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I am submitting herewith a dissertation written by Marc William Mittelman entitled "CHARACTERIZATION OF BACTERIAL BIOMASS CONSTITUENTS, COMMUNITY STRUCTURE, AND METABOLIC ACTIVITY IN DYNAMIC-FLOW TEST SYSTEMS." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

David C. White, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

CHARACTERIZATION OF BACTERIAL BIOFILM BIOMASS CONSTITUENTS, COMMUNITY STRUCTURE, AND METABOLIC ACTIVITY IN DYNAMIC-FLOW TEST SYSTEMS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Marc William Mittelman

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DEDICATION

This work is dedicated to my wonderful daughter, Anjuliee.

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To paraphrase Sir Isaac Newton, "If I appear to have seen far, it is only because I have stood on the shoulders of some tall individuals." First and foremost, I should like to express my gratitude to my major professor, Dr. David C. White. He has provided the tangible support, guidance, and encouragement which have enabled me to realize my goals and make a contribution to science. While emphasizing the importance of applied research in the environmental sciences, David has always infused me with the joy of exploration for the sake of science alone.

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ABSTRACT

Bacterial biofilm formation on inanimate substrata in freshwater, marine, and physiological environments often precedes microbially influenced corrosion and other biofouling activities. Colonization of and attachment to surfaces in these environments is mediated by environmental factors as fluid dynamics, bulk-phase biotic and abiotic constituents, and the physicochemisty of the substrata. A considerable effort has been applied to resolving questions of biofilm effects on hydraulic regimes and substrata properties. However, fundamental research into the biomass constituents, community structure, and activity of bacterial biofilms as a function of bulk-phase and substrata variables has been more limited. In part, this knowledge gap has resulted from a lack of test systems and analytical techniques for examining bacterial colonization and biofilm formation under conditions which approximate those of in situ environments. This dissertation explores the effects of bulk-phase environments and surfaces on bacterial biofilms under dynamic-flow conditions.

Radial-flow, cell adhesion measurement modules (CAMM) were used to demonstrate that changes in cellular concentration, composition, and metabolic activity of a monoculture of Alteromonas atlantica biofilms on 316 stainless steel (SS) were a function of the applied shear

force. At shear forces in the range of between 1 and 10 dynes cm^{-2}, acridine orange direct counts (AODC) on substrata ranged from 0.15 to 57 X 10^6 cells cm^{-2}. Biomass protein and total fatty acids, carbohydrate:protein ratios, and unit area metabolic activity as measured by in situ ^{14}C-acetate incorporation into cellular lipids were positively correlated with the applied shear force. On a per cell basis, however, metabolic activity decreased with shear, indicating a possible shift in metabolism.

A series of laminar-flow adhesion cells were designed, validated, and constructed using qualitative and quantitative measures of fluid dynamics and bacterial biofilm development. The flow cell design incorporated provisions for on-line, non-destructive measurements of open circuit potential (OCP), bioluminescence, and pO_2 for monitoring bacterial colonization and succession as influenced by a systematic change in bulk phase conditions.

The cells consisted of two high density polyethylene blocks, with a 1 mm deep flow channel milled in the top block. A glass viewing window enabled direct observation of a removable, flush-mounted 25 X 50 mm coupon, which was recessed into the bottom block. The overall dimensions of the cells were 32 mm high X 65 mm wide X 178 mm long. Flow laminarity was validated at 1.3 cm s^{-1} (20 mL min^{-1}).

Colonization by monocultures of Pseudomonas fluorescens was reproducible, with 72 h AODC and viable plate counts

values ranging from between 5.1 to 10.4 X 10^7 and 1.4 to 2.2 X 10^7 cells cm^{-2}, respectively, for replicated flow cells (n=20). On-line OCP measurements were stable over 80 h test periods; onset of P. fluorescence visible biofilm formation coincided with a significant perturbation in OCP.

Bulk-phase bacterial composition and successional order after incubation of 4 organisms from continuous culture were found to significantly affect the community structure, biomass constituents, and metabolic activity of biofilms on 316 SS. Laminar-flow adhesion cells were employed in colonization studies which demonstrated that biofilms exposed to sequential seeding of bulk-phase bacteria in the order of P. fluorescens, Hafnia alvei, Desulfovibrio gigas, and B. subtilis had the highest AODC, protein, and unit area metabolic activity. The freshwater, adhesive aerobe, P. fluorescens, was the dominant member of all biofilm communities, with 5-day biofilms containing on the order of 10^8 cells cm^2. Viable B. subtilis were not recovered from SS surfaces; recovery of D. gigas was poor. Significant perturbations in OCP response coincided with visible biofilm formation; however, no significant differences in the shape or magnitude of the response curves between the various communities tested were observed.

No significant effects on biofilm biomass parameters of AODC, viable counts, protein, carbohydrate:protein ratios, or per cell metabolic activity were attributed to

metallurgical inhomogeneities in 316/E308 welded SS coupons in an oligotrophic, laminar-flow environment. The community structure of 5-day biofilms exposed to various successional orders of bulk-phase cultures of P. fluorescens, H. alvei, D. gigas, and B. subtilis was not significantly affected by surface topology or the presence of weldments. Comparison of OCP plots of 1 μm polished/etched coupons and 600 grit polished coupons did not reveal significant differences. Transmission electron micrographs of embedded and thin-sectioned 5-day biofilms taken from various surfaces revealed the presence of extensive intracellular inclusions reminiscent of poly beta-hydroxyalkanoate and polyphosphate granules seen in microbial mats.

Bacterial bioluminescence by a P. fluorescens (lux) construct was utilized as an endpoint for adhesion in a fluid shear gradient. The CAMM were used to differentially colonize the bioluminescent bacteria on glass and 316 SS surfaces. When induced by sodium salicylate, bulk phase and adherent cells were assayed quantitatively with an ammeter-photomultiplier-fiber optic system. The detection limit was 2×10^5 cells cm^{-2} on glass coupons. Light production was found to positively correlate with biofilm lipid synthesis on a per unit area basis. In the range of 10^5 to 10^7 cells cm^{-2}, light was found to correlate directly ($r^2 = 0.826$) with AODC values, providing on-line detection of both biofilm and bulk-phase biomass and metabolic activity when induced.

PREFACE

My interest in the field of biofilm ecology stems from earlier experiences with the damaging effects of biofouling activities in the pharmaceutical industry. It seemed baffling to me at the time (1979) that, despite our best efforts to control bacterial growth in high purity water distribution systems, they could not be maintained in a sterile condition for any length of time. What were the sources of these contaminating bacteria and how could they apparently survive microporous filtration, ultraviolet irradiation, ozonation, and high concentrations of biocides? Subsequent experiences with other critical medical and industrial purified water systems raised additional questions. What were viable bacteria using as carbon and energy sources in 18.2 Mohm·cm waters? Was the failure to maintain these purified waters free from replicating bacteria due to "biocide resistance" or some other factor?

These and other questions related to the fields of applied and environmental microbiology led me to work with Dr. Gill Geesey early in my career. Through Gill, and the likes of such scientists as Dr. Bill Costerton, I was exposed to the seemingly ignored field of biofilm ecology. My professional experiences convinced me that there were major gaps in our understanding of the interactions between planktonic microorganisms and various substrata. Through a

series of workshops on biological fouling, I was introduced to Dr. David White. His analytical approach to dissecting biofilm communities in diverse environmental and industrial ecosystems was interesting and novel.

A few years after meeting David as a fellow workshop speaker, I became his graduate student. During my tenure at the Institute for Applied Microbiology, I have been involved with a number of diverse and interesting projects, many of which are not contained within this dissertation. However, the central focus of my research has always been in the area of bacterial interactions with inanimate surfaces.

This dissertation is organized into a series of six chapters, the first of which is an introduction and review of the pertinent literature. The remaining five chapters address different aspects of bulk-phase and substrata effects on attached bacteria. Development of new test systems and analytical techniques was an important goal of the research as well.

The interdisciplinary aspects of this evolving science should create new opportunities for environmental and medical scientists. I hope that the body of research contained within this dissertation will serve to stimulate interactions between scientists and engineers interested in this rewarding field.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Bacteria possess a number of adaptive mechanisms for responding to those physicochemical factors which define their environment. These factors include nutrient availability, pH, Eh, temperature, organic and ionic content, and the presence of antagonistic agents. Depending upon the types and numbers present, bacteria can effect alterations in their physiology or physical state in response to the environment. Organic and inorganic acid production, heavy metal binding, transformation of xenobiotics, and extracellular polysaccharide production are important adaptive tools for bacteria in this regard. In most ecosystems, these activities are dependent upon the ability of bacteria to attach to surfaces.

Bacteria are a form of self-replicating organic and ionic particulate material that distinguishes them from other, abiological, contaminants. In natural aquatic systems, the majority of bacteria are attached to surfaces. Indeed, surface area is a major limiting factor for microbial growth in nearly every freshwater and marine environment. The ratio of planktonic (free-floating) to sessile (attached) bacteria is a function of several interrelated factors. These include surface energetics, materials of construction, topology, hydraulic factors, and

biofilm chemistry. It is the biofilm which gives rise to biological particulates and by-products of metabolism which are responsible for biological fouling activities.

The impact of these microenvironmental alterations on various surfaces and fluid handling systems of industrial importance can be significant. Biological fouling can be defined in terms of its effects on various products and processes. Mechanical blockage of flowing systems, corrosion activities, product contamination, and impedance of heat transfer processes result from bacterial adhesion processes. The economic effects of these activities can be staggering: it is estimated, for example, that biological fouling of condenser tubes in power generating operations results in annual losses exceeding 1 billion dollars in North America alone.

Despite their omnipresence in most fluid process systems, relatively little is known about those factors which contribute to bacterial growth and replication in these dynamic environments. This chapter addresses the role that attachment processes play in the survival, growth, and replication of bacteria in environmental and industrial systems.

Adaptive Advantages. There are several adaptive advantages which have been ascribed to a sessile existence. Zobell (1943) proposed that solid surfaces not only act to concentrate nutrients by adsorption, but also retard the diffusion of exoenzymes away from the cell--thus promoting the uptake of substrates which must be hydrolyzed extracellularly. Several workers have demonstrated that attachment processes are, in part, a response to nutrient availability. Decreasing bulk-phase carbon-source concentrations in an aqueous system promote the attachment of marine (Marshall, 1988; Morita, 1982) and freshwater bacteria (Brown, et al., 1977). Geesey et al., (1978) showed that the predominant bacterial population in pristine mountain streams was associated with surfaces. Bacteria in industrial purified water systems, which have many similarities to natural oligotrophic ecosystems, also show a preference for surfaces (Mittelman et al., 1987). Oligotrophic environments have been defined by some workers (Poindexter, 1981) as containing <1 mg L^{-1} total organic carbon. However, as Martin and MacLeod (1984) have observed, each bacterium and consortium possess a range of substrate and growth factor affinities. Whether growth in a particular environment will occur is a function of both the specific growth-promoting compounds as well as their

relative concentrations. Attachment appears to be an important adaptive mechanism in what Morita (1982) has called the "starvation-survival" of bacteria in oligotrophic environments. The ability of sessile bacteria to elaborate a polyanionic, extracellular matrix may be an important factor in the concentration of trace nutrients from the bulk-phase environment (Marshall, 1986).

The ability of bacteria to form consortia in aqueous and terrestrial environments is essential for the survival of many species. Microorganisms isolated from natural environments rarely exist as pure cultures. Rather, they live in diverse societies composed of individuals with various physiological and morphological characteristics. Animate and inanimate surfaces provide a matrix for the formation of these societies (Costerton et al., 1985). Many sulfate-reducing, methanogenic, methylotrophic, and sulfur-oxidizing bacteria are only found on surfaces in close association with microorganisms of diverse physiological activities which provide the narrow range of nutrients these microbes can use. For example, the dissimilatory sulfate-reducer, Desulfobacter sp., although frequently isolated from marine and brackish water environments, is difficult to recover as a monoculture. It is, however, easily grown in a consortium (Dowling and White, 1988). Substrate transfer is facilitated by the close association of organisms within these diverse populations (Mah et al., 1977). The role of

consortial members in the formation and activity of biofilms is an important piece of information missing from most adhesion studies. For example, classification of microorganisms involved at various stages of surface succession would be of value. A differential sensitivity to oxidizing biocides exists within biofilm consortia on mild steel (Franklin et al., 1989). This differential sensitivity demonstrates the importance of using mixed cultures in adhesion studies.

Sessile bacteria are afforded a measure of protection from antagonistic agents present in bulk-phase aqueous environments. Protection from lytic bacteria such as Bdellovibrio sps. (Venosa, 1975), the toxic effects of heavy metals (Mittelman and Geesey, 1985), and bactericidal agents (Costerton and Lashen, 1984; Costerton et al., 1981) are important advantages afforded to sessile organisms. This protective feature is a significant factor in many disease processes and biological fouling activities. Bacteria associated with biofilms are more resistant to antibiotic treatments which would otherwise prove effective against free-living populations (Hoyle et al., 1990). Apparent biocide resistance to potable water treatments is a serious problem throughout the world. LeChevallier et al. (1988) showed that attachment of Klebsiella pneumoniae to glass slides increased resistance to free chlorine disinfection by a factor of 150. While the high concentrations and multiple

sites of action associated with many biocides may, for example, overwhelm plasmid-mediated resistance, some biofilm organisms may be resistant by virtue of their ability to form polyanionic extracellular polymeric substances (EPS). Unfortunately, most biocide efficacy studies employ planktonic bacterial populations as challenge organisms. Thus, treatments for biofouling problems very often are ineffective and, in some instances, can exacerbate existing fouling activities (Ruseska, et al., 1982).

Biofilm Development. Bacterial attachment and the subsequent formation of a biofilm appear to take place in a three stage process. In the first stage, surfaces are rapidly coated with an organic "conditioning" film. In the blood, this film might consist of proteinaceous compounds such as albumin (Absolom et al., 1987; Uyen et al., 1990); on teeth, mucopolysaccharides (Embery et al., 1986); in freshwater environments, humic substances (Marshall 1986). In studies of blood interactions with foreign substances, it was found that within 5 s most surfaces become uniformly coated with strongly adherent proteinaceous films (Baier and Dutton, 1969). Rapid modification of clean surfaces in marine waters by glycoprotein and polysaccharide compounds has also been reported (Baier, 1972; Corpe, 1970).

In the second stage of adhesion, single bacterial cells are transported to surfaces and reversible bonds are formed

between the cell wall and surface. Bacterial extracellular polysaccharides (EPS) appear to mediate the attachment of primary colonizers to organic conditioning films associated with animate and inanimate surfaces (Marshall et al., 1971). Corpe (1970) described an "acid polysaccharide" which acts as a polymer bridge between bacterial cell walls and surfaces. It is interesting to note, however, that Allison and Sutherland (1987) have presented evidence that extracellular polysaccharide, while important in the development of surface films, is not directly involved in the initial adhesion process of freshwater bacteria. The high uronic acid content of many types of EPS probably plays an important role in concentrating essential substrates and preventing antagonistic agents from reaching labile cellular processes. Calcium and other divalent cations may be involved in the initial stages of attachment. These ions appear to stabilize the EPS by cross-linking adjoining sugar groups within the polysaccharide polymer. Applegate and Bryers (1991) have described the role that calcium plays in stabilizing EPS to the effects of fluid shear.

The mature or third-stage biofilm consists of the organic conditioning film, a succession of colonizing bacterial consortia with their associated EPS, and various biofilm-associated detrital particles and ionic species. It is this structure which gives rise to the planktonic

bacteria and their by-products (e.g., endotoxins) which act as self-replicating, particulate contaminants.

The question of whether differing substratum surface properties are communicated to the initial or succeeding organisms through the conditioning film is of great interest. Uyen et al. (1990) suggest that substratum properties can be transferred by an adsorbed protein film to adhering eucaryotic or procaryotic cells. They base this supposition on their finding that the amount and surface structure of albumin adsorbed onto inanimate surfaces was a function of substrate wettability (surface free energy). However, the possibility remains that bacteria recognize--and are attracted to--moieties within the conditioning film rather than structures on the underlying surface. Despite recognition of the importance of conditioning films as precursors to biological fouling activities, treatments have not been developed for their control or modification. If, for example, the constituents of a conditioning film elicit a chemotactic response, the chemistry and topology of a given surface might exert a relatively minor influence on biofilm development. The type of conditioning film present may be the major factor involved with the penetration of pathogenic organisms into tissues, tooth surfaces, and implantable medical devices. It may also affect the rate and extent of such surface perturbations as pitting corrosion activities.

Adhesion Mechanisms. Mechanisms for bacterial surface recognition and initial colonization have yet to be fully elucidated. Some workers have described a chemotactic response by bacteria to surface-associated compounds. Chemotaxis is the movement of an organism in response to a chemical gradient. Young and Mitchell (1972) have implicated positive chemotaxis in the initial colonization of bacteria to marine surfaces such as ship hulls. The phenomenon of "negative chemotaxis" in response to surface-associated toxic substances has also been described (Chet et al., 1970; Young and Mitchell, 1973). Models of bacterial chemotaxis to surfaces in flowing aqueous systems have been described (Keller and Segeal, 1971; Lepidus, 1980). Implicit in the chemotaxis mechanistic theory, however, is the ability of bacteria to direct their movement towards the surface. Since non-motile bacteria are also associated with fouling biofilms, chemotaxis can only be used to describe one factor involved in the initial attachment mechanism.

Impedance of flagellar activity by mechanical contact with surfaces appeared to promote attachment and lateral flagella formation in a marine vibrio (Belas and Colwell, 1982). Upon contact with a surface, the polar flagella of Vibrio parahemolyticus ceased to function. Shortly thereafter, lateral flagella formed around the cells, apparently mediating the "irreversible" attachment process.

Pilus mediated adhesion to gastrointestinal surfaces is recognized as a major factor in the toxicity of enteropathogenic Escherichia coli in humans and other animals (Gorbach et al., 1975). Pilus negative E. coli are incapable of attaching to these surfaces and producing pathogenic effects. In most cases, adhesion to host tissues by pathogenic bacteria is a first step in initiating infections (Savage, 1985). Usually, this process is mediated by carbohydrate-specific lectins on the bacterial cell surface (Jann and Hoschutzky, 1990). Ridgway and Olson (1981) presented scanning electron microscopic evidence which indicated that attachment of bacteria to potable water pipelines was mediated by extracellular fibrillar appendages.

Finally, and perhaps most significantly, physical characteristics of bacteria and substrata such as surface energy (Absolom et al., 1983; Baier et al., 1968; Fletcher, 1988, van Dijk, et al., 1988; van Loosdrecht and Zehnder, 1990), hydrophobic effects (Dahlback et al., 1981; van Loosdrecht et al., 1990; Rosenberg and Kjelleberg, 1986), and surface topology (Characklis, 1973b; McCoy et al., 1981; Corpe, 1980) may influence the initial attachment process. The DLVO theory of colloid stabilization, as applied by Marshall, et al. (1971) to bacterial colloids, holds that the separation between bacteria and adsorbents in an electrolyte solution is dependent upon a balance between

attractive and repulsive forces. This relationship can be described by the following equation:

$$V_t=V_r+V_a \quad (1)$$

where, V_t=total energy of interaction

V_r=energy of repulsion

V_a=energy of attraction

The electrical double-layer, which is associated with surfaces in electrolyte or dilute organic solutions, is thought to present a repulsive barrier to bacterial-adsorbent interactions. This repulsion, however, is balanced by the short-range van der Waals forces which attract colloids to surfaces in such environments. The separation distance is minimized under conditions of low surface potential and bacterial affinity for surfaces is maximized at increased salt concentrations. However, above a salt concentration of 0.1 M, Fletcher (1988) found that bacterial attachment began to decline exponentially with increasing cation concentration. Firm ("irreversible") attachment requires some mechanism whereby the finite separation distance imposed by the electrical double-layer is bridged. Corpe (1980) and others believe that EPS are involved in this bridging process.

There is also evidence that hydrophobic interactions play a role in controlling adhesion processes. As bacteria approach a surface, this theory holds that water molecules are displaced, creating a lower free energy of interaction. When two surfaces covered by ordered layers of water molecules approach one another, layers of water are released into the bulk-phase from between the two surfaces. This results in a decrease in free energy and an increase in entropy. Depending upon the degree of interaction between the cell and a surface, this process can favor adhesion (Rosenberg and Kjelleberg, 1986). Surface energetics tends to favor colonization of bacteria to low energy (hydrophobic) surfaces such as Teflon. In general, colonization, but not necessarily irreversible adhesion, is greatest for bacteria possessing hydrophobic cell surfaces interacting with hydrophobic substrata.

The data are often confusing, however, with respect to the mechanisms of bacterial adhesion processes. While surface energy considerations would appear to favor adhesion of bacteria to low energy (hydrophobic) surfaces such as Teflon (Fletcher and Loeb, 1979), Absolom et al. (1983) pointed out that preferential adhesion to relatively high energy (hydrophilic) surfaces can occur when the surface energy of the bacteria is greater than that of the suspending medium. Clearly, the surface energies of

substrata (and their associated conditioning films), bacterial cell surfaces, and bulk-phases are interdependent variables.

FACTORS INFLUENCING ADHESION PROCESSES

Environmental Factors. Studies of environmental and substrata variables which influence the initial attachment of bacteria and the subsequent formation of mature biofilms have generally lacked relevance to the actual in situ conditions. Previous studies by Fletcher et al. (1976,1979,1988), Dexter et al. (1975), Marshall et al.(1971), Rosenberg et al. (1980,1981), and others have focused on physicochemical interactions between bacterial monocultures and glass or polystyrene surfaces. The effects of varying bulk-phase conditions, surface characteristics, in situ conditioning films, and other environmental factors on attachment processes have not been fully explored. Preliminary work by Corpe (1970), Dexter et al. (1975), and Fletcher (1976) did indicate, for example, that organic conditioning films have an effect on attachment processes. The nature of the conditioning films and their relative importance for initial attachment were not described, however.

Bacterial attachment typically occurs under the influence of some type of hydraulic influence. Characklis (1973a, 1973b) and McCoy and Costerton (1982) have demonstrated that such fluid hydraulic parameters as turbulence and fluid velocity have an effect on the nature of biofilms. Below a critical shear force, however, the amount of biomass per unit area does not appear to be significantly reduced at increased linear fluid velocities.

Metabolic Effects. A key to the understanding of adhesion processes is the ability to elicit biofilm function from structure and activity. In studies of biomass formation and mineralization, sessile bacteria have usually demonstrated significantly higher activities than have planktonic populations. For example, a natural population of sessile marine bacteria was found to transport ATP 100 times faster than planktonic organisms on a per cell basis (Hodson et al., 1981). The nature of extracellular polysaccharides also differs between sessile and planktonic bacteria. Valeur et al. (1988) demonstrated that a significant difference existed between the ratio of saturated: unsaturated fatty acids and poly-betahydroxybutyrate (PHB) content between planktonic and sessile bacteria. A subpopulation of the test organism containing a higher ratio of total C18/C16 fatty acids and lower PHB levels selectively adhered to test surfaces. Nichols et al. (1985)

demonstrated that significant shifts in the IR spectra of P. atlantica biofilms as measured by Fourier transform infrared spectroscopy (FTIR) result when the organism is grown under different growth conditions. Environmental factors such as bulk-phase C:N ratio and fluid shear also appear to affect the nature and activity of biofilms on surfaces such as stainless steel.

Bartlett et al. (1988) have shown that extracellular polysaccharide production by P. atlantica is a variable trait, determined by expression of a now defined "eps" locus. Mucoid colonies (eps^+) produce extracellular polysaccharide while the spontaneously reverted crenated colonies (eps^-) apparently lack the ability to synthesize large amounts of extracellular polysaccharide. When recombinant plasmids with a locus for extracellular polysaccharide production are transferred to crenated variants, expression of the mucoid (eps^+) form results. What effect expression of this eps gene has on attachment characteristics is as yet undetermined. A clearer picture of extracellular polysaccharide function in the development of primary and mature biofilms could lead to a better understanding of those factors which control the transition of bacteria from a planktonic to a sessile existence. An alteration of extracellular polysaccharide composition or quantity in response to some environmental perturbation could be an important factor in this transition process.

BIOFILM-MEDIATED CONTAMINATION

Disease Processes. Bacterial biofilms play an important role in disease processes, industrial contamination, and environmental pollution. Antibiotic therapies have, for most part, failed to address the mechanisms associated with initial bacterial colonization and biofilm formation (Anwar et al., 1990). This gap in the approaches to treating gastrointestinal (e.g., enteropathogenic E. coli infections) and pulmonary diseases (e.g., secondary Pseudomonas infections of cystic fibrosis patients) could be narrowed with further research into these mechanisms. Problems related to bacterial colonization of such invasive medical devices as heart valves, catheters, penile prostheses, and intrauterine devices may eventually be resolved by a mechanistic approach to biofilm control (Costerton et al, 1985). This would involve studying mechanisms of surface recognition and initial colonization for various materials of construction. Determinations of attachment efficiency under varying conditions of pH, Eh, organic (protein) concentration, etc. would provide important information for medical device designers as well as physicians. For example, knowledge of the effects of these and other variables on attachment efficacy could be used to predict the probability of device colonization in certain patients. This finding would have implications for researchers

concerned with microbial adhesion to inanimate and biological matrixes.

Purified and Potable Waters. High quality purified water is essential to numerous applications in pharmaceutical, dialysis, medical device, microelectronics, aerospace, and chemical processing industries. Bacterial contamination of these systems remains the most significant threat to product and process quality. Attachment of bacteria and subsequent biofilm formation can occur in waters containing levels of ions and organics below analytical detection limits. The recognition of surface associated organics by bacteria in extremely oligotrophic environments has not been studied. If chemotaxis does indeed play an important role in initial bacterial colonization, such nutrient-limited environments would be ideal systems for studying recognition and uptake mechanisms. The oligotrophic nature and large surface area:volume ratio associated with purified water systems may promote the attachment of bacteria to surfaces.

Ion exchange resins (Flemming, 1987), reverse osmosis and dialysis membranes (Ridgway et al., 1984), storage facilities, and distribution systems (LeChevallier et al., 1987) are subject to biofilm formation. Endotoxin production, product contamination, corrosion activities, and mechanical fouling problems seriously compromise product safety. Novitsky, et al. (1986) demonstrated that bacterial

endotoxins, which are the predominant pyrogenic ("fever-producing") substances in parenteral fluids, preferentially sorb to container surfaces. Removal of these recalcitrant bacterial by-products from surfaces continues to present challenges to pharmaceutical manufacturers. In the microelectronics industry, high bacteria levels in ultrapure deionized water have been associated with yield anomalies in non-metal oxide surfaces, complementary metal oxide surfaces, and bipolar technologies. In addition, biologically mediated failures of back-side metallization adhesion processes have been reported (Craven et al., 1986). As particulates, bacteria have also been implicated in "shadowing" of oxide surfaces during photolithograpy.

The so-called "bacterial regrowth" phenomenon following chlorine treatments of purified and potable water systems is likely a function of biofilm formation rather than resistance in the classical sense of antibiotic resistance (LeChevallier et al., 1988; Ridgway et al., 1984; Wolfe et al., 1988). A combination of the polyanionic nature of many EPS moieties coupled with the inherent biocide demand associated with extracellular slime matrices acts as an inhibitor of biocide actions. Residual concentrations of oxidizing biocides in estuarine power plant effluents can have serious consequences for marine primary producers. Biocide-mediated oxidation of bromide ions in seawater creates a greater threat to living organisms than do the

biocides themselves (Groninger and Mills, 1980). The fact that most power plant condenser waters are treated on a continuous or frequent slug dosage basis is an indication of the recalcitrance of fouling biofilms. A treatment strategy which focused on modifications of the primary condition film and/or initial colonizing bacterial population might obviate the need for toxic biocides, thus removing a significant threat to aquatic ecosystems.

Microbially Influenced Corrosion. The billions of dollars spent in controlling the effects of corrosion and other biologically mediated fouling activities have failed to target the attachment processes which are responsible for the observed effects. Microbially influenced corrosion activities (MIC) are preceded by the development of a biofilm, which can then mediate the formation of chloride concentration cells, development of differential oxygen cells, depolarization of cathodic hydrogen, production of organic acids, and other dissolution processes (Costerton and Lashen, 1984; Thomas et al., 1988). Using electrochemical techniques such as AC impedance spectroscopy, Dowling and White (1989) described the relationship between biofilm development on metal surfaces and corrosion activity. Corrosion rates of mild steels were shown to be significantly accelerated in the presence of fouling microorganisms. It has been estimated that leaks in

the Alaskan oil pipelines related to MIC activities result in losses of one million dollars per day in oil (Lewin, 1984).

The corrosion of containers for high-level radioactive wastes has implications for all of mankind. The materials of construction and design of these containers in geological disposal sites must be sufficient to survive for at least 1000 years (Day et al, 1985). Marsh and Taylor (1988) have considered the theoretical contribution of microbial consortia to the corrosion of these containers. Based upon environmental conditions within the repository, the authors surmised that only the sulfate-reducing bacteria (SRB) of the categories examined would be capable of growth in the progressively anaerobic environment. Employing calculations which assume lactate and/or acetate as sole carbon sources, a "worst case" loss of ca. 13 mm mild steel during the 1000 year lifespan was predicted in addition to the 216 mm lost as a result of "abiological" localized corrosion.

Unfortunately, the influence of biofilms as mediators of corrosion was ignored in these calculations as was the potential autotrophic growth of SRB on H_2 and CO_2. In order to form long term projections on the behavior of such materials some estimation must be made for the water activity of surrounding rock formations. The activity of many microorganisms is severely curtailed in desiccated environments. Most problematic is projection of local

weather trends 1000 years in the future and the effect of the spent fuel thermal output for the first 500 years which will tend to draw water to the repository. Obvious measures such as citing these repositories away from gypsum formations (sulfate sources) must also be considered.

A number of workers have attempted to place a dollar figure on the economic effects of underground pipeline corrosion. In one of the first studies of MIC effects on pipeline corrosion, Greathouse and Wessel (1954) described U.S. economic losses in excess of 100 million dollars per year. Allred et at (1959) found that SRB were responsible for more than 77% of the corrosion of oil-well underground pipelines. Postgate (1984) has noted that despite the "obvious economic importance of anaerobic corrosion, useful estimates of its economic cost are difficult to make."

Biological fouling of heat exchanger surfaces, cooling tower structures, and attendant distribution systems accounts for over 4.5 billion dollars in economic losses in the non-communist world. Losses to U.S. refinery heat exchangers amount to greater than 1.4 billion dollars per year (Knudsen, 1981). Purkiss (1971) has quoted losses in British industrial systems exceeding 300 million pounds per year in 1971 pounds. It is often difficult to separate the effects of fouling phenomena such as mechanical blockages, heat transfer resistance, and corrosion. Within an aerobic layer composed of such slime-forming isolates as Bacillus

subtilis, B. cereus, Flavobacterium spp., Aerobacter spp., and Pseudomonas spp., conditions become ideal for the growth of SRB. Iron oxidizing bacteria such as Sphaerotilus and Crenotrix spp. produce ferric hydroxide, a potent initiator of pitting corrosion in addition to forming differential oxygen cells (Purkiss, 1971).

The fouling and corrosion of jet fuel storage tanks by microbial consortia--principally, fungal contaminants--creates tremendous problems for military and civilian aircraft operations. Severe structural damage resulting from biogenic organic acid attack was commonplace in the 1950's and 1960's, prior to the introduction of biocides and improved measures to prevent water intrusion into the fuel systems. Cabral (1980) analyzed samples of kerosene A1-type jet fuel from aircraft fuel systems in Argentina; 65% of those tested were found to be contaminated with Cladosporium resinae, a ubiquitous, filamentous fungi. While a number of other fungi were isolated, most notably Penicillium spp., only C.resinae was capable of utilizing the fuel as a sole carbon source. Various consortia were tested on a kerosene medium; however, only those mixtures containing C.resinae were able to utilize the kerosene as a sole carbon source. Schiapparelli and Meybaum (1980) have identified dodecanoic acid (a kerosene breakdown product) as a major organic acid involved in the corrosion of aluminum fuel tanks. Other mechanisms of these aluminum fuel tanks include the

differential oxygen cells created by bacteria as result of oxygen uptake in localized areas (Hill, 1984).

The growth and activity of Thiobacillus spp. in concrete pipes is an interesting example of consortial involvement in corrosion accelerated by physically separated members. Microbially influenced corrosion of concrete has resulted in catastrophic failures of the upper (aerated) regions of concrete sewage pipes (Sand and Bock, 1984). Early investigators attributed the rapid corrosion of sewage transport pipelines to the production of hydrogen sulfide in the sewage followed by a "catalytic oxidation" to sulfuric acid vapor. It was thought that this process was abiological in nature, resulting from a chemical reaction catalyzed at the concrete surface (Thistlethwayte, 1972). Milde et al. (1983) and Parker (1947) have described a number of species of thiobacilli which are involved in concrete corrosion.

Treatment by forced aeration of the sewage decreased SRB activity and lowered volatile sulfide production. A decrease in the number of thiobacilli and the associated rate of concrete corrosion then resulted (Bielecki and Schremmer, 1987). With the advent of the National Pollution Discharge Elimination Standards (NPDES) in 1972 controlling discharge of pollutants into waterways, the level of heavy metals in sewage has declined significantly. This change, while having salutary benefits for the environment, has resulted

in an exacerbation of the MIC problem in concrete sewage systems. By removing metals involved in suppressing bacteria concrete corrosion increased several orders of magnitude. Interestingly, the heavy metals appeared to have been effective against the sulfate-reducing bacteria and not against the thiobacilli (A. Walker, L.A. Sanitation Districts, personal communication).

EMERGING TECHNOLOGIES FOR BIOFILM CHARACTERIZATION AND CONTROL

Monitoring. The development of real-time, on-line biofilm monitoring devices will lead to a better understanding of those factors which affect the primary stages of bacterial attachment. Ebersole and Ward (1988) have described an oscillating piezoelectric quartz crystal microbalance (QCM) for measuring particle adhesion on surfaces. This device, may provide a means for detecting initial colonization by bacteria. Perturbations in signal frequency and amplitude measured by the QCM may prove useful for quantitative and qualitative analysis of primary fouling populations. Electrochemical measures of surface activities provide a means for studying interactions between various surfaces and biofilms.

Development of real-time image analysis methods for direct observation of surfaces exposed to flowing liquids

have been described by Caldwell and Germila (1985) and Escher and Characklis (1988). Attenuated total reflectance FTIR spectroscopy has been employed in studies of MIC of copper surfaces in flowing systems. Geesey et al. (1986) was able to measure the erosion of molecular layers of copper on a real-time basis. Bacterial exopolymers were implicated in the dissolution process. Helm et al. (1991) have used FTIR in the attenuated total reflectance mode to "fingerprint" 139 different Gram-negative and Gram-positive bacteria. The classifications are complementary to existing schemes and can be developed rapidly using dedicated computer programs.

A variety of surface scanning analytical techniques have been applied to biofilm studies. Chen et al. (1988) employed scanning Auger microprobe analysis to study biofouling of seawater condenser tubes at a power plant in Taiwan. They found that O, Na, and S were enriched in areas of stainless steel that were influenced by MIC activities. Stainless steel (#304 plate) surfaces were found to have significantly greater corrosion than the titanium. McCoy and Olson (1987) utilized X-ray energy microanalysis in studies of pipe surfaces colonized by bacteria. Their work revealed the presence of Si, P, S, Ca, Fe, Zn, Mg, Al, K, and Mn associated with the biofilm.

