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# Adaptive responses to antimicrobial agents in biofilms

**Barbara Szomolay,[1] Isaac Klapper,[1,2]* Jack Dockery[1,2] and Phil S. Stewart[2,3]**

[1]*Department of Mathematical Sciences, Montana State University, Bozeman, MT 59717, USA.*

[2]*Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717, USA.*

[3]*Department of Chemical and Biological Engineering, Montana State University, Bozeman, MT 59717, USA.*

## Summary

**Bacterial biofilms demonstrate adaptive resistance in response to antimicrobial stress more effectively than corresponding planktonic populations. We propose here that, in biofilms, reaction-diffusion limited penetration may result in only low levels of antimicrobial exposure to deeper regions of the biofilm. Sheltered cells are then able to enter an adapted resistant state if the local time scale for adaptation is faster than that for disinfection. This mechanism is not available to a planktonic population. A mathematical model is presented to illustrate. Results indicate that, for a sufficiently thick biofilm, cells in the biofilm implement adaptive responses more effectively than do freely suspended cells. Effective disinfection requires applied biocide concentration that increases quadratically or exponentially with biofilm thickness.**

## Introduction

Bacteria or yeast that aggregate in dense, surface associated communities called biofilms are protected from antimicrobial agents. The mechanisms of protection are of obvious interest because they may lead the way to new strategies or agents for controlling detrimental biofilms. Some of the phenomena that are postulated to contribute to the biofilm defence include incomplete antimicrobial penetration, slow or no growth of some of the biofilm cells, and the expression of biofilm-specific phenotypes (Mah and O'Toole, 2001; Stewart and Costerton, 2001). Here we explore from a theoretical perspective another potential mechanism that has only rarely been mentioned in the literature: the possibility that some of the cells in a biofilm are able to sense the antimicrobial challenge and actively respond to it by deploying protective stress responses.

Received 10 August, 2004; revised 16 December, 2004; accepted 20 December, 2004. *For correspondence. E-mail klapper@math.montana.edu; Tel. (+1) 406 994 5231; Fax (+1) 406 994 1789.

There is mounting evidence that microorganisms in biofilms do actively respond to antimicrobial challenges. Giwercman and colleagues (1991) reported that *Pseudomonas aeruginosa* biofilms responded to treatment with imipenem by producing a β-lactamase enzyme that deactivates this antibiotic. This response has subsequently been confirmed by Bagge and coworkers (Bagge *et al*., 2004a,b). Sanderson and Stewart (1997) reported that *P. aeruginosa* biofilms increased their capacity to neutralize monochloramine upon exposure to this agent; when biofilms were treated with repeated doses of monochloramine, the second dose was less effective than the first dose even though the biofilm was thinner at the time of the second application. Elkins and colleagues (1999) used a reporter gene fusion for *katB*, a catalase, to show that this enzyme is induced during treatment of *P. aeruginosa* biofilms with 50 mM hydrogen peroxide. The same hydrogen peroxide concentration, when applied to planktonic cells, overwhelmed the bacteria and they were not able to exhibit any detectable response. Two studies have reported that bacteria in biofilms can respond to antibiotic treatment by increasing the synthesis of extracellular polysaccharides that contribute to the matrix of the biofilm (Sailer *et al*., 2003; Bagge *et al*., 2004b).

It is interesting to note that a single microorganism may have multiple possible responses depending on the nature of the antimicrobial agent. For example, *P. aeruginosa* respond to hydrogen peroxide by inducing catalase (Elkins *et al*., 1999), to imipenem by inducing β-lactamase and alginate synthesis (Bagge *et al*., 2004b), and to tobramycin with a distinct genetic response (Whiteley *et al*., 2001).

