

One explanation for the variability of the bacterial suspension test

M.D. Johnston, E.-A. Simons and R.J.W. Lambert

Microbiology, Unilever Research Colworth, Sharnbrook, Bedford, UK

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M.D. JOHNSTON, E.-A. SIMONS AND R.J.W. LAMBERT. 2000. Disinfection kinetic studies of sodium dodecyl sulphate, benzalkonium chloride and sodium hypochlorite against *Staphylococcus aureus* revealed that when a higher inoculum level of *Staph. aureus* than normal was used (approximately 1 log higher), the efficacy of disinfection was severely attenuated. Kinetic analysis using the Hom model for experiments carried out on tests using 3×10^8 organisms ml^{-1} were unable to account for the large increase in disinfection power observed when smaller inoculum levels were used. Since the inoculum was the same in every way except for the numbers used, the large variations in the log reduction/time curves could not be explained by a variation in the resistance of the population to the biocide, as identical log reduction–time curves should have resulted. The level of disinfection achieved for a given concentration of biocide was found to be approximately linearly related to the cell number ml^{-1} of test solution and not to the log number. The variation observed is believed to occur due to intrinsic self-quenching of the biocide by the microbes during the course of the disinfection test. As the level of free biocide decreases, the rate of reaction decreases, giving the tails of the log reduction/time curves. Such intrinsic self-quenching could explain the large variations known to occur in the legally required disinfection suspension tests.

INTRODUCTION

Kronig and Paul (1897) were the first to lay the foundation for a kinetic approach to chemical disinfection. They stated that the rules of chemical kinetics could be applied to disinfection. This later became known as the mechanistic approach (Lee and Gilbert 1918). Kronig and Paul (1897) were also the first to plot logarithms of the surviving organisms against time, which they found gave an approximately linear response. Ten years after their discovery, Madsen and Nyman (1907) further developed the theme of linear log survival/time but during these studies, they encountered departures from true linearity. They put forward the theory that this was due essentially to variability of resistance among cells in a population. This was later to become known as the Vitalist hypothesis. Chick (1908), Watson (1908) and later, Phelps (1911) provided mathematical models correlating the concentration of bactericide to the rate of disinfection of the

test organisms, suggesting an analogy between velocity of microbial disinfection and a unimolecular or first order chemical reaction that has remained the model for subsequent investigations (e.g. Bean and Das 1966; Benarde *et al.* 1967). However, departures from the Chick–Watson law are not uncommon, as inactivation kinetics are dependant on so many variables (Wickramanayake and Sproul 1991).

Departures from linear disinfection kinetics are manifest as ‘lags’ and/or ‘tails’ on graphs of log reduction against time. The legislative tests used to ascertain the efficacy of a biocide, such as the European Suspension Test (EST), do not truly examine the rate of reaction (Anon. 1974; Anon. 1996). The test is concerned with whether a given level of biocide can give a specified log reduction in microbial numbers in a specified time (normally five logs in five minutes). If a biocide exhibited non-linear kinetics, the EST test would fail to detect this.

Crowshaw (1981) reviewed disinfectant testing and concluded that although current tests provide useful data, the results were not reproducible. In the Rideal–Walker test (a phenol coefficient test (Anon. 1934)), a reproducibility of

Correspondence to: Dr Ronnie Lambert, Microbiology, Unilever Research Colworth, Sharnbrook, Bedford MK44 1LQ, UK (e-mail: ronnie.lambert@unilever.com).

$\pm 30\%$ was quoted as being no worse than any other form of testing. Laboratory technique, test organism, type of disinfectant, culture methods and temperature variation all have a role to play in the non-reproducibility. This paper provides evidence for a cause of variability within the suspension test.

MATERIALS AND METHODS

Preparation of bacterial suspensions

Staphylococcus aureus ATCC 6538 was grown overnight at 30 °C, with shaking, in a flask containing 80 ml Tryptone Soya Broth, TSB (Oxoid). The culture was centrifuged at 4000 rev min⁻¹ (510 g, Sigma model 3K-1) for 10 min. The resulting cell pellets were pooled and resuspended in 9 ml 0.1% peptone water to give a cell number of approximately 3×10^{10} , confirmed by Thoma count. Where specific cell numbers were required, cells were diluted to the appropriate number in 0.1% peptone water.

Lysis of up to 10^8 *Staph. aureus* cells

Cells (0.1 ml; maximum level 1×10^{10} ml⁻¹) were added to 10 ml 0.08% SDS at pH 4, and left for 2 h to achieve complete lysis. Viability was checked on the Bioscreen and no survivors were observed. Microscopy also confirmed that the cells had lysed. These disinfectants containing lysed cells were then used in a normal disinfection suspension test.

Preparation of test disinfectants

The following compounds were made up in sterile distilled water at appropriate concentrations (w/v) prior to use: sodium dodecyl sulphate, SDS (Fisons), pH adjusted to 4 using HCl and NaOH as appropriate; benzalkonium chloride, BzK (Aldrich); sodium hypochlorite, NaOCl (Ellis and Everard).

Suspension tests

The method has been described elsewhere (Lambert *et al.* 1998). The term log reductions, logR, used in this paper refers to the log reductions obtained by the Bioscreen methodology.

Monitoring of SDS using the surfactant probe

A surfactant electrode (Orion) was placed into 40 ml disinfectant alongside a reference electrode (Ag/AgCl). While mixing the solution on a magnetic stirrer, 400 µl of a bacterial suspension were added and readings (in millivolts, mV) were monitored over 36 min. The readings were converted to percentage SDS using a previously constructed calibration curve.

RESULTS

SDS disinfection kinetics

The disinfection kinetics of SDS, at pH 4, are markedly non-linear (Fig. 1). It was known that at pH values above 4.5, there was a marked decrease in the biocidal activity of SDS. However, pH monitoring of the solution showed it to be constant throughout. The best fit to Hom's empirical non-linear time model (Hom 1972) is given in Equation 1 for the disinfection of a 3×10^8 ml⁻¹ inoculum:

$$\log R = 10^{2.54}[\text{SDS}]^{1.67}t^{0.25}, r^2 = 0.89, \text{rmse} = 0.031$$

(26 observations) Eqn 1.

However, when the experiment was repeated with an inoculum size of 1×10^7 ml⁻¹, using the same culture as the previous experiment and using the same levels of disinfectant, no survivors were found after 3 min. To obtain survivors over the full 36 min period of disinfection, 0.01% SDS was required, giving 5 log reductions in 36 min. According to Eqn 1, such a level of disinfectant should only have achieved 0.4 log R in that time.

Variation of biocide concentration during disinfection

A surfactant-sensitive electrode was used to examine changes in the bulk concentration of SDS during a disinfection experiment with *Staph. aureus*. A plot of the log reduction data and the measured value of the free level of SDS in solution during

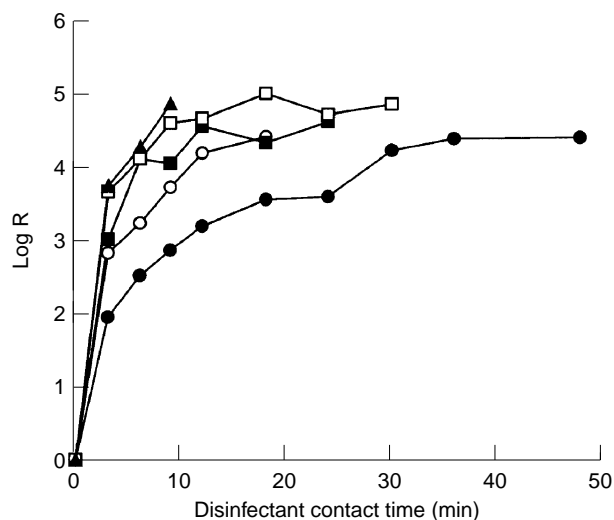


Fig. 1 Disinfection kinetics of SDS on 3×10^8 ml⁻¹ *Staphylococcus aureus*. (●), 0.07% SDS; (○), 0.08% SDS; (■), 0.085% SDS; (□), 0.09% SDS; (▲), 0.095% SDS

the disinfection process suggests that as the level of available biocide decreases, the rate of disinfection falls (Fig. 2).

Dependence of microbial biomass

To study the effect of biomass on the disinfection reaction, a specific concentration of SDS (0.08%) was chosen and various amounts of live cells were added. On leaving for 2 h, complete cell lysis was obtained. A test inoculum of approximately $1 \times 10^8 \text{ ml}^{-1}$ live *Staph. aureus* was then disinfected with these solutions containing the one level of disinfectant and the various known levels of dead cell contents. The effect of this increased biomass on the disinfection rate was then followed. As the level of dead cell contents added to the disinfectant solution decreased, the rate of disinfection increased (Fig. 3). The increase in rate observed was approximately linearly related to the level of lysed cells added and not to their log number. A plot of log reductions achieved at given times against the amount of lysed cells added shows this effect more clearly (Fig. 4).

A range of inocula from $1 \times 10^7 \text{ ml}^{-1}$ to $5 \times 10^7 \text{ ml}^{-1}$ were disinfected with 0.03% SDS. The results (Fig. 5) show that inoculum size has a large effect on the outcome of the experiment. Greater than 5 log reductions were obtained in under 18 min at $1 \times 10^7 \text{ ml}^{-1}$, and less than 2 at $5 \times 10^7 \text{ ml}^{-1}$. An extrapolation of the curves to the abscissa suggests that if the test inoculum level was approximately $6 \times 10^7 \text{ ml}^{-1}$, then no disinfection (i.e. no log reductions) would be observed at this concentration of biocide. We believe that at this level of micro-organisms, there is not enough biocide present per cell to cause rupture. Interestingly, this can give rise to another

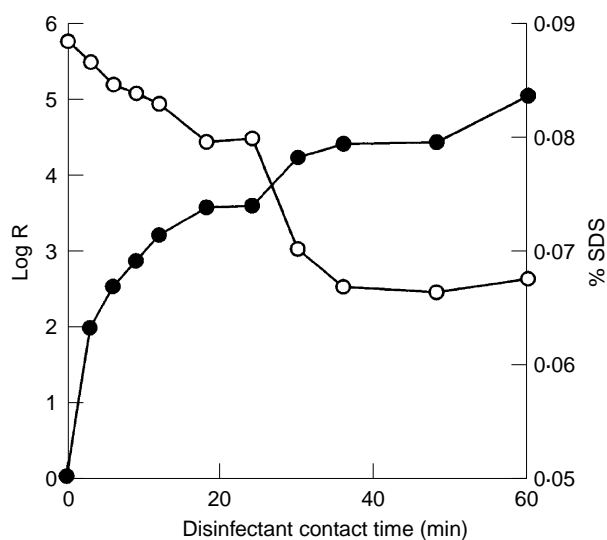


Fig. 2 Comparison of log R and the concentration of free SDS with respect to disinfection time. (●), log R; (○), SDS/%

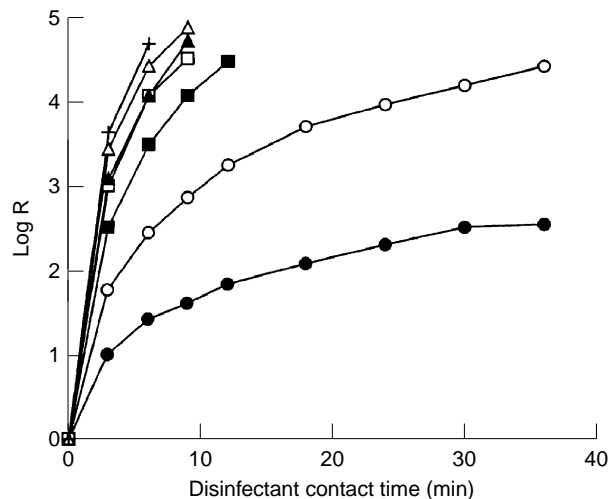


Fig. 3 Disinfection of $1 \times 10^8 \text{ ml}^{-1}$ *Staphylococcus aureus* with 0.08% SDS containing amounts of lysed cells (LC) ml^{-1} : (●), 1×10^8 LC; (○), 5×10^7 LC; (■), 2.5×10^7 LC; (□), 1.25×10^7 LC; (▲), 6.25×10^6 LC; (△), 3.13×10^6 LC; (+), 0 LC (control)

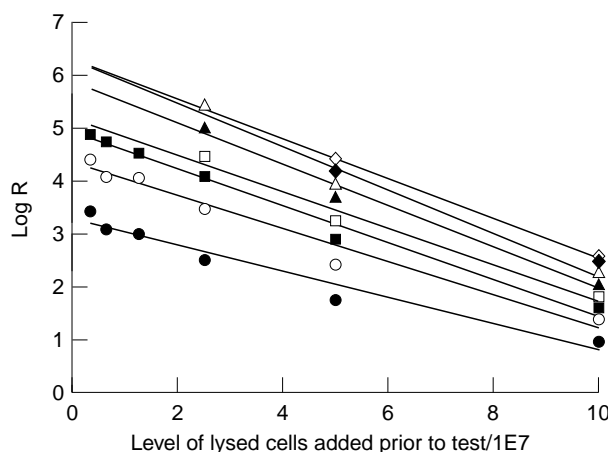


Fig. 4 Relationship between log R and the level of added biomass on the SDS (0.08%) disinfection of *Staphylococcus aureus* ($1 \times 10^8 \text{ ml}^{-1}$). Disinfection contact times: (●), 3 min; (○), 6 min; (■), 9 min; (□), 12 min; (▲), 18 min; (△), 24 min; (◆), 30 min; (◇), 36 min

measure of MIC; for a given level of biocide, what concentration of microbes fails to give any biocidal effect?

Benzalkonium chloride disinfection of *Staph. aureus*

Benzalkonium chloride exhibits non-linear disinfection kinetics. The best fit to a Hom analysis of a $3 \times 10^8 \text{ ml}^{-1}$ inoculum is given in Equation 2:

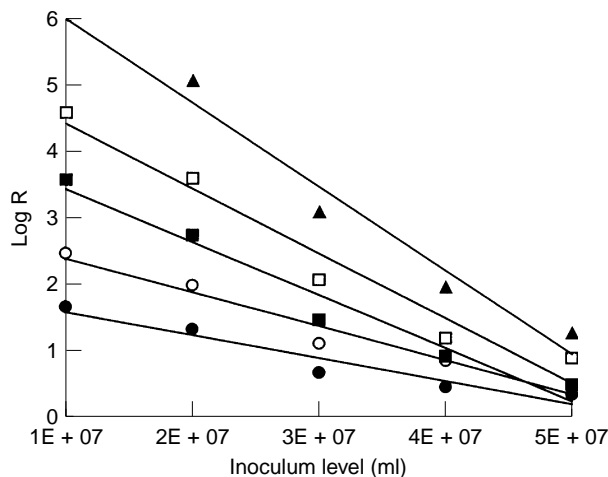


Fig. 5 The effect of inoculum size on the SDS (0.03%) disinfection of *Staphylococcus aureus*. Disinfection contact times: (●), 3 min; (○), 6 min; (■), 9 min; (□), 12 min; (▲), 18 min

$$\log R = 10^{4.28}[\text{BzK}]^{1.47}t^{0.39}, r^2 = 0.91, \text{rmse} = 0.071$$

(64 observations) Eqn 2.

Figure 6 gives the log reductions for the 0.0002% BzK disinfection of *Staph. aureus* for initial inoculum levels between $1 \times 10^7 \text{ ml}^{-1}$ and $6 \times 10^7 \text{ ml}^{-1}$ for different set disinfection times. From the Hom analysis of the disinfection of a $3 \times 10^8 \text{ ml}^{-1}$ culture, in 36 min, 0.0002% BzK would be expected to achieve only 0.28 log reductions.

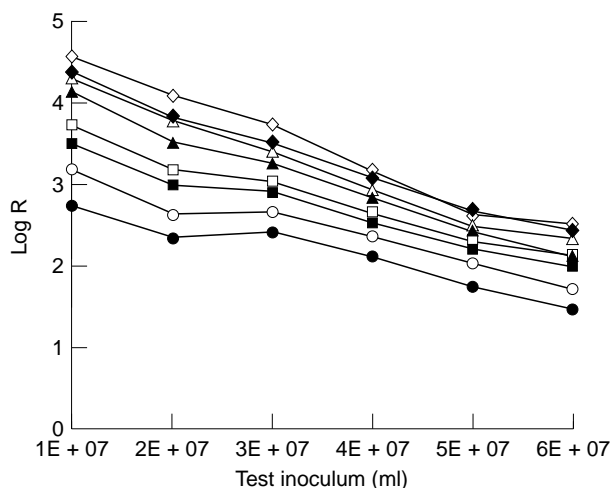


Fig. 6 The effect of inoculum size on the BzK (0.0002%) disinfection of *Staphylococcus aureus*. Disinfection contact times: (●), 3 min; (○), 6 min; (■), 9 min; (□), 12 min; (▲), 18 min; (△), 24 min; (◆), 30 min; (◇), 36 min

Sodium hypochlorite disinfection of *Staph. aureus*

The disinfection kinetics of sodium hypochlorite against $3 \times 10^8 \text{ Staph. aureus ml}^{-1}$ were examined and the best fit to Hom's empirical non-linear time model is given in Equation 3:

$$\log R = 10^{4.28}[\text{Hypo}]^{2.69}t^{0.76}, r^2 = 0.97, \text{rmse} = 0.071$$

(64 observations) Eqn 3.

The amount of non-linearity was not as pronounced as in the previous two cases and this was reflected in the higher time exponent. At an inoculum size of $1 \times 10^7 \text{ Staph. aureus ml}^{-1}$, 0.00125% of hypochlorite achieved approximately 4.5 log reductions in microbial numbers in 18 min. However, higher inocula loadings resulted in smaller log reductions over the same time period. Extrapolation of the data obtained suggested that at approximately $1 \times 10^8 \text{ ml}^{-1}$ of *Staph. aureus*, no disinfection would be expected with this level of hypochlorite. Inserting this concentration value into the Hom analysis, Equation 3 confirms this finding; over a 36 min period, a log R of only 0.14 is predicted.

DISCUSSION

The Chick–Watson model can be applied to linear log survivor/time data. Deviations from this law can be empirically examined using the Hom model. The deviations are, in general, considered to be due to a distribution of resistances with the microbial population, the so called log-normal distribution (Withell 1942a, 2b). In both the Hom and the Withell interpretations, time has been adjusted to fit the observations. In general, the concentration of the biocide is considered constant throughout an experiment; “the reagent in excess is always, of course, the disinfectant” (Lee and Gilbert 1918) and this assumption has become dogma. The concept of utilizing concentration–exposure time data (C.t) as a measure of disinfectant efficacy would not work if the test concentration was dependent on time (see Wickramanayake and Sproul 1991 for a discussion of the C.t concept). It can, however, be clearly shown in the case of the SDS disinfection of *Staph. aureus* that the level of biocide falls during the disinfection procedure itself. This would suggest that in any model examining disinfection rates, the time dependence of the reaction constant (which contains within it the disinfectant concentration dependency) must be taken into account, i.e. with respect to Chick–Watson:

$$-\frac{dN}{dt} = k(t)N$$

If the idea that a log-normal distribution of resistances exists within a given population is accepted, then when various inocula sizes are disinfected with a specific biocide concen-

tration, there should be no alteration of the log reduction/time plot. This is because the population would be expected to have the same distribution of resistance whether there were 1000 or 1×10^7 microbes ml^{-1} . Figure 5 clearly shows this is not the case; a small change in the inoculum size had a dramatic effect on the log R/time plots and this cannot be due to a distribution of resistances.

In this work, it is argued that the reduction in the level of biocide is due to the intrinsic presence of the microbes. We suggest here that the microbes themselves quench, in some way, the action of the biocide. This inactivation can take several forms but is, in general, a physical and/or chemical inactivation. The composition of the quenching agent used in disinfection examinations often includes emulsifying agents such as Tween and lecithin. Microbial membranes contain emulsifiers such as phosphatidyl ethanolamine and triacylglycerols. These constituents are essentially the same types of materials as used in a quenching agent. It is therefore reasonable to predict that if a biocide ruptures a microbe, the cell and membrane contents can quench out the biocide and thus, reduce the effective biocide concentration in solution and the rate of reaction.

This argument works well for surfactant-type biocides such as quaternary ammonium compounds or acid-anionics, but for hypochlorite, another self-quenching phenomenon must be found. For such highly reactive oxidizing agents, any material capable of oxidation will reduce the concentration of the hypochlorite. The material oxidized need not be from a living or viable cell — the hypochlorite obviously cannot distinguish the two. This intrinsic or self-quenching reaction will be dependent on the biomass present per millilitre of solution, i.e. any quenching reaction should be related to cell numbers. The evidence given here suggests that this appears to be the case.

The Hom model allows an estimation of the power of disinfection for a given concentration of disinfectant. The predictive models given were all obtained at a test inoculum size of $3 \times 10^8 \text{ ml}^{-1}$. It is obvious that these expressions are of little use when smaller test inoculum sizes are used. A truly predictive disinfection model must also include a term for the inoculum size dependency of the disinfection, a term completely missing from the current literature.

Implications for the microbial suspension tests

The implications for the legislative biocide suspension tests such as the EST are severe. If a biocide has a non-linear disinfection curve, self-quenching of some sort is assumed to be occurring. Self-quenching is dependent on the actual microbial numbers or biomass present in the test solution. The difference between 1×10^7 organisms ml^{-1} and 5×10^7 organisms ml^{-1} may appear slight on the log scale but will have a fivefold effect on any self-quenching phenomenon.

The legislative tests do not examine biocide rate data (time = 0 and 5 min constitutes a test not an examination). This means that any non-linearity will not be observed. Without this knowledge, large variations in the test results may occur simply by accepting the range of inocula stipulated by legislation without question.

We suggest here that much of the apparent variation observed between laboratories, and on separate days in a single laboratory, may be caused by variation in the inocula size. It is known that the methods of producing the inocula have an effect on the test results. This would be expected if the various methods produced small variations (on a log scale) of the initial starting inocula.

The European committee for standardization CEN TC 216 is currently producing a harmonized biocide test system for Europe. It is accepted in principle that on the implementation of the European Biocides Directive, the CEN tests will be used as the basis for registration. These 'harmonized' tests suffer from the same one-point-in-time testing problems as the current EST system. Therefore, biocides with non-linear kinetics will be most susceptible to the inadequacies of the test. Biocides based on surfactants are at most risk as these are more easily quenched out by the biomass present. The permissible range of test inoculum size for the Basic Bactericidal Test PREN 1040 (Anon. 1993) is between 1×10^7 and 5×10^7 microbes ml^{-1} . The large variations reported with the technique can be easily explained by the intrinsic self-quenching theory. Thus, an allowable difference in inoculum size between two separate tests could mean the difference between passing or failing the disinfectant.

A solution to the problem would be to severely restrict the level of inoculum to an agreed standard inoculum size, e.g. $3 \times 10^8 \text{ ml}^{-1}$ exactly. As the variation observed in the present study occurred over an inoculum range of 1×10^7 – $5 \times 10^7 \text{ ml}^{-1}$, the new proposed legislative range of 1×10^8 – $5 \times 10^8 \text{ ml}^{-1}$ is 50 times greater. A further remedial alteration to the suspension tests would be to carry out a complete kinetic examination over 30 min to obtain true-rate data. The disinfection parameters obtained from such an analysis could then be used for application purposes, as first suggested by Phelps (1911). The greatest barrier to this suggestion is the current method of analysis which, for such suspension tests, is too time-consuming and costly. If the Bioscreen method (Lambert *et al.* 1998) was adopted as a standard method, such tests would become commonplace.

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