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Biofilms in the Aquatic Environment

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The Biofilm Ecology of Microbial Biofouling, Biocide Resistance and Corrosion

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Microbial biofilms were generated reproducibly by pulsing inocula from continuous culture into a non-circulating laminar-flow cell system. Antifouling properties of coupons and coatings were tested under controlled conditions by varying bulk fluid properties such as biocide concentration and fluid shear. Biofilm formation and activity were monitored non-destructively using tryptophan fluorescence (TF) as a measure of biomass, and bioluminescence (BL) as a measure of metabolic status. The effectiveness of antifouling (AF) and fouling-release (FR) coatings were examined in marine systems using bioluminescent *Vibrio harveyi*. By normalizing light measurements to an upstream stainless steel surface, a quantitative ranking of AF efficacy against bacterial attachment and biofilm development was generated. Coatings which demonstrated a TF ratio of 0.65 or less were considered to be effective relative to stainless steel controls. Sublethal toxicity was indicated by a significantly greater BL to TF ratio. FR coatings were ranked according to the percent of biomass stripped from the coating surface after a 15 min exposure to a shear stress of 330 dynes cm^{-2}. The antifoulant zosteric acid (ZA), purified initially from seagrass, inhibited *V. harveyi* biofilm development at a minimum bulk phase concentration of 0.05 mg mL^{-1}. Interestingly, ZA demonstrated little effect upon the growth rate of *V. harveyi* in batch cultures, even in the presence of up to 1 mg mL^{-1}. To study the microbial ecology of pathogens in drinking water distribution system biofilms, a triculture biofilm consisting of a genetically engineered bioluminescent *Pseudomonas aeruginosa* , an *Acidovorax* sp., and a *Bacillus* sp. was utilized as a basic system to which green fluorescent protein (GFP)-containing *E. coli*, *Mycobacterium smegmatis*, naturally fluorescent *Legionella bozemanii*, and acid-fast *Cryptosporidium parvum* oocysts were inoculated. The survival and distribution of the pathogens was monitored in bulk fluid containing hypochlorite. In each case, the survival and persistence when incorporated in the biofilm was much greater than in the pelagic cells and evidence of cell injury (fatty-acid analysis) was markedly decreased. Identification and detection of a single *C. parvum* oocyst, and subsequent infectivity of the oocysts, was determined by signature lipid analysis entrained in microbial biofilms. Microbially influenced corrosion (MIC) was also estimated in this system and shown to correlate with specific biofilm activities. Spatial and temporal congruence between localization of biofilm, metabolic activity, pitting corrosion, and anodic electrochemical activity was clearly demonstrated.

1 INTRODUCTION

Microbes in nature are most often found in biofilms rather than free in pelagic fluids. Biofilms are localized concentrations of microorganisms attached to a substratum and consist of a population of a single species, or more often a multi-species community. Within the biofilm, heterogeneous distribution of organisms and metabolic activities are common.

Attachment in biofilms are advantageous for microorganisms. The benefits include availability of nutrients concentrated at surfaces; access to a flowing system which increases the availability of diffusable nutrients; the ability to generate microniches by metabolic activities such as the generation of anaerobic sites in an aerobic environment; the presence of a glycocalyx matrix to restrict predation by phagocytosis or exposure to toxicants; prevention of transfer to a hostile environment such as mouth to gut; access to metabolic byproducts such as primary photosynthates; and enhanced metabolic prowess by interactions within consortia. The disadvantages of biofilm formation are competition for nutrients and terminal electron acceptors, and the availability to a larger biomass of macroscopic predators able to ingest and breakdown the biofilm.

In biotechnological or bioremediation processes it is often the aim to promote biofilm formation, and maintain active, high density biomass. In other situations, biofouling can seriously restrict effective heat transport, membrane processes, and potentate macrofouling with loss of transportation efficiency. Heterogeneous distribution of microbes and/or their metabolic activity can promote microbially influenced corrosion (MIC) which is a multibillion dollar problem[1].