The objective of this study is to mathematically investigate the potential for an adaptive stress response to contribute to the protection of cells in a biofilm. In particular, we are interested in investigating the additional protection realized in the biofilm state that goes beyond that afforded to free-floating cells. Free-floating cells, after all, have the same adaptive responses coded in their DNA. If an antimicrobial-induced stress response is more effectively deployed in a biofilm, there must be either unique regula-

tion that occurs in the biofilm mode of growth or the conditions in a biofilm must favour induction of the stress response over killing of the cell. We have simulated the latter scenario by modelling the diffusion-limited penetration of a reactive antimicrobial into the biofilm.

## Results

### *Theory*

Consider the action of an antimicrobial agent against a microbial cell that is able to reactively neutralize this agent. The cell may be killed by the antimicrobial, but the cell may also respond to this stimulus by decreasing its susceptibility to killing by the antimicrobial agent. In the following analysis, we will consider the particular case of an antimicrobial whose biological reaction is independent of the viability of the cell (not essential to the model). That is, even dead cells will continue to degrade the antimicrobial agent. Such is the case, for example, for the enzyme-based reactions of hydrogen peroxide (by catalases) or β-lactam antibiotics (by β-lactamases). When antimicrobial-reacting cells are aggregated in a biofilm, the local concentration of the antimicrobial agent will depend on the simultaneous reaction and diffusion of the antimicrobial. These phenomena form the basis for the model described below.

We begin by gathering, in qualitative terms, the central assumptions underpinning the model. We use the term biocide below for convenience, but we note that the theory could apply to any type of antimicrobial agent, antibiotic, biocide, or disinfectant.

Key assumptions:

1. The biofilm geometry is a flat slab with constant total cell density.
2. The biofilm is attached to an impermeable substratum which is non-reactive towards the biofilm.
3. Biocide is applied to the biofilm through the biofilm–bulk fluid interface at a proscribed concentration.
4. Biocide transforms living cells to dead cells at rates proportional to the biocide concentration.
5. Biocide is consumed by reactions with biomass at rate proportional to the biocide concentration.
6. Biocide is transported within the biofilm by Fickian diffusion alone.
7. Living, unadapted cells transform to living, adapted cells in the presence of biocide at a constant rate.
8. Adapted cells are much less susceptible to disinfection than are unadapted cells.
9. The biofilm neither grows nor detaches.

These assumptions and their mathematical translations are detailed and expounded upon below. See Table 1 for a listing of variables and parameters.

**Table 1.** Dimensional variables and parameters.

| | | |
|---|---|---|
| $b_a$ | Adapted cell disinfection rate constant | 1/s |
| $b_u$ | Unadapted cell disinfection rate constant | 1/s |
| $B$ | Local biocide concentration | gm/cm$^3$ |
| $B_0$ | Externally applied biocide concentration | gm/cm$^3$ |
| $D$ | Biocide diffusion constant | cm$^2$/s |
| $k$ | Biocide-cell reaction rate | cm$^3$/cells s |
| $k_a$ | Biocide-adapted cell reaction rate | cm$^3$/cells s |
| $k_u$ | Biocide-unadapted cell reaction rate | cm$^3$/cells s |
| $L$ | Biofilm thickness | cm |
| $r_0$ | Unadapted-adapted cell transformation rate constant | 1/s |
| $X_0$ | Total cell density | cells/cm$^3$ |
| $X_a$ | Adapted cell density | cells/cm$^3$ |
| $X_{ad}$ | Dead adapted cell density | cells/cm$^3$ |
| $X_u$ | Unadapted cell density | cells/cm$^3$ |
| $X_{ud}$ | Dead unadapted cell density | cells/cm$^3$ |

We consider a bacterial population consisting of four constituents:

$$X_u = \text{unadapted cell density}$$
$$X_{ud} = \text{dead unadapted cell density}$$
$$X_a = \text{adapted cell density}$$
$$X_{ad} = \text{dead adapted cell density}$$

subject to the condition $X_u + X_{ud} + X_a + X_{ad} = X_0$ where $X_0$ is constant in space and time. For planktonic systems, we assume the constituent densities are functions of time $t$ only; for biofilm systems, a flat layer of depth $L$ with $0 \le z \le L$ is assumed and in this case the constituent densities are functions of both $z$ and $t$ in general. We designate $z = 0$ as the substratum and $z = L$ as the biofilm–bulk fluid interface. For both planktonic and biofilm systems, we assume that our time scale of observation is sufficiently short so that we can disregard growth.

