

Biofilms in the Aquatic Environment

Edited by

C.W. Keevil

Centre for Applied Microbiology and Research, Porton Down, Salisbury, UK

A. Godfree

Northwest Water Ltd, Warrington, UK

D. Holt

Thames Water Utilities, Reading, UK

C. Dow

Department of Biological Science, University of Warwick, Coventry, UK

The proceedings of the International Conference on Biofilms in Aquatic Systems, held on 13–16 April 1997, at the University of Warwick, UK.

Special Publication No. 242

ISBN 0-85404-758-1

A catalogue record for this book is available from the British Library

© The Royal Society of Chemistry 1999

All rights reserved.

Apart from any fair dealing for the purpose of research or private study, or criticism or review as permitted under the terms of the UK Copyright, Designs and Patents Act, 1988, this publication may not be reproduced, stored or transmitted, in any form or by any means, without the prior permission in writing of The Royal Society of Chemistry, or in the case of reprographic reproduction only in accordance with the terms of the licences issued by the Copyright Licensing Agency in the UK, or in accordance with the terms of the licences issued by the appropriate Reproduction Rights Organization outside the UK. Enquiries concerning reproduction outside the terms stated here should be sent to The Royal Society of Chemistry at the address printed on this page.

Published by The Royal Society of Chemistry,
Thomas Graham House, Science Park, Milton Road,
Cambridge CB4 0WF, UK

For further information see our web site at www.rsc.org

Printed by Bookcraft (Bath) Ltd.

Physiological Heterogeneity and Biofilm Control

G.A. McFeters, P.S. Stewart, C.-T. Huang, E.J. Wentland, K.D. Xu and F.P. Yu

CENTER FOR BIOFILM ENGINEERING AND MICROBIOLOGY DEPARTMENT, MONTANA STATE UNIVERSITY, BOZEMAN, MT 59717, USA

1 ABSTRACT

More knowledge describing the physiological activities of bacteria in attached communities such as biofilms is essential in understanding their numerous important functions and in formulating control strategies. While various recent reports have documented structural and chemical heterogeneities within biofilms, only limited evidence has suggested that physiological heterogeneities also exist within these attached populations. Our current approach involves the application of physiological stains that are fluorogenic to indicate a range of cellular activities followed by cryoembedding, sectioning and observation with fluorescence microscopy. Studies using the respiratory fluorochrome, CTC, have demonstrated the progressive spatial effects of chlorine-based disinfection on cellular respiratory activity over time within biofilms. These findings correspond well with experiments using microelectrodes demonstrating the progressive penetration of chlorine into similar biofilms. Other studies with acridine orange strongly suggest that both bacterial colonies and pure-culture biofilms display a marked gradient in growth rate with depth into cell clusters and suggest that cells in different microzones use alternative metabolic strategies.

More recent studies have investigated the expression of nutrient-limitation responses within attached bacterial communities using the phosphorous starvation system. This approach also provides evidence of physiological heterogeneities within attached bacterial systems including colonies and biofilms. Collectively, these observations indicate that additional research will be fruitful in describing other activity gradients within biofilms and that this information will provide useful insights to guide efforts to optimize beneficial processes mediated by biofilms and control the counterproductive instances of these important microbial communities.

2 INTRODUCTION

Attached microbial communities are found on the surfaces of natural and engineered aquatic systems and account for most of the microbial activity that is seen in those environments. Although they can be used in beneficial applications, there are many problems associated with biofilm buildup on engineered surfaces which include reduced heat transfer efficiency within heat exchangers, increased frictional resistance in pipes, deterioration of water quality within municipal drinking water systems, plugging of pipes, petroleum souring and accelerated biodeterioration of system components.