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

Herein we will describe quantitative measures of microbial biofouling, the ecology of pathogens in drinking water distribution systems, and localization of microbial biofilms and activities with localized MIC.

2 METHODS

2.1 Biofilm Formation

Generation of biofilms in a non-circulating flowing system requires a substratum over which a bulk phase with sufficiently dilute nutrients flows so that only attached microbes can sustain survival and growth. This was done in laminar flow flowcells in which the biofilm can be observed through replaceable quartz windows, maintaining a reproducible shear field[2]. Inocula are added to the stream from continuous culture for a short period at the onset of the experiments. Multiple inocula can be added in sequence from separate continuous cultures[3]. Effects of different substrata or coatings can be tested with flush mounted coupons mounted in the laminar flow[4].

2.2 Microbial Biofouling

For quantitative estimation of AF and FR properties of coatings, stock cultures of *Vibrio harveyi* ATCC 14126 were maintained on artificial seawater (ASW) agar plates and slants supplemented with 0.01% (v/v) glycerol, 0.02% (w/v) casamino acids, and 10 mM tris buffer (pH 7.5) at 25 °C[5]. A 10 ml, 24 h batch culture of *V. harveyi* in ASW was used to inoculate a continuous culture vessel with a working volume of 1 liter and dilution rate of 0.1 h^{-1}. The continuous culture achieved steady-state growth after approximately 48 h and was used to inoculate the laminar-flow cell system for 4 h at a flow rate of 0.5 ml min^{-1}. The cell density of the inoculum was $1.5 \pm 0.24 \times 10^7$ cells ml^{-1} as measured by acridine orange direct counts.

2.2.1 Flow cell system. This will strongly select for biofilm formation in the non-circulating system. The chamber was shaped so laminar flow was generated[6] between two parallel plates, one of which is transparent for observation[7]. The laminar-flow cells[5]) had flow channels measuring 76 mm x 405 mm x 2 mm. The bottom half of the cell was constructed of high molecular weight plastic into which a series of five coupons (35 x 70 x 3 mm) were inserted with long axis perpendicular to the flow and flush with the flow channel. The top half was made with translucent Lexan® with removable, hollow, polypropylene ports; each with a quartz disc inserted at the end. Three ports were positioned directly over each coupon providing viewing areas from which the fluorescence and bioluminescence of a *V. harveyi* biofilm could be measured with fiber optic light cables. Just prior to taking light measurements, the ports were removed, and the quartz discs aseptically wiped free of any biofilm accumulation using isopropyl alcohol and a sterile water rinse. The flow cells were sterilized by exposure to ethylene oxide gas. A flow rate of 10 ml min^{-1} was used during the low-flow portion of the study. The calculated shear stress at this flow rate was 3.3 dynes cm^{-2}.

Biofilm fluorescence was measured using an F212 Fluorolog II fluorometer (Spex Industries Inc., Edison, NJ) equipped with a bifurcated fiber optic light cable and contained dual gratings for both excitation and emission wavelengths[8]. Tryptophan fluorescence (TF) was measured as relative fluorescence at an excitation wavelength of 295 nm and an emission wavelength of 342 nm. Initial background fluorometric measurements were subtracted from both the stainless steel and coated coupons.

V. harveyi bioluminescence was measured with an Oriel liquid light pipe-photomultiplier tube-ammeter light system (Oriel Corp., Stratford, CT) in units of namps as described[5].

2.2.2 On-Line Monitoring. Biofilm formation, succession, stability, and sub-lethal toxicity was monitored with in-line, non-destructive techniques in the flow-through apparatus[9]. Microbial biofilm biomass can be monitored by NADH, tryptophan, or chlorophyll fluorescence[8]. The intrinsic bioluminescence of attached biofilm naturally bioluminescent or genetically engineered bacteria with the *lux* gene cassette can be monitored in flow-through chambers[5] or mapped with a photon counting imaging microscope[10]. The relative bioluminescence per cell (determined by tryptophan fluorescence) provides a non-destructive measure of sub-lethal toxicity[4].