At $t = 0$ a concentration $B_0$ of biocide is applied to the bacterial population. In the case of a planktonic system, the biocide is assumed to be well mixed. In the case of a biofilm, the biocide is applied at the biofilm–bulk fluid interface $z = L$.

In the biofilm case, we assume that biocide concentration $B(z, t)$ satisfies

$$\frac{\partial B}{\partial t} = D\frac{\partial^2 B}{\partial z^2} - k_u(X_u + X_{ud})B - k_a(X_a + X_{ad})B$$

with boundary and initial conditions

$$\frac{\partial B}{\partial z}(0,\ t) = 0, \quad B(L,\ t) = B_0, \quad B(z,\ 0) = 0.$$

The coefficients $k_u$ and $k_a$ are reaction constants. The boundary condition at $z = 0$ indicates that no flux of biocide through the solid boundary occurs; the boundary condition at $z = L$ indicates that biocide concentration in the bulk fluid is fixed. The initial conditions affect the fact that no biocide is present in the biofilm at $t = 0$.

Without affecting qualitatively the conclusions to come we can assume that $k_u = k_a = k$ for some $k$ (in

actual fact one might expect $k_a > k_u$) and thus, using $X_u + X_{ud} + X_a + X_{ad} = X_0$,

$$\frac{\partial B}{\partial t} = D\frac{\partial^2 B}{\partial z^2} - kX_0 B$$
$$\frac{\partial B}{\partial z}(0,\ t) = 0, \quad B(L,\ t) = B_0, \quad B(z,\ 0) = 0. \tag{1}$$

We will assume that in the biofilm system, the biocide concentration profile evolves quasistatically, i.e. $\partial B/\partial t$ in Eq. 1 can be neglected. This assumption amounts to assuming that the balance between biocide diffusion and biocide reaction occurs quickly relative to the time for significant disinfection to occur. Such would be the case for example if disinfection requires an extended intracellular process as compared to biocide reaction involving a relatively quick molecular process.

With regards to the planktonic system, we may regard the biocide to be constantly replenished, i.e. $B(t) = B_0$, or we may regard that biocide is only applied at $t = 0$ (a batch model) in which case

$$\frac{dB}{dt} = -kX_0 B, \quad B(0) = B_0 \tag{2}$$

The equations for the constituent densities $X_u$, $X_{ud}$, $X_a$, $X_{ad}$ take the form

$$\frac{\partial X_u}{\partial t} = -b_u B X_u - r(B)X_u, \quad X_u(z,\ 0) = X_0 \tag{3}$$

$$\frac{\partial X_{ud}}{\partial t} = b_u B X_u, \qquad X_{ud}(z,\ 0) = 0 \tag{4}$$

$$\frac{\partial X_a}{\partial t} = -b_a B X_a + r(B)X_u, \quad X_a(z,\ 0) = 0 \tag{5}$$

$$\frac{\partial X_{ad}}{\partial t} = b_a B X_a, \qquad X_{ad}(z,\ 0) = 0 \tag{6}$$

These equations are the same for the biofilm and planktonic case except that in the planktonic population all densities are independent of space and the derivatives are ordinary rather than partial. The coefficients $b_u$ and $b_a$ are disinfection rate constants and $r(B)$ is the rate of transformation function for unadapted to adapted phenotype. We choose

$$r(B) = \begin{cases} 0 & B = 0 \\ r_0 & B > 0 \end{cases}$$

for some constant $r_0$. We will assume that $b_u >> b_a$, i.e. unadapted cells disinfect at a much faster rate than adapted ones.