The control of counterproductive biofilms is a pervasive and expensive challenge in many industries. Numerous reports in the literature have documented the comparative resistance of bacteria in biofilms when compared with their planktonic counterparts.¹⁻³ Mechanisms have been proposed to explain this enhanced resistance in bacteria that are associated with surfaces. These include reduced penetration of agents into biofilms because of diffusion limitations or reactions with community components and possible unique physiological characteristics of bacteria within biofilms.⁴ Direct measurement of biocide penetration into mature biofilms has provided support for the former hypothesis.⁵ However, very little evidence describing the physiological status of bacteria within biofilms has been reported and virtually nothing was known concerning spatial and temporal heterogeneities of cellular activities within these structured communities. This shortage of information is incongruous in view of the relatively comprehensive current understanding of structural, chemical and physical heterogeneities within biofilms.⁶⁻⁸

This review describes the development of unique methods to describe physiological heterogeneities within biofilms and their application in studies following the spatial and temporal effects of biocide on key cellular activities.

3 APPROACHES FOR STUDYING THE PHYSIOLOGY OF BACTERIA WITHIN BIOFILMS

Approaches that have been applied to the study of disinfection processes have relied on the quantitative removal of the community from the substratum followed by disaggregation and enumeration by colony formation. This traditional approach is limited in a number of ways.

Cultural methods fail to detect some autochthonous bacteria in environments where natural biofilm communities are composed of a wide range of different organisms and allochthonous bacteria can exhibit reduced culturability following environmental exposure. In addition, bacteria exposed to sublethal conditions of antimicrobial treatment often become injured and are incapable of forming colonies on some media.⁹ These limitations are particularly acute when studying biofilms where spatial heterogeneities may be complex. In addition, accurate data describing these structural relationships might be crucial in gaining an understanding of the mechanism of antimicrobial action within the undisturbed community. Methods to remove and desegregate biofilms can also be inefficient. Therefore, the traditional approach of describing microbial populations within biofilms on the basis of biomass removal and colony formation is inadequate and often results in underestimations of the actual viable population of bacteria. In addition, results obtained using conventional methods are community-wide averages and provide no spatial information describing structure and physiological heterogeneities within biofilms following treatment with an antimicrobial agent.

The advent of scanning confocal laser microscopy (SCLM) enables researchers to visualize two and three-dimensional reconstructed images without physically disturbing the biofilm community.^{10,11} The use of SCLM allows horizontal and vertical optical sectioning as well as the rapid virtual reconstruction of the community. However, this approach requires expensive instrumentation and has limited resolution when applied to thicker biofilms. These constraints have had a restricting effect on the wide-spread application of SCLM.

The approach adopted in our laboratory uses a combination of specific fluorescent stains to assess various physiological activities, at the cellular level, and cryosectioning followed by microscopic examination and image analysis. These techniques, which involve less expensive instrumentation and are suitable for the study of thick or opaque biofilms, will be discussed in subsequent sections of this review.

4 CRYOSECTIONING OF BIOFILMS FOR MICROSCOPIC EXAMINATION

Techniques involving physical sectioning have been extensively used with great success in the microscopic study of many biological tissues. For example, cryoembedding and cryotomy (cryosectioning) is routinely applied as a rapid clinical procedure in the diagnosis of certain diseases. This approach was applied in the microscopic examination of biofilm structure through the use of fluorogenic dyes.¹² Binary population biofilms of both *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* grown on stainless steel coupons were used in the initial development of this method. Coupons with stained biofilm were placed on a block of dry ice and commercial tissue embedding medium added to the surface. The frozen, embedded biofilms could then be easily and completely removed from the substratum by gently flexing the coupon. Thin (5 μm) vertical sections of the original biofilm were then obtained with a cryostat operated at -19°C . A schematic of this protocol is shown in Figure 1. These sections were mounted on microscope slides and examined by epifluorescence microscopy. This technique preserved the structural features of the biofilm including an irregular upper surface, water channels or voids and a well defined substratum interface.