White (1988) has reviewed the use of various signature biomarker analyses for <u>in situ</u> biofilm analysis. Community

structure, specific activity, and biomass measures are important indicators of fouling potential. Utilization of fluorescent and ^{32}P-labeled RNA/DNA probes may provide a means for quantitating members of biofilm consortia. The utility of DNA probes in studies of soil consortia, for example, has been described by Ogram and Sayler (1988). Combined with signature biomarker studies, FTIR analysis, and activity measurements, DNA probe technologies could provide information concerning which groups of biofilm bacteria are active under varying environmental conditions. It is possible that these probes can be used in conjunction with biocide efficacy assays to determine the in situ biocide resistance/sensitivity of specific consortial members. The continued development of ultramicro probes for pH, O_2 (redox), and trace ion detection will provide means for monitoring bacterial activity at the surface/biofilm interface. Changes in biofilm community structure, biofilm stability, and biofilm constituents could be rapidly detected with these microprobe tools.

Treatment Considerations. The vast majority of advances in industrial biological contamination control have focused on the treatment of planktonic bacterial populations. More efficient microporous filtration systems (Meltzer, 1987), ultraviolet sterilization devices (Severin, et al., 1983), and novel ion exchange processes (Flemming, 1987), for

example, have all addressed the removal of planktonic microorganisms while virtually ignoring the role of biofilms in their generation. The effective treatment of sessile bacteria and their associated biomass usually requires the application of oxidizing biocides and/or mechanical treatments such as circulating sponge-rubber balls. Mittelman (1987) provides an overview of biofouling treatment processes in industrial water systems.

A patented process developed by Costerton (1983) utilizes a circulating glycol solution to effect ice nucleation within the biofilm. Under controlled thawing conditions, the frozen cells and biomass are stripped from the surface and trapped via side-stream filtration devices. The applicability of this process to a broad industrial market is, however, still to be validated. Future treatment strategies will undoubtedly focus on the prevention of primary bacterial colonization. This will entail alterations in the surface chemistry of both the bulk fluid and the substratum. Although great progress has been made in developing advanced fluid handling materials of construction, mankind still has not developed a surface to which bacteria will not adhere. There exists a real need for process and product compatible biofilm treatments.

RESEARCH NEEDS ADDRESSED BY THIS DISSERTATION

The research described in this dissertation was built around a central theme of exploring the effects of bulk-phase environments and surfaces on bacterial biofilms under dynamic-flow conditions. While there is a plethora of information which describes the effects of biofilms on surfaces (e.g., MIC, impedance of heat transfer efficiency), exchange of bulk-phase components (e.g., wastewater treatment systems, resource recovery), and hydraulic parameters (e.g., fluid turbulence, fluid velocity), much less is known about hydraulic and surface effects on biofilm constituents and metabolic activity. An understanding of these effects is essential to the development of effective antibiotic/biocide chemical treatments as well as novel surfaces for use in medical, industrial and environmental applications.

Fundamental questions of how one type of bulk-phase parameter, fluid shear regimes, affects the biomass constituents and metabolic activity of biofilms have been addressed in chapter II. A number of novel biofilm analytical tools were applied to study the interactions between shear force and biofilm biomass and activity. That bacteria exposed to higher fluid shear forces possess distinctly different biomass constituents and unit metabolic activity may explain, in part, why bacteria tenaciously

adhere to ship hulls, high fluid velocity pipelines, intestinal lumina, and rocks at the bottom of waterfalls.

Difficulties inherent to the development, sampling, and analysis of microbial biofilms have been a major hindrance to advances in understanding biofilm ecology and the all-important transition state between a planktonic and a sessile existence. For lack of better techniques and test systems, many investigators perform quiescent, batch colonization studies with glass slides or polystyrene petri dishes as substrata. The in situ environments associated with natural stream courses, marine benthos, or industrial process operations are rarely quiescent or polystyrene. Therefore, the design, construction and validation of laminar-flow adhesion cells was undertaken and is described in chapter III. The flow cells enable the study of interactions between bulk-phase bacteria and relevant surfaces under realistic hydraulic regimes. Sampling problems alluded to previously are alleviated to some extent by the application of on-line electrochemical and bioluminescence monitoring, also described in chapter III.

Since aqueous environments are rarely composed of monocultures, this research endeavored to evaluate the colonization of inanimate surfaces by a succession of environmentally relevant bacteria. Again, experimental difficulties associated with adhesion studies have often limited investigators to working with one type of

microorganism. The laminar-flow adhesion cells were utilized to compare the constituents and metabolic activity of biofilms on SS surfaces which had been exposed to varying combinations of physiologically distinct bacteria. This research, which is described in chapter IV, demonstrated that the bulk-phase bacterial population does influence biofilm ecology, biomass, and metabolic activity.

In addition to exploring the role bulk-phase conditions play in determining biofilm parameters, the research described in chapter V considered the effects of metallurgical inhomogeneities--surface finish and the presence of welds--on consortial biofilm community structure, biomass, and metabolic activity parameters. Unlike the bulk-phase parameters of fluid shear and planktonic bacterial population, the surfaces evaluated in chapter V did not significantly affect biofilm parameters.

The application of bioluminescent, adherent bacteria as monitors of colonization provides a new tool for exploring the interactions between surfaces and planktonic bacteria. In chapter VI, a bioluminescent P. fluorescens (lux) construct is used in dynamic-flow colonization studies. The applications of this new adhesion monitoring technique are broad and include evaluations of the effects of antifouling coatings on biofilm bacteria. The use of bacterial bioluminescence as an endpoint in adhesion assays should

provide new opportunities to study bulk-phase and surface physicochemical effects on biofilm organisms.

Bacterial biofilms have been recognized for some time as an important, albeit often neglected, part of the environment. The interrelationship between colonized surfaces and the bulk-phase environment, biofilm ecology, has important implications for natural environments, plant and animal life, and a number of industrial process operations. It is hoped that the research described in this dissertation serves to add significant new knowledge to this expanding area of environmental microbiology and toxicology.

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CHAPTER II

DIFFERENTIAL ADHESION, ACTIVITY, AND CARBOHYDRATE:PROTEIN RATIOS OF Alteromonas (Pseudomonas) atlantica MONOCULTURES ATTACHING TO STAINLESS STEEL IN A LINEAR SHEAR GRADIENT.

INTRODUCTION

Ellwood et al. (1982) have presented an elegant description of basic life strategies for obtaining energy and carbon sources. Either organisms can stay in one place and collect growth-promoting factors as they flow by, or they can search them out by moving about in the environment. This latter strategy, which involves a chemotactic response, is an energy-requiring process. While a significant amount of research into the energetics and molecular mechanisms of chemotaxis has been reported (Eisenbach, et al., 1990; Silverman and Simon, 1974), research into the development and activity of this "stationary" strategy has been limited.

A number of theories relating environmental and physiological factors to bacterial adhesion processes have been proposed. Initial colonization and biofilm stability appear to be dependent on physicochemical factors such as interfacial surface energy (Absolom et al., 1983; van Loosdrecht et al., 1989), hydrophobic interactions (Rosenberg and Kjelleberg, 1986), and bulk phase substrate concentrations (Brown et al., 1977; Fletcher, 1988;

Marshall, 1988). Young and Mitchell (1972) showed that chemotaxis in marine systems could play a role in the initial colonization of motile bacteria. Marmur and Ruckenstein (1986) demonstrated that gravitational forces significantly influenced the transport of cells to substrata in quiescent aqueous environments. VanHaecke et al. (1990) have described the role of cell surface hydrophobicity in the adhesion of Pseudomonas aeruginosa to 304 and 316-L stainless steels. Using contact angle measurements, they found that adhesion to electropolished surfaces was correlated with cell surface hydrophobicity. However, two other widely applied measures of hydrophobicity, the salt aggregation and bacterial adhesion to hydrocarbons assays, yielded inconsistent and contradictory hydrophobicity data.

In addition to these physicochemical factors, fluid hydraulic parameters such as shear force and turbulence have also been shown to affect the adhesion process. Sly et al., (1988) found that biofilm formation and manganese deposition rates in a potable water system were significantly increased at a higher water velocity. The deposition rate of Streptococcus sanguis on glass capillary tubes was shown by Rutter and Leach to be a function of both nutrient loading and fluid velocity (Rutter and Leech, 1980). Duddridge et al., (1982) observed decreases in direct microscopic Pseudomonas fluorescens cell counts with increasing fluid shear in the range of 30-130 dynes cm^{-2}.

In the gut and in other organs composed, in part, of mucosal surfaces, surface shear has been recognized as a natural defense mechanism against attachment of pathogenic microorganisms. Despite the presence of shearing forces, pathogenic bacteria do possess mechanisms which enable to adhere to and infect mucosal surfaces. Brooks and Trust (1983) suggested that shear forces can actually potentiate the strength of bacterial adhesion to eucaryotic cells.

However, relatively little is known about what effects shear forces have on the formation and nature of biofilms. While increased fluid velocity (shear) may overcome those electrostatic and covalent forces which result in "irreversible" adhesion, decreased substrate transport rates associated with lower fluid velocities could result in desorption of adhered cells.

In this study, the effects of fluid shear on the biomass constituents and metabolic activity of A. atlantica biofilms attached to polished stainless steel surfaces are reported. Protein, carbohydrate and cell wall fatty acid content were measured as indicators of attached biomass. Incorporation of ^{14}C-1-acetate into membrane fatty acids was measured over a range of applied fluid shears. A radial-flow chamber (Fowler and McKay, 1980) was utilized in these studies, allowing evaluations to be performed on the effects of multiple shear forces on single test coupons.

MATERIALS AND METHODS

Test Organism. The marine bacterium, A. atlantica ATCC 19262 (formerly, Pseudomonas atlantica) was used in all shear studies. The organism was maintained on three-quarter strength 2216 Marine Agar (Difco Laboratories, Detroit, MI) slants at 25 C. A minimal marine medium (MMB) described below, was used to develop inocula for the continuous culture system. The inocula were obtained from "collars" which formed at the air/medium interface in 18-24 h shake flask cultures. This culture isolation process was employed to select for adherent variants within the monoculture.

Continuous Culture Conditions. A Virtis 1 L OmniCulture (Gardiner, NY) system with 600 mL working volume was used to maintain cultures at constant conditions of temperature (25 C), aeration (1 L min^{-1}), agitation (100 rpm), and dilution rate (d = 0.1 h^{-1}). All reagents utilized in this study were of analytical reagent grade, unless otherwise specified. The culture and shake flask medium consisted of the following ingredients in g L^{-1} deionized water: artificial sea salts, 30; galactose, 0.5; NH_4Cl, 0.1; Tris HCl/base, 100 mM; $MgCl_2 \cdot 6H_2O$, 0.4; $K_2HPO_4 \cdot 3H_2O$, 0.4; $FeCl_3$, 4 mM; and 1 mL Wolfe's complex vitamin mixture. The galactose, $FeCl_3$, and vitamin mixture were filter-sterilized and added separately to the autoclaved and cooled medium.

The complete medium had a pH of 7.5 and buffering capacity was sufficient to maintain this pH throughout the test period.

Cell Adhesion Measurement Module (CAMM). A radial flow cell described by Fowler and McKay (Fowler and McKay, 1980) was employed to facilitate the development of a continuous shear gradient across 100 mm diameter, 600-grit finished 316 stainless steel coupons (Fig. 1). Culture medium and suspended bacterial cells contacted the center of the test coupon; shear, τ, was generated radially, in inverse proportion to the radius. Medium and cells exited the CAMM at the outer edge of the coupon, approximately 50 mm from the entry zone. Flow rates through the system were maintained such that a Reynolds number of <2,000, indicating laminar flow, was obtained; flow rates were maintained at a constant value in the range of 60-1,000 mL min^{-1}. Several flow rates in this range were employed to generate data in the experiments described herein. Pulseless pumps and silicone tubing were used throughout the system.

Test Procedure. Following steam-sterilization, the test system was aseptically assembled and filled with sterile culture medium. Following a 24 h sterility test, ca. 1 X 10^9 adherent cells from a shake flask were inoculated into the continuous culture vessel. Dissolved oxygen (DO) levels

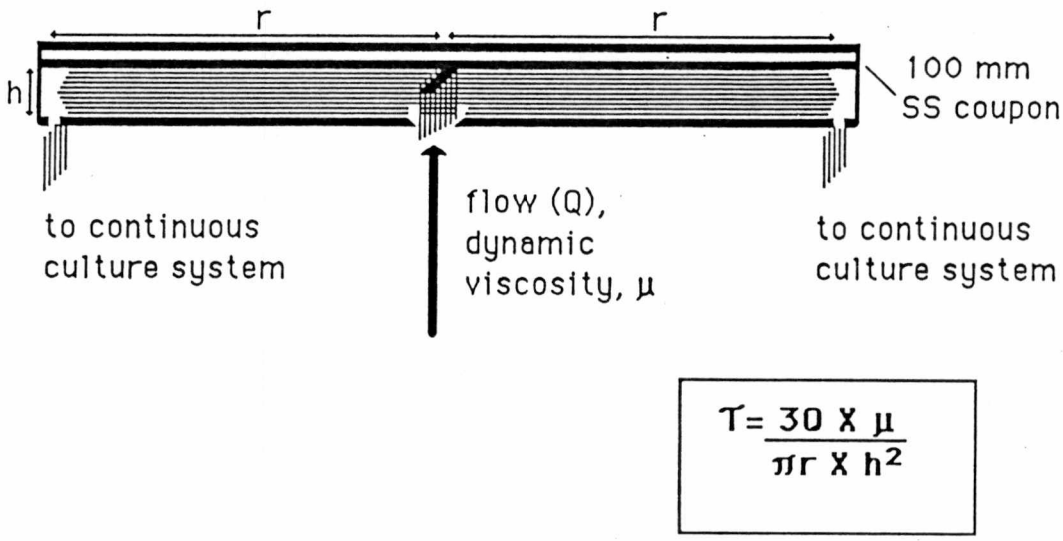


Fig. 1. Vertical section of cell adhesion measurement module showing direction of flow and shear generation. Shear force is inversely proportional to radius, with the greatest shear forces generated near the entry zone. The parallel plate separation distance, h, was maintained at 1 mm.

in the bulk phase were monitored throughout the experiment using a YSI model 50 DO meter/probe (Yellow Springs Instruments, Yellow Springs, OH); DO levels varied from saturation (ca. 8.2 mg L^{-1}) at T = 0 h to 5.75 mg L^{-1} at T = 120 h.

Following the recirculation period, test coupons were aseptically removed from the CAMM and either analyzed immediately (as with acridine orange counts and activity measurements) or frozen at -50 C, lyophilized, and stored in vacuum desiccator until ready for further study.

Analytical Procedures. Coupons for direct observation and microscopic cell counts were stained for 5 min in 0.1 mg mL^{-1} acridine orange (Sigma, St. Louis, MO) in 100 mM phosphate buffer, pH 7.5, containing 2% v/v glutaraldehyde. Coupons were observed directly under epifluorescent illumination. Coupons were also quantitatively extracted for microscopic direct cell counts using 1.2 cm diameter glass o-ring extractors (Kontes Glass, Vineland, NJ). Following addition of 0.6 mL volumes of the minimal medium to the extractors, the surfaces were sonicated to remove adherent cells using three 3-sec pulses at 20% power (Heat Systems sonicator, Plainview, NJ). Preliminary experiments (data not shown) demonstrated that >95% of the adherent cells and associated biomass material could be removed by this extraction procedure. The sonicated suspensions were fixed

in 2% (v/v) glutaraldehyde, then filtered through 0.2 μm polycarbonate filters (Nuclepore Corp., Pleasanton, CA). Dried filters were fixed onto glass slides with immersion oil, then examined under epifluorescence illumination.

Lyophilized and desiccated coupons were analyzed by diffuse reflectance IR spectroscopy (DRIFT) on a Nicolet model 60 SX Fourier transforming infrared spectrophotometer (Nicolet Instruments, Madison, WI) equipped with a standard Globar source, a KBR beam splitter, a narrow band liquid nitrogen cooled HgCdTe detector (Nicolet Instruments), and an Analect Instruments (Utica, NY) DRIFT device. The data were collected as single-sided interferograms with a resolution of 8 cm^{-1} at a mirror retardation velocity of 1.570 cm sec^{-1}. Coupons were scanned at multiple radii; four measurements per radius were taken at 90° angles. Absorbances were measured at reciprocal wavelengths from 800 to 2,000 cm^{-1}, with 250 scans per measurement.

Interferograms were Fourier processed using phase correction and a Happ-Genzel appodization function. A ratio of biofilm absorbances against those obtained from clean stainless steel coupons were determined, baseline corrected, and water vapor subtractions were performed when necessary. The final spectra were converted to Kubelka-Munk units using a five point least squares quadratic smoothing algorithm (Nichols et al., 1985). Coupons manipulations were carried

out in a dry nitrogen atmosphere to limit interferences associated with water vapor and carbon dioxide.

Coupons were quantitatively extracted for protein and total lipids via sonication as described above. Protein was measured using a sodium dodecyl sulfate-modified Lowry procedure (Peterson, 1977) with a bovine serum albumin standard. Total lipids were extracted using the glass o-ring extractors equipped with Kalrez o-rings. Following the recirculation period, the coupons were immediately removed from the CAMM, o-ring extractors were clamped in place, and 0.6 mL of chloroform-methanol-water reagent (Bligh and Dyer, 1959) were added to the surface of each extraction area. Following a 2 h extraction period, the 0.6 mL aliquots were decanted to 13 x 100 mm test tubes. The coupon surfaces were rinsed with two 0.6 mL aliquots of the Bligh-Dyer cocktail. The contents of the 13 x 100 mm test tubes were then transferred to 30 mL separatory funnels; after addition of chloroform and water to effect phase separation, the mixture was allowed to extract for 24 h at room temperature. The organic phase was then subjected to a mild alkaline methanolysis (Ringelberg et al., 1988; Tunlid et al., 1989). Phospholipids were analyzed using a modification of the method of White et al., 1979. Fatty acid methyl esters were quantitated directly using a Hewlett-Packard model 5880 gas chromatograph equipped with a capillary column as described by Ringelberg et al., 1988. Confirmation of fatty acid

identification was made on a Hewlett-Packard model 5996A GC/MS.

Pulse labelling of the biofilm coupons was performed in situ by recirculating 1-^{14}C-acetate through the CAMM at the in situ flow rate. A 100 mL volume of MMB containing 0.5 μCi mL^{-1} sodium acetate with a specific activity of 55.0 mCi mmol^{-1} (New England Nuclear, Boston, MA) was recirculated for 30 min. Following the recirculation period, the coupon was immediately removed from the CAMM. No rinsing of the coupons was performed prior to the extraction. Total fatty acids were extracted from various areas on the labeled coupon surface as described above. The organic phase from the Bligh-Dyer extraction (which contained the labeled lipids) was transferred to 7 mL scintillation vials, made basic by addition of 0.1 M KOH, then dried under a gentle nitrogen stream. Following addition of 4.0 mL Scintillation cocktail (Ecolume, ICN, Irvine, CA), the samples were counted on the LKB model 1212 (Gaithersburg, MD) liquid scintillation counter. The counts were quench corrected using a series of external quench standards (Packard, Downers Grove, IL).

RESULTS

Influence of Shear on Biomass Development. Figure 2 illustrates the spectra of 48 h A. atlantica biofilm at several fluid shear regimes. The peaks appearing at 1,630 cm^{-1} and 1,550 cm^{-1} correspond to the amide I and amide II regions, respectively, of proteins. The region from 1,000 to 1,200 cm^{-1} contains peaks corresponding to the C-O stretch of carbohydrates. A phosphorus band appears at 1,240 cm^{-1}. A significant positive correlation ($P < 0.05$) was observed between fluid shear and the absorbances of these distinctive bands. The absorbance values tended to increase with applied shear up to a critical value which defined the limit of attachment. The transitional area, where absorbance values drop precipitously, typically appeared within 5 to 10 mm of the central impact area.

Direct epifluorescent microscopic observation of the coupon surfaces revealed the presence of greater numbers of bacteria associated with the higher shear areas of the coupon (Table 1). The cells attached to the higher shear areas appeared to be smaller in size than those associated with the low shear areas. Total cell counts were significantly affected by shear in the range of 1 to 13 dynes cm^{-2}. Total bacteria counts ranged from 1.5×10^5 at 1.1 dynes cm^{-2} to 5.7×10^7 cells cm^{-2} at 13 dynes cm^{-2}. Total

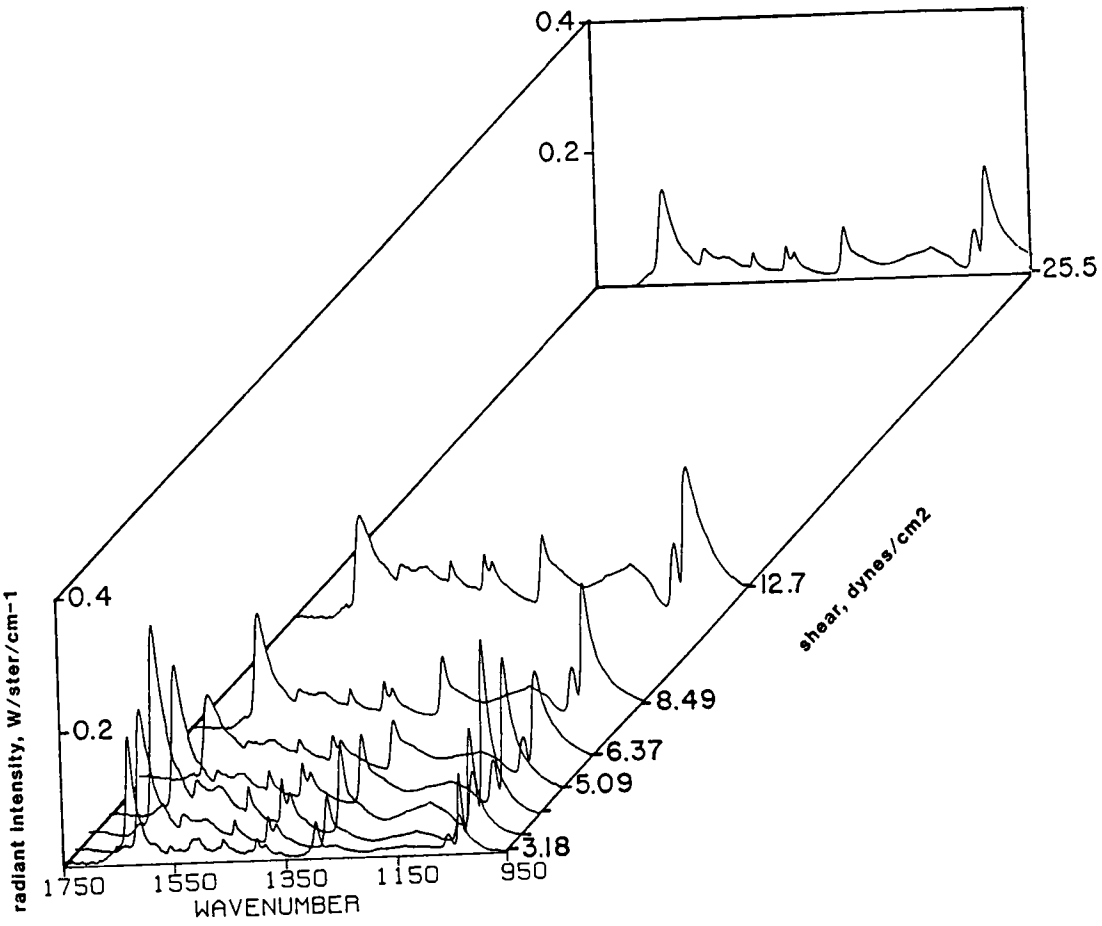


Fig. 2. FTIR spectra of A. atlantica exposed to varying shear stresses. Amide I and II absorbance values are considered indicative of proteinaceous biomass; the CHO absorbances at 1,000-1,200 cm^{-1} are representative of the C-O stretch associated with carbohydrate groups (e.g., polysaccharides).

Table 1. Direct counts and activity of A. atlantica on coupon surfaces exposed to fluid shear.

Midrange Shear[a], dynes cm^{-2} cells cm^{-2}	Mean number of attached bacteria, cells cm^{-2} ($\pm$ 1SD) X 10^{6}[b]	Acetate incorporation, μmoles $cell^{-1}$
1.06	0.15 (0.074)	nt
2.39	0.99 (0.49)	nt
3.62	1.4 (0.79)	17.7 (6.4)
4.97	7.8 (5.5)	2.48 (1.68)
7.96	41.0 (8.8)	0.36 (0.25)
13.0	56.8 (18.6)	nt

[a]Values for shear at the center of 1.2 cm diameter extraction areas are reported.
[b]Average of counts taken from four different extraction areas per shear value; a one-way analysis of variance was performed: $P < 0.01$.
[c]Average of four 30 min uptake measurements taken at each shear value; a one-way analysis of variance was performed: $P < 0.01$.

protein (Fig. 3) and phospholipid fatty acid biomass parameters (Table 2) were significantly influenced by shear effects in the range of 0.2 to 31 and 1 to 8 dynes cm^{-2}, respectively.

Compositional Changes with Shear. An increase in the ratio of carbohydrate to amide II IR absorbance was related to shear in the range of 0.2 to 29 dynes cm^{-2} (Fig. 3). A similar relationship was apparent between the carbohydrate:amide I ratio and shear (data not shown). The results of the DRIFT/FTIR analysis indicate that biomass as carbohydrate and protein on a unit area basis increased with

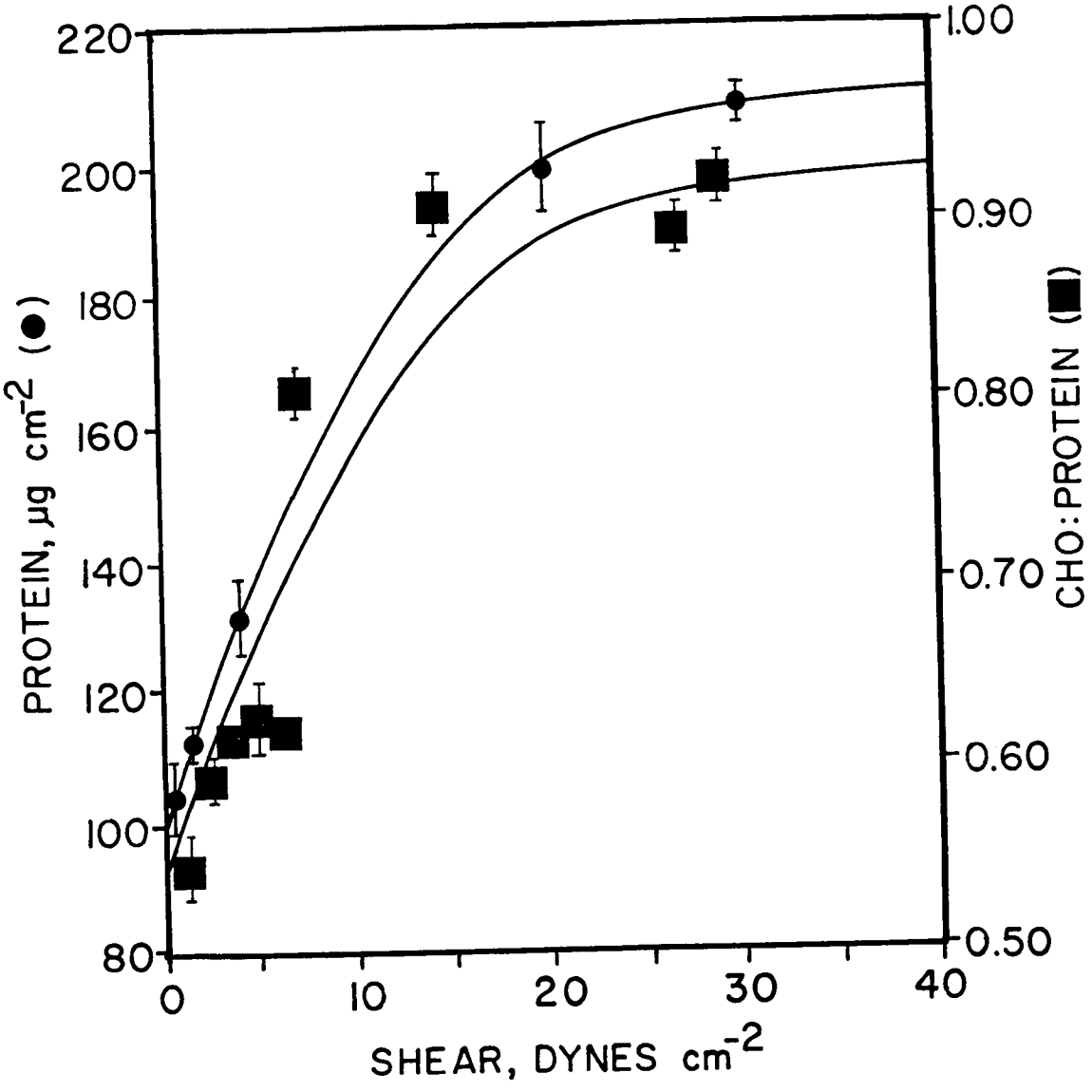


Fig. 3. Ratio of carbohydrate (1,130 cm^{-1}) to amide II (■) (1,550 cm^{-1}) IR absorbances for A. atlantica biofilms exposed to varying shear stresses. Total protein (●) vs. shear; 4 replicates per shear, error bars represent ±1 SD.

Table 2. Total fatty acid biomass and saturated:unsaturated fatty acid ratios vs. fluid shear.

Midrange Shear, dynes cm^{-2}[a]	Total phospholipid fatty acids, pmol cm^{-2} ($\pm$ 1SD)[b]	Saturated:unsaturated fatty acids ($\pm$ 1SD)
1.09	442.0 (143.5)	0.65 (0.09)
3.62	764.4 (138.5)	0.72 (0.07)
4.97	786.1 (84.3)	0.76 (0.07)
7.96	1244.8 (289.9)	0.74 (0.01)

[a]Values for shear at the center of 1.2 cm diameter extraction areas are reported.
[b]Average of measurements taken from four different extraction areas per shear value; a one-way analysis of variance was performed: $P < 0.01$ for total lipids; $P > 0.1$ for saturated:unsaturated fatty acids.

increasing fluid shear. The ratio of saturated:unsaturated fatty acids, a measure of membrane fluidity, was not significantly affected by shear. The ratio varied from 0.75 to 0.85 between 1 and 8 dynes cm^{-2} (Table 2).

Metabolic Activity. The amount of ^{14}C-1-acetate incorporation into lipids increased linearly with fluid shear. Thirty-minute uptake values ranged from 1×10^{-5} to 19×10^{-5} μmol cm^{-2} between 0.15 and 30 dynes cm^{-2}. Normalizing the uptake data with respect to cell counts, however, revealed a statistically significant decline in uptake with increased shear in the range of 1-10 dynes cm^{-2}. A 2.2-fold increase in shear resulted in a 49-fold decrease in acetate incorporation during the 30-minute uptake experiments (Table 1).

Effect of Radius and Time on Biomass. There was little effect of radius on protein or fatty acid biomass parameters (Figs. 4a and b). Tests of significance using first and second order regression coefficients did not reveal a significant effect of radius on bacterial adhesion or acetate incorporation into phospholipids in 1-10 dynes cm-2 ($P < 0.05$; data not shown). The data indicated that location on the coupons did not influence cell density and that shear was the major biomass determinant.

The effect of biofilm age on biomass parameters under varying shear regimes was extremely variable within and among different time periods (data not shown). A statistically significant effect of biofilm age could not be demonstrated for any of the measured biomass development parameters ($P < 0.05$).

DISCUSSION

In most environments, bacterial adhesion processes proceed under some hydraulic influences. Bacterial adhesion to surfaces in these environments is dependent upon a balance between attractive and repulsive physicochemical forces. The results of this study demonstrated that mechanical forces such as fluid shear can potentiate bacterial colonization processes.

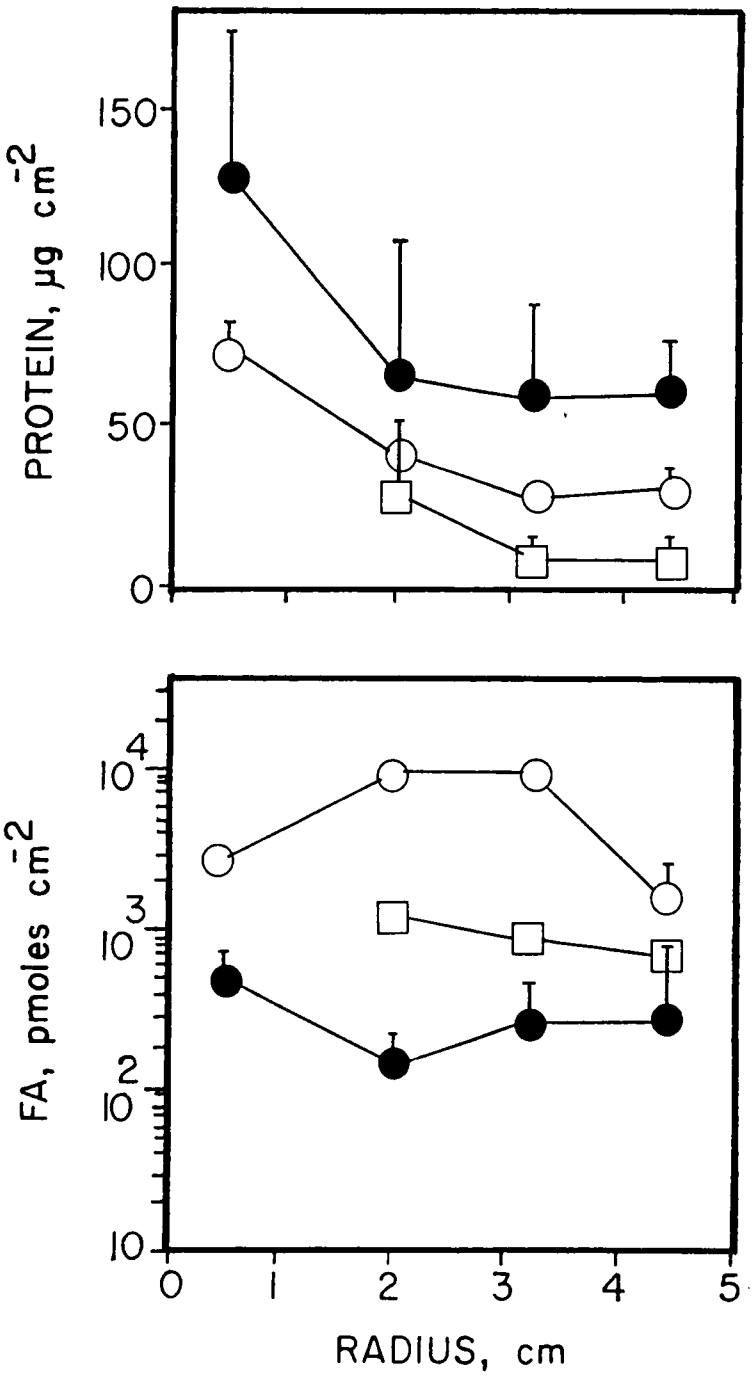


Fig. 4. Protein and total fatty acids as biomass vs. coupon radius. Four replicates from each of the three biofilm development periods were obtained per radius; error bars represent ±1 SD. 48 h (□), 72 h (●), 120 h (○).

One explanation for the observed increase in cell counts with fluid shear could be an interaction between radius and bacterial attachment, that is, a recruitment phenomenon influenced by the frequency of cell-substratum contact and/or substrate transport. If this were the case, a relationship between attachment, biomass formation, and activity and radius should have been apparent. Under the experimental conditions imposed during this study, it is doubtful that recruitment played as significant a role as fluid shear in influencing bacterial attachment to stainless steel.

