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BIOFILM CONTROL BY ANTIMICROBIAL AGENTS

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11.1 INTRODUCTION

One of the hallmarks of the biofilm mode of microbial growth is remarkable resistance to killing by antimicrobial agents. This property frustrates efforts to control detrimental biofouling but it probably also harbors clues to the distinct structure and function of microbial biofilms. In this chapter, quantitative process analysis is applied to several aspects of the treatment of biofilms with antimicrobial agents. This exercise, which is inspired by the approach Bill Characklis established in the first edition of *Biofilms*, is as interesting for the deficiencies in our understanding that it reveals as it is for the areas of insight.

11.1.1 Approaches to Biofilm Control

Mechanical cleaning and antimicrobial chemicals are the most used methods of biofilm control. Mechanical cleaning is effective but can be costly because it often involves equipment downtime or a significant labor expenditure. In many applications, mechanical cleaning is simply not an option because the fouled surface is not physically accessible. Biocides,

Biofilms II: Process Analysis and Applications, Edited by James D. Bryers.
ISBN 0-471-29656-2

antibiotics, and disinfectants have, therefore, been the principal tools in controlling biofouling. This chapter focuses on the issues involved in controlling biofilms with such antimicrobial agents.

Some alternative biofilm control strategies that are mentioned but not discussed further in this chapter are summarized pictorially in Figure 11.1 The stop growth option refers to the possibility of using a biological pretreatment process to remove the nutrients that support biofouling from the water entering a system. In other words, biofilm accumulation is engineered to occur in a reactor where it can be managed rather than in an operating system where it is difficult to control. Many water treatment systems, from applications ranging from desalination to semiconductor manufacture, might benefit by incorporating a properly engineered biological unit operation. The block attachment option envisions an inherently antifouling material, one to which microorganisms either do not adhere or are so weakly bound that they do not accrete. No such generic material has been discovered. One strategy now being pursued is the incorporation of antimicrobial agents into materials or coatings. In the context of biomaterials used in medical devices, it may be economical to design sophisticated surface chemistries that promote tissue integration while retarding microbial attachment or to develop agents that block specific adhesion pathways. The least investigated of the alternative options illustrated in Figure 11.1 is the promote detachment option. Perhaps if we understood more about what holds a biofilm together and how detachment occurs in nature, methods or chemistries to promote this process could be developed.

11.1.2 Quantitative Literature Survey of Reduced Biofilm Susceptibility

Although the diminished susceptibility of microorganisms growing in biofilms to killing by antimicrobial agents is now widely recognized (Costerton, 1984; Costerton et al., 1987; Nichols, 1989; Gilbert et al., 1990; Hoyle and Costerton, 1991; Brown and Gilbert, 1993; Gilbert and Brown, 1995; Carpentier and Cerf, 1993; Eastwood, 1994; Zottola, 1994; Allison and Gilbert, 1995; Gander, 1996; Wilson, 1996; Gilbert et al., 1997; Morton et al., 1998), there is no generally accepted basis for quantifying the degree of this resistance. We propose two alternative measures of biofilm reduced susceptibility. The first resistance factor (which we denote as RF1) is defined as the log reduction measured in the planktonic state divided by the log reduction measured in the biofilm state in response to the same dose. A second resistance factor (denoted by RF2) could be defined as the concentration required to achieve a particular reduction in viable cell numbers (e.g., 99%) in the biofilm state, divided by the concentration needed to achieve the same reduction in the planktonic state for the same dose duration. A survey of resistance factors calculated in these ways from data in the literature are summarized in Table 11.1. This literature review is not comprehensive.

The biofilm resistance factors presented in Table 11.1 reinforce the familiar statement that biofilm reduced susceptibility is observed consistently across a wide range of antimicrobial agents and microbial species. There is considerable variability in the resistance factor even for the same microbial species and the same antimicrobial agent. Indeed, within a single experimental study, the degree of resistance has been noted to depend on biofilm age, biofilm areal cell density, microbial strain, nutrient medium, and antimicrobial dose. This indicates that the precise way in which the biofilm is grown and treated is important in determining the extent of reduced biofilm susceptibility. We caution against attempting to extrapolate any of the resistance factors reported in Table 11.1 to systems different from the one they were determined in. There is no basis at this point for advising use of one resistance factor over another. The consistent use of at least one of these quantitative measures

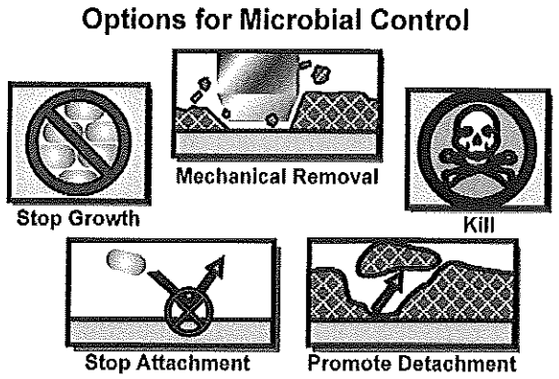

Figure 11.1 Approaches to biofilm control.

of biofilm reduced susceptibility is recommended to all experimenters as once effects are quantified, it becomes possible to search for correlations.

However, it could be argued that use of a biofilm resistance factor such as previously proposed clings unnecessarily to planktonic microbiological methods. Though our ostensible focus is biofilm susceptibility we remain wed, through this factor, to the planktonic result as a reference state. Planktonic methods are familiar and thereby afford a sense of security. But if their utility in understanding biofilm processes is truly so limited (which it is), then why bother at all with assays based on suspended cultures? In many practical applications, the answer to this question may be that there really is no point in performing planktonic tests. Reliable, user-friendly biofilm testing methods need to be developed, adopted by regulatory agencies and professional groups, and disseminated. One reason to continue to study planktonic microbial responses to antimicrobial agents is to elucidate mechanisms of biofilm resistance to antimicrobials. Differences between planktonic and biofilm susceptibility could provide extremely valuable insights if these differences can be linked to specific physical, chemical, and biological phenomena.

11.2 MECHANISMS OF REDUCED SUSCEPTIBILITY

Biofilms evade antimicrobial challenges by multiple mechanisms. These we have grouped into three broad categories: (1) reduction of the antimicrobial concentration in the bulk fluid surrounding the biofilm; (2) failure of the antimicrobial agent to penetrate the biofilm; and (3) adoption of a resistant physiological state or phenotype by at least a fraction of the cells in a biofilm. In the first scenario, the antimicrobial agent is depleted to ineffectual levels before it gets to the biofilm. In the second scenario, the antimicrobial agent is delivered to the surface of the biofilm, but is not effectively transported into the depth of the biofilm. In the third scenario, the antimicrobial agent permeates the biofilm, but is unable to kill microorganisms because they exist in a phenotypic state that confers reduced susceptibility. These mechanisms of biofilm protection are not mutually exclusive. Indeed, it seems likely that combinations of these three general types of resistance work in concert.

The reduced susceptibility of biofilms has not been attributed to the mechanisms—mutation or acquisition of genetic elements coding for specific resistance genes—that

TABLE 11.1. Selected Biofilm Resistance Factors (defined in text) to Antimicrobial Killing

Antimicrobial Agent	Microorganism	RF1	RF2	Reference
Halogens and Oxidants				
Chlorine	*Listeria monocytogenes*	1.8		Ronner and Wong, 1993
	Salmonella typhimurium	1.4		Ronner and Wong, 1993
	Enterobacter aerogenes	4–20		Stewart et al., 1998
	Escherichia coli		50	Ntsama-Essomba et al., 1997
	Staphylococcus aureus	>1.7		Oie et al., 1996
Iodine	*L. monocytogenes*	1.9		Ronner and Wong, 1993
	S. typhimurium	3		Ronner and Wong, 1993
Povidone-iodine	*Citrobacter diversus*	2–8		Stickler and Hewett, 1991
	Enterococcus faecalis	5–7		Stickler and Hewett, 1991
	Pseudomonas aeruginosa	7–14		Stickler and Hewett, 1991
Hydrogen peroxide	*P. aeruginosa*	1–3		Wood et al., 1996
Peracetic acid	*E. coli*		33	Das et al., 1998
	Staphylococcus epidermidis		2	Das et al., 1988
Potassium monopersulfate	*P. aeruginosa*	1–2		Wood et al., 1996
Biguanides				
Chlorhexidine	*C. diversus*	4–6		Stickler and Hewett, 1991
	E. faecalis	8–9		Stickler and Hewett, 1991
	P. aeruginosa	5		Stickler and Hewett, 1991
	E. coli	1.7		Dusart et al., 1994
	A. actinomycetemcomitans	1.3–3		Thrower et al., 1997
	Staphylococcus aureus	>6		Oie et al., 1996
	Streptococcus sanguis		2–8	Millward and Wilson, 1989
	Streptococcus mutans	2–24		Sutch et al., 1995
Polyhexamethylene biguanide	*E. coli*		3–6	Das et al., 1998
	S. epidermidis		3–10	Das et al., 1998
Quaternary Ammonium Compounds				
Benzalkonium chloride	*E. aerogenes*	2–4		Stewart et al., 1998
	S. aureus	>3		Oie et al., 1996
	E. coli		500	Ntsama-Essomba et al., 1997

