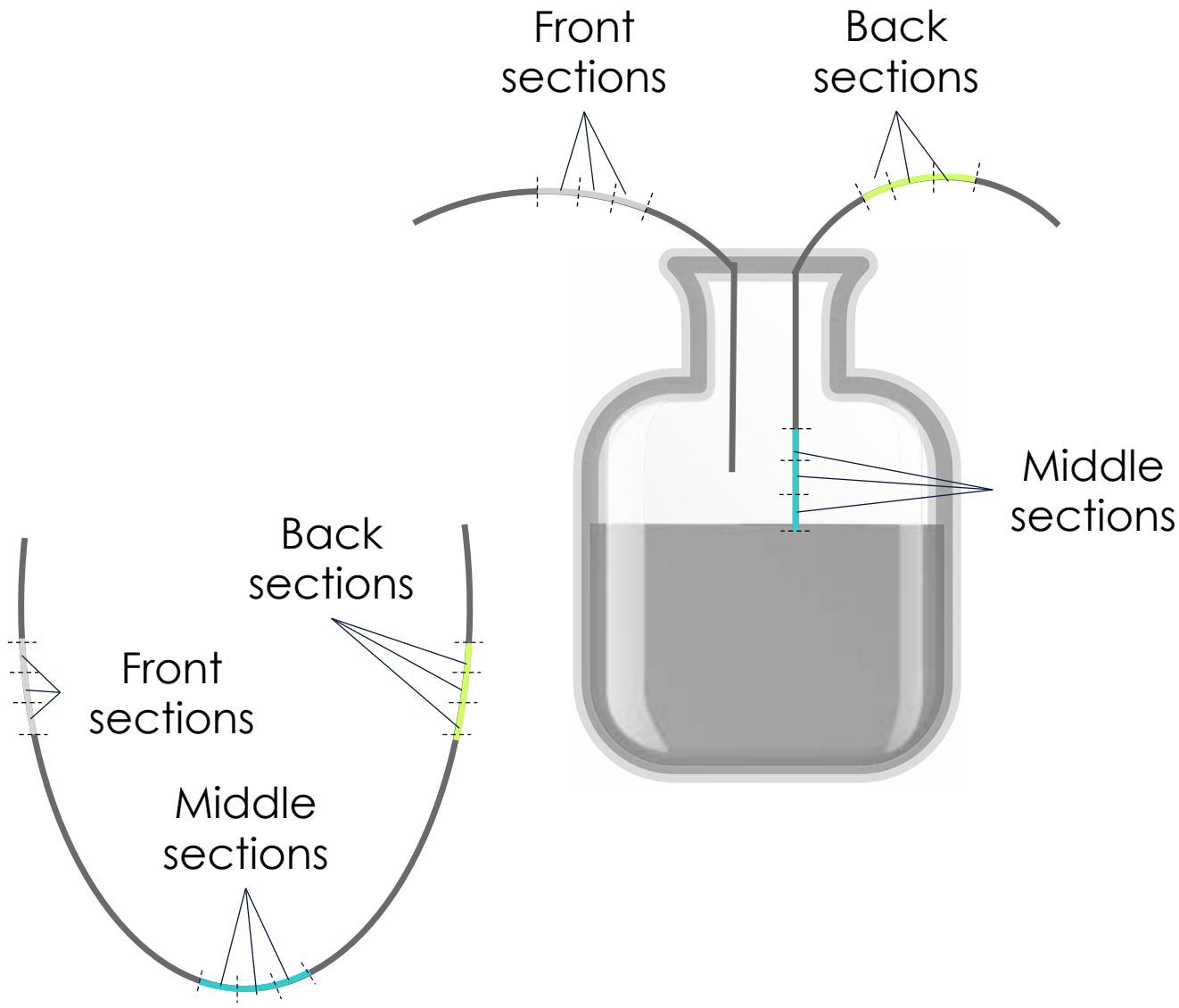




*one replicate only

Regrowth

- Still ongoing experiment
- In most cases, the drain biofilm recovers within one day after treatment at middle and back sections (>5 log_{10} recovered)
- Front section takes longer to regrow (>4 log_{10} reached after 4 days)
- Only PAA treatment prevented the regrowth for more than 4 days (<1 log_{10} recovered)



Disinfectants targeting drain biofilms

- Trap is a perfect environment for microbial growth; it is extremely difficult to control
- Disinfectants are effective against u-bent model after one dose; however it takes at least 3 consecutive daily doses to eradicate biofilm in trap model
- The drain biofilm recovers quickly even after effective treatment

Image source: Pexels

DSB and drain biofilms

Contribute to HCAI

Difficult to eradicate

Patients and staff directly exposed

Composed of dangerous pathogens

Require improved control measures

Dry biofilms on hospital surfaces (DSB)

Wet/hydrated biofilms in drain systems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SPECIAL ISSUE ARTICLE

Artificial dry surface biofilm models for testing the efficacy of cleaning and disinfection

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Significance and Impact of the Study: The widespread presence of biofilms on dry surfaces in healthcare settings has been recently documented. These dry surface biofilms (DSB) present an unprecedented challenge to cleaning and disinfection processes. Here, we describe a practical efficacy protocol based on an *in vitro Staphylococcus aureus* DSB model. The protocol measures reduction in viability, transferability and biofilm regrowth post-treatment to provide altogether a practical assessment of product efficacy against dry surface biofilms.

Keywords
cleaning, disinfection, dry surface biofilm, efficacy test, sodium hypochlorite, test protocol.

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Abstract

Dry surface biofilms (DSB) harbouring pathogens are widespread in healthcare settings, are difficult to detect and are resistant to cleaning and disinfection interventions. Here, we describe a practical test protocol to palliate the lack of standard efficacy test methods for DSB. *Staphylococcus aureus* DSB were produced over a 12-day period, grown with or without the presence of organic matter, and their composition and viability were evaluated. Disinfectant treatment was conducted with a modified ASTM2967-15 test and reduction in viability, transferability and biofilm regrowth post-treatment were measured. Dry surface biofilms produced over a 12-day period had a similar carbohydrates, proteins and DNA content, regardless of the presence or absence of organic matter. The combination of sodium hypochlorite (1000 ppm) and a microfiber cloth was only effective against DSB in the absence of organic load. With the increasing concerns of the uncontrolled presence of DSB in healthcare settings, the development of effective intervention model in the presence of organic load is appropriate for the testing of biocidal products, while the use of three parameters, $\log_{10}$ reduction, transferability and regrowth, provides an accurate and practical measurement of product efficacy.

Introduction

Biofilms are microbial communities embedded in self-secreted extracellular polymeric substances (EPS). Biofilms are significantly more tolerant to antimicrobials when compared to their planktonic equivalents (Akinbobola *et al.* 2017). The vast majority of studies on biofilm resistance to disinfection concerns hydrated biofilms formed and consistently grown in liquid environments or in the presence of high level of moisture (Francolini and Donelli 2010; Bridier *et al.* 2011; Otter *et al.* 2015). Not much attention has been paid to 'dry' biofilms colonizing surfaces with the presence of desiccated micro-organisms, limited moisture and nutrient resource (Vickery *et al.* 2012; Almatroudi *et al.* 2015) despite their widespread presence on healthcare surfaces (Vickery *et al.* 2012; Hu *et al.* 2015; Ledwoch *et al.* 2018).

There is no standardized efficacy test against biofilm published by the European Norm. In the United States, disinfectant efficacy tests against biofilms concern the treatment of hydrated biofilms (US Environmental Protection Agency, 2013). Among common test protocols the Calgary device enables the measurement of the minimal biofilm eradication concentration (MBEC) which

corresponds to the lowest concentration of a biocide or biocidal formulation that kill a monospecies bacterial (hydrated) biofilm (Ceri *et al.* 1999; Ali *et al.* 2006; Azeredo *et al.* 2017); The CDC reactor model and drip flow reactor model have also been used successfully to measure the efficacy of antimicrobials against hydrated biofilms (Schwartz *et al.* 2010; Almatroudi *et al.* 2015). Other nonstandardized biofilm efficacy methods have also been described (Pierce *et al.* 2008; Millhouse *et al.* 2014; Sherry *et al.* 2016). There are, however, no standard protocols to measure the efficacy of biocidal formulations against dry surface biofilms (DSB).

This paper proposes such a test and establishes test parameters to ensure the appropriate control of DSB in practice following cleaning/disinfection interventions.

Results and discussion

Dry surface biofilms are widespread on various surfaces in healthcare facilities (Vickery *et al.* 2012; Almatroudi *et al.* 2015), although their impact on healthcare associated infections has not been yet established. *Bacillus* spp. and *Staphylococcus aureus* were recently identified as the species most commonly associated with DSB formed on hospital surfaces (Ledwoch *et al.* 2018). Furthermore, Vickery *et al.* (2012) showed that DSB can persist on surfaces despite effective cleaning. In their study, samples isolated from ICU unit harboured pathogens including multidrug resistant micro-organisms following terminal cleaning with neutral detergent followed by chlorine 500 ppm disinfection. Equipment and furnishing retrieved from hospital were also positive for the presence of VRE and MRSA (Vickery *et al.* 2012).

Here, we report the development of artificial mono-species DSB grown in the presence of organic load or not, for biocidal product testing. To date there are no such tests reported in the literature and limited existing protocols refer to testing against hydrated biofilms. The number of bacteria recovered from CL-DSB or OL-DSB was the same after the formation of DSB (Fig. 1). There was no statistically significant difference (Two-way ANOVA, $P = 0{\cdot}08821$) in viable count of bacteria ($\log_{10}$ CFU per ml = $7{\cdot}60 \pm 0{\cdot}60$) recovered from each disc between 20 environmental DSB replicates. The average bacterial concentration in CL- and OL-DSB was $7{\cdot}38 \pm 0{\cdot}58$ and $7{\cdot}89 \pm 0{\cdot}60$ $\log_{10}$ CFU per ml, respectively. *Staphylococcus aureus* DSB composition consisted mainly of proteins ($96 \pm 1\%$) with some carbohydrates ($4 \pm 1\%$), with overall little DNA (Fig. 2). No lipids were detected by the colorimetric sulfo-phospho-vanillin method suggesting lipid level was below the detection limit of 35 μg ml^{-1} (Anschau *et al.* 2017). The amount of proteins and carbohydrates remains constant throughout the dry surface biofilm cycles with 95–97% of proteins and 3–5% carbohydrates in both CL and OL- DSB. Surprisingly the addition of BSA during the OL-DSB production did not impact on the composition of the biofilm (Fig. 2). Indeed, there was no statistical difference ($P = 0{\cdot}5317$) between the carbohydrate and proteins ratio of CL- and OL-DSB. More DNA (ANOVA; $P < 0{\cdot}05$) was extracted from DSB grown in the presence of organic load (21 ± 7 ng per disc) compared to without BSA (17 ± 9 ng per disc). Hydrated *S. aureus* biofilms are also mostly composed of proteins, although carbohydrate concentration is higher. Abdallah *et al.* (2014) reported *S. aureus* 24 and 48 h hydrated biofilms formed on stainless steel coupons were composed of 70 and 78% proteins and 30 and 22% of carbohydrates, respectively.

The composition, appearance and viability of our dry surface biofilms were homogeneous (Fig. 3) with $8{\cdot}0 \pm 0{\cdot}6$ $\log_{10}$ CFU per cm^2 after the 12 days process. Abdallah *et al.* (2014) reported similar findings with hydrated *S. aureus* biofilms following 24 and 48 h incubation, $8{\cdot}4 \pm 0{\cdot}2$ and $8{\cdot}2 \pm 0{\cdot}2$ $\log_{10}$ CFU per cm^2, respectively. After the first dry phase, the majority of bacteria were viable (90 and 98% of all bacteria in CL- and OL-DSB, respectively) (Fig. 3). However, in the course of growth and after sequential dry phases, the number of dead bacteria increased (Fig. 3). At the end of dry surface biofilm formation cycle, 42 and 75% of bacteria were viable in CL- and OL-DSB, respectively. SEM of 12-day DSB showed homogenous cluster of bacteria embedded in a matrix and separated by channels. OL-DSB appeared to contain more matrix (Fig. 4). Although uniformity is a positive attribute for reproducibility, these biofilms differ somewhat to DSB isolated from healthcare surfaces. Indeed, environmental DSB form clusters of unevenly scattered bacteria through the colonized surface, thus making disinfectant testing using *in situ* dry surface biofilms inappropriate (Ledwoch *et al.* 2018).