If bacteria are viewed as small particles lacking any purposive behavior (e.g., chemotaxis or polymer production), theoretical considerations dictate that their rate of deposition should increase with fluid velocity. Bowen and Epstein (Bowen and Epstein, 1979) showed that the local particle deposition rate in a parallel plate channel may be described by

$$J_D = DC_o \; X \; \frac{(2/9\tau)^{1/3}}{b\Omega(4/3) + (1/K)(2/9\tau)^{1/3}} \qquad (1)$$

where $\tau = 2Dx/3V_m b^2$ is the dimensionless longitudinal distance from the inlet, $D = kt/6\pi n\alpha$ is the Stokes-Einstein diffusion coefficient, C_o is the particle concentration at the inlet, b is one-half the channel thickness, x the radius from the inlet, V_m the mean velocity of the fluid, k the Boltzmann constant, T the fluid temperature, n the viscosity

of the fluid, α the average particle radius, K the surface reaction rate constant, and Ω a mathematical function. The results of this study agree with these theoretical considerations; namely, that attachment is increased at higher flow rates up to a critical point. Beyond this critical flow (shear) the adhesive forces associated with bacterial substratum interactions are overcome by the "repulsive" forces of fluid shear.

When Rutter and Leech (1980) studied the deposition of Streptococcus sanguis onto glass capillary tubes, they found that deposition was enhanced at higher fluid velocities when the bacteria were grown at a relatively low dilution rate ($0.04\ h^{-1}$). However, at higher dilution rates ($0.2\ h^{-1}$), adhesion was decreased. They attributed this deviation from theory to "polymer bridging." Duddridge et al., (1982) have reported that increased surface shear resulted in decreased attachment of P. fluorescens to stainless steel. However, their adhesion assays were conducted at higher fluid shear values, 60-1,100 dynes cm^{-2}. Observations of biofilms exposed to relatively high shear stresses indicate that the ability of A. atlantica to remain attached to polished stainless steel is greatly reduced beyond a fluid shear of approximately 120 dynes cm^{-2}.

The increase in carbohydrate:protein ratios with shear as measured by FTIR spectroscopy suggests that bacteria able to adhere at high shear force gradients show a shift in

chemical structure. Previous work by Nichols et al., (1985) showed that FTIR spectra of P. atlantica biofilms could be used to infer nutritional status. In the work described herein, a change related to the shear environment has been demonstrated. Perhaps cells which are initially adhered to the coupon surface stabilize the adhesion process via production of additional extracellular substances (EPS). If the 1,130 cm^{-1} band is indicative of EPS carbohydrate biomass, this would suggest that shear force induces the synthesis of EPS. Since the total Lowry protein and total cell count also increased with shear, the carbohydrate: protein ratio may be an indicator of adhesion stability in A. atlantica grown in a defined environment.

The observation that per cell lipid synthetic activity declined with increased shear could be an indication of shear induced stress on the organisms. Alternatively, a shift in biosynthesis from membrane lipids to extracellular adhesive moieties could also be envisioned. The increased per unit area metabolic activity seen with higher shear values correlates well with the observed biomass increases. These findings suggest that microbially influenced surface perturbations may be affected by hydraulic factors.

The research described in this chapter provides powerful tools for assessing the effects of environmental and cultural variables on biofilm development parameters under well defined hydraulic conditions. These methods make

possible studies of complex interactions among consortial members of sessile bacteria present in biofilms. The effects of successional colonization under defined flow regimes are presented in chapters IV and V.

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CHAPTER III

DESIGN, CONSTRUCTION, AND VALIDATION OF LAMINAR-FLOW ADHESION CELLS FOR THE STUDY OF BACTERIAL COLONIZATION AND BIOFILM FORMATION

INTRODUCTION

The study of biofilm development on inanimate substrata in natural environments, industrial fluid distribution systems, wastewater treatment operations, and in laboratory- and pilot-scale test systems has been an active area of research for the past 50 years. Despite an intuitive recognition of their preeminent role in these environments, progress in understanding the dynamics of bacterial colonization and biofilm formation has not kept pace with other developments in environmental microbiology. Henrici's (1933) and Zobell's (1943) pioneering studies of bacterial colonization were carried out by simply exposing glass slides to freshwater and marine ecosystems. A serious gap exists between current analytical techniques for evaluating bacteria and consortial communities and relevant, workable systems for the reproducible development of bacterial biofilms. In many studies, the convenience of using glass substrata in static cultures has outweighed any relevance to the environment which is to be simulated.

Fletcher (1976) and Fletcher and Pringle (1985) have described a spectrophotometric-adhesion assay in which axenic cultures are placed onto polystyrene petri or tissue culture dishes, rinsed, then stained with crystal violet. Differential adhesion in this system is measured by absorbance at 590 nm. Marshall et al. (1971) suspended glass coverslips in natural stream systems and differentiated reversible from irreversible bacterial sorption based upon susceptibility of sorbed cells to a rinsing procedure. Pedersen (1982) utilized a low-flow seawater system to colonize glass coverslips contained within electrochemical cells. Colonization efficiency was determined using the spectrophotometric procedure described above. Much of the experimental variance described in this statistical study was ascribed to the rinsing and staining procedures used. van Pelt et al. (1985) studied differential adhesion of a Streptococcus sp. to surfaces of varying free surface energies in a stirred contactor cell. A series of external rinse and fixation procedures was also required prior to cell enumeration.

Rosenberg and Kjelleberg (1986) have stressed the importance of hydrophobic interactions as determinants of bacterial adhesion. Accordingly, their study systems have utilized substrata with varying degrees of hydrophobicity as colonization surfaces. For example, hydrophobic interaction chromatography (HIC) using octyl- or phenyl-sepharose beads

as the stationary phase (Dahlback et al., 1981) and the bacterial adhesion to hydrocarbons (BATH) assay (Rosenberg et al., 1980) have been widely applied. In both of these batch-type adhesion test systems, a suspension of the bacterial culture of interest is brought into contact with the hydrophobic substratum, and relative adhesiveness determined via endpoints such as turbidity and retention of radiolabeled substrates.

Since passage of static test substrata through an air-liquid interface is often required prior to rinsing and staining procedures, it is important to consider the forces exerted on these sorbed bacterial cells. Sjollema et al. (1989b) have estimated that this force exerted on sorbed cells of an average radius of 1 μm is on the order of 10^{-8} N. The force of adhesion of similarly sized colloidal particles is also on the order of 10^{-8} N (Visser, 1976), passage of sorbed cells through a water (medium)-air interface could significantly influence quantitative and qualitative recoveries. Sjollema et al. (1989b) give the force of rinsing at a fluid velocity of 0.1 mm s^{-1} as 10^{-10} N for spherical particles (equation 1).

$$F = 1.7\ (6\pi\mu RV) \qquad (1)$$

where, F= force onto a spherical particle
μ= fluid viscosity
V= fluid velocity at a distance R from the substratum

Biofilm rinsing procedures are difficult to reproduce within experiments; as such, it is likely that they introduce a significant amount of variability into biofilm recovery procedures.

The application of dynamic-flow adhesion cells to studies of bacterial colonization and biofilm formation provides two distinct advantages: 1) Hydrodynamic parameters of flow velocity, Reynolds number, and shear force can be defined and controlled. Rational comparisons between different treatment effects (surface energy, substratum topology, etc.) and among other model systems can thus be made. 2) Problems associated with inconsistent and ill-defined rinsing of "reversibly" bound cells, culture media, radiolabeled substrates, or vital stains can be obviated: exposure of the biofilm to interfacial forces during passage through the medium-air interface is limited.

A number of dynamic-flow cells have been developed for use in bacterial adhesion studies. (The approximate purchase or manufacturing cost is provided where available) Ruseska et al. (1982) have described the laboratory and field application of "Robbin's Devices" for studies of biofilm development in pipeline systems. The "Robbin's Device" consists of a pipe section into which a series of evenly-spaced, replaceable coupons have been inserted flush with the pipeline interior. Both the pipe section and test coupons can be machined from substrata which are relevant to

the system under study. Additionally, the general design of this biofilm sampling system enables various defined shear stresses and Reynolds numbers to be evaluated. The manufacturing cost of these biofilm samplers is dependent upon desired materials of construction, design operating pressures, and number of sample ports: a reasonable cost estimate can be made of from $300. for a 1 m Plexiglass unit to over $10,000. for high pressure gasoline samplers fabricated from exotic metals and alloys (e.g., Ti). In addition to describing another type of tubular biofilm reactor, Characklis et al. (1982) discuss the application of annular-type reactors for the study of biofilm effects on fluid frictional resistance. The annular reactor consists of two concentric cylinders: a stationary inner and a rotating outer cylinder. Changes in torque resulting from biofilm development are monitored via a shaft-mounted torque-transducer.

There have been real efforts made in recent years to develop test systems which enable direct and real-time evaluation of bacterial colonization on inanimate substrata exposed to dynamic flow conditions. Duxbury (1977) provides information on the construction and operation of a perfusion microculture chamber for studying cell growth. The system allows changes in bulk phase environmental conditions to be made during real-time observation of bacteria. The radial flow cells of Fowler and McKay (1980) were described in

chapter II of this work. The application of these flow cells to on-line monitoring of bacterial adhesion is described in chapter IV of this work. The cost of materials and custom machining for 316 stainless steel "Fowler Cells" is approximately $1,400. per flow cell. Sjollema et al. (1989a) enumerated Streptococcus sanguis cells adhering to a parallel-plate flow cell using on-line image analysis. The cell is relatively simple, consisting of two machined polymethylacrylate plates separated by a 0.06 cm flow channel. The rectangular cells contain fixed lower and upper glass windows for illumination and observation, respectively. Lock and Ford (1983) utilized a jacketed flow cell surrounded by extremely sensitive thermistors to measure heat production of riverine epilithic biota. The cost of a single calorimeter was on the order of $3,500. Bacterial biofilms were developed on germanium crystal surfaces and living biomass constituents monitored on a real-time basis using FTIR in the attenuated total reflectance (ATR) mode (Nichols et al., 1985).

Berg and Block (1984) and Caldwell and Lawrence (1988) have designed small-volume flow cells for continuous monitoring of bacterial colonization under well-defined hydrodynamic parameters. Their cells are roughly based upon Schwobel's (1954) early slide culture flow cell, which consisted of a set of glass coverslips separated by a thin steel support. Exact control over the bulk phase

environment, coupled with the ability to rapidly change biotic communities within the flow stream, enable the study of bacterial adhesion at the level of the in situ microenvironment. While these flow cells for the on-line study bacterial colonization and biofilm formation do offer some advantages over previous designs, they, as with most of their predecessors, are designed for on-line microscopic evaluation of bacterial colonization on glass substrata. Provisions for monitoring biofilm development on other, non-glass substrata, electrochemical methods for studying adhesion, and quantitative assessments of biofilm biomass and metabolic activity have not been made.

The research presented in this chapter addresses these deficiencies in flow cell design and application by describing the design, construction, and validation of laminar-flow adhesion cells with provisions for on-line monitoring of microbial colonization and biofilm development. The goals of this work were three-fold in nature: 1) Design a series of multipurpose flow cells with defined fluid flow characteristics. 2) Demonstrate the practical feasibility of constructing a multi-purpose adhesion cell which is cost effective, simple to construct and easy to clean and sterilize. 3) Utilize independent, quantitative measures of biofilm biomass to demonstrate the reproducibility of bacterial adhesion to stainless steel coupons contained within and among replicate flow cells.

MATERIALS AND METHODS

Design and Hydrodynamic Considerations. The following design criteria were utilized in the development of the flow cells: 1) Flow through the cells should be laminar over the coupon surface. 2) The coupons should be easily replaceable. 3) The cells should be constructed of high-quality materials which are low in extractables, highly corrosion resistant, easy to clean, and offer resistance to repeated chemical and/or gaseous sterilization. 4) The cells should have provisions for continuous macro- and microscopic monitoring of the coupons. 5) They should enable continuous monitoring of bulk phase electrochemical parameters such as pO_2, pH, and Eh. 6) The cells should provide for continuous monitoring of the open circuit potential (OCP) developed at the coupon surface. In addition, it was desirable that these design criteria be consistent with the goal of building 24 or more adhesion cells at a reasonable cost.

Inlet and outlet ports to the flow channel were designed to provide constant velocity across their respective cross sections. This was accomplished by designing the entry and exit sections to flare into a trumpet-shape (Goldstein, 1965). In addition to entry/exit design, another important determinant of fluid dynamics is entry length. Davies and White (1928) demonstrated that for flow in a channel an entry length >54 times the channel

depth was sufficient to allow transition from turbulent to laminar flow. Based upon their calculations, and a 1 mm deep by 25 mm wide flow channel, an entry length of 55 mm between the bottom flare of the inlet and the upstream side of the coupon recess was selected.

The flow channel depth was originally specified at 0.4 mm to enable microscopy with short working distance objectives after Berg and Block (1984). However, machine work at an acceptable level of precision over the entire flow channel would have been prohibitively expensive. Therefore, a 1 mm flow channel was designed with the presumption that longer working distance objectives could be utilized.

Another consideration was the effect of surface roughness on laminar flow, which is described by

$$R_\epsilon = .75R(\epsilon/a)^2 \qquad (2)$$

where,

R_ϵ= Reynolds number due to surface roughness
R= $2au_m/\mu$
a= channel depth
u_m= average velocity
μ= kinematic viscosity
ϵ= maximum height of protuberance

A critical Re, Rc, can be defined such that for Re>Rc, a vortex wake forms behind the protuberance and for Re<Rc, flow closes up behind the protuberance and the effect is negligible. Goldstein (1965) gives the Rc for a flat plate normal to flow at ≈30. Therefore, $\epsilon/a<4/R^{1/2}$ defines the limit for roughness. It can be seen from this derivation

that both channel depth and velocity influence turbulence at the surface imparted by surface inhomogeneities. Since coupons of substrata with varying topologies might be utilized in adhesion assays, selection of a deeper channel would enable use of a broader range of topological heterogeneities which would not effect transition from laminar to turbulent flow.

Based upon a channel depth of 1 mm and the assumption that the limit for laminar flow transition to turbulent flow for a rectangular flow channel is on the order of Re≈1,000-2,000 (Davies and White, 1928), an upper limit on linear fluid velocities could be calculated by

$$u_m = \mu(Re/d) \qquad (3)$$

where,

Re= Reynolds number
u_m= average fluid velocity over the coupon
d= channel depth
μ= kinematic viscosity

Therefore, for an Re<1,000, u_m must be <100 cm s^{-1}. Since u_m= volumetric flow rate/cross sectional area, an upper limit on the volumetric flow rate can be calculated as 1500 mL min^{-1} for the flow cell.

Materials of Construction. High density polyethylene (HDPE) was utilized for all wetted surfaces, with the exception of the viewing window and coupon. This material is intermediate in organic and heavy metal extractables between polypropylene and Teflon. Two other materials were also

considered, 316 stainless steel (SS) and Teflon. Concern over the possibility of microbially influenced corrosion (MIC) of 316 SS--along with its high material and machining costs--made this material unsuitable for use. Teflon was omitted from consideration, primarily because of its relatively high cost and difficulty in machining. Previous experience with prototype Teflon radial flow cells had also revealed problems with the maintenance of a water-tight seal during high flow regimes.

The glass viewing window consisted of a 24 X 50 mm #1 Corning glass coverslip (Corning, New York). The viewing window was fixed into position with a pliable, non-acetic acid containing, silicone sealant (Mexcel Co., Paris). Various SS coupons with dimensions of 2.5 mm X 25.4 mm X 50 mm (depth X width X length) were obtained from Metal Samples (Mumford, AL). As with the glass viewing windows, coupons were fixed into position with the silicone sealant. Viton or n-buna type o-rings (Knoxville Rubber & Gasket Co.) were utilized to ensure a water-tight seal. Thumbscrews and alignment pins were all of 316-type SS. Inlet and outlet connections were threaded 5 mm polypropylene compression- or slip tube-type fittings.

Fittings for electrochemical measurements (e.g., OCP) consisted of 12.5 mm X 38.1 mm polypropylene screws, drilled through the center to form a 4 mm diameter lumen. A 4 mm X

4 mm Vycor glass frit (EG&G, Princeton, NJ) was used as a salt bridge at the screw tip.

Construction of the Flow Cells. Schematics of the complete flow cell are shown in Figs. 1-4. With the exception of the flow channel rendering (Fig. 2), all dimensions are shown at a scale of 1:1. Fig. 1 illustrates the major components of the cell, including the three electrochemical fittings. The fittings were screwed into the top block flush with the flow channel surface. A 3 mm diameter hole was drilled adjacent to the upstream electrochemical fitting to enable insertion of a semimicro oxygen electrode. Fig. 2 shows a cross section of the top block entry-exits and the viewing window recesses. The flow channel is also illustrated, showing the trumpet-shaped entry and exit ports. Figs. 3 and 4 are detailed machining diagrams for the top and bottom blocks, respectively. A total of ten 3.2 X 25 mm thumbscrews were required to seal the cell.

The coupons and glass viewing window were inserted into 3.2 mm and 0.3 mm milled recesses, respectively. The recesses were milled slightly deeper than the actual coupon and glass thicknesses to allow for sealant addition. Following insertion, both surfaces were flush with the flow channel. A thumbscrew inserted through a tapped hole in the bottom block served both as a connection to the working electrode (the coupon) and to facilitate coupon removal.

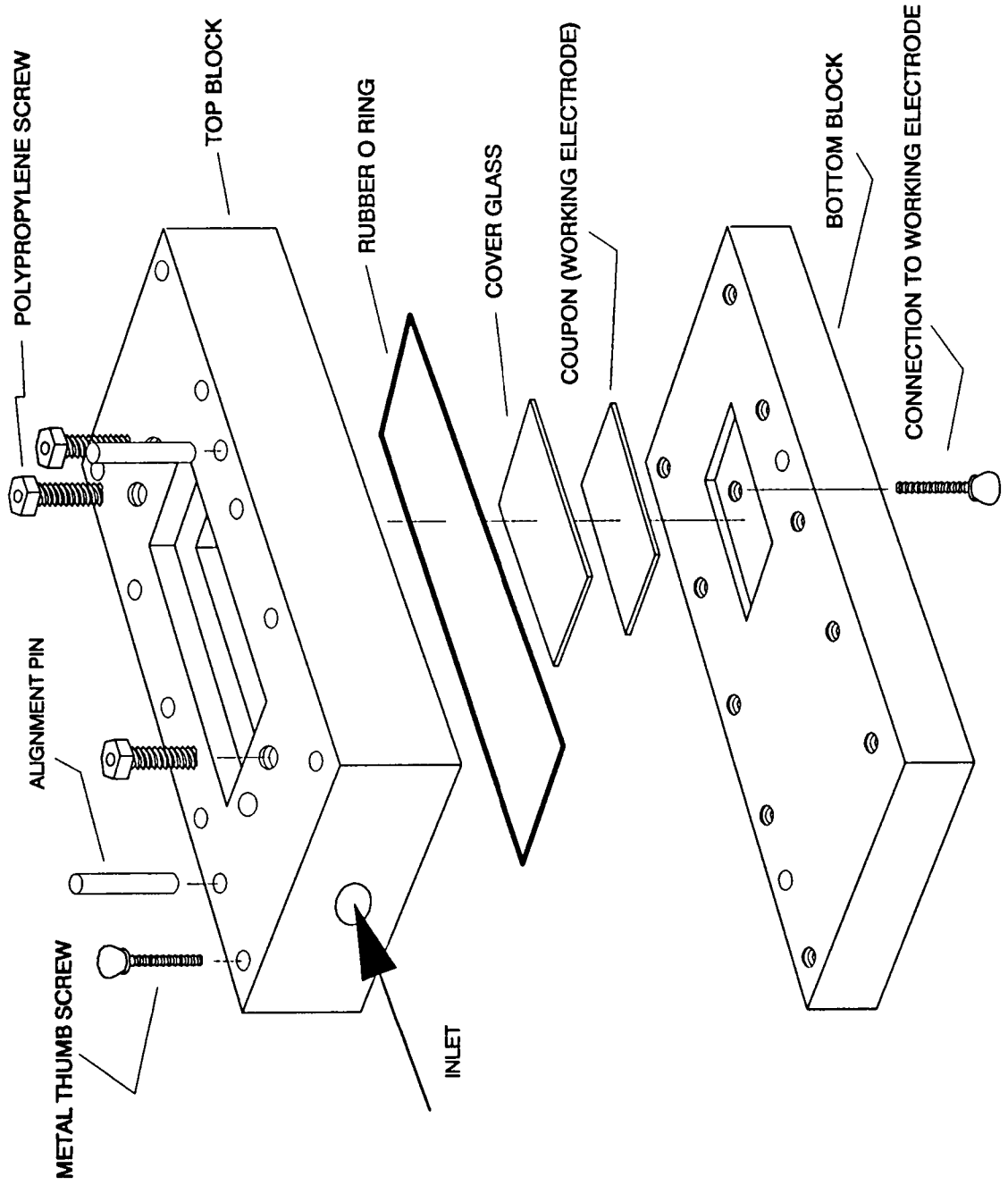


Fig. 1. Exploded view of laminar-flow adhesion cell illustrating cell assembly.

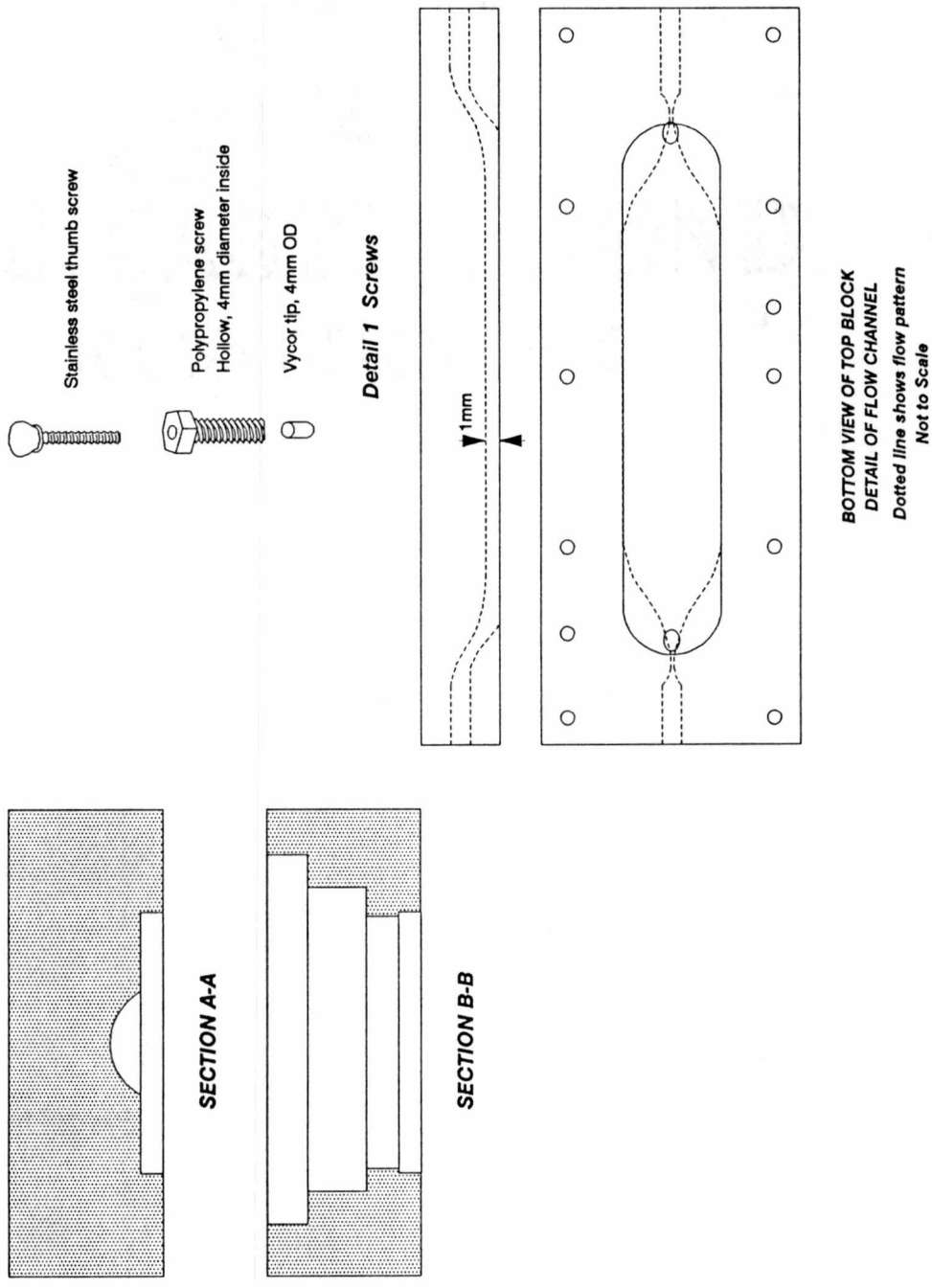


Fig. 2. Schematic of flow channel pattern showing details of electrochemical fittings and channel geometry.

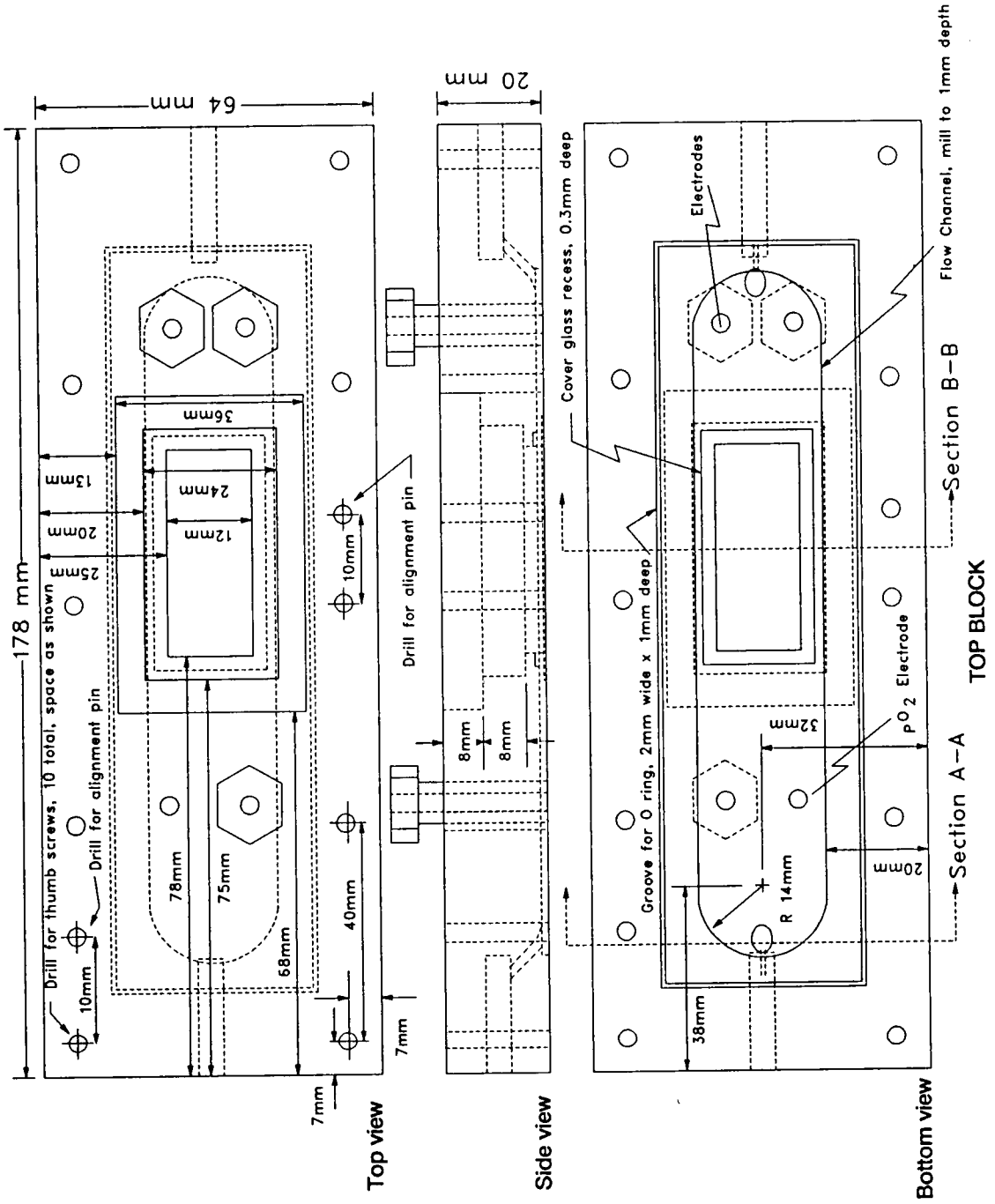


Fig. 3. Schematic diagram of top portion of flow cell containing the flow channel. Dimensions are on a 1:1 scale. Top, side, and bottom views of the cell are illustrated.

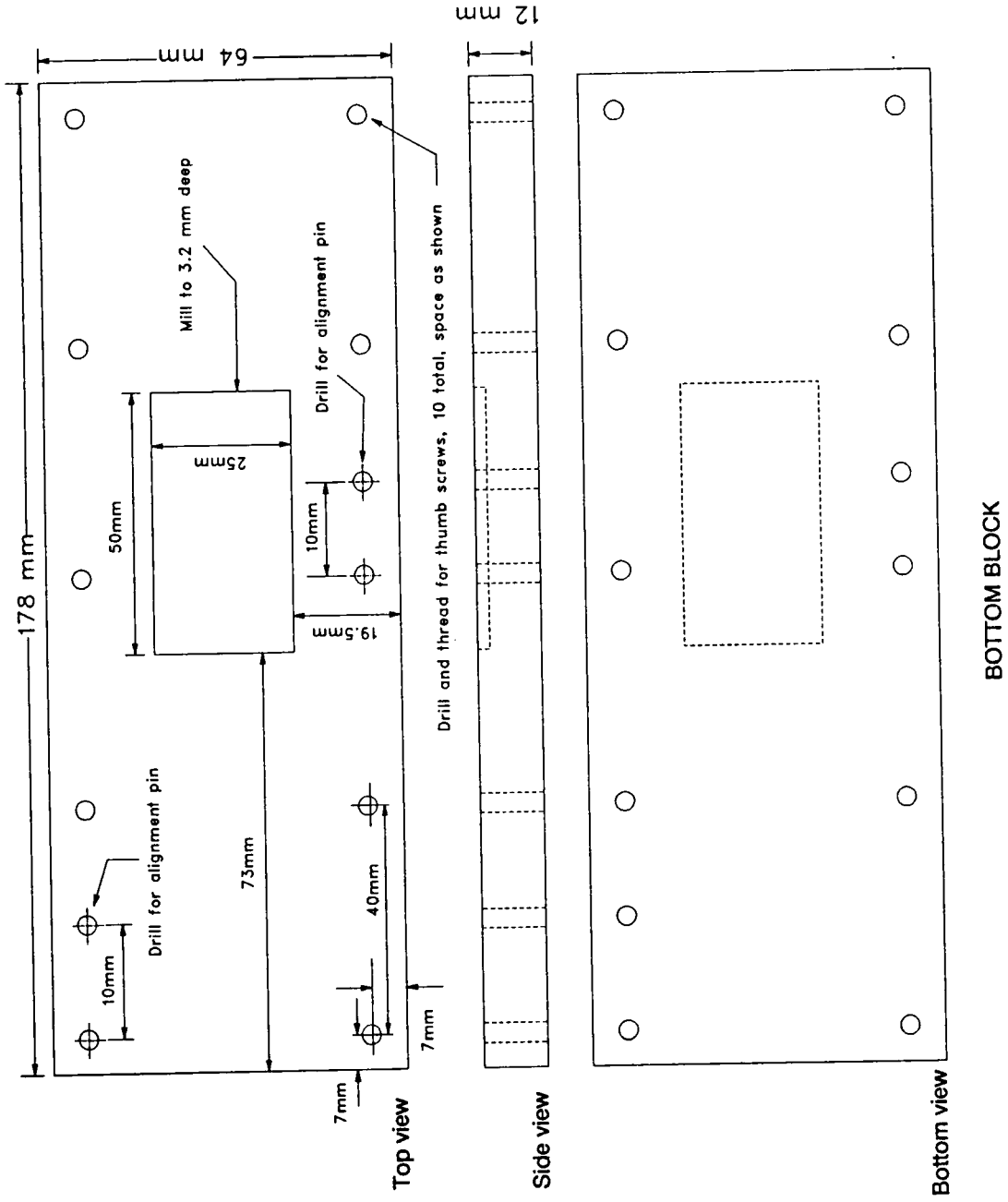


Fig. 4. Schematic diagram of bottom portion of flow cell containing the coupon/working electrode. Dimensions are on a 1:1 scale. Top, side, and bottom views of the cell are illustrated.

A machine tolerance of ± 0.075 mm was specified for all wetted surfaces. The machine work on the flow cells was performed by J & J Machine and Tool Co. (Knoxville, TN).

Reagents. All chemicals used were of analytical reagent grade or better, unless otherwise specified.

Reference Electrode Preparation. Instructions for preparing the Ag/AgCl reference electrodes are given in appendix III-1. The completed electrodes were inserted into a saturated KCl solution contained within the electrochemical fittings. The solution and electrode were sealed by means of a 3 mm X 4 mm rubber stopper.

Preparation of Flow Cells and Colonization Substrata. Prior to initiation of validation experiments, the flow cells were disassembled and critically cleaned in an Alconox soap (Alconox, New York) solution, followed by a solvent rinsing series in the order chloroform, acetone, methanol, deionized water. Coupons for biofilm development were polished to a 600-grit finish, then placed in a sonicator bath and critically cleaned in the same manner as the flow cells. The flow cells were then assembled, wrapped in two layers of Bioshield sterilization material (Baxter, Valencia, CA), and ethylene oxide gas sterilized. Bacillus subtilis var. globigii impregnated spore strips (Baxter, Valencia, CA)

were used as biological indicators to validate sterilization efficacy. While the materials of construction should be compatible with conditions encountered in a steam sterilizer (1.03×10^5 Pa, 121 C), the flow cells were not subjected to steam sterilization. All tubing connections to the flow cells were made under aseptic conditions in a laminar-flow hood.

Bulk Phase Feed System. System components and media composition are described in detail in chapter IV. Sterile, oligotrophic culture media was pumped through the flow cells at a constant volumetric flow rate of 20 mL min^{-1} via a series of Masterflex peristaltic pumps (Cole Parmer, Chicago). T-type connections were made to 20 mm X 150 mm anaerobe tubes to eliminate the pulse-flow which is associated with peristaltic systems. Tubing and connections were of silicone and polypropylene, respectively, throughout the feed system. Effluent from the flow cells was shunted to drain. Injection ports for dye and bacteria addition were located approximately 50 mm upstream from the inlet fittings. The ports consisted of polypropylene 'y' connectors (Cole Parmer, Chicago).

Validation of Flow Laminarity. Two qualitative techniques were employed to validate laminar flow conditions after Goldstein (1965) and Berg and Block (1984). A red silk

thread was affixed to the inlet fitting, passed through the flow channel, and observed through the viewing window. Approximately 10 μL bromophenol blue was injected just upstream of the inlet port and the dye front observed through the viewing window. All flow cells were tested for flow laminarity.

Bacterial Biofilm Development and Analysis. Medium flow was maintained for 18 h prior to bacteria addition and sterility of the bulk phase determined by acridine orange direct counts (AODC). Axenic cultures of Pseudomonas fluorescens (lux), hereafter referred to as 5RL, were maintained in continuous culture at a viable cell density of approximately 10^7 cells mL^{-1}. Details on the phenotypic and genotypic characteristics of this organism may be found in chapter VI. The culture was continuously injected at 0.5 mL min^{-1} for a period of 72 h. Biofilm development on the stainless steel coupon was observed macroscopically through the viewing window. Following establishment of a 72 h biofilm, culture addition was discontinued and the biofilm exposed to flowing sterile medium for 10 min. The coupons were removed and quantitatively extracted using the glass o-ring extractor procedure described in chapter II. Each of the biofilm extracts was assayed for viable cell counts, AODC, and protein. Aerobic plate counts (APC) were performed with yeast extract peptone agar with 14 mg L^{-1} tetracycline as

plating medium. Spread plates were incubated 72 h at 25 C. AODC and micro-Lowry protein assays were performed as described in chapter II. Each coupon was extracted in three, equally spaced 1.13 cm^{-2} areas along its length. Two separate experiments were performed with a total of 4 different flow cells.