2.2.3 Antifouling (AF) and fouling-release (FR) coating efficacy. Stainless steel coupons of the laminar flow cells were colonized by similar numbers of bacteria: determined by TF, bioluminescence (BL), direct and viable cell counts[4, 5]. The AF efficacy of each coating was determined by the ratio of measurements from the coating to those of the stainless steel control coupon inserted just upstream. This design allowed for a direct

comparison of results between experiments, and minimized error due to differences in inoculum density. To detect the effect of coating components released downstream, the ratio of TF and BL measurements obtained from a stainless steel coupon directly downstream of the coating to the upstream stainless steel coupon was determined. Fouling-release (FR) properties of the coatings were determined by fluorescence readings after biofilms were subjected to a flow of 1 liter min^{-1} for 15 min, equivalent to a shear stress of 330 dynes cm^{-2}. Fluorescence readings recorded subsequent to exposure to the increased flow rate were used to determine the percent of the biofilm biomass sheared away and the performance of the coating relative to the upstream stainless steel coupon.

2.2.4 Extraction and enumeration of biofilm from the coupon surface. All glassware used during the extraction process was heated for 4 h at 450 °C to combust residual organic material and sterilize. Glass clamp-on extractors were fastened with an o-ring onto the coupon surface, the biofilm material was sonicated into 1 ml of ASW, the suspension transferred to a sterile screw-capped tube containing glass beads and suspended by vortexing. Three samples (the area viewed by each port) were extracted from each coupon. For direct microscopic evaluation, the sonicated suspension was diluted with ASW, filtered, stained, and examined under epifluorescence illumination as described[4]. Biofilms were analyzed for viable microbes by plating onto appropriate media, direct microscopic counting after recovery from the biofilms, or by signature biomarker analysis (SLB).

2.2.5 Phospholipid fatty acid analyses. Signature lipids can be analyzed after one-phase chloroform/methanol/buffer extraction, fractionation into neutral lipids, glycolipids, and polar lipids on silicic acid columns, derivatizarion and gas chromatography/mass spectrometry as described[11]. These analyses provide insight into the viable biomass, community composition, and nutritional/physiological status[11]. The determination of the total phospholipid ester-linked fatty acids (PLFA) provides a quantitative measure of the viable or potentially viable biomass. Viable microbes have an intact membrane which contains PLFA. The cellular enzymes hydrolyze the phosphate group within minutes to hours of cell death[12]. The remaining lipid is diglyceride with its fatty acids (DGFA). The resulting DGFA contains the same signature fatty acids as the phospholipids, allowing for a comparison of the ratio of phospholipid fatty acids to diglyceride fatty acids (viable to non-viable microbes). Healthy biofilm communities generally have a DGFA/PLFA ratios of < 0.5. Exponentially growing cultures have DGFA/PLFA ratios of < 0.01. The signature lipid analysis also provides insight into the physiological status of the microbial community. The formation of poly β-hydroxyalkanoic acid (PHA in bacteria)[13], or triglyceride (in microeukaryotes)[14] relative to the PLFA provides a measure of the nutritional status. Bacteria grown with adequate carbon and terminal electron acceptors form PHA when they cannot divide because some essential component is missing (phosphate, nitrate, trace metal, etc.). PHA/PLFA ratios of > 0.2 indicate unbalanced growth. Furthermore, specific patterns of PLFA can indicate physiological stress[15]. Exposure to toxic environments can lead to minicell formation and a relative increase in specific *trans* monoenoic PLFA compared to the *cis* isomers. It has been shown that for increasing concentrations of phenol toxicants and organic solvents, the bacterium *Pseudomonas putida* forms increasing proportions of *trans* PLFA[16]. *Trans/cis* 16:1w7c PLFA ratios > 0.1 indicate exposure to toxicants or starvation. Healthy cells have *trans/cis* 16:1w7c PLFA ratios of < 0.05. With incubations inducing stationary-phase growth conditions, cyclopropane PLFA accumulate in Gram-negative heterotrophic bacteria. Increases in cyclopropane PLFA/monoenoic PLFA precursors greater than 0.1 indicate this nutritional stress whereas exponentially

growing cells have cyclopropane PLFA/monoenoic PLFA ratios of < 0.05.

