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Analysis of biocide transport limitation in an artificial biofilm system

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P.S. STEWART, L. GRAB AND J.A. DIEMER. 1998. An alginate gel bead artificial biofilm system was used to assay biofilm susceptibility to four biocides and to analyse the extent to which each agent penetrated the biofilm. Chlorine, glutaraldehyde, an isothiazolone, and a quaternary ammonium compound were tested on alginate-entrapped *Enterobacter aerogenes* in gel beads ranging from 1·8 to 6 mm in diameter. Gel-entrapped bacteria were less susceptible to all four antimicrobial agents than were planktonic micro-organisms. The degree of kill measured in artificial biofilm gel beads depended on the size of the gel bead and the cell density at which it was loaded. Disinfection efficacy decreased as gel bead radius or cell density increased. The manifest dependence of biofilm disinfection efficacy on the physical properties of the artificial biofilm (radius and cell density) suggests the impingement of transport limitation of biocide transport into the biofilm. A previously developed theory of biocide reaction and diffusion in biofilm was tested by calculating an appropriate Thiele modulus. In accordance with the theory, the efficacy of all four biocides decreased, albeit noisily, as the Thiele modulus exceeded 1. This result demonstrates that transport limitation can impact antimicrobial performance against biofilms not only of oxidizing biocides but also of non-oxidizing agents.

INTRODUCTION

Biocides are widely used to control the detrimental formation of microbial biofilms, but their efficacy is always disappointing when compared with disinfection assays performed with planktonic micro-organisms (Costerton *et al.* 1987; Brown and Gilbert 1993). The mechanisms by which biofilms resist disinfection are not all elucidated, but over the past few years, progress has been made in describing and quantifying the extent to which incomplete biocide penetration impacts biocide performance (de Beer *et al.* 1994; Stewart and Raquepas 1995; Chen and Stewart 1996; Xu *et al.* 1996). Retarded delivery of biocide into the biofilm occurs when the biocide is neutralized by reaction with biofilm constituents faster than it diffuses into the biofilm. This simultaneous interaction between reaction and diffusion leads to persistent gradients in the biocide concentration within the biofilm. The surface of the biofilm is exposed to the antimicrobial agent, but the biocide may easily be reduced to ineffectual concentrations in the biofilm interior. Eventually, the biocide can penetrate the biofilm fully, but only after depleting the neutralizing capacity of the biofilm.

Such transport limitation of biocide influx into the biofilm has been convincingly demonstrated for free chlorine at neutral pH. The profoundly retarded penetration of chlorine has been experimentally determined using a chlorine-sensitive micro-electrode in natural (de Beer *et al.* 1994) and artificial (Chen and Stewart 1996; Xu *et al.* 1996) biofilm systems. The penetration time of free chlorine can be hundreds of times longer than for a similarly sized non-reactive solute. For example, applying parameter values measured by Chen and Stewart (1996), the time required to attain 1 mg l^{-1} of chlorine at the substratum beneath a 500 μm thick biofilm exposed to a bulk fluid concentration of 2 mg l^{-1} chlorine would be approximately 114 h for a biofilm with cell mass density of 20 000 mg l^{-1}. This is 9300 times longer than the calculated penetration time if there was no reaction between chlorine and biomass. The degree of transport limitation depends most significantly on the biofilm thickness, density of neu-

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tralizing sites in the biofilm, bulk fluid biocide concentration, and the reaction rate between biocide and neutralizing biomass.

Retarded transport is unquestionably one of the mechanisms rendering biofilms less susceptible to disinfection by chlorine. The rapid reaction of chlorine with biomass, particularly its nitrogenous components, drives this mechanism. Less reactive antimicrobial agents, such as non-oxidizing biocides, would be expected to be less prone to transport limitation in biofilms. The purpose of the work reported in this paper was to perform a preliminary assessment of the extent of transport limitation of three non-oxidizing biocides in an artificial biofilm system. In so doing, the general utility of alginate gel bead artificial biofilm as a flexible and convenient method of biocide screening was further demonstrated (Whitham and Gilbert 1993).

