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# Comparative antimicrobial activity of aerosolised sodium hypochlorite, chlorine dioxide and electrochemically activated solutions (ECAS) using a novel standardised assay.

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## Abstract

The main study aim of this study was to develop a standardised experimental assay to enable differential antimicrobial comparisons of test biocidal aerosols. This study represents the first chlorine-matched comparative assessment of the antimicrobial activity of aerosolised sodium hypochlorite, chlorine dioxide and electrochemically activated solution (ECAS) to determine their relative ability to decontaminate various surface associated healthcare relevant microbial challenges. Standard microbiological challenges were developed by surface associating typed *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* spores or a clinical Methicillin Resistant *S. aureus* strain (MRSA) on stainless steel, polypropylene or fabric. All test coupons were subjected to 20 minute biocidal aerosols of chlorine-matched (100 ppm) sodium hypochlorite, chlorine dioxide or ECAS within a standard aerosolisation chamber using a commercial humidifier under defined conditions. Biocidal treatment type and material surface had a significant effect on the number of microorganisms recovered from various material surfaces post treatment exposure. Under the conditions of the assay, the order of antimicrobial efficacy of biocidal aerosol treatment was: ECAS > chlorine dioxide > sodium hypochlorite. For all biocides, greater antimicrobial reductions were seen when treating stainless steel and fabric compared to plastic associated microorganisms.

**Summary statement:** The experimental fogging system and assay protocol designed within this study was shown capable of differentiating the comparative efficacy of multiple chlorine matched biocidal aerosols against a spectrum of target organisms on a range of test surface materials, and would be appropriate for testing other biocidal aerosol treatments or material surfaces.

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## Introduction

Biocides have a key role to play in decontamination within healthcare environments through disinfection and sterilisation. There is a known linkage between the use of antibiotics and the emergence and spread of antibiotic resistance (1),and the most recent Health Protection Agency (UK) report on healthcare associated infection and antimicrobial resistance indicates the continued rise in both selected antibiotic resistant strains (e.g. Panton-Valentine Leukocidin MRSA) and resistance factors (e.g. carbapenemases) (2). Hence there is an ever greater need to ensure biocides are being used appropriately, to help reduce the level of microbial contamination within the healthcare environment to acceptably safe levels and consequently reduce the risk of infection (3). There are numerous classes of biocides currently utilised for this purpose, with the exact formulation for a given practical application being dependent on convention and/or local standard operating procedures (4).

Effective biocidal action of any given active agent is reliant on concentration, exposure time, composition of contact surface (with rough and/or absorbent surfaces requiring a longer contact time) and presence of organic loading (5). It is important to utilise a delivery mechanism that will maximise the antimicrobial potential of a biocide, and they are conventionally applied in liquid form. Aerosol delivery technology utilises solid particles or liquid droplets suspended in a gas (i.e. biocide droplets carried in air) (6), in contrast to vapours in which the substance itself is in the gas phase. Early research into aerosol delivery mechanisms focused mainly on disinfection of airborne bacteria and spores (7-10).However, more recent research studies have assessed the use of aerosolised biocides for environmental decontamination, although the literature is relatively mixed in terms of its assessment of the benefit of aerosols (6). Nonetheless, the antimicrobial potential of certain soluble biocidal agents has been proven when delivered as an aerosol, including; sodium hypochlorite (11), peroxyacetic acid (12), quaternary ammonium

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compounds (6),lactic acid (13),hydrogen peroxide (14-17), as well as pre-mixed combinations (peroxyacetic acid and hydrogen peroxide)(18). Advancements in aerosolisation technology as a means of biocide delivery has found application in the decontamination of; material surfaces (12) and fresh produce (19) in the food processing industry, commercial and residential buildings materials (11), as well as potential utility within healthcare environments (14).

For environmental decontamination applications within habitable spaces, clearly certain biocides are too toxic (e.g. phenolics and glutaraldehyde), flammable (e.g. alcohols) or have the potential to leave unwanted residues on surfaces (e.g. iodophors). Chlorine containing biocides are widely used for the decontamination of surfaces, usually in the form of hypochlorite ($ClO^-$), being inexpensive to produce and having proven antimicrobial activity (20). Chlorine dioxide ($ClO_2$) is a highly oxidative biocide originally developed for water disinfection applications, the disinfection activity of which is known to be less influenced by pH and results in less harmful by-products than traditional chlorine treatment (21). Chlorine dioxide has also been effectively used for environmental decontamination, particularly within food processing environments, being generated on site using 'sachet' based systems (22). Interestingly although gaseous or liquid systems are widely used, there is little/no literature on the delivery of aqueous chlorine dioxide as a biocidal aerosol. Electrochemically activated solution(s) (ECAS) are currently emerging as novel chlorine containing biocides with numerous potential applications, including potable water disinfection (23), within the food industry (24), and within the healthcare sector (25). ECAS are produced by electrolysis of typically low concentration salt (NaCl) solutions within an electrochemical cell, the resultant anolyte solution ($ECAS^a$) usually having a high redox (oxidising) potential, a low pH (which can be neutralised by internally reconfiguring the electrochemical cell) and a variable chlorine concentration depending on set operating