### *Scaling*

We scale the independent variables $z$ and $t$ by $\tilde{z} = z/L$, $\tilde{t} = t/(b_u B_0)^{-1}$. Note that $0 \le \tilde{z} \le 1$ and that $(b_u B_0)^{-1}$ is the disinfection time scale of unadapted bacteria exposed to full biocide concentration. The dependent variables are scaled by $\tilde{B} = B/B_0$ and $\tilde{X}_u = X_u/X_0$, $\tilde{X}_{ud} = X_{ud}/X_0$, $\tilde{X}_a = X_a/X_0$, $\tilde{X}_{ad} = X_{ad}/X_0$, so that all range between 0 and 1. Dropping tildes for convenience, Eqs 1 and 3–6 transform to

$$\varepsilon\phi^2 \frac{\partial B}{\partial t} = \frac{\partial^2 B}{\partial z^2} - \phi^2 B, \ \frac{\partial B}{\partial z}(0,\ t) = 0, \ B(1,\ t) = 1, \ B(z,\ 0) = 0 \tag{7}$$

$$\frac{\partial X_u}{\partial t} = -(B + \lambda H(B))X_u, \qquad X_u(z,\ 0) = 1 \tag{8}$$

$$\frac{\partial X_{ud}}{\partial t} = BX_u, \qquad X_{ud}(z,\ 0) = 0 \tag{9}$$

$$\frac{\partial X_a}{\partial t} = -\delta B X_a + \lambda H(B)X_u, \quad X_a(z,\ 0) = 0 \tag{10}$$

$$\frac{\partial X_{ad}}{\partial t} = \delta B X_a, \qquad X_{ad}(z,\ 0) = 0 \tag{11}$$

where $\phi^2 = kX_0L^2D^{-1}$ is the Thiele modulus, $\varepsilon = b_uB_0/kX_0$, $\lambda = r_0/b_uB_0$, and $\delta = b_a/b_u$. $H(B)$ is the Heaviside function defined here as

$$H(B) = \begin{cases} 0 & B = 0 \\ 1 & B > 0 \end{cases}$$

Note that $\sqrt{D/kX_0}$, which has units of length, is the depth into the biofilm to which biocide can diffuse before a significant fraction is depleted by reaction. $((kX_0)^{-1}$ is the biocide reaction time scale and for a given time interval of length $T$, say $T = (kX_0)^{-1}$, $\sqrt{DT}$ is approximately the distance a diffusing quantity can spread in significant concentration.) Hence $(\phi^2)^{-1/2} = \sqrt{D/kX_0}/L$ is the depth in scaled variables of the disinfection layer, the layer within the biofilm in which most disinfection occurs. $\varepsilon = b_uB_0/kX_0$ is the ratio of the time scale $(kX_0)^{-1}$ over which significant biocide depletion occurs to the time scale $(b_uB_0)^{-1}$ over which significant disinfection occurs. We suppose that $\varepsilon << 1$ for the large density $X_0$ occurring in the biofilm (see Discussion after Eq. 1) but $\varepsilon$ may be much larger in the planktonic case. We also suppose that $\delta$, the ratio of adapted to unadapted disinfection rates is small for both biofilm and planktonic cases. See Table 2 for a listing of non-dimensional parameters.