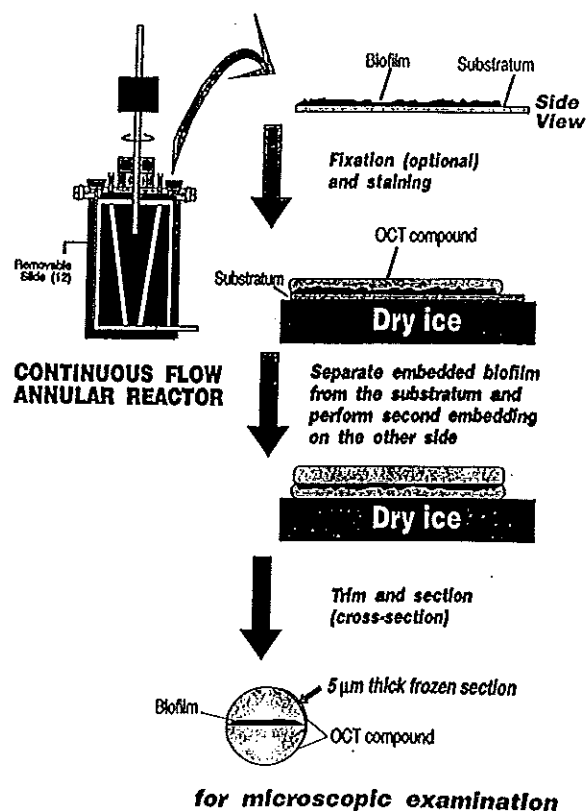


Figure 1. Diagram of cryoembedding and cryosectioning procedure. (from reference # 12, with permission)

These same features have been observed using SCLM.^{10,11} This technique has also been successfully applied to the microscopic examination of biofilms of different thicknesses on various substrata where striking images of the community showing individual cells are clearly resolved when stained with fluorogenic compounds.¹³⁻¹⁵ Hence, this method provides a relatively rapid and simple approach for examining individual cells while also revealing spatial physiological and structural features within biofilms.

5 ASSESSMENT OF PHYSIOLOGICAL ACTIVITIES IN BIOFILMS WITH FLUORESCENT STAINS

Conventional methods for enumerating bacteria are inadequate to determine the viability and activity of microorganisms in biofilms because of their dependence on the disruption of the native structure by scraping and disaggregation followed by colony formation. This results in physical disruption of spatial relationships within the natural community structure of biofilms. Our historical reliance on this approach has resulted in imprecise concepts of the potential viability and activity of bacteria in biofilms that lack spatial inference. However, direct microscopic observations of biofilms stained with fluorogenic stains can provide a description of the physiological architecture of biofilms with minimal physical disruption.¹⁶

Acridine orange (AO) is a fluorogenic dye that is widely applied by microbiologists in the acridine orange direct count (AODC) method and has been interpreted with putative physiological implications. As reviewed elsewhere,¹⁷ numerous workers have used the resulting color of AO-stained bacteria to distinguish between active and inactive cells. While that concept is based on the well established differential spectrofluorescence of single stranded versus double stranded nucleic acids complexed with AO, there was a paucity of specific published information to test that hypothesis by correlating established physiological indices with the AO staining reaction in bacteria of defined physiological activity. The results of such a study were eventually reported using *Escherichia coli* under controlled laboratory conditions.¹⁷ The findings indicate that the AO staining reaction is influenced by a number of variables including undefined characteristics of other bacteria in environmental systems as well as staining and fixation variables. However, AO is potentially useful as an index of physiological activity in carefully controlled experiments.

Experiments were done using AO to visualize and quantify spatial variations in growth rates within colonies and biofilms of *K. pneumoniae*.¹⁴ Colonies grown on membrane filters were used as a model bacterial community to develop the method and to collect control growth rate data. These data were used to determine the specific relationship between intracellular acridine orange reaction colour and growth rate. Spatial patterns of color and intensity were quantified using confocal microscopy and image analysis of stained cross sections of colonies following cryosectioning. Colonies sampled in exponential growth phase were thin, had relatively high average growth rates ($\mu > 1 \text{ h}^{-1}$) and all of the cells stained bright orange. Colonies sampled in the same way after more than 24 h of growth were thicker ($> 200 \text{ }\mu\text{m}$) and were growing slowly ($\mu < 0.15 \text{ h}^{-1}$). Microscopy revealed that these older colonies were characterized by distinct bands of orange at the colony edges and dark green interiors. Colony-average growth rates, determined by calculating the local slope of the cell accumulation versus time data, were correlated with colony-average fluorescence intensities. Growth rate correlated with the orange:green intensity ratio ($r^2 = 0.57$). The resulting

