RECENT DEVELOPMENTS IN BIOFOULING CONTROL

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OXFORD & IBH PUBLISHING CO. PVT. LTD.
New Delhi Bombay Calcutta

Emerging Techniques for the Evaluation of Bacterial Biofilm Formation and Metabolic Activity in Marine and Freshwater Environments

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Test systems have recently been developed that enable the evaluation of bacterial biofilm formation and metabolic activity under conditions simulating those of the in-situ environment. A series of laminar-flow adhesion cells were constructed with provisions for on-line, nondestructive measurements of open circuit potential, bioluminescence, and pO_2 for monitoring colonization and succession as influenced by a systematic change in bulk phase and substratum conditions. Bioluminescence and fluorescence by *Vibrio harveyi* biofilms were used as endpoints for adhesion in evaluations of antifouling ship hull coatings. These test systems facilitated studies of materials compatibility and antifouling efficacy in diverse ecosystems.

INTRODUCTION

Bacterial biofilm formation on inanimate substrata in freshwater, marine, and physiological environments often precedes microbially influenced corrosion and other biofouling activities. The impact, world-wide, of these activities amounts to billions of U.S. dollars each year (Dowling et al. 1991). Colonization of and attachment to surfaces in these environments are mediated by a number of interrelated environmental factors such as fluid dynamics (Mittelman et al. 1990), bulk-phase biotic and abiotic constituents (Marshall 1988), and the physicochemistry of the substrata (Absolom et al. 1983). To a great extent, research targeted to adhesion processes has been limited by a lack of test systems and analytical techniques for examining bacterial colonization and biofilm formation under conditions that approximate those of in-situ environments.

The ability to colonize replica test substrata reproducibly with relevant bacterial populations is a necessary component of antifouling efficacy studies. Laboratory and field applications of the Robbins device have been described by Ruseska et al. (1982), and Characklis et al. (1982) have developed annular-type reactors for the study of biofilm effects on fluid frictional resistance. Most of the systems developed were designed for microscopic evaluation of bacterial colonization on glass substrata. Provisions for monitoring biofilm development on other nonglass substrata, electrochemical/luminescent methods for studying adhesion, and quantitative assessments of biofilm biomass and metabolic activity have not been made in most of the existing systems.

This paper describes the design and application of laminar-flow adhesion cells designed for studies of biofilm formation and determinations of antifouling coating efficacy. These cells enabled determinations of bacterial colonization and succession using bioluminescence and shifts in electrochemical potential as endpoints for adhesion.

METHODS

Flow Cell Design: The flow cells consisted of an upper block of transluscent, laminated Lexan and a lower block of ultra-high molecular weight polyethylene. Overall dimensions of the cells were 15.0 cm width × 28.5 cm length × 3.3 cm height. The upper block was milled to provide a 2-mm deep flow channel: in addition, it contained a series of removable polypropylene screws with 12-mm diameter quartz glass discs at their base, flush-mounted with the flow channel (figure 1). This arrangement enabled direct observation of a series of removable, flush-mounted coupons recessed into the bottom block. Open circuit potential (OCP) measurements were facilitated by means of an Ag/AgCl reference electrode installed in the top block. Laminar-flow conditions were validated in dye studies and by observing a silk thread normal to the flow channel as described by Berg and Block (1984).

Test Substrata: Uncoated 316 stainless steel coupons polished to a 600 grit finish were used in validation and OCP experiments. Three coatings were evaluated for antifouling efficacy: 15% (w/w) dinitrophenol (DNP), and two copper-based paints, Navy F-121 and International Paints (IP) BRA-640. All coatings were applied to an epoxy basecoat. The finished dimensions of the test substrata were 3.5 cm width × 7.0 cm length × 0.3 cm height.

Continuous Culture Conditions: A continuous culture of the bioluminescent bacterium, *Vibrio harveyi* (ATCC 14126), was used to colonize polymer coated test coupons with and without antifouling additives (figure