Cetylpyridinium chloride	*Actinobacillus actinomycetemcomitans*	1–4		Thrower et al., 1997
Quat-256	*L. monocytogenes*	1.9		Ronner and Wong, 1993
	S. typhimurium	1.6		Ronner and Wong, 1993
Other Biocides				
Bronopol	*Legionella pneumophila*	3–6		Wright et al., 1991
Glutaraldehyde	*Pseudomonas fluorescens*		>4	Eagar et al., 1988
Glutaraldehyde	*Legionella bozemanii*	1.8–5		Green and Pirrie, 1993
Glutaraldehyde	*E. aerogenes*	2–10		Stewart et al., 1998
Isothiazolone (Kathon)	*L. pneumophila*	3–6		Wright et al., 1991
Isothiazolone (Kathon)	*E. aerogenes*	1–11		Stewart et al., 1998
Phenoxyethanol	*E. coli*		1–2	Das et al., 1998
	S. epidermidis		1–2	Das et al., 1998
Phenolics				
Phenol	*E. coli*		1–2	Ntsama-Essomba et al., 1997
Chloroxylenol	*E. coli*		1	Das et al., 1998
	S. epidermidis		1	Das et al., 1998
Antibiotics				
Ampicillin	*E. coli*		>4	Morck et al., 1994
Amoxicillin	*Lactobacillus acidophilus*		>16	Muli and Struthers, 1998
Ciprofloxacin	*P. aeruginosa*	14		Jass and Lappin-Scott, 1996
	P. aeruginosa	5		Ishida et al., 1998
	P. aeruginosa	1.3		Vrany et al., 1997
	S. epidermidis	1.4		Hamilton-Miller and Shah, 1997
Metronidazole	*Gardnerella vaginalis*		4	Muli and Struthers, 1998
	Porphyromonas gingivalis		160	Wright et al., 1997
Tobramycin	*P. aeruginosa*	1–10		Anwar et al., 1989b
	P. aeruginosa	18–24		Anwar et al., 1989a
	P. aeruginosa		20	Nickel et al., 1985
Vancomycin	*S. aureus*	>14		Williams et al., 1997
	S. epidermidis	>17		Evans and Holmes, 1987
	S. epidermidis		4	Gristina et al., 1989

account for conventional antibiotic resistance. For these mechanisms to explain biofilm resistance, the genetic modifications would have to be present in the biofilm but absent in the planktonic state. This does not appear to be the case in general. Even clearly susceptible microorganisms acquire marked resistance in the biofilm mode of growth (Table 11.1). When dispersed from a biofilm, however, these microorganisms rapidly return to a susceptible state (Nickel et al., 1985; Eagar et al., 1988; Anwar et al., 1989b; Ntsama-Essomba et al., 1997; Williams et al., 1997).

11.2.1 Antimicrobial Depletion in the Bulk Fluid

If a biofilm exerts a chemical demand for the antimicrobial agent with which it is being challenged, then it is possible for these neutralizing reactions to reduce the bulk fluid concentration of the agent. Consider, for example, an antimicrobial susceptibility test performed first against a dilute suspension of planktonic cells and then against a heavily fouled biofilm specimen. It is not difficult to imagine that the antimicrobial concentration could be maintained during the planktonic test but significantly decreased during the course of the biofilm test. One could argue that this phenomenon is not a true resistance mechanism, but simply an unfair comparison. The biofilm is not being subjected to the same antimicrobial concentration as the planktonic reference test. However, it seems that this straightforward mechanism has been often overlooked. It would pay to keep antimicrobial depletion in the bulk fluid in mind as a possible explanation for poor antimicrobial performance against a biofilm. What this mechanism lacks in glamor it may recoup in practical importance.

Bulk fluid antimicrobial depletion can be diagnosed experimentally by measuring antimicrobial residual concentrations during both planktonic and biofilm tests. In some experimental systems, biofilm and planktonic disinfection can be measured in the same fluid, an approach that elegantly eliminates the possibility of unequal treatment concentrations.

11.2.2 Transport Limitation of Antimicrobial Penetration

Failure of an antimicrobial agent to rapidly or completely penetrate a biofilm is perhaps the most intuitively appealing explanation for biofilm resistance. Antoni van Leeuwenhoek invoked this mechanism more than three centuries ago in his seminal studies of dental plaque (which he called "scurf"):

> From whence I conclude, that the Vinegar with which I washt my Teeth, kill'd only those Animals which were on the outside of the scurf, but did not pass thro the whole substance of it.
>
> —A. van Leeuwenhoek, 1684

We distinguish two versions of the transport limitation resistance mechanism. The first postulates that the biofilm matrix constitutes a barrier to the inherent mobility of antimicrobial agents. According to this hypothesis, the matrix physically excludes antimicrobial compounds from the biofilm. While powerful and generic in its ability to explain antimicrobial resistance, this mechanism poses the paradox of how such a matrix barrier allows nutrients to pass while excluding biocidal molecules of similar size. Measurements of effective diffusion coefficients in biofilms (Stewart, 1998) indicate that, while diffusion is somewhat retarded in biofilms, diffusive transport nevertheless proceeds at rates of the same order of magnitude of those in pure water. Even molecules of the size of oligonucleotide probes, lectins, and others readily penetrate intact biofilms as evidenced by their

ability to stain the interior regions of biofilm specimens. The direct experimental demonstration of permeation of certain antimicrobial agents (Dunne et al., 1993; Darouiche et al., 1994; Kumon et al., 1994; Shigeta et al., 1997; Suci et al., 1994; Vrany et al., 1997) through biofilm also argues against a generic barrier to antimicrobial agent access.

The second and more plausible version of antimicrobial transport limitation in biofilms requires an interaction between the antimicrobial agent and the biofilm that neutralizes antimicrobial activity. The barrier to penetration in this case is reactive rather than physical: The rate of deactivation of the antimicrobial exceeds the rate of diffusive penetration. This mechanism is supported by experimental evidence in the case of hypochlorite (de Beer et al., 1994; Chen and Stewart, 1996; Xu et al., 1996), is likely to be important for other highly reactive oxidants, such as ozone and hydrogen peroxide, and may be a factor for some nonoxidizing biocides as well (Stewart et al., 1998). It is also realistic for certain antibiotics, such as the β-lactams, that are subject to rapid enzymatic degradation (Nichols et al., 1989; Nichols, 1991).

11.2.3 Physiological Limitation of Antimicrobial Efficacy

There are many instances in which a biofilm is too thin or is insufficiently reactive with the antimicrobial agent to manifest either of the preceding resistance mechanisms. In these cases, biological explanations for the reduced susceptibility of biofilm microorganisms are called for. We distinguish two general types of biological limitations to biofilm susceptibility. The first type of biological limitation to biofilm susceptibility requires that at least some of the cells in a biofilm experience nutrient limitation and therefore exist in a slow-growing or starved state (Brown et al., 1988; Gilbert and Brown, 1995). Such slow- or non-growing cells are hypothesized (or have been shown experimentally) to be less susceptible to many antimicrobial agents. The second type of biological limitation of biofilm susceptibility invokes the existence of a distinct, and relatively resistant, biofilm phenotype. This phenotype is not the result of a nutrient limitation. The hypothesized biofilm phenotype is adopted by a subset of the microbial population in a biofilm as a result of some other stimulus, for example, contact with a solid surface or attainment of a threshold cell density. Specific biological factors and mechanisms that may be involved in modulating biofilm susceptibility are discussed elsewhere (Gilbert et al., 1990; Gilbert and Brown, 1995).

11.3 PROCESS ANALYSIS

The action of an antimicrobial agent against a biofilm involves the complex interaction of multiple processes. Among the constituent phenomena that must be considered are: (1) bulk fluid flow into and out of the system, (2) consumption of the antimicrobial by reaction with biomass, (3) substrate utilization, (4) diffusive transport within the biofilm, (5) microbial disinfection, and (6) biofilm detachment. Additional processes that might confound the problem include corrosion, accumulation of abiotic particles in the biofilm, and mutation or adaptation. The interactions between these phenomena and the functional outcome of those interactions are not intuitive.

Precisely because of its complexity, the problem of antimicrobial control of biofilm is one that invites quantitative engineering analysis and modeling. Mathematical modeling is one way to organize and integrate phenomena systematically. A model is a means of both formulating a hypothesis and exploring its implications. Ultimately, using a model could be an effective way of performing design and scale-up tasks, such as selecting an appropriate

antimicrobial agent for a particular situation or developing optimal antimicrobial dosage protocols. A few examples of mathematical biofilm models that incorporate antimicrobial action can be found in the literature (Nichols et al., 1989; Al-Hoti et al., 1990; Rossman et al., 1994; Stewart, 1994; Lu et al., 1995; Dibdin et al, 1996; Stewart et al., 1996; Sanderson and Stewart, 1997). Rather than present a comprehensive model of this type, we have elected to focus here on the quantitative analysis of the constituent phenomena. These analyses constitute submodels that could be mixed and matched to compose an integrated overall model.

In this section, a series of example calculations are presented to illustrate the concepts presented in the preceding section, their real-world significance, and the application of process analytical approaches to describe these phenomena.

11.3.1 Antimicrobial Reaction

The following examples address the possibility that reaction between an antimicrobial agent and biofilm could reduce the bulk fluid concentration of the agent in all or part of the biofilm system. These analyses do not consider the gradient in antimicrobial concentration that may occur within the biofilm. This aspect is addressed separately in Section 11.3.2.2.

