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BIOFILM ECOLOGY: ON-LINE METHODS BRING NEW INSIGHTS INTO MIC AND MICROBIAL BIOFOULING

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Microbial biofilms were formed on coupons with defined coatings in once-through laminar flow fields of controlled bulk-phase composition and shear. Dilute media were utilized to select for biofilm growth. The formation, succession, and stability of the biofilms were monitored with non-destructive on-line methods (fluorescence, bioluminescence, attenuated total reflectance Fourier transform infrared spectrometry [ATR-FTIR] and electrochemical impedance spectroscopy) and by high resolution destructive analysis (viable and direct counts and phospholipid fatty acid signature methods) at the termination of the experiments. Biofilms of reproducible composition can be formed and the order of inoculation of multi-component biofilms affects their composition at harvest. The corrosion rates of mild steel depended on the biofilm composition but not the attached biomass. Examination of biofilms with the scanning vibrating electrode in a microscope field showed effects of heterogeneity in biofilm structure which promoted localized anodic activity. *Pseudomonas* stains were engineered to contain the *lux* gene cassette as a "reporter" and the formation of the exopolymer alginate was shown not to promote attachment of the strain or secondary colonization by *Vibrio*. Examination of mutants forming different alginate structures showed differential attachment and biofilm structure. Studies of mutants of lipopolysaccharide structure showed differential attachment to substrata. Specific antifouling and fouling-release coatings showed a wide range of attachment and release properties as well as sublethal toxicity.

KEYWORDS: biofilm, biofouling, corrosion, heterogeneity, on-line monitoring, toxicity

INTRODUCTION

Biofilms are localized concentrations of microorganisms attached to a substratum. The biofilm can consist of a population of a single species or more often, a multi-species community. Within the biofilm population or community, heterogeneities in the distribution of organisms and in their metabolic activities are common. This report will review the progress in microbial biofilm ecology that new methods have made possible.

Microbes easily attach to surfaces to form biofilms and in nature most microorganisms are attached rather than free in bulk fluids. The importance of microbial biofilms has been established in aquatic, soil, and clinical environments (Costerton *et al.*, 1981a,b; Fletcher & Marshall, 1982; Lappin-Scott & Costerton, 1989). Attachment in biofilms can lead to advantages for microorganisms, such as the availability of nutrients concentrated at surfaces, access to a flowing system which can increase availability of diffusable

nutrients for life at low Reynolds numbers, the ability to generate microniches by multi-species metabolism such as the generation of anaerobic sites in an aerobic environment, the presence of a glycocalyx matrix to restrict predation by phagocytosis or exposure to toxicants, prevention of transfer to a hostile environment such as from mouth to gut, conservation of byproducts of neighbour's metabolism such as primary photosynthate, and provision of enhanced metabolic prowess for digestion by interactions within consortia. The disadvantages of biofilm formation are competition for nutrients and terminal electron acceptors and the availability to a larger biomass of macroscopic predators able to ingest and breakdown the biofilm.

Biofilm formation in clinical medicine is a disaster as the natural defenses, even supplemented by potent antibiotics, are often ineffective (Costerton *et al.*, 1981a). If "the race to colonize the new surface" is won by microbes, then implanted prostheses must be removed if possible or an intractable infection will result. In biotechnological or bioremediation processes it is often an advantage to promote biofilm formation and maintain the active biomass at very high densities. In other situations, biofouling can seriously restrict effective heat transport, membrane processes, and potentiate macrofouling with loss of transportation efficiency. Inhomogeneous distribution of microbes and/or their metabolic activity can promote microbially influenced corrosion (MIC) which is a multibillion dollar problem (Odom & Singleton, 1993).

Consequently it is important that biofilm microbial ecology be understood so it can be manipulated rationally. It is usually easy to select organisms that form biofilms by flowing a medium considerably more dilute than supports optimal growth over a substratum and propagating the organisms that attach. To examine the biofilm most expeditiously, biomass accumulation, and desquamation and metabolic activities need to be monitored on-line and non-destructively. This on-line monitoring becomes even more valuable if the activities can be locally mapped in time and space within the biofilm.

METHODS EMPLOYED IN BIOFILM ECOLOGY

Biofilm Formation

Generation of biofilms requires a substratum over which a bulk phase with sufficiently dilute nutrients flows so that only microbes attached can sustain survival and growth. This can be done in a flow-through apparatus in which the biofilm can be observed through replaceable windows, and in which the laminar flow dynamics maintain a reproducible shear field (Mittelman *et al.*, 1992a). Inocula are added to the stream from continuous cultures for a short period at the onset of the experiments. Multiple inocula can be added in sequence from separate continuous cultures (Jack *et al.*, 1992). The effects of different substrata or coatings can be tested with flush mounted coupons mounted in the laminar flow apparatus (Arrage *et al.*, 1995).

On-line Monitoring

Biofilm formation, succession, stability, and sub-lethal toxicity can be monitored with in-line, non-destructive techniques in the flow-through apparatus (Nivens *et al.*, 1995). Microbial biofilm biomass can be monitored by NADH, tryptophan, or chlorophyll fluorescence (Angell *et al.*, 1993). The intrinsic bioluminescence of attached biofilm,

naturally bioluminescent or genetically engineered bacteria with the *lux* gene cassette, can be monitored in flow-through chambers (Mittelman *et al.*, 1993) or mapped with a photon-counting imaging microscope (Angell *et al.*, 1994b). The relative bioluminescence per cell (determined by tryptophan fluorescence) provides a non-destructive measure of sub-lethal toxicity (Arrage *et al.*, 1995). It is possible to examine the shifts in the chemistry of biofilms by monitoring the changes in infrared spectra. An attenuated total reflectance Fourier transform infrared spectrometer (ATR-FTIR) in which three channels can be monitored simultaneously has been utilized to examine biofilm formation and shifts in composition (Nivens *et al.*, 1993). This apparatus has been utilized in conjunction with a fiberoptic bioluminescence detector to monitor specific biodegradation of solvents in a biofilm (Schmitt *et al.*, 1995). On-line electrochemical monitoring of biofilm activity includes changes in the open circuit potential, small amplitude cyclic voltametry, and electrochemical impedance spectroscopy (EIS), (Dowling *et al.*, 1989a). One of the most exciting new technologies for on-line observation of microbes in biofilms is confocal laser microscopy (CLM). CLM combines fluorescence detection through a pinhole barrier to create a thin (0.3 μm) plane-of-focus in which the out-of-focus light is eliminated. With computer analysis of laser activated fluorescence, a three-dimensional image of the biofilm can be generated (Caldwell *et al.*, 1992). CLM has provided revolutionary images of the structure of biofilms.

High Resolution Destructive Monitoring

At the completion of experiments, the biofilms can be analyzed for viable microbes by plating onto appropriate media, direct microscopic counting after recovery from the biofilms, or by signature biomarker analysis. Biomarkers often utilized are nucleic acids which can be amplified enzymatically with the polymerase chain reaction (PCR) or by analysis of the membrane lipids. These analyses can be combined to provide both genetic and phenotypic insight into the viable biomass, community composition, and nutritional/physiological status (White & Macnaughton, 1996). The determination of the total phospholipid ester-linked fatty acids (PLFA) provides a quantitative measure of the viable or potentially viable biomass. Viable microbes have an intact membrane which contains PLFA. The cellular enzymes hydrolyze the phosphate group within minutes to hours of cell death (White *et al.*, 1979). The remaining lipid is diglyceride (DG). The resulting DG contains the same signature fatty acids as the phospholipids, allowing for a comparison of the ratio of phospholipid fatty acids to diglyceride fatty acids (viable to non-viable microbes). The signature lipid analysis also provides insight into the physiological status of the microbial community. The formation of poly *β*-hydroxyalkanoic acid (PHA in bacteria) (Nickels *et al.*, 1979; Findlay & White, 1983), or triglyceride (in microeukaryotes) (Gehron & White, 1982) relative to the PLFA provides a measure of the nutritional status. Bacteria grown with adequate carbon and terminal electron acceptors form PHA when they cannot divide because some essential component is missing (phosphate, nitrate, trace metal, *etc.*). Furthermore, specific patterns of PLFA can indicate physiological stress (Guckert *et al.*, 1986). Exposure to toxic environments can lead to minicell formation and a relative increase in specific *trans* monoenoic PLFA compared to the *cis* isomers. It has been shown that for increasing concentrations of phenol toxicants, the bacterium *Pseudomonas putida* forms increasing proportions of *trans* PLFA (Heipieper *et al.*, 1992).

RESULTS AND DISCUSSION

Reproducible Formation of Biofilms

The reproducible generation of biofilms requires control of the three major components that affect biofilm ecology, *viz.* the bulk fluid, the substratum and the inoculum in a flow-through system. The bulk fluid should have a chemical composition of sufficiently dilute nutrients that pelagic growth is not possible. This will strongly select for biofilm formation in the once-through system. The chamber should be shaped so laminar flow is generated (Davies & White, 1982) and between two parallel plates one of which is transparent for observation (Berg & Block, 1984). It is very useful for interchangeable windows to maintain a clean viewing port for coupons which serve as the substratum (Mittelman, 1991; Mittelman *et al.*, 1990; 1992b; 1993). The flow-rate should be controlled to determine the shear forces on the biofilm. Strength of adhesion can be tested by increasing the flow up to 100-fold. This has proved extremely useful in testing fouling-release coatings (Arrage *et al.*, 1995). The effects of changes in substratum structure and chemistry are conveniently tested with a series of coupons with a standard surface proximal and one distal to the test material (Arrage *et al.*, 1995). In this way the test substratum can be assessed in comparison to the proximal placed standard surface for relative fouling rates, succession, and biofilm stability. The coupon downstream from the test coupon can be monitored for effects of the test surface on the standard surface. In the authors' studies, 316 stainless steel polished with a 600 grit material has been utilized as a standard substratum. The third feature in reproducible biofilm formation is the inoculum. Continuous culture vessels for each bacterium that will form the biofilm community are maintained in medium like the bulk phase but with nutrients sufficient to maintain pelagic growth. These are then used as a pulsed inoculum into the flow chamber where the biofilm is maintained. In experiments involving *Pseudomonas fluorescens, Hafnia alvei, Desulfovibrio gigas*, and *Bacillus subtilis*, the order of the inoculation from the continuous cultures affected the composition and viable biomass of the resulting biofilm as determined at harvest after 5 d (Mittelman, 1991). *P. fluorescens* dominated the biofilm and if it was utilized as the initial inoculant, the biofilm had the highest biomass (about 10^8 cells cm^{-2}). The biomass and community composition of biofilms of *Bacillus* sp. *Pseudomonas* spp. and *Acidovorax* sp. recovered from drinking water biofilms (defined by similarity of fatty acid patterns to the MIDI data base) also depended on the order of inoculation (Arrage, unpublished experiments).