3 RESULTS AND DISCUSSION

3.1 Antifouling Efficacy

The TF ratio between two stainless steel coupons in the laminar-flow system was 0.92 ± 0.24 over a 4 day period, which indicated a similar rate of biofilm development on both surfaces during that time period. Tryptophan fluorescence as a biomass measure was confirmed by correlation with direct epifluorescent microscopic counts. Bioluminescence measurements indicated that the attached cells were metabolically active. Based on these results, coatings which demonstrated a TF ratio of approximately 0.65 or less within one standard deviation were considered effective at inhibiting biofilm biomass. Coatings releasing biocides such as Sea Nine 211 (C9211), a dichlorinated-keto-sulfur-heterocyclo-hexamine with an 8 carbon saturated side chain, showed inhibition of *V. harveyi* biofilm growth as demonstrated by TF ratios of 0.18 to 0.42 respectively. Generally, the BL ratios for the test coatings showed greater overall light intensity from the biofilm cells on the coatings than cells attached to the upstream stainless steel coupon, suggesting that the BL yield was greater from cells attached to these coatings compared to cells attached to stainless steel. It was proposed that the biocide had a detrimental effect on cellular metabolism, which results in changes in luciferase activity similar to the effect of cyanide on *V. harveyi* as reported by[17]. They suggested that inhibition of the electron transport system by cyanide and other agents increased BL. Toxicants such as C9211, when incorporated in silicone coatings, show a dose-related ability to increase the bioluminescent light production per cell[4]. The progressive increase in bioluminescence characteristic of sublethal toxicity with increased exposure to the toxicant was reflected by a progressive increase in the formation of *trans* 16 and 18 carbon monoenoic fatty acid esters from the *cis* precursors in the phospholipids of the cell membranes. This system provides an excellent system for examination of mechanisms of cellular injury. Zosteric acid (ZA), a component discovered in the seagrass *Zostera marina*[18], is bacteriostatic in that it appears to prevent attachment but not biofilm growth in attached biofilm bacteria. This result has also been observed with AF coatings containing furan compounds.

Some of these coatings showed a downstream leaching effect on biofilm cells as determined by calculating the TF ratio of the stainless steel coupon downstream of the coating to that of the upstream stainless steel coating. The leaching ratio was greater than 1.0 at all C9211 concentrations. The leaching ratio for stainless steel was 0.86 ± 0.16. No significant leaching was detected from the polydimethylsiloxane (PDMS) coatings (which exhibited TF leaching ratios of 0.65 - 0.76), nor were there any differences between the amount of biomass on the coatings and that on the downstream steel coupons.

To test the strength of biofilm adhesion after 4 days, polydimethylsiloxane (PDMS) coatings were subjected to a shear stress of 330 dynes cm^{-2} for 15 min, mediated by an increased flow rate to 1 liter min^{-1}. The amount of biomass stripped from each coating varied between 60 and 70%. Biomass measurements from STL decreased by an average of 55%. The TF ratios were generally decreased after high-flow exposure. Microscopic count ratios generally correlated with the final TF ratios from all coatings tested.

The advantages of the on-line, biofilm monitoring system were the ability to spatially segregate cell populations attached to coating and control stainless steel surfaces,

and to obtain on-line measurements from these populations. By measuring biofilm biomass and activity non-destructively (via TF and BL respectively) and normalizing the values for coatings to those of a stainless steel control surface, determinations on coating performance and cell response to the coating could be made independent of factors such as flow rate and inoculum density, and without the necessity of scraping and/or rinsing biofilm material from the substratum.