11.3.1.1 Antimicrobial Reaction—Depletion of a Biocide along the Length of a Pipe. A biocide introduced into a pipe colonized with biofilm can be depleted by reactions with the attached biomass. This reduces the bulk fluid concentration of the biocide as it flows along. Although the reaction may be relatively slow, the cumulative loss of antimicrobial may become significant in the case of a pipe whose length is measured in kilometers. The decay in bulk fluid concentration can be modeled by a differential material balance with the following form:

$$-v\frac{dC}{dy} = \frac{2kC}{R} \tag{11.1}$$

where C denotes biocide concentration (M L^{-3}); k, biocide surface reaction rate coefficient (L T^{-1}); R, pipe radius (L); v, average axial fluid velocity (L T^{-1}); and y, axial distance (L).

This model assumes ideal plug flow and reaction kinetics that are first order with respect to antimicrobial concentration. The surface reaction rate coefficient has units of velocity and can be understood as combining kinetic and mass transfer properties (Rossman et al., 1994). The two terms in the balance represent advection and surface reaction, respectively. The solution to this equation, taking $C = C_o$ at $y = 0$, is simply

$$\frac{C}{C_o} = \exp\left(-\frac{2k}{vR}y\right) \tag{11.2}$$

In other words, the biocide concentration decays exponentially with distance down the pipe. This phenomenon can be anticipated to occur for many biocides when introduced into a fouled pipe.

Example Calculation. Estimate the chlorine residual in a drinking water distribution pipe a distance 3 km (y) downstream from a point where it is measured to be 1 mg L^{-1} (C_o). The pipe diameter ($2R$) is 20 cm, the average velocity (v) is 50 cm s^{-1}, and the surface re-

action rate coefficient (k) has been estimated to be 5×10^{-4} cm s^{-1}. The reaction rate coefficient could account for reactions between chlorine and concrete, metallic iron or its corrosion products, as well as with attached biomass. The argument of the exponential is -0.6 and the calculated concentration is 0.55 mg L^{-1}. This represents a significant decrease in the bulk fluid residual which could be a factor in the survival of biofilm microorganisms in the distal regions of a pipe system. Rossman et al. (1994) describe more sophisticated calculations of this type.

11.3.1.2 Antimicrobial Reaction—Depletion of a Biocide in a Well-Mixed, Continuous Flow System. Here we outline an analysis of the reaction of a biocide introduced into a system whose hydrodynamics approximate those of a continuous stirred tank reactor (CSTR). A material balance of the following form can be derived:

$$V\frac{dC}{dt} = q_f - QC - kAC \tag{11.3}$$

where the variables and parameters are C, bulk fluid biocide concentration (M L^{-3}); t, time (T); V, system fluid volume (L^3); Q, total fluid flow rate out of system (L^3 T^{-1}); k, surface reaction rate coefficient (L T^{-1}); and q_f, biocide feed rate (M T^{-1}). The terms in Eq. (11.3), from left to right, represent the rate of accumulation of biocide in the circulating water, continuous feed rate of biocide, loss of biocide by fluid flow out of the system as either blowdown (water intentionally purged from the system) and drift (water droplets carried out of the system by wind), and loss of biocide by surface reaction. At steady state, the residual biocide concentration is

$$C = \frac{q_f}{(Q + kA)} \tag{11.4}$$

Example Calculation. Estimate the residual biocide concentration in a recirculating cooling water system to which the biocide is continuously fed. It is difficult to imagine a better environment for growing biofilms than cooling water towers. In these ubiquitous devices, warm water is circulated over packing material with a high surface area to volume ratio to facilitate evaporative cooling of the water (Fig. 11.2). Cooling water systems are prone to scale formation and biofouling. Consider the illustrative case specified as follows: system fluid volume, V, 200 m^3; combined blowdown and drift flow rate, Q, 2 m^3 h^{-1}; surface area to volume ratio, A/V, 50 m^{-1}; surface reaction rate coefficient, k, 0.01 m h^{-1}; biocide feed rate, q_f, 50 g h^{-1}. Then the steady state residual biocide concentration is calculated to be 0.49 g m^{-3} from Eq. (11.4). What this result shows is that surface associated reaction can easily control the residual biocide concentration that can be maintained in a cooling water system.

11.3.2 Antimicrobial Penetration

Penetration of antimicrobial agents into biofilm is determined by the interaction of diffusion, sorption, and reaction processes. Reaction in this case refers to any deactivating chemical modification or irreversible sequestration of the antimicrobial agent. Sorption refers to any reversible binding of the antimicrobial agent to a biofilm component, either biotic or abiotic. As illustrated by the examples below, when an antimicrobial agent reacts in a

biofilm its penetration can be retarded. While sorption can also retard penetration in theory, this aspect is not considered further here because in practice reaction has a far greater potential to limit biofilm penetration than does sorption.

11.3.2.1 Penetration of a Noninteracting Agent. We begin by considering the diffusion of a noninteracting antimicrobial into biofilm. By noninteracting it is meant that neither reaction nor sorption processes act on the antimicrobial agent within the biofilm. In this and other penetration models presented in this section, convective transport within the biofilm is assumed to be negligible. The transient diffusion of an antimicrobial (or any solute for that matter) into a slab biofilm in response to a step increase in the bulk fluid concentration of the solute is given by

$$\frac{\partial C}{\partial t} = D_e \frac{\partial^2 C}{\partial z^2} \tag{11.5}$$

$$\text{at } z = L_f,\ C = C_o \text{ for all } t > 0 \tag{11.6}$$

$$\text{at } z = 0,\ \frac{\partial C}{\partial z} = 0 \text{ for all } t > 0 \tag{11.7}$$

$$\text{at } t \leq 0,\ C = 0 \text{ for all } 0 \leq z \leq L_f \tag{11.8}$$

where z is a spatial coordinate indicating depth in the biofilm. Equation (11.6) is the diffusion equation, which constitutes an unsteady mass balance on the antimicrobial agent. The term on the left-hand side represents the local accumulation (or, if negative in value, disappearance) of the solute, and the term on the right-hand side represents the net change in concentration due to diffusion. Equations (11.6) and (11.7) are boundary conditions that impose constant concentration at the biofilm-bulk fluid interface and a no-flux condition at the substratum, respectively. In physical terms, the no-flux condition means that the sub-

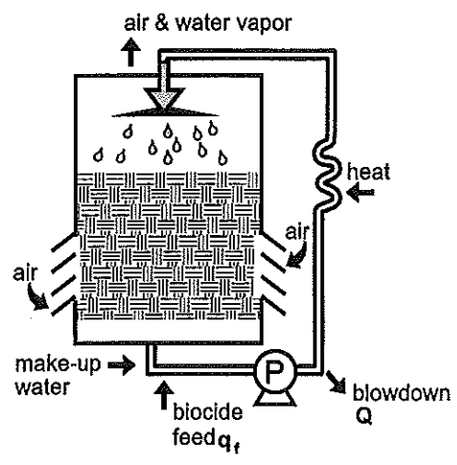

Figure 11.2 Hydraulics of a recirculating cooling water system.

TABLE 11.2. Diffusion Coefficients of Antimicrobial Agents in Water at 25°C

Solute	D_{aq} in cm²/s
Alkyl (C_{10}-C_{12}) dimethyl benzyl ammonium chloride	4.3×10^{-6}
Benzylpenicillin	5.0×10^{-6}
Bromine (Br_2)	1.3×10^{-5}
Ceftazidime	3.8×10^{-6}
Chlorhexidine	3.7×10^{-6}
Chlorine (Cl_2)	1.4×10^{-5}
5-Chloro-2-methyl-4-isothiazolin-3-one	9.1×10^{-6}
Ciprofloxacin	5.1×10^{-6}
Gentamicin	4.6×10^{-6}
Glutaraldehyde	9.3×10^{-6}
Hydrogen peroxide[a]	1.4×10^{-5}
Hypochlorous acid[b]	1.9×10^{-5}
Monochloramine	1.9×10^{-5}
Methylenebisisothiocyanate	8.9×10^{-6}
2-Methyl-4-isothiazolin-3-one	1.0×10^{-5}
Ozone	1.9×10^{-5}
Piperacillin	3.9×10^{-6}
Tobramycin	4.2×10^{-6}
Vancomycin	2.1×10^{-6}

[a]Van Stroe-Biezen et al., 1993.
[b]Comparison to ion mobilities of Cl^-, ClO_3^-, and ClO_4^- (Horvath, 1985).
Note: Unless otherwise noted, estimates are based on the Wilke-Chang correlation.

stratum is impermeable. Equation (11.8) is an initial condition that specifies zero concentration throughout the biofilm at time zero.

The parameter D_e is the effective diffusion coefficient in the biofilm. The value of D_e will be reduced compared to the diffusion coefficient in water, D_{aq}, due to the presence in the biofilm of microbial cells, extracellular polymeric substances, abiotic particles, and gas bubbles. Experimental measurements of the ratio D_e/D_{aq} have recently been reviewed (Stewart, 1998) and that article presents guidelines and formulae for estimating D_e/D_{aq}. D_e/D_{aq} depends on the physical-chemical nature of the diffusing solute (its size and whether it is charged) and the biomass density in the biofilm. Estimates and experimental measurements of aqueous diffusion coefficients for a number of antimicrobial agents are given in Table 11.2

The solution to Eqs. (11.5) through (11.8) is

$$\frac{C}{C_o} = 1 - 2\sum_{n=0}^{\infty} \frac{(-1)^n}{(n + 1/2)\pi} \exp[-(n + 1/2)^2 \pi^2 \alpha] \cos\left[(n + 1/2)\frac{\pi z}{L_f}\right] \tag{11.9}$$

which is reproduced graphically in Figure 11.3. The extent of penetration at any point depends on a single parameter, α, given by

$$\alpha = \frac{tD_e}{L_f^2} \tag{11.10}$$

Two example calculations of diffusive penetration times based on the scenario comprising Eqs. (11.5–11.8) and their solution (Eq. (11.9) Fig. 11.3) are presented next. These cal-

culated times corresponds to the most rapid possible diffusive delivery of antimicrobial into the biofilm as the analysis incorporates neither reaction nor sorption. As mentioned previously, reaction and sorption processes both work to impede or limit penetration of a diffusing solute.