Microbially Influenced Corrosion

An aerobic *Bacillus* spp., the fermentative *Hafnia alvei* and the sulfate-reducing anaerobe *Desulfovibrio gigas* induced differential rates of microbially influenced corrosion (MIC) of mild steel. Coupons were exposed in sterile dilute medium resembling a fresh water lake, but containing 0.4 mM sulfate, in a flow through system to which separate continuous cultures were added, as monocultures or mixtures to an aerobic system. Corrosion rates (Admittance, in mhos $\times 10^{-3}$ cm^{-2}) estimated from electrochemical impedance spectra were about 0.5 for the sterile control, 0.5–1.0 for monocultures, 0.0–1.2 for two of the bicultures, 1.2 for the triculture and 2.5 for the *H. alvei* + *D. gigas* biculture. Corrosion was assessed using a 4-sided electrode (Nivens *et al.*, 1992) as the change in open circuit potential (OCP) and as the reciprocal of the polarization resistance derived from electrochemical impedance spectroscopy (EIS) (Dowling *et al.*, 1989a). The 4-sided electrode technology allowed tests of reproducibility of general corrosion rates.

EIS and OCP were established to not affect growth rates or metabolic activities of microbial biofilms on metal coupons (Franklin *et al.*, 1991a). The monocultures each induced a greater initial corrosion rate than the uninoculated control but with time the rates decreased to that of the sterile controls. The *D. gigas* culture formed a biofilm but was unable to grow in the aerobic bulk phase. In combinations, the presence of *D. gigas* always resulted in a greater rate of corrosion. The consortium containing *H. alvei* and *D. gigas* showed a significantly higher corrosion rate than the triculture or the other bicultures. At the end of the experiment the microbes attached to the coupons were examined by viable counts, MPN estimations and PLFA analysis. The rates of corrosion were not directly related to the total microbial biomass or the number of species on the coupon. The rate of corrosion did not depend on the ratio of heterotrophic to sulfate-reducing bacteria (SRB) or the absolute number of SRB. The PLFA analysis showed the microbes recovered from the biofilm were more metabolically stressed than those recovered from the bulk phase of the inocula. Clearly different combinations of bacteria forming biofilms in the same bulk phase and growing on the same substrata can induce very different corrosion rates. The maximum corrosion rate was within 70% of a total enrichment with a community composition determined by PLFA analysis like that found in native corrosion tubercles. This system proved an efficacious way to test the resistance of materials to specific MIC conditions and the effectiveness of mitigation procedures (Franklin *et al.*, 1991b).

Inhomogeneities in the substratum surfaces induce differences in microbial biofilm distribution and MIC activities (Guezennec *et al.*, 1992a) and weldments are especially vulnerable (Dowling *et al.*, 1989b). To reproducibly localize MIC to a specific area, a concentric electrode system was developed in which a small area was separated by a teflon ring from a larger circumferential area with 100 times the area of the same material. The small central area was then driven electrochemically as an anode compared to the large circumferential area in an anaerobic flow-through system. The system was inoculated and the potential between the anode and cathode shut off. The current between the electrodes was then monitored with a zero resistance ammeter (Guezennec *et al.*, 1992b). The presence of bacteria, including SRB, resulted in stabilization of a corrosion current between the anode and cathode (Campaignolle *et al.*, 1993). With this technology, it proved possible to induce MIC of 304 stainless steel anaerobically in the anode area reproducibly (Angell *et al.*, 1994a). In this system, a biculture of an SRB and a *Vibrio* spp. maintained a current of about 3 $\mu A\ cm^{-2}$ for > 200 h after a 72 h period after an imposed 11 $\mu A\ cm^{-2}$ current was removed. No current was maintained in the sterile control or with inocula of monocultures of these two bacteria which formed biofilms on the concentric electrodes. With the biculture, the charge transfer resistance > $100k\Omega\ cm^{-2}$ on the cathode (measured with EIS) was contrasted with <1 $k\Omega\ cm^{-2}$ at the anode. In this system removal of sulfate or the addition of 10 mM azide did not affect the sustainability of the current after an initial 30 h. Microbial metabolic activity is necessary for initiation processes and once started, the corrosion proceeds independently of the microbial metabolic activity (Angell *et al.*, 1994c). This should be an excellent system to study the microbiology of MIC initiation.

MIC is a localized process leading to pitting corrosion. Localized concentrations of microbes can lead to localized corrosion (Guezennec *et al.*, 1994). With application of a scanning vibrating electrode across a coupon, the charge density can be mapped and localized anodic areas detected in time and space (Franklin *et al.*, 1991c). The scanning vibrating electrode can be utilized in a epi-illuminated microscope with a photon-counting camera and bioluminescent organisms to establish the congruity between the localization of microbes in microcolonies, their metabolic activity as indicated by their

bioluminescence and the development of an anode detected in the charge density field (Angell *et al.*, 1994b).

Exopolymer Formation

The elaboration of extracellular polymers (glycocalyx) is an important feature of microbial biofilm formation (Costerton *et al.*, 1981b). One of the best studied is the formation of the uronic acid polymer alginate by *P. aeruginosa*. The committed step in the biosynthesis of alginate, the conversion of GDP-mannose into GDP manuronic acid, is expressed from the gene *algD* for GDP mannose dehydrogenase which is widely distributed in bacteria recovered from corrosion tubercles in nature (Wallace *et al.*, 1994a). Bacteria may be genetically engineered to contain the *lux* gene cassette under the control of specific promoter sequences (King *et al.*, 1990) as a reporter for activity of the gene sequence. A *Pseudomonas* was constructed with the *lux* gene cassette under the promoter for *algD* (Wallace *et al.*, 1994b) then utilized in biofilm studies. The effects of salt concentration and nitrogen balance in the formation of alginate can be detected in biofilms by the bioluminescence resulting from induction of the synthesis of alginate (Rice *et al.*, 1995). The brightness of the bioluminescent response in the engineered bacteria can be modulated by factors other than the promoter effectiveness at inducing *lux* gene cassette mRNA biosynthesis (Heitzer *et al.*, 1994). Factors such as ionic strength, pH, trace metal concentration, Fe, carbon source, and plasmid multiciplicity affect the brightness of bioluminescence. Monitoring bioluminescence during biofilm formation shows that bioluminescence appears then fades after induction, although alginate biosynthesis can continue (unpublished experiments). At the present time, in the bacterial bioluminescent system, there is only one practical wavelength so only one property can be monitored at a time. A new development involves the utilization DNA complementary for the jellyfish *Aequorea victoria* green fluorescent protein which can be expressed in bacteria (Chalfie *et al.*, 1994). Fluorescence at 509 nm can be detected after excitation at 395 nm. This small protein can be utilized as a reporter that is independent of exogenously added substrates or co-factors and can be detected at a different wavelength from bioluminescence.

ATR-FTIR provides an on-line, real-time monitoring and "inside out" view of the biofilm within the approximate 1 μm base layer within the evanescent wave. This technique monitors the entire area of the internal reflectance unit. With this technology it was possible to monitor the induction for the lipid storage polymer poly β-hydroxyalkanoate in biofilms of *P. cepacia* (Nivens *et al.*, 1995) (Fig. 1) and the differences in the biofilm structure of mutants of *P. aeruginosa* with lesions in the alginate biosynthetic pathway (Nivens *et al.*, 1994). It is possible to combine the ATR-FTIR monitoring of the chemistry of the attached biofilm with bioluminescence if the biofilm contains bioluminescent organisms whose activity is induced (Schmitt *et al.*, 1995).

Differential Attachment

With the laminar flow apparatus and the on-line detection system it is possible to measure the effects of changes in the surface chemistry of bacteria on the adhesion to substrata. The R5 mutant of *Pseudomonas aeruginosa* O6 (Dasgupta *et al.*, 1994) showed much more rapid and extensive biofilm formation on 316 stainless steel in laminar flow cells than the Wild Type with complete core and O6 A and B O-antigens (Flemming & Palmer, unpublished). The A-28 mutant with a complete core but loss of the B chain in

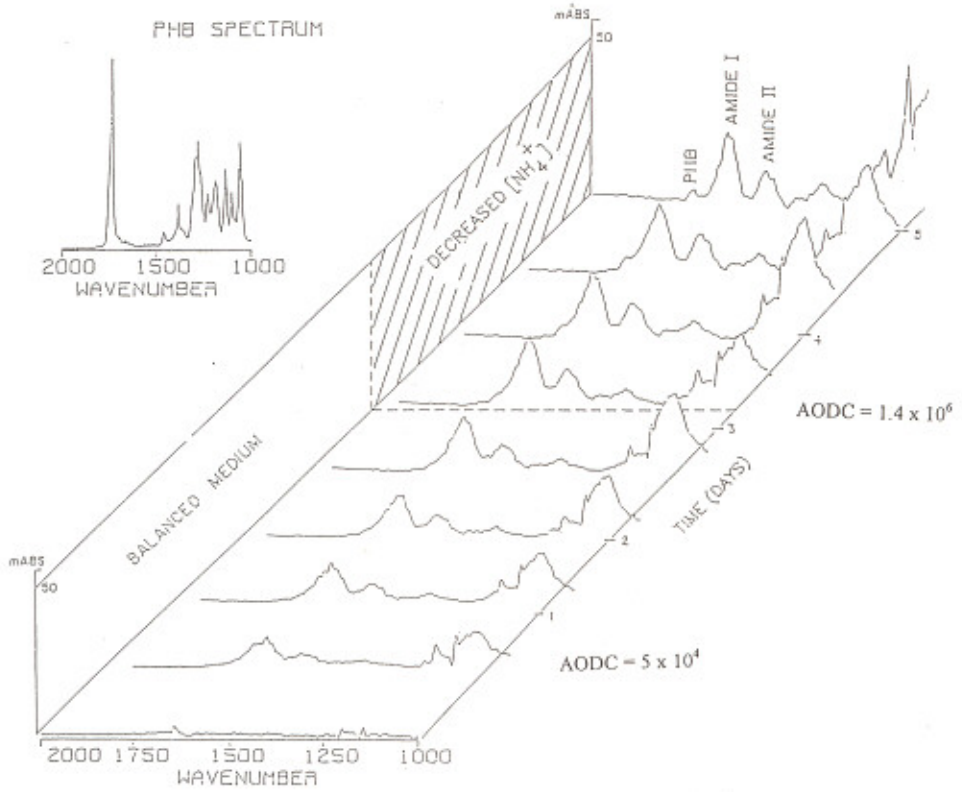

Figure 1

Fig. 1 Three-dimensional plot of time-dependent ATR-FT/IR spectra showing poly β-hydroxyalkanoate (PHA) production by a *Pseudomonas* developed on a germanium crystal in the laminar flow apparatus (Nivens *et al.* 1993) showing bands for amide I at 1650 cm^{-1}, amide II at 1548^{-1}, carboxylate ion 1400 cm^{-1}, and the P-O, C-O stretches at 1080 cm^{-1}. PHA formation was stimulated by significantly lowering the ammonium ion concentration in carbon rich bulk fluid flowing through the cells.