regression was used to predict specific growth rate profiles within the colonies stained with AO. Figures 2 and 3 show the relationship between growth rate and position within young and older colonies, respectively. These profiles indicated that bacteria grew more rapidly near the air and agar surfaces and more slowly, if at all, in the center of colonies when thicker than about 30 μm . The younger colony was thinner and demonstrated a higher overall growth rate with less variation, as a function of position within the colony, than the older colony where the cells in the interior are much less active. Note the different scales in the x-axes of the two graphs (Figures 2 and 3). Stained mature biofilms of the same organism displayed similar orange bands at the biofilm-bulk fluid and substratum interfaces as well as a green interior. The dimensions of orange bands ranged from 20 to 30 μm , which indicate the location of the actively growing regions within the biofilms. These observations are in agreement with the hypothesis that bacterial cells within the interior of thick biofilms (ie. > 100 μm) might display greater resistance to the action of biocides because of their reduced anabolic activity. This kind of data is also of predictive value when applied in models and adds new information to our understanding of the physiological architecture of mature biofilms.

CTC (5-cyano-2,3-ditolyl tetrazolium chloride) and rhodamine 123 (Rh 123) have only recently been used by microbiologists as cellular indicators of bacterial activity. Active cellular respiration results in the reduction of CTC and the accumulation of intracellular fluorescent crystals that can be detected microscopically and by flow cytometry. Intracellular rhodamine 123 fluoresces in proportion to cellular proton motive force. Biofilms of *K. pneumoniae* were used to determine the utility of CTC and Rh 123 as indices of interfacial bacterial activity by comparing those results against PC and *in situ* Direct Viable Count (DVC) data.¹⁸ Our findings demonstrated that the response of bacteria within actively growing biofilms using the DVC method, Rh 123 and CTC were statistically comparable and approximately 90% of the total cell

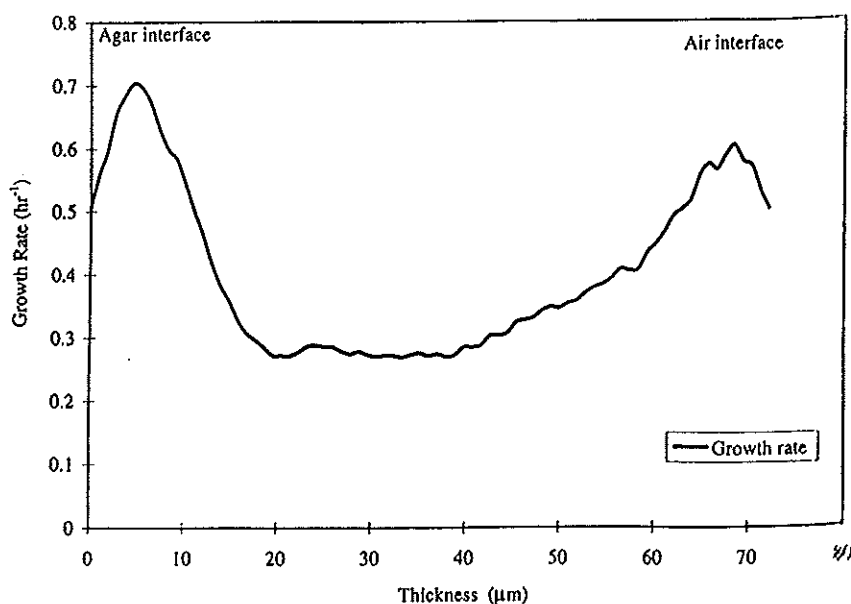


Figure 2. Estimated growth rate profile of a *K. pneumoniae* colony that was 8.17 hours old using AO staining.