Example Calculation. Estimate the time required for the antibiotic ciprofloxacin to attain, at the base of a 250 μm thick biofilm, one-half the bulk fluid concentration bathing the biofilm. In terms of parameters, we have $C/C_o = 0.5$ at $z = 0$, $L_f = 2.5 \times 10^{-2}$ cm, and $D_{aq} = 6.87 \times 10^{-6}$ cm^2 s^{-1}. From Figure 11.3, this degree of penetration corresponds to $\alpha = 0.38$. In the absence of system-specific information, a reasonable estimate of D_e/D_{aq} is 0.4 (Stewart, 1998). The effective diffusion coefficient of ciprofloxacin within the biofilm is then estimated to be 40% of 6.87×10^{-6} cm^2 s^{-1} or 2.75×10^{-6} cm^2 s^{-1}. The aqueous diffusion coefficient used here is slightly higher than the value quoted in Table 11.2 because it has been estimated at a temperature of 37°C to correspond to the presumed physiological condition. Then, solving Eq. (11.10) for t

$$t = \alpha \frac{L_f^2}{D_e} = 86s$$

That is, the antibiotic should penetrate this biofilm within a minute and a half. This time scale has been confirmed by experimental measurements of rapid penetration of fluoroquinilone antibiotics into *Pseudomonas aeruginosa* biofilms (Suci et al., 1994; Vrany et al., 1997). Certainly when compared to the duration of antimicrobial chemotherapy, which might be 5 or 10 days, this delay in delivery of the agent is insignificant. This calculation suggests that, in the case of an antibiotic that neither sorbs nor reacts in the biofilm, the barrier posed by diffusion alone is not a viable mechanism of biofilm resistance to prolonged antimicrobial chemotherapy.

However, there are many situations in which antimicrobial agents are normally applied for relatively brief durations. Examples could include rinsing with an antibacterial mouthwash, sanitizing a toilet bowl, or disinfecting a hospital counter top. In such cases it is im-

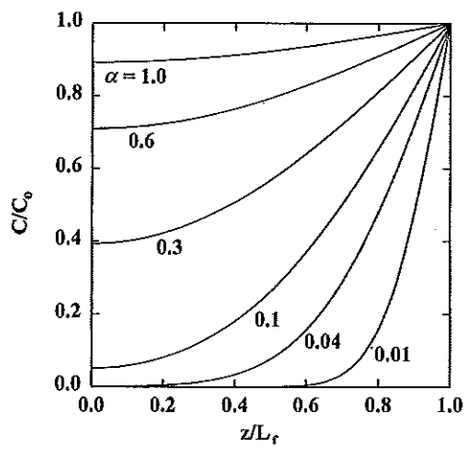

Figure 11.3 Transient diffusion of a noninteracting solute into a slab biofilm.

portant that the dose last sufficiently long to effectively reach the entire depth of the biofilm. The solution to the diffusion equation just presented can be used to estimate the minimum dose duration required.

Example Calculation. Estimate the minimum dose duration necessary to deliver a quaternary ammonium compound based disinfectant, at 90% of the bulk fluid concentration, to the base of a 1.5 mm thick biofilm growing in a crevice on a food-handling surface. The minimum dose duration is calculated by assuming that the disinfectant neither reacts nor is adsorbed within the biofilm; in other words, the result shown in Figure 11.3 applies. Ninety percent penetration at the substratum corresponds approximately to $\alpha = 1$ (Fig. 11.3). Taking 40% of the diffusion coefficient given in Table 11.2, the minimum dose time calculated from Eq. (11.10) is a remarkably long 3.6 h.

11.3.2.2 Penetration of a Reacting Antimicrobial Agent—Catalytic Reaction. A chemical reaction that deactivates an antimicrobial agent as it diffuses into a biofilm impedes effective penetration into the biofilm. In this section, we consider the case of a catalytic reaction. That is, the deactivating reaction proceeds without consuming any component of the biofilm. Examples of such catalytic deactivation include destruction of β-lactam antibiotics by β-lactamase enzymes and disproportionation of hydrogen peroxide by catalase enzymes. This physical situation is mathematically modeled by deriving a differential material balance that incorporates the processes of diffusion and reaction. The formulation and the solution to this problem depend on the intrinsic kinetics of the deactivating reaction. As a simple illustrative case, consider the simultaneous reaction and diffusion of an antimicrobial agent subject to zero-order reaction kinetics. Zero-order reaction kinetics mean that the reaction rate is independent of the antimicrobial concentration. This approximation can be justified, in the case of an enzyme reaction, when the bulk fluid concentration of antimicrobial agent is much greater than the Michaelis-Menten half saturation coefficient. The material balance takes the form

$$D_e \frac{d^2C}{dz^2} = k_o \tag{11.11}$$

where the two terms, from left to right, correspond to diffusion and reaction, respectively. The parameter, k_o, is a zero-order reaction rate coefficient with units of concentration per time. There is no time derivative here as there is in Eq. (11.5) because steady state has been assumed. The steady state solution represents the maximal extent of penetration that can be achieved. Boundary conditions on this balance are the same as those given in Eqs. (11.6) and (11.7). In this case of a catalytically reacting antimicrobial, distinct gradients in antimicrobial concentration persist within the biofilm (Fig. 11.4). The solution to this problem is conveniently framed in terms of a single dimensionless parameter known as the Thiele modulus and denoted here by ϕ, where, for the particular case of zero order kinetics,

$$\phi = \left(\frac{k_o L_f^2}{2C_o D_e}\right)^{1/2} \tag{11.12}$$

This parameter is actually the ratio of the maximum rate of reaction to the maximum rate of diffusion. When ϕ is small (< 1), diffusion is fast compared to reaction and the solute

penetrates well. When ϕ is large (> 1), diffusion is slow compared to reaction and the biofilm is never fully penetrated by the solute. Clearly the situation of $\phi > 1$ would afford an excellent explanation for failure of an antimicrobial agent to kill a biofilm completely: A portion of the biofilm in the deep layers is never exposed to the antimicrobial, no matter how long the exposure period.

The reactions that deactivate the antimicrobial agent might continue long after the microbial cell is dead. For example, neither of the two enzyme reactions mentioned previously are energy-dependent. Provided that the enzyme remains in the cell or trapped in the extracellular matrix, it will continue to catalyze deactivation of the antimicrobial independent of the physiological status of the microorganism from whence it came. In other words, dead cells could contribute to the protection of their viable neighbors because the reactive barrier to penetration is not tied to viability.

Example Calculation. Estimate the penetration depth of 50 mM hydrogen peroxide into a 100 μm thick *P. aeruginosa* biofilm. When zero-order kinetics prevail, the concentration of the penetrating solute will decrease to zero inside the biofilm if it is sufficiently thick. Let the penetration depth, denoted by a, be defined as the depth at which hydrogen peroxide is completely depleted. This distance is given by

$$a = \left(\frac{2C_o D_e}{k_o}\right)^{1/2} \tag{11.13}$$

Take D_e to be 50% of the value given in Table 11.2, or 7.15×10^{-6} cm^2 s^{-1}. Combining a typical biofilm protein density with specific catalase activities in *P. aeruginosa* (Brown et al., 1995), the order of magnitude k_o can be estimated to be 50 mM s^{-1} (Brown et al., 1995). Then the calculated penetration depth is 38 μm. Poor penetration could explain biofilm resistance to disinfection in this case, because 60% of the biofilm will never be exposed to the antimicrobial agent.

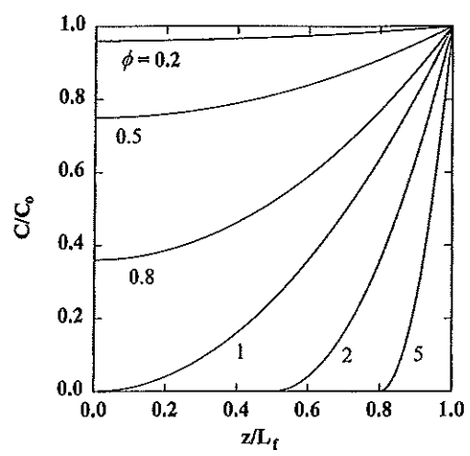

Figure 11.4 Simulated steady state concentration profiles within biofilm for a solute subject to consumption by a zero-order reaction.

Solutions to reaction-diffusion problems of the type outlined can be found in the literature for other kinetic models, such as first order and Michaelis-Menten kinetics. There are also several examples of modeling studies that apply these calculations to the problem of antimicrobial penetration in particular (Nichols et al., 1989; Dibdin et al., 1996).