Accounting for the previous remarks, the biofilm system Eqs 7–11 simplifies to

$$\frac{\partial^2 B}{\partial z^2} = \phi^2 B, \qquad \frac{\partial B}{\partial z}(0,\ t) = 0, \ B(1,\ t) = 1, \tag{12}$$

$$\frac{\partial X_u}{\partial t} = -(B + \lambda H(B))X_u, \quad X_u(z,\ 0) = 1, \tag{13}$$

$$\frac{\partial X_{ud}}{\partial t} = BX_u, \qquad X_{ud}(z,\ 0) = 0, \tag{14}$$

$$\frac{\partial X_a}{\partial t} = \lambda H(B)X_u, \qquad X_a(z,\ 0) = 0. \tag{15}$$

Note that $B = B(z)$, i.e. $B$ is independent of time, and that $X_{ad}(t) = 0$ for this system. For planktonic populations, the same system is used except that Eq. 7 is replaced by either $B(t) = 1$ for the replenished case or, in the non-replenished case,

**Table 2.** Non-dimensional constants.

| | |
|---|---|
| $\delta = b_a/b_\mu$ | Unadapted disinfection time/adapted disinfection time |
| $\varepsilon = b_u B_0/kX_0$ | Biocide reaction time/unadapted disinfection time |
| $\lambda = r_0/b_u B_0$ | Unadapted disinfection time/cell transformation time |
| $\phi^2 = kX_0L^2/D$ | Ratio squared of biofilm depth to disinfection layer depth |

$$\frac{dB}{dt} = -\frac{1}{\varepsilon}B, \quad B(0) = 1 \tag{16}$$

Note that for large $\varepsilon$, $B(t) \approx 1$ again.

*Solutions*

Equation 12 has solution

$$B(z) = \frac{\cosh(\phi z)}{\cosh(\phi)} \tag{17}$$

on $0 \le z \le 1$. Here $\phi = \sqrt{\phi^2}$. Noting that $B(z) > 0$ on $0 \le z \le 1$ so $H(B) = 1$, then

$$X_u(t) = e^{-(B+\lambda)t} \tag{18}$$

$$X_{ud}(t) = \frac{B}{B+\lambda}(1 - e^{-(B+\lambda)t}) \tag{19}$$

$$X_a(t) = \frac{\lambda}{B+\lambda}(1 - e^{-(B+\lambda)t}) \tag{20}$$

Equations 17–20 are for the biofilm case. For large time,

$$X_{ud} \cong \frac{B}{B+\lambda}$$

$$X_a \cong \frac{\lambda}{B+\lambda}$$

and $X_u \cong 0$. The planktonic solution is the same except with $B = 1$ instead of Eq. 17.

In the case of a planktonic population, we see that for long times disinfection dominates for $\lambda << 1$ and resistance dominates for $\lambda >> 1$ (recall $\lambda = r_0/b_u B_0$ is the ratio of disinfection time scale to adaptation time scale). In particular, for large times the ratio of adapted to unadapted (dead) cells is $X_a/X_{ud} = \lambda \sim B_0^{-1}$.

In the biofilm case we distinguish two regimes, namely $\phi^2 << 1$ (biofilm depth small compared to depth of the disinfection layer) and $\phi^2 >> 1$ (biofilm depth large compared to depth of the disinfection layer). First, if $\phi^2 << 1$ then

$$B(z) = \frac{\cosh(\phi z)}{\cosh(\phi)} = 1 - O(\phi^2) \approx 1$$

and we effectively reduce to the planktonic case. That is, a thin biofilm behaves like a planktonic population. See Fig. 1. If on the other hand $\phi^2 >> 1$, then we proceed by dividing the biofilm into two layers. In the disinfection layer, roughly $1 - \phi^{-1} < z < 1$, we see from Eq. 17 that again $B(z) \approx 1$ and hence the biofilm responds as in the planktonic case. However, in the protected layer, roughly