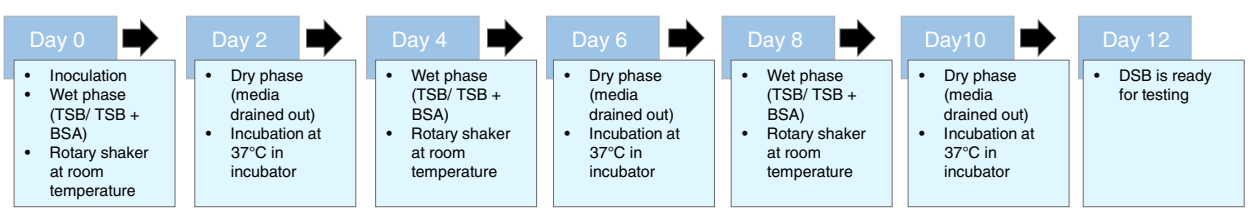

Figure 1 Scheme of dry surface biofilm formation and growth. [Colour figure can be viewed at wileyonlinelibrary.com]

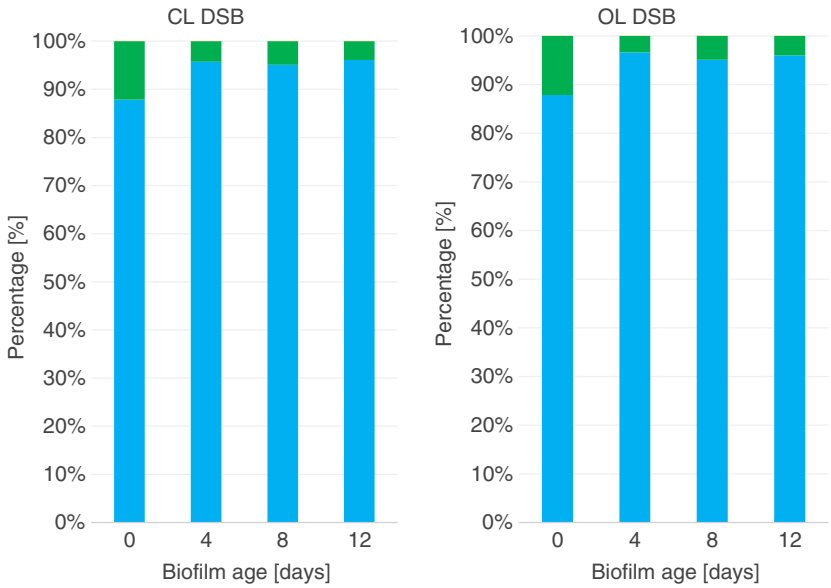

Figure 2 Composition of clean (CL; left) and organic load (OL; right) DSB. DNA: ■, Carbohydrates: ■, Proteins: ■. Time "0" indicates planktonic bacteria. No lipids were recovered with the protocol used in this study. [Colour figure can be viewed at wile yonlinelibrary.com]

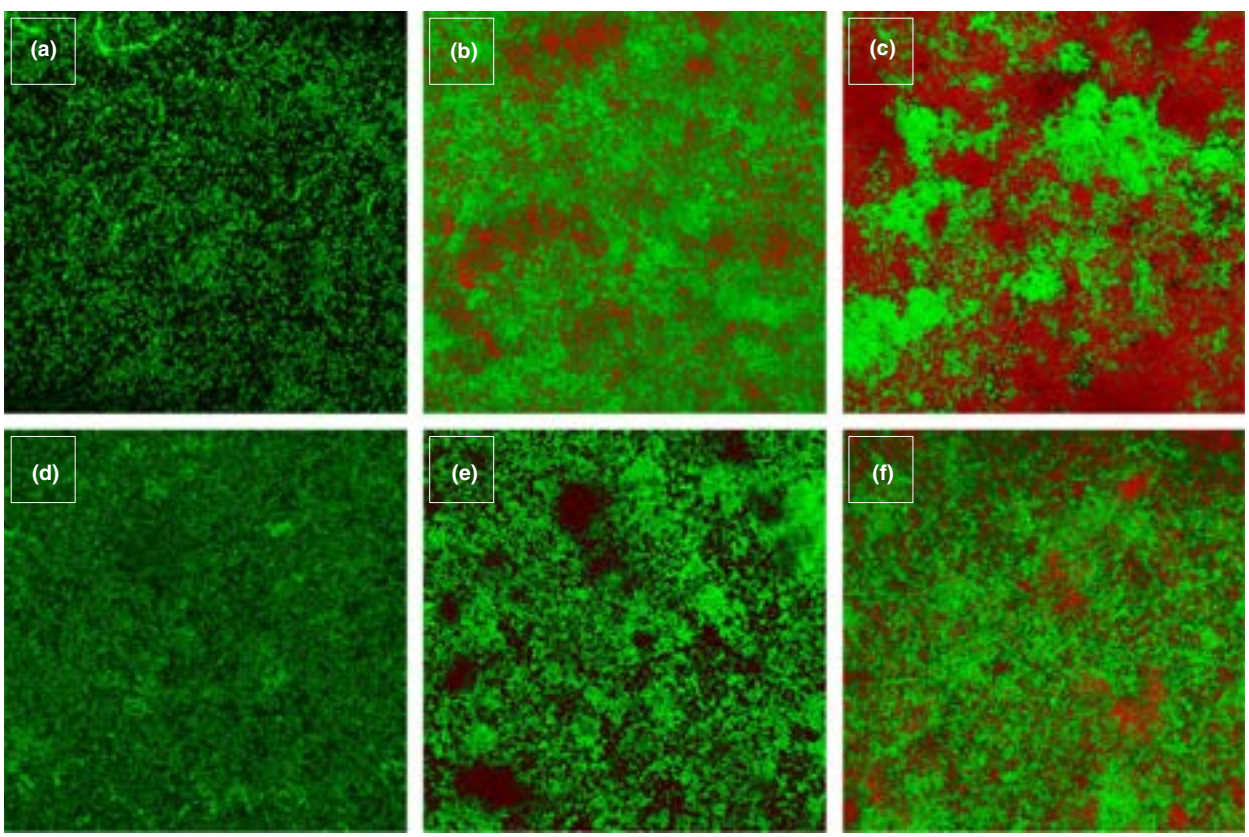

Figure 3 Live (green)/dead(red) fluorescence images of Syto 9/propidium iodide stained clean dry biofilm (CL, top) and Syto 9/propidium iodide stained organic load dry biofilm (OL, bottom) after 4, 8 and 12 days of cultivation. ×63 magnification, Zeiss LSM880 Airscan Confocal Microscope. Representative images from three fields of two samples. CL-DSB after 4 (a), 8 (b) and 12 (c) days; OL-DSB after 4 (d), 8 (e) and 12 (f) days. [Colour figure can be viewed at wileyonlinelibrary.com]

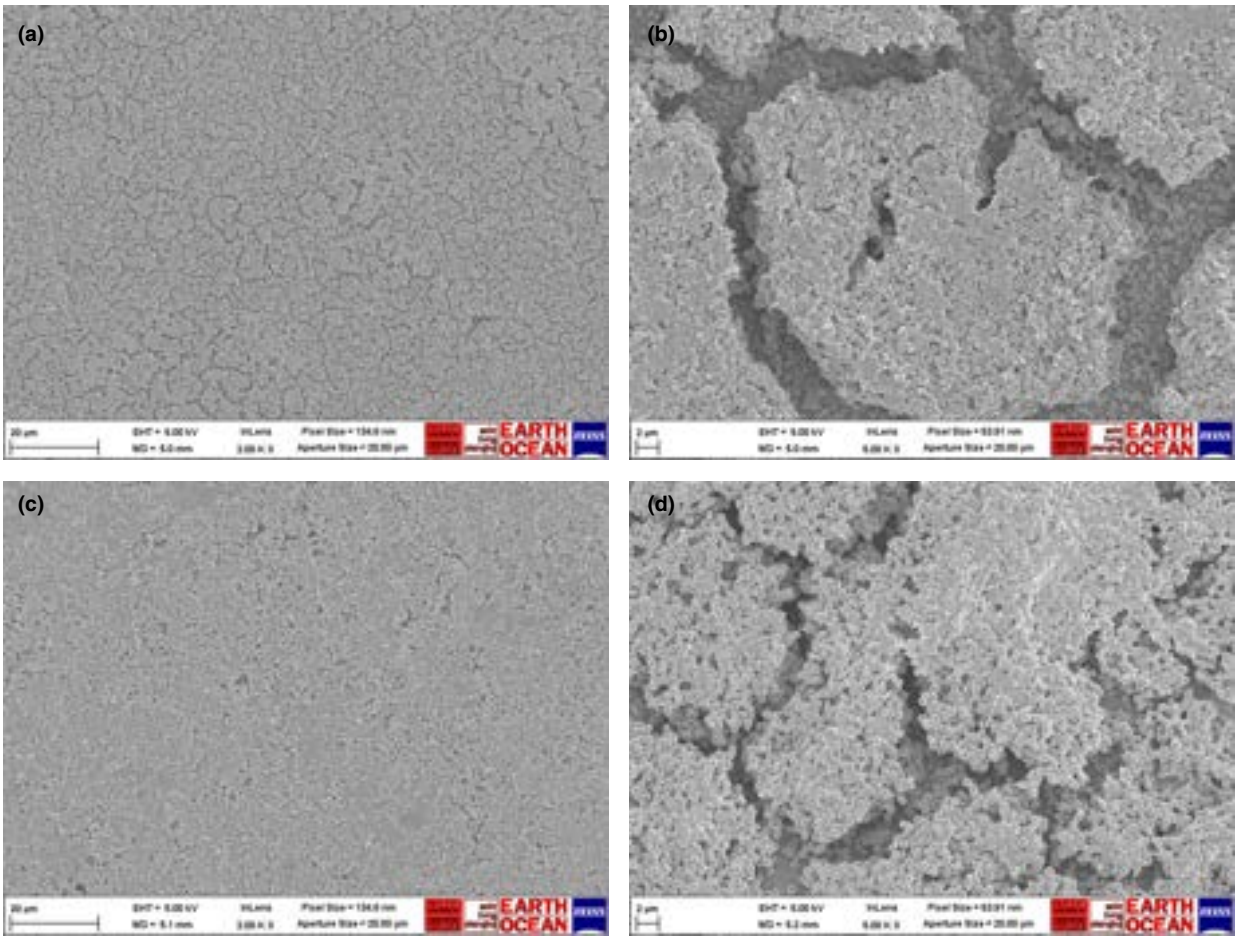

Figure 4 Scanning electron microscope images of clean (CL) dry biofilm and organic load (OL) dry biofilm, ×2000 and ×5000 magnifications. Images presented are representative for the whole disc surface. CL-DSB at ×2000 (a) and ×5000 (b) magnification, OL-DSB at ×2000 (c) and ×5000 (d) magnification. [Colour figure can be viewed at wileyonlinelibrary.com]

Considering potential product usage in practice (Sattar and Maillard 2013), it seemed appropriate to combine the use of the wiperator (ASTM26987-15, 2015) with our DSB. The ASTM2697-15 (2015) was preferred to the EN16615-15 (2015) protocol, as it was recently shown to be a more stringent protocol (Wesgate *et al.* 2018). To provide a sensible and useful measurement of product efficacy against DSB, we decided to measure several criteria indicative of product efficacy: (i) reduction in viability as a result of bactericidal activity or/and removal of bacteria from the surface, (ii) transferability of bacteria post-wiping and (iii) DSB regrowth indicating the frequency of product application needed to render the surface safe. Using such an approach we first identified that DSB produced in the presence of OL, were more difficult to control than those produced in the absence of organic load, despite that NaOCl (1000 ppm) in combination with the microfiber cloth decreased *S. aureus* number in DSB by >4 $\log_{10}$ (Table 1). More bacteria within DSB could be removed/killed when the biofilm was grown in the absence of organic load. Almost 6 $\log_{10}$ reduction was achieved which could be compared to results showed by Almatroudi *et al.* (2016) where more than 7 $\log_{10}$ of bacteria in DSB were removed/killed by treatment with 1000 ppm NaOCl. Although, NaOCl treatment significantly lowered (Two-way ANOVA; $P < 0{\cdot}05$) the transfer of bacteria from dry surface biofilms compared to the absence of treatment, it was significantly less effective when DSB were formed in the presence of organic load (Table 1). Likewise, time for regrowth post NaOCl exposure was much shorter in the presence of organic load (Table 1). Hence, measuring additional parameters to the traditional viability one, provide additional stringency. Transferability post-treatment is particularly important to consider notably in relation to hand hygiene compliance. Indeed, DSB have been shown to be widespread in healthcare settings (Hu *et al.* 2015; Ledwoch *et al.* 2018), acting as a potential transmission reservoir. As median hand

Table 1 Effectiveness of NaOCl (1000 ppm) on clean (CL) and organic load (OL)- DSB. Impact of disinfectant on reduction in bacteria, transferability and regrowth

	NaOCl 1000 ppm	No treatment
Log_{10} reduction in bacteria (CFU per cm^2) ± SD		
CL-DSB	5·83 ± 1·25	–
OL-DSB	4·26 ± 1·26	–
Transferability (%) ± SD		
CL-DSB	1 ± 2	95 ± 8
OL-DSB	68 ± 37	100 ± 0
Regrowth (days)		
CL-DSB	5·4 ± 3·3	1 ± 0
OL-DSB	2·8 ± 0·8	1 ± 0

hygiene rate from 96 empirical studies is only 40% (Erasmus *et al.* 2010), the risk of transmitting pathogens from DSB is high. It is thus conceivable that, despite the reduction in viability following, here, exposure to NaOCl (1000 ppm), bacteria embedded in a dry surface biofilm can still be easily transferred. Chowdhury *et al.* (2018) also reported on the persistent nature of DSB; in their study, treatment with neutral detergent had a little effect on bacterial transferability from DSB. The regrowth parameter, although linked somewhat to a reduction in viability, provides information on how long the surface would be biofilm-free post-treatment. We are proposing that the dry surface biofilm model formed and grown in the presence of organic load, as well as the parameters investigated, are suitable to measure the efficacy of cleaning and/or disinfectant treatments.