11.3.2.3 Penetration of a Reacting Antimicrobial Agent—Stoichiometric Reaction. Here we consider the possibility that the biofilm contains a finite amount of antimicrobial neutralizing capacity that is depleted stoichiometrically as the antimicrobial agent is deactivated. An example of such a stoichiometric reaction might be the neutralization of hypochlorous acid by reaction with nitrogenous organic matter and polysaccharides. In the stoichiometric reaction scenario, the antimicrobial must deplete the neutralizing capacity of the biofilm layer by layer before it can fully penetrate the film. Stewart and coworkers have published full mathematical models of the penetration of a stoichiometrically reacting solute (Stewart and Raquepas, 1995; Stewart, 1997). Presented here is a simplified model that adequately simulates slow penetration. In this conceptual model, the antimicrobial agent diffuses through a continuously expanding zone of the biofilm in which deactivating capacity has been depleted. If the deactivating reaction is very fast, the concentration of the antimicrobial agent will approach zero at the distal side of this depleted zone. With these assumptions, the advance of the antimicrobial front through the biofilm is described by the following differential equation:

$$\frac{X_b}{Y_{xb}}\frac{da}{dt} = D_e\frac{C_o}{a} \tag{11.14}$$

which balances the rate of depletion of neutralizing capacity (left-hand term) with the diffusive flux of antimicrobial through the neutralizing capacity-depleted zone of the biofilm (right-hand term). In Eq. (11.14), a is the penetration depth of the antimicrobial, X_b is the biomass density in the biofilm, and Y_{xb} is a stoichiometric coefficient determining the amount of biomass in which neutralizing capacity is depleted per amount of antimicrobial neutralized. With an initial condition of $a = 0$ at $t = 0$, the solution to this equation can be written in terms of the time required to fully penetrate the biofilm, which is given by

$$t = \frac{X_b L_f^2}{2D_e C_o Y_{xb}} \tag{11.15}$$

Example Calculation. Estimate the time required for hypochlorite bleach at 10 mg/L chlorine to fully penetrate a 400 μm thick biofilm. Suppose that the biofilm biomass density is 10,000 mg/L and the effective diffusion coefficient of hypochlorite in the biofilm is 1×10^{-5} cm^2 s^{-1}. The yield coefficient for chlorine interacting with bacterial biofilm is approximately 1 mg biomass per mg chlorine (Characklis, 1990; Chen and Stewart, 1996). From Eq. (11.15), the calculated penetration time is 80,000 s or 22 hours. Such long penetration times for chlorine are consistent with direct experimental measurements of profoundly retarded penetration of this antimicrobial agent (de Beer et al., 1994; Chen and Stewart, 1996; Xu et al., 1996).

To be able to use either of the penetration theories outlined previously, one must first determine whether the reaction between antimicrobial and biofilm is catalytic or stoichiometric. Performing a calculation requires an estimate of either a reaction rate coefficient or a stoichiometric coefficient, depending on which type of reaction occurs. For most antimi-

crobial agents, these coefficients are unknown and data in the literature are inadequate even for the purpose of making rough estimates.

11.3.2.4 Penetration of a Reacting Antimicrobial Agent—Uncharacterized Reaction. Because the kinetics and stoichiometry of antimicrobial reactions with biofilm are largely uncharacterized, the preceding penetration theories are difficult to apply in most instances. It is possible, nevertheless, to make an assessment of the potential for penetration limitation by comparing the observed overall rate of reaction of the antimicrobial with an estimate of its characteristic diffusion rate. This comparison is made by calculating an observable modulus defined by

$$\Phi = \frac{R_o L_f^2}{D_e C_o} \tag{11.16}$$

where R_o is the observed volumetric reaction rate of antimicrobial agent within the biofilm (Bailey and Ollis, 1986). The units of R_o could be, for example, mg of biocide per cm^3 of biofilm per second. Φ is dimensionless. When its numerical value is clearly greater than one, incomplete penetration is likely. When Φ is clearly less than one, biocide penetrates the biofilm effectively.

Example Calculation. Evaluate the potential for biocide penetration limitation in the hypothetical example considered in Section 11.3.1.2. Assume a biofilm thickness of 1 mm. The measured bulk residual concentration of biocide is 0.5 g m^{-3}. The observed rate of reaction in this case is

$$R_o = \frac{q_f - QC}{AL_f} \tag{11.17}$$

and R_o is calculated to be 4.9 g m^{-3} h^{-1}. The observable modulus, Φ, has a value of 2.7 from Eq. (11.16) indicating probable incomplete penetration of the biocide.

11.3.2.5 Antimicrobial Penetration—Summary. Biofilms are mostly water and diffusion occurs within a biofilm at rates not too different from that in pure water. Diffusion alone, therefore, does not pose a significant barrier to antimicrobial penetration except in the case of thick biofilms subject to relatively brief challenges. However, if the antimicrobial agent reacts with biofilm constituents in a way that destroys its biocidal properties, then profound penetration failure is possible. A biofilm acts like a porous but reactive sponge that deactivates antimicrobial in the surface layers of the biofilm faster than it can diffuse in. This resistance mechanism is clearly operative in the case of reactive oxidants such as hypochlorite and hydrogen peroxide and contributes to reduced biofilm susceptibility to these agents (de Beer et al., 1994; Chen and Stewart, 1996; Xu et al., 1996; Liu et al., 1998).

11.3.2.6 Antimicrobial Penetration—Biofilm Structural Heterogeneity and External Mass Transfer Resistance. Two other issues that require comment when discussing transport phenomena in biofilms are biofilm structural heterogeneity and external mass transfer resistance. Some biofilms can exhibit remarkable structural heterogeneity with clusters of microorganisms separated by water channels through which fluid flows

(see Chapter 3). This observation appears to invalidate the assumptions common to the preceding penetration models of a uniformly thick slab biofilm in which no convective transport occurs. We contend that these simple models are still useful. Structural heterogeneity alters the geometry of the problem, but does not change the fundamental phenomena at work: diffusion and reaction. Within a cell cluster, diffusion is the dominant transport process (de Beer and Stoodley, 1995). Slab biofilm models correctly simulate penetration phenomena, at least qualitatively if not quantitatively. There are also ways to improve the quantitative accuracy of such models. The obvious way to deal with structural heterogeneity in a penetration model is to assume or specify a more realistic biofilm geometry and then solve the equations for this geometry. The math may be somewhat more complex but in the age of computers this does not represent an obstacle.

Example Calculation. Repeat the calculation of diffusive penetration time of a quaternary ammonium compound present in 11.3.2.1 for a biofilm with the heterogeneous structure illustrated in Figure 11.5. The cell clusters have a radius of $R = 3308$ μm. If the biomass in the heterogenous biofilm were spread out into a uniformly thick biofilm it would be 1500 μm thick, the same depth used in the original calculation. In other words, the biomass in the slab biofilm has simply been redistributed into a heterogenous structure. An exact analytical solution to the problem of unsteady diffusion in spherical coordinates is available (Bird et al., 1960). This solution yields a 90% penetration time of 6.0 h, considerably longer than the 3.6 h calculated for a uniformly thick (1500 μm) biofilm. This comparison suggests that heterogeneity will tend to lengthen diffusive penetration times for noninteracting solutes.

When the antimicrobial reacts in the biofilm and a reaction-diffusion model is called for, different approaches for accounting for biofilm structural heterogeneity are appropriate. One of these is to use the slab biofilm model result but replace biofilm thickness with the biofilm volume to surface area ratio. The surface area is that of the biofilm–fluid interface,

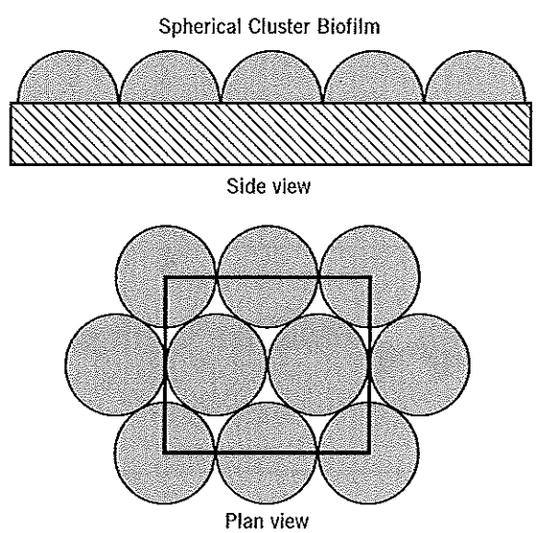

Figure 11.5 Model of a structurally heterogenous biofilm. The biofilm consists of hemispherical cell clusters arranged in a regular array on the substratum.

not the substratum surface area. The theoretical basis for this substitution is well established (Aris, 1957; Bird et al., 1960). In this case, structural heterogeneity enhances access of antimicrobial agent to the biofilm and increases the extent of penetration.

Example Calculation. Repeat the calculation of the penetration depth of hydrogen peroxide presented in 11.3.2.2 for a biofilm with the heterogeneous structure illustrated in Figure 11.5. The cell clusters have a radius of $R = 221$ μm. If the biomass in the heterogeneous biofilm were spread out into a uniformly thick biofilm it would be 100 μm thick, the same depth used in the original calculation. An exact analytical solution to the problem of simultaneous zero-order reaction and diffusion in spherical coordinates can be derived. This solution yields a penetration depth of 40 μm, which corresponds to exposure of 46% of the biofilm volume to the antimicrobial agent. For comparison, recall that 38% of the equivalent slab biofilm was exposed to hydrogen peroxide. Substituting the biofilm volume to surface area ratio, which in the case of a hemisphere is $R/3$, into the slab biofilm model as suggested previously predicts that 51% of the biofilm would be exposed. This correction may be adequate for many purposes. The enhancement of antimicrobial penetration due to heterogenous biofilm structures is relatively modest if this calculation is representative.