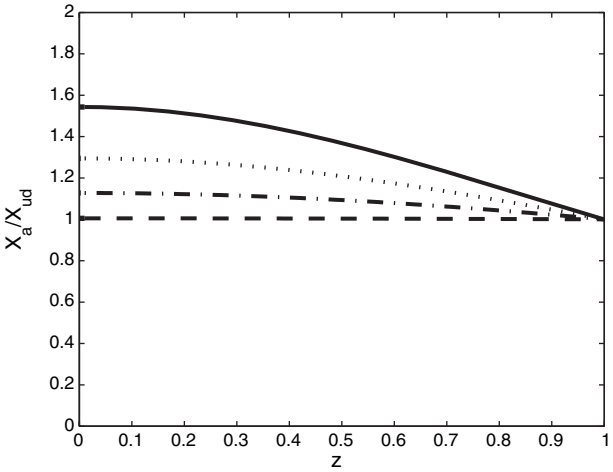


**Fig. 1.** Ratio $X_a/X_{ud}$ as a function of depth $z$ at long time for various smaller values of $\phi$, $\phi = 1$ (—), $\phi = 0.75$ (· · ·), $\phi = 0.5$ (· ▬ ·), $\phi = 0.1$ (– –). In each case $\lambda = 1$. and the ratio for a planktonic population would thus be $X_a/X_{ud} = \lambda = 1$, nearly indistinguishable from the $\phi = 0.1$ biofilm.

$0 < z < 1 - \phi^{-1}$, we see from Eq. 17 that $B(z) << 1$ and hence disinfection may be suppressed. For example, at the bottom $z = 0$ of the biofilm $B(0) = (\cosh\phi)^{-1} \approx e^{-\phi}$ for large $\phi$. From Eq. 20 disinfection dominates for $\lambda << e^{-\phi}$ but adaptation dominates for $\lambda >> e^{-\phi}$. See Fig. 2. The biocide profiles for the curves in Figs 1 and 2 are shown in Fig. 3.

From the point of view of applied biocide concentration $B_0$ as variable in $\lambda = r_0/b_u B_0$, we can summarize as follows. In the planktonic and thin biofilm cases

$B_0 >> C \Rightarrow$ disinfection

$B_0 << C \Rightarrow$ adapted transformation

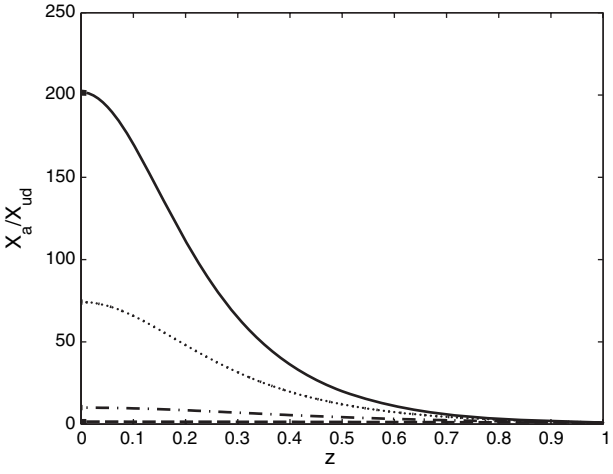


**Fig. 2.** Ratio $X_a/X_{ud}$ as a function of depth $z$ at long time for various larger values of $\phi$, $\phi = 6$ (—), $\phi = 5$ (· · ·), $\phi = 3$ (· ▬ ·), $\phi = 1$ (– –). In each case $\lambda = 1$. Note the dominance of the adaptive phase below the disinfection layer for larger $\phi$.