Materials and methods

Bacterial growth and maintenance

Staphylococcus aureus NCTC10788 was propagated in tryptone soya broth (TSB; Oxoid Limited, Hampshire, UK) at 37°C overnight and washed in tryptone sodium chloride following centrifugation at 1400***g***. Tryptone sodium chloride was prepared by mixing 1 g of tryptone (Oxoid Limited) and 8·5 g of sodium chloride (Sigma-Aldrich®, Dorset, UK) in 1 l of distilled water followed by autoclaving. The bacterial suspension was adjusted to 1×10^6 CFU per ml.

Dry biofilm models

The bacterial growth approach in our DSB model is based on alternating hydrated (growth) phases with desiccation phases as described by Almatroudi *et al.* (2015). In our model we utilized a sedimentation protocol to form and grow DSB, as described below. Bacteria were initially cultured in normal hydrated conditions to allow initial adherence and biofilm formation. This was followed by cycles of dry and hydrated phases for a total duration of 12 days (Fig. 1).

Stainless steel discs AISI 430 (0·7 ± 0·07 mm thickness; 10 ± 0·5 mm diameter, Goodfellow Cambridge Limited, Huntington, UK) were used as a support. Sterile discs were placed in Corning™ Costar™ flat bottom cell culture plates (Fisher Scientific, Loughborough, UK), and each well was inoculated with 1 ml of TSB containing 5% anhydrous D-glucose (Fisher Scientific) with 10^6 CFU per ml washed *S. aureus* suspension. Bacteria were first allowed to attach and form a biofilm on the disc surface over 2 days period at 25°C under gentle agitation using an Orbit P4 plate rocker (Labnet International, Edison); i.e. the hydrated phase. The solution was then drained from the wells and plates were incubated at 37°C for 48 h. Following this dry phase, 1 ml of TSB was added into each well containing stainless steel disc and a new 'hydrated phase' began for 48 h. Hydrated and dry phases alternate every 48 h for a period of 12 days, ending with biofilm in a dry phase (Fig. 1).

Two models of *S. aureus* dry surface biofilms were developed: (i) a clean (CL) DSB grown in TSB only, and (ii) an organic load (OL) DSB grown in 3 gl^{-1} bovine serum albumin (BSA; Sigma® Life Science, Dorset, UK). BSA was added to each wet phase during the 12 days period.

Biofilm composition

Carbohydrate

Carbohydrate content in DSB models was measured by dinitrosalicylic (DNS; Fisher Scientific Ltd) colorimetric assay (Miller 1959). Briefly, disc containing biofilm was placed in McCartney bottle with 1 g of glass beads and 3 ml of sterile water. The disc was vortexed with Fisherbrand® vortex shaker (Fisher Scientific) for 10 min prior to the analysis. Two gl^{-1} of phenol (Fisher Scientific Ltd) was added to DNS reagent to intensify the colour density and increase the sensitivity of the method. Three ml of DNS reagent was added directly to vortexed culture or directly to an overnight planktonic suspension of *S. aureus* and covered with lid to prevent liquid evaporation. The mixture was heated for 15 min at 90°C in Fisherbrand water bath (Fisher Scientific) until the colour developed. Colour was stabilized by adding 1 ml of 40% potassium sodium tartrate solution (Fisher Scientific Ltd). Absorbance at 575 nm was read after the mixture cooled down to the room temperature and compared against the standard curve to evaluate carbohydrates content.

Protein analysis

Discs with *S. aureus* DSB were placed in sterile 30 ml capacity flat bottom glass bottles containing 1 g of glass beads and 2 ml of sterile water and vortexed for 10 min.

The suspensions were then centrifuged in Biofuge Primo R centrifuge (Heraeus, Thermo Fisher Scientific, Newport, UK) at 1400***g*** and 20°C for 10 min. The supernatant was then discarded, and the remaining bacterial pellets were weighted. Proteins were extracted using the Total Protein Extraction Kit (Chemicon®, Millipore Limited, Watford, UK). Briefly, 2·5 ml TM buffer was added to 1 g of the pellet and put on ice for 5 min. Bacterial cells were homogenized three times by vortexing for 20 s and incubation on dry ice for 15 s. The mixture was then centrifuged at 11 000***g*** at 4°C for 20 min using Avanti™ J-20 XP centrifuge (Beckman Coulter, High Wycombe, UK). Collected supernatant was quantified for proteins content. Quantification was carried out with Folin–Ciocalteu reagent (Lowry *et al.* 1951) by using Pierce™ Modified Lowry Protein Assay (Thermo Scientific™, Loughborough, UK). One ml of Modified Lowry Reagent was added to each tube containing 0·2 ml of supernatant. The mixture was well homogenized with vortex shaker (Fisherbrand®, Fisher Scientific Ltd) and incubated at room temperature for 10 min. One hundred μl of 1X Folin-Ciocalteu Reagent was then added and the sample vortexed for 5 s. Sample was covered and incubated for 30 min at room temperature. The absorbance at 750 nm was measured and the amount of the proteins in the sample was evaluated by using standard curve prepared according to manufacturer instructions using diluted albumin (BSA) standards.

Lipid analysis

Lipids were extracted with the chloroform-free Lipid Extraction Kit (Abcam®, Cambridge, UK). Discs with *S. aureus* DSB were vortexed for 10 min in Mccartney bottles containing 1 g of glass beads and 2 ml of sterile water. The suspensions were then centrifuged at 1000***g*** at 20°C for 5 min. Supernatant was discarded, and the pellets were washed and resuspended in 25 μl of phosphate buffer saline (PBS; 8 g of sodium chloride (Sigma-Aldrich®), 0·2 g of potassium chloride (Fisher BioReagents®, Fisher Scientific Ltd), 1·44 g of sodium phosphate dibasic heptahydrate (Thermo Fisher Scientific) and 0·24 g of potassium phosphate monobasic (Thermo Fisher Scientific) in up to 1 l water). pH was adjusted to 7·4. Five hundred μl of Abcam extraction buffer containing 60% hexane and 40% isopropanol (Abcam®) was added to the samples which were vortexed for 2 min. The mixture was agitated on Orbit P4 plate rocker (Labnet International) at room temperature for 20 min. The tubes were centrifuged for 5 min at 10 000***g*** and the supernatant was collected and weighted. The tube with supernatant was dried overnight in Thermo Heraeus Herasafe™ safety cabinet (Thermo Fisher Scientific) at 37°C. The analysis of lipids was carried out following the colorimetric sulfo-phospho-vanillin (SPV; Fisher Scientific Ltd) method (Cheng *et al.* 2011). One ml of chloroform: methanol solvent was added per 15 mg of sample followed by 100 μl of sulphuric acid (Fisher Scientific Ltd). Samples were heated at 90°C for 10 min on a stirring hotplate (Fisher Scientific Ltd) and then placed on ice to cool them down to room temperature. One hundred μl vanillin-phosphoric acid reagent was added to the sample to develop the colour. Absorbance at 540 nm was measured after 5 min to determine the lipid content.

DNA analysis

Discs with DSB were placed in 30 ml capacity flat bottom glass bottle with 1 g glass beads and 0·5 ml TSB and vortexed for 10 min to remove the biofilm from disc surface. 0·5 ml of 4 mol l^{-1} guanidine isothiocyanate (UltraPure™, ThermoFisher Scientific, Newport, UK) was added to the sample and further vortexed for 1 min. One ml of mixture was transferred to a 2 ml tube with cap and Fisherbrand® O-ring (Fisher Scientific) with 1 g of 0·1 mm diameter zirconia/silica beads (Thistle Scientific, Glasgow, UK) and homogenized in bead bug (Benchmark Scientific, Cole-Parmer®, St Neots, UK) at 2800 rev min^{-1}. DNA amplification was carried out with Maxwell® 16 Instrument (Promega, Southampton, UK). The amount of extracted DNA was quantified with Quibit® 3.0 fluorometer (Thermo Fisher Scientific).

Scanning Electron Microscopy (SEM) imaging

Staphylococcus aureus DSB samples were prepared by overnight incubation of discs in 2·5% glutaraldehyde solution (Fisher Scientific) followed by immersion in successive concentrations of 10, 25, 50, 70, 90 and 100% ethanol (Honeywell, Fisher Scientific Ltd) for 10 min each. Prior to SEM scanning, samples were coated with 20 nm AuPd coating with sputter coater (SC500, Biorad, UK). 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 ×2000 and ×5000 magnification and 5–7 mm working distance.

Live/dead staining

Staining of dry surface biofilm was carried out with LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, Thermo Fisher Scientific) with Syto 9 and propidium iodide in 1 : 1 ratio. Prior to staining, each disc was mildly washed with 1 ml sterile water for 5 s to remove any planktonic or loosely adhered cells. Stained discs were imaged with Zeiss LSM880 Airscan Confocal Microscope (Carl Zeiss Ltd., Cambridge, UK).

LIVE/DEAD cells ratios were evaluated using BioFilmAnalyzer ver. 1.0 software with the procedure developed by Bogachev *et al.* (2018). Prior to analysis, nonhomogenous colour

distribution of obtained images in the studied colour channels was resolved by preliminary image colour normalization using GNU Image manipulation program (GIMP 2.8).

ASTM E2967-15 test

The effectiveness of sodium hypochlorite (NaOCl 1000 ppm; Fisher Scientific Ltd) combined with Rubbermaid® HYGEN™ disposable microfiber cloth (Rubbermaid Products, Surrey, UK) allowing 2·5 ml of disinfectant per 1 g of wipe was evaluated against CL- and OL-DSB controls consisted of untreated samples. Disinfection tests were performed according to a modified ASTM E2967 test (2015). The surface of the disc was wiped with the Wiperator (Filtaflex Ltd, Ontario, Canada) from both sides using separate wipes. Dry surface biofilms were wiped for 10 s under 500 g pressure, left at room temperature for 2 min, and then the wiped discs neutralized by placing each disc into 1 ml Dey-Engley (DE) neutralizing broth (Neogen® Corporation, Ayr, UK) for 2 min. Inoculated broth was then incubated overnight at ambient temperature. Transfer of viable bacteria from used wipes to clean a sterile disc was not performed.

Log_{10} reduction in bacteria embedded in DSB

Reduction in bacterial viability (Log_{10} reduction in CFU per ml) gave the number of bacteria that were removed or and killed following wiping. Following wiping, samples were placed in a solution containing 1 g of glass beads (Fisher Scientific), 2 ml DE neutralizing broth and 100 μg ml^{-1} proteinase K (Fisher Bioreagents™) for 1 h at 37°C. After incubation, samples were vortexed for 2 min, serially diluted and 3 × 10 μl drops of each dilution plated onto tryptone soya agar (TSA; Oxoid, Thermo Fisher Scientific). Log_{10} reduction was calculated as the difference between the number of bacteria recovered from untreated (control) and treated samples.

Transferability test following disinfection

Transfer test was conducted to investigate the transferability of surviving bacteria from the dry surface biofilm following wiping. The test was designed to imitate the touch of a finger onto treated surface. Following wiping and 2 min contact time, discs were pressed 36 separate times with 100 g pressure on the surface of DE agar. Following the transfer test, DE agar was incubated overnight at 37°C. Positive growth/adpression was recorded and transferability calculated as the number of positive contact/number of adpressions.

Dry surface biofilm regrowth test following treatment

Regrowth measures the time needed for the DSB to recover following treatment. Wiped samples were placed in 30 ml capacity flat bottom glass bottle with 2 ml of DE neutralizing broth (Acumedia®, Neogen® LabM, Lancashire, UK). The number of days for the DE broth colour to change from purple to yellow indicative of bacterial growth was recorded.

Statistical analysis

Statistical significance of data sets was evaluated with GraphPad PRISM® (ver. 7.04) using two-way ANOVA. All measurements, if not stated otherwise, were performed in triplicates. The sample standard deviation was evaluated with Bassel's correction.

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Conflict of Interest

K. Ledwoch is employed by GAMA Healthcare on a part time basis.

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Beware biofilm! Dry biofilms containing bacterial pathogens on multiple healthcare surfaces; a multi-centre study

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SUMMARY

Background: Wet biofilms associated with medical devices have been widely studied and their link with healthcare-associated infections (HCAIs) is well recognized. Little attention has been paid to the presence of dry biofilms on environmental surfaces in healthcare settings.

Aim: To investigate the occurrence, prevalence, and diversity of dry biofilms on hospital surfaces.

Methods: Sixty-one terminally cleaned items were received from three different UK hospitals. The presence of dry biofilm was investigated using culture-based methods and scanning electron microscopy (SEM). Bacterial diversity within biofilms was investigated using ribosomal RNA intergenic spacer analysis (RISA)—polymerase chain reaction and next-generation sequencing.

Findings: Multi-species dry biofilms were recovered from 95% of 61 samples. Abundance and complexity of dry biofilms were confirmed by SEM. All biofilms harboured Gram-positive bacteria including pathogens associated with HCAI; 58% of samples grew meticillin-resistant *Staphylococcus aureus*. Dry biofilms had similar physical composition regardless of the type of items sampled or the ward from which the samples originated. There were differences observed in the dominance of particular species: dry biofilms from two hospitals contained mostly staphylococcal DNA, whereas more *Bacillus* spp. DNA was found on surfaces from the third hospital.

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Conclusion: The presence of dry biofilms harbouring bacterial pathogens is virtually universal on commonly used items in healthcare settings. The role of dry biofilms in spreading HCAIs may be underestimated. The risk may be further exacerbated by inefficient cleaning and disinfection practices for hospital surfaces.

Introduction

Healthcare-associated infections (HCAIs) affect ~20% of National Health Service patients in the UK, causing significant mortality and financial losses with yearly cost for the healthcare systems estimated to be at least $1 billion [1,2].

A sizable proportion of HCAIs are preventable through improved practices such as environmental hygiene, hand hygiene, use of personal protective equipment, and screening and isolation; some estimate this preventable portion to be 20–30% [3,4]. Cleaning is used to reduce microbial burden on surfaces, but methods vary widely between hospitals. Most UK hospitals use detergents for routine cleaning. Chlorine-based disinfectants are usually reserved for terminal or specialized cleaning of areas exposed to antibiotic-resistant bacteria and *Clostridium difficile*, or during high-risk incidents or outbreaks. Efficacy of the cleaning process is dependent upon type of equipment, consumables, microbicidal activity, allotted time and motivation of cleaning and/or nursing staff [5,6]. There is evidence that enhanced cleaning is cost-effective [7].

Although in studies 98% of patients felt that their hospital room or ward was cleaned properly, visual audits used in monitoring the cleanliness of institutions are insufficient [8,9]. The best evidence for the role of the environment comes from studies showing the risk of infection for patients admitted into a cleaned room recently vacated by a patient with the same pathogen [10]. Microbial pathogens can persist on surfaces for days, months and even years unless removed by some cleaning or disinfection process [11]. Bacterial biofilms have been identified on some medical devices and are associated with the presence of moisture and/or liquid, for example, biofilms on medical devices such as urinary catheters, tracheal tubes, breast implants and endoscopes. Around 65% of HCAIs are associated with 'wet' biofilms [12]. However, more recently, biofilm has been discovered on dry surfaces, despite effective infection control measures. These biofilms are referred to as 'dry' biofilms [13,14].

To date, evidence of the presence of dry biofilms on surfaces in healthcare settings remains limited. This study aims to provide a better appreciation of the scale, extent, and composition of dry biofilms on surfaces that are regularly touched by healthcare workers.

Methods

Samples

Samples were collected from different wards and departments from three different hospitals in Wales (hospital A), Scotland (hospital B) and England (hospital C), including trauma and orthopaedics, adult intensive care, joint assessment unit, acute admission unit, kidney and transplant, nephrology, cardiology, gastroenterology, intensive therapy unit, and haematology (Table I). Items collected included hand sanitizing bottles, keyboards, patient folders and clipboards. In addition, one hospital provided a commode, a chair and wooden tray (part of a food trolley). Due to usage of electronic notes, hospital C was unable to provide any patient folders. All samples were kept in sterile bags to avoid cross-contamination during transportation. Sample processing was carried out under aseptic conditions.

Selection of sampling area

A key was removed from each keyboard and tested separately. The keys selection was made based on English letter frequency; letters with similar frequencies were tested (E, T, A, and O). Folders, clipboards, the chair, commode, sanitizing bottles and the trolley were sampled from the 'high touch' areas. In order to ensure that the same surface area was investigated for the different materials, 1.5 × 1.5 cm squares were cut from each sample.

Sample rinse and 'sterility' testing

Samples were placed in 50 mL polypropylene conical Falcon™ tubes (Fisher Scientific, Loughborough, UK) containing 30 mL of sterilized water and mixed with a Fisherbrand® vortex shaker (Fisher Scientific, Loughborough, UK) for 1 min. After three consecutive rinsing steps, a sterile cotton swab (ThermoFisher Scientific, Newport, UK) was streaked over the surface of the sample and incubated at 37°C overnight on to PP0280 TSA plate (EO Labs, Bonnybridge, UK). A sample was presumed free from planktonic bacteria if there was no bacterial growth observed after rinsing.

Determination of the presence of sessile bacteria

Following the rinsing step, samples were placed into a 50 mL polypropylene conical tube containing 30 mL of tryptone soya broth (TSB; Oxoid Ltd, Basingstoke, UK) and incubated in Sanyo orbital incubator (ThermoFisher Scientific) at 37°C at 180 rpm. The broth was examined for signs of turbidity every 24 h.

Microbial species determination by genotypic analysis

Ribosomal RNA (rRNA) intergenic spacer analysis (RISA) is a method of microbial community analysis to compare samples without culture-dependent bias. Samples were prepared as follows: turbid broths were incubated overnight and centrifuged at 1400 *g* for 10 min at 20°C. The supernatant was discarded, and the pellet was resuspended with 500 μL of TSB. Five hundred microlitres of 4 M guanidine isothiocyanate (UltraPure™, ThermoFisher Scientific) was added and the suspension was mixed with Wizard™ infrared vortex mixer (Fisherbrand) at 2000 rpm. One millilitre of culture mix was added into the tubes containing 1 g of 0.1 mm diameter zirconia/silica

Table I
Samples and time for bacterial regrowth in tryptic soya broth following rinsing

Hospital/department/ward	Sample no.	Sample type	Regrowth (days)[a]
Hospital A			
Gastroenterology	1	Folder	1
	2	Wooden tray (food trolley)	No regrowth[b]
	3	Hospital commode	1
	4	Leather chair seating	1
	5	Fabric chair back support	1
Trauma and orthopaedics	6	Patient folder	6
	7	Patient folder	1
	8	Patient folder	1
	9	Patient folder	1
	10	Patient folder	1
	11	Patient folder	2
	12	Patient folder	1
	13	Patient folder	1
	14	Patient folder	1
	15	Patient folder	1
Adult intensive care	16	Keyboard	1
	17	Keyboard	1
	18	Keyboard	1
Acute short-stay medicine	19	Keyboard	2
	20	Keyboard	1
	21	Keyboard	2
Gastroenterology	22	Keyboard	4
	23	Keyboard	1
	24	Keyboard	4
Nephrology	25	Keyboard	6
	26	Keyboard	9
	27	Keyboard	1
Trauma and orthopaedics	28	Keyboard	7
	29	Keyboard	1
	30	Sanitizing bottle	1
	31	Sanitizing bottle	1
Hospital B			
Microbiology	32	Patient folder	1
	33	Patient folder	1
	34	Patient folder	1
	35	Patient folder	1
	36	Patient folder	4
	37	Patient folder	5
	38	Patient folder	1
Biochemistry	39	Patient folder	1
	40	Patient folder	1
Pharmacy	41	Patient folder	1
General medicine	42	Keyboard	1
	43	Keyboard	1
	44	Keyboard	1
	45	Keyboard	1
	46	Keyboard	1
	47	Keyboard	1
	48	Keyboard	1
	49	Keyboard	1
	50	Keyboard	1
	51	Keyboard	1
Hospital C			
Acute admission unit	52	Keyboard	No regrowth[b]
Kidney and transplant	53	Keyboard	4
Cancer services and haematological malignancies	54	Keyboard	6

(*continued on next page*)

Table I (*continued*)

Hospital/department/ward	Sample no.	Sample type	Regrowth (days)[a]
Kidney and transplant	55	Keyboard	1
	56	Keyboard	1
Cardiology	57	Keyboard	1
	58	Keyboard	7
Acute medicine	59	Keyboard	1
Haematology	60	Keyboard	1
	61	Keyboard	No regrowth[b]

[a] Time for turbidity to develop following incubation in tryptic soy broth.
[b] No regrowth following >14 days incubation.

beads (Thistle Scientific, Glasgow, UK) and suspensions were mixed in Bead Bug homogenizer (Benchmark Scientific, Cole-Parmer, St Neots, UK) at 2800 rpm shaking speed for 2 min. DNA was amplified with Maxwell 16 Instrument (Promega, Southampton, UK) and quantified by Quibit® 3.0 fluorometer (ThermoFisher Scientific). The internal transcribed spacer (ITS) bacterial region between the 16S rRNA and 23S rRNA subunit genes was amplified with 1406F (TGYACACACCGCCCGT; Eurofins Genomics, Ebersberg, Germany) and 23SR (GGGTTBCCCCATTCRG; Eurofins Genomics, Ebersberg, Germany) primers by running RISA–PCR in thermal cycler (Bio-Rad, Watford, UK) [15].

Microfluidic separation was performed with a 2100 Bioanalyser Instrument (Agilent Technologies Ltd, Craven Arms, UK) using DNA 7500 chip (Agilent Technologies Ltd, Craven Arms, UK) [16].

Polymerase chain reaction bands of 55 different hospital samples were cluster-analysed with Bionumerics software (Applied Maths, Gent, Belgium) to determine the similarity of RISA–PCR profiles between hospitals and wards. Similarity was determined using the Pearson coefficient [17].

Microbial species determination by DNA analysis (next-generation sequencing)

DNA was extracted and quantified as described above. Next-generation sequencing (NGS) and quality analysis of FASTQ sequence reads was performed by BaseClear Group, the Netherlands. The 16S rRNA gene (V3–V4) was PCR-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.1.5.1).

Scanning electron microscope analysis

Three randomly selected samples from keyboards, folders, hospital commode and chair seating were analysed with scanning electron microscopy (SEM) to visualize the presence of dry biofilms. Samples were prepared by overnight incubation 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 SEM, samples were coated with 20 nm AuPd coating with sputter coater (SC500, Bio-Rad). 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 ×10,000 magnification and 5–7 mm working distance with the help of Earth and Ocean Sciences Department, Cardiff University, UK.

Statistical analysis

The strength of linear association between PCR–RISA profiles of dry biofilms recovered from hospital samples was calculated using Pearson coefficient with Bionumerics software (Applied Maths). Higher linear association between two data sets is presented by higher absolute value of Pearson coefficient [17]. The Pearson coefficient values were re-calculated into percentages to better reflect the correlation between PCR–RISA data sets.

Results

Bacterial presence on hospital sample surfaces

No planktonic bacteria were recovered from any of the 61 samples investigated after multiple rinsing steps with sterile water. Bacterial growth in a nutrient-rich broth was observed in 95% of samples (58/61) (Table I). The assumption was made that bacterial growth resulted from the presence of sessile bacteria embedded within the dry surfaces, which was confirmed by SEM analysis. Most of the samples (76%) produced growth within 24 h (Table I).

DNA analysis (RISA–PCR)

RISA–PCR bands of bacterial cultures recovered from samples were analysed to determine the consistency of microbial composition between wards from the same hospital and between hospitals (Figure 1). Cluster analysis showed that the majority of bacterial cultures shared a part of the genome, regardless of the sample type or origin. The PCR profiles of hospital samples were convergent in 49%, meaning that the similarity between samples was high. Similarity of bacterial cultures was consistent within samples originating from hospitals A and B with 53% and 45% similarity, but lower for hospital C with 38% similarity.

DNA analysis: next-generation sequencing

The DNA analysis of the bacterial species recovered from broth cultures following incubation of hospital samples was performed by NGS, which allowed more precise and detailed analysis of bacterial cultures' composition than did RISA–PCR (Figure 2). Bacterial species found from the hospital samples

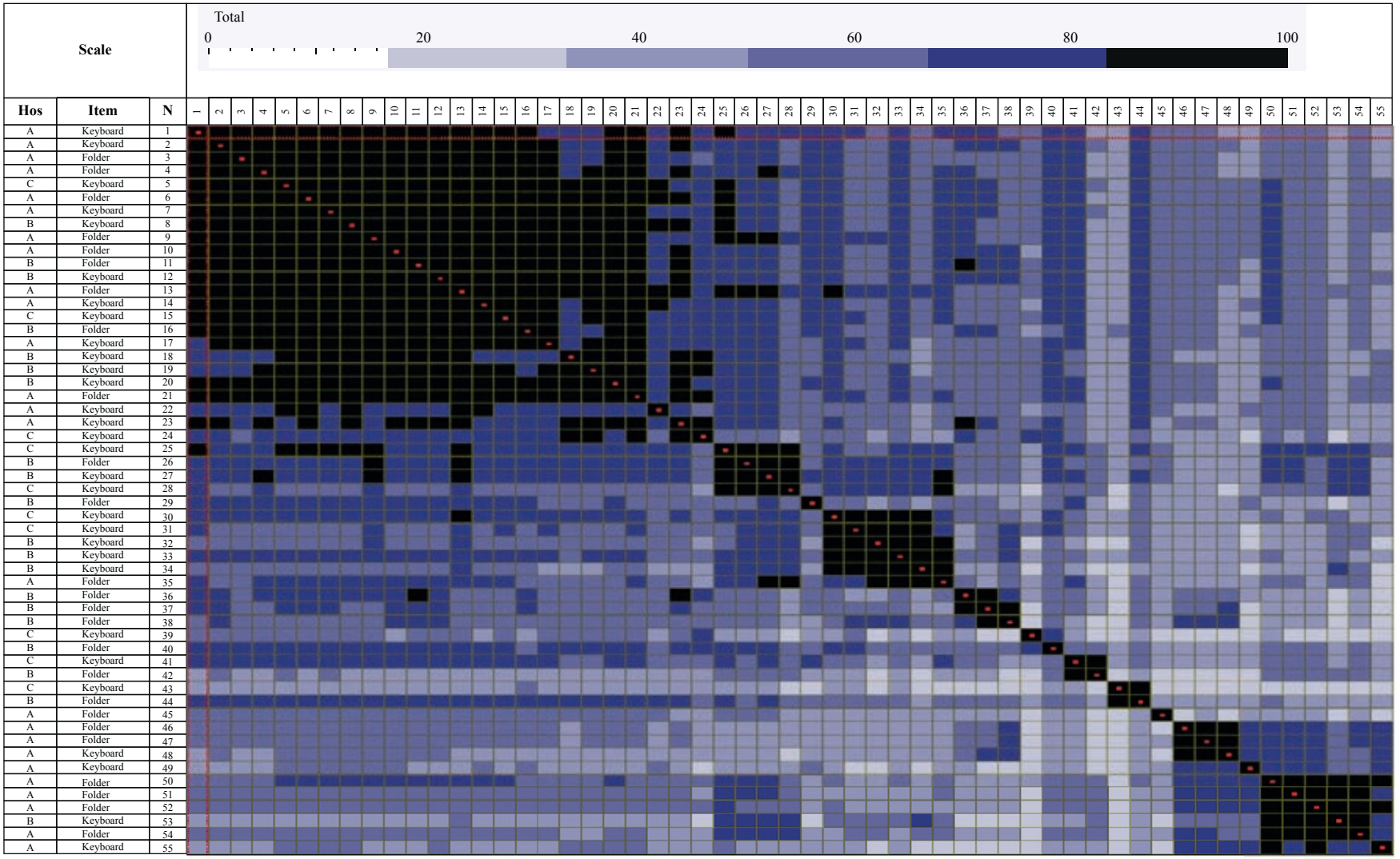

Figure 1. Cluster analysis of ribosomal RNA intergenic spacer analysis—polymerase chain reaction (RISA—PCR) bands of cultures recovered from hospital samples (keyboards and folders). The scale indicates the similarity between a pair of two samples (in a range of 0—100%, where 0% indicates no correlation, and 100% indicates complete convergence in RISA—PCR profiles). Red dots indicate pairs of the same samples, which are not taken into account in analysis.

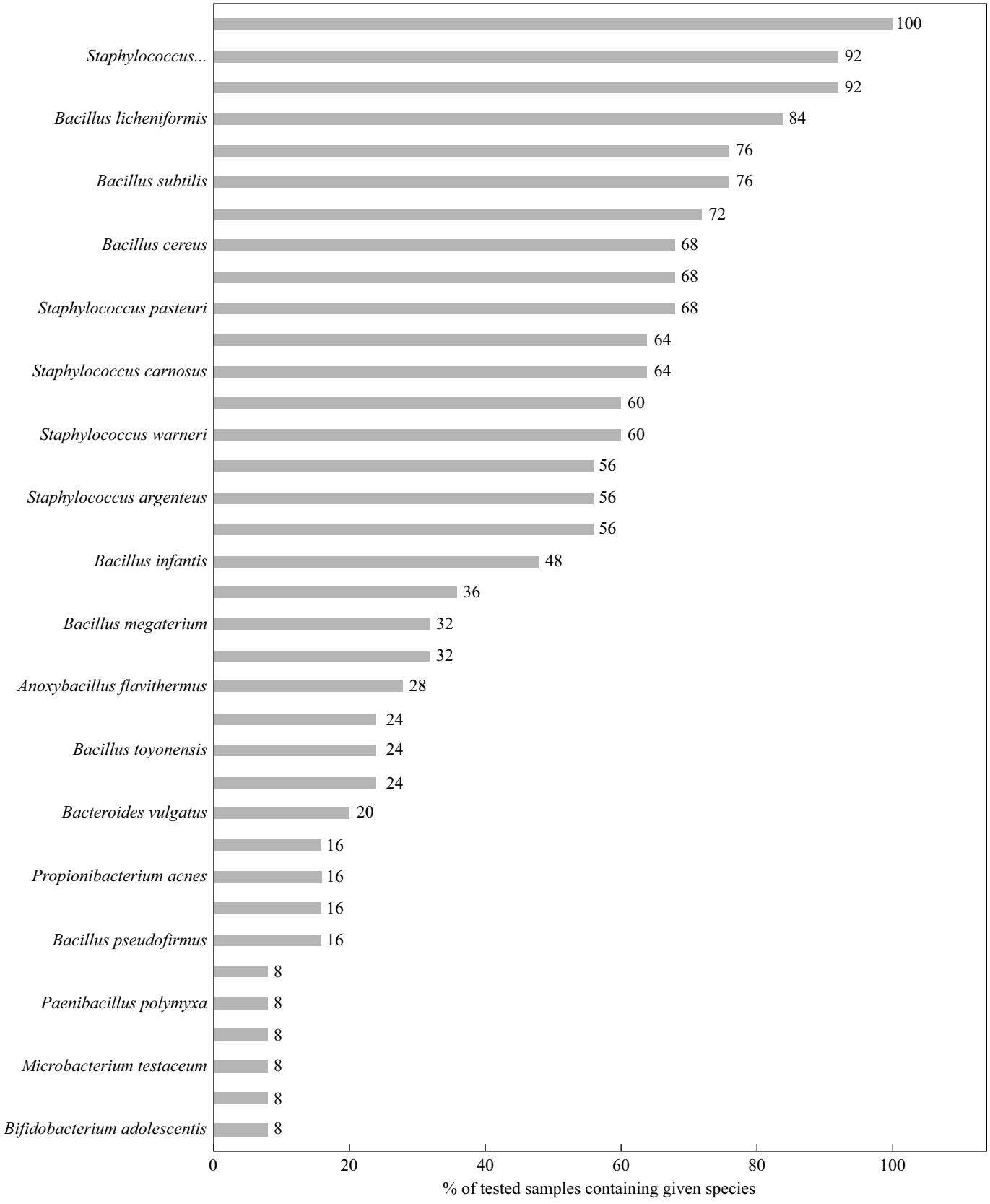

Figure 2. Percentage of samples containing given species following DNA analysis; all samples pooled.

contained mainly staphylococci and *Bacillus* spp. The most common bacteria identified were *S. aureus*, *S. saprophyticus* and *S. epidermidis*, with *B. licheniformis* and *B. subtilis* being the most common *Bacillus* spp. The only Gram-negative DNA isolated was that of *Pseudomonas* spp. (Figure 2).

On average, 18 different bacterial species were present on each surface analysed from the dry surface samples.

Dominance of particular species was associated with their origin (Figure 3). Samples isolated from hospitals A and B mostly contained *S. aureus* and *S. epidermidis* (34.6% and 24.6% in hospital A, 23.8% and 24.2% in hospital B, respectively). The most common bacteria found from hospital C samples were *S. saprophyticus* and *B. subtilis* (20.6% and 19.1%, respectively).

Staphylococci generally dominated on hospital A and B surfaces (66% and 53% of dry biofilm composition, respectively), whereas in hospital C, *Bacillus* spp. were dominant (68% of dry biofilm composition).

Scanning electron microscope analysis

The previous experiments indicated the presence of multiple sessile bacterial species on the surfaces from dry hospital surfaces. SEM was used to visualize the presence and complexity of dry biofilms formed on these surfaces. Samples varied in their composition and presence of matrix. These dry biofilms formed random clusters of bacterial biofilm on surfaces (Figure 4).

Discussion

A biofilm is a complex community of micro-organisms embedded in self-produced extracellular polymeric matrix, markedly different from their planktonic state equivalent [18]. Most biofilm studies are associated with wet biofilm sessile micro-organisms grown in aqueous habitats. Little attention has been paid to biofilms grown in dry environments, despite their presence on dry hospital surfaces [19]. There is no standardized method for biofilm detection on surfaces [12]. Nevertheless, an approach was developed to identify the presence of dry biofilm on healthcare surfaces. No viable bacteria were grown following rinsing dry surface samples, suggesting that no planktonic bacteria were present. However, by immersing the sample surfaces in nutrient broth and following incubation, we detected multiple bacterial

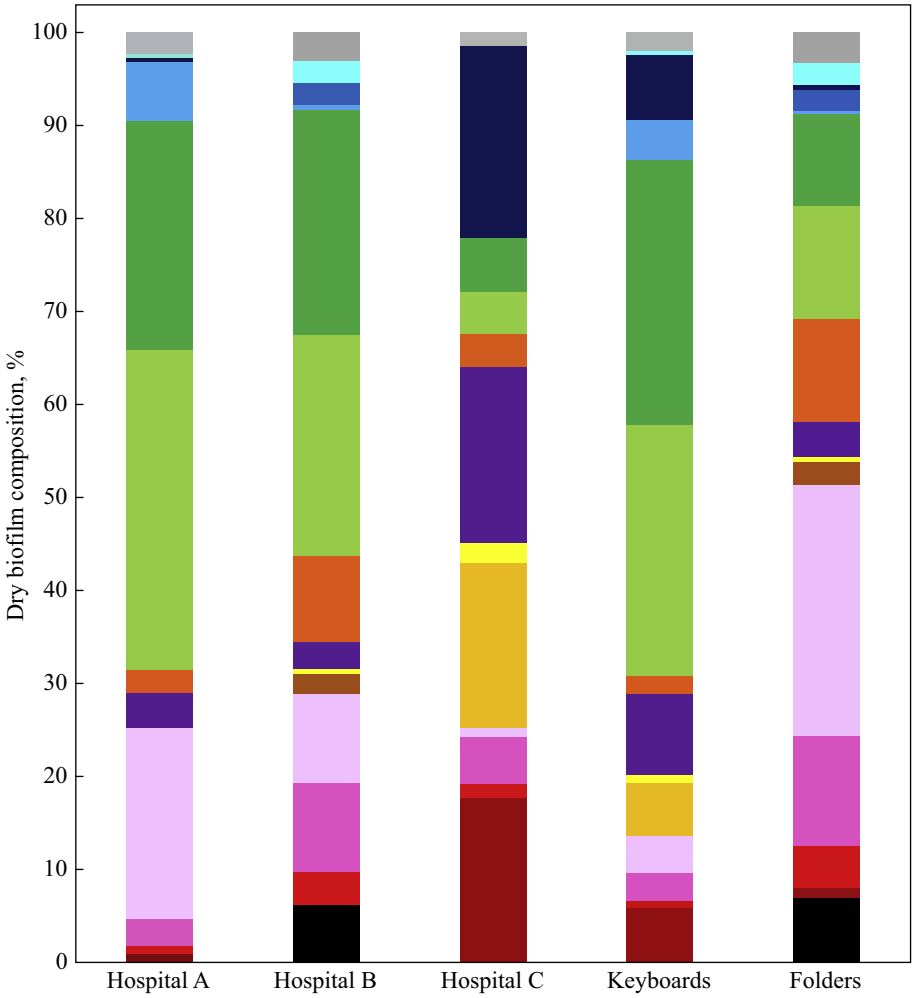

Figure 3. Difference in 'dry' biofilm composition between hospitals. ■, *Anoxybacillus flavithermus*; ■, *Bacillus amyloliquefaciens*; ■, *Bacillus anthracis*; ■, *Bacillus cereus*; ■, *Bacillus licheniformis*; ■, *Bacillus megaterium*; ■, *Bacillus pumilus*; ■, *Bacillus* sp.; ■, *Bacillus subtilis*; ■, *Bacillus thuringiensis*; ■, *Staphylococcus aureus*; ■, *Staphylococcus epidermidis*; ■, *Staphylococcus lugdenensis*; ■, *Staphylococcus pasteuri*; ■, *Staphylococcus saprophyticus*; ■, *Staphylococcus warneri*; ■, other.

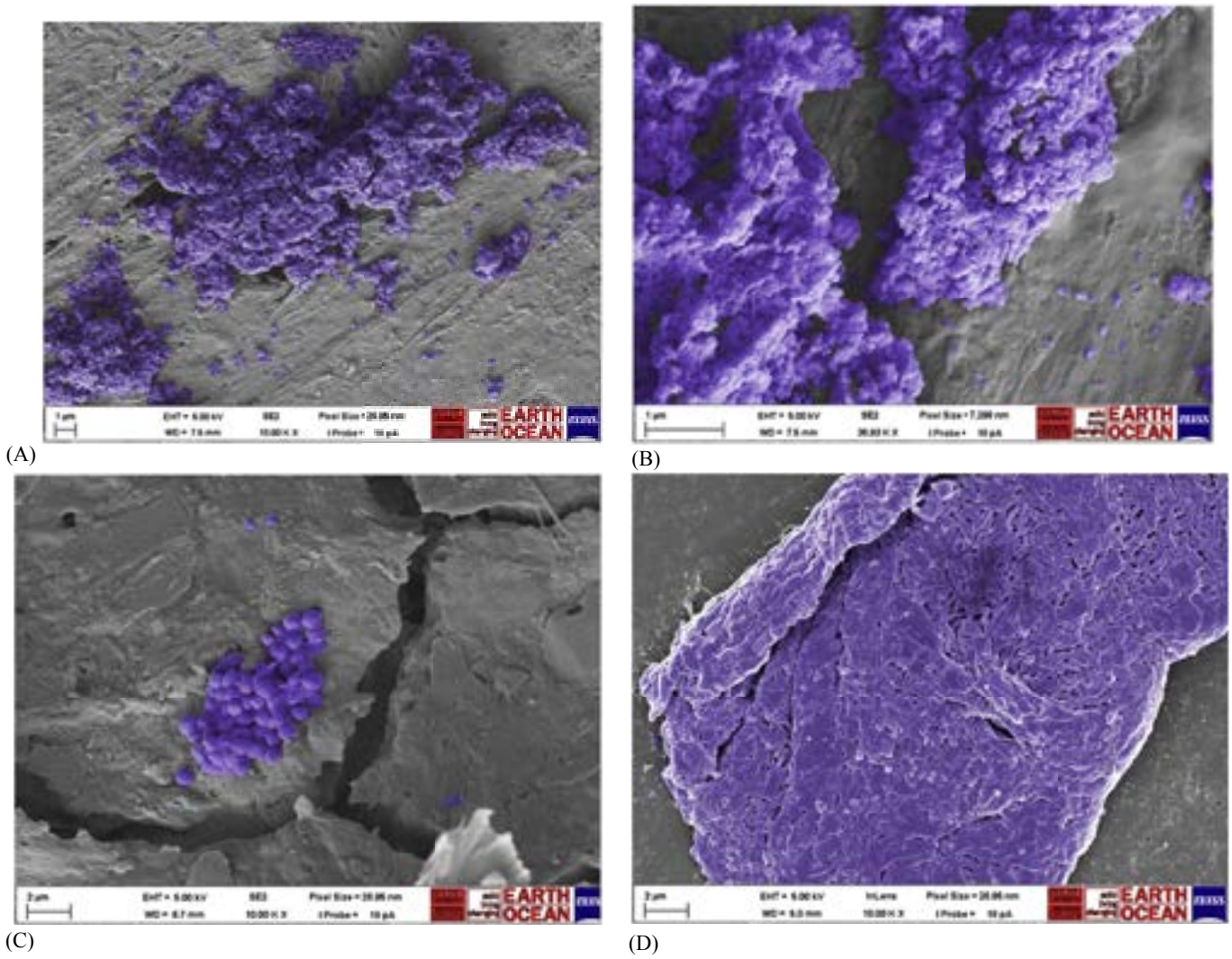

Figure 4. Examples of 'dry' biofilms recovered from surfaces; magnification ×10,000. (A, B) Patient folders, (C) patient chair, (D) keyboard key. Images of biofilms were coloured in purple to help visualization and contrast using GNU Image manipulation program (GIMP 2.8) software. Images were not otherwise altered.

species from the surfaces, and scattered random microbial biofilms were observed by SEM. Indeed, almost all samples (95%) harboured dry biofilms. Similar results were obtained by Hu *et al.*, who confirmed the presence of biofilm on 41/44 (93%) hospital surface samples [14]. It is conceivable that the rinsing step including vortexing may have contributed to dislodging loosely attached bacteria/biofilm [20]. Our results showed, however, that the majority of surfaces bear dry biofilms that were not removed or loosened by the multiple rinsing steps.

Our study shows staphylococci and *Bacillus* spp. to be the main bacterial genera recovered from the dry biofilms. It was surprising to see some variability between hospitals whereby in one study hospital *Bacillus* spp. were the dominant bacteria. These differences may be explained by hospital size, antimicrobial consumption, ventilation and patient infections (since patients shed pathogens on to surfaces around them), cleaning and disinfection regimens (which will impact on soiling), and possibly differences in the item sampled [21]. Here, the three hospitals varied in size: hospitals A and C are 1500-bed teaching hospitals in Wales and in London, whereas hospital B is a Scottish district general hospital with 550 beds. Detergents are used for routine cleaning in all three hospitals, although, in hospital A, there is proportionately greater use of chlorine-based disinfectants. All three hospitals used a terminal cleaning regimen with bleach-type agents for areas exposed to known pathogens such as carbapenemase-producing Enterobacteriaceae, norovirus, *C. difficile*, MRSA and vancomycin-resistant enterococci. We cannot comment on the type of patients or their infections, as that information was not collected during this study. Despite these differences, it was remarkable that dry biofilms on the sampled surfaces were widespread and that there was a good similarity of the dry biofilm microbial composition from two hospitals. The local environment (urban vs rural) could also play a role. For example, external building work has been associated with increased number of bacillus spores in healthcare facilities [22,23]. Here, no external building work was reported near or within hospital C at the time

of sampling, which would have explained the high level of *Bacillus* spp. observed.

Staphylococcus aureus, *S. saprophyticus* and *S. epidermidis* were the dominant *Staphylococcus* spp. MRSA was cultured from a high proportion of samples; the clinical risk associated with this finding is yet to be determined. Among *Bacillus* spp., the prevalent species were *B. licheniformis* and *B. subtilis*. These results differ from the study by Hu *et al.*, in which no *Bacillus* spp. were reported, although the authors analysed only samples from the critical care environment [14]. Nevertheless, it is clear that composition of the isolated biofilms is a mixture of environmental, skin and gut microflora, including pathogens. The complexity of composition has been reported for biofilm from different surfaces, although overall composition depends on the object sampled [14]. Our study showed some variability in the detailed composition of the biofilm for the same object between different hospitals. However, variability in microbial composition decreased between different objects from the same hospital. It is likely that each hospital has its own unique 'environmentome', which is reflected in the composition of biofilm on items and surfaces.

We and others have not addressed the formation of complex dry biofilm on environmental surfaces in healthcare settings [13,14]. Bacterial stress such as desiccation and exposure to chemicals may enhance the production of extracellular polysaccharides, which can protect biofilms from adverse chemical and physical effects [24–28]. More concerning is the report of ineffectiveness of disinfection, notably chlorine-based agents, against dry biofilms, and how this impacts on infection control [14,29]. The high prevalence of *S. aureus*, especially of MRSA, from dry biofilm from the surfaces sampled adds to that concern, since MRSA is known to be transmitted through environmental contamination [30]. The presence and transmission of pathogens in dry biofilms from healthcare surfaces warrants further examination [31,32]. The risk associated with bacterial pathogens recovered in dry biofilms and HCAIs is yet to be determined. Some studies link the survival rates of biofilm-forming strains with persistent nosocomial infections and outbreaks [33,34]. The role of *Bacillus* spp. in protecting bacterial pathogens in biofilm from disinfection also needs to be addressed [34–38].

In conclusion, our study provides more evidence that complex dry biofilms containing bacterial pathogens are virtually universal on hospital surfaces, despite regular cleaning and disinfection. These dry biofilms were shown to occur in clusters on different materials and were predominantly formed by Gram-positive bacteria, although occasional *Acinetobacter* spp. were identified. Unlike other studies, we identified a large proportion of *Bacillus* spp. The role of *Bacillus* spp. in protecting a mixed biofilm community from environmental conditions and disinfection should be further investigated [38]. Although these dry biofilms are clearly harbouring pathogens, their role in transmission needs to be established. This is especially important in view of the failing of disinfection to control these biofilms and the general absence of testing and biocidal products claiming efficacy against dry biofilms.

Conflict of interest statement

K. Ledwoch is partly funded by GAMA Healthcare Ltd. J.A. Otter is a consultant to GAMA Healthcare Ltd, and has been a consultant to Pfizer in the last three years.

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materials

MDPI

Article

Candida auris Dry Surface Biofilm (DSB) for Disinfectant Efficacy Testing

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Abstract: *Candida auris* is an emerging pathogen that needs to be controlled effectively due to its association with a high mortality rate. The presence of biofilms on dry surfaces has been shown to be widespread in healthcare settings. We produced a *C. auris* dry surface biofilm (DSB) on stainless steel surfaces following sequential hydration and desiccation cycles for 12 days. The ASTM2967-15 was used to measure the reduction in viability of 12 commercially wipe-based disinfectants and sodium hypochlorite (1000 ppm) against *C. auris* DSB. We also evaluated *C. auris* transferability and biofilm regrowth post-treatment. A peracetic acid (3500 ppm) product and two chlorine-based products (1000 ppm available chlorine) were successful in reducing *C. auris* viability and delaying DSB regrowth. However, 50% of the products tested failed to decrease *C. auris* viability, 58% failed to prevent its transferability, and 75% did not delay biofilm regrowth. Using three different parameters to measure product efficacy provided a practical evaluation of product effectiveness against *C. auris* DSB. Although $\log_{10}$ reduction in viability is traditionally measured, transferability is an important factor to consider from an infection control and prevention point of view as it allows for determination of whether the surface is safe to touch by patients and hospital staff post-treatment.

Keywords: *Candida auris*; dry-biofilm; disinfection; peracetic acid; sodium hypochlorite; chlorine dioxide; sodium dichloroisocyanurate; transferability; regrowth

1. Introduction

Candida auris was first isolated and identified in Japan in 2009 [1]. *C. auris* is an emerging pathogen responsible for many life-threating infections and it can be associated with high mortality rates [2]. *C. auris* infections are difficult to treat mostly due to the unpredictable resistance profile of the yeast to anti-fungal agents, frequent misidentification, non-aggregative phenotype, and its ability to form biofilm [3]. Higher risk of candidemia occurs in immunocompromised patients, patients that have undergone antibiotic or anti-fungal therapy, patients after surgeries, and patients with central venous catheters [4].

Contaminated surfaces in healthcare settings contribute to the transmission of infectious diseases [5–7]. Vickery and colleagues [8] showed that pathogens embedded in dry surface biofilms (DSB) remain on hospital surfaces despite rigorous surface decontamination. The widespread presence of dry surface biofilms on healthcare surfaces has now been established [9,10]. *C. auris* can persist on surfaces for weeks [11,12] and transmission of *C. auris* in healthcare settings have been reported [2,4,13,14]. The elimination of *C. auris* from surfaces is therefore important to consider. However, not many studies have investigated the effectiveness of disinfectants against *C. auris* [15]. Public Health England guidance [16] for the management and infection prevention and control of *C. auris* recommends using hypochlorite at 1000 ppm available chlorine to terminally clean room or bed space after the discharge of a *C. auris* infected or colonized patient. Not surprisingly, the majority

of efficacy studies against *C. auris* relates to chlorine-releasing agents [14,17–19], although other biocides have been considered, such as quaternary ammonium compounds [14,18], acetic acid [18], peracetic acid [19], and hydrogen peroxide [17]. All these studies but one studied planktonic (suspension of) *C. auris* [20]. None of these studies investigated the transferability of *C. auris* to other surfaces post-treatment. Here, we investigated the efficacy of 12 commercially available products and sodium hypochlorite 1000 ppm against *C. auris* DSB using a modified product efficacy test protocol ASTM2967-15 [21] to measure decreases in viability, transferability, and biofilm regrowth post-treatment. The evaluation of three different parameters provides a better and more practical understanding of product efficacy against this pathogen.

2. Materials and Methods

C. auris Growth and Maintenance

C. auris (DSM 21092) was propagated overnight in malt extract broth (MEB, Oxoid, Thermo Scientific™, Loughborough, UK) at 25 °C and the pellet was re-suspended in MEB following centrifugation at 1200× *g*. The yeast suspension was adjusted to 1×10^6 CFU/mL.