Electrochemical Monitoring. Each electrochemical fitting was tested for continuity with both Ag/AgCl and saturated calomel electrodes (SCE) (Corning, New York) connected to a voltmeter (Sycopel Scientific, East Bolden, England). Vycor tips observed to be leaking, plugged, or yielding unstable voltage readings were discarded. Monitoring of OCP was performed at approximately 8 h intervals for 80 h in one uninoculated and one inoculated flow cell.

A semimicro dissolved oxygen polarographic electrode (Microelectrodes, Londonderry, NH) monitored by an Orion (Cambridge, MA) pH/ion monitor, was tested in one flow cell for stability and maintenance of calibration. An oxygen sensitive, Teflon membrane was employed for all measurements The probe was zeroed in a 5% hydrogen in nitrogen atmosphere; oxygen saturation was obtained in an air-saturated atmosphere. The monitor was calibrated for a 100% response at an oxygen saturation of approximately 8.5 mg L^{-1}.

Bioluminescence Measurements. Biofilms continuously induced with sodium salicylate were monitored for bioluminescence using a liquid light pipe-photomultiplier tube-ammeter light monitoring system (Oriel, Stratford, CT). The 0.25 cm^2 detector head was manually moved across 9 different viewing window areas on each of three flow cells. All measurements were performed in the dark. Chapter 6 provides further details concerning the application of bioluminescence measurements to on-line biofilm monitoring.

RESULTS

Problems with Early Prototypes. Earlier prototypes of the flow cells described above either had problems with leaks or failed to provide laminar-flow conditions across the coupon surfaces. These prototypes variously lacked sealing o-rings, sufficient thumbscrews, utilized poor-quality viewing windows, or contained significant variations in surface smoothness across the flow channel. This latter problem, which was resolved by the use of thicker top and bottom blocks, resulted in non-uniform flow across the coupon surfaces. This was detected as visible channeling of flow via both the silk thread and dye front assays. In addition, biofilms appeared to be discontinuous across coupon surfaces. In order to meet the final design specifications,

it was necessary that all wetted surfaces be milled using digitally controlled lathes (rather than analog equipment).

Fluid Flow Observations. The silk thread observed at the center of the viewing window remained fixed in one location and did not demonstrate any lateral or vertical movement about its axis. One flow cell of the 24 tested failed this test. This failure resulted from a small machine tool recess (approximately 0.2 mm) which was found in the upstream flow channel.

The dye front test proved rather difficult to interpret. At the flow rate used, the dye front moved very quickly across the viewing window area. However, after repeated dye additions, it was possible to determine that the velocity of dye front movement was constant across the coupon surface. A significant difference in flow pattern was observed in the failed cell described above.

Bubble formation in several of the flow cells created discontinuities within the biofilms. Bubbles appeared to form 2-4 h prior to the development of visible biofilms on the coupon surfaces. Dye injection demonstrated that flow appeared to be disturbed by the bubbles, which formed between the coupon and viewing glass window. In most instances, however, the bubbles dispersed upon elevation of the downstream end by approximately 45° from normal.

Reproducibility of Colonization. Results of the biofilm analyses for 4 flow cells are shown in fig. 5. Each coupon was sampled in 3 locations; no significant effect of sample location on AODC, APC, or protein values was found. An analysis of variance for the AODC and APC data revealed that no significant differences in biofilm cell numbers existed among the 4 flow cells examined. Cell counts ranged from between 5.1 X 10^7 and 10.4 X 10^7 cells cm^{-2} for AODC, and 1.4 X 10^7 and 2.2 X 10^7 cells cm^{-2} for APC. Protein recoveries among the 4 flow cells were significantly different ($P<0.01$), however. A students t-test revealed that flow cell 2 contained a significantly lower amount of protein per unit area than the other three cells. It is difficult to explain this apparent anomaly, since the AODC and APC for this flow cell were not significantly different from the other three tested.

Electrochemical Measurements. The open circuit potentials for an uninoculated flow cell was extremely constant over an 80 h time frame (Fig. 6). OCP varied by approximately 12 mV for the duration of this control experiment. Following inoculation of a second flow cell run in parallel with the sterile control, a transient decrease in OCP was observed (table 1). This was followed by an increase through the 76 h test period. A net change of 53 Mv was observed for the inoculated flow cell.

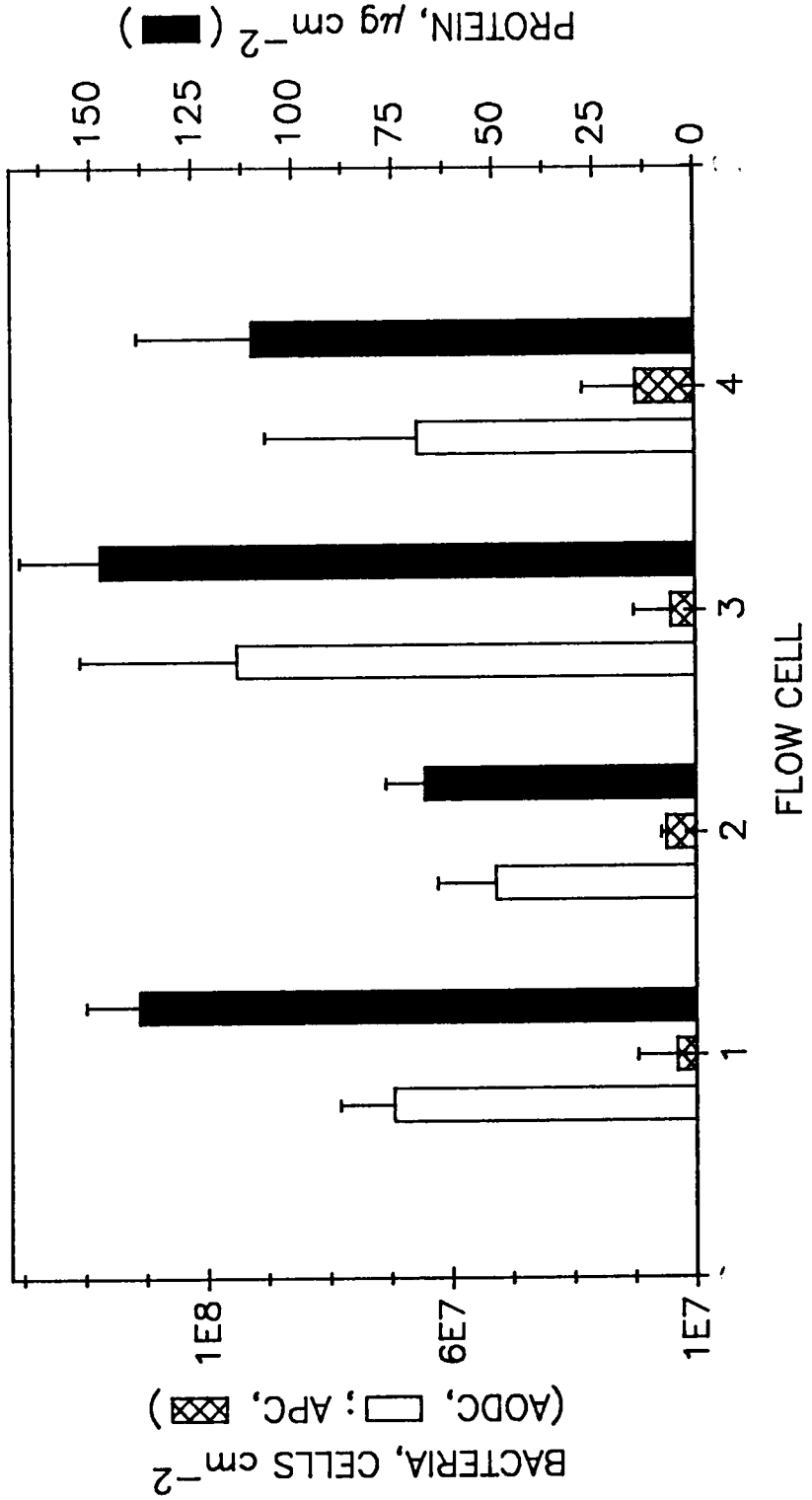


Fig. 5. Biomass measured on replica flow cells. Error bars represent ±1 SD.

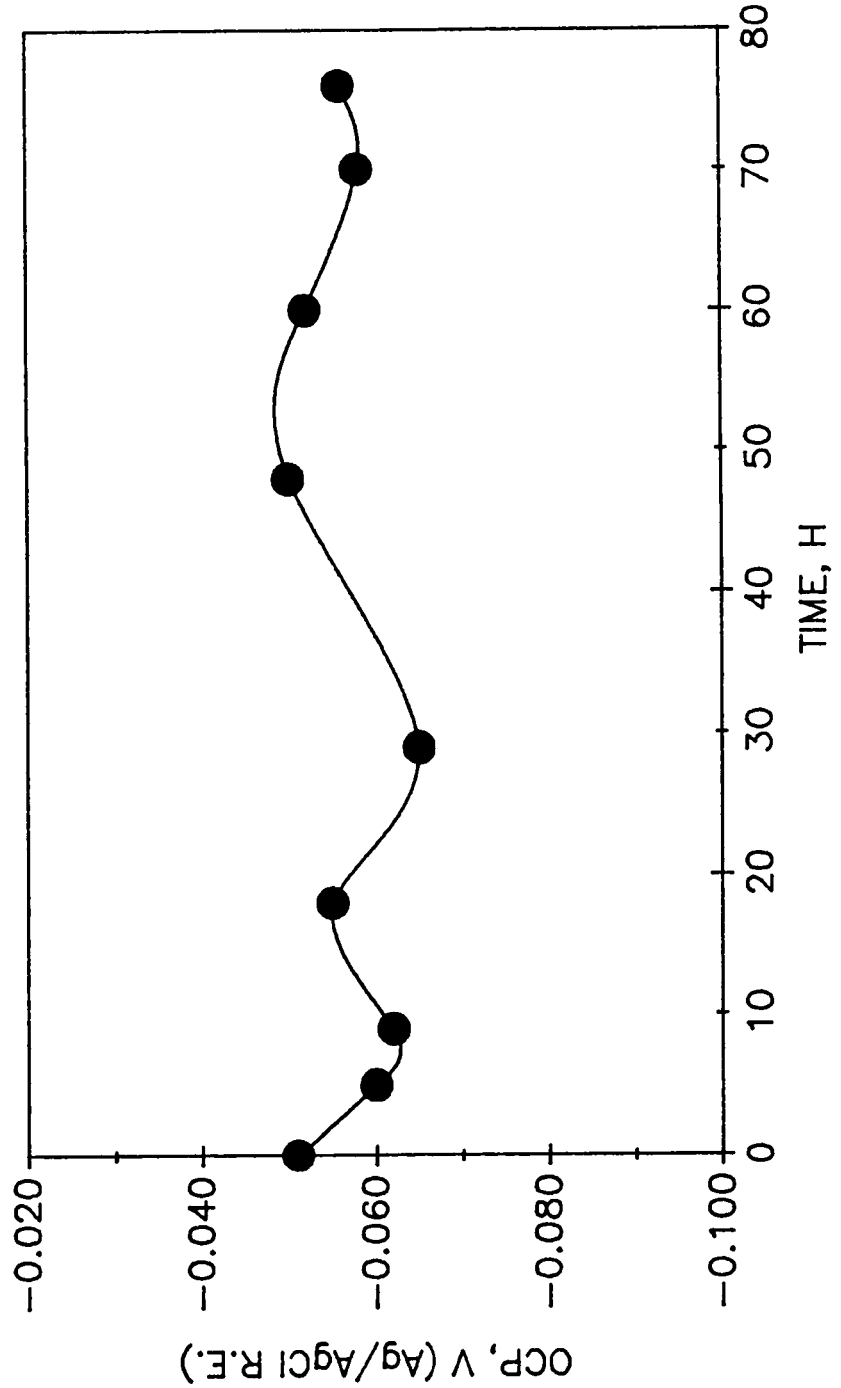


Fig. 6. OCP of 316 SS coupon exposed to 20 mL min^{-1} flow of sterile, oligotrophic culture medium at ambient temperature.

Table 1. OCP of 316 SS colonized with 5RL[a].

Time, h	OCP, Mv (Ag/AgCl/Kcl)
0	40
12	42
24	41
36	38
48	30
60	42
70	62
72	73
76	76

[a]Bacteria addition was begun following the 36 h measurement.

Any physical or electrical (e.g., actuation of pump motors) perturbations tended to shift the OCP values dramatically. Bubble formation at the Vycor tip created anomalies in OCP, which were quickly remedied by gently tapping the cells to dislodge bubbles. Inclination of the cells as described above significantly reduced the effect of bubble formation on OCP.

The oxygen electrode remained in calibration for the duration of a 72 h experiment. Oxygen levels in the flow cell monitored ranged from approximately 6.0 to 0.5 mg L^{-1}; probe calibration was not affected by the presence of viable cells when measured at the end of the experiment (data not shown).

Bioluminescence of 5RL Biofilms. Bioluminescence was consistent within the individual flow cells monitored and among the 4 replicate cells tested (table 2). Both the within and among group readings were very consistent. Background light leakage was insignificant, with typical readings of 0.01-0.02 namps obtained during flow cell measurements. While the contribution of bulk phase bioluminescent bacteria to the light readings obtained was minimal, colonization of the glass viewing windows did impart a significant effect. Following replacement of the colonized viewing windows with clean glass coverslips, the light measured decreased by a factor of 2.

Table 2. Bioluminescence of induced 5RL biofilms on 316 SS[a].

Flow Cell	Average Bioluminescence, namps[b]	Standard Deviation	Coefficient of Variation
1	4.7	0.77	16.0
2	7.9	1.4	18.0
3	9.2	1.6	17.0
4	8.0	1.0	13.0

[a]Sessile bacteria were continuously induced with sodium salicylate; the contribution of bulk-phase bioluminescent bacteria to the coupon readings ranged from approximately 0.05-0.10 Namps.
[b]An average of 9 readings were taken from the upstream, central, and downstream portions of the coupon.

Variations in the vertical distance from the probe tip to the viewing window were a source of experimental error. Light readings decreased with small increases in this distance. The problem was resolved by fixing the probe tip in one location by means of a clamp-ringstand arrangement, and manually moving the flow cells under the probe tip.

DISCUSSION

A series of multipurpose, laminar-flow adhesion cells has been designed, constructed, and validated using qualitative and quantitative measures of fluid dynamics and biofilm development. The flow cell design incorporated provisions for on-line measurements of bacterial colonization and bulk-phase environmental conditions. Colonized substrata could be easily removed for off-line non-destructive and destructive biofilm analysis. Cleaning, sterilization, use in extended flow studies, and disinfection did not appear to adversely affect the appearance or operation of the cells.

The trapping of gas bubbles between the coupons and glass viewing windows can account for a potential source of analytical error. Apparently, changes in surface tension at the biofilm-substratum are partly responsible for this phenomenon (R. Baier, Univ. of Buffalo, personal

communication). Trapping of gas bubbles by sterile coupons was not observed. Berg and Block (1984) have recommended autoclaving or otherwise degassing the flowing medium prior to introduction. However, this would render the bulk phase relatively anoxic; reintroduction of an oxygen/inert gas could resolve this complication. Since large volumes of media are required for these types of flow experiments, an alternative solution to this problem seems advisable. Inclination of the downstream ends decreased the residence type of trapped bubbles. It is likely that increasing the flow channel depth would also diminish bubble trapping.

With the exception of the apparently anomalous protein recoveries from one flow cell, biofilm biomass measured by 4 different quantitative analyses was consistent among the individual cells tested. The reproducibility of the data was surprising given the difficulties inherent to the recovery of bacterial cells and biomass from surfaces. Unfortunately, the literature does not reveal comparable validation data for other adhesion cell designs. Berg and Block (1984), Caldwell and Lawrence (1988), Characklis et al. (1982), Duxbury (1977), Pedersen (1982), and Sjollema et al. (1989b) describe the construction and application of various types of adhesion cells, but provide limited data on the reproducibility of colonization. With the exception of the radial flow cells described in chapter II, the majority of designs contain provisions for only one type of biofilm

measurement. This is typically direct microscopic observation of colonization. While microscopical methods provide invaluable information on the kinetics, spatial distribution, and numbers of colonizing bacteria, they generally cannot yield more detailed information on the biomass constituents, community structure, metabolic activity, or physicochemical interactions between the biofilm and substratum.

The flow cells described in this chapter have potential for application to a number of biofilm-related studies. Studies of MIC would be facilitated by these flow cells, since electrochemical parameters could be monitored continuously along with biofilm formation. Modification of the cells to include counter electrodes (e.g., Ti) would enable AC impedance spectroscopy and small amplitude cyclic voltammetry.

Their use in evaluating novel, non-polluting antifouling hull coatings has drawn interest from the U.S Navy (ONR, personal communication). Determinations of biocide efficacy against sessile bacteria would also be facilitated, particularly if bioluminescence can be utilized as an endpoint for adhesion and or metabolic activity determinations. The use of bioluminescent organisms in toxicity assays has been previously described (Dutka et al., 1988). Perhaps sublethal changes in metabolic activity in biofilms could be measured in much the same manner using an

adaptation of the techniques described in this chapter. Colonization effectiveness could also be monitored continuously with the appropriate video hardware and image analysis system. Marshall (1986) describes various microscopic techniques which would be applicable to the adhesion cells.

The manufactured cost of a completed flow cell was approximately $200., when they were machined in lots of 8-10 cells. As with many precision-engineered apparatus, the unit manufacturing cost is very often closely tied to the number produced. This relatively low unit cost makes possible the type of replicate analyses which are essential to the challenging--and often frustrating--study of bacterial-substratum interactions.

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CHAPTER IV

BIOFILM DEVELOPMENT ON 316 STAINLESS STEEL SUBSTRATA EXPOSED TO SUCCEEDING AXENIC CULTURES OF FRESHWATER BACTERIA IN AN OLIGOTROPHIC, LAMINAR-FLOW ENVIRONMENT.

INTRODUCTION

In natural freshwater and marine environments substrata are exposed to a variety of bulk phase microbiota. In some environments, colonization has been found to proceed in a somewhat predictable manner. Corpe (1972) described the importance of primary bacterial colonization for the development of complex eucaryotic epiphyte communities. He found that glass slides immersed in several near-shore marine environments were colonized in the order of chemoorganotrophs (pseudomonads)-stalked/appendaged procaryotes (Caulobacter, Hyphomicrobium, Saprospira-diatoms-stalked protozoa (Zoothamnium). In an earlier work, Corpe (1970) observed that Pseudomonas atlantica was often the primary colonizing bacterial species in Pacific waters. McCoy et al. (1981) showed that development of a layer of exopolymer-producing cells in a tubular reactor system preceded colonization by filamentous bacteria. Interestingly, this observations was made when the linear fluid velocity was high (265.4 cm s^{-1}); at lower fluid

velocities (on the order of 138 cm s^{-1}) these organisms were able to colonize "cleaner", non-fouled surfaces.

This apparent dependence of biofilm community structure on some type of successional colonization can have serious implications for biofouling activities, materials degradation, and environmental health. For example, in the work cited above, McCoy et al. (1981) showed that the "climax" population of filamentous bacteria was directly responsible for energy losses associated with biofilms. Bacteria colonizing surfaces in marine ecosystems can either act as a stimulus (Kirchman et al., 1982; Mitchell and Kirchman, 1984) or as an inhibitor (Maki et al., 1988) of marine invertebrate macrofoulants. Specific bacteria have been identified which appear to either promote or retard barnacle and oyster formation on surfaces exposed to marine environments. Weiner et al. (1985) described a specific Gram-negative bacterium which promotes settlement of oyster larvae. Of 18 species of marine bacteria tested by Maki et al. (1988), 7 species were inhibitory, 10 showed no effect, and 1 species was stimulatory for cyprid barnacle (Balanus amphitrite) attachment.

Clearly, the presence of specific bacteria within bacterial biofilms is an important factor in macrofoulant colonization. The primary colonizers of reverse osmosis membranes in a secondary wastewater treatment facility were almost exclusively fast-growing mycobacteria spp. (Ridgway

et al., 1984). This group of biofilm organisms, which possessed an apparent in situ resistance to sodium hypochlorite treatment, was succeeded by a diverse population of biocide-sensitive, heterotrophic bacteria common to wastewater streams. This study raised the important issue of biocide resistance as a function of biofilm community structure.

There is large gap in understanding between the observed effects of bacterial biofilms on substrata and the community structure, biomass constituents, and metabolic activity of the biofilm consortia. For the most part, studies of bacterial biofilms have involved only single organisms (Dawson et al., 1981; Fletcher, 1988) or undefined consortia from natural environments (Ruseska et al., 1982). Difficulties associated with the maintenance of diverse bacterial populations in continuous culture--combined with a lack of appropriate adhesion test systems--have limited the scope of consortial biofilm studies.

In chapter III the development of multipurpose laminar-flow adhesion cells was described. Axenic cultures of the aerobic pseudomonad, Pseudomonas fluorescens (lux) (5RL), were employed to demonstrate the applicability of the flow cells to the study of bacterial adhesion. In the research presented in this chapter, the flow cells were used to develop biofilms on 316 stainless steel (SS) substrata which had been exposed to succeeding populations of

physiologically distinct, freshwater bacteria. Experiments were performed to challenge the following hypotheses: 1) Physiologically distinct bulk phase microorganisms will differentially colonize 316 SS substrata. 2) The order of axenic culture addition to the flowing bulk phase will affect the biofilm community structure, biomass constituents, or metabolic activity. 3) Greater physiological diversity in bulk phase bacteria will result in a similarly diverse biofilm in terms of community structure, biomass constituents, and metabolic activity. 4) The bulk phase bacterial community structure will be reflected in the biofilm community structure.

Open circuit potential (OCP) measurements were made on a continuous basis during the colonization experiments. This technique was utilized to follow bacterial colonization in a flow-cell system. In keeping with the overall objectives of this doctoral dissertation, OCP measurements were utilized as an on-line method to 1) Determine the onset of bacterial colonization; 2) Detect changes in biofilm constituents; and 3) Predict the onset of corrosion activities mediated by biofilm bacteria.

MATERIALS AND METHODS

Reagents. All reagents utilized were of reagent-grade or better, unless otherwise specified.

Bacteria. Four physiologically distinct, freshwater isolates were utilized in the colonization studies. Pseudomonas fluorescens (lux), hereafter referred to as 5RL, was isolated from a manufactured gas site soil and was kindly provided by Dr. Gary Saylor. This organism was selected on the basis of its ability to bioluminesce when induced, and its adhesive characteristics in liquid culture. Further details on its construction and growth characteristics may be found in chapter VI. Bacillus subtilis, Hafnia alvei, and Desulfovibrio gigas were isolated from a Tennessee Valley Authority pipeline system. Based upon the adhesive nature of 5RL and the pipeline environment from which the other 3 organisms were isolated, the bacteria utilized in these colonization studies were considered to be putative agents of biofouling/biocorrosion.

Identification of the three aerobic isolates was performed using the Microbial Identification System (MIDI, Newark, DE); D. gigas, an anaerobic, dissimilatory sulfate-reducer, was identified based upon classical biochemical analyses (Widdel and Pfenig, 1984), diagnostic lipid profiles (Dowling, et al., 1988), and its characteristic

morphology in liquid culture. D. gigas was maintained in sulfate-API broth (Difco, Detroit) and transferred bimonthly. The aerobic cultures were maintained on trypticase soy agar slants (Difco, Detroit) and transferred bimonthly.

Continuous Culture Conditions. The four isolates were maintained at a dilution rate of 0.10 h^{-1} in a continuously stirred medium containing the following components (in g L^{-1}): sodium lactate, 0.05; sodium succinate, 0.05; NH_4NO_3, 0.05; Na_2SO_4, 0.12; KH_2PO_4, 0.19; K_2HPO_4, 0.63; $FeCl_3$, 0.33 mL of a 10 mM solution; Hutner's salt solution, 1.0 mL. The completed medium was steam sterilized. The final pH of the culture medium was 7.2. The 50 mg L^{-1} succinate and lactate substrate concentrations were selected based upon preliminary shake-flask studies. This substrate concentration was the lowest amount yielding a firm collar at the flask air/medium interface.

The three aerobic cultures were continuously sparged with breathing-grade air; D. gigas, with 5% H_2/N_2 passed through a heated Cu-reducing column. Selection of adhesive variants of 5RL, B. subtilis, and H. alvei was made by using scrapings from collars which formed at the air/medium interface on 24 h shake flasks as inocula. All experiments were conducted at ambient room temperature.

Adhesion Cells and Test Coupons. The laminar-flow adhesion cells described in chapter III were cleaned, sterilized, and assembled as previously described. Twenty-five by fifty mm 316 SS coupons were polished to a 600 grit finish, then passed through a series of sonication baths as described in chapter III. All flow cells were sterilized in an ethylene oxide chamber.

Flow System. A schematic drawing of the flow cell system is shown in Fig. 1. Because of the large volumes of sterile media required for each experiment--typically, in excess of 1,700 L--sterile medium concentrate was continuously mixed with process filter-sterilized, deionized (DI) water. DI water quality was monitored with conductivity meters on the effluents from the primary and secondary organic-DI resin beds. The ion exchange capacity of the polishing DI resins (Baxter, Valencia, CA) was sufficient to produce approximately 2,500 L of 15 Mohm·cm DI water. Infrared level sensors (Ryan Herco, Burbank, CA) were used to control a feed water solenoid valve and pumps for the DI water and medium concentrate. The same medium described above for the continuous culture system was prepared continuously by maintaining a constant ratio of medium concentrate:DI water in the sterile feed vessel. Preliminary experiments demonstrated that the flow system could produce sterile medium for 5 or more days without replacement of DI resins

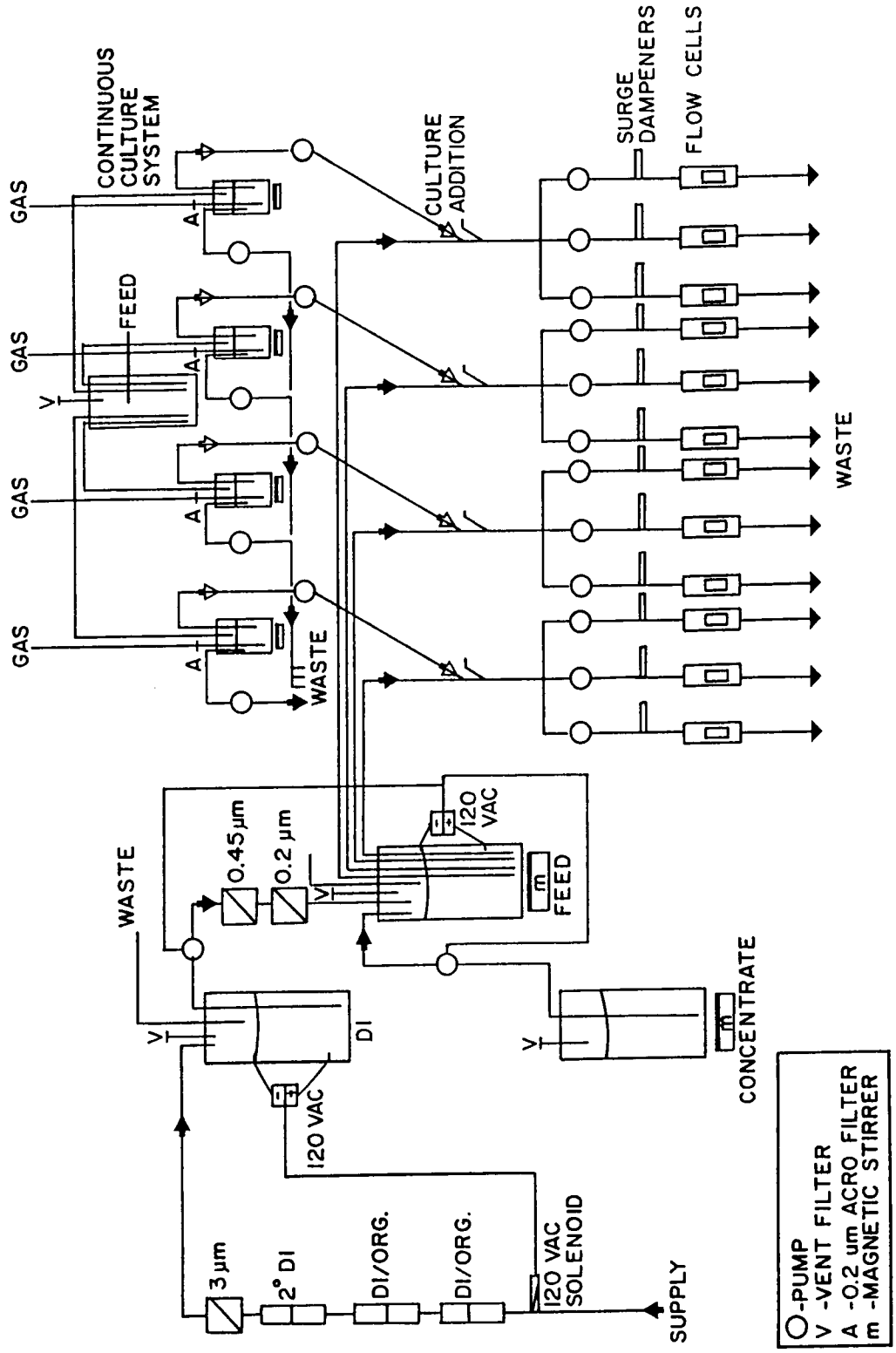


Fig. 1. Schematic of flow system illustrating sterile media production, continuous culture, and flow cell components.

or microporous membrane filters. Pressure tubes were used as surge dampeners for the Masterflex peristaltic pumps (Cole Parmer, Chicago). A volumetric flow rate of 20 mL min^{-1} (linear velocity of 1.31 cm s^{-1}) was maintained for the duration of the experiments. Effluent from the flow cells was collected in a 10 L polypropylene carboy into which flowed approximately 0.5 mL min^{-1} of a 5.25% sodium hypochlorite solution (Chlorox). Disinfected effluent was drained to sewer from a spigot at the base of the carboy. Viable organisms were not detected in this sewered effluent.

Bulk Phase Inocula. Addition of the bulk phase organisms to the flow cells was begun approximately 36 h following inoculation of the continuous culture system. (The D. gigas were cultured for an additional 12 h prior to inoculation.) Viable plate counts and OD readings demonstrated that the three aerobic cultures had reached a steady state. Aliquots from the D. gigas continuous culture were inoculated into a 3-tube most-probable-number (MPN) series at the end of the experiment.

Various axenic cultures were added in succession to the sterile, flowing medium. Table 1 lists the various "climax" communities and the order in which their component members were added to the flow stream. Each culture was injected into the sterile, flowing medium at a rate of 0.4 mL min^{-1}. Single-member communities were injected for 72 h; two-member

communities, 48 h each; and four-member communities, 24-48 h each (Table 1). Each community was tested in triplicate, with triplicate experiments performed for the four-member communities, and duplicate experiments for the single- and two-member communities.

Table 1. Bulk-phase bacterial additions used in colonization studies.

Community Designation	Colonization Order[a]	Development Time, h
1	5RL/axenic	72
2	5RL-*B. subtilis*-*H. alvei*-*D. gigas*	120
3	5RL-*D. gigas*-*H. alvei*-*B. subtilis*	120
4	5RL-*H. alvei*-*B. subtilis*-*D. gigas*	120
5	5RL-*H. alvei*-*D. gigas*-*B. subtilis*	120
6	5RL-*B. subtilis*	96
7	5RL-*D. gigas*	96
8	*H. alvei*/axenic	72
9	5RL-*H. alvei*	96
10	*B. subtilis*/axenic	72
11	*D. gigas*/axenic	72

[a]In communities 2-5, 5RL was injected for 48 h, with succeeding organisms injected for a period of 24 h each.

Pulse-Labeling. A mixture of 1,4-^{14}C-succinate and U-^{14}C-lactate (New England Nuclear, Boston) was used to pulse label biofilm populations. *D. gigas* is incapable of using succinate (or glucose) as a carbon source. While this organism can use acetate, *D. gigas* requires the presence of H_2 as an electron-donor.

At the end of the biofilm development period (Table 1), injection of bulk-phase organisms was discontinued. Sterile

medium was allowed to flow through the adhesion cells for an additional 10 min to flush out bulk-phase organisms and remove "reversibly" bound cells from the coupon surfaces. The inlets to triplicate cells were then connected to 125 mL Erlenmeyer flasks containing sterile, C-free medium and flushed with approximately 30 mL each at the in situ flow rate of 20 mL min^{-1}. The inlets and outlets were then connected to 125 mL Erlenmeyer flasks containing 100 mL sterile, C-free medium, which was recirculated at the in situ flow rate of 20 mL min^{-1} for 15 min. These last two steps were designed to remove C-sources from the biofilm environment in preparation for label uptake.

Four-hundred and 200 μL volumes of the labeled succinate (100 μCi mL^{-1}, 60 mCi $mmole^{-1}$) and lactate (12.5 μCi mL^{-1}, 177.3 mCi $mmole^{-1}$), respectively, were added to 100 mL volumes of sterile, C-free medium contained in 125 mL Erlenmeyer flasks. Immediately following the final flushing procedure, the inlets and outlets to triplicate adhesion cells were connected to the flasks and the label solution was recirculated for 60 min at the in situ flow rate of 20 mL min^{-1}. At the end of the labeling period, inlets to the adhesion cells were connected to 125 mL Erlenmeyer flasks containing sterile, C-free medium. The cells were then flushed with approximately 30 mL each at the in situ flow rate of 20 mL min^{-1}.

All manipulations (e.g., tubing and pump changes) performed during the flushing and labeling procedures were carried in such a manner as to minimize perturbations of the biofilm. The coupons remained wetted at all times during the labeling procedure, and passed through an air-medium interface only once, immediately prior to biofilm extraction. Each manipulation of the adhesion cells required approximately 30 s for completion.

Biofilm Extraction. Coupons were quantitatively extracted using the glass o-ring extractors and sonication procedure described in chapters II and III. Each coupon was extracted in three 1.13 cm^2 areas corresponding to the upstream and downstream ends, and the coupon center. Approximately 5 cm^2 of each coupon were not extracted. Aliquots from the sonicates were transferred to a series of fired, 13 X 100 mm glass screw-top tubes for cell counts and biofilm analysis. The extracted coupons were immediately placed in a -50 C freezer for further processing for Fourier transform infrared/diffuse reflectance (FTIR/DRIFT) spectroscopy.

Biofilm Cell Counts. Immediately following extraction, sonicate aliquots were serially diluted in 10 mM, pH 7.2 phosphate buffer and plated in duplicate onto yeast-extract-peptone agar containing 14 mg L^{-1} tetracycline (YEPG-tet), MacConkey agar (Mac) (Difco, Detroit), and phenylethanol

agar (PEA) (Difco, Detroit). Each of the three aerobic cultures was plated on the three selective media. Preliminary experiments demonstrated good selectivity--and recovery vs. trypticase soy agar counts--for the above media plated, respectively, with 5RL, H. alvei, and B. subtilis. While some growth of the 5RL on Mac was observed, colonies were easily differentiated on the basis of H. alvei's unique ability to ferment lactose and produce brick-red colonies. As described in chapter VI, the Tn5 transposon used in the construction of 5RL contains a gene sequence which encodes for tetracycline resistance. Plates were incubated at 25 C for 72 h.