External mass transfer resistance refers to the diffusive resistance presented by the slow-moving fluid immediately adjacent to the biofilm. Even in a turbulent flow, flow velocities close to the biofilm are greatly reduced. An antimicrobial agent in the bulk fluid must pass through this quiescent fluid layer before it can move into the biofilm. The additional retardation of antimicrobial penetration posed by external mass transfer resistance was not incorporated in any of the preceding analyses of biofilm penetration. Obviously, external mass transfer resistance, when present, exacerbates the limitation to antimicrobial agent penetration arising from any of the mechanisms discussed previously. External mass transfer resistance can be quantitatively accounted for in any of these penetration models by replacing the boundary condition Eq. (11.6) with a so-called matching flux condition of the form

$$D_e \frac{dC}{dz}\bigg|_{z=L_f} = k(C_o - C_s) \tag{11.18}$$

where C_o is the bulk fluid concentration, C_s is the concentration at the biofilm-bulk fluid interface, and k is a mass transfer coefficient. The mass transfer coefficient can be estimated

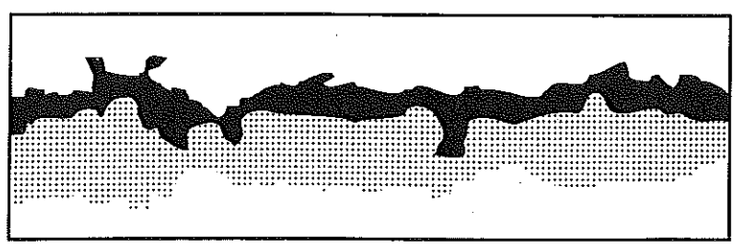

Figure 11.6 Physiological heterogeneity within a *Klebsiella pneumoniae* biofilm cross-section revealed by staining with acridine orange. Dark shading indicates a region where bacteria are growing and light shading indicates a region where bacteria are growing slowly or not at all. The substratum was at the bottom and nutrient medium at the top. After Wentland et al., 1995.

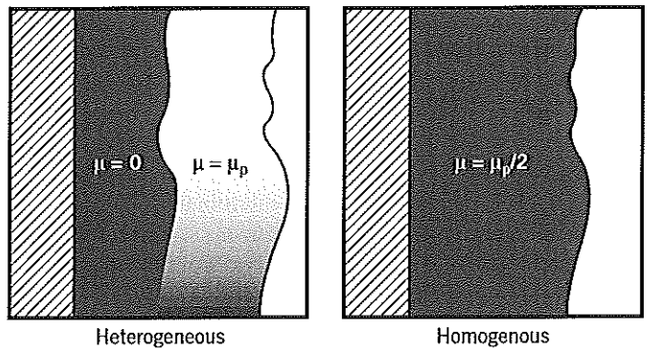

Figure 11.7 Alternative conceptual models for biofilm growth. The planktonic growth rate is denoted by μ_p. The substratum is on the left and the bulk fluid on the right.

by existing correlations or by reference to experimental measurements of this parameter in biofilm systems.

11.3.3 Biofilm Physiology

The physiological status of microorganisms within a biofilm probably plays a central, perhaps dominant, role in governing biofilm susceptibility to antimicrobial agents. Experimental data to date suggest that antimicrobial agent penetration limitation is at best only a partial explanation for reduced biofilm susceptibility (Stewart et al., 1998). Microorganisms are remarkably flexible in the phenotypes they adopt. To complicate matters, we can anticipate microscale spatial heterogeneity in physiological status within biofilms, even within pure culture biofilms (Fig. 11.6). In this section we attempt to provide a quantitative framework for modeling and understanding the potential for physiological limitation of biofilm susceptibility to disinfection.

A basic model for microbial disinfection is

$$r_{\text{dis}} = -k_{\text{dis}}XC \tag{11.19}$$

where r_{dis} is the rate of disinfection, X is the viable cell density, and k_{dis} is a disinfection rate coefficient. It is important to recognize that the disinfection rate coefficient, in this or in any other model of disinfection, can not be regarded as a constant. Indeed in all likelihood, k_{dis} is hugely variable, not only between organisms, but with genotype and phenotype for a particular species. The disinfection rate coefficient could change depending on physiological status, the implementation of an adaptive stress response, or by mutation. Other models of microbial disinfection are described elsewhere (Wickramanayake and Sproul, 1991).

11.3.3.1 Disinfection of a Physiologically Heterogeneous Biofilm. The spatial heterogeneity of physiological status within a biofilm may be a crucial issue in determining susceptibility to antimicrobial agents. To illustrate this point, consider the action of

an antimicrobial agent whose killing is growth rate-dependent. The disinfection model is modified in this case to incorporate dependence on the local specific growth rate of the microorganisms, denoted by μ:

$$r_{dis} = -k_{dis}\mu XC \tag{11.20}$$

Now consider two distinct scenarios regarding microbial growth rate profiles within the biofilm as depicted in Figure 11.7. In the homogeneous scenario, the whole biofilm grows at the same rate, which is reduced by a factor of two from the planktonic growth rate. In the heterogeneous scenario, the top half of the biofilm grows at the planktonic rate while the bottom half of the biofilm is nongrowing. The average growth rate of the biofilm is the same in both cases, namely, one-half the planktonic growth rate. The response to a growth-rate dependent antimicrobial agent is expected to be drastically different in these two scenarios, as shown in Figure 11.8. The growing half of the heterogeneous biofilm will be killed, but the nongrowing (yet still viable) half is impervious to killing. The maximum kill that can be realized in the heterogeneous biofilm is therefore 50%, which corresponds to a log reduction of about 0.3. The homogeneous biofilm can be completely killed, albeit at half the rate of the planktonic cells. This thought experiment underscores the need to go beyond the use of community averages of biofilm activity to a fuller understanding of physiological heterogeneity and its implications.

11.3.3.2 Growth Rate-Dependent Killing in a Nutrient-Limited Biofilm. Nutrient limitation and growth rate variations within biofilms are well known and these phenomena have been mathematically modeled by civil and environmental engineers for nearly 30 years. Such models are based on an analysis of simultaneous reaction and diffusion exactly like that presented in Eq. (11.11), except that the reaction and diffusion processes pertain to the growth-limiting nutrient rather than to an antimicrobial agent. By coupling such a growth rate model to a model of disinfection, such as Eq. (11.20), one can predict the outcome of biofilm disinfection by a growth rate-dependent antimicrobial agent.

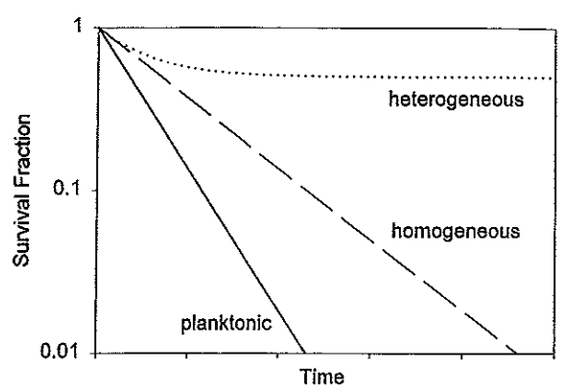

Figure 11.8 Expected killing of planktonic, physiologically homogeneous biofilm, and physiologically heterogeneous biofilm by a growth rate-dependent antimicrobial agent.

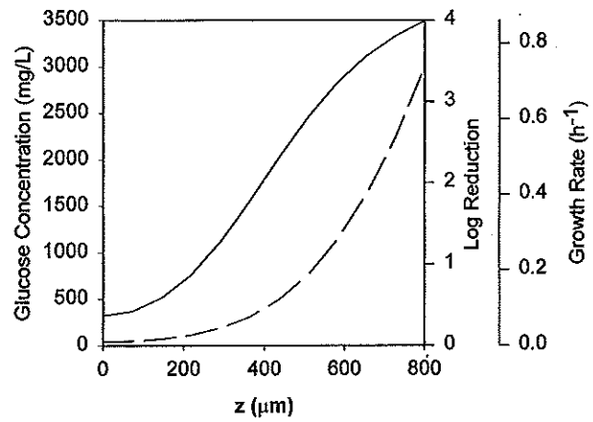

Figure 11.9 Simulated glucose concentration profile (dashed line, left axis), growth rate profile before treatment (solid line, second right axis), and survival profile after treatment (solid line, first right axis) within a biofilm subject to killing by a growth rate-dependent antibiotic.

Example Calculation. Determine the pretreatment growth rate profile and posttreatment survival profile within a sugar-fermenting biofilm subjected to a 4 h dose of ampicillin. The biofilm is 800 μm thick, the biofilm cell density is 20,000 mg L^{-1}, and the limiting nutrient is glucose with a bulk fluid concentration of 3000 mg L^{-1}. Independent measurements with planktonic cells indicate a Monod half saturation coefficient, K_m, of 500 mg L^{-1}, a maximum specific growth rate of 1 h^{-1}, a growth yield, Y_{xs}, of 0.5 mg mg^{-1}. The antibiotic dose results in a 4 log reduction when applied to planktonic cells growing in the bulk fluid. The balance of glucose diffusion and reactive utilization by the microorganisms is expressed by

$$D_e \frac{d^2C}{dz^2} = \frac{\mu_{max} C}{C + K_m} \frac{X_b}{Y_{xs}} \tag{11.21}$$

Boundary conditions Eqs. (11.6) and (11.7) complete the mathematical statement of this problem. This problem does not have an analytical solution, but can be solved numerically by any number of commercially available software packages. The resulting solution, which takes the form of concentration versus distance information, is substituted into the Monod kinetic expression to solve for the specific growth rate as a function of depth in the biofilm. This growth rate profile is plotted in Figure 11.9 for the case at hand. As expected, the growth rate declines with depth into the biofilm: It is calculated to be 0.08 h^{-1} at the substratum which is less than 10% of the planktonic growth rate of 0.86 h^{-1}.