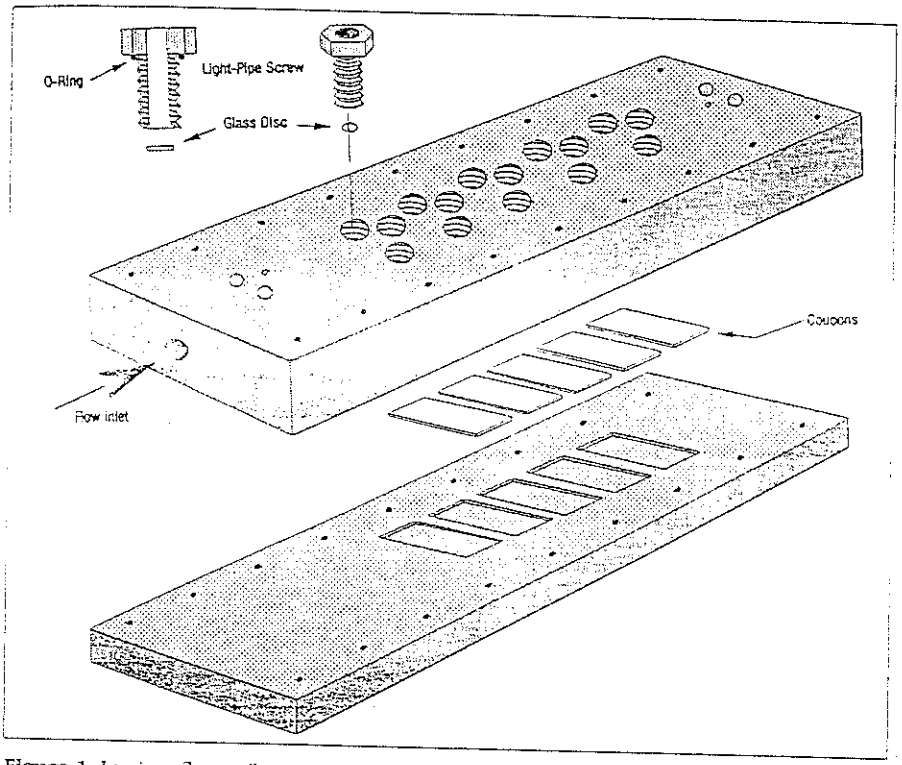

Figure 1. Laminar-flow adhesion cell. Holes drilled at entry and exit ends of the cell provide access for electrochemical monitoring.

2). An artificial seawater medium (ASTM 1986) with the addition of 0.01% glycerol, 0.02% casamino acids, and 10 mm tris buffer (Sigma Chemical, St. Louis, Missouri) at pH 7.5 was used throughout the experiments. All experiments were performed at ambient temperature (23–25°C).

Bioluminescence Measurements: Bioluminescence was measured in situ with an Oriel (Stratford, Connecticut) liquid light pipe-photomultiplier tube-ammeter light monitoring system through a 6-mm lumen in the polypropylene screws. The quartz glass window-polypropylene screw assembly was replaced prior to bioluminescence measurement to eliminate contributions from glass associated biofilms.

Fluorometric Measurements: Preliminary on-line fluorometric monitoring of biomass and activity was performed with a Spex Instruments Fluorolog II spectrofluorometer (Edison, New Jersey) equipped with a quartz fiberoptic cable.

Electrochemical Measurements: The OCP of uncoated stainless steel coupons was monitored with a Keithley (Keithley Instruments, Cleveland, Ohio) model 706 multichannel scanner and measured on a Hewlett Packard

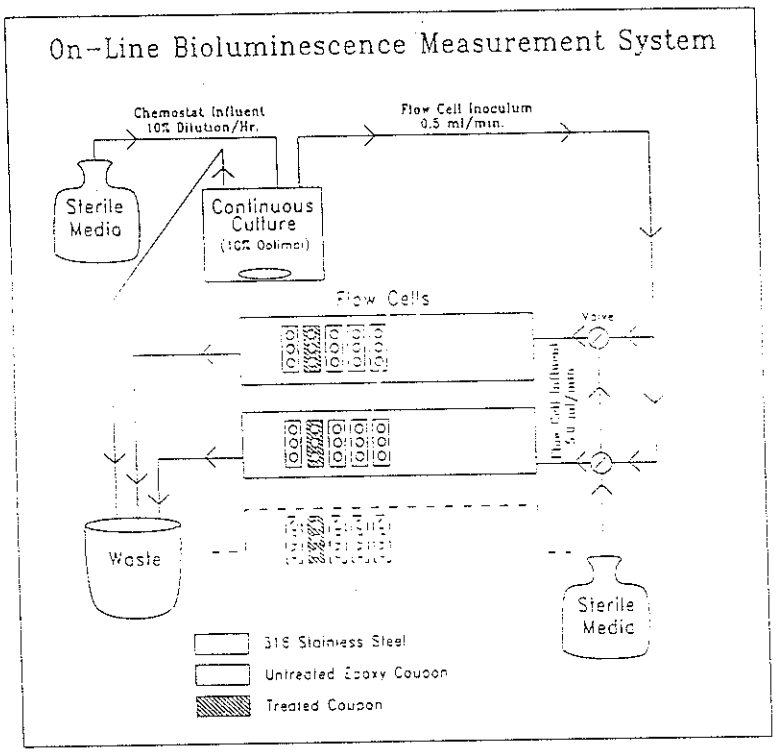

Figure 2. Flow diagram for studies of antifouling coating efficacy.

(Palo Alto, California) model 3458A voltmeter interfaced with a GPIB board and IBM clone personal computer. The test coupons served as the working electrodes; a stainless steel thumbscrew provided the connection to the working electrode.

Biofilm Analyses: Reproducibility of colonization was determined by direct counting of acridine-orange stained bacteria (AODC) and by viable counts on marine agar.

RESULTS AND DISCUSSION

Replica experiments with *V. harveyi* biofilms demonstrated reproducible colonization on coupons 3–5. Bioluminescence, AODC, and viable counts were reproducible for 5 ml min^{-1} flow rates. A significant positive correlation was established between bioluminescence and viable/direct bacteria counts (figure 3). The Lexan tops will enable fouling studies with cyanobacteria, diatoms, and other photosynthetic organisms. In addition, replica bioluminescence/fluorescence data can be obtained from three different areas of each coated/uncoated test coupon.

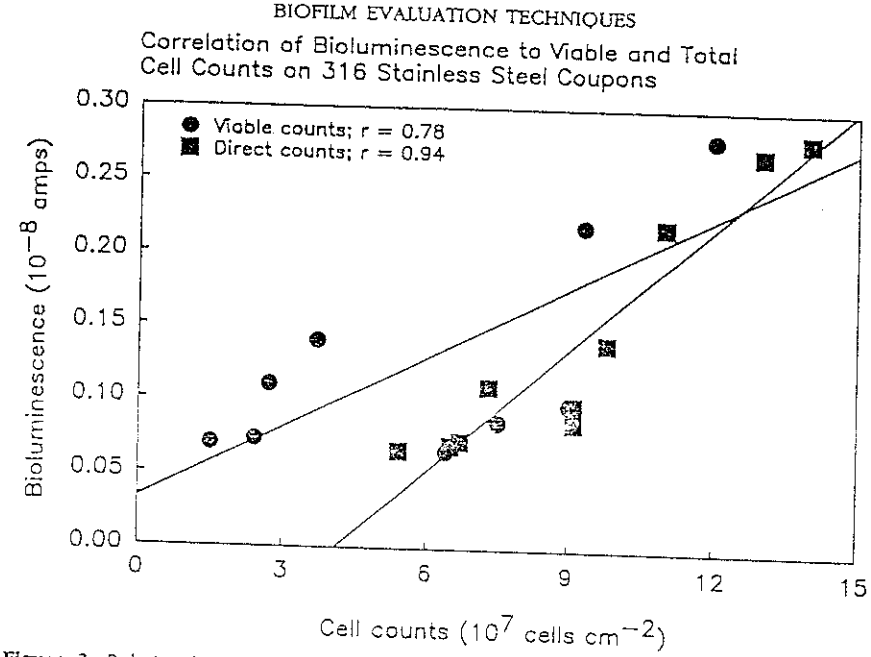

Figure 3. Relationship between bioluminescence and viable and total bacteria counts on uncoated 316 stainless steel coupons.

Resistance to colonization of *V. harveyi* was noted in the order of F-121 (Navy) > BRA 640 (IP) > 15% DNP (figure 4, table 1). There were good agreements between bioluminescence, viable count, and direct count data. The effects of AF compound release on non-AF containing coatings