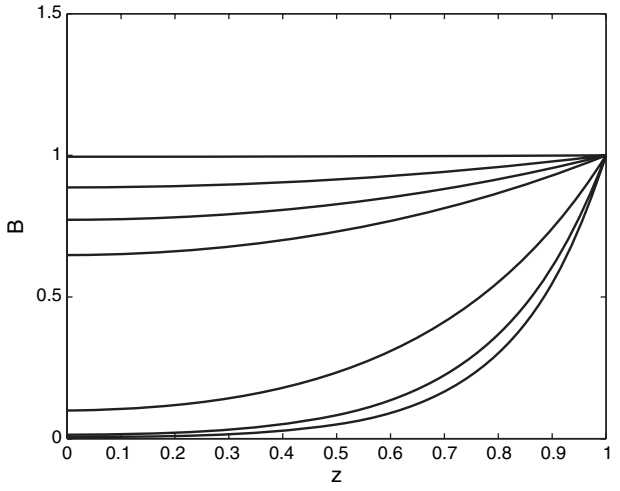


**Fig. 3.** Biocide $B$ as a function of depth $z$. Profiles are for ratios show in Figs 1 and 2, from top to bottom: $\phi = 0.1$, $\phi = 0.5$, $\phi = 0.75$, $\phi = 1$, $\phi = 3$, $\phi = 5$, $\phi = 6$.

where $C = r_0/b_\mu$ is a killing concentration threshold. In the thick biofilm case,

$B_0 >> Ce^{\phi} \Rightarrow$ disinfection

$B_0 << Ce^{\phi} \Rightarrow$ adapted transformation (21)

Thus, regarding $\phi$ as linear in biofilm thickness $L$, for a deep biofilm (relative to the disinfection depth) exponentially large in $L$ amounts of biocide are required for disinfection. If less biocide is applied, then an adapted layer will form. Although we remark that Eq. 21 is, for very deep biofilms, probably too pessimistic as this bound relies on first order (in $B$) reaction kinetics in Eq. 1. In fact for sufficiently large concentration $B_0$ of applied biocide, saturation can be expected in which case reaction kinetics will approach zeroth rather than first order. Inclusion of these more realistic kinetics only affects Eq. 12 and the profile $B(x)$. Straightforward analysis demonstrates then that the disinfection concentration eventually grows quadratically with increase in $L$ without otherwise effecting the presented results.

In order to directly compare disinfection in biofilm versus planktonic systems we follow Stewart and Raquepas (1995) and define the disinfection efficacy

$$\eta = \frac{\log(AF_b)}{\log(AF_p)}$$

where $AF_b$ is the long time adapted fraction in the biofilm case and $AF_p$ is the long time adapted fraction in the planktonic case, in particular

$$AF_b = \int_0^1 \frac{\lambda}{B(z) + \lambda} dz$$

$$AF_p = \frac{\lambda}{1 + \lambda}$$

$\eta$ measures effectiveness of disinfection in biofilm relative to disinfection in planktonic populations at the same biocide concentration. Observe that $\eta \leq 1$ necessarily. The model predicts $\eta \cong 1$ for thin biofilms and $\eta \cong 0$ for thick biofilms (Fig. 4).

## Discussion

One of the ways that microorganisms in biofilms evade killing by antimicrobial agents is by sensing the challenge and deploying adaptive responses. Here we have derived a simple mathematical model of this process and shown that the model predicts that an adaptive response can provide greater protection to cells in a biofilm than to free-floating cells. Differential protection afforded to the biofilm can be understood in the following way. The outcome of exposure of a microbial cell to an antimicrobial substance is a race between killing of the cell and deployment of an adaptive response that reduces susceptibility of the cell. This race is run in a planktonic suspension of cells just as it is in a biofilm, and both planktonic cells and biofilm cells have the same adaptive responses coded into their DNA. However, in a biofilm if there is some other protective mechanism that retards killing relative to adaptation, e.g. a reaction-diffusion interaction (Stewart and Raquepas, 1995), the stress response will be more effectively expressed in the biofilm state. In other words, incomplete penetration of a reactive antimicrobial agent into a biofilm buys time for at least some of the cells (and perhaps many) to adapt to the antimicrobial. Our result is illustrated in Fig. 5. Protective adaptation (darker shading) is favoured in biofilms when reaction of the antimicrobial limits its penetration (larger $\phi$).