When the disinfection submodel, Eq. (11.20), is applied to the growth rate profile described previously, the local killing is calculated with the result shown in Figure 11.9. The growing region of the biofilm near the biofilm-bulk fluid interface is readily killed, but the slow growing interior region of the biofilm experiences poor killing. The average log reduction across the entire biofilm is 0.97. Since the same treatment applied to planktonic microorganisms would result in a 4 log reduction, the calculated biofilm resistance factor (see Section 11.1.2) is 4.1

Specific growth rate is a familiar physiological index that can be quantified and modeled. Certainly for antimicrobial agents for which a clear growth rate dependence has been established, such as for some antibiotics, slow growth in some regions of a biofilm affords a powerful explanation for biofilm reduced susceptibility.

11.3.3.3 The Resistant Biofilm Phenotype. We explore here the possibility that a subpopulation of the microorganisms in a biofilm adopts a resistant phenotype. Consider a biofilm in which both susceptible and resistant phenotypes are expressed, possibly in a stratified manner as sketched in Figure 11.10. The spatial orientation of these layers is not critical to the following derivations, but is consistent with recent demonstrations of physiological heterogeneity within biofilms (Wentland et al., 1996; Xu et al., 1998). Since we assume complete penetration of the antimicrobial in the following analyses, the exact spatial distribution of the two phenotypes is irrelevant. The two phenotypes each constitute a fraction of the total biofilm population. Let ε_r denote the resistant fraction; $1 - \varepsilon_r$ is, therefore, the susceptible fraction. Suppose that the disinfection rate coefficient of the susceptible population is k_{dis} and the disinfection rate coefficient of the resistant subpopulation is given by pk_{dis}. The parameter p is a fraction less than one that reflects the fact that the disinfection rate of the resistant population is reduced compared to the susceptible population.

When this conceptual model is married to a disinfection model, such as Eq. (11.20), killing curves with a characteristic bilinear shape are obtained as illustrated in Figure 11.11. What is interesting is that clear examples of such bilinear killing of biofilm microorganisms can be found in the literature (Frank and Koffi, 1990; Oie et al., 1996). Of course, biphasic killing is also commonly encountered in planktonic disinfection. The difference is that much higher resistant fractions can be inferred from biofilm disinfection data. While no more than circumstantial evidence, these observations are consistent with the existence of

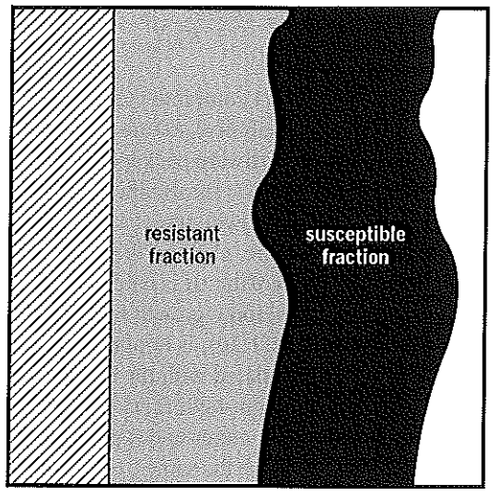

Figure 11.10 Conceptual model of distribution of resistant and susceptible biofilm cells. The substratum is on the left and the bulk fluid on the right.

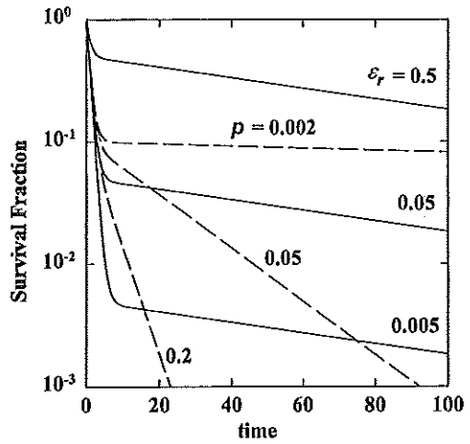

Figure 11.11 Simulated microbial survival in a completely penetrated biofilm expressing susceptible and resistant phenotypes in various proportions. The resistant fraction is denoted by ε_r and the relative susceptibility of the resistant fraction by p. Solid lines show simulations for three different values of ε_r and a fixed value of p (0.01) while the dashed lines show simulations for three different values of p and a fixed value of ε_r (0.1).

a generically resistant biofilm phenotype and should motivate continued investigation of this possibility.

11.3.4 Detachment

Detachment occurs continuously and naturally in every biofilm system. In most cases detachment is the primary process balancing microbial growth and driving the biofilm to a steady state level of accumulation. Despite the central importance of detachment in biofilm development, very little is known about the biological, chemical, and physical mechanisms of detachment. In many biofouling situations, a clean surface is the desired endpoint of a biofilm control program. Certain antimicrobial agents achieve this indirectly by stopping growth and allowing the natural detachment process to slowly remove the biofilm. In this section, detachment subsequent to antimicrobial treatments is discussed.

One of the common mathematical submodels of biofilm detachment, and one for which some phenomenological justification can be argued (Stewart, 1993), states that detachment rate is proportional to the square of biofilm thickness:

$$r_{\text{det}} = k_{\text{det}} L_f^2 \tag{11.22}$$

The order of magnitude of $k_{\text{det}} L_f$ is 0.1 h^{-1} or less, which means that the time scale for detachment is 10 h or more. Detachment is a slow process. It is certainly much slower than disinfection, which occurs over a time scale of minutes or seconds. Consider, for example, a biofilm that has been completely killed by an antimicrobial treatment. The balance that describes this situation is

$$\frac{dL_f}{dt} = -k_{\text{det}} L_f^2 \tag{11.23}$$

and its solution, given the initial condition $L_f = L_{fo}$ at $t = 0$, is

$$\frac{L_f}{L_{fo}} = \frac{1}{1 + k_{\text{det}} L_{fo} t} \tag{11.24}$$

This result is plotted in Figure 11.12 for an illustrative case. According to this simulation, treatment of a biofilm with an antimicrobial agent, even if it completely disinfects the biofilm, will not lead to immediate removal of the biofilm. Rather, dead biomass will gradually slough off over a period of many hours. There is some experimental data consistent with this model (Srinivasan et al., 1995; Sanderson and Stewart, 1997).

Stewart (1993) has suggested that part of the detachment process may be growth rate-dependent. If this is the case, then detachment after a killing antimicrobial treatment would be slower than predicted by Eq. (11.24). Other data (Tijhuis et al., 1995) indicate that some portion of biofilm remains attached even after prolonged periods of no microbial growth. This case could be simulated by a detachment model of the form

$$r_{\text{det}} = \begin{cases} k_{\text{det}}(L_f - L_{f*})^2 & L_f > L_{f*} \\ 0 & L_f \leq L_{f*} \end{cases} \tag{11.25}$$

where L_{f*} is the thickness at which detachment ceases. An illustrative result is shown in Figure 11.12.

However, some antimicrobial treatments might be expected to induce detachment, of at least a part of the biofilm, much more rapidly than predicted by the above models. This possibility could be modeled by assuming that a fraction of the biofilm thickness, denoted by

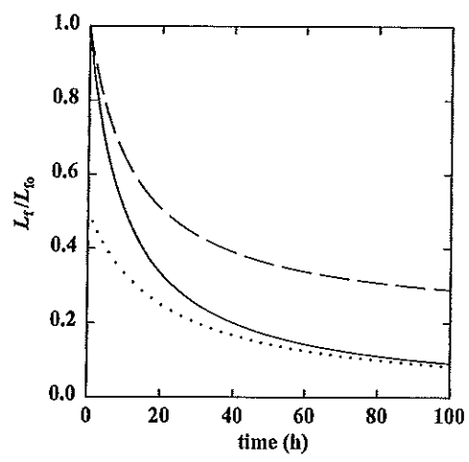

Figure 11.12 Biofilm detachment following a lethal antimicrobial treatment as predicted by three different models. Basic model (Eqs. (11.22) to (11.24), $k_{\text{det}} L_{fo} = 0.05\ \text{h}^{-1}$), solid line; basic model with a fraction of the biofilm that never detaches (Eq. (11.25); $L_{f*} = 0.2\, L_{fo}$), dashed line; basic model with instantaneous detachment of a fraction of the biofilm (Eq. 11.26; $A = 0.5$), dotted line.

A, detaches instantaneously upon antimicrobial treatment. This leads to the following equation for a completely killed biofilm

$$\frac{L_f}{L_{fo}} = \frac{1 - A}{1 + k_{\text{det}} L_{fo}(1 - A)t} \tag{11.26}$$

which is also illustrated in Figure 11.12.

These models are mostly speculation. Clearly, experimental data on detachment rates following antimicrobial treatment are needed to advance the ability to model this process.

11.4 PRACTICAL CONCERNS

The preceding examples of process analysis, many of which contain inaccessible parameters and hypothetical mechanisms, might well frustrate practitioners faced with the challenge of controlling biofouling in the real world. This section attempts to link the ideas regarding fundamental biofilm resistance mechanisms presented previously to more practical aspects of biofilm control. In particular, we discuss possible new approaches and technologies for improved biofilm control and conclude with some remarks about the need for further development of biofilm testing methods.