3.2 Multispecies Biofilm Ecology

The reproducible generation of biofilms requires control of the three major components that effect biofilm ecology: the bulk fluid, the substratum and the inoculum in a flow-through system. The bulk fluid should have a chemical composition of sufficiently dilute nutrients that pelagic growth is not possible. The third feature in reproducible biofilm formation is the inoculum. Continuous culture vessels with one for each bacteria that will form the biofilm community are maintained in medium like the bulk phase but with nutrients sufficient to maintain pelagic growth. These are then used as a pulsed inoculum into the flow chamber where the biofilm is maintained. In experiments involving *Pseudomonas fluorescens*, *Hafnia alvei*, *Desulfovibrio gigas*, and *Bacillus subtilis*, the order of the inoculation from the continuous cultures affected the composition and viable biomass of the resulting biofilm as determined at harvest after 5 days[3]. *P. fluorescens* dominated the biofilm and if it was utilized as the initial inoculant, the biofilm had the highest biomass (about 10^8 cells cm^{-2}). In later experiments a genetically engineered bioluminescent *Pseudomonas aeruginosa* isolated from soil, a *Bacillus* sp., and an *Acidovorax* sp. recovered from drinking water biofilms (defined by similarity of fatty acid patterns to the MIDI data base) also depended on the order of inoculation[4].

3.2.1 Ecology of pathogens in drinking water biofilms. The triculture of *P. aeruginosa*, *Bacillus* sp., and *Acidovorax* sp. formed a reproducible biofilm in the laminar flow apparatus described above in a flowing system fed by a 1:1000 dilution of tryptic soy broth. The biofilm was established from a continuous culture of the triculture with a flow of 10 ml min^{-1}. The system was operated in triplicate and was exposed to 1 and 5 ppm of chlorine. The triculture biofilm showed effects of exposure to chlorine. After 96 hours the control showed $43 \pm 8 \times 10^8$ cells cm^{-2}, $9 \pm 6 \times 10^8$ cells cm^{-2} (1 ppm), and $2.8 \pm 2 \times 10^8$ cells cm^{-2} (5 ppm), of which about 10-20 % were detectable with viable plate counts. Into this background a series of pathogens and pathogen-surrogates were tested.

A mycobacterial pathogen, *M. smegmatis* provided by Dr. Vojo Deretic (University of Texas Health Science Center) was engineered to contain green fluorescent protein (GFP). It was inoculated at 10^8 cells over 5 minutes from a continuous culture. Monoculture biofilms of *M. smegmatis* formed a thin biofilm. In contrast the mycobacteria readily colonized the biofilm to at least a 10-fold higher density and were slowly lost over the timecourse of 96 hours. Examining the lipid biomarkers of the *M. smegmatis* monoculture biofilm showed an increase in the DGFA/PLFA of 0.4 to 0.7 with chlorine, in contrast to the ratio of 0.1 for the control and both chlorine concentrations when the mycobacteria was a part of the triculture biofilm. Evidence of toxicity exposure was evident in the *M. smegmatis* monoculture biofilm with *trans/cis* 18:0 ratios of 0.6 to 0.8 in contrast to the ratios of < 0.1 in the mixed culture biofilm in the control and both 1 and 5 ppm chlorine. Evidence of growth phase shift to stationary phase was detected in the cy17:0/16:1w7c ratio in the *M. smegmatis* monoculture biofilm of 0.5 in contrast to the ratio of 0.2 in the mixed

culture biofilm. In the mixed culture biofilm the *M. smegmatis* was fully protected (Table 1).