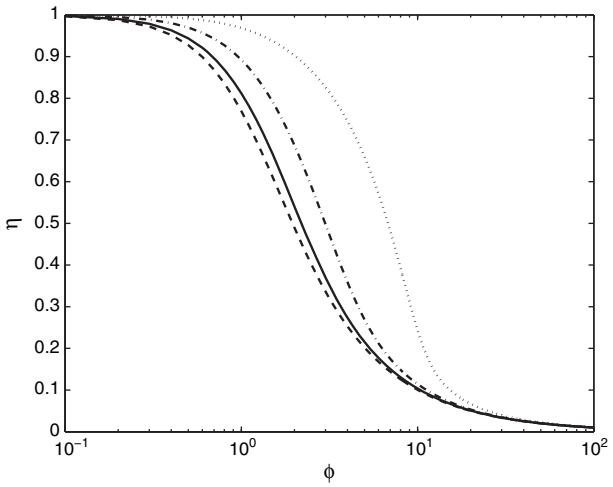


**Fig. 4.** Efficacy $\eta$ as a function of $\phi$ at long time for various large values of $\lambda$, $\lambda = 1$ (—), $\lambda = 10$ (– –), $\lambda = 0.1$ (· ▬ ·), $\lambda = 10^{-4}$ (· · ·). Note that even for very small $\lambda$, i.e. very strong biocide concentration $B_0$, efficacy may be poor for moderately sized biofilms.

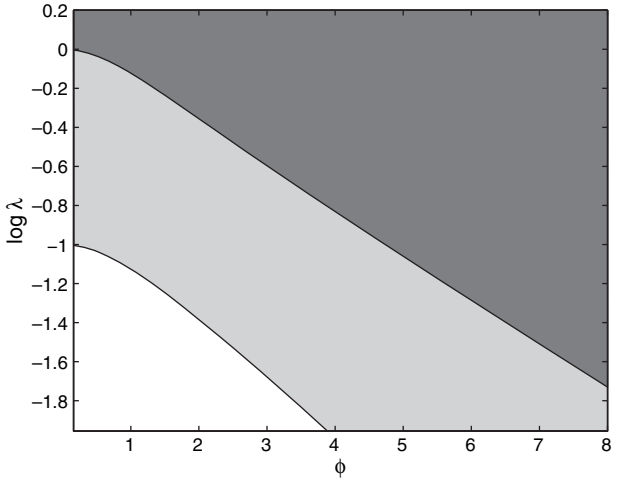


**Fig. 5.** Map of cell adaptation versus non-dimensional numbers log λ and $\phi$. λ measures ratio of biocide disinfection time versus cell transformation (to adapted state) time, i.e. smaller λ indicates faster acting biocide. $\phi$ measures ratio of biofilm depth to disinfection layer depth, i.e. smaller $\phi$ indicates thinner biofilm. Three bands are distinguished in the map: lower white band is the region where 10% or less of cells are able to adapt, middle light band is the region where between 10% and 50% of cells are able to adapt, upper dark band is the region where more than 50% of cells are able to adapt. The axis $\phi = 0$ is effectively the same as the planktonic case.

As a remark, we generalize this conclusion to predict that in a similar manner many mechanisms of protection that operate in a biofilm will facilitate the implementation of an adaptive response by biofilm cells. For example, a non-reactive antibiotic can be expected to penetrate the entire biofilm diffusively, that is, its concentration will be $B(z, t) = B_0$. However, antimicrobial action of the antibiotic will depend on the metabolic status of the cell and hence on the presence of some limiting metabolic substrate. In this case the reaction-diffusion Eq. 1 for $B(z, t)$ in the present theory would be replaced by an essentially identical reaction-diffusion equation for substrate concentration $C(z, t)$. Similarly, Eqs 2–6 in the present theory would be replaced by essentially identical equations except with $B$ replaced by $C$. Thus behaviour of the various quantities of interest, cell densities in particular, would be qualitatively the same for a non-reactive biocide as in the present reactive biocide system.

This analysis should motivate continued experimental investigation of adaptive responses to antimicrobial agents in biofilms.

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