the O-antigen showed an intermediate ability to form a biofilm under the same conditions. With a 10-fold increase in flow, all the stains showed a loss of biofilm stability with the R5 mutant showing the greatest loss. This laminar flow test system will be an excellent system for characterizing the attachment of specifically modified bacteria to polymer coatings with known surface chemistry.

Antifouling and Fouling-release Coatings

The detection of bioluminescence generated with a photomultiplier and a fiberoptic probe can provide for the on-line non-destructive monitoring of bacteria in biofilms. The naturally bioluminescent bacterium *Vibrio harveyi* readily forms biofilms (Makemson *et al.*, 1992). This attachment propensity has been exploited in a laminar flow of dilute medium in seawater inoculated with *V. harveyi* from continuous culture used to monitor

biofilm formation by bioluminescence in tests of antifouling coatings (Mittelman *et al.*, 1993). This once-through device utilizes quartz windows which may be changed aseptically for viewing the test substratum and up-stream and down-stream control surfaces activity with fiberoptic probes. The biomass of the biofilm can be monitored non-destructively with tryptophan fluorescence (Angell *et al.*, 1993), and the bioluminescence, a measure of metabolic activity, can be related to the biomass as a measure of sublethal toxicity (Arrage *et al.*, 1995). In this system the shear gradients can be controlled, which allows the study of both antifouling and fouling-release effectiveness of various surface treatments as well as the effects of adding other bacteria or diatoms to the system (Arrage *et al.*, 1995).

Sublethal Toxicity

Utilizing laminar flow cells the effects of toxicants incorporated into coatings can be tested. The system has the distinct advantage that on-line determination of biomass is available through the measurement of fluorescence with tryptophan and the metabolic activity of the microorganisms can be monitored on-line by the luminescence in bioluminescent bacteria (Angell *et al.*, 1993, Mittelman *et al.*, 1993). By comparing the biomass and activity it has been possible to demonstrate that some compounds in coatings such as BRA640 (a copper (I) oxide containing antifouling paint) has a bactericidal effect, with low biomass and low activity. The bioluminescence pathway is known to be competitive for ATP with the normal metabolic pathways. With sublethal inhibition of synthesis of polymers apparently more of the ATP derived products are transferred to the bioluminescence pathways. Tests with known metabolic inhibitors of the electron transport chain such as carbon monoxide and sodium azide have shown that light output is enhanced upon treatment of bacteria with these compounds (Mittelman *et al.*, 1993). Alternatively, other toxicants such as Sea Nine 211, a dichlorinated-keto-sulfur-heterocyclo-hexamine with an 8 carbon saturated side chain, (Rohm & Hass Company) shows a dose-related ability to increase the bioluminescent light production per cell when incorporated in silicone coatings (Arrage *et al.*, 1995). The progressive increase in bioluminescence characteristic of sublethal toxicity with increased exposure to the toxicant is reflected by a progressive increase in the formation of *trans* 16 and 18 carbon monoenoic fatty acid esters from the *cis* precursors in the phospholipids of the cell membranes (Arrage, unpublished). This system will be excellent for examination of the mechanisms of cellular injury. Zosteric acid, a component discovered in the seagrass *Zostera marina* (Todd *et al.*, 1993), is bacteristatic in that it appears to prevent attachment, but not biofilm growth in biofilm bacteria.

Heterogeneity in Biofilm Composition and Activity

Generally, microscopy is thought of as a static approach to microbiology; the investigator examines preparations, typically single slides, and some photographs may be taken to document particular fields of interest. However, microbial growth and physiology can be examined microscopically in a time-resolved, non-destructive manner. Combining microscopy and the scanning vibrating electrode can be utilized to establish congruence between localization of microbial colonies, metabolic activity and formation of anodic electrochemical activity in MIC (Angell *et al.*, 1994b). Biofilms can be grown in flowcells (small "microbial perfusion chambers") that permit the investigator to not only establish particular environmental conditions, but also to alter the conditions during the course of an experiment.

An early paper demonstrating the concept of the flowcell as a microscopy tool was that of Berg and Block (1984). Since then, two groups have used flowcells extensively in their work, and both groups have used digital image processing to analyze images captured on video. The kinetics of particle adhesion (Sjollema *et al.*, 1989; Bos *et al.*, 1985) and the relationship of substratum to cell surface (Sjollema *et al.*, 1988; Sjollema & Busscher, 1990; Sjollema *et al.*, 1990) have been studied by Sjollema, Busscher and colleagues. Caldwell, Korber, and Lawrence have concentrated on more general ecological phenomena such as biofilm structure (Lawrence *et al.*, 1992) and growth rates of attached cells (Korber *et al.*, 1989). The latter group pioneered the application of laser confocal microscopy to microbial ecology (Lawrence *et al.*, 1991; Caldwell *et al.*, 1992); this major advance was made possible in part by using flowcells. Confocal microscopy, flowcells, and image analysis are now used in environmental microbiology (Assmus *et al.*, 1995; Wolfaardt *et al.*, 1995) and in biomedical microbial ecology (Rundegren *et al.*, 1992; Korber *et al.*, 1994b; Palmer & Caldwell, 1995). The techniques await direct application to industrial processes, although the gulf between the laboratory bench and the industrial plant is, in theory, easy to bridge. A simple approach is to connect a flowcell as a separate line, for example, in a bioreactor (Korber *et al.*, 1994a). The conditions within the reactor are mimicked in the flowcell, and the biofilms that form in the flowcell can be microscopically examined without disconnection from the line: true

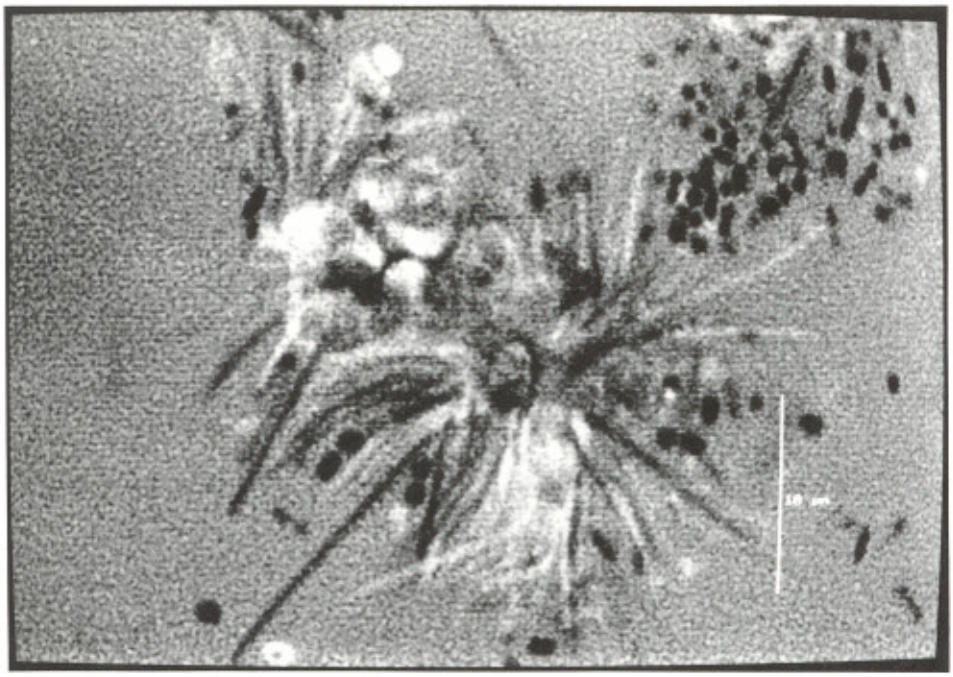

Fig. 2 A composite view (projection, "through-view") of a mixed species microcolony within a plaque-analogue biofilm. The biofilm was grown in a flowcell using whole saliva as inoculum. The image is a composite of eight 0.3-µm-thick optical sections collected at 1-µm intervals through the colony. The cells were stained with fluorescein; some cells take up the stain and appear bright whereas others exclude the stain and appear dark. The "star-burst" colony of filamentous organisms is anchored at the center by a packet of sarcenoid cells. Cocci are visible throughout and in a separate colony in the upper right. The filamentous cells have differential stain uptake; this could reflect biochemical differences between cells of (presumably) a single organism.

on-line monitoring. A less satisfactory approach (because continuous real-time data are precluded) would be to build removable sampling devices into the plant lines at points of interest. The surfaces of these samplers could be examined for microbial colonization, and it is conceivable that they could be replaced so that temporal studies could take place. Once sampling of the system can be carried out temporally, image analysis will greatly enhance the collection of quantitative data from fields-of-view within the sample. Object counting, volume determinations, quantitative fluorescence measurements, and other image analysis techniques will be used to describe numerically what was previously only appreciated visually.