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parameters (25). ECAS have broad spectrum activity, including spores (26), and have been shown to be extremely fast acting, even in comparison to other commonly used biocides (27). The antimicrobial potential of aerosolised ECAS has been previously investigated using a portable electrostatic aerosol applicator which delivers large volumes of liquid in a spray, whereby sufficient wetting occurred for a mop to be required to dry the laboratory floor post treatment (28, 29).Significant reductions in the microbial load of ceramic tiles were observed post-treatment (28); however, since no appropriate control (non-biocidal fog) was described, the effects of physical removal and kill cannot be disentangled.

The main study objective was to further our understanding of the antimicrobial potential of aerosolised biocides through the development of a standardised experimental model and defined microbiological challenge, enabling differential antimicrobial comparisons of various test biocides. This study represents the first chlorine-matched comparative assessment of the antimicrobial activity of aerosolised sodium hypochlorite, chlorine dioxide and ECAS to determine their relative ability to decontaminate various surface materials (stainless steel, polypropylene and cellulose fabric) when loaded with a range of bacterial contaminants relevant to healthcare environments.

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## Materials and Methods

### Preparation of test biocides

Liquid $ClO_2$ (Selectrocide™, Selective Micro Technologies, MA, US) was prepared at 500 ppm according to manufacturer's instructions, and both sodium hypochlorite (Sigma-aldrich, UK) and liquid $ClO_2$ were diluted in deionised water to 100 ppm for experimental use. Acidic electrochemically activated solution(s) (ECAS[a]) were generated by the electrolysis of 1% (w/v) NaCl solution within a commercial ECAS generator (Bridge Systems Ltd.,Fife, UK). The free chlorine level of ECAS was determined using the DPD test (Palintest Ltd., Gateshead, UK) and standardised to 100 ppm by altering the operating parameters of the ECAS generator (specifically the flow rate and salinity of bulk solution passed through the electrolytic cell). The redox potential and pH of ECAS[a] were measured during production using inline probes and validated using external probes before experimentation.

### Growth and maintenance of target micro-organisms

Methicillin Sensitive *Staphylococcus aureus* (MSSA; ATCC 6538), Methicillin Resistant *Staphylococcus aureus* (MRSA; SMH 11622, kind gift from Southmead Hospital, Bristol, UK) and *Pseudomonas aeruginosa* (ATCC 15442) were stored at -80°C, and recovered onto Tryptone Soya Agar (TSA; Oxoid Ltd, Basingstoke, UK) when required. *Bacillus subtilis* subsp. spizizenii (ATCC 6633) spores were prepared by using the protocol described in BS EN ISO 14347:2005 (30). Briefly, spread plate cultures were prepared, and then incubated for 3 days at 37°C followed by 7 days at 30°C. Plate cultures were scraped into sterile distilled water and resultant suspension washed by centrifugation. Spores suspensions were washed in isopropanol for 3h to kill any remaining vegetative cells, and washed a further three times in distilled water to remove cell debris.

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**Aerosolisation test chamber**

The chamber was constructed from clear acrylic polymer sheets held together by tongue and groove jointing, and mounted on a plastic base plate supported on a metal frame. All edges were sealed using a silicone rubber compound, with the exception the front door panel which was hinged and sealed with metal latches to allow for introduction and removal of test equipment and sample material. The final internal dimensions of this chamber were 450 x 380 x 380 mm, which created a fogging space with a nominal volume of 65 L. Fog was introduced via an inlet port cut into the bottom base plate (Ø = 55 mm).

**Preparation of material test surfaces**

Three test surface materials were chosen. Stainless steel (type 1.4301 with Grade 2 B finish; FC Hammonds, Bristol, UK) and plastic sheets (polypropylene; Sirane, Telford, UK) were cut into 15 mm diameter coupons. Surfaces were cleaned and sterilised according to BS EN ISO 13697:2001(31). Briefly, coupons were soaked in 5 % (V/V) alkaline detergent solution (Decon 90; Decon Laboratories Limited, East Sussex, UK) for 60 minutes and then immediately rinsed with deionised water. Coupons were disinfected by immersing in 70 % (V/V) iso-propanol for 15 min, and then dried by evaporation within a laminar flow hood. Cotton gauze coupons (10 $mm^2$) were cut from larger pre-sterilised sheets (Velveteen; Bel-art Products, New Jersey, USA).