Table 1 *Signature lipid shifts indicating cell lysis (DGFA/PLFA), exposure to toxicity (trans/cis PLFA), and induction of stationary phase (Cyclopropane PLFA/monoenoic PLFA) in pathogen infected triculture drinking water biofilms*

M. smegmatis

	Monoculture			*Triculture*		
Chlorine Level	0 ppm	1 ppm	5ppm	0 ppm	1 ppm	5 ppm
DGFA/PLFA	0.4	0.6	0.7	0.1	0.1	0.1
trans/cis PLFA	0.6	0.7	0.8	<0.1	<0.1	<0.1
Cy/Monoenoic PLFA	0.5	0.5	0.5	0.2	0.2	0.2

E.coli

	Monoculture			*Triculture*		
Chlorine Level	0 ppm	1 ppm	5ppm	0 ppm	1 ppm	5 ppm
DGFA/PLFA	0.004	0.007	0.01	0.005	0.006	0.006
trans/cis PLFA	0.005	0.005	0.05	<0.005	<0.005	<0.005
Cy/Monoenoic PLFA	3.2	2.1	2.0	1.0	1.1	1.0

A similar experiment was performed with the triculture biofilm and *E. coli* engineered to contain GFP. Comparison of the viable count to the total count showed that the ratio dropped from 1.2 in the control to 0.1 when exposed to 1 ppm chlorine and to 0.05 when exposed to 5 ppm chorine for 15 minutes. Allowing the *E. coli* to form biofilm enabled the cells to maintain a ratio of viable to total count of 0.05 for 4 days. However, with exposures of 5 ppm chlorine, the biofilm contained no viable cells after 4 days. When the *E. coli* was protected in the triculture biofilm, the viable to total counts were 0.3 (1 ppm chlorine) and 0.2 (5 ppm chlorine) after 5 days. The triculture biofilm increased the survival of *E. coli* more than 2-fold in chlorine relative to liquid culture. Lipid analyses paralleled the viable count data. Exposure to chlorine increased the DGFA/PLFA ratio to a greater extent in the in the *E. coli* monoculture biofilm from 0.0045 (control), 0.007 (1 ppm chlorine) and 0.01 (5 ppm chlorine) in contrast to 0.005-0.006 for control and 1 ppm and 5 ppm chlorine in the triculture + *E. coli*. The toxicity estimated as the ratio of *trans/cis* 16:1w7c PLFA was 0.005 for the control and 1 ppm chlorine and 0.035 for 5 ppm chlorine in the *E. coli* monoculture biofilm. This stands in stark contrast to the *trans/cis* 16:1w7c PLFA ratio of < 0.005 for the control and both chlorine exposures in the triculture + *E. coli*. The shift to stationary phase was reflected in the cy 17:0/16:1w7c PLFA ratio: in the control *E. coli* monoculture biofilm the ratio was 3.2, 2.1 in the cells exposed to 1 ppm chlorine and 2.0 in the cells exposed to 5 ppm chlorine. In the triculture biofilm + *E. coli* the cy 17:0/16:1w7c PLFA ratio was 1.0 to 1.1 for the control and both chlorine exposed

biofilms. Chlorine exposure apparently induced continued growth over the 4 day exposure in contrast to the control. This effect was not detected in the *E. coli* in the triculture biofilm. The SLB data clearly indicate the protective effects of the biofilm on the lysis, toxicity exposure, and nutritional status of the *E. coli*.