MATERIALS AND METHODS

Micro-organism and culture method

Enterobacter aerogenes ATCC 13048 was used in pure culture. This micro-organism was grown on Bacto Brain Heart Infusion (BHI) agar (Difco) plates at 37 °C for 24 h. Colonies were then harvested and resuspended in a 0·85% sodium chloride solution; suspensions were used within 1 h. The density of micro-organisms in the inoculum suspension was controlled by the number of colonies harvested and was roughly assayed by an optical density measurement.

Biocides

Four biocides were used: glutaraldehyde (1,5-pentanedial) (Union Carbide); an isothiazolone mixture (3:1, 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) (Rohm and Haas); alkyl (C_{14}, 50%; C_{12}, 40%; C_{16}, 10%) dimethyl benzyl ammonium chloride (ADBAC, Lonza); and chlorine (hypochlorous acid/hypochlorite, pH 7, Clorox bleach). Biocide molecular weights and estimated diffusion coefficients are given in Table 1.

Table 1 Diffusion coefficients of biocides in water at 25 °C calculated using the Wilke-Chang correlation (Perry and Chilton 1973)

Agent	(g mol^{-1}) MW	(cm^2 s^{-1}) $D_{aq} \times 10^6$
Chlorine	51·5	19·0
Glutaraldehyde	100·1	9·32
Isothiazolone	141·0	9·35
ADBAC	330	4·27

Alginate gel bead preparation

Alginic acid, sodium salt-low viscosity, was purchased from Sigma. A 4% (w/w) aqueous solution of alginate was prepared by suspending 4 g alginate in 100 g of sterile Millipore water. Complete dissolution of the alginate was effected by stirring this solution for 4–6 h. The micro-organisms were incorporated into the alginate matrix by mixing 10 g of the 4% alginate suspension with 10 g of a microbial inoculum (see above) yielding a final alginate concentration of 2% (w/w).

Alginate gel beads containing entrapped bacteria were prepared by dropping the alginate-bacteria mixture into stirred 50 mmol l^{-1} $CaCl_2$ as described elsewhere (Smidsrød and Skjåk-Bræk 1990). The alginate-micro-organism mixture was delivered from a distance of approximately 20 cm above the $CaCl_2$ using a sterile 10 ml syringe with a needle attached. The beads that formed were gently stirred for 30 min, filtered to remove the $CaCl_2$ solution, and stored in 5 mmol l^{-1} $CaCl_2$ at ambient temperatures until they were used. The diameter of the beads formed was controlled by the size of the needle attached to the syringe and by application of a coaxial air stream according to the method of Smidsrød and Skjåk-Bræk (1990). For example, 1·8 mm beads were formed using a 23 gauge needle, 3·5 mm beads were formed using a 16 gauge needle, and 6 mm beads were formed with 3·5 mm (i.d.) Tygon tubing attached to the syringe. The average bead diameter was measured by lining up 20 beads along the edge of a plastic ruler. The gel beads thus prepared were loaded with bacteria at concentrations ranging from about 10^7 to 10^{10} cfu ml^{-1}.

Planktonic disinfection experiments

Enterobacter aerogenes ATCC 13048 was grown out on BHI agar plates at 37 °C for 24 h. Bacteria were harvested by centrifugation and resuspended in 0·85% saline solution to prepare inocula containing between 10^8 and 10^{10} cfu ml^{-1}. A 1 ml sample of this inoculum was then added to a series of sterile test tubes at 25 °C containing 9 ml of 0·1 mol l^{-1} phosphate buffer (pH 7·5) and the desired concentration of biocide. After 1, 2, 3, 5 and 24 h contact periods under continuous stirring, surviving bacteria were enumerated by standard pour plating of serial dilutions on BHI agar. Residual biocide was removed by 100-fold dilution followed within 1 min by transfer of the diluted sample to a pour plate where the rich medium further diluted and neutralized biocide.