**Preparation of surface associated standard microbiological challenge**

*S. aureus* (MSSA and MRSA) and *P. aeruginosa* cell suspensions were prepared by emulsifying fresh plate colonies (grown on Tryptone Soya Agar incubated for <24h) into diluent (0.1% [w/v] Tryptone + 0.85% [w/v] NaCl), and adjusting the cell density to 3 x $10^9$cfu per mL according to previously determined spectrophotometric calibration curves ($OD_{620nm}$). *B. subtilis* spore

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suspensions were prepared (as previously described), enumerated by viable counting and adjusted to 1 x $10^9$ spores per mL. Sterile coupons of each test surface (three coupons per species per test condition) were inoculated with 10 µL of bacterial test suspensions (deposited as a single drop). Inoculated coupons were left to dry (≤ 1 h) in a class II biological safety cabinet.

**Aerosolisation test procedure**

For each aerosolisation experiment, three coupons of each test surface for each species were transferred to 10 mL Letheen broth (BD, Oxford, UK) immediately following inoculation to provide untreated control data. A separate set of coupons (n=3 per species of each test surface) were placed on a sterile plastic drainage grid (test samples) and transferred to the test aerosolisation chamber. A HU-25OG humidifier (Contronics, Sint-Oedenrode, Netherlands) was used to deliver aerosolised liquid. Prior to aerosolisation, the HU-25OG was filled and drained with the control or test solution to be delivered, and then refilled immediately before challenging inoculated coupons. The HU-25OG was set with the fan to maximum and relative humidity at 50% (delivering a droplet size of 1-3 µm). The aerosol was introduced to the chamber via 40 mm diameter tubing. The chamber was positioned within an extracting fume hood providing a constant air-draw, and ventilated via a 12 mm diameter hole on the sides of the chamber. Inoculated test sample coupons were exposed to test biocide aerosol for 20 minutes, followed by a 10-minute settle time. All aerosolised biocides and a water control were independently tested according to the above protocol a minimum of three times. Following drainage the HU-25OG was filled and drained three times with deionised water to remove any residual biocide from within the humidifier.

**Microbial sampling and recovery method**

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Following treatment, test coupons were immediately transferred to 10 mL Letheen broth containing sterile glass beads, shaken for 20 minutes (200 rpm) to neutralise any remaining biocide and vortexed for 5 seconds to ensure maximal recovery of microbial survivors from the test surfaces (neutraliser was validated before use). Viable counts were performed on neat and diluted recovery suspensions using a spiral plater (Don Whitley Scientific, Shipley, UK) onto TSA recovery plates, incubated at 37ºC aerobically for 24 hours and counted to determine the numbers of colony forming units per coupon (cfu coupon$^{-1}$). In addition, a 1 mL TSA pour plate of the neat recovery suspensions was performed to ensure an accurate minimum detection limit for the assay of 3 x $10^2$ organisms, and an absolute detection limit of 1 x $10^1$ organisms.

**Analysis of results**

To determine whether aerosolisation treatment resulted in significant reductions in viable bacterial cells, an Analysis of Variance (ANOVA) was performed on the microbial recovery counts followed by a Dunnett's Multiple Comparison Test against the control (water) recovery counts, and a Tukey's test to compare different biocidal aerosol treatments, with a $p<0.05$ regarded as significant. A two way ANOVA was also performed to elucidate any significant effects of both treatment type and material surface on antimicrobial efficacy. Graph construction, and statistical analyses were conducted with the use of GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

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197 **Results**

198 Table 1 shows the recovery of viable cells from the surface of test materials immediately
199 following preparation of surface associated standard microbial challenges (i.e. post drying but
200 before treatment). On stainless steel or plastic coupons, there was no significant reduction in the
201 number of viable MSSA, MRSA and *B. subtilis* (spores) recovered compared to the inoculum;
202 however, the study protocol resulted in a significant reduction in viable *P. aeruginosa* cells from
203 the surface of both stainless steel (2.71 log reduction; $p<0.001$) and plastic coupons (1.37 log
204 reduction; $p<0.001$). When recovering from fabric, there was a significant reduction in the
205 number of MSSA (0.33 log reduction; $p<0.001$), MRSA (0.28 log reduction; $p<0.001$), *P.*
206 *aeruginosa* (2.08 log reduction; $p<0.001$) and *B. subtilis* (spores; 0.69 log reduction; $p<0.001$)
207 after drying compared to the inoculum. It was found that the microbial inoculum dried faster on
208 stainless steel coupons (~30 minutes) compared to plastic coupons (~60 minutes) and hence a
209 staggered inoculation protocol was required for subsequent experiments.