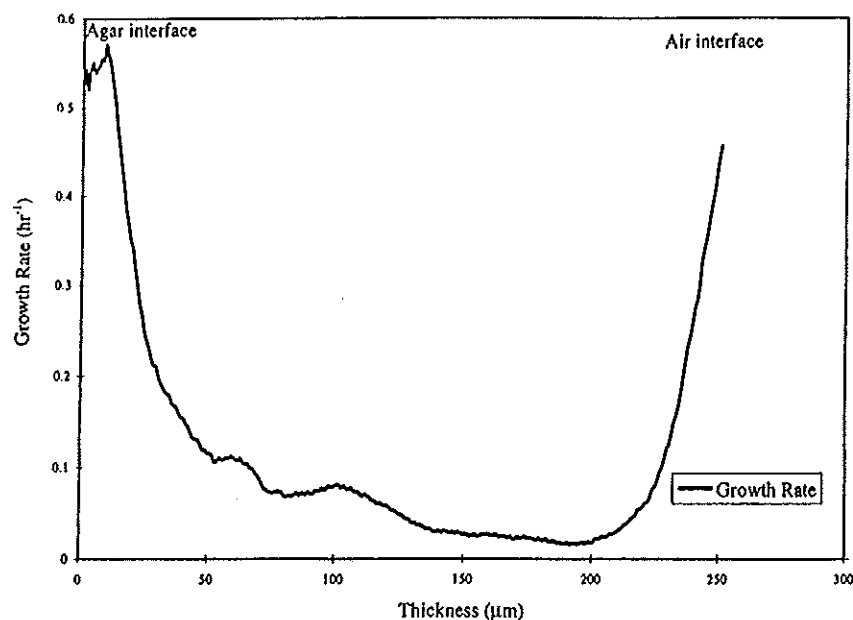


Figure 3. Estimated growth rate profile of a *K. pneumoniae* that was 30.42 hours old using AO staining.

population appeared active. Bacterial population densities determined using these indices of cellular activity were also at least two-fold higher than with PC enumeration data.¹⁹ A similar result was reported by another group studying the use of CTC to describe autochthonous biofilms in drinking water although they observed a lower fraction of the bacteria with active respiration.²⁰ These results collectively indicate that staining with CTC and Rh 123 accomplish the rapid and accurate (*i.e.* comparable with DVC data) *in situ* enumeration of active bacteria. They also allow the assessment of specific physiological properties of bacteria within biofilms to an extent that is not possible with conventional techniques. Hence, these fluorescent probes can be useful in revealing physiological insights when applied to bacterial biofilms and may be used in studies of biofilm control through the use of biocides.

More recent and ongoing studies are investigating the spatial expression of specific enzymatic activities within mature biofilms. Enzyme-labeled fluorescence (ELF) reagents for phosphatase (Molecular Probes) were applied to mature *K. pneumoniae* and *Pseudomonas aeruginosa* biofilms in both phosphate-containing and phosphate-limited media to determine the community architecture with respect to expression of phosphatase. When a *P. aeruginosa* biofilm was shifted from a medium containing phosphate to one lacking phosphate, only a layer (ca. 15 μm) of bacteria on the aspect of the biofilm exposed to the bulk fluid became induced. However, only a relatively small fraction of the total community scattered throughout mature biofilms of *K. pneumoniae* became induced. This observation suggests that the ELF reagents penetrated the entire depth of the communities and that these two bacteria respond quite differently to environmental conditions that induce phosphatase expression.

There is an impressive array of fluorogenic reagents utilized by biologists studying eukaryotic cells in a wide variety of physiological applications.^{21,22} By comparison, there are only a few microbiological uses of this analytical approach. These observations suggest that there is a great potential for the expanded application of fluorescent probes to determine bacterial physiological and metabolic activities. This position is supported by recent advances in SCLM, cryosectioning, digital image analysis and the availability of highly sensitive cameras. Such a strategy provides information describing the physiological status of individual bacteria and assessment of specific metabolic activities and gene expression and positioning *in situ* within communities as well as enumerative data. Some of these assays may also be done nondestructively and in real time. The resulting information provides exciting new physiological capabilities to complement the recently developed genetic/molecular analyses that are currently used in many areas of microbiology.