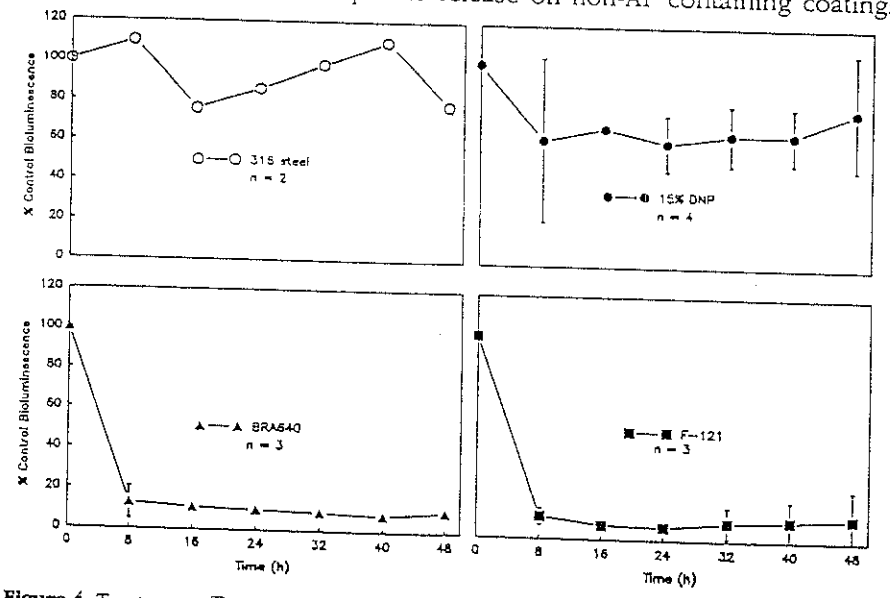

Figure 4. Treatment efficacy of coatings expressed as a percentage of control bioluminescence versus time.

are shown in table 1. There was decreased attachment from AF coatings release in the order of F-121 > 15% DNP ≈ BRA 640 (IP).

The SPEX system will enable low-level detection of both bioluminescence (photon counting) as well as fluorescence emissions from tryptophane and other aromatic amino acids and nucleotides (figure 5). Tryptophane was detected in bulk phase cultures and in-situ biofilms of

Table 1. Treatment and release effects on *Vibrio harveyi* colonization.

Treatment	n	Treatment Efficacy[a]		Effect of Release[a]	
		Viable	AODC[c]	Viable	AODC[c]
DNP	4	38 (±1)[b]	*64 (3.5)	*28 (25)	*48 (5.8)
IP BRA-640	3	* 0.73 (0.23)	*32 (19)	*25 (39)	74 (17)
F-121	3	*<0.0003	*12 (3.8)	*11.2 (5.4)	*36 (16)

[a]expressed as a percent of control value; [b]( ) = standard deviation; [c] = acridine-orange, stained bacteria

*significantly different from the control (nontreatment), P<0.05

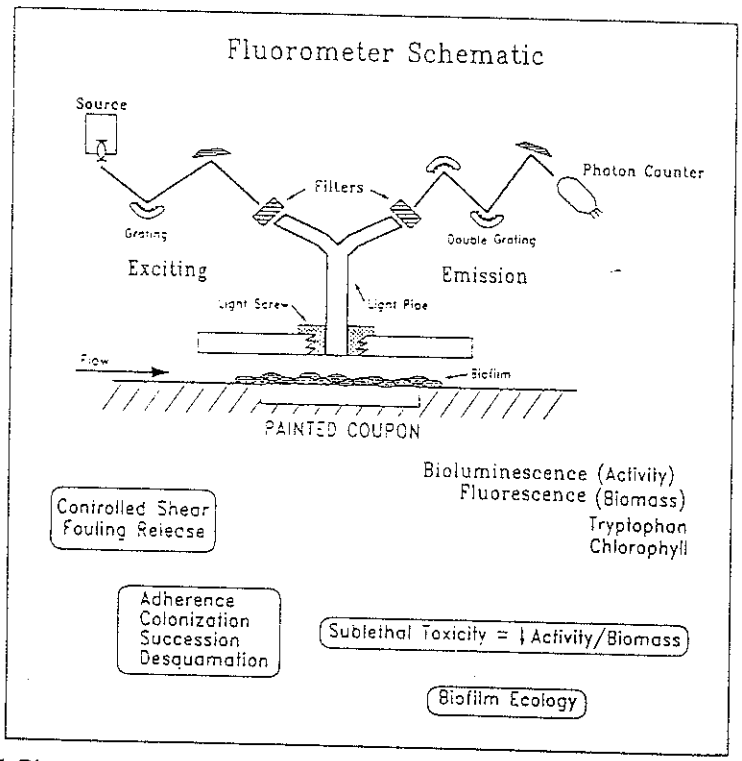

Figure 5. Diagrammatic representation of fluorometer application to antifouling coating efficacy studies.

V. harveyi associated with 316 stainless steel surfaces (figure 6). Biomass and metabolic activity can be monitored on AF coated and control surfaces in situ on a real-time basis. Several compounds show promise as biomass and metabolic activity markers within biofilms (table 2).

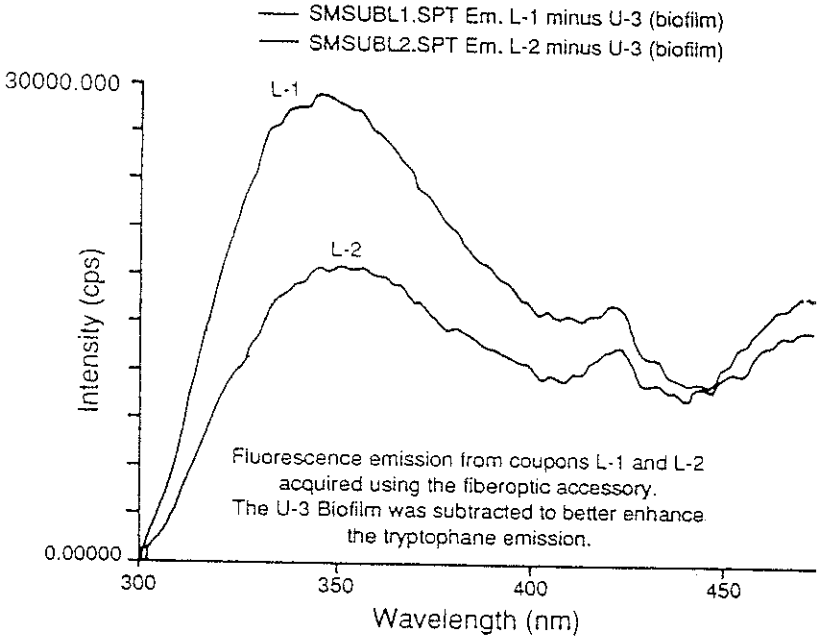

Figure 6. Detection of the aromatic amino acid tryptophane in *Vibrio harveyi* biofilms associated with 316 stainless steel.

Table 2. Examples of relevant wavelengths for fluorometry of fouled surfaces.

Biomass	Excitation	Emission
TRP, TYR, PHE	260–280	303, 348, 282
Activity		
bioluminescence	–	490
ATP	272	380
NADH	340	460
Algae		
chlorophyll *b*	480	640

OCP values, which provide an indication of surface potential, were significantly perturbed by the addition of *V. harveyi*. However, neither the magnitude nor the onset of the observed perturbations was diagnostic for

biomass quantity or community structure (figure 7). Changes in potential preceded visible biofilm formation and bioluminescence production.

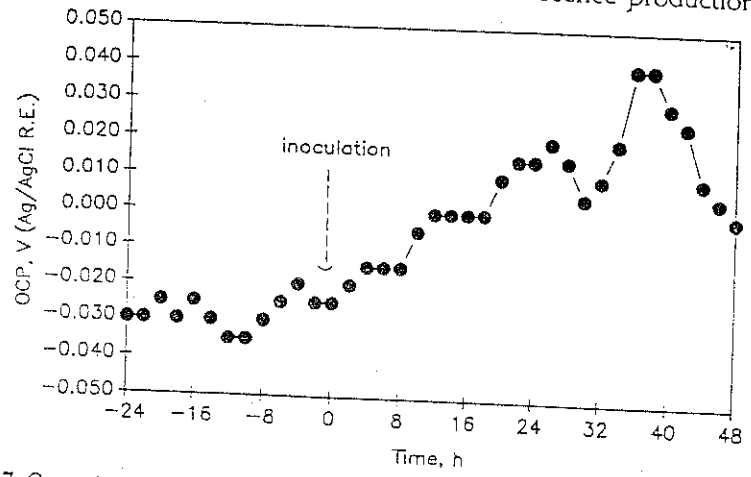

Figure 7. Open circuit potential (OCP) of *Vibrio harveyi* biofilms associated with 316 stainless steel.

These multipurpose, laminar-flow adhesion cells provided a reproducible means for colonizing various surfaces and measuring biomass accumulation. Bioluminescence measurements were used to determine antifouling coating efficacy and the effect of coating release on sessile marine bacteria. Future research will use this test system for studies of sublethal toxicity effects on microbial monocultures and consortia.

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