An 0.5 mL sonicate aliquot from each extraction was transferred to 1 mL microfuge tubes (Eppendorf, Westbury, NY) and centrifuged in a refrigerated centrifuge (Eppendorf, Westbury, NY) at 3,000 X g. The supernatant was carefully decanted and the pellet resuspended in 0.1 mL RNAase stabilization buffer (Gen-Probe, San Diego). This suspension was probed with all-eubacterial and Desulfovibrio genus-specific 16s rRNA specific DNA oligonucleotides. Summaries of the hybridization and detection procedures, as well as control experiments, are provided in appendix IV-1. The relative-light-units detected were converted to viable cell equivalents using titered control cultures.

Sonicate aliquots for AODC were diluted in filter-sterilized phosphate buffer, filtered through 0.2 μm

Nuclepore membrane filters (Nuclepore, Pleasanton, CA), and processed as described in chapter II. A total of 25, randomly selected, 0.01 mm^2 microscopic viewing fields were counted for each sample at 1000X total magnification.

Biomass Constituent Analysis. Biomass protein was quantitated via the micro-Lowry procedure described in chapter II. Bovine serum albumin (BSA) was used to prepare a standard calibration curve.

Biomass carbohydrate:protein ratios were estimated from FTIR/DRIFT absorbance values. Frozen coupons were lyophilized overnight, desiccated in vacuo, and the unextracted areas analyzed as described in chapter II. Twelve spectra were obtained for each triplicate coupon examined, corresponding to upstream, downstream, and central areas. All spectra used in the calculation of carbohydrate: protein ratios were water-subtracted, baseline-corrected, and ratioed against cleaned and polished 316 SS coupons.

Label Incorporation Assay. Incorporation of ^{14}C labels into cellular lipids was determined as described in chapter II. Extracts were subjected to a Bligh-Dyer (1959) chloroform/methanol/water extraction. Following phase separation, dried lipid extracts were processed for liquid scintillation counting as previously described. CPM values

were quench corrected, and the data expressed as DPM uptake $cell^{-1}$ using the corresponding AODC data.

OCP Monitoring. Preparation of the flow cells for electrochemical monitoring is described in further detail in chapter III. Following installation of Ag/AgCl reference electrodes in electrochemical fittings containing a saturated KCl solution, the reference electrodes and working electrodes (the coupons) were connected to a Keithley (Keithley Instruments, Cleveland) model 706 multichannel scanner. OCP measurements were taken by an HP model 3458A voltmeter (Palo Alto, CA) which was interfaced with a GPIB board (National Instruments, Austin, TX) to an IBM clone personal computer. Data acquisition and manipulation were facilitated by Lotus Measure (National Instruments), with measurements taken every 15 min for the duration of the experiments. Communities 2-5 were monitored continuously in duplicate experiments. Sterile media flowed through the cells for approximately 20 h prior to addition of the 5RL.

Electrochemical Noise Measurements. Electrochemical noise analysis was performed on the free corrosion potential at the beginning and end of the OCP monitoring. Data were retrieved to a Keithley model 706 scanner (Keithley, Cleveland) coupled to a Hewlett-Packard model 3458A multimeter. The multimeter was set to retrieve measurements

with a sampling interval of 1 second for a total of 2000 measurements. Data were retrieved from the multimeter to an IBM clone 286 personal computer via an IEEE-1978/GPIB connection employing a National Instruments PC11 card. The controlling program was a macro written for Lotus-Measure.

The raw data were transformed using the Mat-Lab (Math-Works, South Natick, MA) math package. An initial fast Fourier transformation (FFT) was made of the raw data. Adjacent records were averaged to obtain more reliable spectral estimates. Confidence limits on the auto and cross-spectra were also plotted. The spectral analysis was carried out on the "real" and "imaginary" components obtained from the FFT. The sequences of 2000 points in length were divided into sections of 250 points in length. Successive sections were multiplied by a Hanning window (Math-Works, South Natick, MA). Confidence intervals were estimated by calculating the variance of the unaveraged spectral estimates under the assumption of a normal distribution.

Statistical Analyses. Comparisons of treatment effects on biofilm biomass and metabolic activity were made via one-way analysis of variance (ANOVA) and a posteriori, step-wise Student-Neuman-Keuls (SNK) comparison of means tests (Sokal and Rohlf, 1969). Tests of significance for the SNK test were performed using Studentized range values (Rohlf and

Sokal, 1969) at the 0.01 error level. Pair-wise comparisons of means were made via a Student's t-test (Sokal and Rohlf, 1969). All computations were made on Lotus spreadsheets containing the experimental data.

RESULTS

Continuous Culture Titers. Viable counts of the 5RL, H. alvei, and B. subtilis were maintained at approximately 2 X 10^7, 1 X 10^7, and 4 X 10^6 cells mL^{-1} for the duration of the adhesion studies. The D. gigas culture reached a maximum titer of approximately 5.5 X 10^5 cells mL^{-1} within 48 h of growth in continuous culture. These concentrations were reproducible from experiment-to-experiment within a factor of approximately 2. Viability of the inocula through the flow system was confirmed by comparing titers obtained from the pre-disinfection effluents with those in the continuous culture vessels. When the dilution factor of 0.4/20 was taken into account, viable counts from paired samples taken from the two locations were comparable ($P<0.05$).

Cell Counts and Biomass. Although B. subtilis remained viable in the bulk-phase throughout the colonization periods, this organism was not recovered in a viable state from any of the 6 communities in which it was a member.

Based upon the dilutions employed for viable counts, <200 B. subtilis cm^{-2} were recovered. Although direct AODC observations did reveal some presumptive spore-forming morphologies, further efforts to identify spore-formers in the extracts using a sudan-black spore stain were not successful.

The results of 72 h colonization studies with axenic cultures of the 4 isolates demonstrated that only 5RL and, to a lessor extent, H alvei, colonized the coupons. The community structure for each of the 9 biofilm communities is shown in Fig. 2. D. gigas were recovered in communities 2 and 4, as measured by 16s rRNA estimates. With exception of community 9, the aerobic pseudomonad, 5RL, was the predominant member of all biofilm communities in which it was a member, reaching a "climax" population level of between 5×10^7 and 1×10^8 cells cm^{-2}. The 5RL-H. alvei community (#9) contained approximately equal numbers of each bacterium, at $6.5 \times 10^7 +/- 1.5 \times 10^7$ cells cm^{-2} (Fig. 2). The number of viable 5RL recovered was not significantly affected ($P<0.05$) by either the community composition or the order of addition. An ANOVA did not reveal a significant difference ($P<0.05$) between either the 5RL or the H. alvei populations in communities 2-5.

Despite the relatively high degree of variability in the data, an ANOVA revealed a significant treatment effect

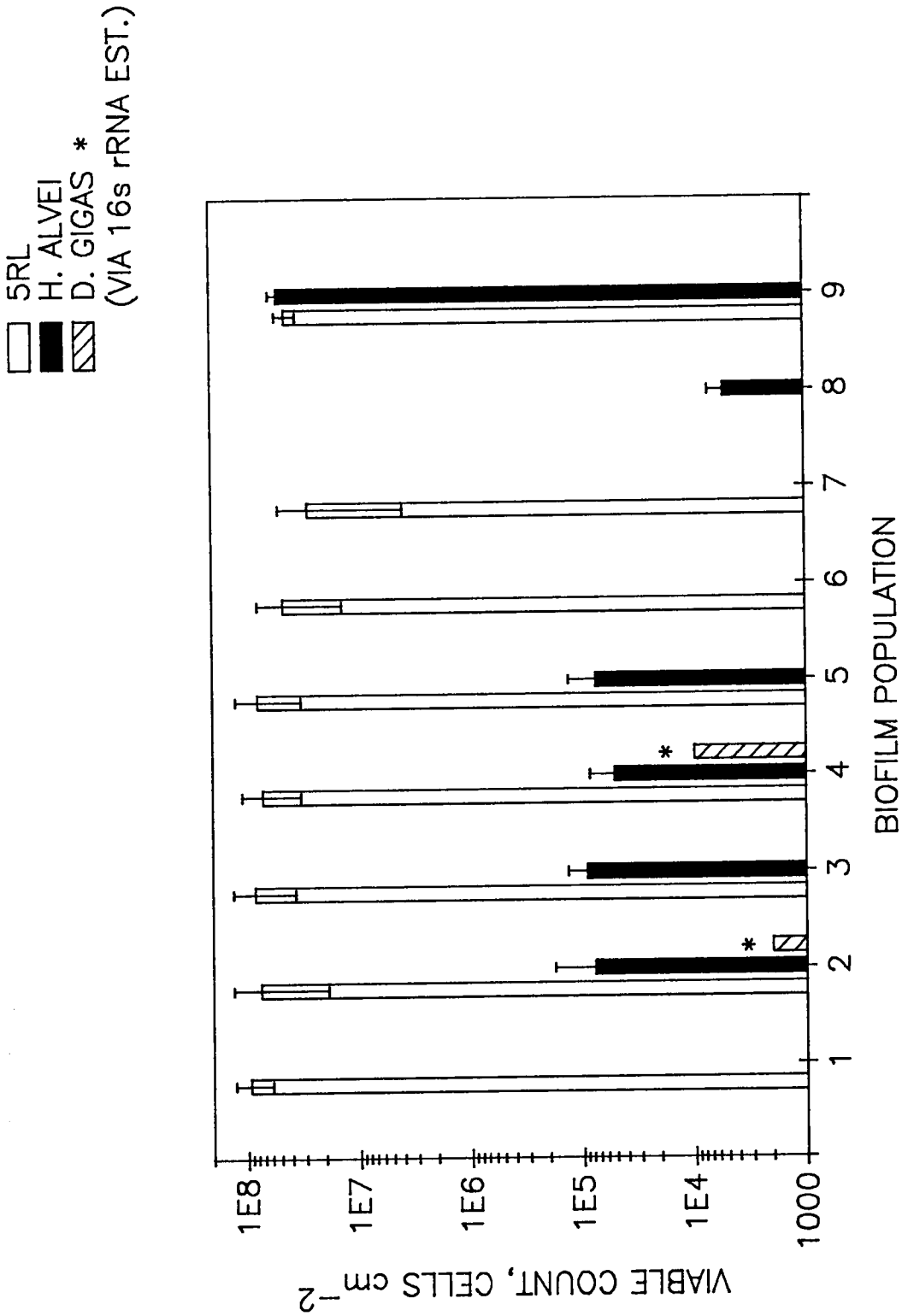


Fig. 2. Relative community structure of biofilms from 9 different communities. Error bars are ±1 SD.

($P<0.01$) of community on AODC and protein biomass values (Fig. 3). Since community 8 yielded relatively low AODC values and non-detectable protein, it was not included as part of the ANOVA in order to subject the data to a more conservative statistical analysis.

AODC values were significantly influenced by order of addition at $P<0.05$, but not at $P<0.01$. Community 5 resulted in significantly higher total counts than communities 2-4 at $P<0.05$, but not at $P<0.01$. No differences in AODC values were seen between communities 2-4 at $P<0.05$. Protein biomass was significantly influenced ($P<0.01$) by order of addition. Community 5 contained significantly more protein per unit area than did communities 2-4, which did not vary significantly ($P<0.05$) from one another.

Carbohydrate:Protein Ratios. Figure 4 gives a comparison of the carbohydrate:amide I absorbances--interpreted as carbohydrate:protein ratios--for each of the 8 communities tested. The mean ratios were compared using an SNK step-wise test; the results of this iterative test are shown below. Any group of means enclosed by the range of any one line is not significantly different.

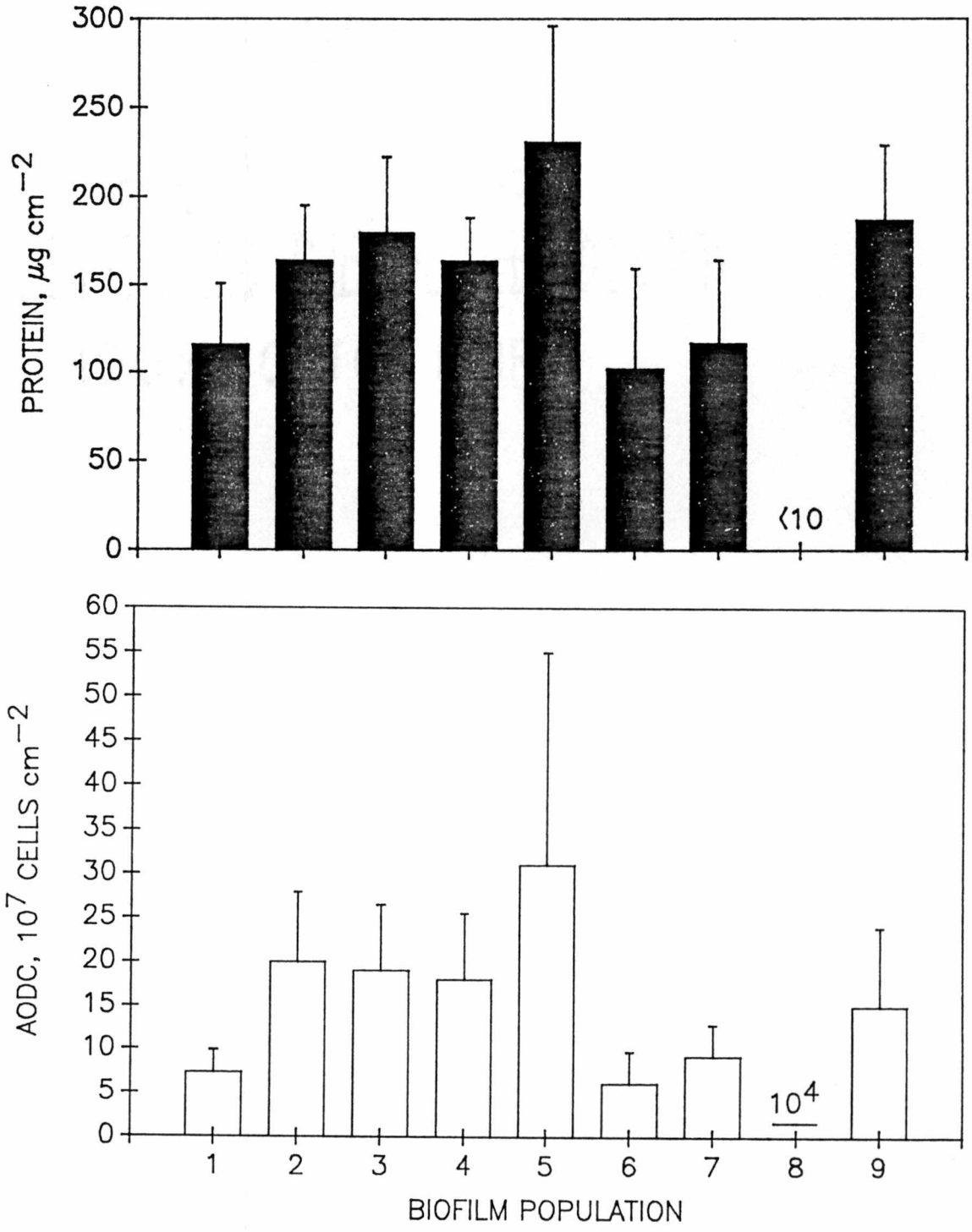


Fig. 3. AODC and protein biomass values for the 9 biofilm communities. Error bars are ±1 SD.

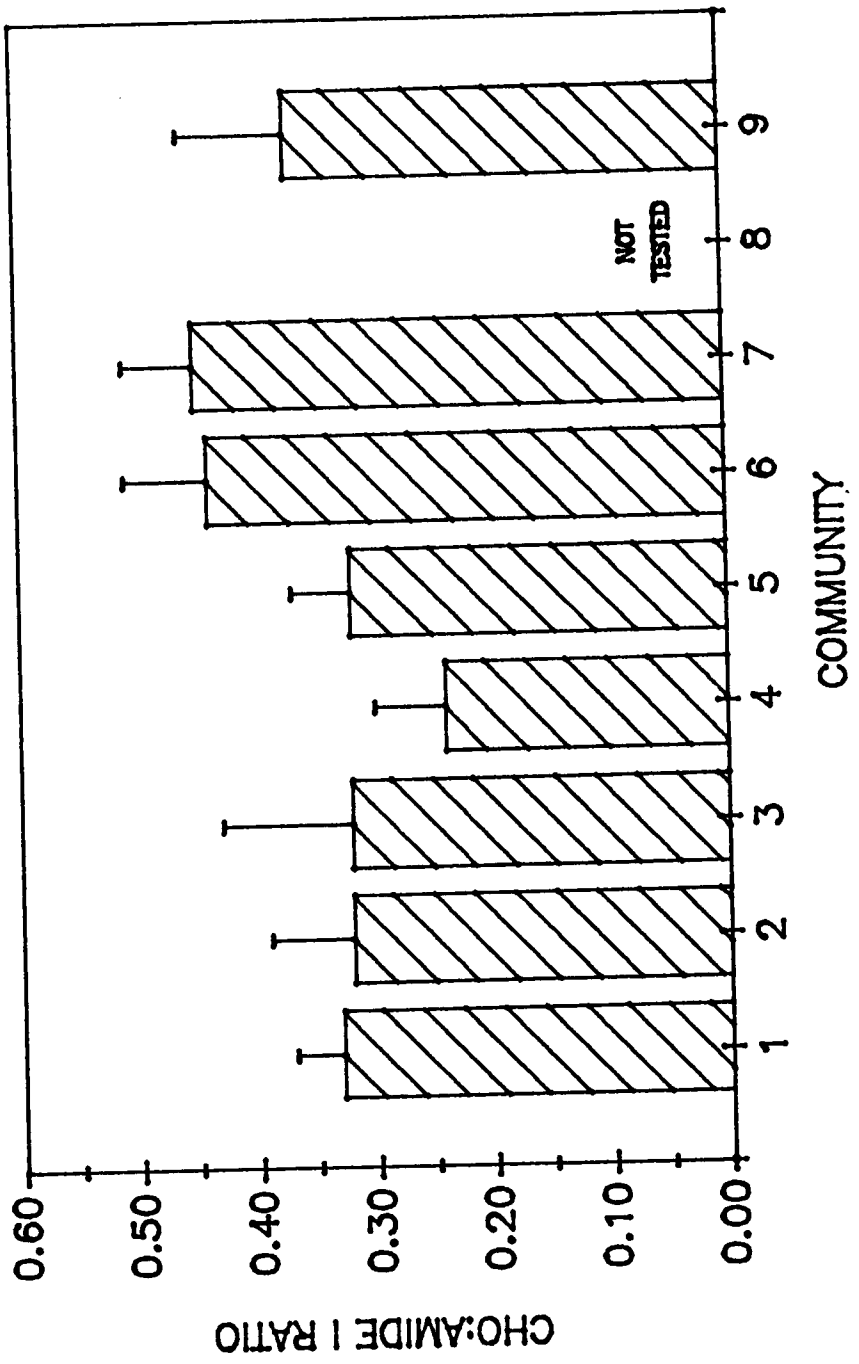
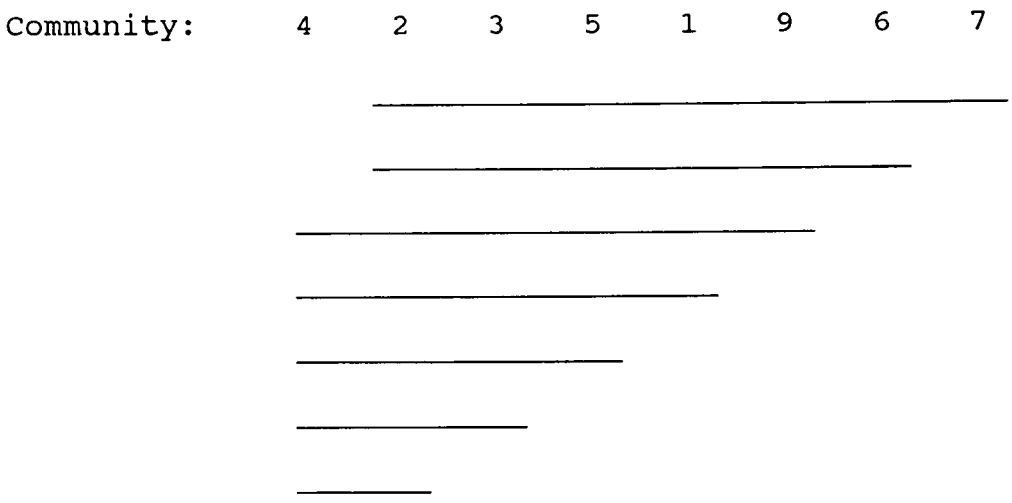


Fig. 4. Carbohydrate:protein ratios from FTIR/DRIFT absorbance values. Error bars are ±1 SD.



The carbohydrate:protein ratios were grouped into three significantly different ($P<0.01$) populations. Community 4 contained a significantly lower carbohydrate:protein ratio ($P<0.01$) than either community 6 or 7. Communities 2,3,5,1,9,6,7 were not significantly different ($P<0.05$) from one another, nor were communities 4,2,3,5,1, and 9. The results of this qualitative analysis did not suggest that order of addition significantly affects ($P<0.05$) carbohydrate:protein ratios.

Metabolic Activity. As with the cell count and biomass data, significant differences ($P<0.01$) between the various communities were observed. The CPM obtained for community 8 was not significantly above background. This was not unexpected, given the relatively low AODC and viable Hafnia alvei counts of approximately 10^4 cells cm^{-2}. (Community 8 was not included in the determination of statistical

significance.) Since it was not possible to determine relative uptake efficiencies of the individual radiolabeled substrates, data were expressed as DPM $cell^{-1}$ using AODC as a normalization factor.

Communities 4 and 5 showed the highest metabolic activities, 2.0×10^{-4} and 3.3×10^{-1} DPM $cell^{-1}$, respectively (Fig. 5). Order of addition was also a significant factor ($P<0.01$) for the per cell metabolic activity. Communities 4 and 5 elicited significantly higher activities than did 2 and 3.

Electrochemical Monitoring. Plots of OCP vs. time for the 4 communities monitored are shown in Fig. 6. In each case, the formation of a visible biofilm was preceded by a change in OCP. An increase in potential was followed by a period of transient instability. Net increases of between 50 and 60 mV were observed for the four communities. No correlation between changes in OCP and biomass or metabolic activity was apparent.

Electrochemical noise measurements were made on each of the 4 communities monitored for OCP. Figure 7 illustrates a power spectrum from community 2. The shifts in ECN observed at the diagnostic lower frequencies were not indicative of corrosion activities. No significant differences in frequency shifts were seen among the 4 communities or between the sterile (data not shown) and colonized coupons.

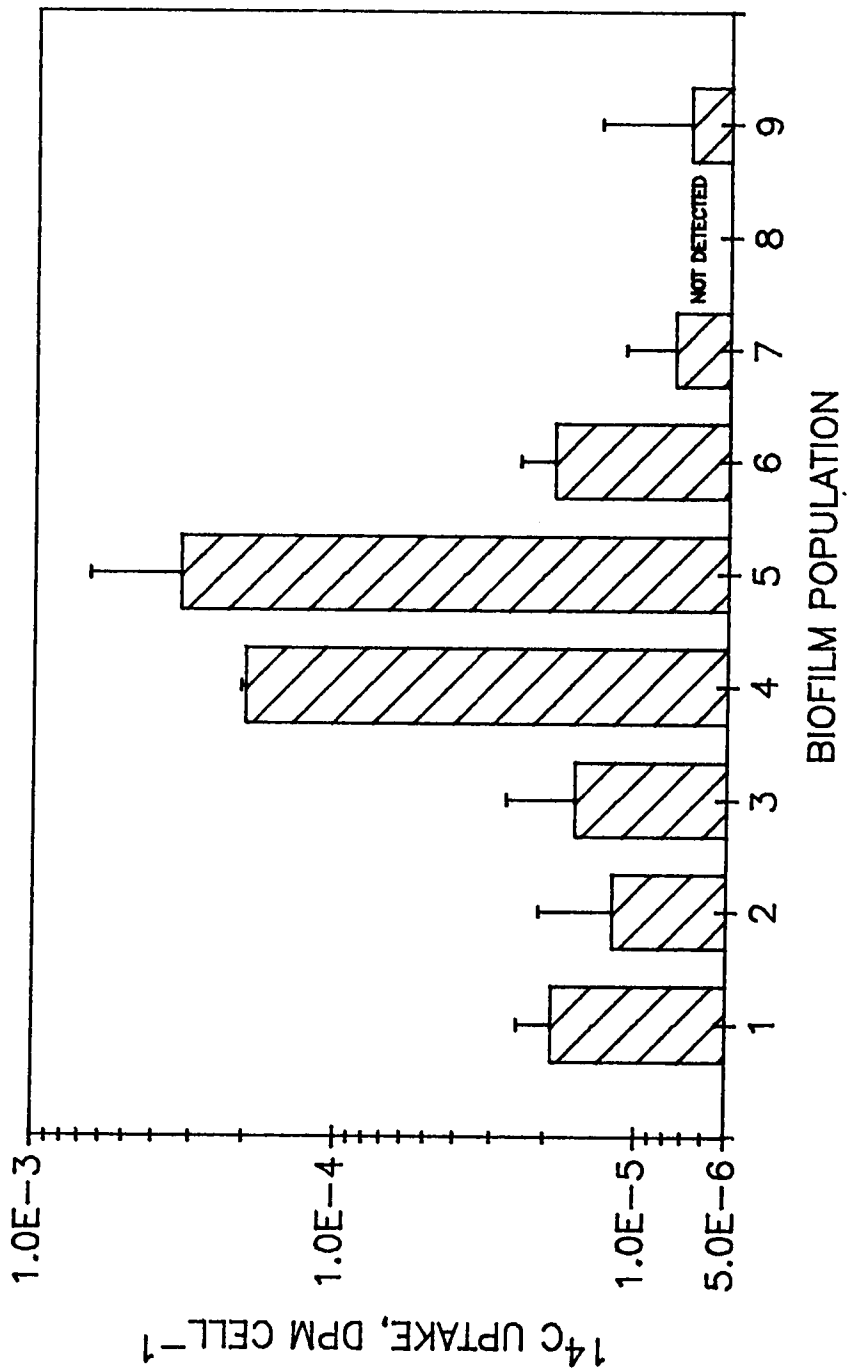


Fig. 5. Metabolic activity of the biofilm communities as measured by 60 min pulse labeling with ^{14}C-succinate. Error bars are ±1 SD.

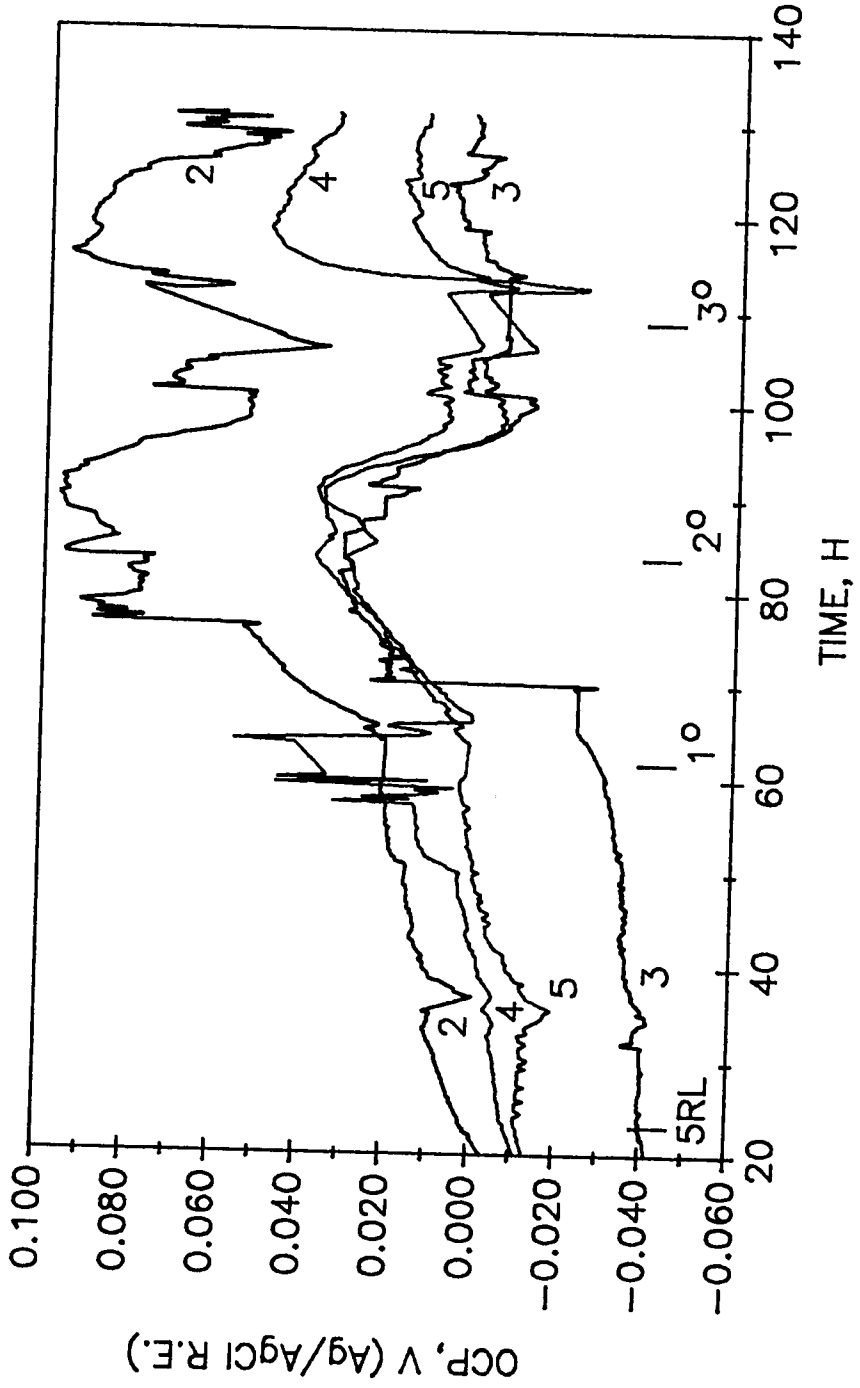


Fig. 6. OCP of communities 2-5 monitored over 136 h. Measurements were taken at 15 min intervals. Additions of 5RL and the other 3 bulk-phase organisms are indicated by the vertical bars.

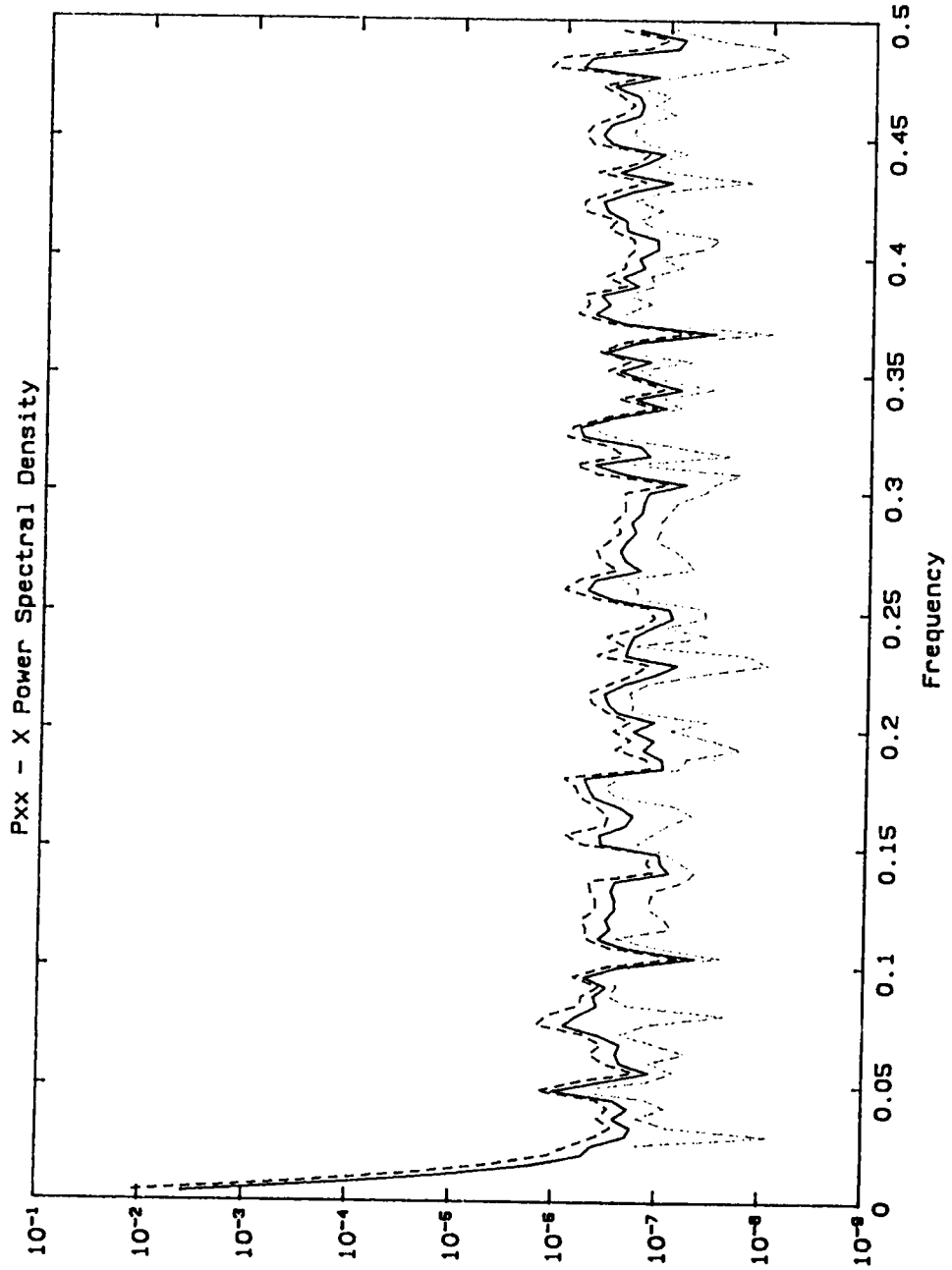


Fig. 7. ECN power spectrum of community 2 following the 136 h colonization period. Dotted line represents 95% UCL.

DISCUSSION

The results of this research have demonstrated that bulk-phase bacterial community structure is an important determinant of biofilm biomass and metabolic activity. The order in which physiologically distinct bacteria were exposed to 316 SS substrata significantly affected the biofilm biomass and unit metabolic activity. In addition, biofilms containing two or three viable members contained significantly higher total cell counts and protein biomass than did biofilms consisting of single species. The flow regime employed in these experiments was selected on the basis of relevance to *in situ* conditions in an industrial water system as well as practical considerations of media production and the maintenance of sterility.

The 20 mL min^{-1} volumetric flow rate is approximately equivalent to a volumetric flow rate of 2 gpm (126 mL s^{-1}) through a 4" (10.2 cm) diameter pipeline.

The failure to recover viable *B. subtilis* from coupon surfaces was surprising. Although some *Bacillus* sp. do have complex growth requirements, the fact that viability in the continuous culture system was maintained for the duration of the experiment suggests that the bulk phase could support their growth. One explanation for the lack of *B. subtilis* colonization could lie in the nature of Gram positive cell surface energetics. Although specific colonization studies

employing B. subtilis in similar environments have not been reported, Busscher et al. (1986) and others (Absolom et al., 1983) have shown that the Gram positive streptococci tend to favor more hydrophilic surfaces than do most Gram negative organisms. Interestingly, Ronner et al. (1990) have reported that both vegetative cells and spores of B. subtilis adhered poorly to stainless steel and glass surfaces, irrespective of substratum free surface energy. Stainless steel has a relatively low free surface energy, corresponding to a surface with hydrophobic characteristics. Whether this difference in cell surface-substratum energetics is an important factor in the paucity of Gram positive organisms isolated from most aquatic environments is unknown at this time.

