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Test systems for determining antifouling coating efficacy using on-line detection of bioluminescence and fluorescence in a laminar-flow environment

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Summary

Test systems have been developed which enable the evaluation of bacterial biofilm formation and metabolic activity under conditions simulating those of in situ environments. A series of laminar-flow adhesion cells were constructed with provisions for on-line, non-destructive measurements of bioluminescence, fluorescence, open circuit potential, and pO_2 to monitor colonization and succession as influenced by a systematic change in bulk-phase and substratum conditions. Bioluminescence by biofilms of the bioluminescent marine bacterium, *Vibrio harveyi* was utilized as an endpoint for adhesion in evaluations of antifouling (AF) ship hull coatings. Resistance to colonization of *V. harveyi* was noted in the order of F-121 (Navy)> BRA 640 (IP)> 15% DNP. Statistically significant ($p < 0.05$) correlations were obtained between bioluminescent, viable, and direct bacteria counts. Tryptophane was used as a biomass marker in fluorometric measurements of *V. harveyi* biofilms. These methods enabled on-line evaluations of biofilm formation and AF coating efficacy.

Key words: Antifouling paint; Bioluminescence detection; Flow cell; Fluorometry; On-line biofilm monitoring; *Vibrio harveyi*

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 [1]. In addition to their direct involvement in fouling and corrosion activities, bacteria (and other microorganisms) can have an impact on the settling and adhesion of macrofouling organisms to engineered surfaces. For

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example, Kirchman et al. [2] and Weiner et al. [3] described specific bacteria which promoted barnacle and oyster formation on surfaces exposed to marine environments.

Colonization of and attachment to surfaces in these environments is mediated by a number of interrelated environmental factors such as fluid dynamics [4], bulk-phase biotic and abiotic constituents [5], and the physicochemistry of the substrata [6]. 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 which approximate those of in situ environments.

The ability to reproducibly colonize replica test substrata 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. [7] and Characklis et al. [8] have developed annular-type reactors for the study of biofilm effects on fluid frictional resistance. Most of the systems developed thus far have been designed for microscopic evaluation of bacterial colonization on glass substrata. Provisions for monitoring biofilm development on other, non-glass, 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. Therefore, these methods are limited in their application to comprehensive evaluations of bacterial colonization and the efficacy of antifouling compounds/surfaces.

Bioluminescent bacteria have been employed in a few different types of biofouling and toxicity assessments. Mittelman et al. [9] utilized *lux* constructs of *Pseudomonas fluorescens* in an on-line assay, using bioluminescence as an endpoint for adhesion. Jassim et al. [10] have described an in vivo bioluminescence technique for evaluating biocide effects on planktonic bacterial populations. King et al. [11] utilized a bioluminescent reporter plasmid to evaluate aromatic hydrocarbon utilization in contaminated soils.

The design and application of laminar-flow adhesion cells developed for studies of bacterial biofilm formation and determinations of antifouling (AF) coating efficacy are described herein. These cells enabled determinations of bacterial colonization and succession using bioluminescence, fluorometry, and shifts in electrochemical potential as endpoints for adhesion.

Materials and Methods

Flow cell design

The flow cells consisted of an upper block of translucent, laminated Lexan and a lower block of ultra-high molecular weight polyethylene. The translucent upper block was intended for future applications employing diatoms and other photosynthetic microorganisms. Overall dimensions of the cells were 15.0 cm W × 28.5 cm L × 3.3 cm H, with a working (void) volume of 75 ml. 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 (Fig. 1). This arrangement enabled direct observation of a series of removable, flush-mounted coupons recessed into the bottom block. Open circuit

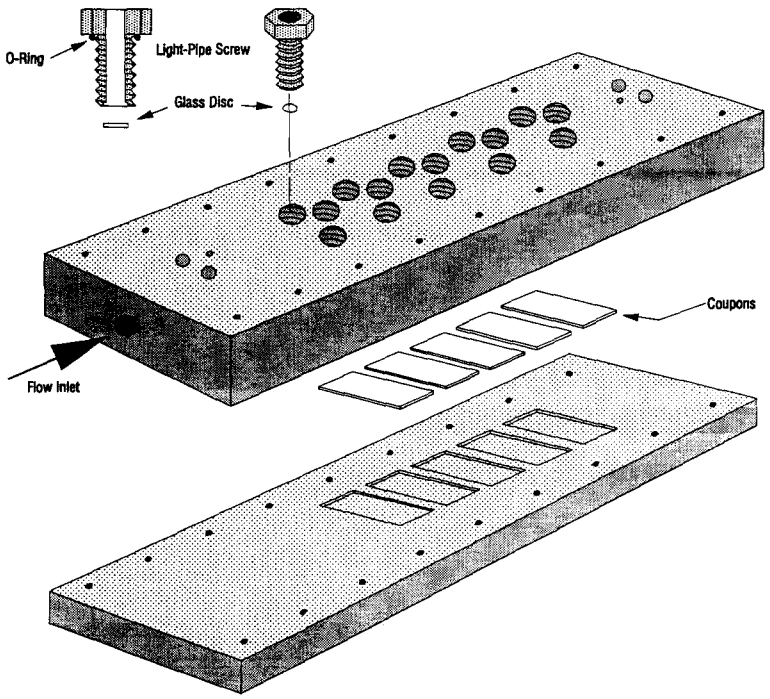

Fig. 1. Laminar-flow adhesion cell. Holes drilled at entry and exit ends of the cell provide access for electrochemical monitoring.

potential (OCP) measurements were facilitated by means of a Ag/AgCl reference electrode installed in the top block. The upper block also contained provisions for oxygen monitoring via a 3 mm diameter semi-micro amperometric probe. 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 [12].