210 The effect of a 20 minute aerosolisation treatment regimen of water (control) or active
211 biocidal aerosol of sodium hypochlorite, chlorine dioxide or ECAS (each prepared to 100 ppm
212 free chlorine) against a standard steel, plastic or fabric surface associated microbiological
213 challenge are shown in figures 1-4. Recovery of a clinical Methicillin Sensitive *S. aureus*
214 (MSSA) type strain from the surface of material surfaces after being subjected to control or test
215 aerosol treatments is shown in figure 1. When water alone was used to treat surfaces there was
216 no significant reduction in the number of viable recoverable survivors compared to that after
217 drying (see table 1) for any of the test material surfaces. Sodium hypochlorite elicited a
218 significant antimicrobial effect compared to water alone when used to treat steel and fabric
219 surface associated MSSA; however, had no significant effect against plastic surface associated

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MSSA (figure 1b). Chlorine dioxide elicited a significant antimicrobial effect compared to water alone when used to treat all three material surfaces, and was significantly more effective than sodium hypochlorite when used to treat steel and plastic material surfaces (figure 1b). ECAS elicited a significantly greater log reduction compared to water, sodium hypochlorite and chlorine dioxide when used to treat steel and plastic surface associated *S. aureus*, and a significantly greater log reduction compared to water and sodium hypochlorite when used to treat fabric associated MSSA (figure 1b). When comparing the log reductions achieved for a given biocide against a specific surface type, the greatest log reductions were seen when treating fabric and steel, compared to plastic surface associated MSSA.

Recovery of a clinical Methicillin Resistant *S. aureus* (MRSA) strain from the surface of material surfaces after being subjected to control or test aerosol treatments is shown in figure 2, and similar trends were seen to that observed for theMSSAtype strain (figure 1). When water alone was used to treat surfaces there was no significant reduction in the number of viable recoverable survivors compared to that after drying (see table 1) for stainless steel and plastic coupons; however, a significant 0.38 log reduction ($p<0.01$) was seen for fabric. Sodium hypochlorite elicited a significant antimicrobial effect compared to water alone when used to treat steel and fabric surface associated MRSA (figure 2b); however, the reductions were significantly greater than that observed for the MSSA type strain (figure 1). Sodium hypochlorite elicited no significant antimicrobial effects compared to water alone when used to treat plastic surface associated MRSA (figure 2b). Chlorine dioxide elicited a significant antimicrobial effect compared to water alone when used to treat all three surface associated MRSA challenge materials, and is significantly more effective than sodium hypochlorite in all cases (figure 2b). In fact complete kill (as determined by the limit of detection of the test) was observed when used to

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243 treat fabric associated MRSA (figure 2a). ECAS elicited a significantly greater log reduction
244 compared to water and sodium hypochlorite when used to treat all three surface associated
245 MRSA challenge types, whereby complete kill was observed against steel and fabric associated
246 MRSA, and was significantly more effective than chlorine dioxide when used to treat plastic
247 associated MRSA (figure 2). Similarly to MSSA far greater log reductions were achieved for a
248 given biocide when used to treat fabric and steel, compared to plastic surface associated MRSA.

249 Recovery of a typed *P. aeruginosa* strain from the surface of material surfaces after being
250 subjected to control or test aerosol treatments is shown in figure 3. When water alone was used
251 to treat surfaces there was no significant reduction in the number of viable recoverable survivors
252 compared to that after drying for stainless steel and plastic coupons; however, a significant 0.50
253 log reduction ($p<0.01$) was seen for fabric. Sodium hypochlorite elicited a significant
254 antimicrobial effect when used to treat all three surface types, with no detectable survivors being
255 recovered from the surface of steel or fabric (figure 3a). Chlorine dioxide elicited a significant
256 antimicrobial effect compared to water alone when used to treat all three surface types, and was
257 significantly more effective than sodium hypochlorite when used to treat plastic surface
258 associated *P. aeruginosa* (figure 3b). ECAS elicited a significantly greater log reduction
259 compared to water and sodium hypochlorite when used to treat plastic surface associated *P.*
260 *aeruginosa* (figure 3b), but was similarly effective to sodium hypochlorite and chlorine dioxide
261 when used to treat steel and plastic since all three biocides elicited complete kill (figure 3a).
262 Similarly to the results obtained for MSSA and MRSA surface associated microbiological
263 challenges, far greater log reductions of *P. aeruginosa* were achieved for a given biocidal aerosol
264 when used to treat fabric and steel, compared to plastic.

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Recovery of *B. subtilis* spores from the surface of material surfaces after being subjected to control or test aerosol treatments is shown in figure 4. When water alone was used to treat surfaces there was no significant reduction in the number of viable recoverable survivors compared to that after drying (see table 1) for any test material surface. Compared to this water control, sodium hypochlorite elicited a significant sporicidal effect when used to treat steel and plastic associated *B. subtilis* spores; however, there was no significant reduction when used to treat fabric associated *B. subtilis* spores (figure 4b). Chlorine dioxide elicited a significant sporicidal effect compared to water alone when used to treat all three surface types; however, the log reduction was not significantly different to that observed when treating with sodium hypochlorite (figure 4b). ECAS also elicited a significant sporicidal effect compared to water alone when used to treat all three surface types, with log reductions not significantly different from both sodium hypochlorite and chlorine dioxide, except on plastic where it was found to be significantly more effective than sodium hypochlorite (figure 4b).

