Testing the efficacy of topical antimicrobial treatments using a two- and five-species chronic wound biofilm model.

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Running title: chronic wound treatments in a complex biofilm model

Abstract

Aims: The effectiveness of commercially available wound dressings and a HOCl gel formulation was tested against two- and five-species biofilms in a dynamic *in vitro* chronic wound infection model.

Method: Two- species biofilms (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) were cultured using a biofilm flow device and treated with either wound dressings containing silver, iodine, polyhexamethylene biguanide (PHMB), crystal violet, or HOCl gel at 5h. Five-species biofilms (*P. aeruginosa, S. aureus, Enterococcus faecalis, Streptococcus pyogenes* and *Escherichia coli*) were similarly cultured and treated with HOCl gel at 5h and 24h. Multidose experiments used two- and five-species biofilms with HOCl applied at 24h, 48h and 72h.

Results: None of the treatments completely disrupted the biofilms and, with the exception of silver, bacteria recovered in number post-treatment. HOCl was most effective when applied to 24h established biofilms with most activity against *P. aeruginosa*. Recovery post-treatment was negligible with HOCl applied at 24h and multiple doses indicated that bacteria were not becoming tolerant to treatment.

Conclusions: Realistic models are necessary to test the effectiveness of antimicrobial wound treatments to ensure findings are clinically translatable. HOCl gel shows promise as a new topical antimicrobial for wounds, especially due to its ability to inhibit *P. aeruginosa*.

Significance and impact of study: This study highlights a need for robust *in vitro* data to support development and use of wound treatments that can only be obtained from the refinement of realistic infection models. Further, it indicates the potential use of HOCl gel for chronic wound management.

Introduction

In the UK, chronic wound management costs the NHS over £5.3 billion per year; in the USA this figure exceeds $28 billion (Guest et al. 2020). These costs are comparable to resources apportioned to high-profile health disorders such as obesity or heart disease. Consequently, chronic wounds are increasingly considered a "silent epidemic", imposing an alarmingly high burden on national healthcare systems. Antimicrobial dressings and topical formulations routinely applied in the management of these wounds contain broad spectrum antimicrobial agents such as iodine, silver, and polyhexamethylene biguanide (PHMB), aiming to target the majority of pathogens most commonly implicated in persistent infections (Wounds UK, 2013; Sood et al. 2014; National Institute for Health and Care Excellence [NICE], 2016). However, while many of these products show high levels of efficacy *in vitro*, clinical experience has shown that their use *in vivo* contributes little to the resolution of chronic wound infections (Hinchliffe et al. 2008; Hussey et al. 2019.).

Currently, there are no standardised methods to test the efficacy of antimicrobial wound dressings or topical formulations for chronic wound management, and compelling data to support the use of specific dressings are lacking (National Institute for Health and Care Excellence [NICE], 2016). Many *in vitro* approaches rely on models that do not effectively represent the chronic wound environment, making it difficult for observations to translate readily into clinical success. Greater than 80% of chronic wounds are infected with a multi-species consortium of microorganisms growing as a biofilm (Omar et al. 2017). Complex microbial interactions between biofilm members increase tolerance to antimicrobial treatments, enhance virulence, and persistence, making chronic wound infections exceptionally difficult to overcome (Peters et al. 2012; Nabb et al. 2019; Orazi and O'Toole

2020). *Staphylococcus aureus* predominates initially in chronic wounds, but other aerobic and anaerobic bacteria and fungi such as *Pseudomonas aeruginosa, Bacteroides* spp. and *Candida albicans*, respectively, are often co-isolated (Bowler et al. 2001). Whilst is it acknowledged that chronic wounds are indeed colonized by multiple species of bacteria, there are conflicting studies to support conclusions that the biofilm is truly polymicrobial (Johani et al. 2017; Kirketerp-Møller et al. 2020).

As wound infection progresses towards chronicity the composition of the microbial community shifts to a primarily Gram-negative population, where *P. aeruginosa*, a characteristic late coloniser of non-healing wounds, becomes dominant. Detection of this pathogen is common in chronic infection and is associated with treatment failure. Although the Gram-negative shift is observed *in vivo* and recognized by clinicians, it is not seen in many of the *in vitro* models used to test the efficacy of chronic wound infection treatments, which limits their usefulness in guiding clinical applications (Altoparlak et al. 2004; Kostenko et al. 2010; Guggenheim et al. 2011; Pastar et al. 2013; Said et al. 2014).

In order to evaluate more rigorously the potential contributions of wound dressing formulations we have used here a polymicrobial wound biofilm model that incorporates elements of the chronic wound environment, including a dynamic flow of media to represent a heavily exuding wound (Mulder 1994; Lipp et al. 2010), and the Gram-negative shift (Duckworth et al. 2018). Four conventional antimicrobial products were tested that have been previously reported to have excellent anti-biofilm activity in a variety of *in vitro* models, and compared to a gel of hypochlorous acid (HOCl).

Materials and Methods

Bacterial strains and agar

Staphylococcus aureus EMRSA-15*, Pseudomonas aeruginosa* ATCC 9027*, Streptococcus pyogenes* 75784*, Escherichia coli* ATCC 10418 and *Enterococcus faecalis* ATCC 19433 were used in this study and routinely cultured at 37°C on nutrient agar (NA) at 1.5% concentration (Sigma-Aldrich, USA). *S. aureus* and *P. aeruginosa* used in this study are type strains originally of skin origin; *S. pyogenes* was originally isolated from a wound; *E. coli* and *E. faecalis* are type strains originally from enteric origin to represent typical wound contamination but they are not wound adapted strains. For mixed-species biofilms, each bacterial species was equilibrated to an OD of 0.1 (A_{650}), equivalent to CFU=$1x10^8$ and then prepared to a 1:1 ratio. For total viable counts, bacteria were recovered on the following media: *S. aureus* on Baird Parker Agar (Oxoid, UK), *P. aeruginosa* on Cetrimide Agar (Sigma-Aldrich, USA), *S. pyogenes* on Streptococcus Selective Agar (Sigma-Aldrich, USA), and *E. coli* and *E. faecalis* on UTI Chromoselect Agar (Sigma-Aldrich, USA).

Culture of biofilms using a biofilm flow device

Set up of the biofilm flow device used the method of Duckworth *et al,* with the addition of a 0.22µm syringe filter placed in the inlet tubing to prevent contamination of the fresh media (Duckworth et al, 2018). A bacterial cocktail in a 1:1 ratio, was prepared by mixing 48µl of each bacterial suspension to a sterile microcentrifuge tube, to allow the bacterial species to be added to the device simultaneously. Twelve disks of noble agar at 1.5% concentration were cut, using an 8mm biopsy punch, and added to the device using sterile forceps. One 13mm 0.22µm cellulose filter (Millipore, UK) was placed on top of each agar, using sterile forceps, and was inoculated with 20µl of the bacterial cocktail. The device was placed in an

incubator at 33°C and connected to the peristaltic pump with a flow rate of 0.322mL min^{-1}. At the end of each experiment the device was sterilised using Gerrard Ampholytic Surface Active Biocide (GASAB) disinfectant at a 1:100 concentration, submerged for 24 hours and then placed in deionised water. The tubing was washed through with GASAB disinfectant, before autoclaving.

Biofilm recovery and total viable count

At 24, 48 and 72 hours, cellulose filters were collected, and individually placed into a 15ml falcon tube containing 1ml of phosphate buffered saline or 0.1% (w/v) sodium thiosulphate to deactivate the HOCl, and vortexed (2200rpm, 30s) to homogenise the biofilm. Serial dilutions were prepared from 10^{-1} to 10^{-12}. 10µl of each dilution was pipetted in triplicate onto appropriate selective agars and incubated at 37°C 24 h for total viable counts (TVC).

Antimicrobial treatment and assessment of viability

Mixed-species biofilms were prepared as described above. Commercially available wound dressings were cut to size using sterile dissecting scissors. HOCl gel was formulated by Briotech Incorporated. 0.19 g of HOCl gel was weighed and transferred to each treated biofilm (sufficient to cover the entire biofilm and filter). Biofilms were allowed to establish for 5h or 24h without treatment, the nutrient flow was then paused to allow addition of topical treatments and allowed to run for a further 48-72h, with sampling at 24h time points. At these time points treatments were removed from the cellulose filters and enumeration of the biofilm occurred as described previously. For the untreated control, biofilms were cultured for 72h and tested as described, without the addition of any treatment.

Sequential dosing with HOCl gel

Biofilms were cultured in the DBD as described with HOCl gel applied at 5h. After 24h and 48h growth, biofilms were scraped from the filters, resuspended in 0.1% (w/v) sodium thiosulphate and adjusted to an OD of 0.1 (A_{650}). These bacterial suspensions were used to seed fresh biofilms that were cultured for 24h before application of HOCl gel, and enumeration by TVC, as previously described.

Results

Effectiveness of antimicrobial wound dressings against two-species (mixed) biofilms

Biofilms were established for 5h before the application of a wound dressing as the Gram-negative shift between *P. aeruginosa* and *S. aureus* occurs at approximately 10h in our model (Duckworth et al. 2018), and we intended that treatment should impair or prevent this phenomenon. A dressing with no antimicrobial ingredient was used as a control. Log changes in bacterial number at 24, 48 and 72h post-treatment were calculated relative to an untreated biofilm, cultured and sampled over the same period (Table 1 and Supplementary 1).

Iodine, crystal violet and silver dressings were most effective against both bacteria, with PHMB resulting in a negligible reduction that was not statistically different to the control ($p>0.05$). Despite their efficacy, analysis of relative competitive indices (defined by ratio of species in the output population divided by the corresponding ratio in the inoculum) revealed that neither the crystal violet nor the silver dressing prevented a Gram-negative shift from occurring, resulting in a population predominated by *P. aeruginosa* (Figure 1).

Effectiveness of HOCl gel against two-species biofilms

HOCl gel was tested against single and two-species biofilm of *S. aureus and P. aeruginosa,* with treatment applied at 5h to determine whether the Gram-negative shift could be impaired or prevented, and 24h to determine effectiveness against an established biofilm in which the Gram-negative shift has occurred in our model (Table 2).

In single-species biofilms, HOCl gel was more effective against *S. aureus* than *P. aeruginosa* at both application time points (Table 2 and Supplementary 2). In the two-species biofilm, HOCl gel slightly decreased the amount of *P. aeruginosa* when applied at 5h ($p>0.05$) (Table 2 and Supplementary 2). At 24h, the decrease of *P. aeruginosa* was equivalent to that of the single species ($p<0.05$). HOCl gel was less efficacious against *S. aureus* in a two species biofilm when applied at 5h and 24h, and earlier treatment resulted in increased numbers of recoverable *S. aureus* (Table 2 and Supplementary 2). Compared to the commercially available dressings, HOCl gel applied at 5h was more effective than iodine, PHMB and crystal violet at preventing recovery of *P. aeruginosa* 24h and 48h post-treatment and as effective as the silver treatment (Figure 2A). HOCl gel applied at 5h did not stop *S. aureus* from recovering post-treatment but did so when applied to the 24h established biofilm. *P. aeruginosa* recovery was also prevented when applied at the 5h and 24h time points (Figures 2A and 2B).

Effectiveness of HOCl gel against a five-species biofilm

Five species biofilms comprised of *S. aureus, S. pyogenes, E. faecalis, E. coli* and *P. aeruginosa* were cultured for 5 and 24h prior to the application of HOCl gel. Untreated

biofilms were established to determine the effect of HOCl gel on the microbial community (Figure 3A). In the untreated biofilm four of the five bacteria were detectable at 72h, with a shift between *S. aureus* and *P. aeruginosa* occurring between 48-72h. The presence of *E. coli* diminished over time and was undetectable at the 72h time point. The application of HOCl gel at 5h resulted in significantly ($p<0.05$) reduced numbers of *P. aeruginosa* but relatively stable growth of the other four bacteria, with all five detectable at each time point (Figure 3B). When HOCl gel was applied at 24h the numbers of detectable bacteria for each species were significantly ($p<0.05$) reduced with *E. faecalis, E. coli* and *S. pyogenes* not detectable at 48h (Figure 3C). However, *S. aureus, P. aeruginosa* and *S. pyogenes* recovered in number at 24h post-treatment. Interestingly, no shift between *S. aureus* and *P. aeruginosa* was observed.

Susceptibility of HOCl treated bacteria to subsequent doses of HOCl gel

Three separate doses of HOCl gel were applied to the biofilms at 5h, 24h and 48h, with TVCs recorded at 24h, 48h and 72h (Figures 4 A and 4B) to test for recovery. For both two- and five-species biofilm a similar growth pattern to that seen in Table 2 and Figure 3B was observed after the first dose of HOCl gel. However, subsequent doses of HOCl led to a continued reduction in bacterial numbers; in the five-species biofilm *S. aureus* was no longer detectable after three doses of HOCl gel (Figure 4B). No bacteria recovered in number between doses.

Discussion

Numerous and varied *in vitro* models have been used to assess the efficacy of topical antimicrobial treatments for wounds. These primarily include modified antibiotic disk

diffusion approaches, static batch biofilms, constant-depth film fermenters (CDFF), and drip-flow reactors (DFR) (Hill et al. 2010; Lipp et al. 2010; Junka et al. 2017; Anjum et al. 2018). The different parameters of these models give different results in terms of antimicrobial efficacy, and two factors, flow and a mixed-microbial community, appear to afford significantly greater tolerance of biofilms to treatment. Many of these models do not closely mimic the Gram-negative shift seen in chronic wound infections in clinic and limit the translation into the clinic (Altoparlak et al. 2004; Guggenheim et al. 2011; Miller et al. 2017; Alves et al. 2018). In this study we show that wound dressings impregnated with iodine, silver, PHMB or crystal violet do not prevent a Gram-negative shift between *S. aureus* and *P. aeruginosa* from occurring in our *in vitro* chronic wound biofilm model. With the exception of PHMB, all of the dressings tested were more effective against *S. aureus* than *P. aeruginosa*. However, PHMB dressings did not significantly reduce the number of either bacteria in the biofilm. We also demonstrate that an HOCl gel is effective at reducing bacterial numbers, with best activity against *P. aeruginosa* when applied at 5h.

Prior studies with iodine and silver against established biofilms of *P. aeruginosa* have given conflicting results (Hill et al. 2010; Hoekstra et al. 2017; Roche et al. 2019). Under static conditions, complete biofilm clearance is observed, however, in a DFR no significant change in biomass is seen (Hill et al. 2010; Bourdillon et al. 2017). Clinically, silver dressings effectively reduce the bioburden of chronic wound pathogens including *S. aureus, P. aeruginosa* and members of the Enterobacteriaceae family, improving clinical outcomes in "mild infection", however there is little data for their efficacy against established chronic wounds (National Institute for Health and Care Excellence [NICE], 2016; Lázaro-Martínez et al. 2019). The clinical use of iodine (povidone iodine or cadexomer iodine) remains

241 contentious due to mixed findings describing efficacy for reducing bacterial load and
242 delayed healing (Bigliardi et al. 2017; Bourdillon et al. 2017; Hoekstra et al. 2017; Roche et
243 al. 2019). Data from our model agrees with clinical observations, with demonstrable
244 reduction in bioburden using silver and iodine dressings, but incomplete biofilm clearance.
245 This reiterates the importance of incorporating the dynamic wound environment *in vitro* to
246 establish antimicrobial efficacy for wound treatments that is more clinically relevant.
247
248 Our data indicate that PHMB did not significantly reduce bacterial counts, in contrast to
249 previous studies using planktonic culture, single and mixed biofilms treated statically which
250 showed significant reduction in bioburden (Lipp et al. 2010; Rembe et al. 2016). Models in
251 which bacteria are immobilised in a collagen matrix that better represents the biofilm
252 environment suggest the efficacy of PHMB has been overestimated with smaller reductions
253 in bacterial bioburden in agreement with our results (Shoukat et al. 2015). Crystal or gentian
254 violet (CV/GV) was re-evaluated in 1992 as a dermatological antiseptic and approved by FDA
255 in 2013 for the treatment of diabetic foot ulcers and pressure ulcers, both of which are
256 classed as chronic wounds (Maley and Arbiser 2013; Edwards 2016). Case studies have
257 demonstrated foam CV/GV dressings to more effectively inhibit *S. aureus* than iodine
258 dressings in some ulcers, and *in vitro* CV/GV inhibits the growth of *P. aeruginosa*, including
259 biofilms (Woo and Heil 2017). Despite this, there is scant data from robust *in vitro* testing or
260 randomized clinical trials. Our data shows CV/GV to perform similarly to silver against both
261 *S. aureus* and *P. aeruginosa* indicating support for clinical use. However, controversy
262 regarding the oncogenic potential of CV/GV means it has limited usage which is unlikely to
263 change and makes clear the need for new methods of treatment.
264

HOCl is a broad spectrum, low toxicity antimicrobial regularly used in both dentistry and ophthalmology and as a wound cleanser (Haws et al. 2018; Boyar 2020; Gold et al. 2020). It has a role in the mammalian innate immune response to infection, being generated *in vivo* by myeloperoxidase released by neutrophils. Studies have shown that HOCl promotes the healing process in wounds and scar mitigation (Haws et al. 2018). Solutions of HOCl have excellent activity against single species biofilms of *P. aeruginosa* and *S. aureus* in static biofilm models, and in our single species biofilm model resulted in a 3-7 log decrease in bacterial numbers (Harriott et al. 2019). When applied at 5h in a two-species biofilm (prior to the Gram-negative shift), we found that HOCl gel significantly reduced numbers of *P. aeruginosa,* which did not recover post-treatment. *S. aureus* increased immediately post-treatment, indicating recovery. However, upon multiple dosing experiments both *P. aeruginosa* and *S. aureus* decreased in the two-species and five-species biofilms. Several studies have described a scenario in which *P. aeruginosa* effectively outcompetes *S. aureus* in *in vitro* co-culture by secreting 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), which is regulated by the Pseudomonas Quinolone Signal (PQS) system and has a role in quorum sensing, virulence, iron acquisition, and modulation of host immune responses (Filkins et al. 2015; Hotterbeekx et al. 2017; Radlinski et al. 2017; Orazi et al. 2019). In combination with membrane disrupting antimicrobials, HQNO renders *S. aureus* highly susceptible to treatments in early (6h) *in vitro* biofilms (Orazi et al. 2019). It is possible that HOCl reduces numbers of *P. aeruginosa* in the early biofilm by acting as an antimicrobial and simultaneously, modifying (e.g., oxidation or chlorination) secreted molecules such as HQNO or QS signals, thus impeding biofilm development. This could provide any remaining *S. aureus* post-treatment with conditions that are favourable for recovery. In the five-species biofilm a similar effect was observed. Applied at 5h, HOCl significantly reduced the

numbers of *P. aeruginosa* which was maintained as a minor biofilm member. When HOCl gel was applied to a 24h established biofilm this effect was not observed, and all members of the biofilm were reduced in number 24h post-treatment suggesting the HOCl gel was biofilm-disruptive.

Models describing bacterial interactions in multispecies biofilm models are scarce, and because they vary, highly differing bacterial interactions have been reported. The majority of models exploring interactions between bacteria in chronic infection have used two species. Our five-species model showed that over 72h, *S. aureus, P. aeruginosa, S. pyogenes* and *E. faecalis* co-existed, with *E. coli* quickly outcompeted. Studies using two-species biofilms suggest that *E. coli* can co-exist with each of the other microorganisms, in some cases growing preferentially in co-culture (Culotti and Packman 2014). However, we do not know at present what interactions underpin the growth phenomenon observed for *E. coli* in our model but suggest that inhibition might be due to competitive exclusion by *P. aeruginosa*, since *E. coli* and the other three bacteria grew in a steady state when *P. aeruginosa* numbers were limited by early HOCl gel treatment.

We demonstrate that repeat doses of HOCl gel at 24h intervals in a two- and five-species biofilm continually reduced all bacterial numbers, suggesting that the organisms were not becoming tolerant to treatment over time. This indicated that recovery of *P. aeruginosa, S. aureus* and *S. pyogenes* observed in the five-species biofilm, post-treatment, was likely due to diminishing levels of the active chlorine, which is known to react quickly, rather than adaptation to HOCl.

313 The most effective disruption and killing of bacteria in our mixed species models occurred
314 with multiple doses of HOCl gel. It is likely that a similar effect might be observed if the
315 commercially available antimicrobial wound dressings were similarly applied, however
316 published studies using silver indicate effectiveness of treatment is diminished with daily
317 transfer, and the design of our model meant that it was not possible to investigate this
318 further without inadvertent mechanical disruption of the biofilm (Kostenko et al. 2010).
319 Clinically, wound dressings might be changed daily or kept in place for a number of days,
320 depending on individual assessment of the wound, and rotation of antibacterial dressings
321 has been suggested as a strategy to reduce adaptation to a specific treatment (National
322 Institute for Health and Care Excellence [NICE], 2016). A lack of robust data hampers
323 evidence-based prescribing, and the Cochrane reviews on wounds highlight the dearth of
324 good-quality evidence. A such, few clinical or *in vitro* studies investigating the effect of
325 multiple doses/or dressing changes on the wound bioburden currently exist. Despite this the
326 use of antimicrobial dressings are advised by the British National Formulary to reduce levels
327 of bacteria at the wound surface, with iodine and silver recommended where clinical
328 infection is suspected (Joint Formulary Committee, 2020). Whilst it is acknowledged that
329 vigorous randomised control trials for chronic wound treatments are difficult, realistic
330 human-relevant *in vitro* models could offer a means to provide translatable evidence for the
331 efficacy of topical antimicrobial treatments and wound dressings. It is therefore vital that
332 work towards refining accurate chronic wound models continues. This study has highlighted
333 the need for more realistic models, like the one used here, to produce results that align
334 better with clinical cases to improve the translation of treatments from *in vitro* to *in vivo.*
335 Moreover, this model indicates that an HOCl gel has promise as a treatment for complex
336 wound biofilms, being especially effective against the chronic wound pathogen *P.*

aeruginosa. Adding HOCl to the clinical armoury for chronic wound infection has the potential to improve healing, treatment and healthcare costs for chronic wounds worldwide.

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Conflict of interest

LIR is a consultant to Briotech Incorporated.

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487 **Author contributions:**

488 AGN, RLP and SEM undertook experimental work. All authors contributed to design of the
489 experiments. All authors contributed to writing and proof-reading of the manuscript.

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	Log change vs untreated control					
	Staphylococcus aureus			*Pseudomonas aeruginosa*		
	24h	48h	72h	24h	48h	72h
Non-antimicrobial dressing	-0.34	-0.67	+0.48	-0.96	-0.75	-0.99
Iodine	-3.14*	-4.74*	-3.17*	-3.76*	-1.35	-0.28
PHMB	-0.72	-0.55	-0.72	-1.25	-1.66	-1.37
Crystal violet	-4.35*	-5.51*	-3.37*	-1.56	-2.51*	-2.46*
Silver	-4.97*	-5.26*	-5.33*	-2.23*	-2.61*	-3.27*

Table 1. Log change in recoverable bacteria from two-species biofilms following treatment with a wound dressing at 5h. Statistically significant changes are indicated by *

	Log change vs untreated control					
	HOCl treatment at 5h			HOCl treatment at 24h		
	24h	48h	72h	24h	48h	72h
P. aeruginosa	-2.37*	-2.28*	-3.22*	-2.89*	-2.94*	-3.01*
S. aureus	-6.65*	-6.48*	-5.61*	-5.86*	-5.41*	-7.46*
P. aeruginosa (mixed)	-1.15	-1.70	-1.57	-2.96*	-3.68*	-3.02*
S. aureus (mixed)	+1.97	+2.18*	+1.14	-2.07*	-3.18*	-4.20*

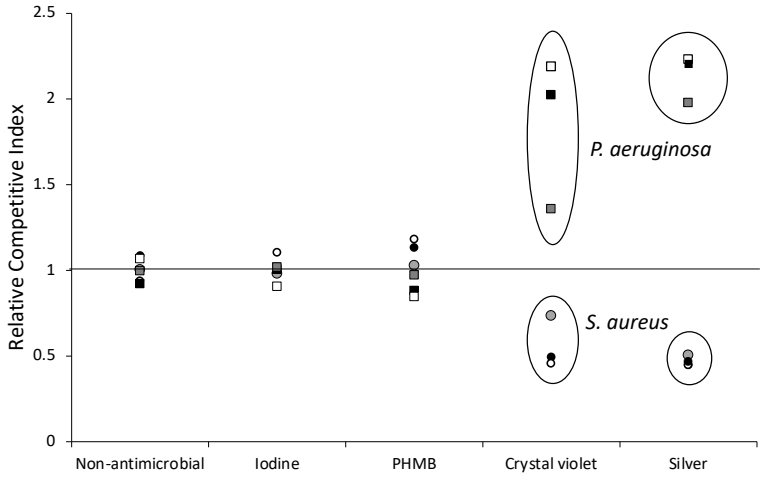
Table 2. Log change in recoverable bacteria from single and two-species biofilms following treatment with HOCl gel applied after 5h or 24h of biofilm growth. Statistically significant changes are indicated by *

Figure 1. Relative competitive index of the two-species population at 24h, 48h and 72h post-treatment with an antimicrobial dressing where "1" signifies no competition between species. Values above 1 indicate the predominant constituent of the population, values below 1 indicate the minor constituent of the population. *S. aureus* 24h (black filled circle), 48h (open circle), 72h (grey filled circle); *P. aeruginosa* 24h (black filled square), 48h (open square), 72h (grey filled square).

Figure 2. Relative recovery of bacteria post-treatment. (A) *S. aureus* (B) *P. aeruginosa*. Grey: 24h post-treatment; black: 48h post-treatment. Above zero indicates higher than original inoculum (growth); below zero indicates lower that original inoculum (decrease). Bacterial recovery above the level of the original inoculum is highlighted with an oval.

Figure 3. Five species biofilms (A) untreated; (B) treated with HOCl at 5h biofilm development; (C) treated with HOCl at 24h biofilm development. Arrows indicate application of HOCl. *S. aureus* (solid black); *S. pyogenes* (open black); *E. faecalis* (round dotted); *E. coli* (short dashed); *P. aeruginosa* (long dashed).

Figure 4. Multiple doses of HOCl gel applied at 5h, 24h and 48h (indicated by arrows) to (A) two-species and (B) five-species biofilms. The three doses of HOCl are indicated by arrows. *S. aureus* (solid black); *S. pyogenes* (open black); *E. faecalis* (round dotted); *E. coli* (short dashed); *P. aeruginosa* (long dashed).



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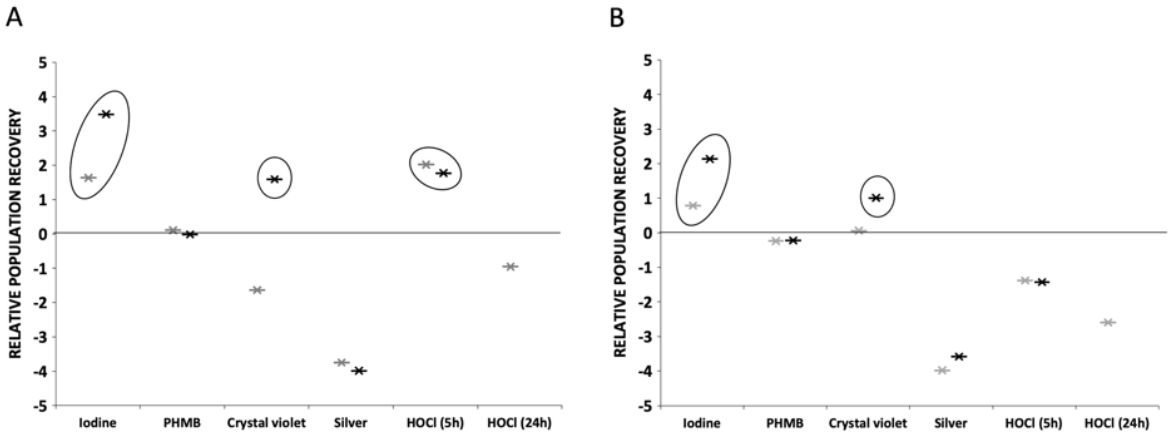

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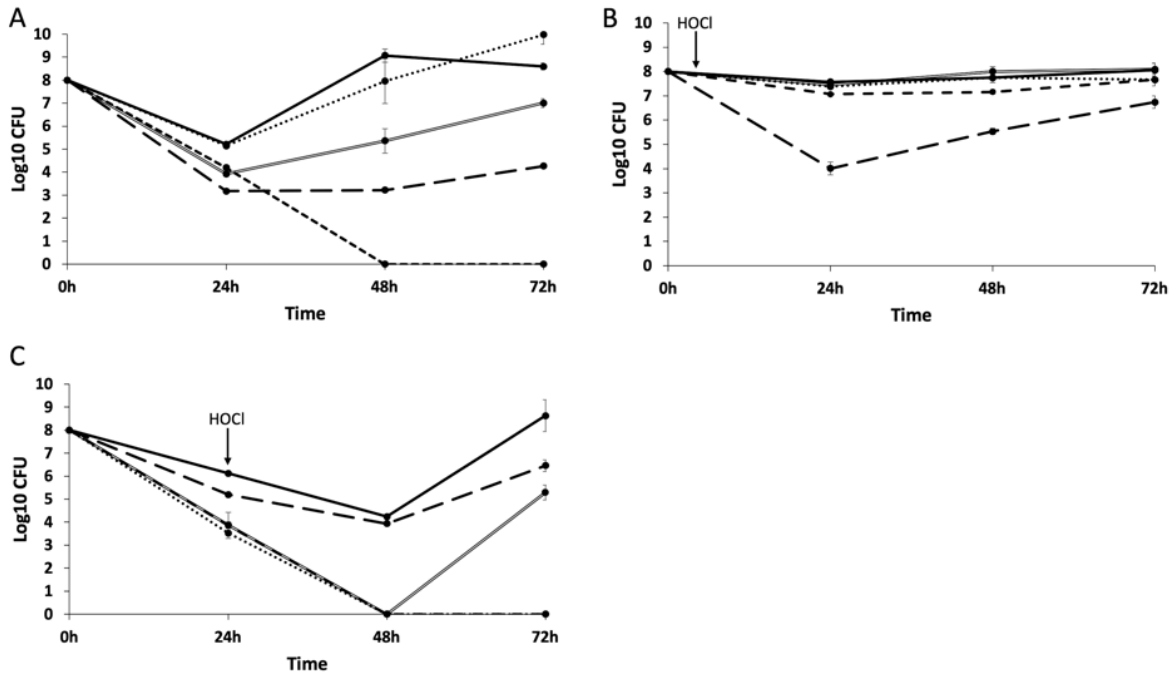

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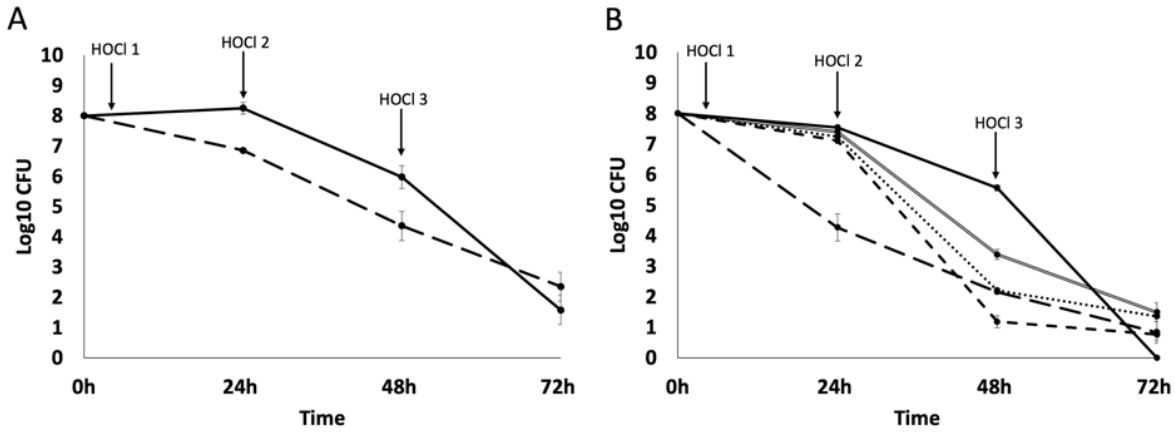

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	Log_{10} total viable counts post-treatment					
	Staphylococcus aureus			***P. aeruginosa***		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**
Non-antimicrobial dressing	7.66	7.33	8.48	7.04	7.25	7.01
Iodine	4.86*	3.26*	4.83*	4.24*	6.65	8.72
PHMB	7.28	7.45	7.28	6.75	6.34	6.63
Crystal violet	3.65*	2.49*	4.63*	6.44	5.49*	5.54*
Silver	3.03*	2.74*	2.67*	5.77*	5.39*	4.73*

Supplementary 1. Log_{10} Total Viable Counts of bacteria recovered from two species biofilms 24h, 48h and 72h post-treatment with a topical antimicrobial dressing. Start inoculum log_{10} TVC = 8. Statistically significant changes are indicated by *

	Log_{10} total viable counts post-treatment					
	HOCl treatment at 5h			**HOCl treatment at 24h**		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**
P. aeruginosa	5.63*	5.72*	4.78*	5.11*	5.06*	4.99*
S. aureus	1.35*	1.52*	2.14*	2.14*	2.59*	0.54*
P. aeruginosa (mixed)	6.85	6.30	6.43	5.04*	4.32*	4.98*
S. aureus (mixed)	9.97	10.18*	9.14	5.95*	4.82*	3.80*

Supplementary 2. Log_{10} Total Viable Counts of bacteria recovered from two species biofilms 24h, 48h and 72h post-treatment with HOCl applied to biofilms at 5h or 24h. Start inoculum log_{10} TVC = 8. Statistically significant changes are indicated by *