Test substrata

Uncoated 316 stainless steel (SS) coupons polished to a 600 grit finish were used in validation and OCP experiments. Three free-association coatings were evaluated for AF efficacy: 15% (w/w) dinitrophenol (DNP), and two copper-based paints, Navy F-121 and International Paints (IP) BRA-640. Epoxy coatings free of any AF agents were used as experimental controls. The finished dimensions of the test substrata were 3.5 cm W × 7.0 cm L × 0.3 cm H.

Continuous culture conditions

A continuous culture of the bioluminescent bacterium, *Vibrio harveyi* (ATCC 14126), was used to colonize test substrata with and without AF additives (Fig. 2). An artificial seawater medium (ASW) [13] with the addition of 0.01% glycerol, 0.02% casamino acids, and 10 mM Tris buffer (Sigma Chemical, St. Louis, MO) at pH 7.5 was used throughout the experiments. A working volume of 1 l was employed with a

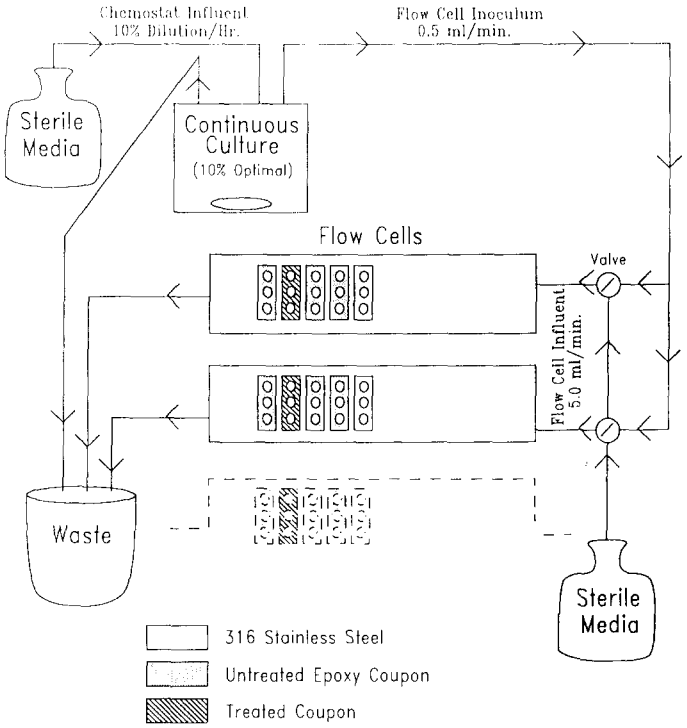

Fig. 2. Flow diagram for studies of antifouling coating efficacy.

dilution rate of 0.1 h^{-1}. The maximum growth rate of the culture was 1.45 cells h^{-1}. Aeration was achieved by continuous agitation of the cultures at a stir rate of ca. 300 rpm. All experiments were performed at ambient temperature (23–25°C).

A flow-rate of 5 ml min^{-1} for the sterile media was maintained through the flow cells for the duration of the experiments. *V. harveyi* maintained at 10^8 cells ml^{-1} was continuously injected into the sterile media at a flow rate 0.5 ml min^{-1}.

Bioluminescence measurements

Bioluminescence was measured in situ with an Oriel (Stratford, CT) liquid light pipe-photomultiplier tube-ammeter light monitoring system through a 10 mm lumen in the polypropylene screws. Sterile, cell-free medium was flushed through the cells at the in situ rate for 30 min prior to each bioluminescence measurement. The quartz glass window-polypropylene screw assembly was replaced prior to bioluminescence measurement to eliminate contributions from glass-associated biofilms. Replica bioluminescence/fluorescence data were obtained from three different areas of each coated or uncoated test coupon.

Fluorometric measurements

Preliminary on-line fluorometric monitoring of biomass and activity was performed with a Spex Instruments Fluorolog II spectrofluorometer (Edison, NJ)

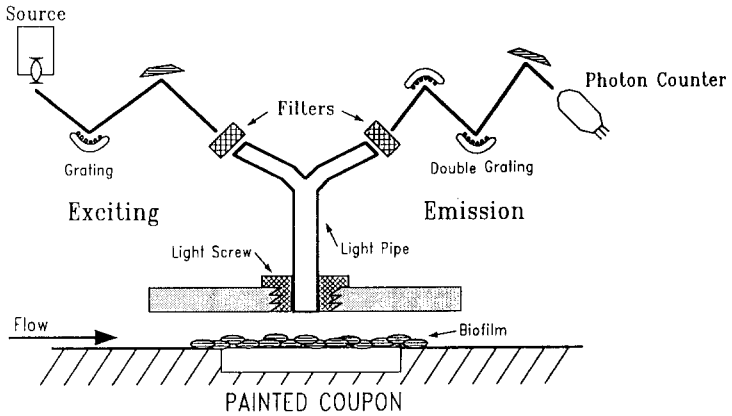

Fig. 3. Diagrammatic representation of fluorometer application to antifouling coating efficacy studies.

equipped with a bifurcated quartz fiberoptic cable. The quartz glass windows were also replaced prior to fluorescence measurements. A schematic of the test system is shown in Fig. 3.

Electrochemical measurements

The OCP of uncoated SS coupons was monitored with a Keithley (Keithley Instruments, Cleveland, OH) model 706 multichannel scanner and measured on a Hewlett Packard (Palo Alto, CA) model 3458A voltmeter interfaced with a GPIB board and IBM clone personal computer. The test coupons served as the working electrodes; a SS thumbscrew provided the connection to the working electrode. