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Review

Surface-attached cells, biofilms and biocide susceptibility: implications for hospital cleaning and disinfection

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SUMMARY

Microbes tend to attach to available surfaces and readily form biofilms, which is problematic in healthcare settings. Biofilms are traditionally associated with wet or damp surfaces such as indwelling medical devices and tubing on medical equipment. However, microbes can survive for extended periods in a desiccated state on dry hospital surfaces, and biofilms have recently been discovered on dry hospital surfaces. Microbes attached to surfaces and in biofilms are less susceptible to biocides, antibiotics and physical stress. Thus, surface attachment and/or biofilm formation may explain how vegetative bacteria can survive on surfaces for weeks to months (or more), interfere with attempts to recover microbes through environmental sampling, and provide a mixed bacterial population for the horizontal transfer of resistance genes. The capacity of existing detergent formulations and disinfectants to disrupt biofilms may have an important and previously unrecognized role in determining their effectiveness in the field, which should be reflected in testing standards. There is a need for further research to elucidate the nature and physiology of microbes on dry hospital surfaces, specifically the prevalence and composition of biofilms. This will inform new approaches to hospital cleaning and disinfection, including novel surfaces that reduce microbial attachment and improve microbial detachment, and methods to augment the activity of biocides against surface-attached microbes such as bacteriophages and antimicrobial peptides. Future strategies to address environmental contamination on hospital surfaces should consider the presence of microbes attached to surfaces, including biofilms.

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Introduction

Microbes tend to attach to available surfaces and form biofilms readily.[1–3] Biofilms are problematic in healthcare settings, where they are thought to be involved in 65% of nosocomial infections, and are usually reported in relation to indwelling medical devices and prostheses, water lines and tubing on endoscopes, and on wounds.[1–3] In these settings, biofilm persistence can be prolonged, periodically 'sloughing off' and releasing planktonic bacteria that may act as a source of infection. Biofilms are a common problem on liquid–hard surface interfaces, and in areas of a hospital that are usually wet or damp, such as taps and sink drains.[4] The recent problems caused by *Pseudomonas aeruginosa* in water supplied to intensive care units, which resulted in changes to UK national guidance, illustrates this problem.[4]

A biofilm is a community of micro-organisms attached to a substrate producing extracellular polymeric substances (EPS) and exhibiting an altered phenotype compared with corresponding planktonic cells, especially regarding growth, gene transcription, protein production and intercellular interaction.[1–3,5,6] Biofilms comprising various micro-organisms, including bacteria, viruses, fungi and other micro-organisms, can form on almost any biological or inanimate surface, and have been identified in various industrial and clinical settings.[1,7] Not all microbes attached to surfaces meet the definition of a biofilm, and the transition from a planktonic culture through surface attachment to an established biofilm is likely to be a continuum rather than a stepwise process (Figure 1).[1–3]

Microbes including bacterial spores, vegetative bacteria, fungi and viruses can also survive on dry surfaces for extended periods.[8–10] Contaminated environmental surfaces are an increasingly recognized reservoir in the transmission of certain healthcare-associated pathogens.[11–13] Whilst this extended survival is not surprising for the metabolically inert bacterial endospores, survival of some vegetative bacteria that is measured in years rather than days challenges our understanding of bacterial physiology.[10,14] The structural and physiological state of microbes dried on to hospital surfaces has not been studied in detail, but it seems likely that bacteria attach to surfaces to some degree, and may form biofilms. Indeed, a recent study from Australia by Vickery *et al.*[15] 'destructively sampled' (i.e. cut the materials out of the hospital environment and undertook laboratory analysis) several hospital surfaces after cleaning and bleach disinfection. Scanning electron microscopy was used to examine the surfaces for biofilms, which were identified on five of six surfaces. Furthermore, viable meticillin-resistant *Staphylococcus aureus* (MRSA) was identified in the biofilm on three of the surfaces.

This article will review in-vitro studies that explore the structure, physiology and biocide susceptibility of microbes dried on to hard surfaces in the context of surface attachment and biofilm establishment, and discuss the potential implications for hospital cleaning and disinfection.[16]

Search strategy

Pubmed was searched with no date restrictions using the search terms 'biofilm and biocide', 'biofilm and reduced susceptibility', 'biofilm and [MRSA, VRE, *C. difficile*, *Acinetobacter*, *E. coli*, *Pseudomonas*]' and 'susceptibility planktonic biofilm biocide' (see Figure 1). The reference lists of articles identified via the Pubmed searches were hand searched to identify other relevant literature.

Resistance and reduced susceptibility

Biofilms constitute a protected mode of growth, allowing bacteria to survive in hostile environments and conferring

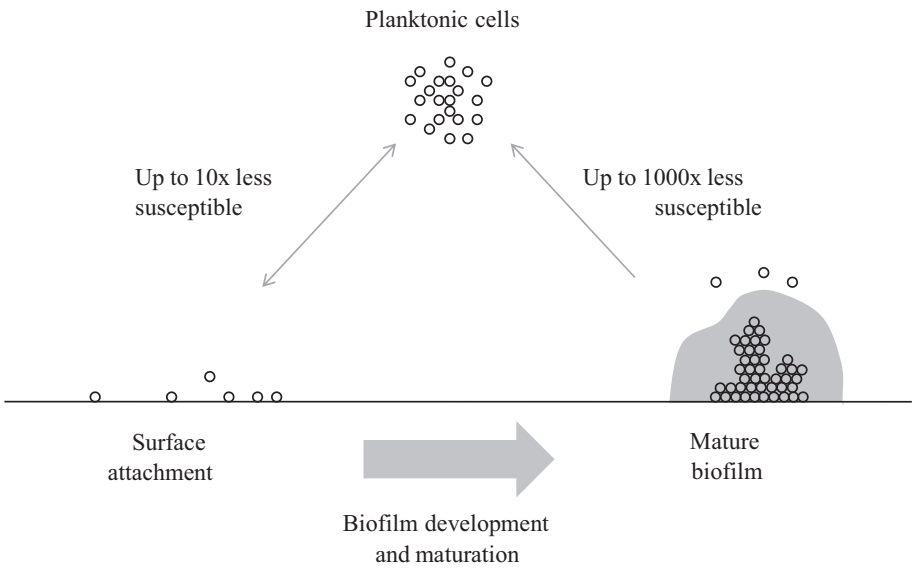

Figure 1. Schematic of surface attachment, biofilm formation and biocide susceptibility. This illustrates bacterial attachment to surfaces, development and maturation of biofilms, and implications for microbial susceptibility. The grey shading around the mature biofilms illustrates EPS. The biofilm development and maturation process is a complex step-wise process, simplified here as a single step.[2,3] Whilst the reduced biocide susceptibility associated with surface attachment and biofilms will be determined by a number of factors, not least the biocide, microbe and testing conditions, bacteria in mature biofilms are consistently less susceptible than biofilms attached to surfaces, often by several orders of magnitude.[18–20]

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reduced susceptibility to dehydration, phagocytosis, metal toxicity, acid exposure, antibiotics and biocides.[1,7,17] Microbes attached to surfaces that have not formed an established biofilm appear to represent an intermediate step, with reduced susceptibility to biocides compared with planktonic cells, but increased susceptibility relative to biofilms (Figure 1).[18–20]

Mechanisms of reduced susceptibility

Causes of reduced susceptibility to antimicrobial agents in biofilms are multi-factorial, including reduced penetration (particularly due to changes in cell density and the production of EPS), slow growth (and subsequent reduced metabolism of antimicrobial agents), modulation of the stress response and other metabolic processes, and changes in quorum sensing.[5,21–23] It seems likely that these mechanisms also explain reduced biocide susceptibility in surface-attached cells that have not yet formed biofilms.

Biocide susceptibility

Many studies have evaluated the impact of established biofilms on biocide susceptibility, and fewer studies have evaluated the susceptibility of surface-attached cells that have not yet formed established biofilms (Figure 1). Table I summarizes studies that have investigated organisms and biocides relevant to disinfection in healthcare settings that include data comparing susceptibility in planktonic culture with surface-attached cells and/or biofilms. Studies have evaluated a range of organisms (both alone and in combination), various suspending media, and several methods of attaching cells to surfaces and producing biofilms on different substrates; all of these factors are likely to influence biocide susceptibility. One important factor is the maturation of the microbes tested, which ranges from cells attached to surfaces for hours to samples extracted from continuously fed biofilm reactors that are weeks old.[24,25] Furthermore, few studies have controlled for cell density in attached cells or biofilms compared with planktonic culture.[26] Thus, although several studies have suggested that cell density alone does not explain the reduced susceptibility of biofilms to biocides, it is difficult to be certain of the impact of the biofilm phenotype independent of cell density in many studies.[20,27–29] A number of different approaches have been taken to quantify growth, including both direct microbial culture and indirect measures, such as live/dead viability assays.[20,30–32] Finally, different approaches to compare susceptibility in planktonic culture and biofilms have included measuring the amount of biocide required to inhibit growth [minimum inhibitory concentration (MIC)] or kill cells [minimum bactericidal concentration (MBC)],[7,18,20,33] or measuring the survival time at a given concentration of biocide;[20,32,34,35] this makes comparison of studies difficult.

Notwithstanding difficulties in comparing studies, the phase of the surface-attached cells influences biocide susceptibility. In general, bacteria in planktonic culture are more susceptible than attached cells, which are, in turn, more susceptible than established biofilms (Figure 1).[18–20] Meanwhile, detached biofilm cells revert to the susceptible phenotypic state.[19,36,37] Similarly, growth phase affects biocide susceptibility of planktonic culture.[38–40] Reduced susceptibility in surface-attached cells ranges widely from two-fold to >1000-fold.[26,41] For example, clinical isolates of MRSA and *P. aeruginosa* were grown as biofilms on discs of common materials in the hospital environment, and treated with three commonly used hospital biocides: benzalkonium chloride (1% w/v), chlorhexidine gluconate (4% w/v) and triclosan (1% w/v).[7] The MBCs of all biocides for planktonic cultures of both organisms were considerably less than the concentrations recommended for use by the manufacturer. However, when isolates were grown as biofilms, the biocides were ineffective at killing bacteria at the concentrations recommended for use. The MBCs of all three biocides were found to be 10–1000-fold higher than the same isolates grown in planktonic culture for MRSA and *P. aeruginosa*. Following biocide treatment, up to 11% of cells in MRSA biofilms survived, and up to 80% of cells in *P. aeruginosa* biofilms survived. Another study evaluated the susceptibility of four *Candida* spp. and two *Escherichia coli* strains to sodium hypochlorite, ethanol, hydrogen peroxide and iodine.[20] Strains were tested in planktonic culture, as attached cells and as biofilms in microtitre plates. Whilst susceptibility varied by organism and biocide, biofilms were less susceptible than attached cells, which were less susceptible than planktonic cells. MICs for biofilms were up to >10-fold higher for 5-min and 24-h exposures compared with planktonic cells. These studies suggest that although biocides may be effective against planktonic populations of bacteria, some biocides currently used in hospitals may be ineffective against nosocomial pathogens when attached to surfaces or in biofilms, and thus fail to control this reservoir for hospital-acquired infection.[7]

However, whilst surface-attached microbes and biofilms are generally less susceptible to biocides than bacteria in planktonic culture, the degree of reduced susceptibility is not always this stark. For example, a study reported no difference between planktonic culture and biofilms of *Klebsiella pneumoniae* exposed to sodium hypochlorite and monochloramine.[42] Other studies have not identified reduced susceptibility for all biocides or organisms tested.[20,24,43,44] It is difficult to determine the relative importance of organism, biocide and testing conditions in these studies that found little or no reduced biocide susceptibility associated with biofilms.

The composition of the biofilm also influences susceptibility. For example, high-nutrient, high-density biofilms are less susceptible to biocides than low-nutrient, low-density biofilms.[18,31,45] This seems particularly important in the context of biofilms that may be present on hospital surfaces, which are likely to be low-nutrient, low-density biofilms in most cases. However, gross contamination with body fluids could provide an environment in which high-nutrient, high-density biofilms could form on hospital surfaces. Indeed, three-quarters of the biofilms reported by Vickery *et al.* had very thick EPS despite having a low density of microbes in most cases, perhaps in response to desiccation.[15,46,47]

The microbial ecology of the biofilm is another factor influencing susceptibility. Biofilms composed of multiple species are less susceptible than single-species biofilms, although this is not always the case with the corresponding planktonic cultures.[19,36,37,48]

Some biocides are more effective than others at inactivating bacteria in biofilms, although conflicting data have been reported, which may be explained by differences in experimental conditions.[20,24,35,49,50] In one study, susceptibility varied by phase, organism and biocide.[20] In another study, the oxidizing agents sodium hypochlorite and peroxygens were more effective than a range of other chemicals (including alcohols, biguanides, halogens, phenols and quaternary ammonium compounds) for inactivating *P. aeruginosa* and *S. aureus* biofilms.[35] In other studies, sodium hypochlorite was more

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Table I
Biocide susceptibility of planktonic vs surface-attached and/or biofilm mode

Author	Organisms (*N* isolates)	Biocides	Methods	Findings
Condell 2012[18]	*Salmonella enterica* (189)	Seven common food contact surface biocides	Tested in planktonic culture, dried on surfaces and as established high-nutrient (2-day) or low-nutrient (7-day) biofilms on microtitre plates	Susceptibility rank: high-nutrient biofilm < low-nutrient biofilm < surface dried < planktonic
Behnke 2012[37]	*Pseudomonas aeruginosa* (1); *Burkholderia cepacia* (1)	Chlorine dioxide	Tested in single- and binary-species planktonic culture, attached (4-day) and detached biofilm	Susceptibility rank: attached biofilm < detached biofilm = planktonic cells. Binary cultures were less susceptible than single-species cultures
Xing 2012[49]	*Staphylococcus aureus* (13)	Chlorhexidine and harmaline	Tested in planktonic culture and in 2-day biofilms on microtitre plates	Biofilms were 10 to >100 times less susceptible to chlorhexidine and >2 times less susceptible to harmaline. Synergy noted for most strains
Leung 2012[20]	*Candida* spp. (4); *Escherichia coli* (2)	Sodium hypochlorite, ethanol, hydrogen peroxide and iodine	Tested in planktonic culture (low- and high-titre), attached cells (90 min) and 1-day biofilm in microtitre plates; 24-h and 5-min contact times compared	Susceptibility rank: biofilm < attached cells < high-titre planktonic cells ≤ low-titre planktonic cells. MICs for biofilm vs planktonic cells up to >10-fold higher for 5-min and 24-h exposures
Behnke 2011[36]	*P. aeruginosa* (1); *B. cepacia* (1)	Sodium hypochlorite	Tested in single- and binary-species planktonic cultures, attached (4-day) and detached biofilms	Susceptibility rank: attached biofilm < detached biofilm = planktonic cells. Binary-species cultures were less susceptible than single-species cultures for attached and detached biofilms, but the reverse was true for planktonic cells
Xu 2011[34]	*Neisseria gonorrhoeae* (3)	Atmospheric pressure non-equilibrium plasma	Tested dried on glass surfaces or 4-day biofilm on glass	Bacteria in biofilm survived approximately twice as long as bacteria dried on surfaces
Wong 2010[44]	*S. enterica* (1)	Six biocides	Tested in planktonic culture or 3-day biofilm on microtitre plates	Bacteria in biofilm were less susceptible than planktonic cells for all but sodium hypochlorite
Tote 2010[35]	*S. aureus* (1); *P. aeruginosa* (1)	12 biocides	Tested in planktonic culture or in 1-day (*P. aeruginosa*) or 3-day (*S. aureus*) biofilm on microtitre plates	Most disinfectants tested did not eliminate bacteria in the biofilm after 60-min contact. Only hydrogen peroxide and chlorine had an impact on the biofilm matrix
Lee 2009[24]	Meticillin-resistant *S. aureus* (2)	Three denture-cleaning biocides	Tested in planktonic culture, sessile biofilm (4 h), established biofilm (24 h) or mature biofilm (120 h) on resin	Two of three biocides were less effective for the inactivation of bacteria in biofilm. NaOCl was the most effective against biofilm
Hendry 2009[103]	*S. aureus* (1); meticillin-resistant *S. aureus* (1); *P. aeruginosa* (1); *E. coli* (1); *Candida albicans* (1)	Eucalyptus oil, '1,8-cineole' and chlorhexidine	Tested in planktonic culture or 2-day biofilm on microtitre plates	Biofilm MICs and MBCs were 10 to >100 times less susceptible than planktonic culture. Synergy between chlorhexidine and the other agents was noted against some organisms
Smith 2008[7]	Meticillin-resistant *S. aureus* (8); *P. aeruginosa* (8)	Benzalkonium chloride, triclosan and chlorhexidine	Tested in planktonic culture or 1-day biofilms on metal or plastic discs	MBCs for MRSA biofilms were 100 to 1000 times greater than for planktonic cells; MBCs for *P. aeruginosa* biofilm were 10 to 100 times greater than for planktonic cells

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Table I (*continued*)

Author	Organisms (*N* isolates)	Biocides	Methods	Findings
Brandle 2008[19]	*Enterococcus faecalis* (1); *Streptococcus sobrinus* (1); *C. albicans* (1); *Actinomyces. naeslundii* (1), *Fusobacterium nucleatum* (1)	Calcium hydroxide	Tested in planktonic culture, adherent cells, single-species 5-day biofilm and mixed-species 5-day biofilm on dentin and detached biofilm	Susceptibility rank: mixed species biofilm < single-species biofilm < adherent < planktonic = detached biofilm
Nett 2008[26]	*C. albicans* (2); *Candida parapsilosis* (2); *Candida glabrata* (1)	Ethanol, hydrogen peroxide and sodium dodecyl sulphate	Tested in planktonic culture, planktonic culture with adjustment to match the cell density of the biofilm and 1-day biofilm on microtitre plates	Concentrations required to inhibit growth in biofilm were 2- to 10-fold higher; lower concentrations of hydrogen peroxide prevented biofilm formation than the other agents tested
Karpanen 2008[102]	*Staphylococcus epidermidis* (2)	Chlorhexidine gluconate, tea tree oil, eucalyptus oil and thymol	Tested in planktonic culture or 3-day biofilm on microtitre plates	MICs/MBCs were elevated up to 16-fold for biofilm; synergy was noted between chlorhexidine and eucalyptus oil
Bjarnsholt 2007[104]	*P. aeruginosa* (1)	Silver	Tested in planktonic culture or 4-day biofilm	Biofilm was 10–100 times less susceptible than planktonic cells
Tabak 2007[40]	*Salmonella typhimurium* (3)	Triclosan	Tested in planktonic (log and stationary phase) culture and in 1-day biofilm on microtitre plates	Susceptibility rank: biofilm < stationary phase planktonic < log phase planktonic. 8-log difference in bacteria surviving in biofilm vs planktonic log phase
Surdeau 2006[105]	*E. coli* (1); *Enterococcus hirae* (1); *P. aeruginosa* (1); *S. aureus* (1)	Novel disinfectant (*Oxsil* 320N)	Tested in planktonic culture and 1-day biofilm on stainless steel	Disinfectant concentration required to achieve a 5-log reduction was approximately 10 times more for biofilm vs planktonic culture
Theraud 2004[30]	Five fungi from patient (3) and environment (3)	Five antiseptics, three disinfectants and UVC	Tested in single- and mixed-species planktonic culture, and single- and mixed-species 1-day biofilms on microtitre plates	UVC and 3% hydrogen peroxide were not fungicidal in initial suspension tests. Agents were less effective against mixed suspensions. Only chlorhexidine was effective against biofilms
Simoes 2003[106]	*Pseudomonas flourescens* (1)	Ortho-phthalaldehyde	Tested in planktonic culture and 6-day biofilm on glass	Biofilm was less susceptible than planktonic cells based on respiratory activity
Bardouniotis 2003[33]	*Mycobacterium fortuitum* (1); *Mycobacterium marinum* (1)	Seven biocides	Tested in planktonic culture and biofilm on microtitre plate assessed over 14 days	MBECs were up to 40-fold higher than MBCs for *M. fortuitum*, but not for *M. marinum*
Elvers 2002[107]	*Alcaligenes denitrificans* (1); *Pseudomonas alcaligenes* (1); *Stenotrophomonas maltophilia* (1); *Flavobacterium indologenes* (1); *Fusarium oxysporum* (1); *Fusobacterium solani* (1); *Rhodotorula glutinis* (1)	One biocide (isothiazolone compound)	Tested in single-species planktonic culture, and single- and mixed-species 1-day biofilms on glass	Biofilms were less susceptible than planktonic cells. Mixed-species biofilm, particularly for the bacterial species, offered greater protection

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Table I (*continued*)

Author	Organisms (*N* isolates)	Biocides	Methods	Findings
Peng 2002[31]	*Bacillus cereus* (1)	Sodium hypochlorite and quaternary ammonium compounds	Tested in planktonic culture, attached to stainless steel chips (4 h) and 8-day biofilm on stainless steel with or without milk	Susceptibility rank: milk biofilm < biofilm < attached < planktonic. 5-log difference between planktonic cells and milk biofilm
Bardouniotis 2001[108]	*Mycobacterium phlei* (1)	Seven biocides	Tested in planktonic culture and 5-day biofilm on microtitre plate	MBECs were higher than MBCs after 30-min and 120-min exposure to most agents tested
Joseph 2001[32]	*Salmonella* spp. (2)	Chlorine and iodine	Tested in planktonic culture and 10-day biofilms on plastic, cement and stainless steel	Biofilms were less susceptible to both disinfectants; survival time no more than 10 min in suspension vs > 25 min in biofilm
Cochran 2000[29]	*P. aeruginosa* (1)	Monochloramine and hydrogen peroxide	Tested in planktonic culture and 3-h to 3-day biofilms on alginate beads and glass slides	Biofilms were less susceptible to both disinfectants. Reduced diffusion of biocide in biofilm did not explain reduced susceptibility
Elasri 1999[53]	*P. aeruginosa* (1)	UVA, UVB and UVC	Strain tested in planktonic culture or biofilm in alginate beads assessed over 1 day	Biofilm transmitted only a small amount of UV radiation (13% of UVC, 31% of UVB and 33% of UVA), meaning biofilm was less susceptible than planktonic cells
Das 1998[43]	*S. epidermidis* (1); *E. coli* (1)	Five biocides	Tested in planktonic culture and 6–24-h biofilms on microtitre plates	Biofilms were up to 33-fold less susceptible to the disinfectants tested, apart from chloroxylenol and cetrimide (*E. coli* only)
Stewart 1998[45]	*Enterobacter aerogenes* (1)	Four biocides	Tested in planktonic culture and high- and low-density biofilms on alginate beads assessed over 5 h	Susceptibility rank: high-density biofilm < low-density biofilm < planktonic
Yu 1993[42]	*Klebsiella pneumoniae* (1)	Sodium hypochlorite and monochloramine	Tested in planktonic culture and biofilm on stainless steel discs	No difference identified between planktonic and biofilm cells
Eginton 1998[56]	*S. epidermidis* (1); *P. aeruginosa* (1)	Sodium hypochlorite and dodigen; SDS and Tween-80	Tested in planktonic culture and 16-h biofilms on glass and stainless steel	Biofilms were up to >1000-fold less susceptible than planktonic cells; attachment to the surfaces was loosened
LeChevallier 1988[25]	*Pseudomonas picketti*, *Pseudomonas paucimobilis* *Moraxella*[a]; *K. pneumoniae* (1)	Hypochlorous acid, hypochlorite, chlorine dioxide and monochloramine	Tested in planktonic culture and 3-week biofilms on granular activated carbon, metal or glass	Biofilms were 150 to 3000 times less susceptible to hypochlorous acid, and 2- to 100-fold less susceptible to monochloramine

MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MBEC, minimal biofilm eradicating concentration; UV, ultraviolet.

Search strategy: Pubmed search for 'susceptibility planktonic biofilm biocide' performed on 15th November 2013. Of 44 results, 35 were selected for review and 21 were included. A further 10 articles were included following review of the reference lists. Articles were included if they tested organisms and biocides relevant to disinfection in healthcare facilities, and included data comparing planktonic with surface-attached and/or biofilm mode susceptibility.

[a] Population from de-ionized water system: composition 70% *P. picketti*; 18% *Moraxella* spp.; 12% *P. paucimobilis*.

effective than chlorhexidine for inactivating *Enterococcus faecium* and MRSA in biofilms,[24,50] whereas chlorhexidine was found to be effective against yeast biofilms when sodium hypochlorite was not effective.[30] In general, oxidizing agents target multiple biofilm components and microbial targets, whereas other biocides such as chlorhexidine only target cell wall components; thus, oxidizing agents tend to have a higher level of efficacy against biofilms.[20,24,35,49,50] The variations in performance of biocides under different experimental conditions may have implications for practice, where the same biocide could have a different impact on biofilms in different settings.

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Comparing biocides may be further confounded by the 'dose–response' type relationship that has been shown between biofilm susceptibility and biocide concentration.[35,51,52] For example, one study showed that 10% hydrogen peroxide was considerably more effective for inactivating bacteria in biofilms compared with 6% hydrogen peroxide.[51] Biofilms have also been shown to reduce the susceptibility of microbes to physical processes such as exposure to ultraviolet (UV) radiation, most likely due to poor penetration of UV into the biofilm.[53] This may have implications for automated room disinfection systems using UV radiation.[54] To the authors' knowledge, no studies have evaluated the impact of hydrogen-peroxide-based automated room disinfection systems against biofilms, although emerging data suggest that liquid hydrogen peroxide, as an oxidizing agent, targets both the biofilm matrix and microbes in the biofilm.[35]

Aside from the inactivation of microbes attached to surfaces, the chemical properties of biocides also seem to be important in terms of preventing, promoting or dismantling biofilms.[26,35,55] One study showed that only sodium hypochlorite and hydrogen peroxide damaged both the bacteria within the biofilm and the biofilm matrix itself.[35] Also, hydrogen peroxide was more effective than other agents at preventing *Candida spp.* biofilm formation.[26] In another study, exposure to chlorhexidine and benzalkonium chloride inhibited biofilm formation for *E. coli, K. pneumoniae* and *P. aeruginosa*, but promoted biofilm formation in *Staphylococcus epidermidis*, suggesting that microbial factors are important.[55] It is possible, therefore, that one microbe in a biofilm may be inactivated by a biocide, but another less susceptible microbe may survive and then grow to replace the microbe that was inactivated.

Antibiotic susceptibility

Bacteria in biofilms are usually less susceptible to antibiotics than bacteria in planktonic culture, and many of the mechanisms for reduced susceptibility to biocides and antibiotics are shared.[5,6] Furthermore, bacteria acquired from surfaces in biofilm mode with reduced biocide susceptibility may retain reduced susceptibility to antibiotics.

Physical removal

The protected mode of growth offers physical protection to cells within biofilms, and makes the physical breakdown of biofilms challenging.[5] Although biofilm attachment appears to be loosened by some biocides,[35,56] several studies have illustrated how difficult it can be to remove bacteria in biofilms through cleaning and/or inactivation through disinfection. For example, regular and extended detergent cleaning did not remove a *Bacillus cereus* biofilm *in vitro*; a modified procedure including heating to 70°C was required.[31] Clearly, heating to 70°C is not feasible for the cleaning and disinfection of hospital surfaces in clinical areas. Similarly, attached, viable *Pseudomonas fragi* were detected on stainless steel surfaces after two cleaning and disinfection procedures were tested under 'worst-case' conditions at 50% in-use disinfectant concentrations.[57] An acid-detergent-based method was more effective at removing attached cells than an alkaline-detergent-based method. However, these studies were performed using mature biofilms which may not be representative of the biofilms present on hospital surfaces.

Surface-attached cells and biofilms are clearly not the only reason for failures in hospital disinfection, given the difficulty in achieving adequate distribution and contact time using manual methods.[11,13,54,58] However, both reduced biocide susceptibility (Table I) and increasing resilience to physical removal by cleaning are likely to contribute to failures in hospital cleaning. This could partly explain why disinfectants that are effective for the inactivation of planktonic bacteria in laboratory tests are not effective for the eradication of a considerably lower load of the same bacterial species from hospital surfaces.[11,13,58–60] In support of this, it is noteworthy that the biofilms identified by Vickery *et al.* were on surfaces that had been cleaned with detergent and then disinfected using 500 ppm chlorine.[15] These findings may have implications for infection control practices within hospitals, and on the choice of appropriate disinfectants used to decontaminate surfaces.[11,13,54]

The presence of biofilms on dry hospital surfaces could also interfere with attempts to recover microbes through environmental sampling.[15,61–63] This could mean that an environmental reservoir of a pathogen remains undetected, or the concentration of contamination and degree of associated risk is underestimated.

Persistence

Vegetative bacteria dried on to surfaces can survive for weeks to months (or more) *in vitro*, despite the lack of a nutrient source or water (aside from ambient humidity).[8,9] Biofilms may explain this surprising propensity of vegetative bacteria.[8–10] This is supported by a recent study which found that biofilm-forming strains of *Acinetobacter baumannii* survived for longer on dry surfaces than non-biofilm-forming strains (36 vs 15 days; $P < 0.001$).[64] In-vitro studies evaluating the persistence of dried inocula did not supply any water or nutrients.[8–10,14,65] However, in the hospital environment, daily and terminal cleaning or disinfection does provide a supply of water, and some bacteria may be able to metabolize some constituent parts of detergents and even disinfectants, providing a nutrient source for the growth in biofilms.[66–69]

Transfer of plasmids and development of antimicrobial resistance

Biofilms are suited for horizontal gene dissemination because they are a mixed population at high bacterial density, which facilitates metabolic activity in the harshest environments, albeit at a reduced rate. Horizontal transfer of plasmids does occur through conjugation, as illustrated by the transfer of extended-spectrum β-lactamase (*CTX-M-15*) and carbapenemase (*NDM-1*) plasmids between *Enterobacteriaceae* when dried on surfaces.[70,71] Furthermore, the mutation rate (the rate at which DNA replication mistakes occur during cell division) of bacteria in biofilms is increased.[6,72] Thus, both horizontal transfer of resistance determinants such as plasmids and increased mutation rates could result in the acquisition or *de-novo* development of reduced susceptibility to antimicrobial agents and other important microbial capabilities, such as increased virulence.

Tackling surface-attached cells and biofilms

Surface-attached cells, especially established biofilms, present a difficult challenge to hospital cleaning and disinfection,

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combining protection from physical removal with reduced susceptibility to biocides (Table I).[31,57] A number of different approaches are available to tackle surface-attached cells and biofilms. Using physical methods to dislodge detached bacteria, which can be aided by the use of a detergent, can be effective in removing established biofilms and preventing the development of biofilms.[5,56,73] However, detergent cleaning alone may not be sufficient to remove biofilms.[5,15,31,56,61,73] Tackling the microbes in the biofilm alone (e.g. using some disinfectants or attempts to interfere with quorum sensing) can be effective, but may not reach microbes protected deep in the biofilm matrix. Tackling the biofilm matrix alone (e.g. using enzymatic digestion) will help to reach microbes protected within the biofilm matrix and interrupt persistence of the biofilm, but will not necessarily have direct microbicidal activity. Thus, tackling both the microbes in the biofilm and the biofilm matrix simultaneously (using oxidizing disinfectants or combination approaches) offers the potential to reach microbes protected deep in the matrix and interrupt the persistence of the biofilm. In addition, some biocides have the ability to reduce biofilm formation, which can be assisted by choosing surface materials that do not readily support biofilm formation.

Biocides and biocide adjuvants

Differences between biocides appear to influence their activity against bacteria attached to surfaces and may also promote, prevent or dismantle biofilms. Thus, biocides with the highest activity against bacteria attached to surfaces, and ideally those with the ability to prevent biofilm formation and dismantle existing biofilms, should be selected. Emerging data indicate that oxidizing agents may possess more of these properties than other agents.[35] Similarly, detergent formulations that are better at physical removal should be selected, although there is a paucity of data on the capacity of currently available detergents to address surface-attached cells.[73]

Several novel approaches also warrant consideration as potential additives to hospital detergents or disinfectants to augment their effectiveness against biofilms. Firstly, certain enzymes such as DNase and dispersinB have been shown to dissolve the biofilm matrix.[73–78] For example, detergents supplemented with high concentrations of enzymes were effective against hydrated biofilms, whereas detergents supplemented with low concentrations of enzymes were not.[73] Secondly, quorum-sensing inhibitors have proven successful in increasing antimicrobial susceptibility.[79–81] In one study, drimendiol, a quorum-sensing inhibitor, was found to enhance the effects of copper sulphate on biofilms of *Pseudomonas syringae*.[81] Thirdly, recently discovered human antimicrobial peptides also have antibiofilm activities.[82–84] For example, a range of antimicrobial peptides tested against multi-drug-resistant *A. baumannii* demonstrated direct antimicrobial activity, and enhanced the activity of a range of other antimicrobial agents.[82]

However, the addition of enzymes, quorum-sensing inhibitors or antimicrobial peptides into a cleaning or disinfection solution would result in chemical residues on surfaces with associated health and safety implications, so are not recommended without further study. Another approach is the inclusion of bacteriophages, which have been found to disrupt biofilms.[85,86] For example, *Streptococcus pyogenes* biofilms were degraded by PlyC, a bacteriophage-encoded endolysin, which also acted synergistically with a range of antimicrobial agents.[86] However, the therapeutic use of bacteriophages in human medicine and, by implication, in the clinical environment is controversial due to potential for the rapid development of resistance and the risk that the introduced bacteriophages may play an unintended role in horizontal gene transfer.[85,87]

Surface modification to prevent biofilm formation

Some surface materials are more prone to biofilm formation than others.[71,88] A recent study reviewed attempts to modify the chemical or physical surface properties of medical devices to inhibit or prevent microbial adhesion.[88] These include 'liquid glass' (silicon dioxide), Sharklet pattern,[89,90] advanced polymer coatings [e.g. polyethylene glycol (PEG), superhydrophobic/philic and zwitterionic][91–94] and diamond-like carbon films.[95] Whilst these technologies have the potential to reduce biofilm deposition on hospital surfaces, they are at an early stage of development. The feasibility and cost-effectiveness of scaling up these technologies for use on hospital surfaces needs to be evaluated.

Another approach is the implementation of antimicrobial surfaces. Options include metals such as copper and silver, or chemicals such as organosilanes with quaternary ammonium groups and light-activated antimicrobials.[12,71,96] Copper is the most-studied candidate for antimicrobial surfaces, and has been shown to inactivate microbes and DNA deposited on surfaces and may reduce the transmission of pathogens in the hospital setting.[12,71,97] However, the presence of a conditioning film can greatly reduce the efficacy of antimicrobial surfaces.[98–100] Thus, an antimicrobial surface that combines reduced biofilm formation with direct antimicrobial activity is a promising area for future research. Another challenge in developing an antimicrobial surface for hospitals is the requirement for multiple different surface types (from fabric to hard surfaces) with a range of required functions. Thus, there is unlikely to be a single agent or surface structure that is suitable for all applications.

Implications for susceptibility testing

Surface-attached cells and biofilms are a more accurate reflection of the occurrence of bacteria in nature than planktonic cells.[1,101] However, planktonic culture remains the current model for many microbiological studies and testing standards including susceptibility testing.[1,101] Although quantitative surface tests for evaluation of the bactericidal activity of chemical disinfectants do exist (e.g. BS EN 13697:2001), none have been published for EPS-producing biofilms. Future testing should specify the use of surface-attached cells and consider the use of biofilm models to ensure that the disinfectants tested are as effective in the 'real world' as in laboratory tests.[1] It seems likely that low-nutrient, low-density surface-attached cells would be more appropriate than high-nutrient, high-density established biofilms. Most in-vitro studies measured growth over a 24-h period to evaluate the impact of a chemical biocide to determine the MIC or MBC, using methodology often used to test antibiotic susceptibility (Table I).[7,20,102] One study compared the MICs of four common biocides for *E. coil* and various *Candida* spp. with a 'contact time' of 5 min and 24 h.[20] Unsurprisingly, the concentration required to inhibit growth within 5 min was considerably greater than the concentration required to inhibit growth over

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24 h. Thus, as biocides are only applied for a short period in practice, evaluating the impact of a biocide over a short contact time as per most published biocide testing standards is more suitable for in-vitro biocide studies than measuring the MIC or MBC when microbes are grown in varying concentrations of biocide.

Further research is required to evaluate the prevalence and composition of biofilms *in situ* on hard and soft hospital surfaces, to develop in-vitro models that are representative of those likely to be found on hospital surfaces, and to optimize methods to tackle biofilms on hospital surfaces, which may include new cleaning and disinfection agents and adjuvants, new technologies (such as microfibre or automated room disinfection technology), and surface modification.[15]

Conclusion

Surface-attached cells are likely to be common on dry hospital surfaces, and there is evidence that they also harbour established biofilms. The variety of methods used to create and evaluate in-vitro biofilms makes it difficult to compare studies evaluating antibiofilm biocide activity. Nonetheless, microbes attached to surfaces, especially established biofilms, are less susceptible to chemical biocides, UV radiation and antibiotics than their corresponding planktonic bacteria. The phase of the surface-attached microbes influences susceptibility: attached cells are more susceptible to biocides than established biofilms; low-density, nutrient-limited biofilms make less of an impact on biocide susceptibility than high-density, high-nutrient biofilms; and biocides are less effective for inactivating bacteria in mixed-species biofilms than in single-species biofilms. Biocide-specific issues also influence susceptibility in terms of activity against bacteria in biofilms, and the prevention, promotion and dismantling of biofilms. Reduced susceptibility to biocides combined with protection from physical removal through cleaning is likely to contribute to failures in hospital cleaning and disinfection.

Biofilms may explain why vegetative bacteria can survive for unusually long periods (weeks to months) on dry hospital surfaces. Also, the presence of surface-attached bacteria and biofilms is likely to interfere with attempts to recover bacteria from hospital surfaces, and may lead to underestimation of both the prevalence of contamination with pathogens and the number of bacteria that are on surfaces. This has important implications, particularly for hospital outbreak investigation. Biofilms provide a mixed bacterial community where the horizontal transfer of resistance genes may occur. Attempts to tackle surface-attached microbes and biofilms on hospital surfaces should include: identification and selection of biocide and detergents with the best all-round performance, including the ability to inactivate surface-attached cells and biofilms; ensuring that in-vitro tests are developed to model surface-attached microbes likely to be encountered in the field; harnessing surface science to develop a hospital environment that reduces the chance of biofilm formation; and further research to develop novel approaches to augment the activity of biocides against surface-attached microbes, including established biofilms.

Conflict of interest statement

JAO is employed part-time by Bioquell, and JAGS, JC and SY are employed by Bioquell. All other authors have no conflicts of interest to declare.

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