C. auris Organic Load (OL) Dry-Biofilm Model

The *C. auris* DSB model is based on a recently developed *Staphylococcus aureus* DSB protocol [22]. Briefly, dry-biofilm formation consists of alternating hydration and desiccation phases in the presence of an organic load (OL). Stainless steel AISI 430 discs (0.7 ± 0.07 mm thickness; 10 ± 0.5 mm diameter, Goodfellow Cambridge Limited, Huntington, UK) were used as support. Sterile discs were placed in wells of a Corning™ Costar™ flat-bottom cell culture plates (Fisher Scientific™, Loughborough, UK), containing 1 mL of MEB with 5% anhydrous D-glucose (Fisher Scientific, Loughborough, UK), 3 g/L bovine serum albumin (BSA; Sigma® Life Science, Dorset, UK), and 10^6 CFU/mL washed *C. auris* suspension. Yeasts were first allowed to attach and form a biofilm on the disc surface for 2 days at 25 °C under gentle agitation using an Orbit P4 plate rocker (Labnet International, Edison, NJ, USA). The suspension was then drained from the wells, and plates were incubated at 25 °C for 48 h. Following this dry phase, 1 mL of MEB with 3 g/L BSA was added to each well, and a new hydrated phase began for 48 h. Hydrated and dry phases alternated every 48 h for a period of 12 days, ending with biofilm in a dry phase.

Scanning Electron Microscopy (SEM) Imaging

C. auris DSB samples were prepared by overnight incubation of discs in a 2.5% glutaraldehyde solution (ACROS Organics™, Fisher Scientific, Loughborough, UK) followed by immersion in successive concentrations of 10%, 25%, 50%, 70%, 90%, and 100% ethanol (Honeywell, Fisher Scientific Ltd., Loughborough, UK) for 10 min each. Prior to scanning electron microscopy (SEM, Carl Zeiss Ltd., Cambridge, UK) scanning, samples were coated with 20 nm of AuPd coating with a sputter coater (SC500, Biorad, UK). 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 ×2000 and ×10,000 magnification and a 5 mm working distance. SEM images were false-colored to help visualization and contrast using GNU Image manipulation program (GIMP 2.8) software. Images were not otherwise altered.

Product Tested

The effectiveness of four commercially available wipes and eight commercially available liquid disinfectants was tested against *C. auris* OL dry-biofilm (Table 1). Disinfectants were prepared according to manufacturers' instructions and combined with Rubbermaid® HYGEN™ disposable microfiber cloth (Rubbermaid Products, Surrey, UK), allowing 2.5 mL of disinfectant per 1 g of wipe. Wipes were cut into 3×3 cm^2 squares prior to testing.

Table 1. Disinfectants tested.

Abbreviation	Main Active Ingredient [1]	Excipients (from MSDS) [1]	Concentration of the Main Active Ingredient [4]	pH [5]	Mechanism of Disinfectant Action [6]	Wipe Material
BZK	Benzalkonium chloride, polyhexamethylene biguanide (PHMB)	Didecyl dimethyl ammonium chloride	< 0.5% (<5000 ppm)	5.41	Membrane active agents; damage cytoplasmic membrane and increase permeability [23]	Non-Woven Wipe [7]
ClO_2-1	Chlorine dioxide	Sodium chlorite, sodium dodecyl sulphate, sodium carbonate, citric acid, sodium dichloroisocyanurate	300 ppm	5.05	Affect membrane permeability of the membrane and inhibits cellular respiration [23]	Microfiber cloth [8]
ClO_2-2	Chlorine dioxide	Not mentioned	1000 ppm	4.31		Microfiber cloth [8]
NaDCC-1	Sodium dichloroisocyanurate	Adipic acid, arylsulfonates, sodium fatty acid sarcosides	1000 ppm	6.31	Permeabilization of the cytoplasmic membrane [24], progressive oxidation of thiol groups to disulphides [25] and deleterious effects on DNA synthesis [26]	Microfiber cloth [8]
NaDCC-2	Sodium dichloroisocyanurate	Adipic acid, sodium toluene sulphonate, sodium n-lauroylsarcosinate	1000 ppm	5.93		Microfiber cloth [8]
NaDCC-3	Sodium dichloroisocyanurate	Sulfonic acid	10,000 ppm	5.77		Non-woven wipe [9]
NaDCC-4	Sodium dichloroisocyanurate	Adipic acid, sodium carbonate	1000 ppm	5.86		Microfiber cloth [8]
NaDCC-5	Sodium dichloroisocyanurate	Adipic acid, sodium toluenesulphonate, sodium *N*-lauroyl sarcosinate	1000 ppm	5.64		Microfiber cloth [8]
NaOCl-Ref [2]	Sodium hypochlorite	N/A	1000 ppm	11.31	Biosynthetic alterations in cellular metabolism [27], phospholipid degradation, irreversible enzymatic inactivation in bacteria, lipid and fatty acid degradation [28]	Microfiber cloth [8]
NaOCl-2	Sodium hypochlorite	Sodium hydroxide, sodium chloride	500 ppm	8.68		Non-woven wipe [7]
NaOCl-3	Sodium hypochlorite	phosphoric acid (trisodium salt, dodecahydrate), sodium hydroxide, phosphoric acid	1000 ppm	13.13		Non-woven wipe [7]
PAA-1	Peracetic acid	sodium percarbonate, citric acid	3500 ppm	8.82	Rupture or dislocation of cell wall, disruption of biochemical processes intercellularly [29] and impairment of DNA replication [30]	Non-woven wipe [9]
PAA-2	Peracetic acid	Not mentioned	250 ppm	7.74		Microfibre cloth [8]
Water [3]	N/A	N/A	N/A	6.99	N/A	Microfibre cloth [8]

[1]: Main active ingredient and excipients mentioned in the MSDS information of the commercial products used in this study. [2]: Unformulated sodium hypochlorite (1000 ppm), used as reference. [3]: Sterile deionized water. [4]: Concentration of available chlorine/peracetic acid concentration was measured with Pocket Colorimeter™ (HACH®, Manchester, UK) (regardless of the product claim on label) via the *N*, *N*-diethyl-p-phenylenediamine (DPD) method. [5]: pH was measured by bench top pH meter (HANNA® Instruments, Leighton Buzzard, UK). [6]: Reported mechanisms of action, mainly from studies of bacteria. [7]: Wipe originally moisturized with disinfectant by the manufacturer. [8]: Disinfectant prepared according to manufacturer's instruction and then placed on Rubbermaid® HYGEN™ disposable microfiber cloth (2.5 mL of liquid per 1 g of cloth). [9]: Dry non-woven wipe impregnated with powder particles—needs to be wetted according to manufacturer instructions prior to use.

ASTM E2967-15 Test

Disinfection tests were performed according to a modified ASTM E2967-15 test [21]. Briefly, DSB were wiped with the Wiperator (Filtaflex Ltd., Almonte, Ontario, Canada) from both sides for 10 s under 500 g pressure, left for 2 min at 25 °C, and then neutralized in Dey-Engley neutralizing (DE) broth (Neogen® Corporation, Ayr, UK). Transfer of viable yeasts from used wipes to clean sterile disc was not performed.

Reduction in Viability for Yeasts Embedded in Dry Biofilms

Following wiping, samples were incubated for 1 h at 25 °C in 2 mL DE with 100 µg/mL proteinase K (Fisher Bioreagents™, Fisher Scientific, Loughborough, UK) and 1 g of glass beads (Fisher Scientific, Loughborough, UK). After incubation, samples were vortexed for 2 min and then serially diluted, and 3 × 10 μL^2 drops of each dilution was plated onto tryptone soya agar (TSA; Oxoid, Thermo Fisher Scientific, Newport, UK). Reduction in yeast viability, expressed as a $\log_{10}$ reduction, was calculated as the difference between the number of yeasts recovered from untreated (control) and treated samples.

Transferability Test

Following wiping, discs were pressed 36 separate times with 100 g pressure on the surface of DE agar. Following the transfer test, DE agar was incubated at 25 °C for a up to 5 days until colonies appeared. Positive growth/adpression was recorded, and transferability was calculated as the number of positive contact/number of adpressions.

Dry-Biofilm Regrowth

Following wiping, discs were placed in 30 mL capacity flat bottom glass bottle with 2 mL of DE broth. The number of days for turbidity change, which is indicative of growth, was recorded. Samples were plated on TSA to confirm yeast growth and purity.

Statistical Analysis

The statistical significance of data sets was evaluated with GraphPad PRISM® (version 7.04, GraphPad Software, San Diego, CA, USA) using two- and one-way Analysis of Variance (ANOVA). All experiments were performed in triplicates in three independent biological replicates unless otherwise stated. The sample standard deviation was evaluated with Bassel's correction.

3. Results

SEM Analysis of C. auris Dry Surface Biofilm

C. auris formed a thin biofilm that was evenly scattered throughout the stainless-steel disc surface with no evidence of extracellular polymeric substances (Figure 1). There was no statistically significant difference (two-way ANOVA, $p = 0.06$) in viable count of yeasts ($\log_{10}$ CFU/mL = 7.8 ± 0.3) recovered from each disc between four independent biofilm batches.

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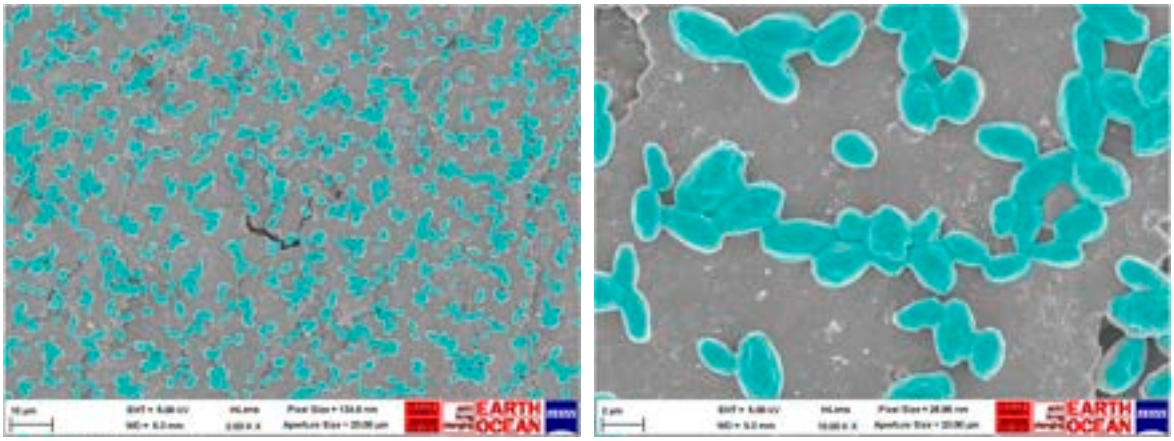

(**a**) (**b**)

Figure 1. Scanning electron microscope images of *C. auris* grown on a stainless-steel (SS) dry surface biofilm (DSB): (**a**) 2000× magnification; (**b**) 10,000× magnification. The images presented are representative of the whole disc surface observations made on three independent replicates of *C. auris* DSB, and whole disc surface (~0.8 cm²) was investigated each time with ~500× magnification. Images of dry surface biofilm (DSB) were colored in green to help visualization and contrast using GNU Image manipulation program (GIMP 2.8) software. Images were not otherwise altered.

Product Efficacy

The most effective treatments including PAA-1 (3500 ppm), NaDCC-5 (1000 ppm), NaOCl-Ref (1000 ppm), and NaOCl-3 (1000 ppm) removed or killed more than 7 $\log_{10}$ of *C. auris* embedded in DSB (Figure 2). Peracetic acid at 3500 ppm combined with a non-woven wipe was significantly (one-way ANOVA, $p < 0.05$) more effective in biofilm eradication than PAA at 250 ppm combined with a microfiber cloth (0.84 ± 0.11 $\log_{10}$ reduction). NaDCC-5 was the most effective (two-way ANOVA, $p < 0.05$) in killing or removing *C. auris* DSB compared to the other NaDCC-based products that all failed to produce a 4 $\log_{10}$ reduction in viability (Figure 2). Chlorine-dioxide-based products overall did not perform very well, achieving less than 2.5 $\log_{10}$ reduction even with ClO_2-2 containing a higher concentration (1000 ppm) of available chlorine (Figure 2). There was no difference (one-way ANOVA, $p = 0.22$) in activity between ClO_2-1 and ClO_2-2. Overall, half of the products tested (ClO_2-1, NaDCC-2, and NaOCl-2) showed either a similar performance (one-way ANOVA, $p > 0.05$) to water combined with the microfiber cloth or performed worse (one-way ANOVA, $p < 0.05$) than water combined with the microfiber cloth (ClO_2-2, NaDCC-3, and PAA-2).