The poor recovery of "viable" D. gigas cells from biofilms may indicate that conditions were not sufficiently anaerobic to enable growth within existing biofilms. It is also likely that extending the colonization periods beyond 5-days would increase numbers of sessile D. gigas. In studies with mild steel exposed to consortia of freshwater bacteria, Desulfovibrio spp. were recovered in much greater numbers (Franklin et al., 1990). However the large amounts of corrosion product associated with mild steels in aqueous environments not only provide additional surface area for colonization, but also act to reduce local oxygen concentrations. The availability of Fe may also be an

important factor. The absence of such corrosion products with stainless steels probably results in a less anoxic environment for anaerobes such as D. gigas.

Given the predominance of 5RL in the four-4 member communities, it is not surprising that order of addition had little affect on carbohydrate:protein ratios. This qualitative biomass ratio could be an indication of EPS production, with the values being indicative of increased production. While media containing a low C:N ratio often tend to select for more adhesive bulk phase organisms (McEldowney and Fletcher, 1986; Mittelman, unpublished results), the relationship between biofilm carbohydrate:protein ratios and biomass quantity is less well defined. In the research described in chapter II, however, this ratio was positively correlated with biomass values.

That the 5RL strain was clearly the predominant colonizer demonstrates that physiologically distinct bacteria in freshwater systems possess differential adhesion characteristics. If the other three organisms had adhered to clean or previously colonized surfaces, that attachment by Marshall's (1971) definition was apparently "reversible" in nature. Since order of addition was also an important determinant of biomass constituents and unit metabolic activity, biofilm development was influenced by successional colonization. Additionally, the presence of two or three--

and perhaps four--members within a biofilm consortium increased the amount of biomass formed per unit area as well as unit metabolic activity. These findings have important implications for both the study and treatment of fouling microorganisms. In particular, they point to the importance of utilizing consortial communities in studies of bacterial colonization and fouling biofilm formation. The importance of consortia in biofouling activities will be discussed further in chapter V.

Changes in OCP were observed following colonization of the coupons with the bulk phase organisms. Although a correlation was not evident between changes in OCP and the other quantitative and qualitative analyses performed, useful information may be obtained from OCP vs. time graphs (Fig. 6). The OCP is a net potential, describing the sum of cathodic and anodic reactions. The cathodic reaction, which predominates in stainless steels exposed to aqueous environment--contrasted with mild or carbon steels, in which the anodic reaction predominates--is described by

$$O_2 + 2H_2O + 4e^- \rightarrow\rightarrow 4OH^- \qquad (1)$$

The OCP is described by two parameters contained within the Nernst equation, pH and oxygen. For the cathodic reaction,

$$E = E(O_2/OH^-) + RT/nF\ (\ln[pO_2]/[OH^-]^4) \qquad (2)$$

Accordingly, there are three possible explanations for the observed net increases in OCP. Firstly, decreased pH at the biofilm-substratum interface. A change in 1 pH unit can account for approximately +60 mV potential. Since the bulk phase pH remained relatively stable at 7.1 ± 0.2, pH could only be a factor if it was reduced on a local scale by bacterial activity. Since H. alvei is capable of producing acid from both succinate and lactate, it is conceivable that a local drop in the biofilm pH could have occurred. However, increases in OCP were observed prior to the introduction of this fermentative organism (Fig. 6). Perhaps some of the transient instability in the later portions of the OCP curves could be attributed to bacteria-induced pH changes. One additional possibility involves the displacement of H^+ ions from the biofilm by divalent cations. Mittelman and Geesey (1985) showed that H^+ ions were released from bacterial extracellular polymeric substances (EPS) upon exposure to Cu^{++} ions. In this regard, the on-line OCP technique could be applied to studies of heavy metal interactions with biofilms.

A second explanation involves increases in oxygen at the substratum surface. The presence of a biofilm comprised primarily of an aerobic heterotroph should reduce oxygen levels at the surface. In addition, stainless steels are resistent to anodic dissolution primarily due to the presence of a passive metal oxide film. However,

discontinuities in this passive film could be imparted by bacterial metabolic activity; e.g., production organic acids or cathodic depolarization of monoatomic hydrogen. The metal surface would then be exposed to oxygen, whereupon repassivation would occur. The presence of differential oxygen cells on SS surfaces has been proposed as one mechanism for MIC (Tiller, 1982). The observed stochastic inhomogeneities in the biofilms (chapter III) could result in favorable conditions for "oxygen attack"; namely, a small anode (non-colonized area) and a large cathode (underneath the biofilm).

Finally, changes in oxygen reaction kinetics at the surface could result in increases and/or decreases in OCP. Johnsen and Bardal (1985) theorized that the presence of heavy metals could influence the activity of oxygen at SS surfaces. Bacterial EPS can concentrate divalent cations--including heavy metals--as was described above. Metals and other divalent cations were present in low concentrations in the bulk-phase, as they are in many oligotrophic, natural environments. Unfortunately, the reactions between heavy metals and SS surfaces in aqueous environments are poorly understood at this time. The results of this research do provide preliminary evidence that biofilms influence some aspect of pH, oxygen, or catalytic dynamics at stainless steel surfaces.

The ECN data provide an estimation of the breaching and "healing" of the passive film associated with all stainless steels. As corrosion activity commences with the short-term removal of this film, the potential of the immediate surface decreases (Simoes and Ferreira, 1987). If the film "repassivates", this is accompanied by a sharp increase in the potential. Thus, a constantly fluctuating potential is associated with a surface that is under distributed attack.

If, however, a pit propagates to form a zone of constant dissolution, current from the surrounding area is fed to a stable pit and although corrosion continues, the "electrochemical noise" decreases in amplitude. Thus, a particular stage of corrosion is associated with large amplitude frequency oscillations.

The ECN frequency plots obtained from this work did not exhibit either of these attributes. Indeed, there was little difference between the noise values for the sterile and the colonized coupons. Given the relatively short-term nature of the colonization experiments and the failure of D. gigas to colonize to any great extent, it was unlikely that MIC activities would be significant. In addition, some investigators (Pierpoline, et al., 1990) have maintained that the presence of 100-200 mg L^{-1} chloride is a prerequisite for initiating corrosion of 316-type stainless steels.

Electrochemical measurements such as OCP and ECN show great potential for use as analytical tools in studies of bacterial colonization. In addition to their application to studies of MIC (Attwood et al., 1990; Iverson, 1981), they may also be useful in evaluations of novel surfaces and surface coatings for medical and industrial applications. Since these types of techniques measure changes occurring at the surface, they are uniquely suited for continuous monitoring of colonization and biofilm activity. Both of these fouling parameters are important in the bacterial "regrowth" phenomenon which occurs in biocide-treated potable water pipelines (LeChevallier, et al., 1987). The research presented in this chapter should provide a starting point for future on-line biocide efficacy analyses. However, further studies will be required to enable differentiation of biotic from abiotic film formation in industrial systems.

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CHAPTER V

EFFECTS OF SUBSTRATUM METALLURGICAL AND TOPOLOGICAL INHOMOGENEITIES ON BACTERIAL BIOFILM COMMUNITY STRUCTURE, BIOMASS, AND METABOLIC ACTIVITY

INTRODUCTION

In the search for surfaces which retard or otherwise inhibit bacterial colonization, research efforts have been primarily focussed on the physicochemical nature of the substrata. For example, McCoy et al. (1981), Characklis (1990), Pedersen (1990) and others have examined the effects of gross topological differences on total biomass formation. van Loosdrecht et al. (1990), Baier (1980), Meyer (1988) and others have described the relationship between interfacial free surface energies and bacterial colonization. In most instances, these studies have been carried out with single cultures on various substrata. Knudsen (1981) examined the effects of various metals on bacterial colonization of ocean thermal energy conversion (OTEC) systems. The fanning frictional resistance imparted by a mixed micro- and macrofauna was not found to be significantly affected by the type of substratum colonized.

In the area of microbially influenced corrosion (MIC), Borenstein (1988) described preferential attack of welds in stainless steels mediated by bacteria. It has been estimated that 60% or more of the weld failures in the

nuclear power industry are mediated by microbial activities (Bob Tatnall, E.I. DuPont, personal communication). Indeed the Nuclear Regulatory Commission (NRC) has recently promulgated a regulation which requires specific attention be paid to MIC-related failures in these areas. The question particular to this biofouling problem is whether the welded areas are more susceptible to MIC attack or are the bacterial communities associated with these areas different from those colonizing the non-welded (base metal) areas.

Gaylarde and Johnston (1986) described accelerated corrosion of mild steel when cultures of D. vulgaris were grown with V. anguillarum. Their results demonstrated the importance of conducting coculture experiments and explain, in part, the relatively low corrosion rates obtained with laboratory SRB monocultures compared with those observed in the environment. Since many estimates of environmental MIC rates are based upon monoculture experiments, it seems likely that most estimates of the economic impact of MIC activities on manufactured materials are overly conservative.

Hamilton (1985) also discussed the importance of mixed cultures in MIC activities. Different physiological groups within the biofilm operate in concert. The obligately anaerobic bacteria, for example, were never found in biofilms without other organisms which scavange oxygen.

Under some conditions, new metal surfaces (produced by mechanical scouring for example) have a large oxygen requirement to maintain the surface oxide layer. This, however is a short term event and seems unlikely to be sustained long enough to provide the anaerobic environment required by obligate anaerobes. Thus other bacteria are required to not only provide the substrates required by sulfate-reducing bacteria, but also to scavange the oxygen which prevents growth of these organisms.

Other studies have shown that copper-nickel alloys are particularly affected when oxygen-consuming bacteria are present (Schriffrin and DeSanchez, 1985). The same authors described a significant increase in corrosion rate when mixed cultures of Pseudomonas sp., Micrococcus sp., and Corynebacterium sp. were introduced into the test system. The deterioration of copper and copper-based alloys has been described as associated with the formation of exopolymers (Geesey and Bremer, 1990) which would tend to support the data of Schiffrin and DeSanchez (1985). This theory is also discussed in chapter IV.

In chapter IV, biomass values and metabolic activity of biofilms associated with 316 SS coupons were shown to be dependent on bulk-phase and biofilm community structure. The order in which substrata were exposed to four physiologically distinct bulk-phase bacterial was also an important determinant of biofilm constituents. In the

research described in this chapter, substratum inhomogeneities were used as a variable in colonization studies. The grain size, elemental composition, and crystal structure all vary across a weld. Whether microorganisms are recruited to these sites (by magnetic or electric fields, for example) or organisms present merely exert a greater corrosive effect has not been established. It is possible that these metallurgical inhomogeneities are more sensitive to corrosion activities, irrespective of microbiological factors. While large numbers of bacteria arranged in tubercules have been observed with many types of welds, their role in the actual deterioration of the weld is not understood (Borenstein, 1988, Tatnall, 1981; Kobrin, 1976).

Welds can be divided into three areas: 1) The base metal, which is unaffected by the weld adjacent to it. 2) The weld metal, which contains the liquified pool of metal behind the heat strike (a mixture of the filler metal and base metal). 3) The heat affected zone (HAZ), an area where dislocations occur due to the proximity of the heat strike, but where no filler metal is incorporated. A duplex structure is characteristic of 300 series stainless steel welds. In this type of arrangement, two crystal structures, ferrite and austenite, are produced by differential cooling-solidification rates after the heat strike has passed, where previously the base metal was fully austenitic. MIC attack

of such welds appears to selectively remove either one phase or the other, but not both (Borenstein and Lindsay, 1988).

Biofilm constituents on base metal, welded, and polished/etched/welded 316 SS coupons were compared for each of the four 4-member communities described in chapter IV. Etching of 316 SS coupons was performed in order to expose the metal-grain boundaries and provide a suitable surface for accelerated corrosion (ASTM, 1988). The same analytical techniques applied in chapter IV were utilized in the work described herein. In this way, meaningful comparisons of colonization to and biomass formation on welded and non-welded surfaces could be made. The principle goals associated with this research were to determine whether substratum metallurgical inhomogeneities could affect colonization and/or metabolic activity, and whether preferential adhesion of particular bacterial communities to these inhomogeneities could be demonstrated.

MATERIALS AND METHODS

Reagents. All reagents utilized were of reagent-grade or better, unless otherwise specified. Bacteriological media and reagent formulations are provided in chapter IV.

Bacteria. The same four-membered communities employed in chapter IV were utilized in the colonization studies described herein. Growth conditions and tranfer regimes for 5RL, *B. subtilis*, *H. alvei*, and *D. gigas* were as described in chapter IV.

Continuous Culture and Flow System Conditions. The same media formulations, dilution rates, and inoculation regimes were used as those described in chapter IV. Continuous production of sterile bulk phase media and disinfection of flow cell effluents were performed as previously described. Volumetric flow rates of 20 mL min^{-1} (linear velocity of 1.31 cm s^{-1}) were employed for the duration of the colonization experiments.

Adhesion Cells and Test Coupons. Adhesion cells were cleaned, sterilized, and assembled as per the procedures described in chapters III and IV. The 316 SS coupons were laterally welded with a 308E filler metal across the center (Metal Samples, Mumford, AL). Coupons were finished to a

600 grit standard, then critically cleaned as described in chapter III. There were no gross topological differences between the base metal, HAZ, or welded areas.

One set of the welded coupons was polished to 1 μm diamond finish, then etched in a glycergia solution consisting of a mixture of 30 mL glycerol, 50 mL HCl, and 10 mL HNO_3. The solution was then sonicated for 5 min in a Bransonic ultrasonic unit (Shelton, CT). The etched coupons were then carefully rinsed in several volumes of 18 Mohm·cm deionized water and stored desiccated *in vacuo*. Photographs of the base metal, heat affected zone (HAZ), and weld sections were taken using an Olympus BH-2 epi-illumination microscope (Southern Micro Instruments, Atlanta). Surface depth profiles were estimated by differential focussing on the "hills" and "valleys" associated with the processed coupons.

Bulk Phase Inocula. Table 1 lists the four groups of organism which were injected into the flow stream. The 5RL was injected for 48 h, followed by 24 h injection periods for the remaining three organisms. Colonization was monitored for approximately 120 h following injection of the 5RL. Each group was tested in triplicate; duplicate experiments were performed.

Table 1. Bulk-phase bacterial additions used in colonization studies.

Community Designation	Colonization Order
1	5RL-*B. subtilis*-*H. alvei*-*D. gigas*
2	5RL-*D. gigas*-*H. alvei*-*B. subtilis*
3	5RL-*H. alvei*-*B. subtilis*-*D. gigas*
4	5RL-*H. alvei*-*D. gigas*-*B. subtilis*

Pulse Labeling and Metabolic Activity Analysis. The concentrations and specific activities of ^{14}C-labeled succinate and lactate were identical to those described in chapter IV. All "climax" communities were pulse labeled for 60 min as per the methods described in chapter IV. Label incorporation determinations were also made as previously described.

Biofilm Quantitative and Qualitative Analysis. As per the methods provided in chapter IV, analyses of biofilm extracts from 1.13 cm^{-2} sections of the base metal and weld metal areas were performed for differential viable counts, AODC, and micro-Lowry protein. Because of the limited area available for replicate extractions, it was not possible to separate the welded from the HAZ extraction areas. The two areas were extracted together and considered as weld-HAZ sections.

Base metal and weld-HAZ areas of etched and non-etched coupons were examined by FTIR/DRIFT using the techniques described in chapter IV.

Electron Microscopy. A modification of Aldrich's (H. Aldrich, University of FL, personal communication) technique for fixation and embedding was used to obtain representative samples from biofilms associated with base metal, HAZ, and welded areas of etched and non-etched coupons. The procedure is outlined in appendix V-1. Embedded biofilms were thin sectioned (1.2 μm sections) and stained in 0.01 M lead citrate and saturated uranyl acetate solutions for 30 and 2 min, respectively. The stained sections were gently washed in deionized water then affixed to EM grids and examined on a Hitachi model H-600 (Hitachi, Tokyo) electron microscope. Various sections from different biofilm depths were examined and representative areas photographed.

Electrochemical Measurements. OCP was monitored continuously as described in chapter IV. Measurements were taken for duplicate etched coupons for each of the 4 communities. ECN measurements were taken of the corresponding etched coupons prior to inoculation and at the end of the 5-day colonization period.

Statistical Analyses. Tests of significance for treatment effects and comparisons of means were made as described in chapter IV.

RESULTS AND DISCUSSION

Surface Characteristics of Colonization Substrata. Photomicrographs of the non-etched and etched coupons are shown in figs. 1 and 2, respectively. Although both types of substrata contained E308 SS filler weld metal, the weld was only visible on the 1 μm polished and etched coupons. There were significant differences in surface topology between the etched and non-etched coupons. The depth of scratches imparted by the 600 grit polishing disc was on the order of 5-10 μm. The 1 μm polished and etched coupons were essentially free from scratch marks; the depth of etched areas was estimated to be on the order of <1-2 μm. Etching revealed the metal crystalline structure as austenitic in the base metal, ferritic in the weld metal and a combination of austenitic and ferritic in the weld-HAZ (ASTM, 1988).

The etching procedure revealed that significant differences in metallurgical structure exist within relatively small, welded substrata. The surface topology associated with the 600 grit polished substrata could afford sessile bacteria protection from the effects of hydraulic

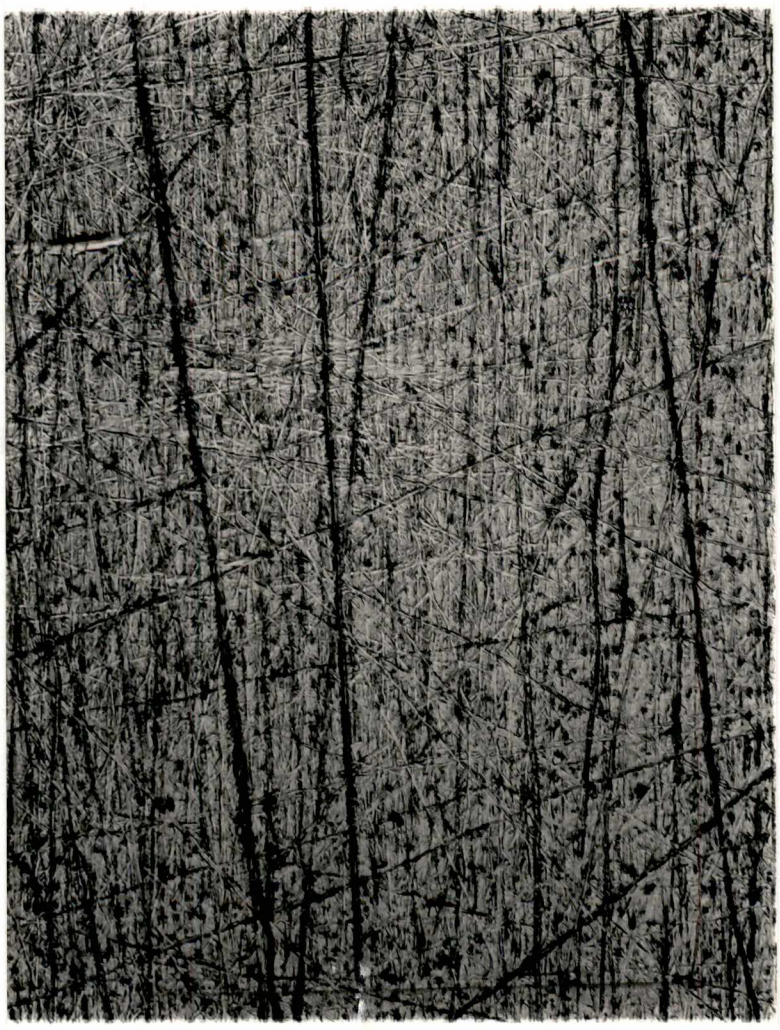


Fig. 1. Photomicrograph of the surface of a 600 grit polished 316 SS coupon, non etched. Magnification at 200 X.

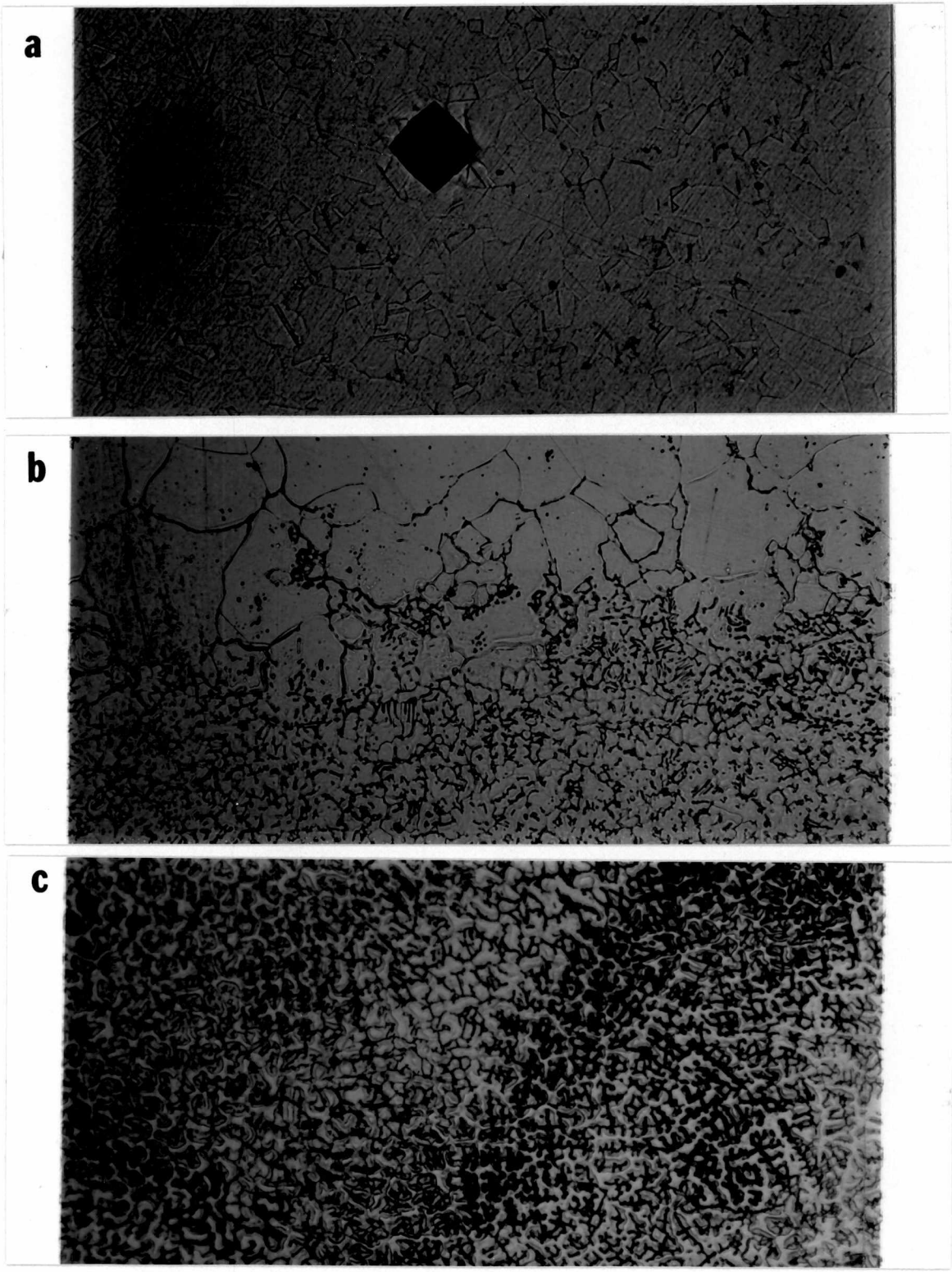


Fig. 2. Photomicrograph of a 316 SS coupon, etched. Sections of base metal (a), HAZ (b), and weld (c) areas shown at 200 X magnification.

shear and or mechanical scouring. In addition, the increased surface area associated with heterogeneous surfaces provides additional areas for colonization.

Biofilm Community Structure of Etched and Non-Etched Base Metal and Welded-HAZ SS Substrata. Differential viable counts on the various surfaces extracted are given in Table 2. As was the case in the colonization studies described in chapter IV, viable B. subtilis were not recovered and recovery of viable D. gigas was poor. Significant differences in community structure on etched and non-etched coupons were not observed ($P<0.05$). The presence of weldaments in either the etched or non-etched coupons likewise had no significant effect ($P<0.05$) on the number or distribution of viable bacteria. Aside from McCoy's (1981) observations on the effects of surface roughness on the morphology of biofilm organisms, there are no data which support the hypothesis that particular bacteria are preferentially--and irreversibly--sorbed to substratum inhomogeneities.

Effects of Metallurgical Inhomogeneities on Bacterial Biofilm Biomass and Metabolic Activity. No significant ($P<0.05$) effects on biomass parameters were attributed to etching and/or the presence of weldaments. Differences in AODC, protein, and metabolic activity values for the 4

Table 2. Biofilm community structure on 316 SS as a function of substratum structure.[a]

Community	Surface Treatment[b]	Cells cm^{-1} 5RL	*Hafnia alvei*	*D. gigas*[d]
1	NEB	1.4×10^8 (4.5×10^7)[c]	1.6×10^5 (1.5×10^5)	NR[e]
	NEW	1.0×10^8 (4.5×10^7)	1.1×10^5 (3.6×10^4)	NR
	EB	7.9×10^7 (4.0×10^7)	4.6×10^4 (2.8×10^4)	NR
	EW	8.1×10^7 (4.4×10^7)	5.8×10^4 (1.0×10^4)	NR
2	NEB	1.4×10^8 (5.4×10^7)	1.4×10^5 (2.5×10^4)	1.2×10^3
	NEW	9.2×10^7 (1.6×10^7)	8.2×10^4 (2.5×10^4)	1.7×10^3
	EB	5.8×10^7 (1.9×10^7)	8.6×10^4 (3.2×10^4)	NR
	EW	4.3×10^7 (2.1×10^7)	5.4×10^4 (1.9×10^4)	1.0×10^3
3	NEB	9.8×10^7 (3.0×10^7)	8.2×10^4 (1.8×10^4)	NR
	NEW	1.1×10^8 (2.8×10^7)	8.4×10^4 (1.7×10^4)	NR
	EB	6.0×10^7 (2.0×10^7)	3.2×10^4 (4.0×10^3)	NR
	EW	3.9×10^7 (3.0×10^7)	1.0×10^4 (3.6×10^3)	NR
4	NEB	8.0×10^7 (8.0×10^7)	7.2×10^4 (1.0×10^4)	8.7×10^3
	NEW	9.7×10^7 (6.0×10^7)	8.7×10^4 (1.0×10^4)	1.0×10^4
	EB	6.4×10^7 (1.5×10^7)	6.0×10^4 (1.3×10^4)	1.0×10^4
	EW	9.5×10^7 (1.9×10^7)	9.1×10^4 (2.3×10^4)	4.9×10^3

[a]Viable *B. subtilis* were not recovered from coupon surfaces.
[b]NEB=non-etched, base metal; NEW=non-etched, weld-HAZ; EB=etched, base metal; EW=etched, weld-HAZ.
[c]Standard deviation.
[d]Estimates of viable counts made from 16s rRNA analysis.
[e]NR=not recovered.

communities were similar to those observed in the experiments described in chapter IV (Figs. 3a-c). The greater variability seen in the AODC, protein, and uptake analyses, relative to those described in chapter IV, is primarily a function of the smaller sample sizes tested in these experiments.

Differences in the carbohydrate:protein ratios (Fig. 4) of biofilms on the various surfaces analyzed were not significant ($P<0.05$). The potential importance of this finding lies in the role that bacterial exopolymers, in particular, acidic polymers such as glucuronic acid, may play in corrosion activities. Geesey and Bremer (1990) have described Cu corrosion activities mediated by bacterial exopolymers. This metal-binding is dependent both upon the concentration of polymer as well as the number and binding strength of the ligand(s).

Although the 5-day "climax" communities were homogeneously distributed across the coupon surfaces--irrespective of surface topology or the presence of metallurgical inhomogeneities--initial differences in colonization were observed across the polished/etched, welded surfaces. After approximately 20 h exposure to the 5RL, visible biofilms began to appear first at the polished/etched, welded surfaces. Within approximately 10 h following this initial observation, the surfaces became uniformly covered with a biofilm. As was previously

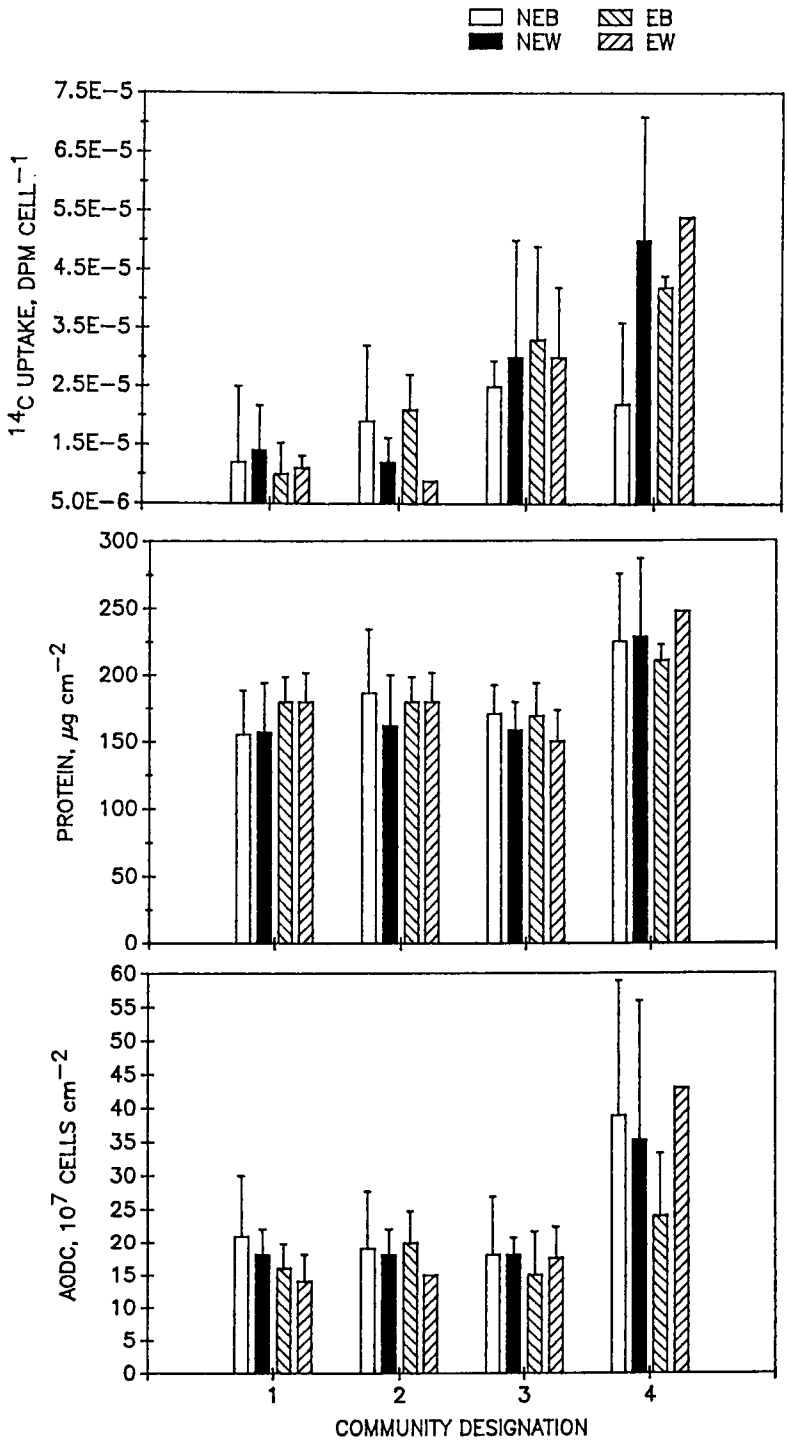


Fig. 3. AODC, protein, and metabolic activity results for the 4 communities. NEB= non-etched, base metal; NEW= non-tched, weld; EB= etched base metal; EW= etched weld. Error bars are ±1 SD.

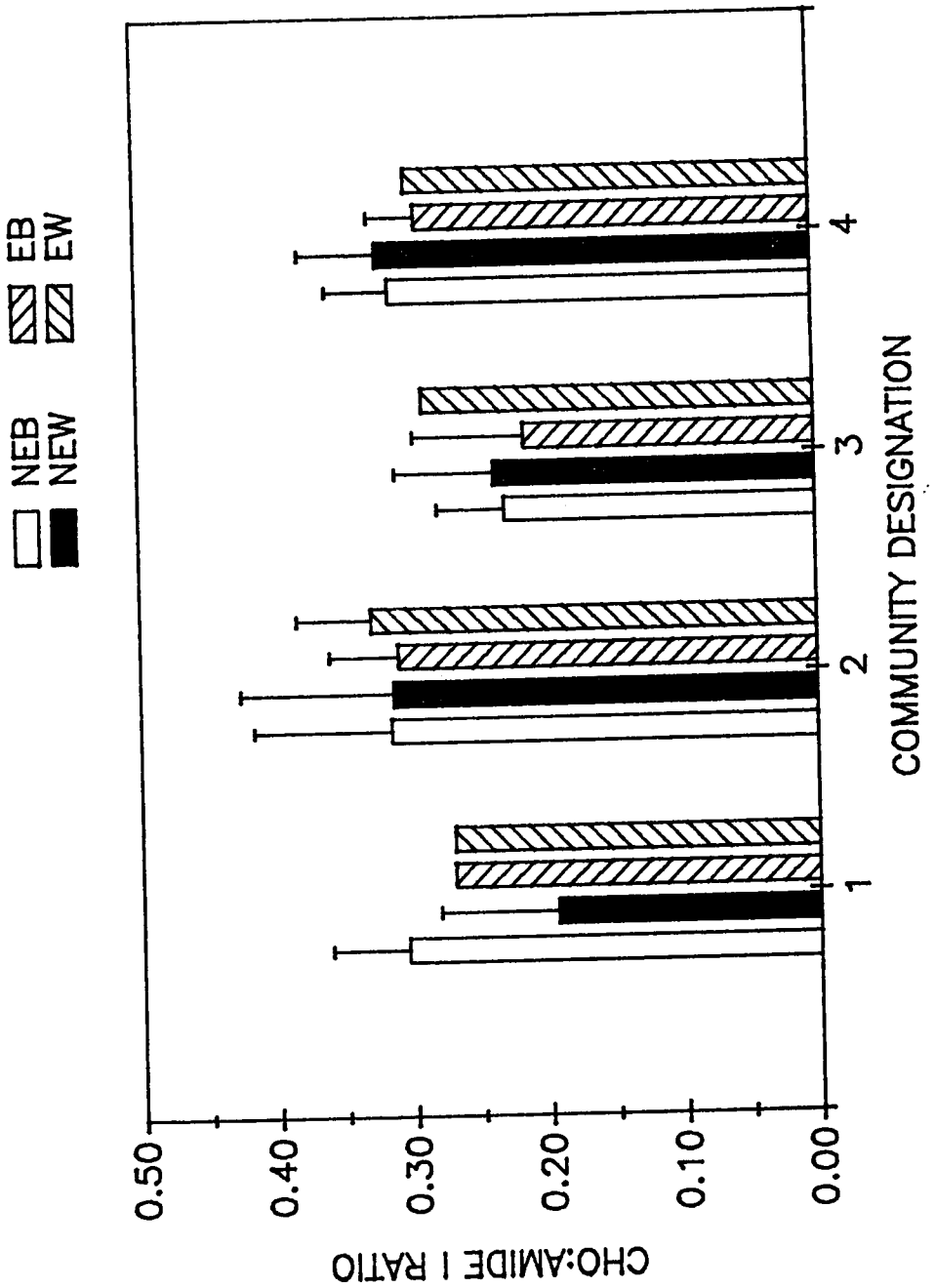


Fig. 4. Carbohydrate:protein ratios for the 4 communities. NEB= non-etched, base metal; NEW= non-etched, weld; EB= etched base metal; EW= etched weld. Error bars are ±1 SD.

described in chapter IV, in all cases development of visible biofilms was preceded by the trapping of extremely small gas bubbles (on the order of 0.5 mm) on exposed surfaces. These observations suggest that differential adhesion may occur on previously uncolonized surfaces as a function of time.

Future studies of metallurgical inhomogeneities effects on colonization should include surface energy analyses. Perhaps differences in surface energy at the exposed weld grain boundaries may account for the observations of initial differential adhesion. Another possibility could be differences in available iron at these exposed, ferritic crystalline sites. Indeed, the notion that iron availability may play an important role in bacterial adhesion to and MIC of metal surfaces has not been adequately tested.

The results of these 5-day studies are in general agreement with the longer-term colonization studies of Pedersen (1990). In studies of biofilm development in a municipal drinking water system (Goteborg), he found that AODC values on hydrophilic SS and hydrophobic polyvinyl chloride (PVC) surfaces were not significantly different after 167 exposure days. The rougher "matt-type" stainless steel had an average of 1.2 times more total bacteria than did "bright annealed" stainless steel. Patel et al. (1990) also found that while differential adhesion of two strains of oral bacteria to titanium could be demonstrated in static

tests, there was no effect of surface roughness (1 μm vs. "sand-blasted" finish) on the level of adhesion for either of the organisms tested. Interestingly, approximately twice as many cells of the periodontal pathogen, Actinobacillus actinomycetemcomitans, adhered to titanium dental implant surfaces as did Streptococcus sanguis, an organism associated with a normal oral flora. Unfortunately, comparable results for the other biofilm parameters reported herein were not available from these authors or others.

Characklis (1990) has noted, however, that "microroughness" may play a role in the convective transport of cells to surfaces. He cites anecdotal evidence that the rate of colonization is greater on rougher surfaces. A number of workers in short-term (several hours) colonization studies have also noted differences in colonization rates for bacteria and substrata of varying free surface energies (Busscher et al., 1984; Fletcher and Loeb, 1979; van Loosdrecht et al. 1990; et al.). Surface structure and interfacial free energy, however, are unlikely to affect colonization of primary, secondary, etc. biofilms by succeeding bulk-phase bacteria.

The results reported in this chapter suggest that SS surfaces with surface topologies differing in magnitude by a factor of 10 do not significantly influence the quantity or nature of biofilm biomass constituents. In addition, the presence of metallurgical inhomogeneities in the form of

weldaments do not significantly influence biofilm biomass or unit metabolic activity. These results have implications for the interpretation of such surface phenomena as MIC activities. For the particular communities tested and experimental conditions described in this research, it is unlikely that MIC of welded SS surfaces can be attributed to either differential colonization, biofilm carbohydrate: protein ratios, or metabolic activity. Additional studies are required before these findings can be extended to other biofilm communities, bulk-phase conditions, hydraulic regimes, or substratum metallurgical inhomogeneities.

Effects of Metallurgical Inhomogeneities on Electrochemical Parameter Associated with Bacterial Colonization. A plot of OCP vs. time for community 3 is shown in Fig. 5. A comparison of this plot with the corresponding plot in chapter IV, did not reveal any apparent differences. Following an initial increase in the OCP, a period of instability was observed. The magnitude and timing of the observed perturbations for the etched coupons did not appear to be distinguishable from those of the non-etched coupons. OCP was again stable for approximately 20 h prior to injection of the 5RL. Similar results were obtained for the other 3 communities (data not shown).

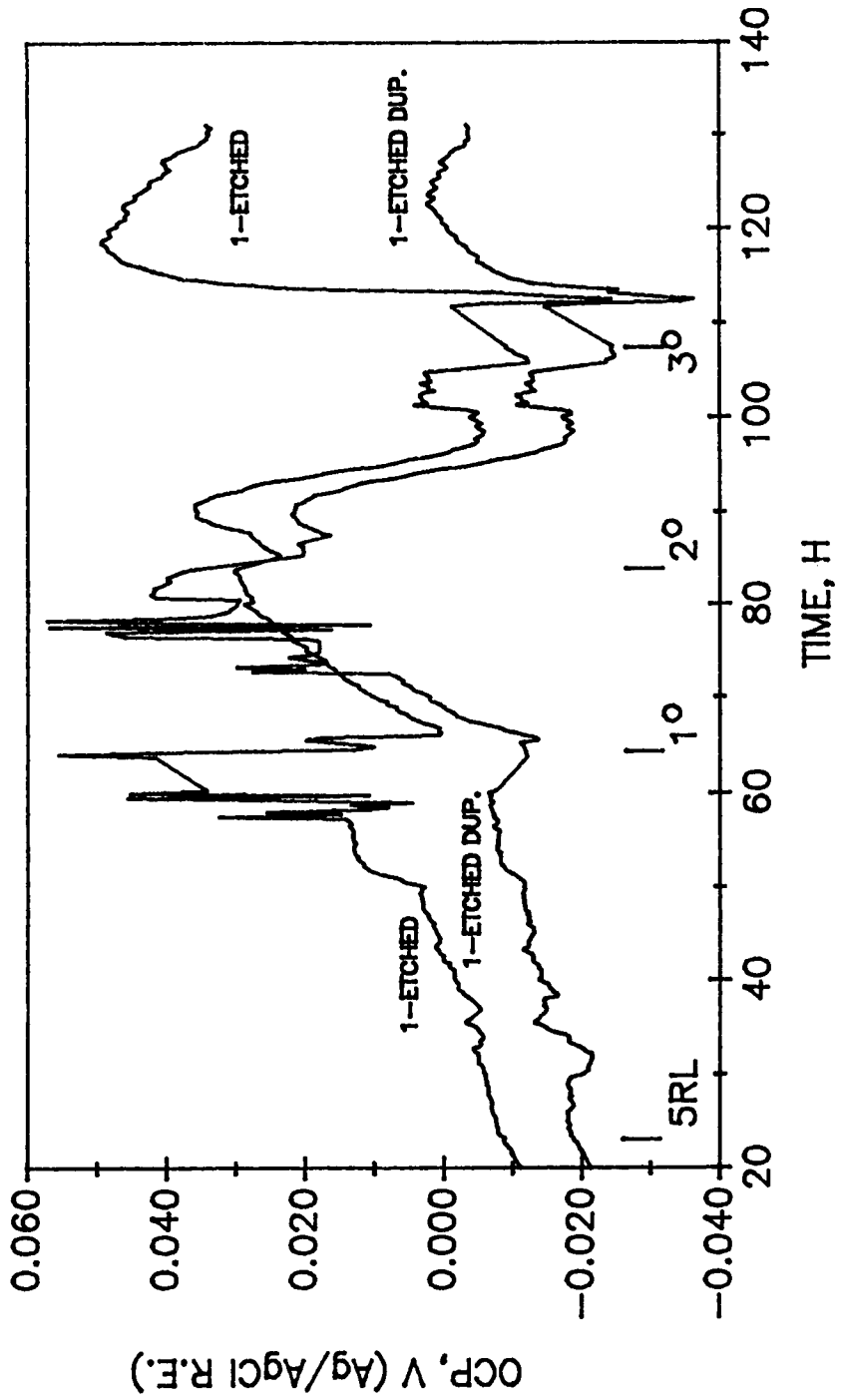


Fig. 5. OCP vs. time for community 3. Addition times for 5RL and the other three organisms are indicated by vertical bars. Measurements were taken every 15 min for 136 h.

ECN plots of etched coupons before (Fig. 6) and after (Fig. 7) colonization with community 1 are not indicative of pitting- or overall-type surface corrosion. A comparison with the ECN plots in chapter IV reveals no significant differences between the etched and non-etched coupons. Despite attempts to accelerate the corrosion of 316 SS by etching, corrosion activities were not observed. In addition to the poor recoveries of the major putative MIC bacterium, D. gigas, the relatively low chloride concentrations present in the bulk phase may have slowed or obviated MIC activities. Chloride concentration cells under biofilms may be an important factor in the initiation and propagation of corrosion activities (Paul Guthrie, TVA, personal communication).

Electron Microscopy of Biofilms Associated with Metallurgical Inhomogeneities. Representative electron photomicrographs of non-etched and etched surfaces are shown in Figs. 8a-f and 9a-f, respectively. The photomicrographs revealed rod-shaped bacteria with multi-laminate cell wall structures typical of Gram-negative bacteria. The majority of the cells contained large numbers of refractile, intracellular inclusions surrounded by a pseudomembrane structure. Previous studies (Mittelman, unpublished results) identified similar structures as poly-beta-hydroxyalkanoate (PHA), which is a polyester compound

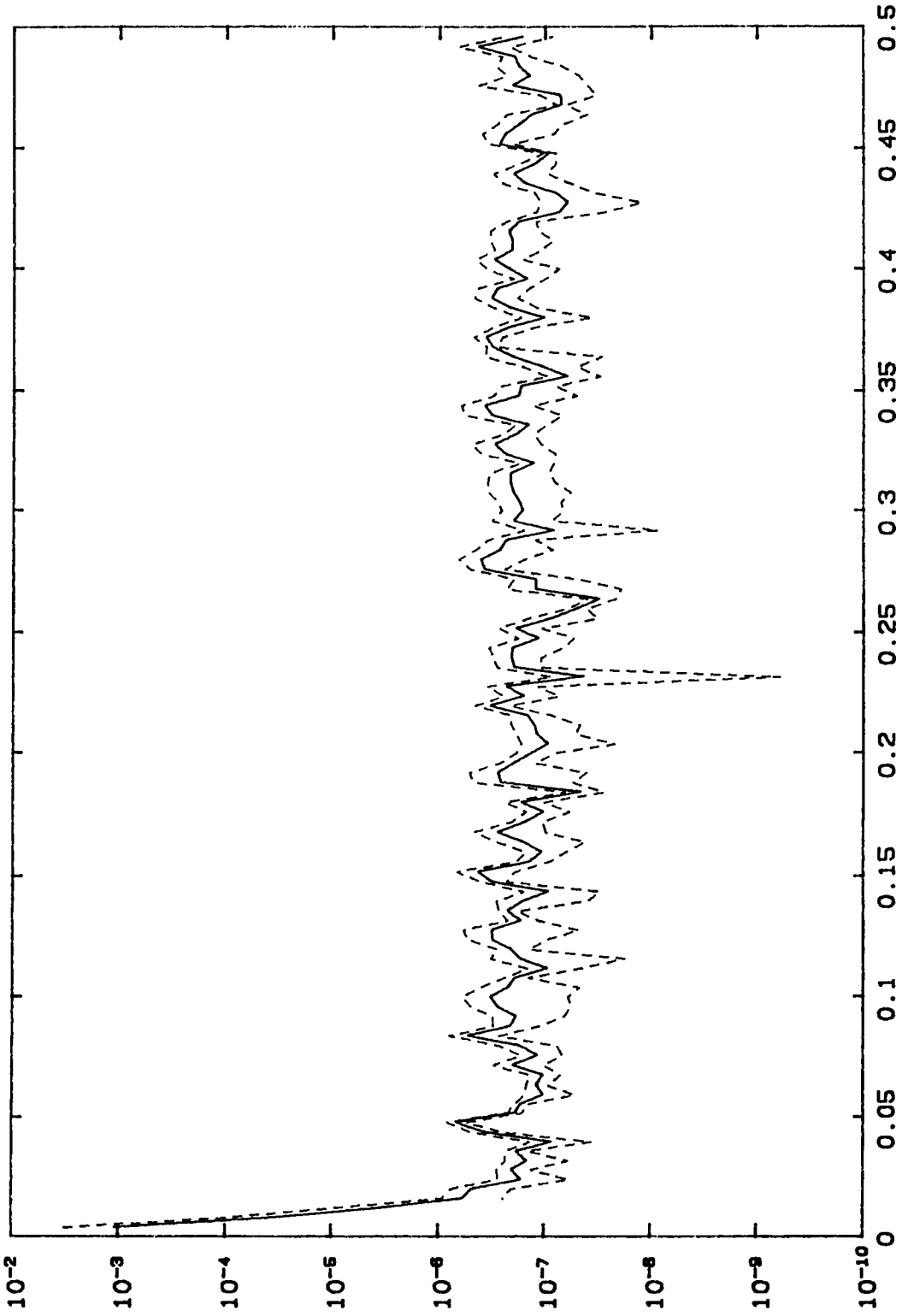


Fig. 6. ECN power spectra for an uncolonized, sterile, etched coupon. Dotted lines represent 95% UCL.

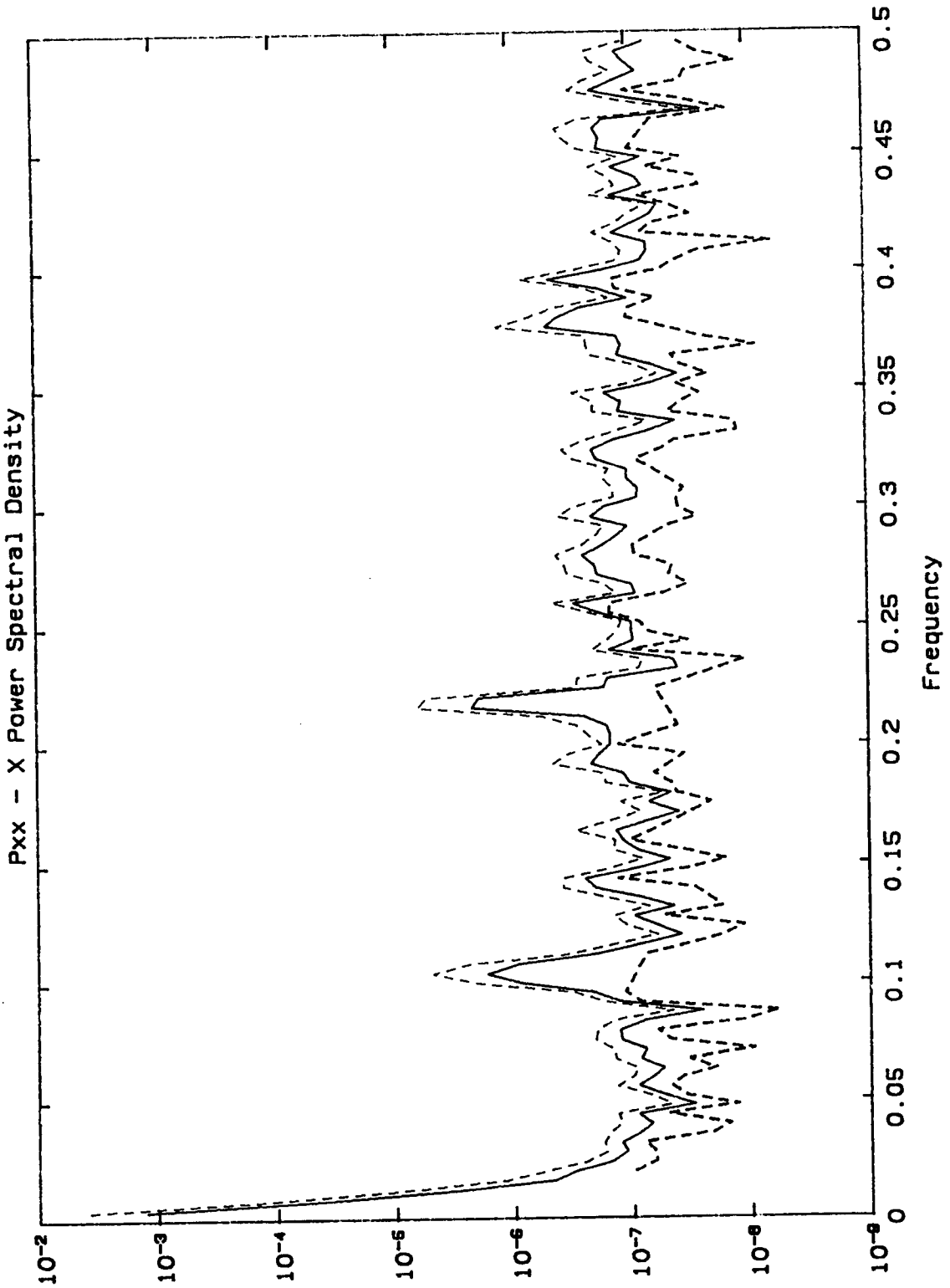


Fig. 7. ECN power spectra for an etched coupon colonized by community 1. Dotted lines represent 95% UCL.

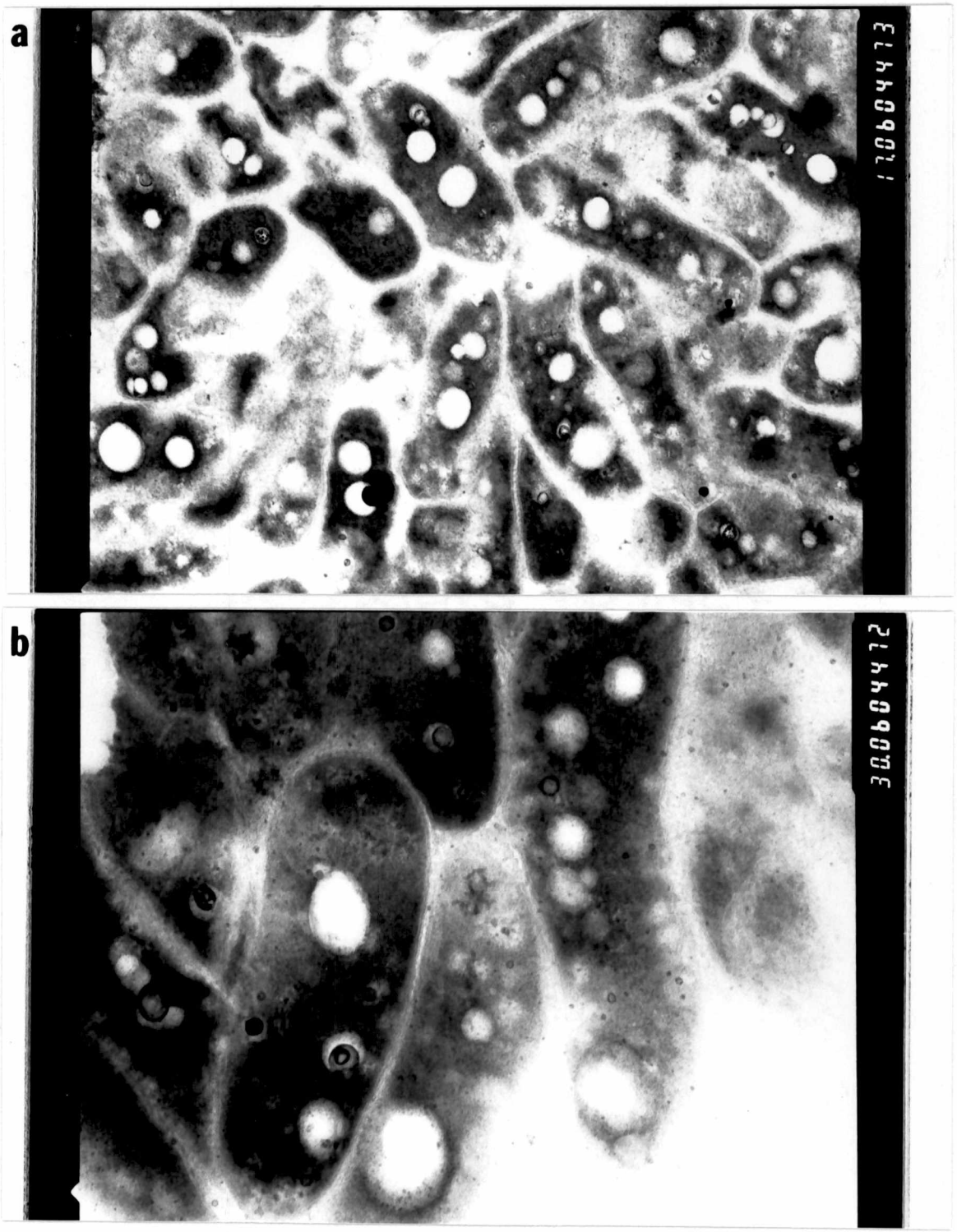


Fig. 8. Electron micrographs of thin sections taken from a 600 grit SS coupon colonized with community 1. Magnification as indicated. Base metal (a) and weld (b) areas from the substratum/biofilm interface are shown.

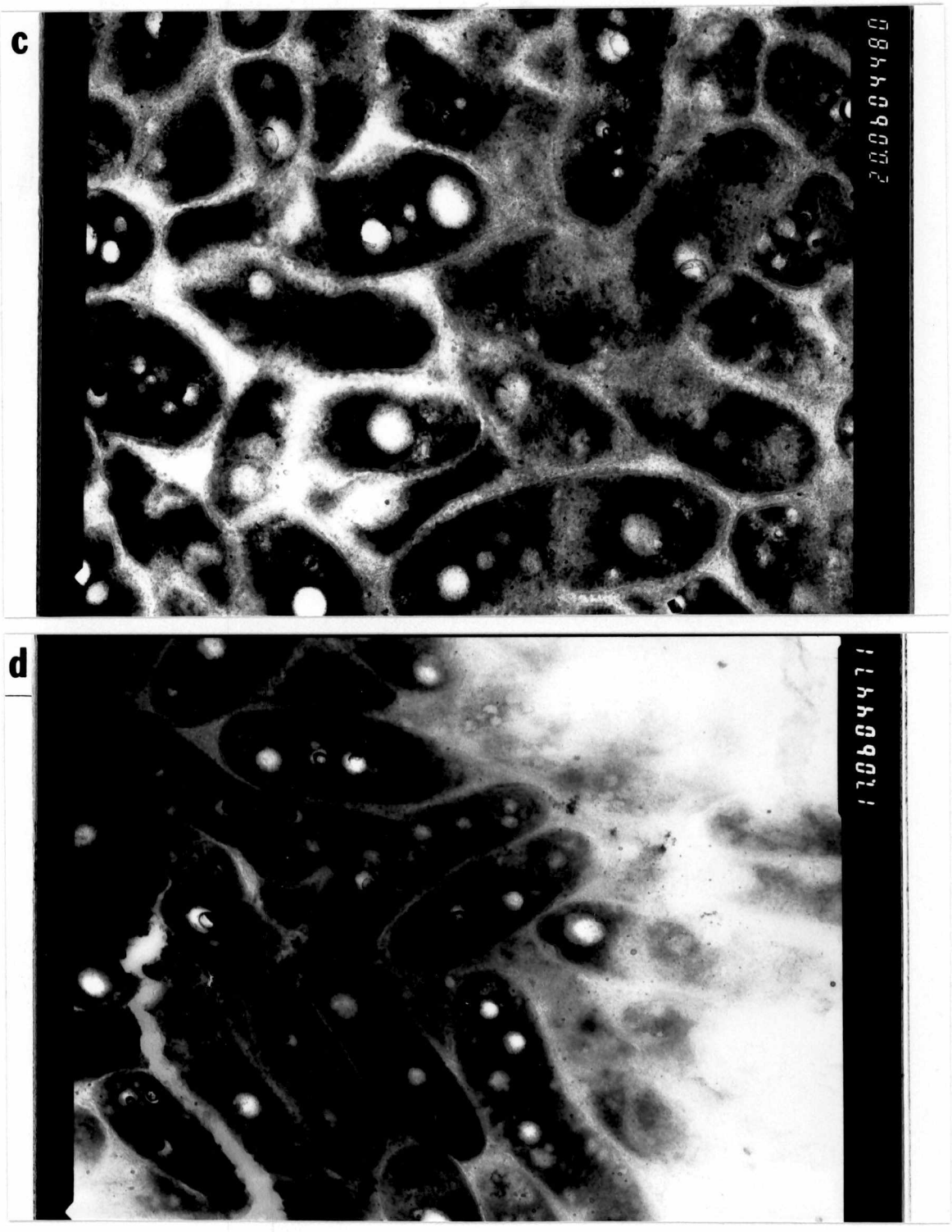


Fig. 8 (continued). Base metal (c) and weld (d) areas sectioned 2 μm from the substratum/biofilm interface are shown.

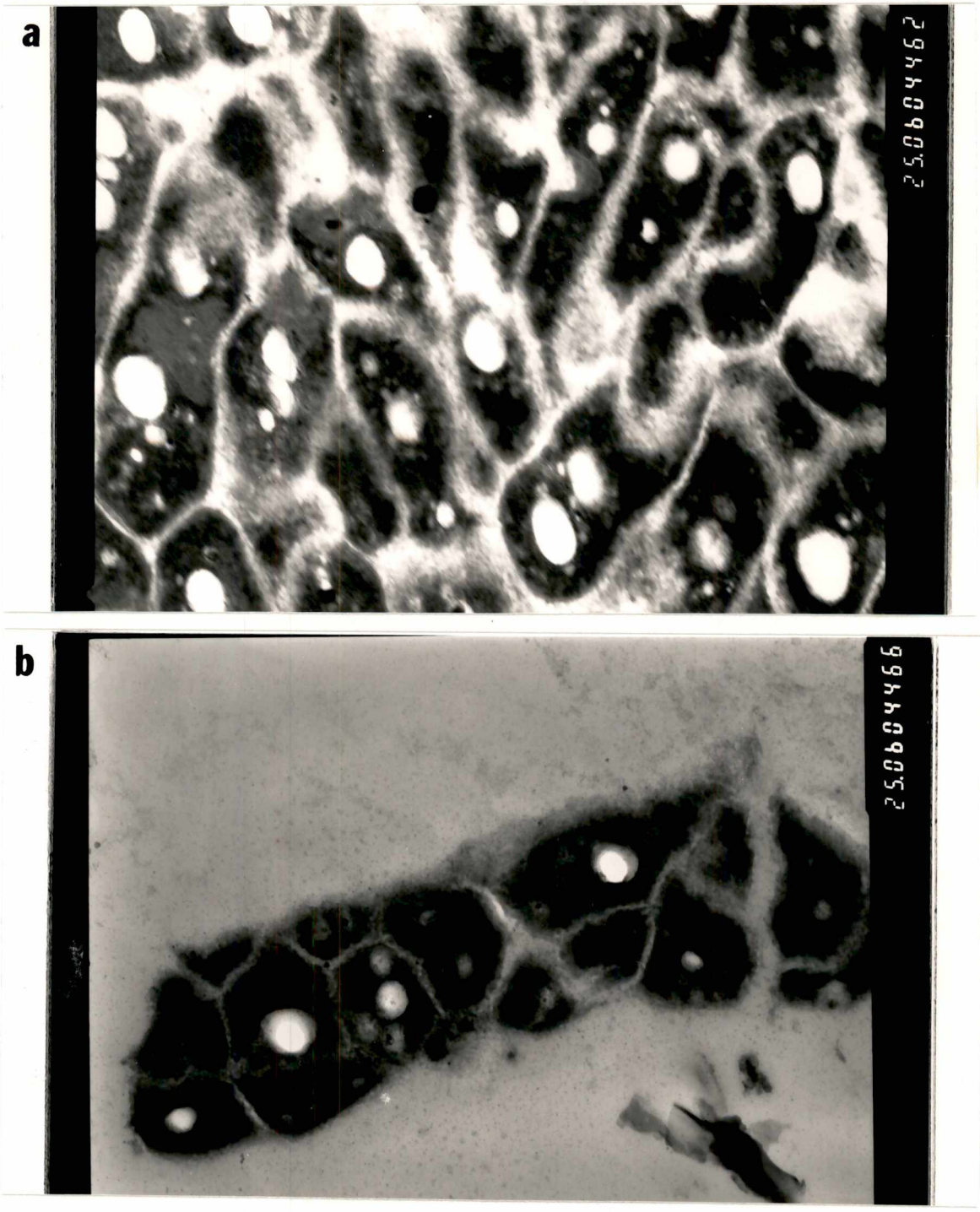


Fig. 9. Electron micrographs of thin sections taken from a 1 μm polished and etched SS coupon colonized with community 1. Magnification as indicated. Base metal (a) and weld (b) areas from the substratum/biofilm interface are shown.

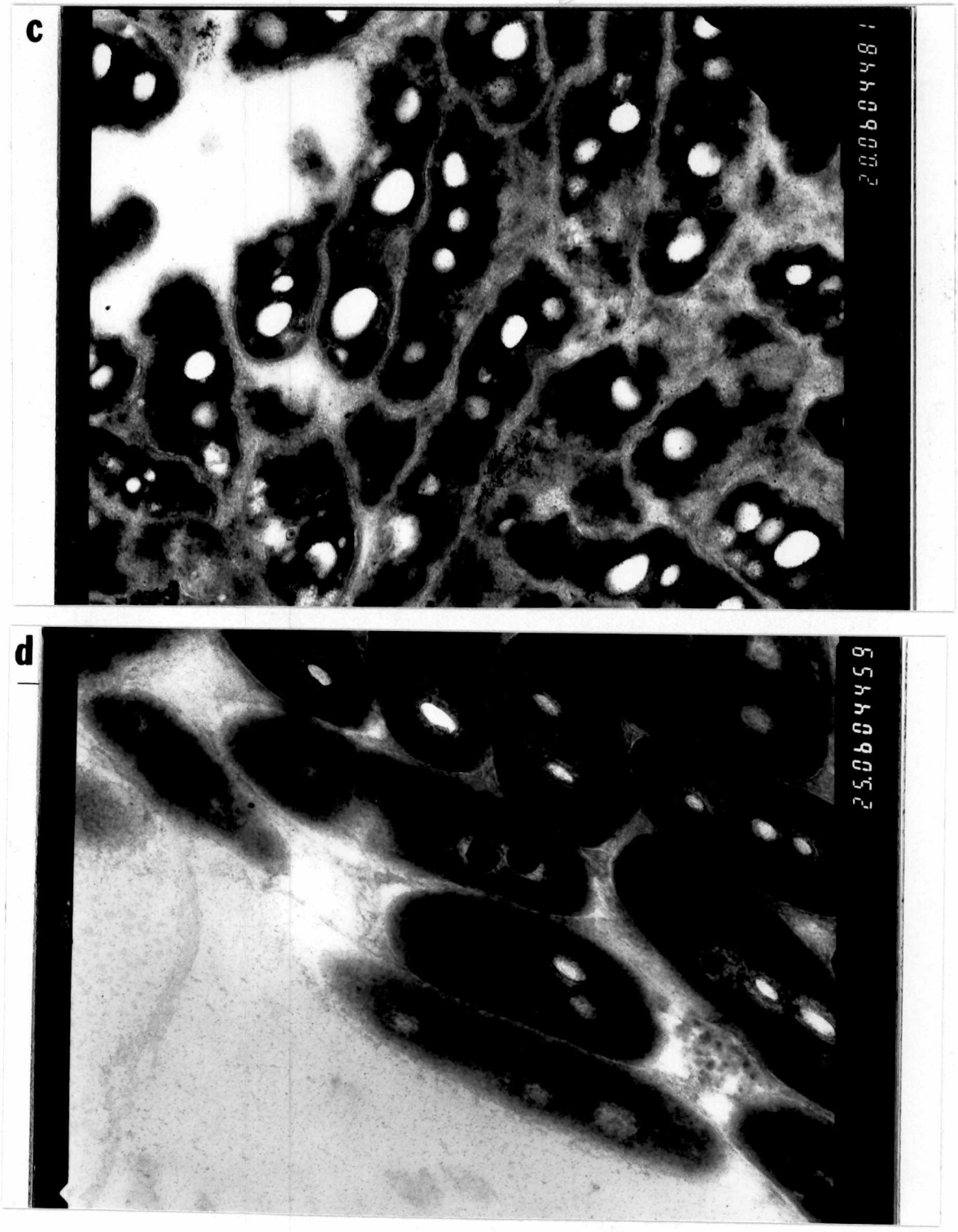


Fig. 9 (continued). Base metal (c) and weld (d) areas sectioned 2 μm from the substratum/biofilm interface are shown.

produced under conditions of carbon sufficiency and nitrogen, oxygen, sulfur, potassium, or phosphorous insufficiency (Dawes, 1984). Smaller, electron dense inclusion were also seen in some of the cells. They were presumptively identified as polyphosphate granules.

The appearance of cells within the various sections examined was relatively consistent. Topological, metallurgical, or spatial factors did not appear to influence biofilm structure. Given the large numbers of viable 5RL recovered in biofilm extracts, it is not surprising that the photomicrographs reveal a fairly homogeneous distribution of cell morphologies.

The overall appearance of the sectioned biofilms is remarkably similar to those of biofilms seen in microbial mats (D'Amelio et al., 1989). D'Amelio et al. (1989) have also presumptively identified refractile intracellular inclusions observed in mat communities as PHB granules. If, as Dawes and Senior (1973) have suggested, the presence of PHA/PHB granules is indicative of unbalanced growth or nutrient stress, cells within these biofilms appeared to be lacking one or more essential growth factors. Anwar et al. (1990) have implicated this altered metabolic state as a factor in the antibiotic resistance of bacteria within biofilms associated with various medical devices.

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CHAPTER VI

ON-LINE DETECTION OF BACTERIAL ADHESION IN A SHEAR GRADIENT WITH BIOLUMINESCENCE BY A *Pseudomonas fluorescens* (*lux*) STRAIN.

INTRODUCTION

Conditions affecting bacterial adhesion with the formation of biofilms are important considerations in many industrial applications. Microbial biofouling leads to increased corrosion rates (Costerton and Boivan, 1988), impedance of heat transfer (McCoy and Costerton, 1982), and detrimental changes in fluid hydraulics (Characklis, 1973). Microbial biofilms are the primary reservoir for planktonic bacteria which contaminate pharmaceutical products (Mittelman et al., 1987), medical devices (Passerini et al., 1990), and microelectronics (Craven et al., 1986).

Recently employed method for detecting *in situ* biofilm activity include confocal laser microscopy (Lawrence et al., 1990), electrochemical impedance spectroscopy (EIS) (Dowling et al., 1988), and attenuated total reflectance Fourier transform spectroscopy (ATR-FTIR) (Jolley et al., 1989). These evolving technologies are complex, however, and involve relatively expensive instrumentation. In addition, they fail to distinguish between viable and non-viable biofilm bacteria and thus are limited in their applicability to studies of metabolic activity.

Bacterial bioluminescence as an indicator of cell number and viability has been applied to several test systems (Stewart, 1990). Jassim et al. (1990) have described an in vivo bioluminescence technique for evaluating biocide effects on planktonic bacterial populations. King et al. (1990) utilized a bioluminescent reporter plasmid to evaluate aromatic hydrocarbon utilization in contaminated soils. Vashitz et al. (1990) optimized continuous culture conditions in a bioreactor using bioluminescence as a biosensor for activity. Ludwicka et al. (1985) described a bioluminescent assay for measuring adhesion of staphylococci to polystyrene. The method involves extracting the surfaces for ATP, which is then measured via the luciferin-luciferase luminescence reaction. A U.S. patent (Ulitzer and Kuhn, 1989) has been issued for the application of bioluminescence to microbial detection and identification.

The goal in the present investigation was the development of a real-time system for measuring bacterial adhesion under conditions which simulate those of in situ environments. A number of cell adhesion experiments were performed which were designed to evaluate the potential utility of bioluminescent bacteria in adhesion studies. The system described herein utilized an engineered light emitting strain to provide not only detection of adhesion, but also defined metabolic activity in the biofilm.

MATERIALS AND METHODS

Bioluminescent Strain Construction. The details of the bioluminescent construct, P. fluorescens 5RL, have been previously described by King et al. (1990). A bioluminescent reporter plasmid for naphthalene catabolism designated PUTK21 was constructed by transposon mutagenesis of a P. fluorescens isolate using the lux transposon, Tn4431 (Shaw and Kado, 1987). Southern hybridization studies demonstrated that the lux transposon was inserted adjacent to the nahH gene in a 1.6 kb Pst I fragment and within the nahG (salicylate hydroxylase) gene (King et al., 1990). Luciferase-mediated light production was induced upon exposure to sodium salicylate. Salicylate was not, however, utilized by this construct as a carbon source.