11.4.1 Suggestions for Improving Biofilm Control When Penetration Is Limiting

In this section we consider practical suggestions for improving antimicrobial performance against a biofilm in the particular case of a poorly penetrating biocide. The first suggestion is to treat the biofilm when it is thin. Biofilm penetration times are more sensitive to biofilm thickness than to any other single parameter. In the case of a stoichiometrically reacting biocide, for example, penetration time is proportional to the square of biofilm thickness (Eq. (11.15)). In other words, a doubling of biofilm thickness would necessitate using four times the biocide concentration or four times as long a dose duration to achieve full penetration.

While treating the biofilm when thin may seem to be an obvious suggestion, it led us to a dosing strategy that was not immediately intuitive. This strategy, which emerged from exploratory simulations with a computer model (Stewart et al., 1996), calls for two sequential and appropriately timed biocide doses. The second dose is delivered when the biofilm is at its thinnest point following the first dose. The time required to reach minimum thickness after the first dose is typically considerably longer than the time required to reach the maximum kill in the biofilm. This results because detachment is a much slower process than disinfection. According to this concept, the second dose will have maximal effect if added when the system is least fouled. This contradicts most current practices, which call for dosing when an unacceptable threshold of planktonic or biofilm cell numbers is reached.

We recently tested this double-dosing strategy experimentally and met with failure (Sanderson and Stewart, 1997). A second dose of monochloramine delivered 40 to 60 h after a first dose, rather than being more effective than the first dose, was actually clearly less effective. This result contradicted the model prediction. It was hypothesized that bacteria were able to adapt after exposure to the first dose to become less susceptible to monochloramine.

A second recommendation to improve reactive biocide penetration and performance is to decrease external mass transfer resistance to biocide delivery into the biofilm. In practical terms, this equates to keeping the bulk fluid moving. If slow penetration of a biocide into biofilm is indeed an issue, then there is no surer way to exacerbate this limitation than by applying the antimicrobial agent in a static soak. The delivery of a biocide to a reactive surface (i.e., biofilm) from a stagnant fluid is highly inefficient.

A third suggestion for improving biocide performance against biofilms is to weigh both biocide disinfecting power and reactivity when selecting a biocide. A strong disinfectant, if it is also highly reactive, may fail to penetrate biofilm well and thus be limited to incomplete disinfection of the biofilm. This phenomenon is underscored by an experimental comparison of chlorine and monochloramine made using alginate gel bead artificial biofilms. Equal quantities of gel beads containing *P. aeruginosa* were added to stirred beakers containing equivalent concentrations of chlorine, one in the form of free chlorine (pH 7) and one in the form of monochloramine (pH 8.9). Free chlorine was consumed much more rapidly than was monochloramine (Fig. 11.13A), indicating more rapid reaction of free chlorine than monochloramine in the gel beads. Monochloramine was able to disinfect the gel beads well, while free chlorine, the stronger disinfectant, scarcely caused any drop in cell numbers in the beads (Fig. 11.13B). The explanation for the poor performance of free

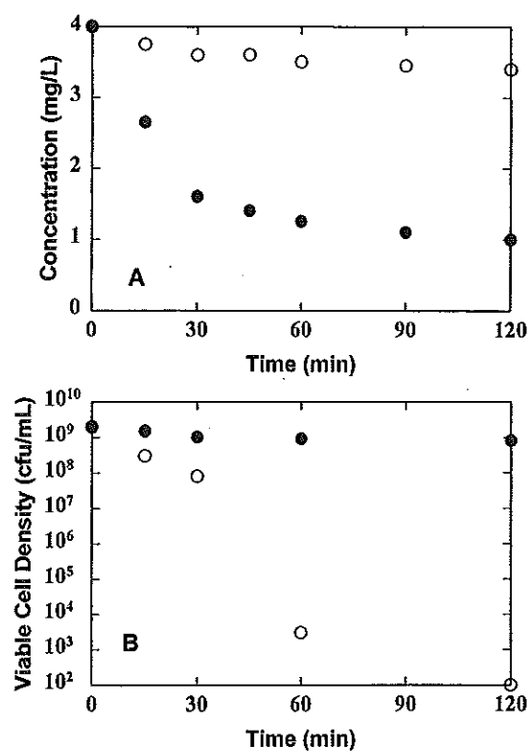

Figure 11.13 Comparison of monochloramine at pH 9 (○) and hypochlorite at pH 7 (●) reaction with (*A*) and disinfection of (*B*) gel bead artificial biofilms. See text for discussion. Unpublished data of Xu and Stewart; see Xu et al., 1996 for methods.

chlorine in this case is that it fails to penetrate the beads because of its high reactivity. The less reactive monochloramine penetrates more effectively and, even though it is a weaker disinfectant than free chlorine, can reach and disinfect the bead interior.

11.4.2 Suggestions for Improving Biofilm Control When Physiology Is Limiting

In this section, we speculate about possible approaches for manipulating or bypassing the physiological status of microorganisms within a biofilm to improve antimicrobial efficacy. The potential to subvert physiological resistance would be greatly increased by knowledge of the specific biological nature of the barrier. For example, if reduced susceptibility stems solely from low specific growth rates in the biofilm, then one could imagine prescribing a pretreatment with nutrients to stimulate growth and place the microorganisms in a more susceptible state. If the resistant biofilm phenotype is based on reduced permeability of the cell envelope, then cotreatment with a permeabilizing agent, such as an organic solvent, might improve antimicrobial performance. Targeted prescriptions such as these await better definition of the particular physiological changes that render biofilms cells less susceptible.

Another approach that can be envisioned for overcoming physiological limitations to biofilm disinfection is to discover alternative antimicrobial agents that are effective against resistant phenotypes. There probably exist antibiotics and biocides that have been dismissed as inferior based on tests against planktonic microorganisms but whose relative efficacy against biofilms would make them superior for this application. As an example, consider the possibility of overcoming the limitations of growth rate-dependent antibiotics by screening for agents based on their efficacy against nongrowing bacteria. Such agents are unlikely to prevail in traditional planktonic or agar plate screening approaches, but might well offer improved performance against slow-growing biofilms. Similarly, we predict the existence of antimicrobial agents or formulations that target resistant biofilm phenotypes and will therefore be more effective than many currently used agents. The challenge is to develop suitable screening protocols centered around biofilm testing methods because existing planktonic methodologies are simply incapable of identifying biofilm-effective antimicrobials.

11.4.3 Novel Control Strategies

A few interesting potential strategies for controlling biofilms have been described in recent years and deserve mention here. Wood et al. (1996) created catalytic surfaces that generate highly reactive hydroxyl radicals when exposed to a suitable biocide precursor such as hydrogen peroxide or potassium monopersulfate. The hydroxyl radicals are generated right at the base of the biofilm where they degrade the matrix and cause detachment of the biofilm. By generating the active agent in-situ, this scheme evades the penetration limitation that would inevitably occur in attempting to deliver a species as reactive as the hydroxyl radical from the bulk fluid.

Costerton and co-workers described the electrical enhancement of antimicrobial efficacy, which they termed the "bioelectric effect" (Blenkinsopp et al., 1992). Subsequent reports have reproduced this effect (Jass et al., 1995; Jass and Lappin-Scott, 1996; Wellman et al., 1996). For example, the efficacy of killing of *P. aeruginosa* biofilm by the antibiotic tobramycin can be enhanced as much as 4 to nearly 8 log reductions by the application of direct current (Wellman et al., 1996). The bioelectric effect is interesting not only as an alternative control strategy but also for the light it may shed on the underlying mechanisms

of reduced biofilm susceptibility. In the case of *P. aeruginosa* and tobramycin, unpublished data from our lab suggest that the mechanism of enhancement is either alleviation of growth limitation by provision of the limiting nutrient (oxygen) or potentiation of antibiotic efficacy by induction of oxidative stress in the cell.

The discovery of a role for cell-to-cell communication in the development of microbial biofilm (Davies et al., 1998) raises the possibility that signaling molecules may mediate biofilm resistance to antimicrobial agents. Davies showed, for example, that a mutant *P. aeruginosa* biofilm unable to synthesize an acyl-homoserine lactone signaling molecule was more susceptible to removal by sodiuim dodecyl sulfate. If signaling does play a role in determining biofilm susceptibility to antimicrobial agents, then it might be possible to find analogues of the natural signaling molecules that would interfere with the communication and thereby render the biofilm more susceptible to disinfection or removal.

11.4.4 Biofilm Testing Methods

At this juncture in the evolution of the biofilm concept it is still impossible to predict the efficacy of an antimicrobial agent against a biofilm based on experiments performed with planktonic microorganisms. The performance of antimicrobial agents against biofilm is modulated by such phenomena as expression of biofilm phenotypes, reactive neutralization of antimicrobial agents, detachment, and diffusion that are manifested distinctly in the biofilm mode of growth. These essential phenomena are not captured by tests that use microorganisms in suspension. To put it very bluntly, planktonic disinfection measurements do not even remotely simulate the biofilm state and should not be used as a basis for designing biofilm control strategies.

Biofilm control will only be advanced by the deliberate application of biofilm-based testing methods. The current lack of recognized standard biofilm testing methodologies poses a real barrier to the discovery, understanding, and development of practical biofilm control technologies. Many biofilm methods dwell in the technical literature, but a new generation of methods is now needed. These methods should be developed with an awareness of the mechanisms of biofilm resistance discussed in this chapter, must be validated by comparison with field or in-vivo systems, and, crucially, must win the attention and acceptance of regulatory agencies.

ACKNOWLEDGMENT

This work was supported by Cooperative Agreement EEC-8907039 between the National Science Foundation and the Center for Biofilm Engineering and by National Science Foundation grant BES-9623233.

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