Now that the ability to examine biofilms non-destructively in four dimensions (x, y, z, t) exists, the "developmental biology" of biofilms can be explored on levels from the single cell to organization of large multicellular colonies. The most promising application of this approach lies in the study of multi-species biofilms. Oral communities (*e.g.*, dental plaque; Fig. 2), marine snow, and microbial mats are prime candidates for real-time non-destructive study because they are reasonably well defined systems that are still complex enough to support a variety of niches. Application of the wide variety of fluorescent probes developed by cell biologists and neurophysiologists will yield new insight into physiology and community composition within biofilms. Two simple examples of differential staining using bacteria-specific probes are presented; Figure 3 shows whole saliva stained with a proprietary preparation that distinguishes Gram-negative from Gram-positive cells, whereas Figure 4 demonstrates the utility of confocal microscopy for examining the interaction of bacteria with eukaryotic cells.

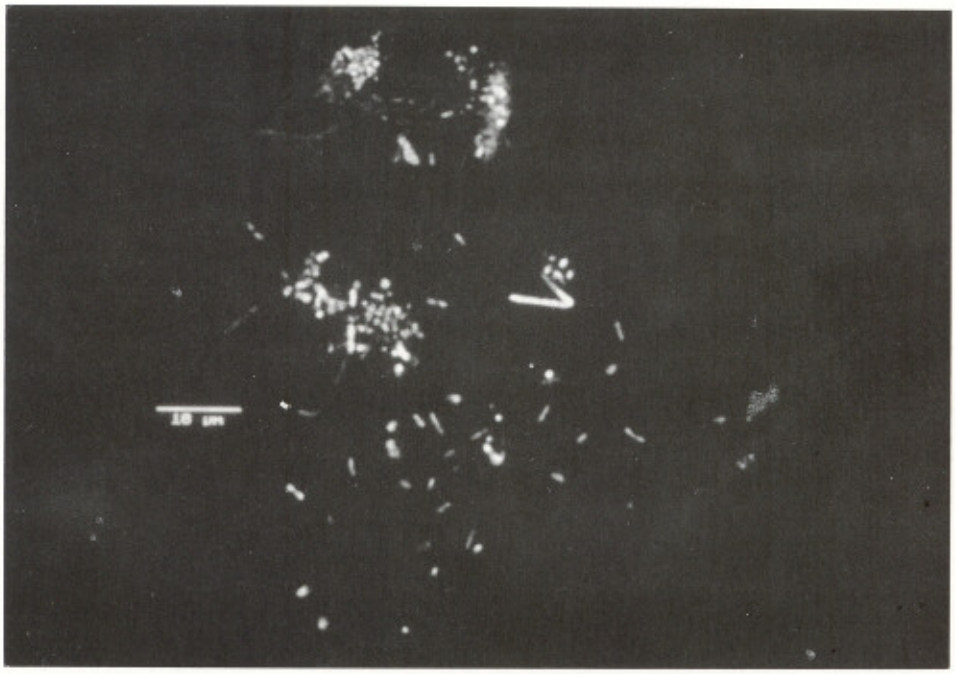

Fig. 3 Saliva stained with BacLight Gram Stain (Molecular Probes, Eugene, Oregon). Gram-positive cells stain red, Gram-negative cells stain green. The preparation was scanned twice; first under conditions resulting in green fluorescence, then under conditions for red fluorescence. The two data sets were then combined to yield a single dual-wavelength data set. (See Color Section.)

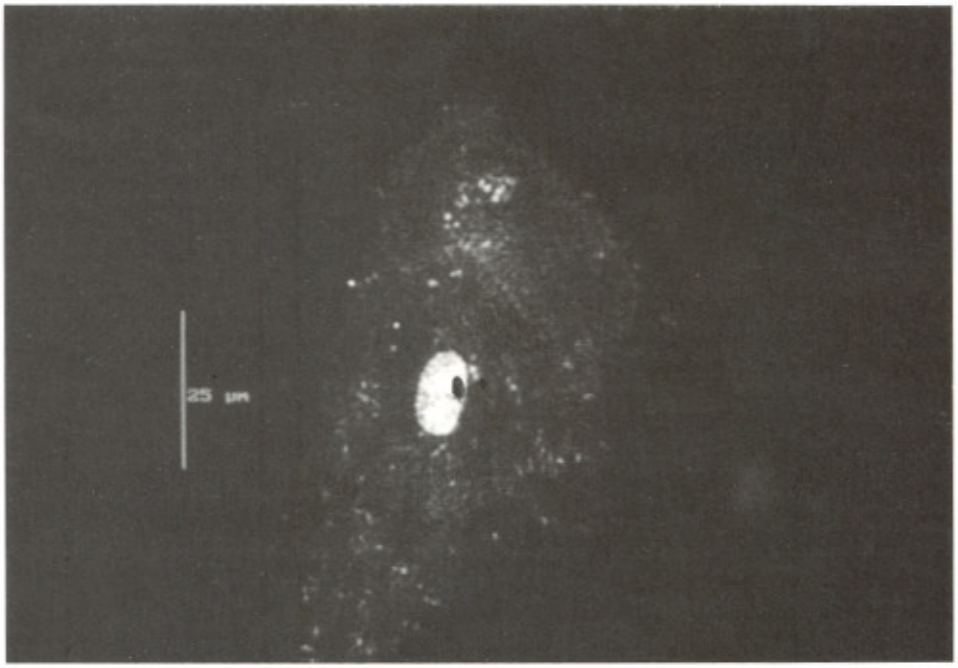

Fig. 4 A cheek epithelial cell stained with BacLight Viability Stain (Molecular Probes, Eugene, Oregon). This composite image consists of 6 0.3-µm-thick optical sections taken at 0.5-µm intervals through the preparation. The large orange-stained cell nucleus is seen in the center of the image. The epithelial cell is defined by the diffuse green-stained material. Bacterial cells are the discrete red and green objects on the cell surface. Red cells are inactive (lacking an active membrane) whereas green cells are active. The image was collected and processed as in Figure 3. (See Color Section.)

Interaction of bacteria with materials is another area ripe for investigation with confocal microscopy. A dual-species biofilms can corrode 304 stainless reproducibly under special conditions (Angell *et al.*, 1995; Palmer, unpublished). What is the relationship in the distribution of the cells to the corroding substratum? Are particular relationships between cells noted? How do corrosion products accumulate in the biofilm? Further, can the utility of confocal/flowcell technology be expanded by introducing a FTIR prism as one of the flowcell substrata? This will permit IR spectra of developing conditioning films to be collected continuously prior to inoculation with microbial communities. After inoculation, recruitment of cells to the conditioning film can be followed, and changes in the IR spectrum that result from biofilm accumulation can be recorded. Other real-time chemical analysis methods can also be integrated with confocal microscopy.

These and other applications of the new technology will rapidly accelerate the insights into biofilm microbial ecology.

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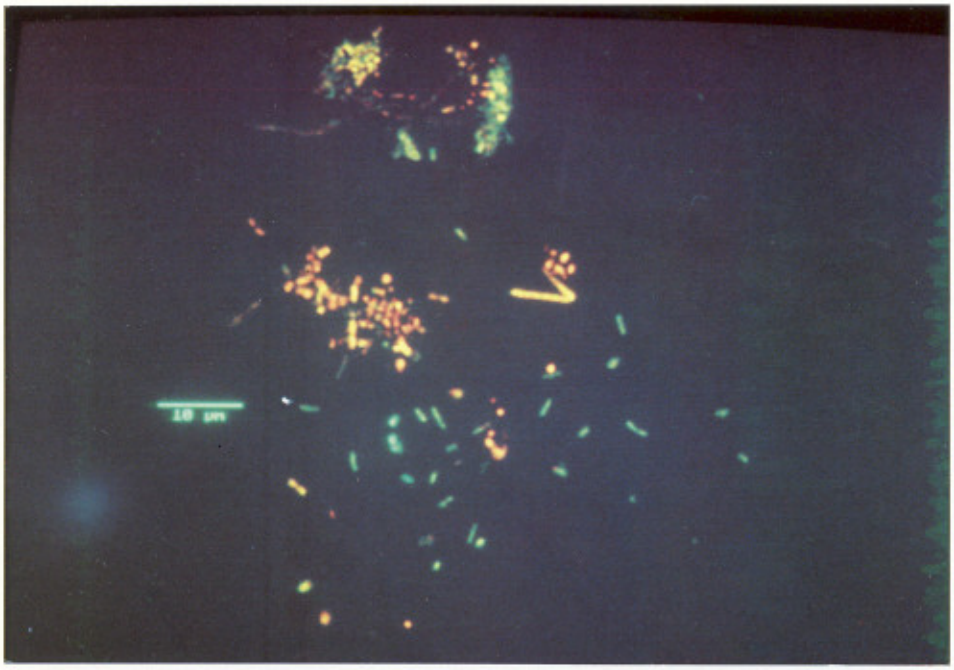
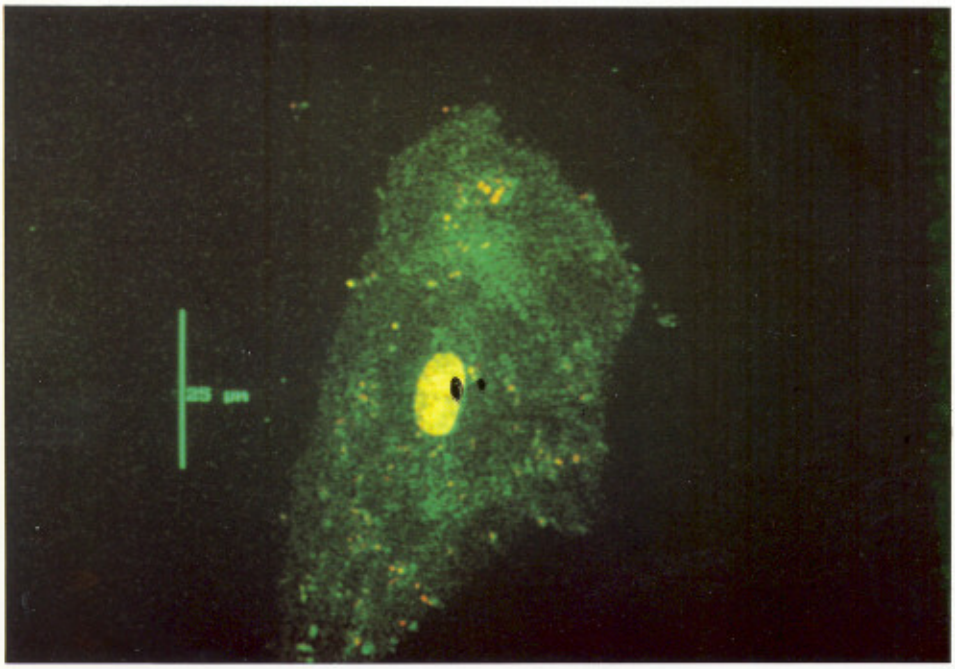
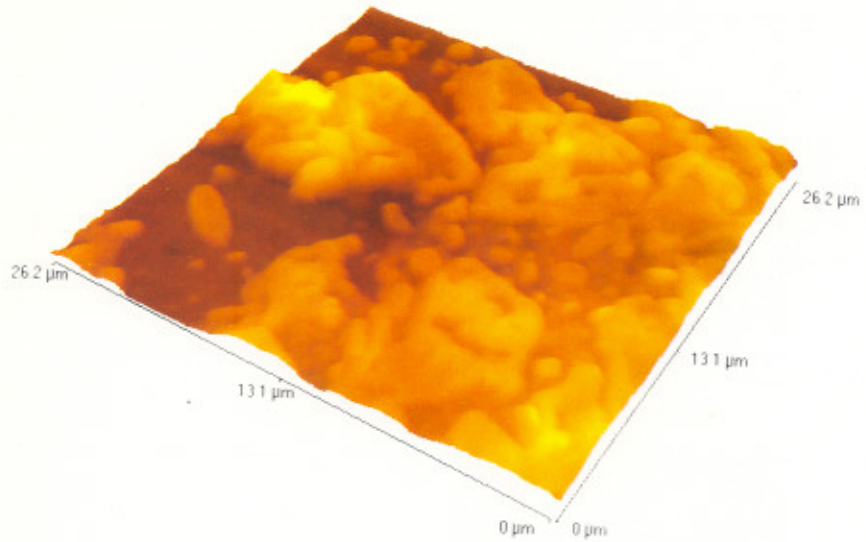


Color Plate I (See Fig. 3 "Biofilm Ecology: On-line Methods Bring New..." by D C White *et al.*)
Fig. 3 Saliva stained with BacLight Gram Stain (Molecular Probes, Eugene, Oregon). Gram-positive cells stain red, Gram-negative cells stain green. The preparation was scanned twice; first under conditions resulting in green fluorescence, then under conditions for red fluorescence. The two data sets were then combined to yield a single dual-wavelength data set.

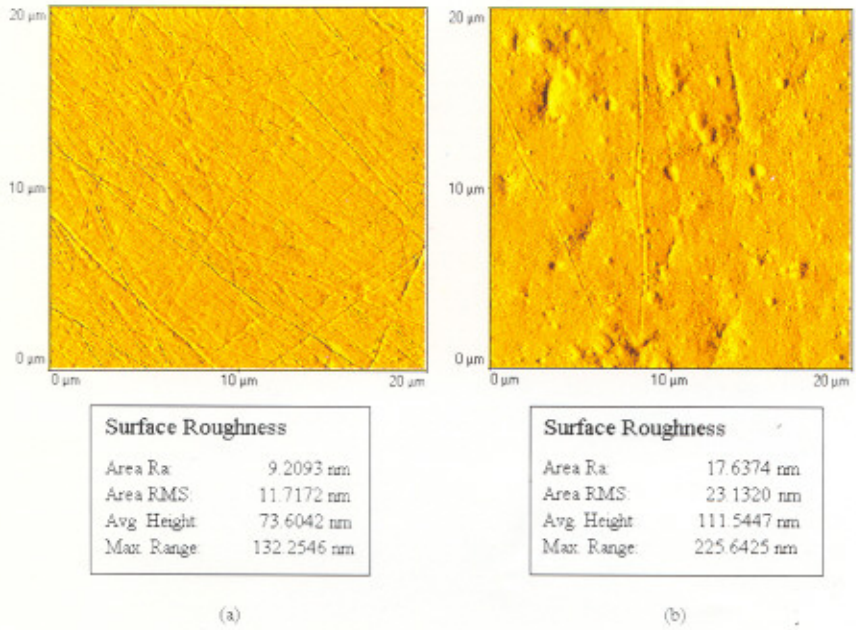


Color Plate II (See Fig. 4 "Biofilm Ecology: On-line Methods Bring New..." by D C White *et al.*)
Fig. 4 A cheek epithelial cell stained with BacLight Viability Stain (Molecular Probes, Eugene, Oregon). This composite image consists of 6 0.3-µm-thick optical sections taken at 0.5-µm intervals through the preparation. The large orange-stained cell nucleus is seen in the center of the image. The epithelial cell is defined by the diffuse green-stained material. Bacterial cells are the discreet red and green objects on the cell surface. Red cells are inactive (lacking an active membrane) whereas green cells are active. The image was collected and processed as in Figure 3.

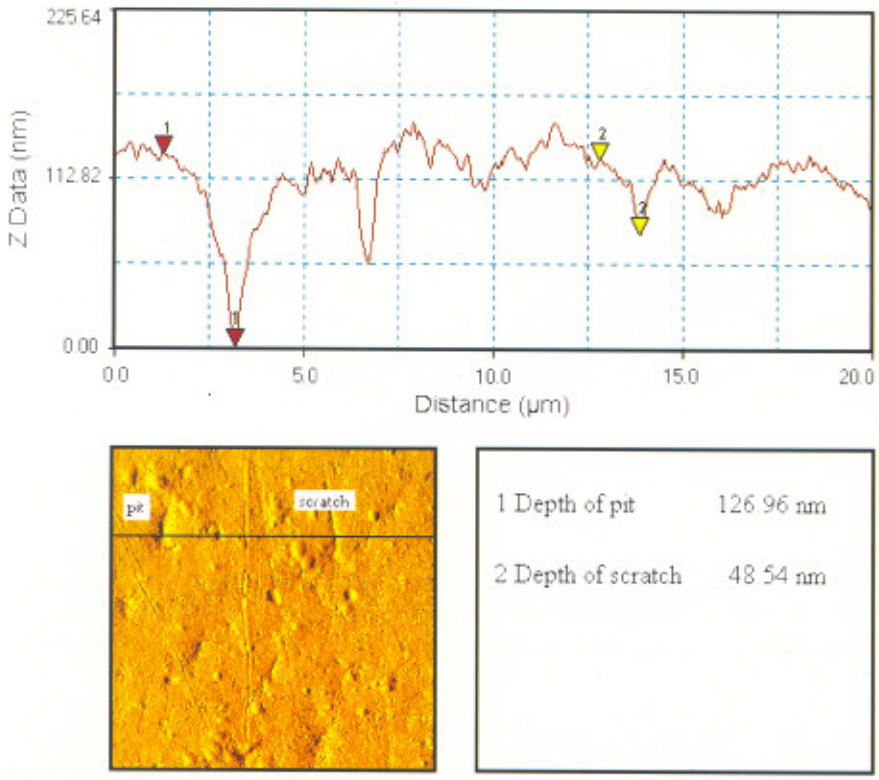
Color Plate III (See Fig. 1 "Comparative Studies of Bacterial Biofilms on Steel..." by I Beech *et al.*)
Fig. 1 Bacterial streamer growth in a disused pyrite mine in North Wales. The main stream is around 0.5 m wide and 20 m long.



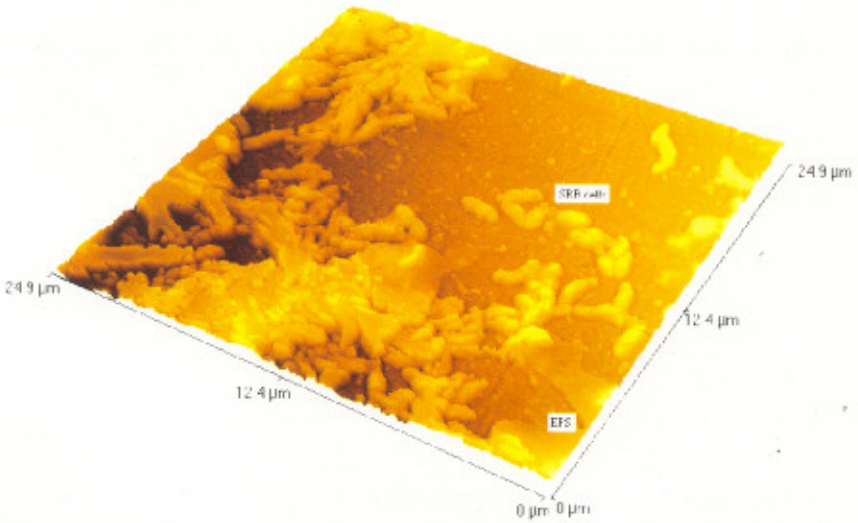
Color Plate IV (See Fig. 3 "Comparative Studies of Bacterial Biofilms on Steel..." by I Beech *et al.*)
Fig. 3 AFM image of acidophilic communities on the surface of AISI 316 stainless steel, following 30 d incubation, showing individual cells of different size and morphology, as well as cells within the EPS matrix.



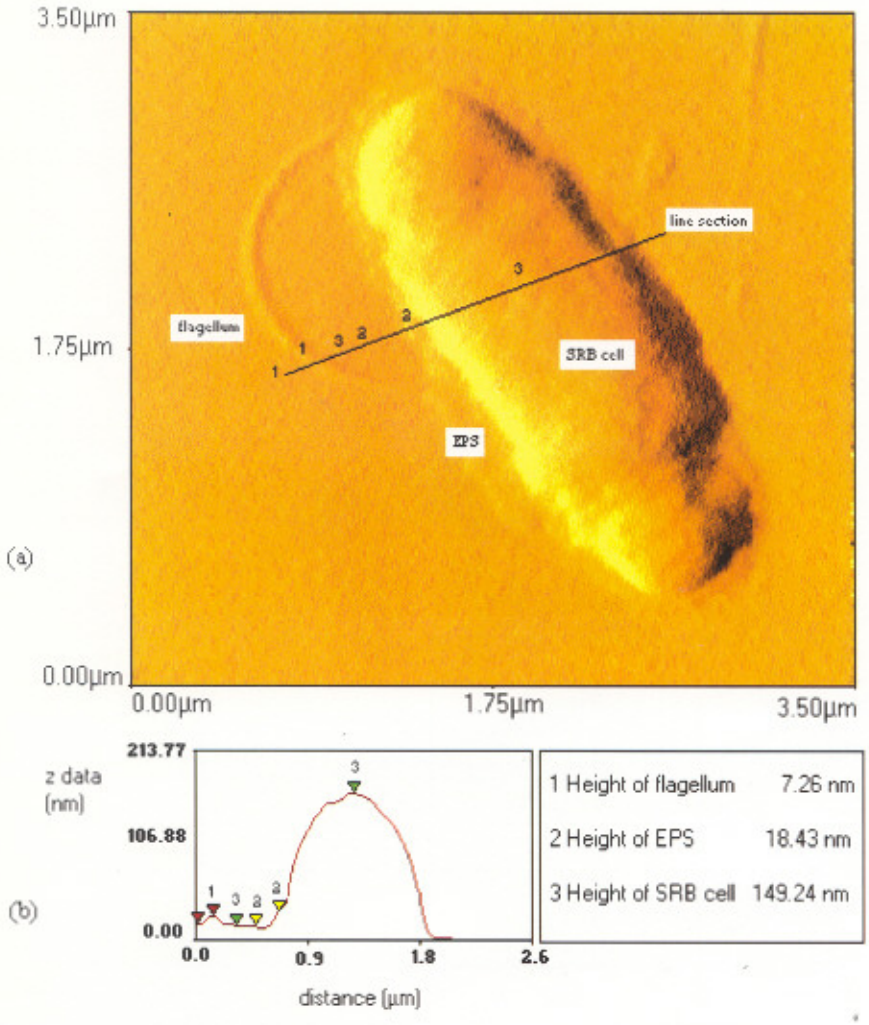
Color Plate V (See Fig. 4 "Comparative Studies of Bacterial Biofilms on Steel..." by I Beech *et al.*)
Fig. 4 AFM micrographs of AISI 316 stainless steel surfaces exposed for 30 d to sterile acid mine water (a) and to acidic streamer (b). The difference in surface topography between the two samples is confirmed following surface roughness measurements, performed using AFM image analysis software.



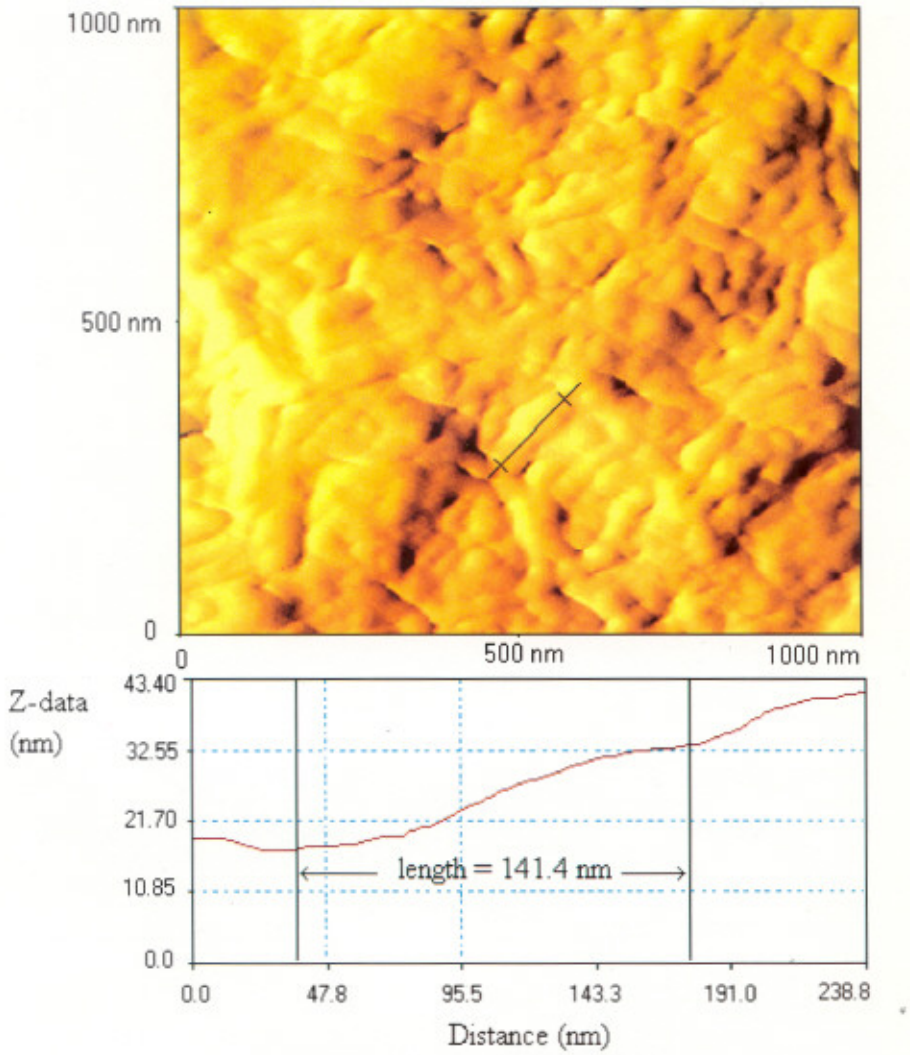
Color Plate VI (See Fig. 5 "Comparative Studies of Bacterial Biofilms on Steel..." by I Beech *et al.*)
Fig. 5 Surface profile of the sample shown in Fig. 4b, demonstrating the ability of AFM to generate quantitative data, such as depth and diameter of micropits and polishing marks.



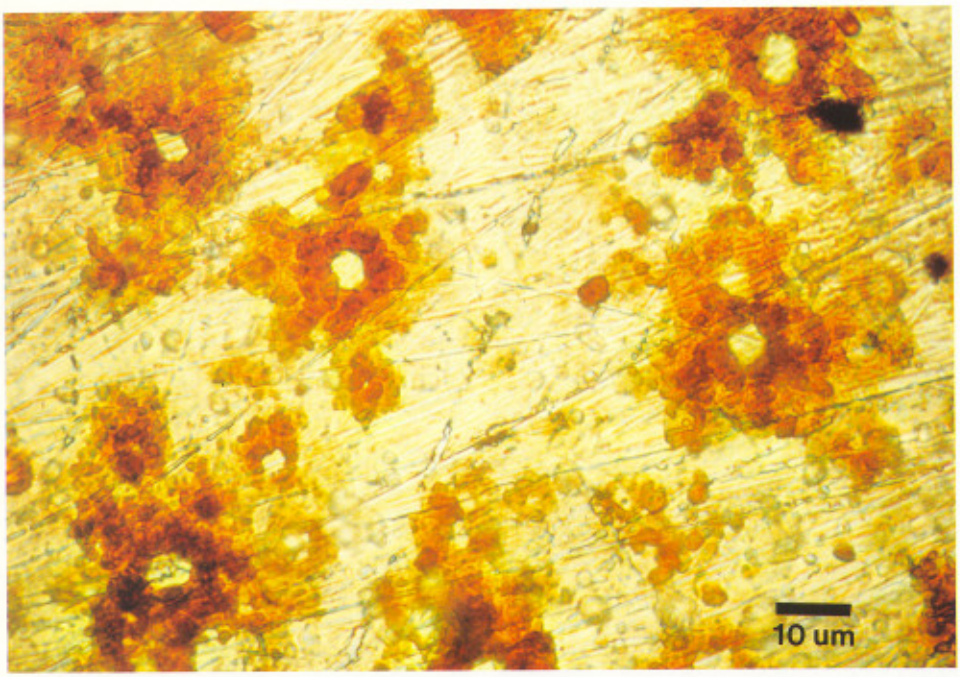
Color Plate VII (See Fig. 7 "Comparative Studies of Bacterial Biofilms on Steel..." by I Beech *et al.*)
Fig. 7 AFM image of a 7 d old Part SRB biofilm developed on the surface of an AISI 316 stainless steel stub in marine Postgate medium C, demostrating patchy coverage of the surface by the layer of bacterial cells and EPS.



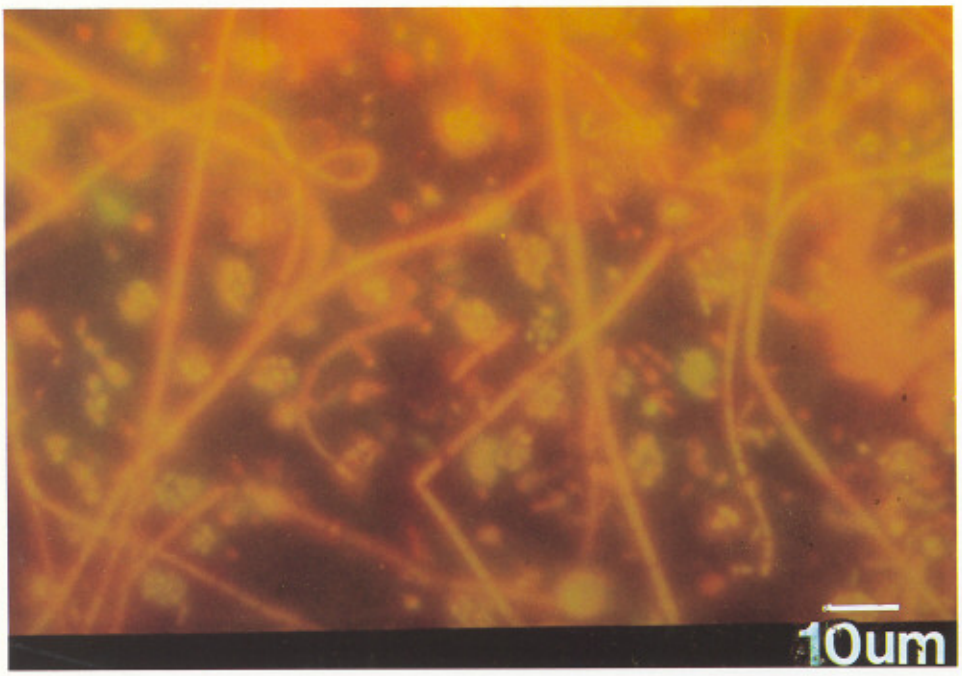
Color Plate VIII (See Fig. 8 "Comparative Studies of Bacterial Biofilms on Steel..." by I Beech *et al.*)
Fig. 8 AFM micrograph of a sessile Port SRB cell on a mica, showing capsular EPS associated with the cell and a polar flagellum (a), Quantitative measurements such as the thickness of the EPS layer and the height of both flagellum and bacterium are also presented (b). These were acquired following image analysis along the line profile.



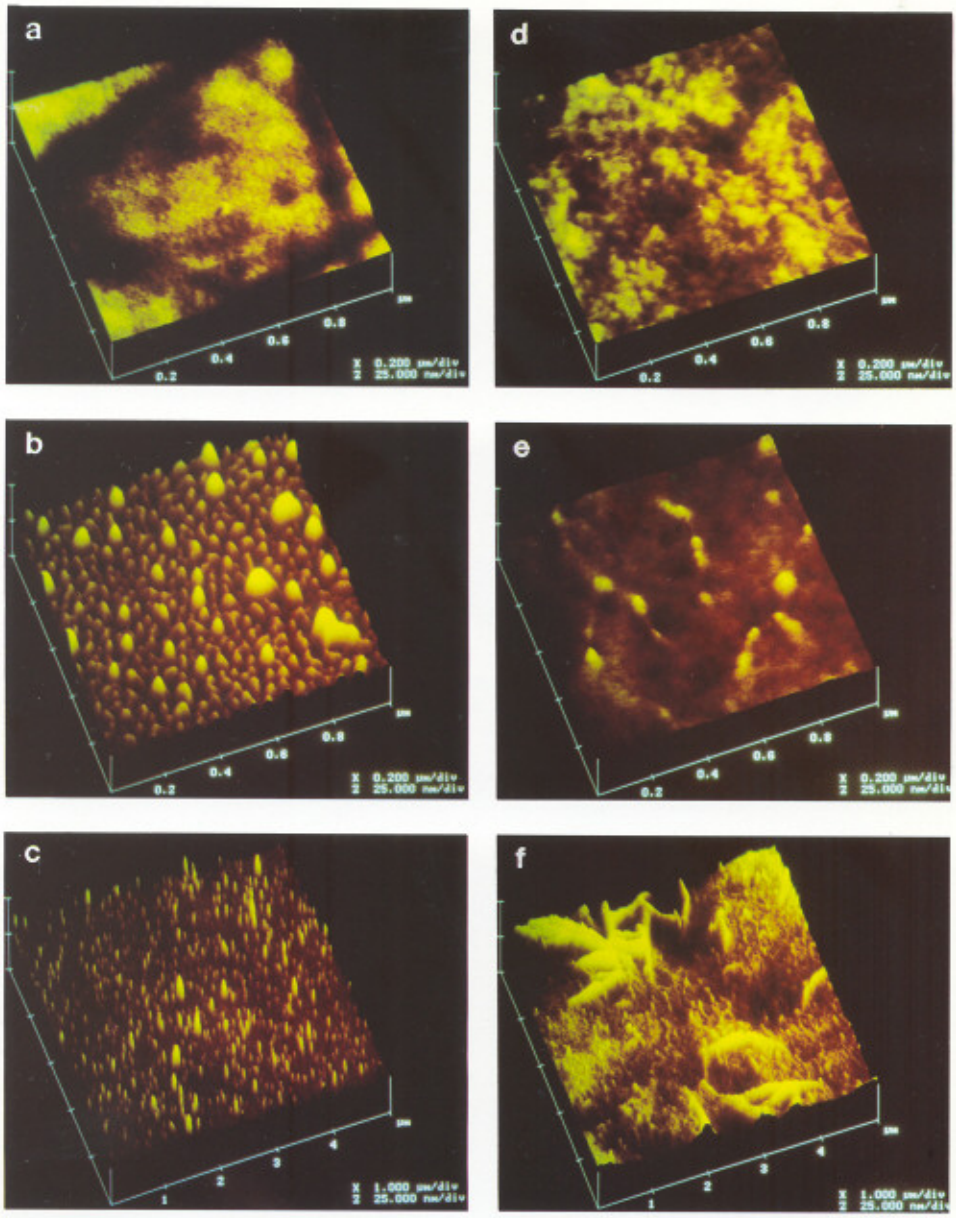
Color Plate IX (See Fig. 9 "Comparative Studies of Bacterial Biofilms on Steel..." by I Beech *et al.*)
Fig. 9 A nanometre resolution AFM image revealing the "mosaic" - like surface structure of a Port SRB cell attached to a mica. Quantification of cell surface features (units) is demonstrated by measurements following the straight line drawn along the unit length.



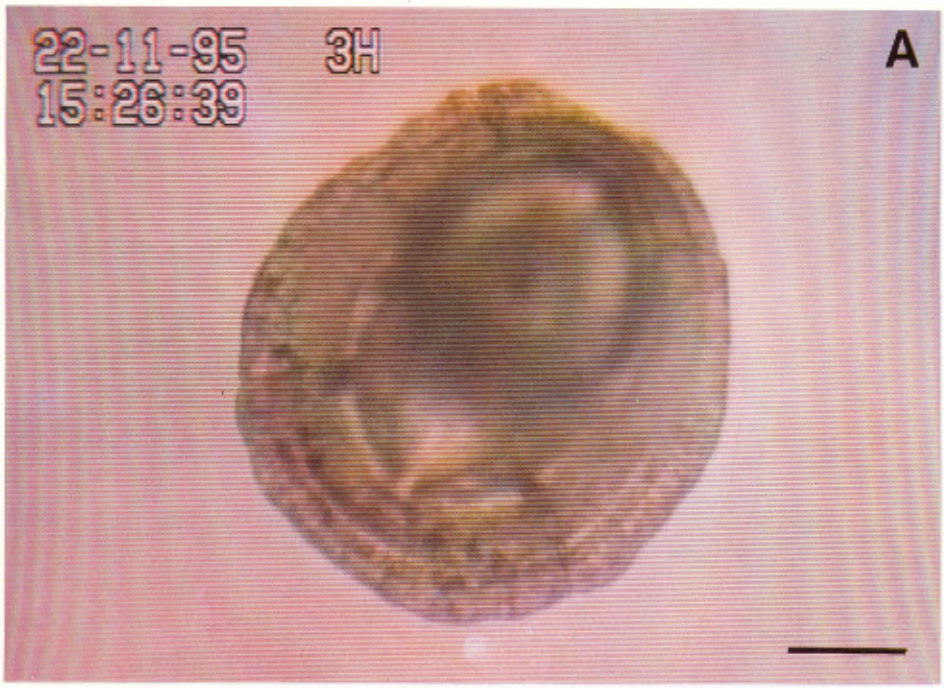
Color Plate X (See Fig. 5 "Manganese Biofouling and the Corrosion..." by W H Dickinson and Z Lewandowski) **Fig. 5** Reflected-light micrograph of annular deposits on 316L stainless steel coupon after 13 d *in situ* exposure to fresh river-water. Stainless steel substratum is visible outside the rings and within the central void.

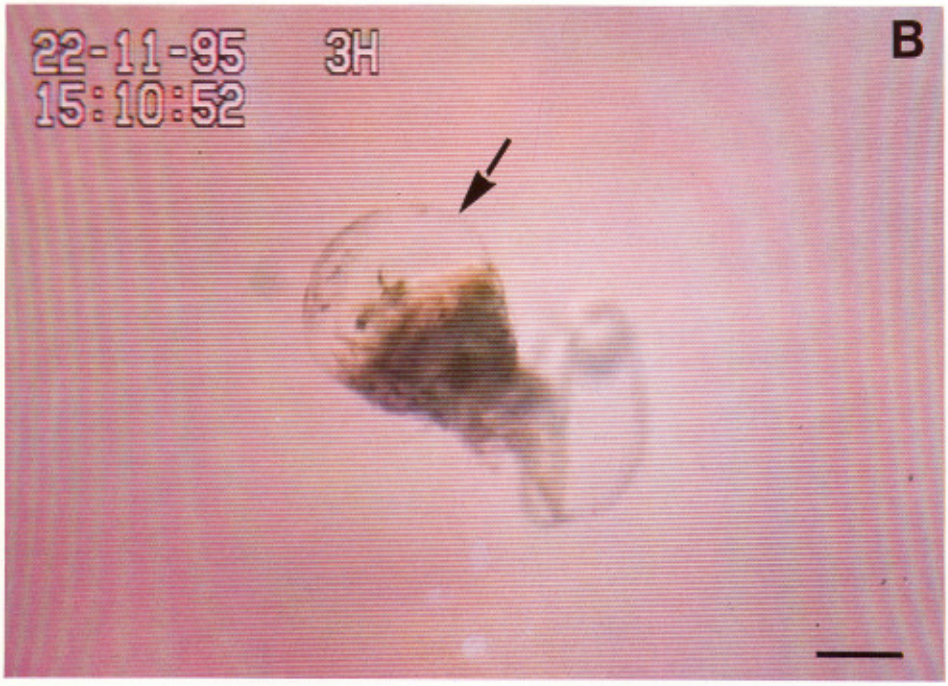


Color Plate XI (See Fig. 5 "Manganese Biofouling and the Corrosion..." by W H Dickinson and Z Lewandowski) **Fig. 6** Epifluorescence micrograph of acridine-orange stained biofilm on 316L stainless steel coupon after 4 d *in situ* exposure to fresh river-water. Individual bacterial cells centrally located within annular deposits as well as sheathed filamentous bacteria can be seen.

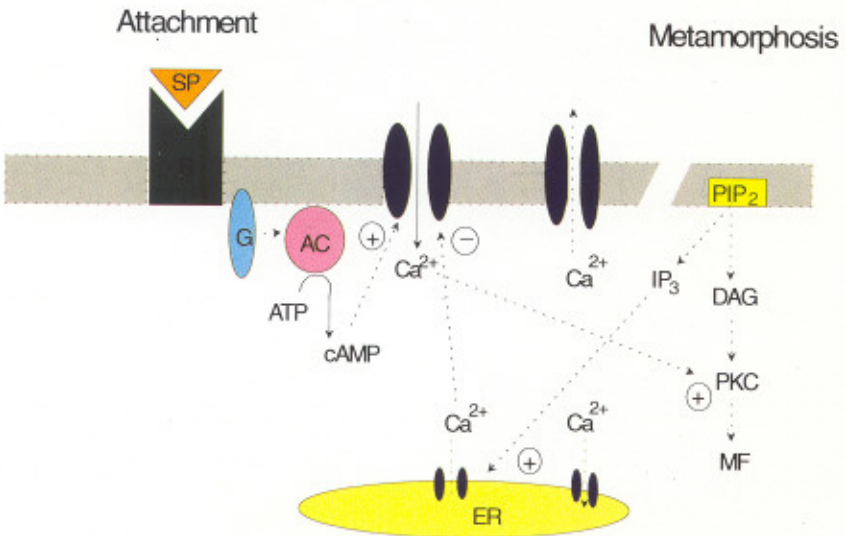


Color Plate XII (See Fig. 1 "Adhesion of Biofilms to Insert Surfaces: A molecular..." by A M Baty *et al.*)
Fig. 1 a,d; b,e; c,f = AFM contour images, Underlying substratum is PS (left side) or POMA (right side). a = 1 μm x 1 μm area of PS before protein adsorption; b = 1 μm x 1 μm area of MAP adsorbed to PS; c = 5 μm x 5 μm area of MAP adsorbed to PS; d = 1 μm x 1 μm area of POMA before protein adsoprtion; e = 1 μm x 1 μm area of MAP adsorbed to POMA; f = 5 μm x 5 μm area of MAP adsorbed to POMA. Protein adsorption was performed in a 50 μg ml^{-1} solution of MAP for 60 min followed by a rinse using fluid displacement (Baty *et al.*, 1995; with permission, *J Colloid Interface Sci*).

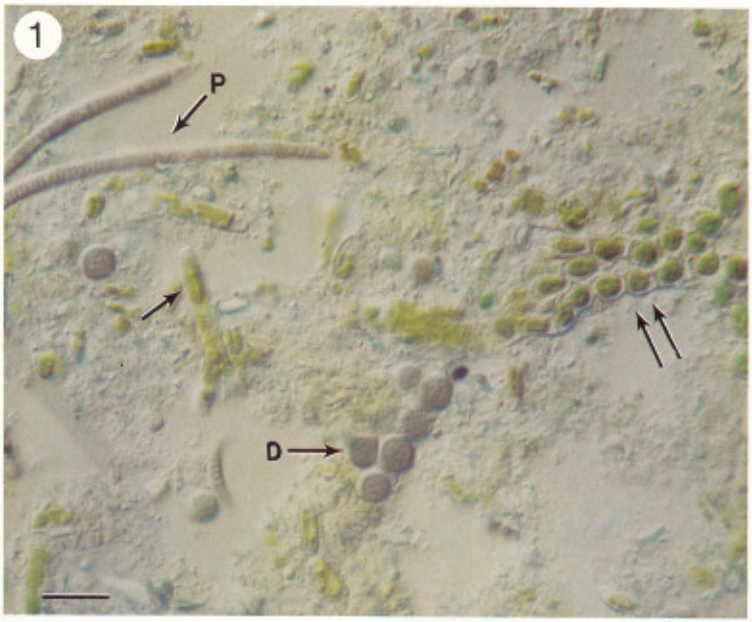




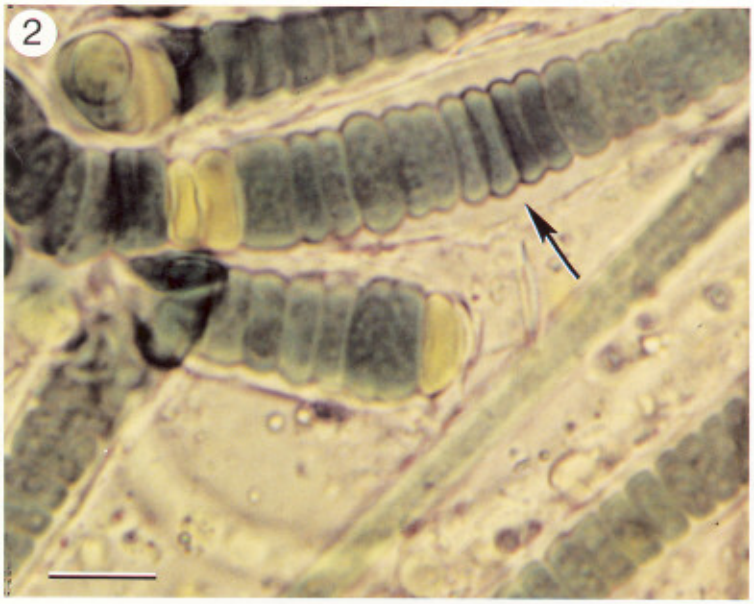
Color Plate XIII (See Fig. 3 "Signal Transduction in Barnacle Settlement: Calcium..." by A S Clare)
Fig. 3 Abnormal metamorphosis in low magnesium seawater. A = irregualr basal margin of spat; B = ballooning of basis (arrow) in juvenile that had metamorphosed at the mensicus. Scale bars = 200 µm.



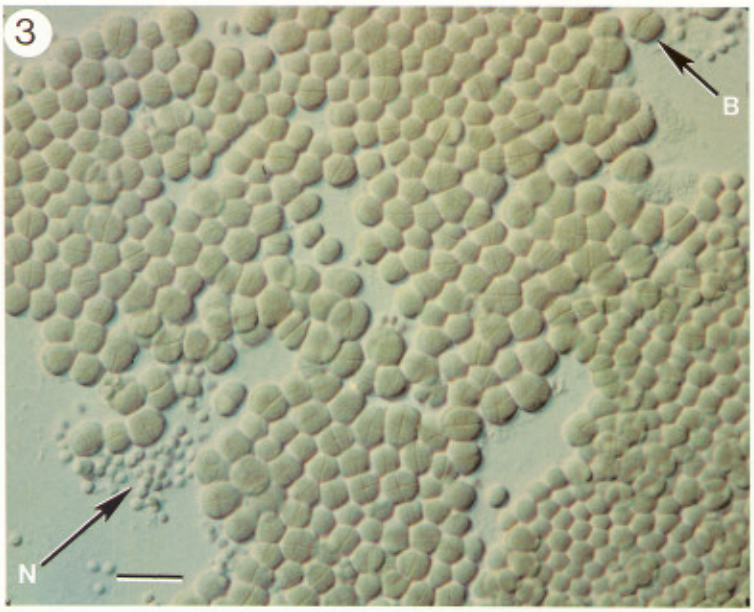
Color Plate XIV (See Fig. 11 "Signal Transduction in Barnacle Settlement: Calcium..." by A S Clare)
Fig. 11 Hypothetical scheme for the signal transduction pathways involved in *B. amphirite* settlement (see text; Clare, 1995; Clare *et al*., 1995; Yamamoto *et al*., 1995). SP = settlement pheromone; G = G protein; AC adenylate cyclase; cAMP = cyclic AMP; ER = endoplasmic reticulum; IP_3 = inositol triphosphate; PIP_2 = phosphatidylinositol bisphosphate; DAG = diaclyglycerol; PKC = protein kinase C; MF = methyl farnesoate, ⊕ = stimulation; ⊖ = inhibition. Blockage of Ca^{2+} efflux from the cell and/or influx to the ER is postulated to explain settlement induction by nifedipine and verapamil; channel blockage would lead to an increase in $[Ca^{2+}]_i$. Alternatively, these organic channel blockers could block calcium channels in the open state. The stimulus to activation of the phospahtidylinositol pathway is not known, but one scenario is for attachment to be induced by water-borne phermone and for metamorphosis to be induced by substratum-bound pheromone. Attachment and metamorphosis are separable pharmacologically, *i.e*. stimulation of PKC leads to metamorphosis without attachment.



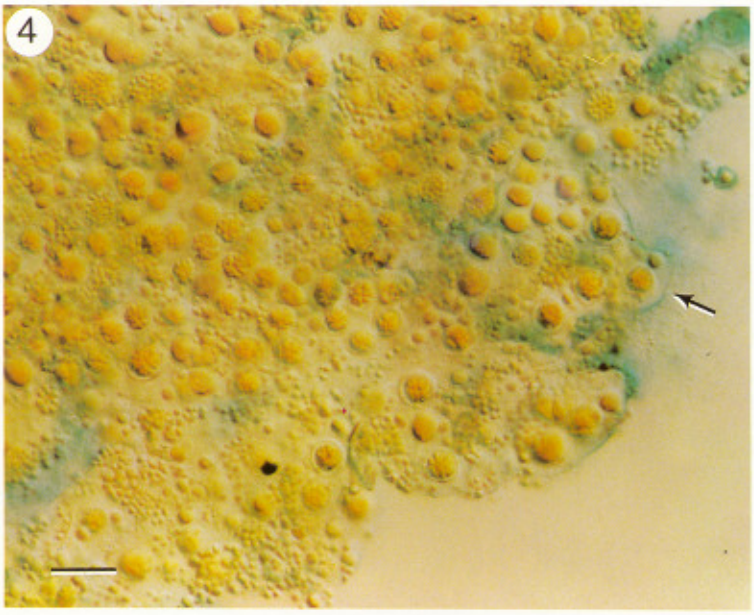
Color Plate XV (See Fig. 1 " Observations on the Mechanisms of Attachment of Some..." by C Scott *et al.*)
Fig. 1 Light micrograph showing a typical early fouling assemblage on a Perspex panel immersed in Langstone Harbour in August 1993, after 4 weeks. The blue-green algae *Plectonema* sp. (arrowed P) and *Dermocarpa* sp. (arrowed D), diatoms (single arrow) and the green alga *Pseudendoclonium submarinum* (double arrows) are early microfoulers. Scale bar = 10 µm.



Color Plate XVI (See Fig. 2 " Observations on the Mechanisms of Attachment of Some..." by C Scott *et al.*)
Fig. 2 Light micrograph showing filaments of *Calothrix* sp. Thick sheath arrowed. The discoid cell shape is typical of the broader blue-green algae (>5 µm.). Scale bar = 10 µm.



Color Plate XVII (See Fig. 3 " Observations on the Mechanisms of Attachment of Some..." by C Scott *et al.*)
Fig. 3 Light micrograph showing a colony of *Xenococcus* sp. Reproduction is by binary fission (arrowed B) and nanocyte (spore) release (arrowed N). Scale bar = 5 µm.



Color Plate XVIII (See Fig. 4 " Observations on the Mechanisms of Attachment of Some..." by C Scott *et al.*)
Fig. 4 Light micrograph of a colony of *Dermocarpa* sp., after staining with alcian blue, showing the acidic polysaccharide nature of the surrounding mucilage (arrowed). Scale bar = 10 µm.