6 SPATIAL RESPONSE OF BACTERIA IN BIOFILMS TO BIOCIDES EXPOSURE

Studies were conducted to describe spatial and temporal patterns of respiratory activity within mature biofilms during disinfection.¹³ Binary population biofilms of *P. aeruginosa* and *K. pneumoniae* were grown on stainless steel coupons in an annular reactor using mineral salts medium plus glucose for 7 to 10 days and exposed to low concentrations (2 and 4 mg/l) of monochloramine. Bacteria in treated biofilms were evaluated for viability by colony formation on a nonselective medium and for physiological activity by CTC reduction followed by cryosectioning and image analysis. Epifluorescent micrographs of CTC-stained sections of biofilms exposed to disinfectant for various times clearly revealed gradients of respiratory activity. Cells near the biofilm-bulk fluid interface lost respiratory activity first and that effect progressed into the biofilms with time of biocide treatment. The total depth of the biofilms also decreased with exposure time. After 2 hours of disinfection, there was a small residual zone of bacteria capable of respiration deep within structure cell clusters. These gradients of respiratory activity with depth in the biofilm community were quantified by the ratio of CTC-positive cells to the total cell population, stained with DAPI, using image analysis and are shown in Figure 4. The resulting quantitative data supported the qualitative observations from the micrographs. These findings further suggest that the increased bacterial resistance seen within thick biofilms is a function of bacteria within dense clusters where biocide penetration is restricted and the cells might exhibit a unique physiological status resembling stationary phase cells under planktonic cultivation. Therefore, by coupling the CTC probe for bacterial respiratory activity with the cryosectioning technique, we were able to resolve spatial patterns of bacterial respiratory activity within a disinfected heterogeneous biofilm community using microscopic observations and image analysis. The selection of CTC as the physiological probe for the action of chlorine-based disinfectants is also judicious since bacterial respiration is known to be one of the primary cellular physiological targets for these disinfectants.²³ These findings are also in qualitative agreement with the results of experiments describing the penetration of chlorine-based disinfectants into biofilms through the use of chlorine-specific microelectrodes.⁵

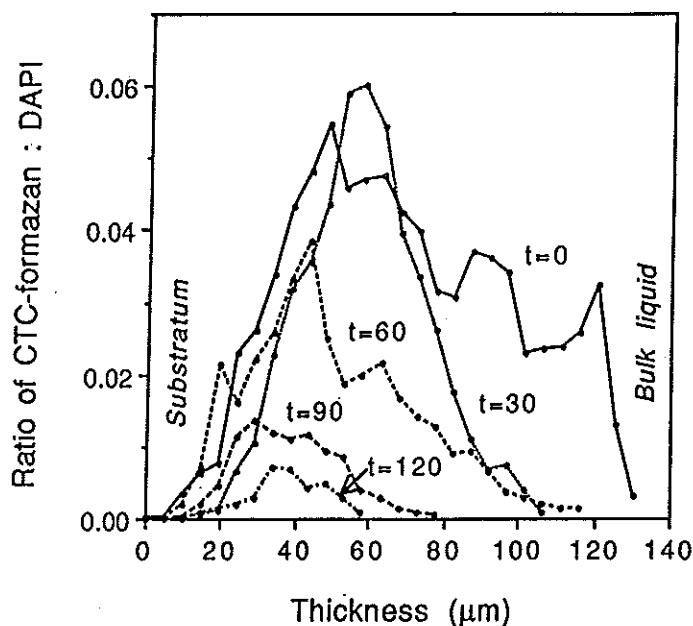


Figure 4. Intensity ratios of CTC-formazan and DAPI obtained from sections of a biofilm composed of *K. pneumoniae* and *P. aeruginosa* exposed to 2 mg of monochloramine per liter. The biofilm thickness was 121 μm before treatment and 116, 106, 125 and 90 μm at 30, 60, 90 and 120 minutes of exposure, respectively. t, time (minutes). (from reference # 15, with permission)

7 SUMMARY

- * Fluorogenic stains that reflect different cellular activities are useful in determining physiological heterogeneities within bacterial colonies and biofilms when coupled with cryosectioning and fluorescent microscopy.
- * Mature biofilms are spatially heterogenous with respect to physiological activities, including growth rate and cells capable of inducing some enzymes.
- * Exposure of mature biofilms to chlorine-based disinfectants results in a progressive loss of respiration within cells near the bulk fluid surface and the erosion of community thickness.
- * More mature bacterial biofilms and colonies contain slowly growing cells within clusters and areas of greater thickness while younger communities have higher overall growth rates that are more uniform.
- * Bacteria within mature biofilms are responsive to environmental stimuli.
- * Information describing the physiological architecture of biofilms provides novel insights that have potential usefulness in predicting the behavior of these communities, their ecology and susceptibility to disinfectants.