Gel bead disinfection experiments

A 1 g aliquot of the micro-organism-containing gel beads was added per 100 ml of sterile 5 mmol l^{-1} $CaCl_2$ at pH 7·5. The solution was stirred and the appropriate quantity of biocide

then added. After 1, 2, 3, 5 and 24 h of contact under continuous stirring, 10 beads were collected and dissolved in 10 ml of sterile 100 mmol l^{-1} sodium citrate solution with stirring. This represented dilution by a factor of between about 10 and 300, effectively reducing the biocide residual concentration. After the beads dissolved, the bacterial populations of the citrate solutions were enumerated by standard pour plating of serial dilutions on BHI agar. The appropriate controls were also run in which no biocide was added to the beads. Micro-organism densities in the beads were calculated by first determining the micro-organism counts per bead in the solution and dividing by the calculated volume of the spherical gel bead. Log reductions were calculated by comparing counts of the biocide-treated beads with the counts of the untreated beads.

Data analysis

Planktonic disinfection efficacy was quantified by fitting data to the equation

$$dX/dt = -k_{dis}BX$$

where k_{dis} = disinfection rate coefficient (l $mg^{-1} h^{-1}$), B = biocide concentration (mg l^{-1}), X = culturable cell density (cfu ml^{-1}), and t = time (h). Estimates of k_{dis} were computed using difference formulae in a spreadsheet. A reaction rate coefficient was analogously calculated using

$$dB/dt = -k_{rxn}BX$$

where k_{rxn} = biomass–biocide reaction rate coefficient (ml $cfu^{-1} h^{-1}$). Disinfection rates in the biofilm were referenced to the planktonic disinfection rate coefficient for the respective biocide to obtain a ratio that expresses the relative efficacy of a biocide against biofilm compared with its efficacy against planktonic cells. This ratio, termed here as the biocide efficacy, was calculated as

$$-\ln (X_5/X_0)/(k_{dis} \int Bt\, dt)$$

where X_5 = culturable cell density after 5 h of treatment and X_o = initial culturable cell density. The biocide efficacy is the observed rate of disinfection in the biofilm divided by the rate of disinfection that would be measured in a planktonic culture for the same biocide concentration.

Mean values of data groups were compared by a two-sample two-sided t-test.

Analytical methods

Chlorine concentration was determined by the *N*,*N*,-diethyl-*p*-phenylenediamine colorimetric standard method using the Hach kit Model CN-66. Glutaraldehyde concentration was determined by gas chromatography using a Hewlett-Packard 5890 Series II instrument equipped with flame ionization detectors and a Hewlett-Packard 3394 A integrator. Isothiazolone concentration was determined by HPLC using a Varian Model 5000 LC equipped with a Supelcosil LC-18 column and a Polychrom 9065 detector set at 280 nm. The mobile phase was 25/75 methanol/0·4% acetic acid. ADBAC concentration was determined by the direct binary couples method using the Hach QAC kit.

RESULTS

All four biocides killed planktonic bacteria effectively. After 5 h of treatment, planktonic cells experienced reductions by factors of approximately 10^8 in 2·5 mg l^{-1} chlorine, 10^7 in 25 mg l^{-1} glutaraldehyde, 10^2 in 0·77 mg l^{-1} chloroisothiazolone and 10^3 in 10 mg l^{-1} ADBAC. Some typical disinfection data are plotted in Fig. 1. Disinfection rate coefficients estimated for planktonic cells are summarized in Table 2.

Biocides were partially neutralized by uncharacterized reactions with cell mass. Reaction rate coefficients for the biocide–cell mass reaction are reported in Table 2. The rate coefficients range by an order of magnitude. Isothiazolone and chlorine were the most rapidly reacted, glutaraldehyde was reacted at intermediate rates, and ADBAC was the most slowly neutralized.

All biocides clearly reduced viable bacterial numbers in alginate gel bead artificial biofilms, but less rapidly than planktonic bacteria were killed (Fig. 1). The degree of kill measured in artificial biofilm depended on the size of the gel bead and the cell density at which it was loaded. Disinfection efficacy decreased as gel bead radius increased (Fig. 2) at least for high cell density (10^9 cfu ml^{-1}) conditions. For initial cell densities exceeding 3×10^9 cfu ml^{-1}, the biocide efficacy measured in 6 mm diameter gel beads was significantly lower ($P = 0{\cdot}039$) than the biocide efficacy measured in 1·8 mm diameter beads. Bacteria loaded at relatively high cell densities (about 10^9 cfu ml^{-1} in large beads) were scarcely affected by any of the biocides (Fig. 1). Bacteria loaded at relatively low densities (about 5×10^7 cfu ml^{-1} in large beads) were killed at rates intermediate between those measured planktonicly and in high cell density artificial biofilm (Fig. 1). No effect of initial viable cell density on artificial biofilm disinfection efficacy could be discerned at cell densities less than approximately 3×10^9 cfu ml^{-1} (Fig. 3). At cell densities of 3×10^9 cfu ml^{-1} or higher, disinfection efficacy in the gel beads was clearly reduced ($P = 10^{-7}$; Fig. 3).

DISCUSSION

Bacteria entrapped in alginate gel beads were less susceptible to disinfection by each of the four biocides tested than were freely suspended cells. This result is in agreement with other studies of susceptibility of gel-entrapped bacteria (Whitham

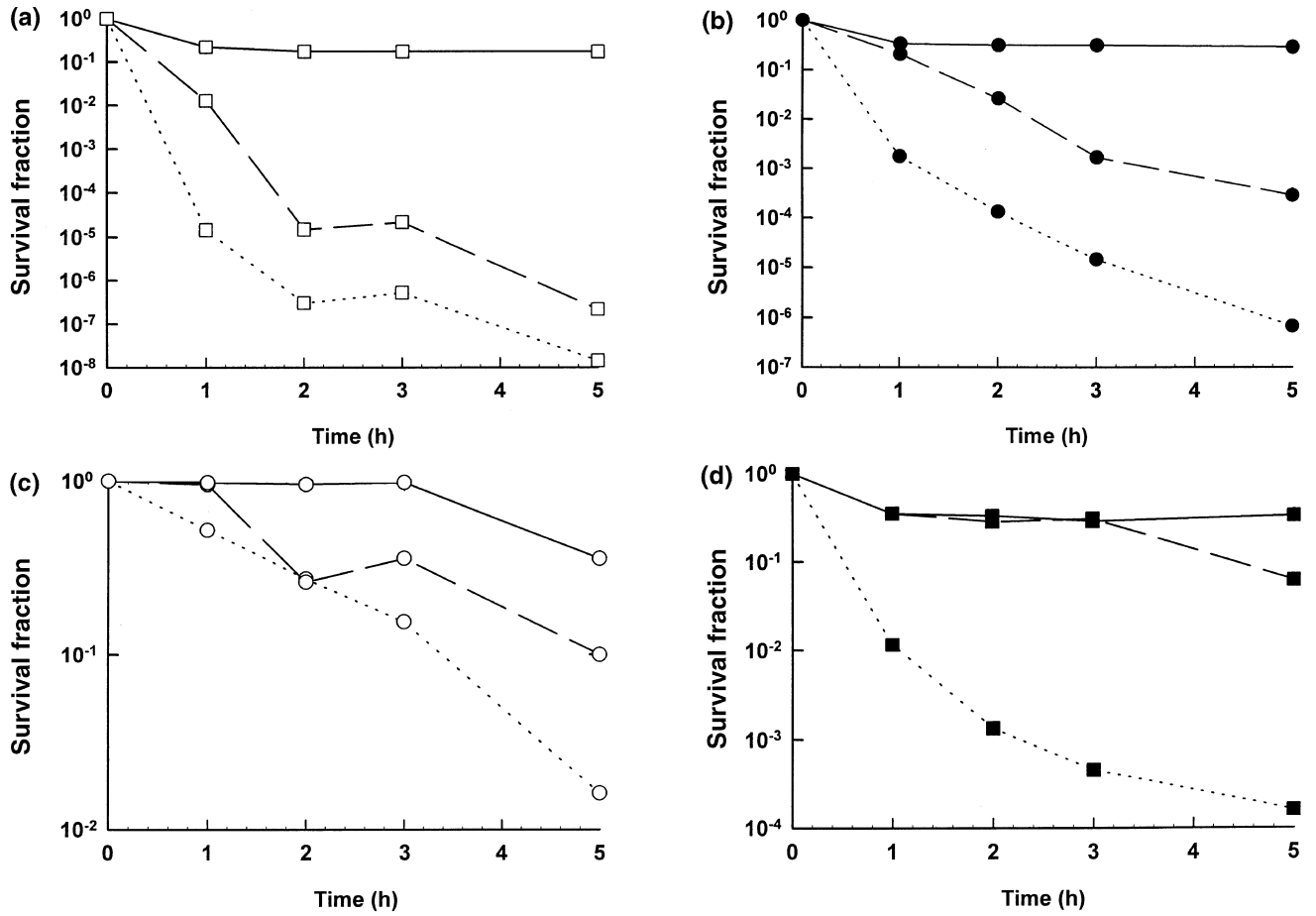

Fig. 1 Comparison of disinfection measured planktonicly (. . .) and in artificial biofilm alginate gel beads (– – –, low cell density; ——, high cell density) exposed to 2·5 mg l^{-1} chlorine bleach (a); 25 mg l^{-1} glutaraldehyde (b); 0·77 mg l^{-1} isothiazolone (c); and 10 mg l^{-1} ADBAC (d). Shown are data for 6·0 mm diameter gel beads that were initially loaded with bacteria at densities between $4{\cdot}2 \times 10^7$ and $6{\cdot}9 \times 10^7$ cfu ml^{-1} (low) or between $4{\cdot}5 \times 10^9$ and $6{\cdot}4 \times 10^9$ cfu ml^{-1} (high)

Table 2 Disinfection and reaction rate coefficients determined from planktonic experiments

Agent	(l mg^{-1} h^{-1}) k_{dis}	(l mg^{-1} $cell^{-1}$ h^{-1}) $k_{rxn} \times 10^{10}$
Chlorine	2·0	19
Glutaraldehyde	0·14	7
Isothiazolone	1·1	29
ADBAC	0·13	1·9

and Gilbert 1993; Jouenne *et al.* 1994; Chen and Stewart 1996; Xu *et al.* 1996) and is consistent with the nearly universally observed reduced susceptibility of biofilms to disinfection. At least with regard to antimicrobial challenge, the alginate gel bead artificial biofilm system does appear to capture some of the features of real biofilms. One aspect of biofilms that the gel bead system surely does not mimic is the structural heterogeneity observed in some natural biofilms (Costerton *et al.* 1995). It may be useful to think of the gel bead as simulating the situation in a cell cluster within a biofilm rather than the biofilm as a whole.

The manifest dependence of biofilm disinfection efficacy on the physical properties of the biofilm (radius and cell density) suggests the impingement of transport limitation of biocide transport into the biofilm. As the radius (analogous to biofilm thickness) increases, the time required for a solute to penetrate into the biofilm increases. As the cell density increases, the biocide neutralizing capacity increases, which in turn leads to poorer penetration. To test this possibility, the data were interpreted using a previously developed theory of biocide reaction and diffusion in biofilms (Stewart and Raquepas 1995). The key parameter in evaluating the degree of transport limitation is a Thiele modulus, denoted by ϕ.

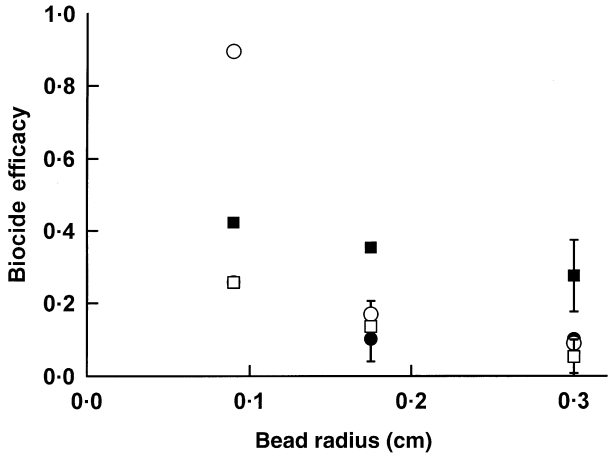

Fig. 2 Effect of artificial biofilm gel bead size on biofilm disinfection efficacy. Symbols denote 2·5 mg l^{-1} chlorine (□); 25 mg l^{-1} glutaraldehyde (●); 0·77 mg l^{-1} isothiazolone (○); and 10 mg l^{-1} ADBAC (■) treatments. The initial cell densities in the gel beads were between $3{\cdot}4 \times 10^9$ and $8{\cdot}3 \times 10^9$ cfu ml^{-1}

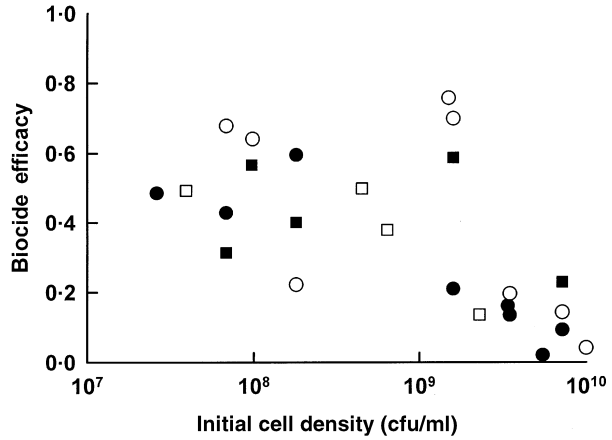

Fig. 3 Effect of artificial biofilm initial cell density on biofilm disinfection efficacy. Symbols denote 2·5 mg l^{-1} chlorine (□); 25 mg l^{-1} glutaraldehyde (●); 0·77 mg l^{-1} isothiazolone (○); and 10 mg l^{-1} ADBAC (■) treatments. The radius of the gel beads was 0·175 cm

The Thiele modulus, which is dimensionless, reflects the relative rates of reaction and diffusion. The Thiele modulus is directly proportional to the biofilm thickness (e.g. gel bead radius) and is proportional to the square root of the biofilm cell density. When $\phi \geqslant 1$, the system is transport limited; when $\phi \leqslant 1$, transport limitation is negligible. The theory predicts that biocide efficacy should begin to drop as ϕ exceeds 1. Biocide efficacy is defined in this case as the ratio of the observed rate of disinfection in the biofilm to the rate of disinfection measured planktonicly (see Materials and Methods). The extent to which this ratio is less than 1 is a measure of the reduced susceptibility of the biofilm.

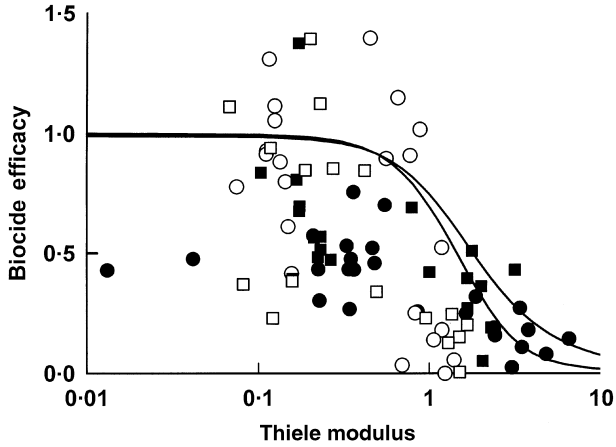

Fig. 4 Test for transport limitation of biocide efficacy in alginate gel bead artificial biofilms. The lines are upper and lower bounds as predicted by the theory of Stewart and Raquepas (1995). Symbols denote chlorine (□); glutaraldehyde (●); isothiazolone (○); and ADBAC (■) treatments. The biocide efficacy is the observed rate of disinfection in the biofilm divided by the rate of disinfection that would be measured in a planktonic culture for the same biocide concentration

Biocide efficacy does indeed decrease, albeit noisily, as the Thiele modulus exceeds 1 (Fig. 4). Biocide efficacies

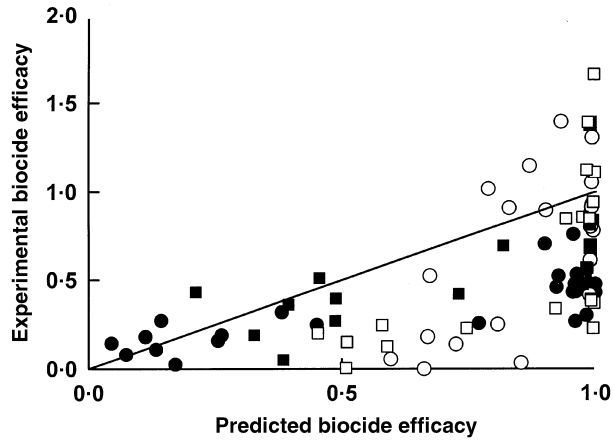

Fig. 5 Predicted *vs* experimental biocide efficacy. The predicted value was generated by the steady-state theory of Stewart and Raquepas (1995). Symbols denote chlorine (□); glutaraldehyde (●); isothiazolone (○); and ADBAC (■) treatments. The line is simply the one to one correspondence that would be expected if the theory were complete and correct. The biocide efficacy is the observed rate of disinfection in the biofilm divided by the rate of disinfection that would be measured in a planktonic culture for the same biocide concentration

measured for Thiele moduli greater than or equal to 1 were significantly lower than biocide efficacies corresponding to Thiele moduli less than 1 ($P = 10^{-13}$). A Thiele modulus of 1 or greater ensures that transport limitation is a factor in reduced biofilm susceptibility. This result demonstrates unequivocally that transport limitation can impact antimicrobial performance against biofilms not only of oxidizing biocides (chlorine) but also of non-oxidizing agents (glutaraldehyde, isothiazolone, quaternary ammonium compounds). The tremendous scatter in the data for which the Thiele modulus was less than 1 suggest that there are additional mechanisms altering the susceptibility of gel bead-entrapped bacteria other than transport limitation. These could include the induction of adaptive stress responses in the entrapped bacterial system and cell density-dependent gene regulation (quorum sensing).

Directly comparing biocide efficacies predicted by the theory of Stewart and Raquepas (1995) with experimentally measured biocide efficacies (Fig. 5) supports transport limitation as a partial, but incomplete, mechanism of biofilm resistance to disinfection. The slopes of the regressed lines through the experimental *vs* predicted values for each individual biocide would be 1 if the theory was correct and completely sufficient to explain biofilm disinfection phenomena. The computed slopes (m) and regression coefficients (r^2) for the four antimicrobial agents were: glutaraldehyde ($m = 0{\cdot}40; r^2 = 0{\cdot}64$); isothiazolone ($m = 2{\cdot}1; r^2 = 0{\cdot}44$), ADBAC ($m = 0{\cdot}59; r^2 = 0{\cdot}37$) and chlorine ($m = 1{\cdot}6; r^2 = 0{\cdot}45$). The fact that the slopes are all positive suggests that some transport limitation occurs. The magnitude of the r^2 values suggests that transport limitation accounts for only about 50% of the difference in disinfection susceptibility between planktonic and artificial biofilm bacteria.

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