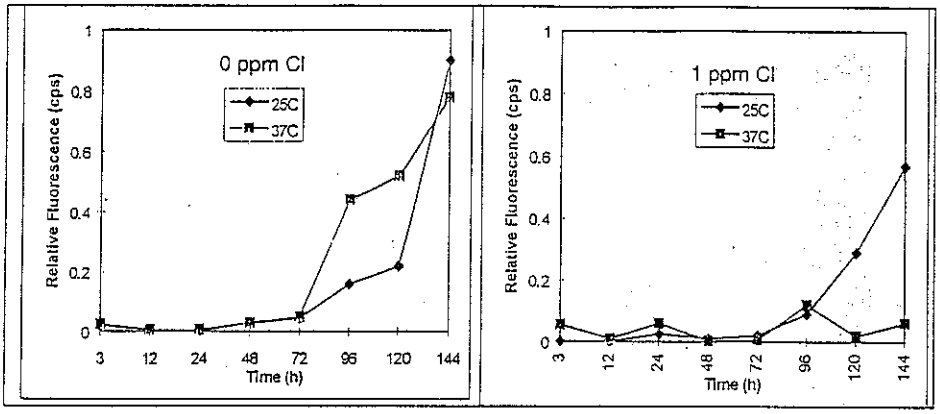
The pathogen *Legionella bozemanii* is naturally fluorescent. However the natural blue fluorescence proved too weak to detect the organisms in the triculture biofilm. The *L. bozemanii* monoculture biofilm did show an interesting response to temperature. When incubated at 25 °C the growth was slower than at 37 °C and it required 144 days to reach equivalent biofilm density. The biofilm formed at 25 °C showed essentially complete resistance to 1 ppm chlorine whereas the biofilm formed at 37 °C was completely inhibited (Figure 1).

The triculture biofilm retained oocysts of *Cryptosporidium parvum* after a pulse of 2.5×10^8 oocysts were injected into the laminar flow apparatus by a factor of greater than 2-fold over a 72 hour period (Figure 2). Research has shown that the infectivity of *C. parvum* ooccysts to neonatal mice can be correlated with the lipid composition[19] and that the shifts in lipid composition may represent metabolism by the oocysts. Possibly the conditions in the drinking water biofilms can be manipulated to greatly depress the infectivity of the entrained oocysts.

3.3 Microbially Influenced Corrosion

An aerobic *Bacillus* spp., the fermentative *Hafnia alvei* and the sulfate-reducing anaerobe *Desulfovibrio gigas* induced differential rates of microbially influenced corrosion (MIC) of mild steel. Coupons were exposed in sterile dilute media resembling a fresh water lake but containing 0.4 mM sulfate in a flow through system to which bacteria grown in separate continuous cultures were added as monocultures or mixtures to an aerobic system. Corrosion rates measured as admittance (in mhos $\times 10^{-3}$ cm^{-2}), which were estimated from electrochemical impedance spectra (EIS), were about 0.5 for the sterile control, 0.5-1.0 for monocultures, 0.0-1.2 for two of the bicultures, 1.2 for the triculture and 2.5 for the *H. alvei* + *D. gigas* biculture[3]. Corrosion was assessed using a 4-sided electrode[20] as the reciprocal of the polarization resistance derived from EIS[21]. The 4-sided electrode technology allowed tests of reproducibility of general corrosion rates. EIS were established to not effect growth rates or metabolic activities of microbial biofilms on metal coupons[22]. The consortium containing *H. alvei* and *D. gigas* showed a significantly higher corrosion rate than the triculture or the other bicultures. At the end of the experiment the microbes actually attached to the coupons were examined by viable counts, MPN estimations and PLFA analysis. The rates of corrosion were not directly related to the total microbial biomass or the number of species on the coupon. The rate of corrosion did not depend on the ratio of heterotrophic to sulfate-reducing bacteria (SRB) or the absolute number of SRB. The PLFA analysis showed the microbes recovered from the biofilm were more metabolically stressed than those recovered from the bulk phase for the inocula. Clearly different combinations of bacteria forming biofilms in the same bulk phase growing on the same substrata can induce very different corrosion rates.