The data presented in this study was collectively analysed by two-way analysis of variance, whereby biocidal treatment and material surface were the defining factors. Both biocidal treatment and material surface had a significant effect on the number of microorganisms recovered from various material surfaces post treatment exposure. Moreover, a greater proportion of the differential kill observed was attributable to biocidal treatment type rather than surface material, indicating that this is the dominant factor.

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## Discussion

Initial experiments focussed on standardising the surface associated microbial challenge including organisms currently used in European standard biocidal assays (EN ISO 13697:2001) (31). It was evident that after inoculation and drying there were significant differences in the recovery rate of different species from different test materials. MSSA, MRSA and *B. subtilis* spores suffered no detrimental effect after drying and recovery from plastic and stainless steel surfaces, in contrast to *P. aeruginosa* which was significantly affected by drying. This is not unexpected since both Gram positive organisms and spores are known to be more resistant to the effects of drying compared to Gram negative organisms (32). In contrast there was a significant reduction in the number of all target microorganisms recovered from fabric after drying. Since both the number of recoverable spores and Gram positive organisms was reduced, it is postulated that this is due to inefficient recovery from this material type rather than the effects of drying. These significant reductions in some microbial populations after drying and recovery were consistent and quantifiable. A viable population of surface associated microbial cells always remained to enable a potential 4 log reduction in microbial numbers to be quantified, sufficient to define a compound as having surface bactericidal activity according to EN ISO 13697:2001(31). Moreover, all effects of test biocidal aerosol treatment were compared to a water treatment which controls for any reductions post-inoculation and drying, as well as ensuring any significant reductions were due to active antimicrobial processes.

Preliminary aerosolisation experiments were performed to standardise the experimental system, and initially a portable electrostatic aerosol generator was used, similar to that in a previous study investigating the aerosol delivery of ECAS (28). This device delivers high volumes of liquid but was found to wet surfaces to an unacceptable level within the

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aerosolisation test chamber, and lead to the investigation and implementation of transducer based fogging devices. These devices use a piezoelectric transducer (resonating frequency ~1.6MHz) whereby ultrasonic waves are focused on water, generating a cold dry-feeling fog with a droplet size of 1-3 µm volume medium diameter (10 fold lower than the portable electrostatic aerosol applicator). Exposure to test biocide aerosols for 20 minutes (followed by a 10 minute settle time) using a standard fog output (50% with maximum fan speed, utilising ~0.5 L of test aerosol solution), resulted in minimal wetting of surfaces and hence would be appropriate for a diverse range of applications. This was therefore chosen as the standard treatment, for all biocide, microbiological and material surface variables.

According to the European Standard quantitative non-porous surface test, bactericidal activity on a surface is defined as the "capability of a product to produce at least a $10^4$ log reduction in the number of viable bacterial cells belonging to the reference strains" (31).This was used as the standard to which the antimicrobial activity of biocidal aerosols against surface associated microorganisms was compared. When testing sodium hypochlorite against MSSA it did not elicit a >4 log reduction on any test surface material, although significant reductions were seen on steel and fabric. Chlorine dioxide elicited a >4 log reduction against MSSA surface associated on steel and fabric, but not against plastic, whereas ECAS elicited a >4 log reduction against MSSA associated on all three test surface materials. A similar trend was observed when treating surface associated MRSA with biocidal aerosols, but greater reductions were seen in all cases. This is perhaps surprising, given that previous studies have shown MRSA strains to be more resistant to certain biocides than MSSA (33), although little can be inferred from this single strain comparison since other studies have assessed the relative sensitivity of a broad range of MSSA and MRSA strains. This highlights the importance of including strains relevant to the in-

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use application of a given biocide when testing new biocides and delivery mechanisms. *P. aeruginosa* was comparatively more sensitive to treatment with all three biocides, with complete kill being observed with all treatment types against steel and fabric associated microorganisms (>4 log reduction), although plastic coupon associated *P. aeruginosa* were again more resistant to biocidal aerosol treatment. This may be due to the relative hydrophobicity of the test surfaces chosen since all test biocides are carried within water droplets as part of aerosol dispersion. The test plastic (polypropylene) material used within this study was found to be hydrophobic, as evidenced by small water droplets forming on the upward facing surface of coupons after treatment, in contrast to the confluent liquid layer that formed on the surface of stainless steel. This again highlights the importance of integrating and testing not only application specific species and strains, but also relevant material types. As determined by two-way ANOVA, a greater proportion of the differential kill observed in this study was attributable to choice of biocidal aerosol treatment rather than surface material. To enable direct comparison of three test chlorine containing biocides, they were free chlorine matched before aerosol delivery. Under these conditions, the order of antimicrobial efficacy of biocidal aerosol treatment was ECAS > chlorine dioxide > sodium hypochlorite. However, it should be noted that there were instances where no significant difference between biocidal aerosol treatments was observed. Overall, sodium hypochlorite had only low level activity when used to treat surface associated microorganisms compared to ECAS and chlorine dioxide, which both produced significant microbial reductions, indicating this would be an effective delivery mechanism for these biocide types. ECAS are generally referred to as hypochlorous acid, although they contain a variety of oxidants and free radicals known to possess antimicrobial properties in addition to free chlorine (25), and may explain the comparative efficacy observed in this study when free chlorine matched with chlorine dioxide. One of the main advantages of ECAS and chlorine dioxide is that

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355 they can be generated on site, although chlorine dioxide traditionally requires manual mixing of
356 'sachets' whereas ECAS generation can be automated where an electrochemical cell, power and
357 salt is provided.

358 In general, when comparing the sensitivity of all target strains tested within this study the
359 order of resistance (irrespective of biocide type) was *B. subtilis* spores >MSSA (type strain) >
360 MRSA (clinical strain) >*P. aeruginosa* (type strain). It is therefore evident that there was only
361 limited activity of all three biocidal aerosols against spores and well below the 4 log fold
362 reduction seen as the threshold of surface active biocidal action, although the European Standard
363 quantitative non-porous surface test from which this value was derived was not developed to
364 assess the sporicidal efficacy of biocides (31). Nonetheless, all biocides elicited small significant
365 reductions (<1 log reduction) with the exception of sodium hypochlorite against fabric associated
366 spores. Spores are known to be more resistant to biocidal treatment compared to vegetative cells
367 due to a diversity of physiological factors (34, 35). It is known that all three biocides are active
368 against spores (25, 36), therefore if biocidal aerosols were to be deployed for their
369 decontamination it is likely that an increased contact time or more potent formulation would be
370 required. For example, with regard to ECAS, the physicochemical parameters are dependent on
371 the operating parameters of the cell. Therefore, since the redox potential (ORP) of ECAS is
372 considered as the most important factor when predicting their antimicrobial efficacy (25), a
373 solution with > 1155 mV (that used within this study) could be used. Interestingly, another
374 biocidal aerosol (hydrogen peroxide) required repeated cycles to be sporicidal (14), and this may
375 also be required for chlorine containing biocidal aerosols.

376 In terms of microbiological decontamination, aerosolised biocides (typical droplet size >
377 1 µm) will fall out of air (according to the sedimentation rate) and be active on material surfaces.

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The efficacy of a given aerosolised biocide when used within a specific application will be dependent on the design of aerosolisation device and hence standardisation of output (fog rate and droplet size), as well as the volume and topography of the environment to be decontaminated. For example, the use of fans has (unsurprisingly) been shown to improve the effectiveness of aerosolised biocides, by increasing the distribution of active fog and reducing the time from production to surface contact (6, 7). Aerosol technology facilitates continuous delivery of biocidal solutions, enabling constant replenishment of active agent on material and has the potential to reach areas inaccessible to traditional 'liquid' cleaning. Aerosol delivery of biocides is not seen as an alternative to thorough and appropriate physical cleaning (12), but as an adjunct to improve the microbial quality of a physical environment. Within healthcare environments this could include integration into infection control programmes through periodic decontamination of clinical wards, intensive care units and/or operating theatres, since removal of surface associated microorganisms is thought to reduce infection rates of patients (3). Moreover, if biocide dosing pumps or an ECAS generator was coupled to fogging devices and left in situ this could occur through automated decontamination regimens similar to the systems in existence for delivery of hydrogen peroxide (37).

The experimental fogging system and assay protocol designed within this study has been shown capable of differentiating the comparative efficacy of multiple chlorine matched biocidal aerosols against a spectrum of target organisms on a range of test surface materials (compared to a standard control treatment), and would be appropriate for testing other biocidal aerosol treatments or material surfaces. As stated by one author, there is need for "an appropriate evaluation framework" to compare decontamination processes (38), and this assay could be utilised as part of standard laboratory (phase 1) testing. Evidently, further application specific

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401 investigations would be required in comparison to existing technology (e.g. hydrogen peroxide
402 vapour) before any biocidal aerosol treatment regimen could be integrated into standard
403 operating procedures, either within healthcare settings or for wider industrial use.

404

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509 **Tables**

510 **Table 1.** Recovery (% and log cfu) of viable methicillin sensitive (MSSA) and methicillin resistant *S.*
511 *aureus* (MRSA), *P. aeruginosa* and *B. subtilis* (spores) compared to initial microbial surface loading after
512 being dried onto various material test surfaces (n=3; values reported to 2 significant figures).

513

| | Stainless steel | | Plastic | | Fabric | |
|---|---|---|---|---|---|---|
| | [%] | [log cfu] | [%] | [log cfu] | [%] | [log cfu] |
| **MSSA** | 98 ± 6.2 | 7.5± 0.03 | 100 ± 8.6 | 7.5± 0.03 | 47 ± 4.6* | 7.2± 0.04* |
| **MRSA** | 78 ± 11 | 7.5± 0.06 | 81 ± 16 | 7.5± 0.09 | 56 ± 23* | 7.3± 0.18* |
| ***P. aeruginosa*** | 0.23 ± 0.04* | 5.0± 0.1* | 4.3 ± 1.1* | 6.3± 0.11* | 0.85 ± 0.27* | 5.6± 0.14* |
| ***B. subtilis*** | 100 ± 15 | 7.1± 0.06 | 100 ± 11 | 7.1± 0.05 | 20 ± 1.6* | 6.4± 0.03* |

514 *significant reduction ($p<0.05$) compared to initial microbial loading

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**Figure headings**

**Figure 1. (a)** Recovery of methicillin sensitive *S. aureus* (MSSA) from the surface of various material surfaces after being subjected to a 20 minute aerosolisation treatment regimen with either sterile water (white bars) or one of three chlorine containing biocides (sodium hypochlorite [dark grey bars]; chlorine dioxide [light grey bars]; electrochemically activated solution [ECAS; black bars]) prepared to a matched free chlorine concentration of 100 ppm (n=6 ± SD). **(b)** Comparative efficacy matrix of the four aerosolisation treatment regimens. A reported value indicates a significant difference ($p<0.05$) in the number of microbes recovered ($log_{10}$ cfu coupon$^{-1}$) when comparing the two treatment regimens (ns = not significant).

**Figure 2. (a)** Recovery of methicillin resistant *S. aureus* (MRSA) from the surface of various material surfaces after being subjected to a 20 minute aerosolisation treatment regimen with either sterile water (white bars) or one of three chlorine containing biocides (sodium hypochlorite [dark grey bars]; chlorine dioxide [light grey bars]; electrochemically activated solution [ECAS; black bars]) prepared to a matched free chlorine concentration of 100 ppm (n=6 ± SD). **(b)** Comparative efficacy matrix of the four aerosolisation treatment regimens. A reported value indicates a significant difference ($p<0.05$) in the number of microbes recovered ($log_{10}$ cfu coupon$^{-1}$) when comparing the two treatment regimens (ns = not significant).

**Figure 3. (a)** Recovery of *P. aeruginosa* from the surface of various material surfaces after being subjected to a 20 minute aerosolisation treatment regimen with either sterile water (white bars) or one of three chlorine containing biocides (sodium hypochlorite [dark grey bars]; chlorine dioxide [light grey bars]; electrochemically activated solution [ECAS; black bars]) prepared to a matched free chlorine concentration of 100 ppm (n=6 ± SD). **(b)** Comparative efficacy matrix of the four aerosolisation treatment regimens. A reported value indicates a significant difference ($p<0.05$) in the number of microbes recovered ($log_{10}$ cfu coupon$^{-1}$) when comparing the two treatment regimens (ns = not significant).

**Figure 4. (a)** Recovery of *B. subtilis* spores from the surface of various material surfaces after being subjected to a 20 minute aerosolisation treatment regimen with either sterile water (white bars) or one of three chlorine containing biocides (sodium hypochlorite [dark grey bars]; chlorine dioxide [light grey bars]; electrochemically activated solution [ECAS; black bars]) prepared to a matched free chlorine concentration of 100 ppm (n=6 ± SD). **(b)** Comparative efficacy matrix of the four aerosolisation treatment regimens. A reported value indicates a significant difference ($p<0.05$) in the number of microbes recovered ($log_{10}$ cfu coupon$^{-1}$) when comparing the two treatment regimens (ns = not significant).

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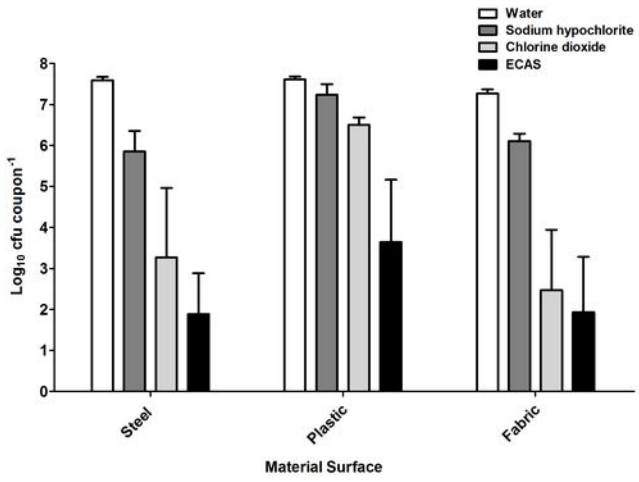

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| | Material Surface | Water | | |
|---|---|---|---|---|
| Sodium hypochlorite | Steel | 1.741 | | |
| | Plastic | ns | | |
| | Fabric | 1.161 | Sodium hypochlorite | |
| Chlorine dioxide | Steel | 4.322 | 2.582 | |
| | Plastic | 1.107 | ns | |
| | Fabric | 4.790 | 3.629 | Chlorine dioxide |
| ECAS | Steel | 5.704 | 3.963 | 1.381 |
| | Plastic | 3.968 | 3.597 | 2.862 |
| | Fabric | 5.338 | 4.177 | ns |

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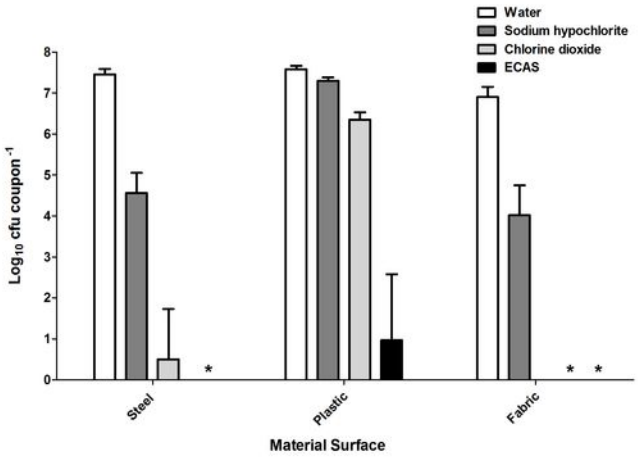

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| | Material Surface | Water | | |
|---|---|---|---|---|
| Sodium hypochlorite | Steel | 2.902 | | |
| | Plastic | ns | | |
| | Fabric | 2.887 | Sodium hypochlorite | |
| Chlorine dioxide | Steel | 6.957 | 4.055 | |
| | Plastic | 1.227 | 0.949 | |
| | Fabric | 6.903 | 4.017 | Chlorine dioxide |
| ECAS | Steel | 7.459 | 4.557 | ns |
| | Plastic | 6.604 | 6.326 | 5.377 |
| | Fabric | 6.903 | 4.017 | ns |

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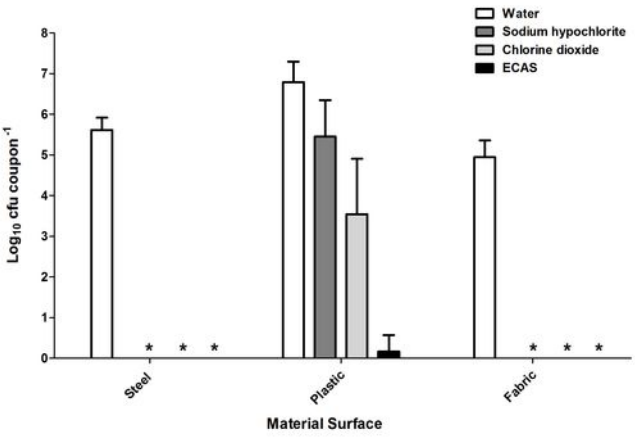

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| | Material Surface | Water | | |
|---|---|---|---|---|
| Sodium hypochlorite | Steel | 5.616 | | |
| | Plastic | 1.338 | | |
| | Fabric | 4.942 | Sodium hypochlorite | |
| Chlorine dioxide | Steel | 5.616 | ns | |
| | Plastic | 3.248 | 1.91 | |
| | Fabric | 4.942 | ns | Chlorine dioxide |
| ECAS | Steel | 5.616 | ns | ns |
| | Plastic | 6.622 | 5.284 | 3.374 |
| | Fabric | 4.942 | ns | ns |

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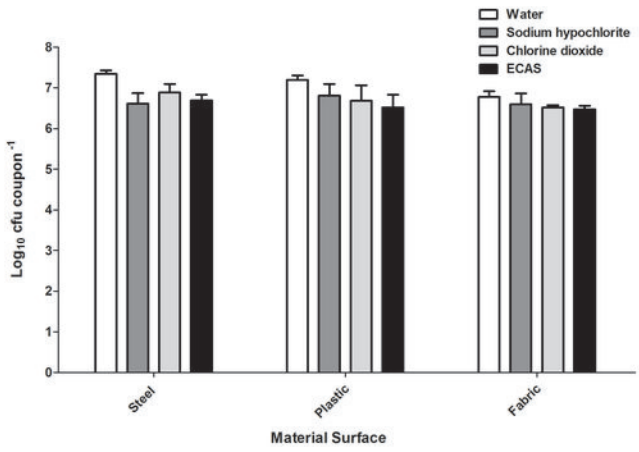

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| | Material Surface | Water | Sodium hypochlorite | Chlorine dioxide |
|---|---|---|---|---|
| **Sodium hypochlorite** | **Steel** | 0.725 | | |
| | **Plastic** | 0.380 | | |
| | **Fabric** | ns | | |
| **Chlorine dioxide** | **Steel** | 0.458 | ns | |
| | **Plastic** | 0.510 | ns | |
| | **Fabric** | 0.260 | ns | |
| **ECAS** | **Steel** | 0.652 | ns | ns |
| | **Plastic** | 0.668 | 0.289 | ns |
| | **Fabric** | 0.305 | ns | ns |