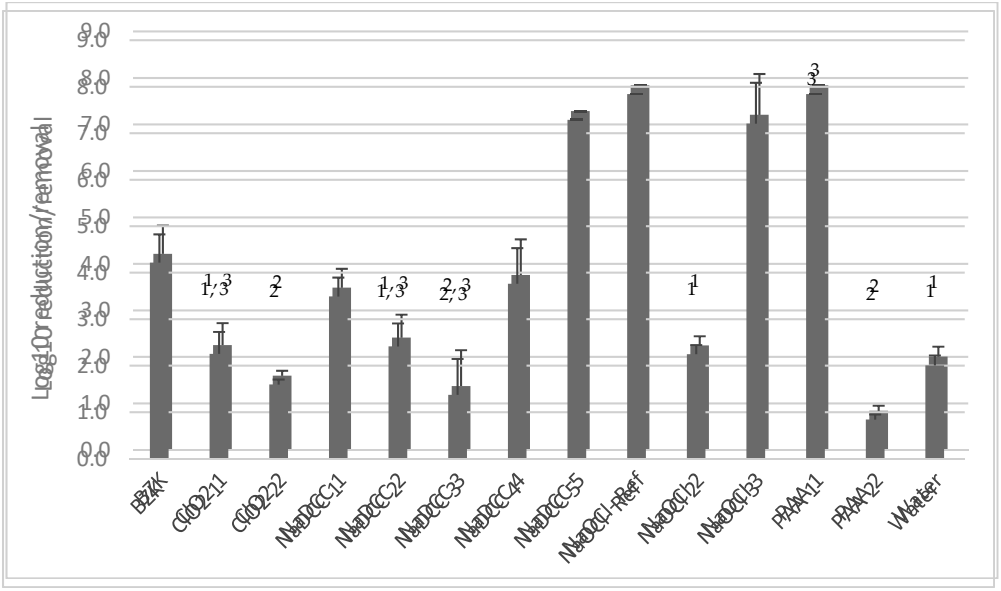

Figure 2. Product efficacy in killing/removing *C. auris* embedded in a DSB. 1: indicates no statistical difference (one-way ANOVA, $p > 0.05$) in $\log_{10}$ reduction/removal from surfaces; 2: $\log_{10}$ reduction/removal lower than wiping with water (one-way ANOVA, $p \leq 0.05$); and 3: indicates that only two biological replicates were performed.

Only two products, PAA-1 and NaOCl-3, prevented *C. auris* transfer after treatment (Figure 3). Seven out of 12 commercially available disinfectants were not effective in lowering the transferability of *C. auris* from DSB post-wiping. For four product/materials combinations (ClO_2-2, NaDCC-2, NaDCC-3, and NaOCl-1), there was no statistically significant difference (one-way ANOVA, for each pair $p > 0.05$) between their performance and that of water (Figure 3). The remaining three treatments (NaDCC-4, NaOCl-2 and PAA-2) were even less effective than wiping with water (one-way ANOVA, for each pair $p < 0.05$).

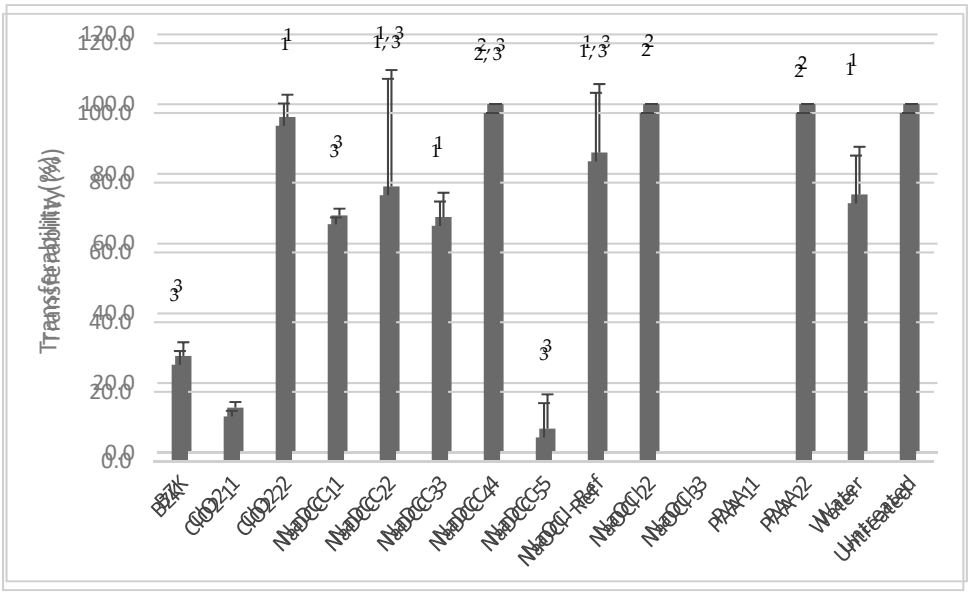

Figure 3. Product efficacy in preventing *C. auris* transferability post-wiping. 1: indicates no statistical difference (one-way ANOVA, for each pair $p > 0.05$) in transferability; 2: higher transferability (one-way ANOVA, for each pair $p < 0.05$) than with water control; and 3: indicates that only two biological replicates were performed.

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The best commercial products, NaDCC-3, NaDCC-5, and PAA-1, delayed the recovery of biofilm post-treatment for more than 4 days (5.0 ± 0.0, 4.7 ± 1.2 and 6.5 ± 2.1 days, respectively; Figure 4). Such activity was similar (two-way ANOVA, $p = 0.53$) to NaOCl-Ref which delayed regrowth by 4.5 ± 0.7 days. Nine out of 12 commercial treatments failed to prevent the regrowth of *C. auris* DSB for more than 2 days (Figure 4).

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The less-reactive chemistry (as compared to the oxidizing chemistries) based on a quaternary ammonium compound produced a 4 $\log_{10}$ reduction in *C. auris* on surfaces and reduced transferability post-wiping to 20%.

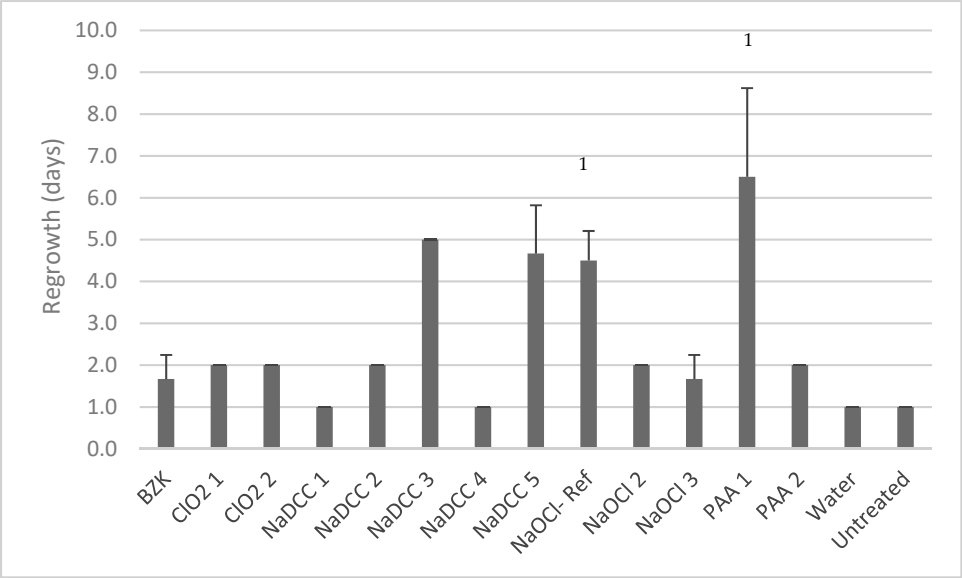

Figure 4. Efficacy of products in preventing regrowth post-wiping. 1: indicates that only two biological replicates were performed.

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4. Discussion

Environmental surfaces play an important role in the transmission of infection [5,6]. Infection control regimens include the combination of cleaning and disinfection processes [16] and are often based on the use of a disinfectant or cleaner combined with diverse materials [31]. Performance of biocidal products still relies, however, on testing the efficacy of the formulation and not the combination of formulation and material [22]. A number of US-based protocols to test wipe activity have been described, but all had severe limitations in their setting or performance [31]. Recently, the ASTM2967-15 and the EN16615-15 protocols [32] have been recommended for evaluating the efficacy of antimicrobial wipes, although EN16615-15 has recently been shown to lack stringency [33]. Both tests evaluate the reduction in microbial number from the test surface and the transfer of micro-organisms during wiping. Attaining a > 5$\log_{10}$ reduction in microbial inoculum following treatment has been, until now, deemed to provide enough assurance that all micro-organisms would be killed on surfaces in practice. Although this might be the case where surfaces are contaminated with a low number of microorganisms, this might not be correct with surfaces contaminated with a high number of microorganisms or when dry surface biofilms are present. Hence, the evaluation of microbial transferability post-treatment is important to take into consideration to provide reassurance that a surface would be safe to touch. The presence of DSB on healthcare surfaces has been established [8–10], and the resilience of DSB to disinfection has been described [8]. The presence of *C. auris* DSB has not been yet established in healthcare settings, although its persistence in healthcare settings has been described [11,12].

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Here, we have successfully produced reproducible dry surface biofilms of *C. auris* on stainless steel surfaces, for which appearance and characteristics are not dissimilar to artificial DSB of *Staphylococcus aureus* (Ledwoch, Said and Maillard, unpublished results) or DSB isolated from

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aureus (Ledwoch, Said and Maillard, unpublished results) or DSB isolated from endoscopes [34]. These dry surface biofilms of *C. auris* provided a platform for testing the efficacy of commercially available wipe-based products or formulation combined with a microfiber cloth. Here, we observed that the majority of commercially available chlorine-releasing agents widely used by hospitals do not effectively eradicate a *C. auris* DSB or lower its transferability. The ability of *C. auris* to form dry surface biofilm on surfaces contribute somewhat to its resistance against disinfection. We showed that *C. auris* inactivation was product dependent. NaOCl-containing products are widely used in healthcare settings, although the efficacy of NaOCl against *C. auris* dried on surfaces differs in the literature. While only 2.5–3 $\log_{10}$ of *C. auris* were killed by NaOCl (1000 ppm) within 5 min [20], a 3000 ppm NaOCl solution resulted in reducing *C. auris* dried on surfaces for 2 h by 6 $\log_{10}$ in 1 min [18]. An 8000 ppm NaOCl solution, however, produced a 6 $\log_{10}$ reduction in 10 min for *C. auris* dried on surfaces for 1 h [14]. Here, NaOCl-Ref (1000 ppm) and NaOCl-3 (1000 ppm) produced a 7 $\log_{10}$ reduction in number within 2 min following wiping.

The microfiber cloth loaded with sterile water enabled the removal of *C. auris* (2 $\log_{10}$ reduction) but failed to prevent transfer or biofilm re-growth post wiping. Interestingly, a number of products performed similarly to water, indicating either that the combination with the Rubbermaid® HYGEN™ disposable microfiber cloth was incompatible with the product formulation, or that the product activity was caused by the material only. Wesgate et al. [33] recently showed that the type of material might have a small impact on the formulation presumably because the concentration of the active ingredient was high. In this study, it was interesting to observe that lower concentration and, possibly, the type of material affected the efficacy of peracetic acid and NaOCl. It has also been reported that the formulation itself impacts activity [35]. Here, different NaOCl formulations produced different results. While the use of unformulated NaOCl (NaOCl-Ref) was effective in reducing counts of *C. auris* on surfaces and prolonged biofilm regrowth, it failed to prevent *C. auris* transfer. The formulated NaOCl product (NaOCl-3) containing the same concentration of available chlorine produced a high reduction in count, thus preventing transfer but was less efficacious in delaying biofilm regrowth. NaOCl-2 containing a lower concentration of available chlorine was not effective in reducing *C. auris* count, preventing transfer, or delaying regrowth. The impact of formulation is also evident with NaDCC-based products. Overall, the peracetic acid wipe product containing 3500 ppm of PAA performed the best against *C. auris* DSB.

5. Conclusions

We successfully developed a dry surface biofilm in vitro model of *C. auris*. Although *C. auris* has not yet been isolated from environmental DSB, its presence on surfaces and associated high pathogenicity highlights the need to select an efficient infection control regimen. The use of a product efficacy test such as ASTM2967-15 is essential to evaluate the efficacy of formulated products. The additional evaluation of transferability of microorganisms post-wiping provides important information on a product's overall efficacy as well as reassurance that surfaces are safe to touch post-treatment. Here, we observed that measuring $\log_{10}$ reduction in viability was not enough to discriminate between product efficacy. Importantly, we observed that a number of commercially-available formulations combined with a microfiber cloth, or products, failed to control dry surface biofilms of *C. auris*. It was also clear that high concentration and an appropriate formulation of the active ingredient was key for efficacy, with the PAA-based product performing better.

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