Media Composition and Adhesive Strain Selection. Preliminary studies utilized collar formation in shake flasks incubated 48 h at 25 C as a criterion for adhesive selection. A yeast extract-polypeptone-glucose medium (YEPG), containing (in g L^{-1}) yeast extract, 0.20; polypeptone, 2.0; NH4NO3, 0.20 at Ph 7.1 produced a significant collar from which an adherent strain was selected. Tetracycline HCl was added at 14 mg L^{-1} to maintain selective pressure on the organisms. The light induction medium consisted of 0.2 g L^{-1} sodium succinate in a minimal medium composed of (in g L^{-1}) sodium

succinate, 0.10; D-glucose, 0.10; yeast extract (Difco, Detroit, MI), 0.10; NH_4NO_3, 0.023; $MgSO_4 \cdot 7H_2O$, 0.10; KH_2PO_4, 0.68; K_2HPO_4, 1.73; Wolfe's complex vitamins/minerals, 0.1 ml. The complete medium had a pH of 7.1-7.2. Viable plate counts were performed on YEPG agar with 14 mg L^{-1} tetracycline. All reagents employed in this study were obtained from Sigma Chemical (St. Louis, MO) or Difco Laboratories (Detroit, MI) and were of analytical reagent grade, unless otherwise noted.

Continuous culture-adhesion cell system. A series of 2 L reaction vessels containing 1 L working volume were connected in parallel to cell adhesion measurement modules (CAMM) fitted with 100 mm diameter (5.7 mm thick) borosilicate glass coupons. The CAMM system provided a means by which fluid shear across the coupons could be continuously varied in direct proportion to flow rate. The system as described in chapter II was modified by inclusion of a glass coupon to permit in situ light detection (Fig. 1). Glass coupons were washed in a detergent solution (Alconox, New York) followed by a chloroform-acetone-methanol-deionized water solvent series prior to use. With the exception of heat labile pump heads and flow meters which were ethylene oxide sterilized, all system components were steam sterilized prior to media introduction and inoculation.

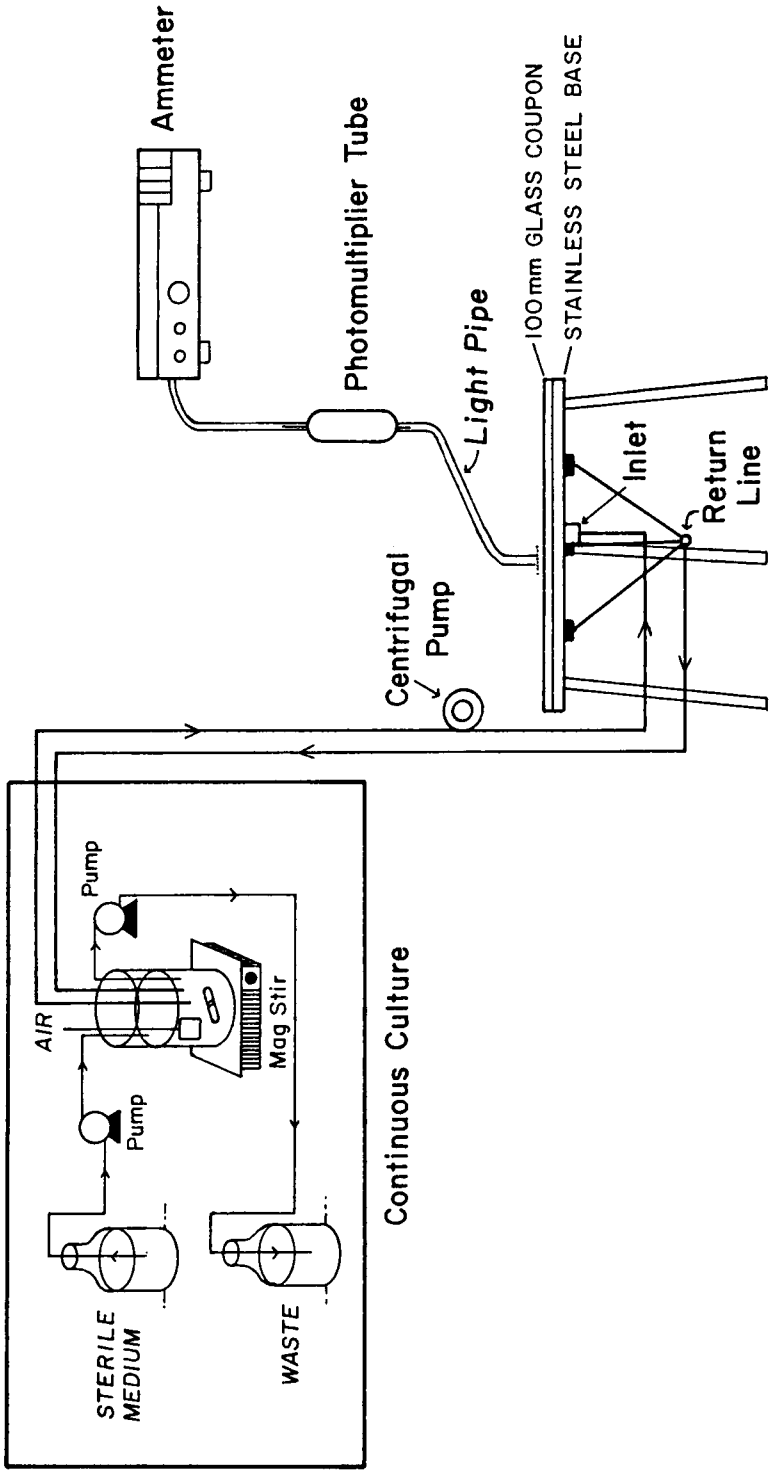


Fig. 1. Schematic of <u>in situ</u> CAMM biofilm monitoring system in which the shear force is inversely proportional to the radius of the interaction. The bioluminescence is measured through a glass plate on the CAMM upper surface.

Following inoculation of the reaction vessels with 1 mL of an 18 h broth culture, the suspension was allowed to recirculate through the CAMM without dilution for 4 h at volumetric flow rates and fluid shear stresses ranging from 0.25 to 1.3 L min^{-1} and 1.0 to 40 dynes cm^{-2}, respectively. At the end of the 4 h period, the YEPG dilution rate was maintained at 0.2 h^{-1}. Aeration of the vessels was maintained at ca. 0.25 L min^{-1}. Following recirculation periods ranging from 18-87 h, the CAMM were flushed at the in situ flow rates with 1.5 l of the salicylate induction medium. Cell-free salicylate medium was then recirculated through the CAMM for 3 h, with replacement of the medium at 1 h intervals. All experiments were conducted at 25 C in a laminar-flow hood.

Light measurement. Bioluminescence was measured in situ through the glass coupons as well as via grab samples of the bulk phase. A flexible liquid light cable and collimating beam probe (0.25 cm^2 collection area) were utilized for light acquisition. Detection of photoelectric-induced current was made with an Oriel (Stratford, CT) photomultiplier-digital readout system (model 77340;7070). The light detection system was calibrated electronically in a light-proof container prior to and following each sampling. In situ coupon measurements were obtained by inserting the beam probe through a light-proof fabric placed

at the top of each CAMM (Fig. 1). Light measurements were taken in triplicate at several radii on each coupon by manually directing the probe across the coupon surfaces.

Immediately following the coupon measurements, 2 ml samples were taken from the bulk liquid phase and placed in 1 cm pathlength polystyrene cuvettes; light measurements were made within 30 s of sampling. The probe was placed directly in contact with the cuvettes which were contained within light-proof cuvette holder assemblies.

Enumeration of Bacteria. Viable plate counts of bulk phase organisms were performed on YEPG agar. Plates were incubated at 25 C for 72 h prior to counting.

Extracts from glass coupons were obtained via sonication in a 10 mM, pH 7.2 disodium pyrophosphate buffer solution. The CAMM stainless steel surfaces underlying the glass coupons were quantitatively scraped with cell-free Teflon scrapers and sterile cotton-tipped swabs saturated with 10 mM, pH 7.2 disodium pyrophosphate buffer solution. The swabs were then placed in small volumes of pyrophosphate buffer containing 2% (v/v) glutaraldehyde as a preservative, and sonicated for several seconds to disaggregate cells from the swab surfaces.

The coupon and CAMM extracts were processed for AODC by filtration through 0.2 μm polycarbonate filters (Nuclepore Corp., Pleasanton, CA), staining for 5 min in 0.1 mg mL^{-1}

acridine orange (AO) in 100 Mm phosphate buffer, pH 7.2, then examination under epifluorescent illumination. Direct epifluorescent microscopic examination of AO stained coupons following extraction revealed that greater than 95% of the adherent cells were removed during the sonication procedure.

Metabolic activity. A 200 ml volume of the cell-free succinate medium containing 0.25 μCi ml^{-1} of D-[^{14}C(U)]glucose (New England Nuclear, Wilmington, DE) with a specific activity of 340 mCi $mmol^{-1}$ was recirculated through the CAMM at the in situ flow rates. The cells in the CAMM were induced prior to labeling as described above. Following a 60 min labeling period, the coupons were immediately removed from the CAMM, drained of residual bulk phase liquid, placed in a sealed 150 mm petri dish containing 1 ml 25% (v/v) glutaraldehyde, then refrigerated overnight.

The pulse-labeled coupons and CAMM underlying stainless steel surfaces were processed as described above for cell counts. A Bligh-Dyer lipid extraction protocol, which employed chloroform-methanol-water extraction reagents (Bligh and Dyer, 1959), was utilized for radiolabeled lipid extraction. Lipid extracts were counted in 4.0 ml Ecolume (ICN Biomedicals, Irvine, CA) on a LKB model 1212 (Gaithersburg, MD) liquid scintillation counter. The counts were quench corrected using a series of external standards

(Packard, Downers Grove, IL). Further details of this procedure may be found in chapter II.

RESULTS AND DISCUSSION

Initial light production in the bulk phase was evident within approximately 25 min after salicylate induction of an 18 h continuous culture (Fig. 2). After approximately 2 h following onset of induction, bulk phase light production increased to ca. 28 namps. Replacement of the bulk phase with cell-free media resulted in an immediate drop in light production. Light production in the biofilm, however, was not significantly influenced by the flushing procedure (Fig. 2). Biofilm light production stabilized within 2 h following sodium salicylate induction.

The interference of turbidity due to growth in the bulk phase is eliminated by the flushing process under conditions in which the cells are still not attached. Figure 3 illustrates the relationship between light measured in the 1 cm lightpath cuvette and that measured <u>in situ</u> through the coupon during a 2 h recirculation at 1 L min^{-1}. A linear equation describing the influence of bulk phase light production on the <u>in situ</u> measurements was obtained from a least squares linear regression of the data (r^2= 0.998). The equation, y= 0.142x-5.44 X 10^{-12}, was employed as a

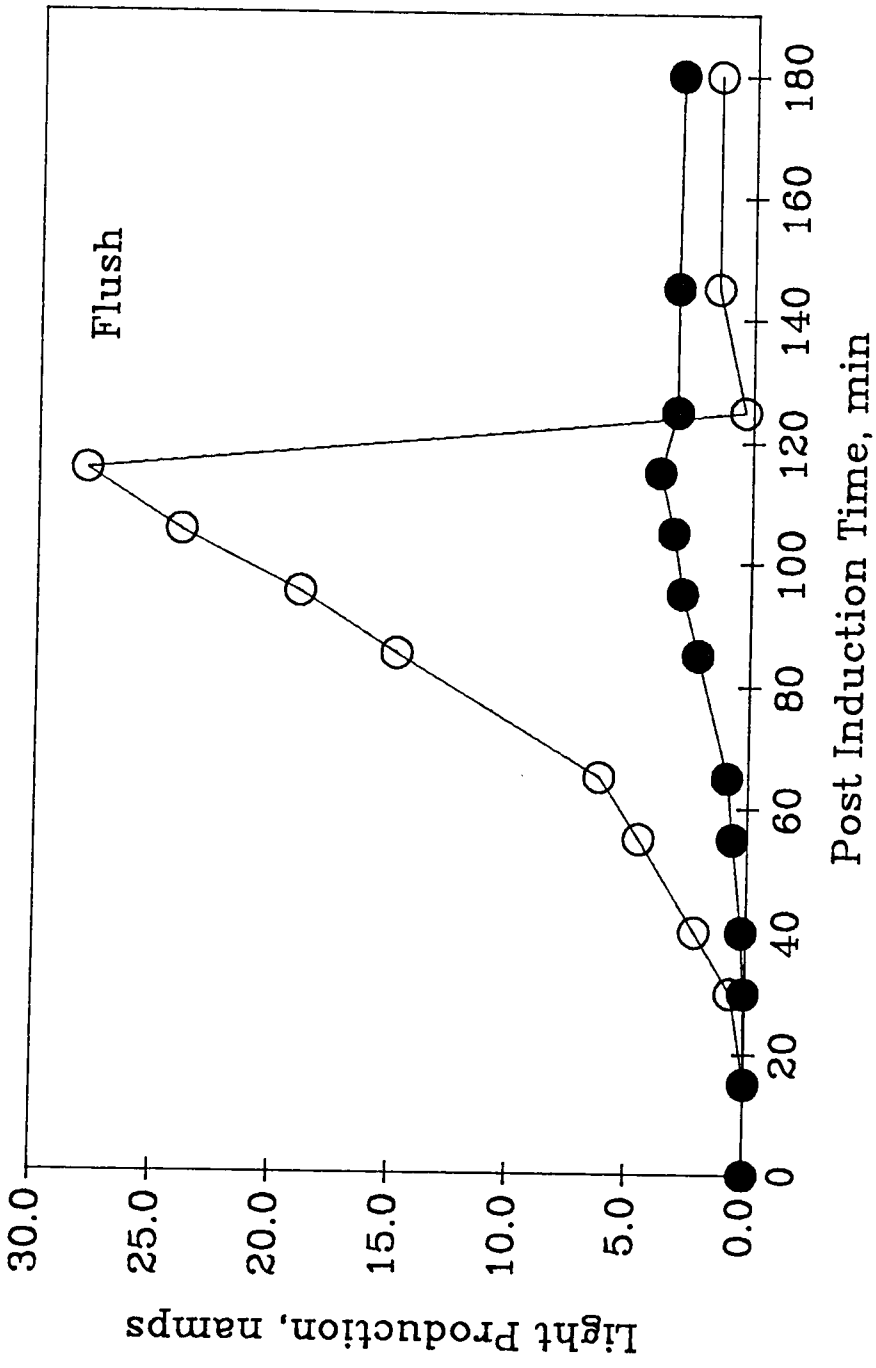


Fig. 2. Effect of flushing procedure on bulk phase and biofilm bioluminescence. Coupons were flushed with cell-free medium containing sodium salicylate for 2 h following induction.

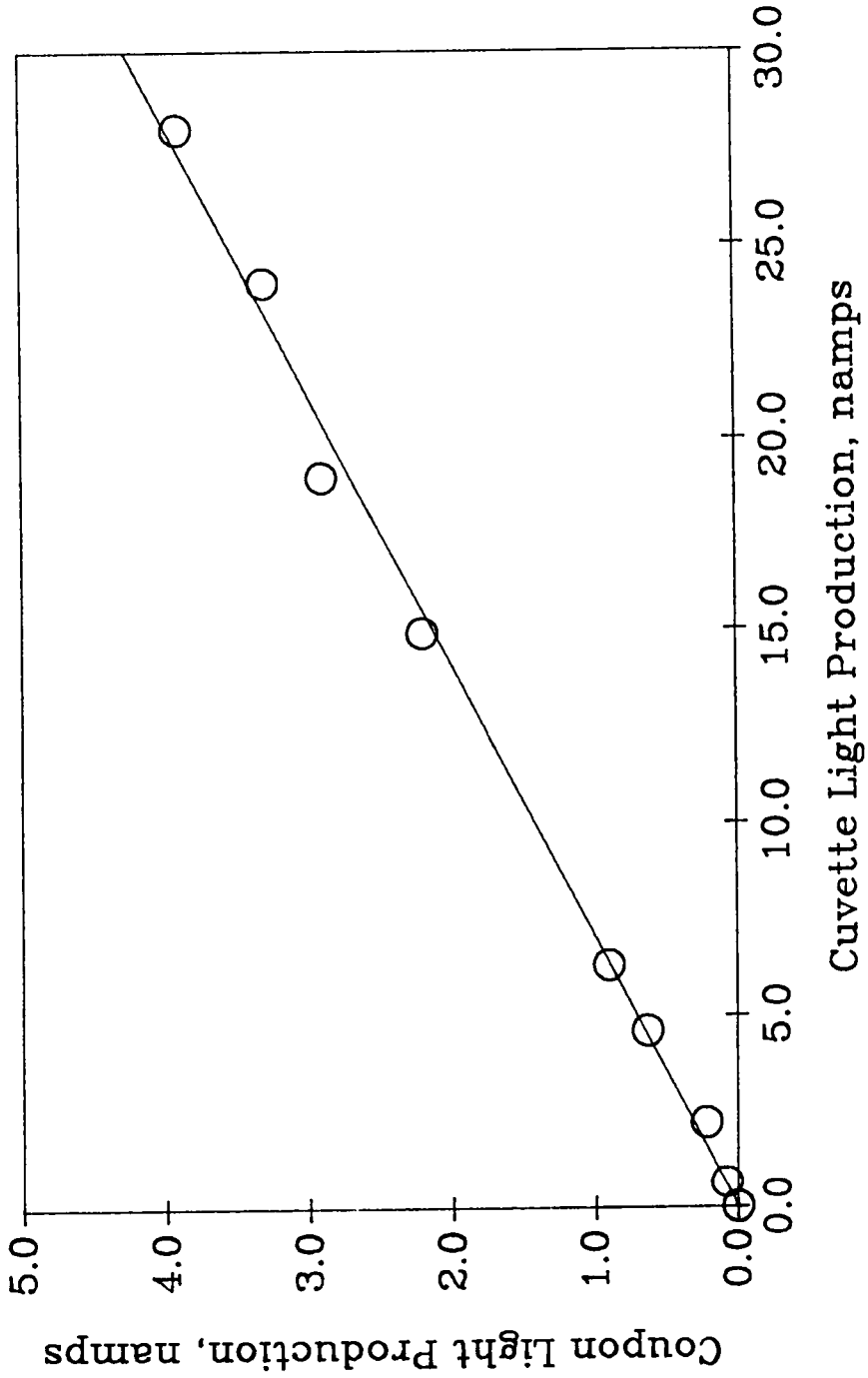


Fig. 3. Relationship between bulk phase and biofilm bioluminescence under flowing conditions.

correction factor to compensate for the bulk phase effect on biofilm light production values (x).

Bioluminescence detected through the glass coupon was a function of attachment to both the CAMM glass and stainless steel substrata. Light output, cell counts, and per cell metabolic activity were are shown in Table I for 72 h biofilms. On a per cell basis, the activities of the colonized cells were equivalent on the two surfaces. Bioluminescence originating in the stainless steel biofilms was distinguished from that produced on glass coupons by replacing the colonized glass with a clean coupon prior to light measurement.

Table 1. Effect of biofilm metabolic activity on light production.

Substratum	uptake cm^{-2}[a]	light production, μamps
glass	3.0×10^{-8} (7.1×10^{-9})[b]	5.2×10^{-4} (1.0×10^{-4})
stainless	4.0×10^{-7} (2.0×10^{-7})	7.6×10^{-4} (1.2×10^{-4})
	cells cm^{-2}[c]	uptake, μmoles $cell^{-1}$
glass	2.6×10^{5} (2.5×10^{5})	1.2×10^{-13} (1.2×10^{-13})
stainless	3.3×10^{6} (1.3×10^{6})	1.2×10^{-13} (3.1×10^{-13})

[a] μmoles glucose per 60 min pulse-labeling
[b] () = standard deviation
[c] acridine orange direct counts

A comparison of light production between bulk phase and glass biofilm organisms revealed that per cell light production was approximately the same, from 1.1-2.4 X 10^{-7} namps $cell^{-1}$ and 2.1-4.0 X 10^{-7} namps $cell^{-1}$. respectively.

Both the glass and stainless steel substrata were colonized to approximately the same extent; colonization decreased with exposure time (Table II) in a shear force gradient.

Table 2. AODC of biofilm organisms extracted from glass and stainless steel substrata.

Exposure Time, h	AODC, Cells cm^{-2}	
	Glass	Stainless Steel
18	7.7 X 10^6 (6.8 X 10^6)[a]	1.5 X 10^7 (4.3 X 10^6)
36	1.8 X 10^6 (1.9 X 10^6)	8.6 X 10^6 (1.2 X 10^6)
60	8.7 X 10^5 (3.2 X 10^5)	7.3 X 10^5 (1.8 X 10^5)
87	3.9 X 10^5 (1.4 X 10^5)	4.1 X 10^5 (4.2 X 10^4)

[a]()= standard deviation.

A total of 36 light and AODC measurements were taken from 18-87 h induced biofilms exposed to 1-40 dynes cm^{-2} fluid shear. Biofilm light production was positively correlated (r^2= 0.826; significant at $P < 0.01$) with biofilm cell number on glass substrata in the range of 1 X 10^5 to 1 X 10^7 cells cm^{-2} (Fig. 4). The cell counts obtained represent extracts from glass coupons exposed to different shear forces and exposure times. The threshold light detection value in this test system was 0.05 namps, which

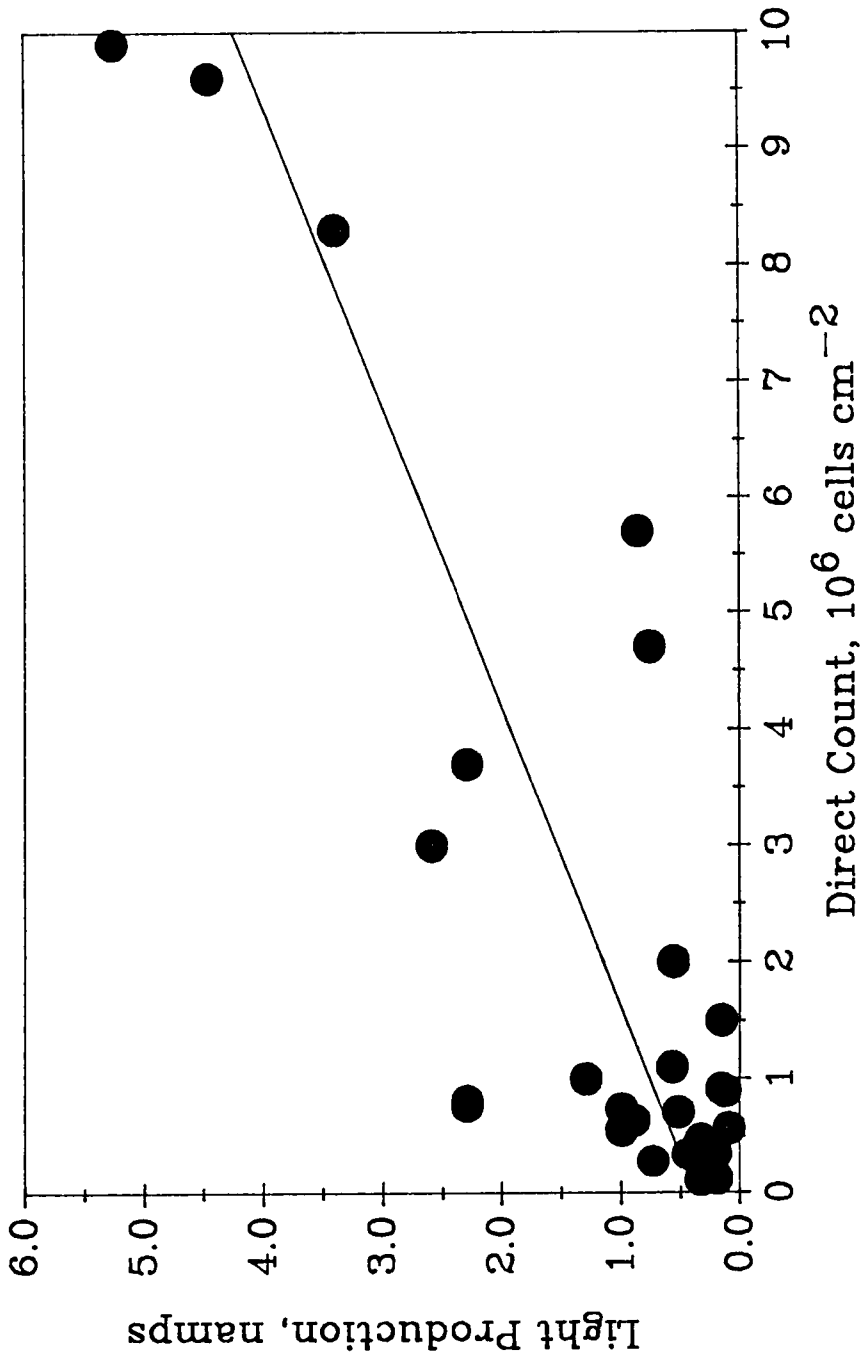


Fig. 4. Relationship between bioluminescence and AODC of induced P. fluorescens (lux) attached to glass under flowing conditions. Average shear stress was calculated at approximately 20 dynes cm^{-2}.

corresponded to 10^5 cells cm^{-2} on the glass coupon with an equivalent contribution from the stainless steel plate.

This threshold value is based upon total cell counts rather than viable counts. Recovery of viable cells from intact biofilms is inefficient and, for fastidious and stressed environmental isolates, may be negatively biased (Colwell et al., 1985). The use of direct counts in establishing calibration curves provides a conservative estimate of viable biofilm bacterial numbers.

The results reported herein compare favorable with those obtained with other, on-line biofilm detection methods. Nivens et al. (1990) showed that 10^4 and 5 X 10^5 cells cm^{-2} could be detected using ATR-FTIR and a piezoelectric quartz crystal microgravimetric technique, respectively. Detection of bioluminescent reporter strains should improve with the application of more sophisticated light measuring systems such as the CCD imaging device described by Hooper et al. (1990).

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APPENDICES

APPENDIX III-1

REFERENCE ELECTRODE PREPARATION

The half-cell used for the reference electrodes was Ag/AgCl/KCL, in which the Nernst potential is given by

$$E = E_o + 0.0591 \log a_{Cl^-} \quad (E_o = -0.224\ V)$$

The electrodes were prepared by successively coating Ag wire with a AgOH slurry, followed by repeated heatings, and an anodic polarization. The silver hydroxide coating was prepared by mixing equal weight portions of $AgNO_3$ and NaOH on the surface of a Whatman #1 filter, then rinsing with copious quantities of deionized water to remove excess NaOH. Silver wire (Johnson Matthey, Ward Hill, MA) was covered with a thin layer of AgOH, then placed in a 600 C oven for approximately 3 min to form a porous silver deposit on the surface of the 0.5 mm diameter wire. This coating and heating procedure was repeated twice. The coated wires were next anodically polarized at approximately 1A cm^{-2} for 20 h in a 1M Hcl solution. Anodic polarization under these conditions leads to the formation of a AgCl deposit on the surface. Using the same chloride concentrations in two half-cells, the Ag/AgCl electrode potentials were measured with a commercial $Hg/HgCl_2$ electrode (Corning, New York). Relative to the $Hg/HgCl_2$ electrode, the Ag/AgCl electrodes yielded an average potential of -0.045V ± -0.005 V.

APPENDIX IV-1

16s rRNA BIOMASS ANALYSIS PROCEDURE

The Gen-Probe (San Diego) 16s rRNA hybridization protection assay (HPA) was utilized to detect D. gigas extracted from test coupons. In addition, an all-eubacterial probe was employed to quantitate coupon biomass from approximately 80 sample extracts. The assay involved the following steps:

1. Pelleted samples were resuspended in 100 μL resuspension reagent containing sodium dodecyl sulfate and a proprietary RNAse stabilization reagent.
2. Following a thorough mixing, the resuspended sample was added to an acridinium ester-linked oligonucleotide DNA probe contained within 5 X 50 mm borosilicate glass tubes.
3. The mixture was incubated in a water bath at 60 C for 1 h.
4. Selection of bound (hybridized) probe was made via a differential hydrolysis with a proprietary reagent at 55 or 60 C for 10 min. The hydrolysis procedure rendered the non-bound acridinium group non-chemiluminescent. Probe bound to the target 16s rRNA sequence is protected from hydrolysis.
5. Direct detection of hybridized probe was made within a luminometer (Gen-Probe) immediately following hydrolysis with H_2O_2 under basic conditions. Chemiluminescence was

measured over a 2 s integration period and was transformed into relative light units (RLU).

6. D. gigas from 72 h sulfate-API broth cultures was titered in a sulfate-API broth MPN series prior to the HPA analysis. The MPN values were utilized to relate RLU values to viable bacterial numbers.

7. AODC values from approximately 80 biofilm extracts were compared with all-eubacterial probe RLU values. The resulting graph is presented in Fig. IV-1-1. The correlation coefficient of 0.759 was found to be significant at $P < 0.01$.

The use of 16s rRNA probes to quantitate biofilm biomass shows promise as a new analytical technique. The method obviates the necessity for culturing biofilm organisms prior to enumeration and enable rapid quantitation and identification within approximately 90 min. The analytical sensitivity as given by the manufacturer is on the order of 5×10^3 cells. The per assay reagent cost is in the range of \$10-\$15.00, which makes this technique economically feasible relative to existing viable- and direct-count methods.

Further studies are required, however, to relate RLU to viable cell numbers. There is some question as to the relationship between metabolic state--as inferred by ribosome copy number--and RLU. According to the manufacturer, copy number varies by a factor of 2-3.

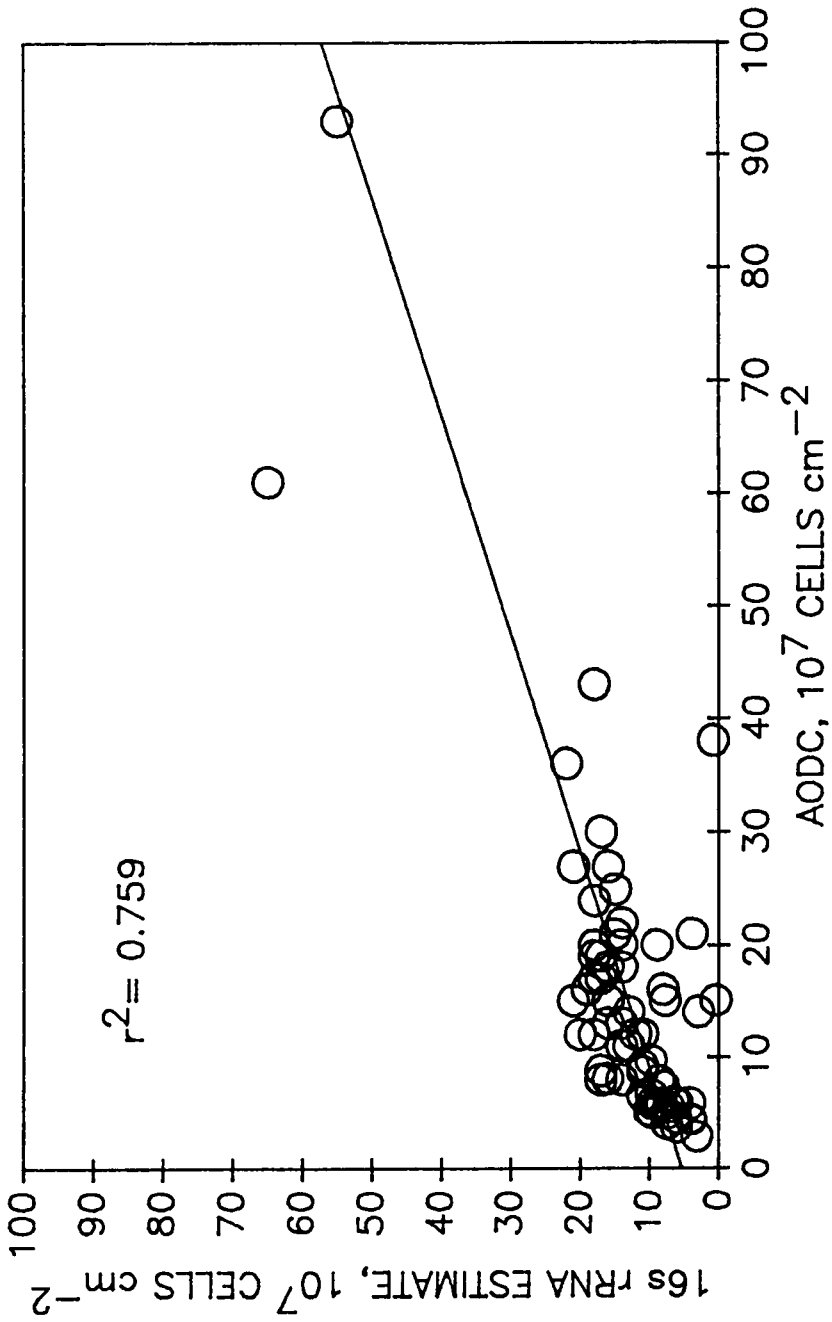


Fig. IV-1-1. Relationship between RLU values obtained from Gen-Probe hybridization protection assay and AODC values. An all-eubacterial probe was employed for the HPA.

APPENDIX V-1

BIOFILM FIXATION AND EMBEDDING FOR TRANSMISSION ELECTRON MICROSCOPY

Biofilm specimens were processed for transmission electron microscopy using a modification of Aldrich's procedure (H. Aldrich, University of Florida, Gainesville). The following steps were employed for biofilms associated with stainless steel sustrata:

1. Coupons were fixed in a 2.5% (v/v in pH 7 PBS) EM-grade glutaraldehyde (Sigma, St. Louis) solution for 15 min at room temperature.
2. The fixed coupons were taken through an ethanol drying series with pH 7 PBS as diluent: 25%, 5 min; 50%, 10 min; 75%, 10 min; 100%, 1 h.
3. The coupons were next immersed in a 30:70 mixture (w/v in 100% ethanol) of LR white medium-grade resin (Ted Pella Co., Redding, CA) for 30 min at room temperature.
4. The coupons were then immersed in a 70:30 mixture of the LR white for 30 min at room temperature.
5. The coupons were immersed in 100% LR white for 10 min at room temperature.
6. The coupons were removed to small glass jars and sparged with nitrogen for 5 min.
7. The jars were immediately sealed and placed in a 65 C oven for 12 h.

8. The hardened resin was separated from the coupons by freezing at -80 C for approximately 5 min, followed by immersion in room temperature deionized water.

9. The resin was then carefully peeled from the coupons using a razor blade.

10. Sections of the hardened resin were then cut with a hack-saw.

11. The sections were then processed for transmission electron microscopy following thin sectioning and staining as described in chapter V.

VITA

Marc William Mittelman was born in Los Angeles, California on January 15, 1957. He graduated from Del Norte High School in Crescent City, California, in May, 1975. He received the Bachelor of Science degree in Microbiology from California State University, Long Beach (CSULB), in 1979. He was employed for three years as a pharmaceutical microbiologist by Riker Laboratories in Northridge, California. He returned to CSULB and completed an M.S. degree in microbiology (thesis title: "Heavy Metal Binding Capacity of the Exopolymer from a Freshwater Sediment Bacterium") in 1983, with Dr. Gill Geesey. Mittelman was the founding partner of an analytical laboratory, Applied Microbiological Services, Inc. in Signal Hill, CA, where he served as president from 1983-1986. From 1986-1988, Mittelman was technical director for Water Micro Associates in San Diego, CA, a consulting and publishing firm. He entered the laboratory of Dr. David White in the summer of 1988.