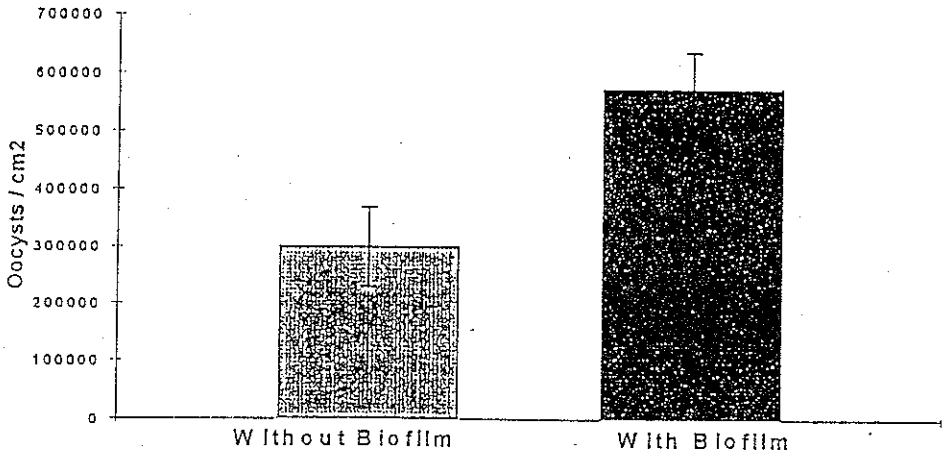
Inhomogeneities in the substratum surfaces induce differences in microbial biofilm distribution and MIC activities[23], and weldments are especially vulnerable[24]. To reproducibly localize MIC to a specific area, a concentric electrode system was developed in which a small area was separated by a Teflon ring from a larger circumferential area with 100 times the area of the same material. The small central area was then driven



No chlorine: Legionella growth rate higher at 37°C
Similar biomass levels after 144 h

Chlorine exposure: Decreased growth rate at 25°C
Little growth at 37°C

Figure 1 *Tryptophan fluorescence of Legionella bozmanii monoculture biofilms grown at 25 °C and 37 °C in the presence of 0 and 1 ppm chlorine on stainless steel coupons in the laminar-flow fouling test apparatus*[4, 5]



Biofilm increased number of oocysts retained in the system

Figure 2 *Comparison of retained Cryptosporidium parvum oocysts on stainless steel coupons determined microscopically in the presence and absence of the drinking-water derived triculture biofilm after 72 hours*

electrochemically as an anode compared to the large circumferential area in an anaerobic flow-through system. The system was inoculated and the potential between the anode and cathode shut off. The current flow between the electrodes was then monitored with a zero resistance ammeter[25]. The presence of bacteria including SRB resulted in stabilization of a corrosion current between the anode and cathode[26]. With this technology it proved possible to induce MIC of 304 stainless steel anaerobically in the anode area, reproducibly[27]. In this system a biculture of an SRB and a *Vibrio* sp. maintained a current of about 3 $\mu A\ cm^2$ for > 200 h after a 72 hour period after an imposed 11 $\mu A\ cm^2$ current was removed. No current was maintained in the sterile control or with inocula of monocultures of these two bacteria which formed biofilms on the concentric electrodes. With the biculture, the charge transfer resistance > 100 $k\Omega\ cm^2$ on the cathode (measured with EIS) was contrasted with < 1 $k\Omega\ cm^2$ at the anode. In this system removal of sulfate or the addition of 10 mM azide did not effect the sustainability of the current after an initial 30 hours. Apparently the microbial metabolic activity is necessary for initiation processes and once started the corrosion proceeds independent of the microbial metabolic activity[28]. This should be an excellent system to study the microbiology of MIC initiation.

MIC is a localized process leading to pitting corrosion. Localized concentrations of microbes can lead to localized corrosion[29]. With application of a scanning vibrating electrode across a coupon the charge density can be mapped and localized anodic areas detected in time and space[10]. The scanning vibrating electrode can be utilized in an epi-illuminated microscope with a photon counting camera and bioluminescent organisms to establish the congruity between the localization of microbes in microcolonies, their metabolic activity as indicated by their bioluminescence and the development of an anode detected in the charge density field[10].

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