8 ACKNOWLEDGMENTS

The preparation of this manuscript was supported by funds from the National Aeronautics and Space Administration, National Science Foundation/Office of Polar Programs, U.S. Army and the Center for Biofilm Engineering at Montana State University (cooperative agreement ECC-8907039).

9 REFERENCES

1. M. R. W. Brown and P. Gilbert, *J. Appl. Bacteriol.*, 1993, **74**, 87S.
2. K. L. Cargill, B. H. Pyle, R. L. Sauer and G. A. McFeters, *Can. J. Microbiol.*, 1992, **38**, 423.
3. J. W. Costerton, K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta and T. J. Marrie, *Ann. Rev. Microbiol.*, 1987, **41**, 435.
4. P. S. Stewart, *Antimicrob. Agents Chemother.*, 1994, **38**, 1052.
5. D. de Beer, R. Srinivasan and P. S. Stewart, *Appl. Environ. Microbiol.*, 1994, **60**, 4339.
6. N. B. Ramsing, M. Kuhl and B. B. Jorgensen, *Appl. Environ. Microbiol.*, 1993, **59**, 3840.
7. Z. Lewandowski, S. A. Altovelli and E. Fukushima, *Biotechnol. Prog.*, 1993, **9**, 40.
8. J. W. Costerton, Z. Lewandowski, D. E. Caldwell, D. R. Korber and H. M. Lappin-Scott, *Ann. Rev. Microbiol.*, 1995, **49**, 711.
9. G. A. McFeters, 'Drinking Water Microbiology', Springer-Verlag, New York, 1990, Chapter 23, p. 478.
10. D. E. Caldwell, D. R. Korber and J. R. Lawrence, 'Advances in Microbial Ecology', Plenum Press, New York, 1992, Vol. 12, p. 1.
11. D. E. Caldwell, D. R. Korber and J. R. Lawrence, *J. Microbiol. Meth.*, 1992, **15**, 249.
12. F. P. Yu, G. M. Callis, P. S. Stewart, T. Griebel and G. A. McFeters, *Biofouling*, 1994, **8**, 85.
13. C.-T. Huang, F. P. Yu, G. A. McFeters and P. S. Stewart, *Appl. Environ. Microbiol.*, 1994, **61**, 2252.
14. E. J. Wentland, P. S. Stewart, C.-T. Huang and G. A. McFeters, *Biotechnol. Prog.*, 1996, **12**, 316.
15. C.-T. Huang, G. A. McFeters and P. S. Stewart, *Biofouling*, 1996, **9**, 269.

16. G. A. McFeters, F. P. Yu, B. H. Pyle and P. S. Stewart, *J. Microbiol. Meth.*, 1995, **21**, 1.
17. G. A. McFeters, A. Singh, S. Byun, P. R. Callis and S. Williams, *J. Microbiol. Meth.*, 1991, **13**, 87.
18. F. P. Yu and G. A. McFeters, *J. Microbiol. Meth.*, 1994, **20**, 1.
19. F. P. Yu and G. A. McFeters, *Appl. Environ. Microbiol.*, 1994, **60**, 2462.
20. G. Schaule, H.-C. Flemming and H. F. Ridgway, *Appl. Environ. Microbiol.*, 1993, **59**, 3850.
21. W. T. Mason, 'Fluorescent and Luminescent Probes for Biological Activity', Academic Press, London, 1993.
22. R. P. Haugland, 'Handbook of Fluorescent probes and Research Chemicals', 6th ed., Molecular Probes Inc., Eugene, Oregon, 1996.
23. A. K. Camper and G. A. McFeters, *Appl. Environ. Microbiol.*, 1979, **37**, 633.