Assessment of resistance towards biocides following the attachment of micro-organisms to, and growth on, surfaces

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Aims: To develop a rapid method for the assessment of biocidal activity directed towards intact biofilms.

Methods and Results: Escherichia coli and Staphylococcus epidermidis were cultured for up to 48 h within 96-well microtitre plates. The planktonic phase was removed and the wells rinsed. Residual biofilms were exposed to various concentrations of chloroxylenol, peracetic acid, polyhexamethylene biguanide (PHMB), cetrimide or phenoxyethanol for 1 h. At 15-min intervals, biocide was removed, and the wells washed in neutraliser and filled with volumes of fresh medium. Re-growth of the cultures was monitored during incubation at 35°C in the plate reader. Times taken for the treated wells to re-grow to fixed endpoints were determined and related to numbers of surviving cells. Time–survival curves were constructed and the survival of the attached bacteria, following exposure to the agents for 30 min, interpolated for each biocide concentration. Log–log plots of these survival data and biocide concentration were constructed, and linear regression analysis performed in order to (i) calculate concentration exponents and (ii) compare the effectiveness of the biocides between variously aged biofilm and planktonic cells. From such analyses iso-effective concentrations of biocide (95% kill in 30 min) were calculated and expressed as planktonic : biofilm indices (PBI).

Conclusions: PBI varied between 1Æ02 and 0Æ02, were relatively unaffected by age of the biofilms but differed significantly between organism and biocide. Notably those compounds with the higher activity against planktonic bacteria (PHMB and peracetic acid) were most prone to a biofilm effect but remained the most effective of the agents selected.

Significance and Impact of the Study: The endpoint method proved robust, enabled the bactericidal effects of the biocides to be assessed against in-situ biofilms, and was suitable for routine screening applications.

INTRODUCTION

Microbial biofilms are notorious for their high level of resistance towards biocidal treatments (Brown and Gilbert 1993; McBain and Gilbert 2001). The mechanisms associated with such resistance are complex. These mechanisms involve not only the reaction-diffusion limitation of biocide-access to the underlying cells (Stewart 1994; Stewart et al. 1994; Huang et al. 1995), but also the expression of spatially heterogenous, less susceptible phenotypes, caused either by growth as a biofilm per se (Brown et al. 1988; Gilbert et al. 1990a) or through the expression of high cell density (Davies et al. 1998), or starvation, phenotypes (Foley et al. 1999). Whilst there have been many publications that support the assertion of biofilm recalcitrance, there have been few studies made which generate kinetic data that is capable of systematic evaluation of the relative extent of this phenomenon across a range of chemical biocides. This largely

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reflects the lack of bactericidal methodologies that are suitable for screening purposes. Das et al. (1998) reported a microtitre plate methodology that enabled the growth inhibitory effects of a range of biocides to be simultaneously monitored for both planktonic and attached bacteria. Such methods showed that susceptibility changed markedly within minutes of cellular attachment to a surface and that the extent of change was dependent upon the nature of the biocidal agent. A vast majority of the reported methodologies for estimating the hygienic disinfection of surfaces, particularly those associated with attached biofilms, rely on the physical removal and separation of the attached bacteria, post-treatment (Gilbert et al. 1998; Wirtanen et al. 1998). This necessarily stresses the sublethally injured microorganisms further and only considers those surviving microorganisms that have been successfully removed from the surface. Such procedures will inevitably lead to overestimation of disinfection. A number of direct observation methodologies have been described that employ vital staining (Wood et al. 1998), ATP-levels (Kinniment and Wimpenny 1992; Jakubovics and Dow 1997), or reporter genes, such as Lux, for cellular vitality (Walker et al. 1992; Duffy et al. 1995), but these are labour intensive, generally require that particular reporter constructs are available or are variable in their outcome. Another option is to submerge the test pieces, post-treatment, and to measure the recovery of the surviving cells either by turbidometric (Lambert and van der Ouderaa 1999; Lambert et al. 1999) or conductimetric (Connolly et al. 1994; Johnston and Jones 1995) methods. In the present study we have developed a turbidity endpoint method that is suitable for the generation of kinetic data describing the disinfection of biofilms. This has enabled comparisons of activity/resistance kinetics to be made for a wide range of different biocidal agents directed against variously aged biofilms.

**METHODS**

**Organisms and culture**

Smooth, clinical isolates of *Escherichia coli* (Dyson) and *Staphylococcus epidermidis NCTC11047* were used throughout. Cultures were maintained at room temperature on nutrient agar slants in a darkened cupboard and replaced at monthly intervals.

Organisms were routinely cultured in chemically defined liquid media that caused entry into stationary phase through depletion of carbon substrate (glycerol) with all other nutrients being present at a five-times excess (Al-Hiti and Gilbert 1983; Duguid et al. 1992). Overnight cultures (16 h) were obtained in 100-ml volumes of media held within 250-ml Erlenmeyer flasks shaken at 100 osc. min⁻¹ at 35°C. Cell suspensions were prepared by centrifugation (13 000 g, 10 min), washing and re-suspension to an appropriate optical density in sterile saline (0.9% w/v).

**Chemicals and biocides**

Phenoxyethanol and cetrimide USP (tetradecyltrimethyl ammonium bromide) were obtained from Sigma Chemicals (Poole, UK). Chloroxylenol (2-chloro-4,5-dimethyl phenol, 99%) was obtained from Aldrich Chemicals (Gillingham, UK). Peracetic acid (Proxatane 4002) was obtained from Solvay Interox Ltd., (Warrington, UK). Polyhexamethylene biguanide (Vantocil IB) was obtained from ICI Organics Division, Speciality Chemicals (Blackley, UK). All other reagents and chemicals were of the purest available grade and were purchased from BDH (Poole, UK) or Sigma Chemicals (Poole, UK).

**Plate counts**

Viable counts were made by spreading 0.2 ml aliquots of appropriate decimal dilutions, made in sterile saline (0.9% w/v), onto the surfaces of triplicate, predried nutrient agar (Oxoid, Basingstoke, UK) plates. These were incubated at 35°C for 24 h and the colonies counted.

**Turbidity endpoint determination of viable count**

Serial dilutions of mid-log and stationary phase cultures were prepared in simple salts media (1–1 × 10⁸ cfu ml⁻¹) and viable counts made using plate counts (above). Aliquots (0.1 ml) were placed, in quadruplicate, into the wells of 96-chamber, polystyrene microtitre plates. These were placed within an incubated (35°C, moderate shaking) microtitre plate reader (Anthos-Hill HTL.III., Labtech Instruments) and turbidities (492 nm) monitored at 30-min intervals over 24 h. Initial absorbance readings for the filled plates were subtracted from the subsequent data set, and the turbidity (mean of four wells) plotted as a logarithmic function of time. The times taken for each set of wells to achieve specified optical density values were found to relate linearly (r² 0.985–0.997) to the plate count of the original suspension. Whilst significant differences were noted in the slopes of these relationships between the two organisms, no significant difference was found either between log- and early stationary-phase inocula, or in the curve-fits (t-statistic 1.67, P = 0.95, n = 40) determined for various turbidity endpoints within the range 0.01–0.03.

**Sub-lethal injury of micro-organisms**

Survivors of sublethal biocide treatments are likely to be metabolically injured and might therefore have recovery times in liquid growth media that are different to healthy...
cells (Gilbert 1984). Calibration curves were therefore re-determined using cell suspensions that had been pre-treated with various biocides before dilution into appropriate neutraliser solutions (below). Whilst pretreatments using exposure to the biocides that generated 90–95% killing of the test populations gave 1–2 h increases in the lag phases of the surviving fraction, there were no losses in linearity between turbidity-endpoints of viability and conventional plate counts. Indeed there was no significant difference between treatments with the different biocides, and the regression lines obtained for healthy and sublethally damaged cells (Fig. 1) were not significantly different from one another ($P = 0.95$). Separate calibration graphs relating turbidity–endpoint time and viable count were therefore unnecessary for healthy and biocide-treated suspensions.

**Bactericidal activity determinations**

Experiments were conducted to determine the effects of biocide concentration, exposure time and culture age upon the susceptibility of biofilm bacteria towards the five agents. 96-well microtitre plates were inoculated ($0.1 \text{ ml well}^{-1}$) with overnight (16 h) simple-salts broth cultures that had been diluted $1:100$ with fresh sterile medium. These were incubated within a static incubator at $35^\circ C$ for 16 h, 24 h or 48 h. The planktonic contents were carefully removed by pipette and replaced with volumes ($0.15 \text{ ml}$) of an appropriate solution of biocide. Sixteen wells were designated for each biocide treatment or control. At 15-min intervals, over a 1-h period, biocide solution was removed by pipette from quadruplicate wells and replaced with neutraliser solution ($0.2 \text{ ml}$). Volumes ($0.05 \text{ ml}$) of the removed samples were transferred to tubes containing neutraliser solution ($4.95 \text{ ml}$). After 15 min the neutraliser solution was removed from the sampled wells which were subsequently washed twice with volumes ($0.2 \text{ ml}$) of saline. The wells were finally filled with fresh growth medium and the plates incubated within the microtitre plate reader. Growth was monitored and the turbidity endpoints calculated (count A). Samples of the saline washes, removed neutraliser solution and neutralized biocide solution were bulked and subjected to conventional viable counting as colony-forming units (count B). Estimates of the effectiveness of the killing process were made by adding the total number of cfu remaining in each well after treatment, neutralization and washing (count A) to count B associated with that well and representing viable cells removed from the biofilm, during and post-treatment. Results were expressed as percentage values of the cfu associated with control wells subjected to saline rather than biocide treatment.

**Neutralization of biocides**

The antimicrobial effects of chloroxylenol and phenoxyethanol were arrested by dilution ($> 1:100$) whilst those of polyhexamethylene biguanide and cetrimide were arrested by the addition of, or transfer to, Soybean Lecithin (1% w/v)/Tween 80 (2% w/v) broth. The antimicrobial properties of peracetic acid were neutralized by the addition of or dilution into sodium thiosulphate (2% w/v).

**RESULTS AND DISCUSSION**

Preliminary experiments utilizing inoculated microtitre plates (Fig. 1) established linear relationships between inoculum size ($10^{-7} \text{ cfu ml}^{-1}$) and the times taken for individual wells to achieve defined turbidity endpoints within the range $0.1–0.3$ optical density units within 1–24 h. Within these limits no significant differences were noted between logarithmic phase inocula and those taken from 16-h-old stationary phase cultures. Separate calibration curves were required for each organism [$\log \text{ cfu} = 6.75–0.329 \text{ h}, E. coli, r^2 = 0.997, n = 16$; $\log \text{ cfu} = 6.35–0.213 \text{ h}, Staph. epidermidis, r^2 = 0.985, n = 16$] but these gave good estimates of the viable count determined by the plate method. It was our intention to use the approach to measure the survival of biofilm populations following exposure to
sublethal biocide treatments. It was probable that the treated cells would be sublethally injured and in this respect grow more slowly or exhibit extended lag-phases relative to healthy populations. In either instance the effect would be for the turbidity endpoint determinations of viability to be underestimates. In order to accommodate for this, cell suspensions, prepared from broth cultures, were sublethally treated (90–99% kill) with each of the five biocides and the numbers of survivors estimated by the turbidity endpoint method and by plate counts. Results, illustrated in Fig. 1 for _Staph. epidermidis_, indicate a high degree of covariance between the two data sets, irrespective of the nature of the biocide. The relationships obtained for sublethally treated and untreated control suspensions were not significantly different (95% confidence level). Additionally, 24-h-old biofilms were established within 96-well microtitre plates and exposed to various concentrations of the five agents for 30 min. After sequential replacement of the biocide with neutraliser, rinse solution and fresh medium then the numbers of survivors was assessed for each either as a turbidity endpoint for the residual attached biofilm or as a plate counts made on the residual biofilm population after re-suspension. Re-suspended biofilms were prepared by vigorous, and repeated, filling and discharge of an automated pipette (0.1 ml saline) into the rinsed wells. Viable count data generated on successive re-suspensions of a single well indicated that a single re-suspension treatment recovered greater than 99% of cells (data not shown), paired Students _t_-tests indicated that the two methods gave comparable results. Regression equations generated using microtitre plate, turbidity-endpoint determinations of viability for the combined data for each organism were therefore used to directly estimate the survival of biofilm exposed to the various biocide treatments.

Biofilms of _E. coli_ and _Staph. epidermidis_ were established for 16 h, 24 h and 48 h within microtitre plates prior to exposure to various concentrations of the biocides. The survival data obtained at each time interval represented the net killing of the treated biofilm, since the number of surviving cells removed from the surfaces, during treatment and subsequent rinsing (count B, above), was added to the estimate of the residual attached population determined by turbidity endpoint (count A, above). Time–survival-curves, typical data for which are illustrated in Fig. 2, were constructed for each biocide over 1 h exposure periods. The data were interpolated to give estimates of the survival of the attached bacteria at 30 min and the extent of killing then expressed as logarithmic functions of biocide concentration (Figs 3 and 4). Log–Log plots of bactericidal activity such as these (Hugo and Denyer 1987) have slopes that reflect the concentration exponent (dilution coefficient) of the biocides (Table 1). In the majority of cases, regression equations fitted to these data were not significantly different between the variously aged biofilms or between biofilm and planktonic cells. With the exception of the two cationic biocides, PHMB and cetrimide, concentration exponents reflected those values found in the literature (Table 1). Concentration exponents for these agents were significantly higher than the previously published values (Broxton _et al._ 1983; Gilbert and Al-Taee 1985). This probably reflects the mode of growth and preparation of the inocula which differs substantially between the current and previous studies. Concentration exponents generated for the cationic biocides were also significantly different for biofilm and planktonic

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Fig. 2. Time survival data generated for variously aged (▲, 16 h; ■, 24 h; ●, 48 h) biofilms of (a) _Staphylococcus epidermidis_ and (b) _Escherichia coli_ exposed to polyhexamethylene biguanide (PHMB, 0.015 μmol l⁻¹) and Chlorxylenol (3 mmol l⁻¹), respectively.
populations of \( E. \) coli. Differences in iso-effective concentrations of biocide between planktonic and biofilm bacteria, determined from Figs 3 and 4, were greatest for the most active agents and decreased with decreasing activity (\( \text{PHMB} > \text{peracetic acid} > \text{cetrimide}, \text{chooroxylenol and phenoxyethanol} \)). For the majority of biocide : bacteria combinations the data presented a continuum of susceptibility with little significant difference in susceptibility or concentration exponent with respect to the mode or duration of growth. For this reason, and for clarity of presentation, the data in Figs 3 and 4 do not indicate the age of the biofilm cultures, rather all of the biofilm-related data points have been collated. Concentration exponents have been suggested to reflect, not only the mechanism of action of biocidal agents (Hugo and Denyer 1987), but also the ease of displacement of materials (i.e., cations and lipopolysaccharides) from the cell envelope (Cowles 1939). Chemically reactive biocides (i.e., thiol interactive, oxidizing) tend to have concentration exponents that approximate the chemical laws of mass action (concentration exponents of 1–2, a doubling in concentration gives a doubling in activity). Membrane active antimicrobial agents, particularly those which perturb membrane potential, are much more concentration dependent reflecting a multiplicity of interactive sites and mechanism (concentration exponents \( c \approx 6–20 \)). Concentration exponents quantify the extent of reduction in antimicrobial activity of biocidal agents on dilution. On dilution, activity is reduced by the dilution factor raised to the power of the concentration exponent. Lack of change in concentration exponent, generally observed in the present study, suggests that for these biocides and bacteria, the critical lethal actions associated with cell death remain unchanged and that differences in susceptibility reflect access. The significant differences noted in the concentration exponents for both of the cationic biocides against the Gram–negative test organisms suggests that there have been fundamental changes in the physiology of these organisms, affecting the critical lethal site(s), associated with maturation as a biofilm. These changes most probably relate to cation-exchange reactions between the biocides and the extracellular polymeric matrix of the biofilm and the cell envelopes. PHMB and peracetic acid were the most active of the chosen biocides towards planktonic cells and demonstrated the greatest attenuation of activity when used against biofilm populations. The concentration exponent of peracetic acid, a strong oxidizing biocide, was unaffected by such changes. The large percentage change in iso-effective concentration in this instance is a reflection of the low concentrations of peracetic acid required for biocidal action and the relatively high reaction-capacity of the glycolalox. Earlier work, with \( Staphylococcus aureus \), has also concluded that major adaptation in physiology was unlikely to account for the increased resistance of attached cells to peracetic acid (Johnston and Jones 1995). PHMB, on the other hand, has an activity which depends upon the relative abundance of acidic phospholipids within the cytoplasmic membrane (Broxton et al. 1984), and which accesses the cell envelope through self-promotion (Hancock 1981) requiring the displacement of divalent cations from the lipopolysaccharide (Gilbert et al. 1990b). Previously, Das et al. (1998) had shown that the growth inhibitory activity of PHMB, more than any other of the biocides tested was subject to a rapid and significant attenuation of action following attachment of the cells to a surface. This agent showed a marked difference in concentration exponent, suggesting major changes in the primary killing mechanism (i.e., acidic membrane lipids) or
in the number and disposition of cation binding sites associated with biofilm and planktonic bacteria.

Iso-effective concentrations (95% killing, 30 min) towards the planktonic and biofilm populations were calculated from the combined data for each biocide and expressed as planktonic : biofilm indices (PBI). These values are tabulated in Table 2 and show the PBI to vary between 1 and 0.2, indicating a 50-fold increase in susceptibility of biofilm grown cells, and 0.02, indicating a 50× decrease in susceptibility. The indices differed significantly between organism and biocide type. It is notable that whilst the high activity biocides (PHMB, peracetic acid) possessed the greatest PBI and biocide type. It is notable that whilst the high activity biocides (PHMB, peracetic acid) possessed the greatest PBI they also remained amongst the most effective of the agents against biofilm cells.

The endpoint method itself proved robust, and enabled the bactericidal effects of the biocides to be assessed against intact biofilms. We believe that the approach might be useful for routine screening applications.

**REFERENCES**


