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Bacterial resistance to microbicides: Development of a predictive protocol

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Abstract

Regulations dealing with microbicides in Europe and the United States are evolving and now require data on the risk of resistance development in organisms targeted by microbicidal products. There is no standard protocol to assess the risk of resistance development to microbicidal formulations. This study aimed to validate the use of changes in microbicide and antibiotic susceptibility as initial markers for predicting microbicide resistance and cross-resistance to antibiotics. Three industrial isolates (*Pseudomonas aeruginosa, Burkholderia cepacia, Klebsiella pneumoniae*) and two *Salmonella enterica* serovar Typhimurium strains (SL1344 and 14028S) were exposed to a shampoo, a mouthwash, eye make-up remover and the microbicides contained within these formulations (chlorhexidine digluconate; CHG and benzalkonium chloride; BZC), under realistic, in-use conditions. Baseline and post- exposure data were compared. No significant increases in minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) were observed in any strain after exposure to the three formulations. Increases in the MIC and MBC of CHG and BZC of up to 100-fold were observed in SL1344 and 14028S but were unstable. Changes in antibiotic susceptibility were not clinically significant.

The use of MICs and MBCs combined with antibiotic susceptibility profiling and stability testing generated reproducible data that allowed for an initial prediction of microbicide resistance development. These approaches measure characteristics that are directly relevant to the concern over resistance and cross-resistance development following use of microbicides. These techniques are low cost and high-throughput, allowing manufacturers to provide data to support early assessment of risk of resistance development to regulatory bodies promptly and efficiently.

Keywords: microbicides, resistance, predictive protocol, regulation

INTRODUCTION

Microbicides have been extensively used in the control of bacteria for decades, and are commonly incorporated into a variety of products including disinfectants, cosmetics, preservatives, pesticides and antiseptics. Despite this ever-increasing use, bacteria generally remain susceptible to microbicides when they are used correctly. However, the indiscriminate use of microbicides in a wide range of environments has raised concerns about the selection of microbicide and antibiotic-resistant bacteria (1, 2). Despite the establishment of the European Union (EU) biocidal product regulation (BPR) (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:167:0001:0123:EN:PDF accessed 24th November 2014) to regulate the authorisation and use of biocidal products throughout the EU, the total amount of microbicide use in the EU remains unknown (2).

Of particular concern are formulations that contain microbicides at low concentrations which may increase the risk of selection for resistance amongst target or non-target microorganisms (2). Resistance or reduced susceptibility to microbicides and/or antibiotics as a result of exposure to low microbicide concentrations has been demonstrated extensively in the laboratory setting (3-7). Despite the lack of *in vivo* or *in situ* studies reporting a link between microbicide exposure and antibiotic resistance development, *in vitro* studies have clearly demonstrated the possibility of microbicide and antibiotic resistance development in bacteria. This has lead committees such as the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) to produce reports and opinions on the knowledge gaps and research concerns associated with resistance. In their 2010 opinion paper SCENIHR stated that data on microbicide usage are lacking together

with an understanding of the microbicides most at risk for the development of bacterial resistance (http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_028.pdf, accessed 24th November 2014). SCENIHR recommended the standardisation of methodologies used to monitor resistance levels and suggested the development of a standard protocol that could determine the risk of resistance development in a particular microorganism as a result of microbicide exposure.

In support of the requirement for such a protocol, the new BPR (EU 528/2012) states that it is a requirement of biocidal product manufacturers to provide information on the likelihood of resistance development to their product in target organisms. In particular the following articles state:

"(13) Active substances can, on basis of their intrinsic hazardous properties, be designated as candidates for substitution with other active substances, whenever such substances considered as efficient towards the targeted harmful organisms become available in sufficient variety to avoid the development of resistances amongst harmful organisms…"

"(25) … The use of low-risk biocidal products should not lead to a high risk of developing resistance in target organisms."

"(33) When biocidal products are being authorized, it is necessary to ensure that, when properly used for the purpose intended, they are sufficiently effective and have no unacceptable effect on the target organisms such as resistance...".

In addition, the U.S. Food and Drug Administration (FDA) has also issued a proposed rule to require manufacturers of antibacterial hand soaps and body washes to demonstrate that their products are safe for long-term daily use, more effective

than plain soap and water in preventing the spread of certain infections and do not select for resistance (http://www.gpo.gov/fdsys/pkg/FR-2013-12-17/pdf/2013-29814.pdf accessed 24th November 2014). A standard protocol that could determine the risk of resistance development would allow microbicidal product manufacturers to provide this information to the BPR and FDA promptly and efficiently.

Our work focuses on the development of such a protocol and has involved the assessment of several laboratory techniques that can be used to measure microbicide resistance (e.g. minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC) determination, antibiotic susceptibility testing, and phenotype stability testing) in terms of ease of use, high throughput, cost and reproducibility. Our recommended protocol encompasses MIC, MBC and antibiotic susceptibility determination combined with bacterial phenotype stability testing as initial markers of bacterial microbicide resistance or antibiotic cross-resistance. This study aims to validate the use of these techniques in a *combination* protocol with the testing of three commercially available formulations and the corresponding active microbicides contained therein.

MATERIALS AND METHODS

Bacterial strains. A range of Gram-negative bacteria was selected for the testing of three antimicrobial formulations and the corresponding microbicides contained within each formulation. The bacteria included *Salmonella enterica* serovar Typhimurium strains SL1344 and 14028S (obtained from the University of Birmingham, UK), *Burkholderia cepacia* (UL2P; Unilever culture collection, UK),

Klebsiella pneumoniae (UL13; Unilever culture collection, UK) and *Pseudomonas aeruginosa* (UL-7P; Unilever culture collection, UK). The 3 Unilever strains were selected as challenge organisms due to their routine use, propagation and handling in Unilever laboratories.

Culture and storage of bacteria. Liquid cultures of all strains were grown in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) at 37°C (± 1 °C). Strains were stored on protect beads (Fisher Scientific, Loughborough, UK) at -80 °C (± 1 °C) and restricted to a maximum of 2 subcultures from the original freezer stock prior to exposure to a given microbicide. Test inocula were prepared from harvesting an overnight TSB culture centrifuged at 5000 g for 10 min and re-suspended in deionised water (diH_20).

Formulations, actives and neutraliser. A mouthwash (2 mg/mL chlorhexidine digluconate; CHG), eye make-up remover (1 mg/mL CHG) and a shampoo (5 mg/mL benzalkonium chloride; BZC) were tested. Selection of these products was based on the fact that they are commonly used home and personal care products. The microbicides CHG and BZC (Sigma-Aldrich, Dorset, UK), the only microbicides contained within the three formulations, were also tested. The neutraliser used was composed of Tween 80 (30 g/L) and Asolectin (3 g/L) (both Sigma-Aldrich, Dorset, UK). Neutraliser efficacy for mouthwash, shampoo and eye make-up remover, and toxicity towards all strains was determined as described previously (3).

Antimicrobial susceptibility testing

Suspension testing: Test strains were exposed to each formulation and each microbicide at a concentration resulting in a 1-3 $\log_{10}$ reduction in CFU/mL, leaving sufficient survivors for further antimicrobial susceptibility testing. Suspension tests were carried out following the British Standard EN 1276 2009 protocol (8). Briefly, bacterial suspensions in deionised water (diH20) produced from overnight cultures were standardised to 1 x 10^8 CFU/mL. Suspensions were used within 15 minutes of preparation. One mL of standardised suspension was added to 9 mL of the desired formulation or active (diluted in diH20) at 1.25 times the required concentration. Concentrations tested were as follows: 0.000125 mg/mL mouthwash/CHG, 0.00015 mg/mL shampoo/BZC and 1 mg/mL eye make-up remover/CHG. After exposure for 1 min (the estimated length of time spent using each formulation by the consumer), 1 mL of this suspension was removed and added to 9 mL of neutraliser. After neutralisation, suspensions were centrifuged at 5000 g for 10 min and the supernatant discarded. The remaining cells were then used in further antimicrobial susceptibility testing experiments. *S. enterica* strains SL1344 and 14028S were also exposed to low BZC and CHG concentrations ranging from 0.0001– 0.004 mg/mL for 5 min.

Determination of the minimum inhibitory concentration (MIC). The MIC of each formulation/microbicide was determined for all strains before and after suspension test exposure to a given formulation/active, following the BS EN ISO: 20776-1 (9) protocol. Briefly, a 96 well microtitre plate (Sterilin Ltd, Newport, UK) containing doubling dilutions of a given formulation/active in TSB was set up. Concentration ranges were as follows: Mouthwash/CHG 2 – 0.001 mg/mL, shampoo/BZC 1.25– 0.001 mg/mL, eye make-up remover/CHG 0.5 – 0.00048 mg/mL, CHG/BZC

(*Salmonella* strains only) 40 – 0.019 mg/mL. An overnight broth culture of each strain was standardised to 1 x 10^8 CFU/mL and 50 µL volumes of this were added to the microtitre plate. The plate was incubated for 24 h at 37°C. The MIC was defined as the lowest concentration of a formulation/microbicide at which no bacterial growth was observed visually on the microtitre plate. (Approximate cost to test one microbicide and one bacterium in triplicate: < 1€).

Determination of the minimum bactericidal concentration (MBC). The MBC of each formulation/microbicide was also determined before and after suspension test exposure of each strain to a given formulation/active. Twenty µL of suspension was removed from each well of the MIC microtitre plate where no bacterial growth was observed and the two lowest formulation/active concentrations at which growth was observed, and added to 180 µL of neutraliser. Twenty-five µl of this suspension was then spotted on to tryptone soya agar (TSA) and incubated at 37°C for 24 h. The minimum bactericidal concentration was defined as the lowest formulation/active concentration where no bacterial growth was observed on the agar plate. (Approximate cost to test one microbicide and one bacterium in triplicate: < 1 €).

Antibiotic susceptibility testing. The susceptibility of each strain to one or more of the following antibiotics was determined before and after suspension test exposure to a given formulation/microbicide following the British Society for Antimicrobial Chemotherapy (BSAC) disk diffusion protocol (10): chloramphenicol (50 µg), ampicillin (10 µg), ciprofloxacin (1 µg), ceftriaxone (30 µg), piperacillin (30 µg), ceftazidime (30 µg), imipenem (10 µg), meropenem (15 µg), tobramycin (10 µg),

aztreonam (30 µg) (all from Oxoid, Baskingstoke, UK). These antibiotics were selected due to their use as therapeutic agents in the treatment of infection with the organisms chosen for this study. There are no available BSAC susceptibility breakpoints for *Burkholderia* spp., so breakpoints for *Pseudomonas* spp. were used instead in the case of strain UL2P (*B. cepacia*). (Approximate cost to evaluate susceptibility of 1 strain to 6 antibiotics: < 2 €)

Phenotype stability testing. The stability of any alterations in antimicrobial susceptibility observed after 5 min exposure of *S. enterica* strains SL1344 and 14028S to a range of low CHG and BZC concentrations was investigated via the 24 h subculture of surviving organisms through TSB +/- a low concentration of CHG or BZC as described previously (3).

Data reproducibility. In order to determine the reproducibility of baseline and post-exposure data obtained, the above experiments were performed on 3 separate occasions (each using 3 biological replicates) over a 6 month period, resulting in data values being a mean of 9 results.

Statistical analysis. A Students t-test was used to compare MIC, MBC and antibiotic zone of inhibition sizes before and after microbicide exposure.

RESULTS

Three formulations and their corresponding microbicides were tested on three separate occasions over a 6 month period in order to determine if exposure to a given microbicidal product or microbicide resulted in an alteration in microbicide or

antibiotic susceptibility in test organisms. Data obtained on each occasion were compared in order to determine the reproducibility of the MIC, MBC and antibiotic susceptibility tests, and therefore validate the use of these tests as a high throughput and low cost initial approach in the determination of the risk of resistance development. The mean MIC and MBC for each test organism before and after exposure to mouthwash, eye make-up remover or shampoo and their corresponding microbicides (CHG, CHG, BZC) at the same concentration as that contained within the product are presented in FIG.1. Exposure to one of three formulations or their corresponding microbicides resulted in both increases and decreases in MIC and MBC in individual strains. In the case of shampoo and eye make-up remover an accurate MBC could not be determined as all 5 strains grew in the highest testable concentration of the formulation. The greatest increases in MBC were observed in *S. enterica* strain 14028S after exposure to 0.005 mg/mL CHG and mouthwash, and 0.015 mg/mL BZC, all of which were found to be significantly different from baseline MBC values. However when considering the post-exposure MBC values observed (0.08, 0.05 and 0.05 mg/mL respectively) it is clear that these values are still below or equal to the concentrations of CHG and BZC present in the relevant formulations when considered as a worst case scenario of product dilution by the consumer. 'Worst case' dilution factors of 1 in 40 (mouthwash) and 1 in 100 (shampoo) were estimated based on product use, e.g. rinsing with water. This would result in 0.05 mg/mL CHG in mouthwash and 0.05 mg/mL BZC in shampoo. An MBC of 0.50 mg/mL for BZC is also of less concern as the primary function of BZC in the shampoo is not as an antimicrobial, but as a surfactant. Very few of the remaining observed changes in MIC or MBC were found to be statistically

significant ($p \leq 0.05$), nor did they approach the microbicide concentrations found in the formulations tested after 'worst case' product dilution by the consumer.

An important factor in the validation of the use of MIC and MBC determination in an initial assessment of the risk of resistance development was the reproducibility of the data obtained. It is clear from FIG. 1 that both the baseline and post-exposure mean MIC and MBC values were highly reproducible across the 3 separate experiments, as indicated by the small standard deviations observed for each strain and formulation/pure active. Our protocol is based on performing MIC/MBC in two fold dilutions. Standard deviations were calculated based on the MIC or MBC values, which means an increase or decrease in MIC or MBC by one fold dilution will result in a large standard deviation. Error bars (representing SD) on the graphs displayed in FIG. 1 may only indicate an increase or decrease of one doubling dilution.

There was no clinical change in susceptibility to any of the antibiotics tested after 1 min exposure to all 3 formulations and their corresponding microbicides, in the case of all 5 strains (according to BSAC susceptibility breakpoints for Enterobacteriaceae/*Pseudomonas* spp. (10) (data not shown). In the case of some strains and antibiotics, statistically significant changes in the zone of inhibition size were observed. However these differences were often due to an increase in the mean zone of inhibition size and therefore an increase in antibiotic susceptibility [e.g. ciprofloxacin, chloramphenicol, ceftazidime in *K. pneumoniae* after exposure to mouthwash (0.050 mg/mL CHG) or ceftazidime in *P. aeruginosa* after exposure to shampoo (0.015 mg/mL BZC)]. A statistically significant reduction in the mean zone of inhibition size for aztreonam was observed in *P. aeruginosa* after exposure to

0.005 mg/mL CHG, 0.015 mg/mL BZC and 1 mg/mL CHG. However *P. aeruginosa* was already resistant to this antibiotic prior to microbicide exposure and therefore no clinical susceptibility change was observed. It was not possible to clearly determine if clinical changes in susceptibility were observed in *B. cepacia*, as there were no available breakpoints provided in the BSAC protocol, and clinical susceptibility was therefore based on *Pseudomonas* spp.

Carrying out this experiment on 3 separate occasions over a 6-month period also allowed for an assessment of the reproducibility of the results obtained. The BSAC method produces consistent and reproducible baseline and post-exposure data (data not shown).

S. enterica strains SL1344 and 14028S were also exposed to a range of low concentrations of CHG and BZC for 5 min before the antimicrobial susceptibility of surviving organisms was determined. Tables one and two show the baseline and post exposure values for SL1344 and 14028S respectively after 5 min exposure to a range of low CHG and BZC concentrations.

In the case of both strains post-exposure MIC and MBC values for CHG and BZC were all significantly different from baseline MIC and MBC values ($p \leq 0.05$). For strain SL1344 the greatest increases in MIC and MBC were observed after 5 min exposure to 0.004 mg/mL CHG and 0.004 mg/mL BZC (Table 1). For strain 14028S exposure to 0.001 mg/mL CHG and 0.004 mg/mL BZC resulted in the greatest increases in MIC and MBC in surviving organisms (Table 2). The data appear highly reproducible across all 9 repeats in the case of both strains, as indicated by the low standard deviation values, supporting our recommendation of the use of MIC and MBC determination as an initial indicator of resistance development in bacteria. (As

discussed for FIG. 1, occasions where standard deviations appear larger are due to the use of doubling dilutions of a given microbicide/formulation during MIC/MBC testing). Susceptibility to a range of antibiotics was also determined for strains SL1344 and 14028S before and after exposure to low CHG and BZC concentrations. No alterations in antibiotic susceptibility were observed (data not shown).

The stability of the increases in MBC observed after 5 min exposure of SL1344 and 14028S to a range of low CHG and BZC concentrations was investigated via the 24 h subculture of surviving organisms through TSB +/- a low concentration of CHG or BZC. Table 3 and 4 show the mean MBC values after 1, 5 and 10 subcultures of surviving organisms through TSB +/- CHG or BZC for SL1344 and 14028S respectively. The high MBC values observed after the initial 5 min exposure to CHG or BZC were lost after 1 subculture in the absence of CHG or BZC. In the presence of a low CHG or BZC concentration, MBC values also returned to baseline levels after 10 subcultures. This was thought to be due to cumulative damage to the cell or the fact that maintaining a high MBC was detrimental to cell survival. The instability of the increased MBC values suggested a low risk of stable resistance development to CHG or BZC in either *S. enterica* strain at the concentrations tested. The values obtained from the phenotype stability tests were reproducible between repeats (as indicated by the low standard deviation values in Tables 3 and 4) and the data therefore supports our recommendation of the use this technique as part of a protocol to predict microbicide resistance development.

DISCUSSION

The principle aim of this work is to design a protocol that can predict bacterial microbicide resistance and antibiotic cross-resistance and give an indication of the risk of resistance development. The purpose of this study was to validate the use of MIC, MBC and antibiotic susceptibility determination before and after microbicide exposure, and phenotype stability testing for use in the initial prediction of bacterial microbicide resistance.

The use of existing standard protocols for MIC, MBC and antibiotic susceptibility measurement (i.e. EN 1276, ISO 20776-1, BSAC disk diffusion method) helps to avoid data variability which has been observed previously with MIC values obtained using different methodologies. Schurmaans *et al.* (11) found that MIC values could vary by a factor of up to eight if small alterations were made to the method used. Phenotypic variability was avoided through the use of overnight broth cultures for susceptibility testing, rather than selecting single colonies from an agar plate, which has been demonstrated to result in phenotypic variability in *Burkholderia cepacia* (12), illustrating the importance of consistent inoculum preparation when performing susceptibility tests. In the work carried out here the inoculum was re-suspended in diH20 instead of tryptone sodium chloride (TSC) buffer as TSC has been seen to interfere with log reduction results due to carry over from the inoculum (unpublished data). However the inoculum was used within 15 min of preparation in diH_20 to avoid subjecting bacterial cells to osmotic stress.

The MIC, MBC and antibiotic susceptibility values for mouthwash, shampoo, eye make-up remover, CHG and BZC were found to be reproducible between separate experiments at the concentrations tested in all 5 test strains, confirming the appropriateness of using these standard protocols. We concluded that there is a very low risk of resistance development to the formulations and corresponding pure

337 actives tested, even in the case of the elevated MICs and MBCs observed in strains
338 SL1344 and 14028S as these values were not stable in the absence or presence of
339 CHG or BZC.
340 The use of MIC and MBC in resistance prediction and making a comparison
341 between baseline and post-exposure susceptibility data is supported by our previous
342 work investigating the effect of cationic microbicide exposure on *B. lata* strain 383
343 (3). Our protocol allows the testing of any isolate of interest as data are always
344 compared for the individual isolate rather than general data for the given bacterial
345 species.
346
347 One of the criticisms of *in vitro* techniques used in microbicide resistance
348 measurement is that experimental parameters such as microbicide concentration,
349 exposure time, dilution on application and bioavailability are not reflective of in-use
350 conditions (1, 13). In our work we attempted to accurately reflect product use in
351 terms of exposure time and product concentration (i.e. any dilution of the product as
352 a result of its use). For the purpose of protocol development test concentrations used
353 were considerably lower than those found in the original formulations (i.e.
354 concentrations low enough to obtain surviving organisms), but should be kept
355 realistic when using the techniques recommended here to predict and assess the risk
356 of resistance development. Both formulations and the corresponding active
357 microbicides were tested during protocol development in order to validate the
358 different techniques used, but it must be emphasised that using such a protocol to
359 predict resistance to pure actives alone may be of less relevance than testing the
360 formulation as a whole, as multiple components of a formulation often contribute to
361 the overall microbicidal effect, or could prove antagonistic in the formulation.

Although better representative of microbicide use, long-term (≥ 6 months) studies investigating the effect of exposure to commonly used household microbicides on antimicrobial susceptibility, have failed to demonstrate resistance development in isolated bacteria (14-17). These studies are also costly and do not allow for a prompt response to regulatory bodies. This suggests that in light of new regulatory expectations a compromise may be required, allowing the rapid generation of data and preliminary assessment of risk, using *in vitro* techniques based on existing standard methods whilst controlling parameters such as microbicide formulation, contact time and concentration in order to bring realism to the evaluation. The protocol proposed in this study aims to achieve this.

A further recommendation of Maillard *et al.* (1) and SCENIHR (2) in the event of the observation of a reproducible change in microbicide susceptibility is the execution of further tests to understand the nature of the change. This could include molecular techniques to investigate changes to the transcriptome and proteome as a result of microbicide exposure. Genotypic alterations as a result of microbicide exposure and their potential as resistance markers have been investigated by numerous groups (18-20), and molecular techniques such as PCR and microarray technology have been successfully used to define microbicide resistance mechanisms. Although useful, molecular techniques can be complex, costly and time consuming and we therefore do not recommend them as a core part of this predictive protocol. Taking this in to account, FIG. 2 shows the proposed protocol steps in the form of a decision tree, as well as potential steps in the event of observed, reproducible resistance. A stable increase in MIC or MBC or change in antibiotic susceptibility could result in risk of resistance development. It must be

emphasised that the exact level of risk can only be determined through further assessment. For example, a stable increase in MBC may not constitute a high level of risk if this new MBC does not approach the concentration of a particular microbicide intended for use (FIG. 2). Some microbicides have a long history of use, and there is a large amount of literature studying their efficacy and any observed bacterial resistance, e.g. chlorhexidine, triclosan, benzalkonium chloride. For these microbicides there may be sufficient evidence available in the literature to support a weight of evidence assessment of the risk of resistance development, before considering the generation of new data on resistance (21, 22).

Our findings and proposed approach for assessment of risk can be applicable to the wider use of microbicides in various settings where such compounds are applied. This approach is preventative and aimed at being predictive, thereby ensuring that microbicide-containing formulations are safe by design with regards to resistance and cross-resistance risks, either by enabling omission of an ingredient identified by the protocol as undesirable or by using the improved understanding of resistance and cross-resistance mechanisms to design a formulation with an ingredient preventing the expression of a microbicide-relevant resistance mechanism (e.g. efflux pump inhibitors). Such a strategy has already been investigated and documented to decrease bacterial resistance to antibiotics (23).

With regulatory bodies such as the US FDA and EU BPR requiring information on the propensity of microbicidal products to select for resistant bacteria, it is imperative that relevant, cost-effective, high throughput techniques are available in order for product manufacturers to provide this information. As global harmonisation

of protocols used to measure changes in microbicide susceptibility is now considered a key requirement in moving microbicidal research forward (1,2), we recommend, and here demonstrate, the efficacy of a protocol that allows the prediction of resistance development using simple, low cost and high throughput techniques.

Conflict of Interest

This project conducted by Cardiff University was sponsored by Unilever Safety & Environmental Assurance Centre that provided a PhD studentship to L Knapp.

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TABLE 1: Mean baseline and post-exposure MIC and MBC values for strain SL1344 after 5 min exposure to a range of low CHG and BZC concentrations. N=9

	Biocide concentration (mg/mL) ± SD							
MIC/MBC (mg/mL)	**Baseline**	**0.004 CHG**	**0.001 CHG**	**0.0005 CHG**	**0.0001 CHG**	**0.004 BZC**	**0.001 BZC**	**0.0001 BZC**
CHG MIC	0.03 ± 0.03	0.80 ± 0.00	0.80 ± 0.00	0.40 ± 0.00	0.80 ± 0.00	0.50 ± 2.00	0.40 ± 0.00	0.80 ± 0.00
CHG MBC	0.10 ± 0.06	2.00 ± 0.90	2.00 ± 0.00	0.40 ± 0.00	1.00 ± 0.40	3.00 ± 0.00	2.00 ± 0.00	2.00 ± 1.00
BZC MIC	0.03 ± 0.00	2.00 ± 0.00	0.30 ± 0.20	0.10 ± 0.00	0.70 ± 1.00	3.00 ± 1.00	0.80 ± 0.00	0.70 ± 1.00
BZC MBC	0.03 ± 0.03	2.00 ± 0.00	0.50 ± 0.20	2.00 ± 2.00	1.30 ± 2.00	8.00 ± 0.00	2.00 ± 0.00	3.00 ± 2.00

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522 TABLE 2: Mean baseline and post-exposure MIC and MBC values for strain 14028S after 5 min exposure to a range of low CHG and BZC concentrations. N=9

	Biocide concentration (mg/mL) ± SD				
MIC/MBC (mg/mL ± SD)	**Baseline**	**0.005 CHG**	**0.001 CHG**	**0.015 BZC**	**0.004 BZC**
CHG MIC	0.030 ± 0.03	0.10 ± 0.00	1.00 ± 0.00	0.40 ± 0.00	0.80 ± 0.00
CHG MBC	0.06 ± 0.03	1.00 ± 0.90	20.00 ± 0.00	50.00 ± 0.00	3.00 ± 0.00
BZC MIC	0.04 ± 0.03	0.80 ± 0.00	0.10 ± 0.00	0.80 ± 0.00	2.00 ± 0.00
BZC MBC	0.08 ± 0.02	1.00 ± 0.00	2.00 ± 0.60	1.00 ± 0.00	20.00 ± 0.90

523 TABLE 3: Mean baseline and post-exposure MBC values for strain SL1344 after 1, 5 and 10 subcultures in TSB +/- 0.004 mg/mL CHG or BZC.

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525 SC = subculture * = significantly different from baseline ($p \leq 0.05$)

	Baseline MBC (mg/mL)	5 min CHG 0.004	1 SC	5 SC	10 SC	1 SC (CHG)	5 SC (CHG)	10 SC (CHG)
CHG MBC (mg/mL ± SD)	0.10 ± 0.90	5.00 ± 0.00*	0.08 ± 0.00	0.09 ± 0.00	0.06 ± 0.00	0.15 ± 0.40	0.10 ± 0.40	0.10 ± 0.00
BZC MBC (mg/mL ± SD)	0.03 ± 0.00	1.50 ± 0.00*	0.04 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.19 ± 0.00*	0.50 ± 0.20*	0.06 ± 0.00
	Baseline MBC (mg/mL)	**5 min BZC 0.004**	**1 SC**	**5 SC**	**10 SC**	**1 SC (BZC)**	**5 SC (BZC)**	**10 SC (BZC)**
CHG MBC (mg/mL ± SD)	0.10 ± 0.90	5.00 ± 0.00*	0.20 ± 0.30	0.10 ± 0.00	0.10 ± 0.00	0.80 ± 0.40*	0.80 ± 0.40*	0.10 ± 0.00
BZC MBC (mg/mL ± SD)	0.03 ± 0.00	3.00* ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.78 ± 0.00*	0.60 ± 0.20*	0.03 ± 0.00

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TABLE 4: Mean baseline and post-exposure MBC values for strain 14028S after 1, 5 and 10 subcultures in TSB +/- 0.004 mg/mL CHG or BZC.

SC = subculture * = significantly different from baseline ($p \leq 0.05$)

	Baseline MBC (mg/mL)	5 min CHG 0.001	1 SC	5 SC	10 SC	1 SC (CHG)	5 SC (CHG)	10 SC (CHG)
CHG MBC (mg/mL ± SD)	0.06 ± 0.03	5.00 ± 0.00*	0.01 ± 0.00	0.06 ± 0.00	0.09 ± 0.00	0.80 ± 0.40*	0.80 ± 0.40*	0.06 ± 0.00
BZC MBC (mg/mL ± SD)	0.08 ± 0.02	3.00 ± 0.00*	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.19 ± 0.00*	0.20 ± 0.00*	0.06 ± 0.00
	Baseline MBC (mg/mL)	**5 min BZC 0.004**	**1 SC**	**5 SC**	**10 SC**	**1 SC (BZC)**	**5 SC (BZC)**	**10 SC (BZC)**
CHG MBC (mg/mL ± SD)	0.06 ± 0.03	5.00 ± 0.00*	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.40 ± 0.20*	0.70 ± 0.70*	0.06 ± 0.00
BZC MBC (mg/mL ± SD)	0.08 ± 0.02	3.00 ± 0.00*	0.07 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.19 ± 0.00*	0.20 ± 0.00*	0.06 ± 0.00

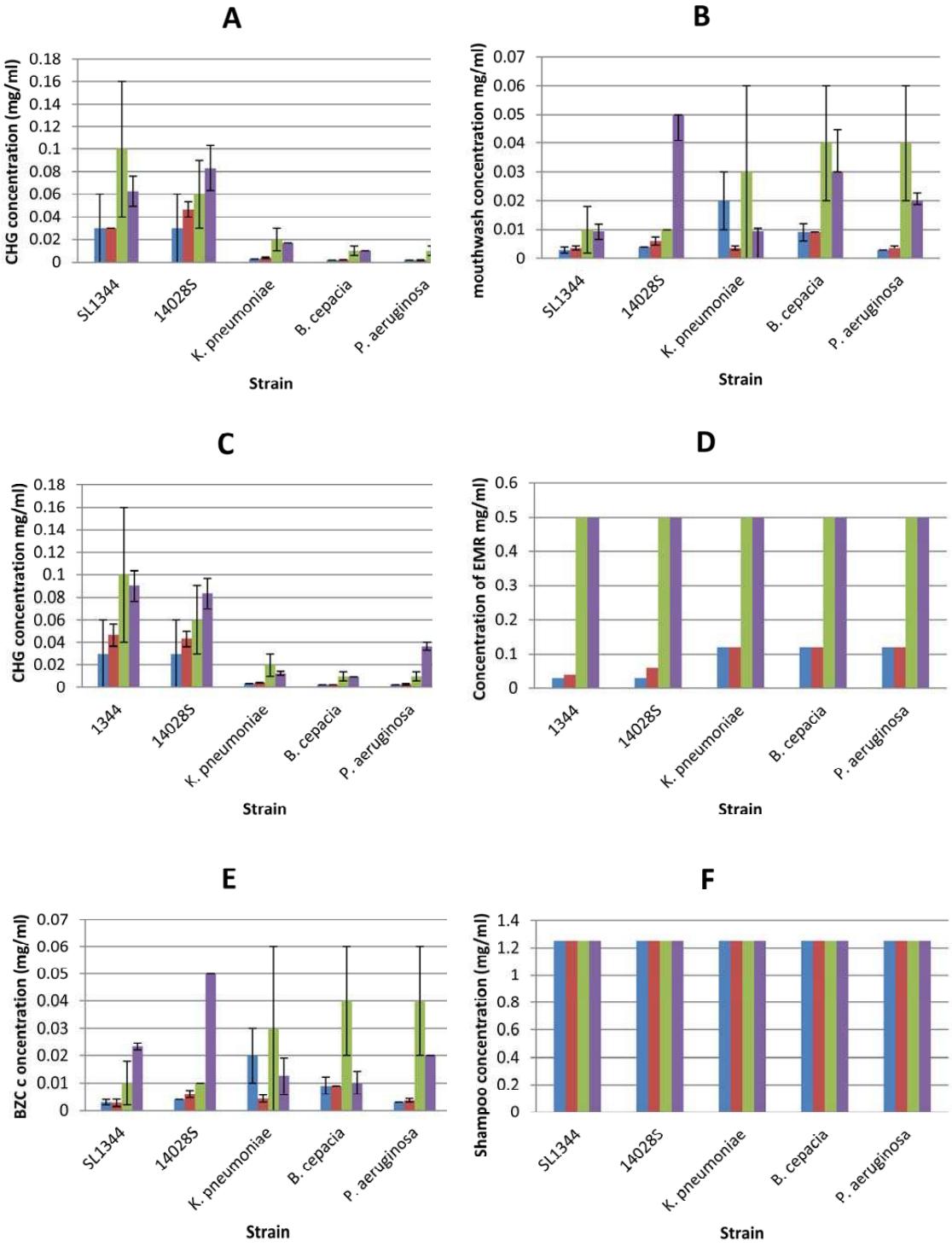
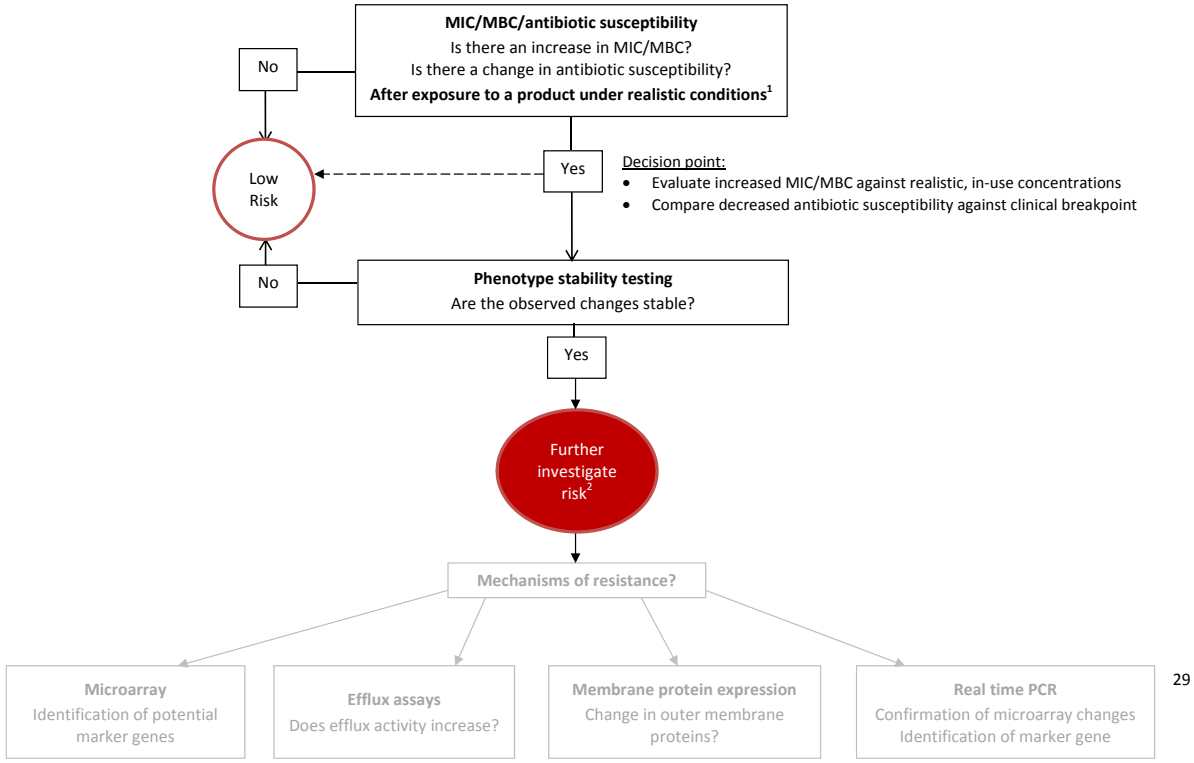


FIG 1: MIC and MBC values for 5 test organisms re and after exposure to 3 formulations and their corresponding pure actives. N=9. Blue = baseline MIC. Red = post-exposure MIC. Green = baseline MBC. Purple = post-exposure MBC. Error bars correspond to the SD. MIC and MBC were performed in two fold dilution (see text for detailed information). A) 0.005 mg/ml CHG; B) mouthwash (0.005 mg/mL CHG); C) 1 mg/mL CHG; D) Eye-maker remover (neat: 1 mg/mL CHG); E) 0.015 mg/mL BZC; F) Shampoo (0.015

Figure 3: Proposed protocol for use in the prediction of bacterial microbicide resistance. Grey boxes are examples of further work that could be carried out to investigate mechanisms behind changes in antimicrobial susceptibility.



Footnotes for figure 3

[1] Realistic conditions refers to those under which the product will be used. Factors such as concentration, contact time and product formulation should be considered in order to represent product use as accurately as possible.

[2] If reproducible and phenotypically stable changes in antimicrobial susceptibility are observed after exposure to a particular product under realistic, in-use conditions, further investigation into the risk can be carried out. This may involve the elucidation of possible mechanisms behind susceptibility changes such as the examples shown in the grey boxes in figure 3, leading to better understanding of the level of risk. This investigation could be extended beyond the examples given in figure 3, and could include the exploration of additional resistance markers and the use of additional techniques.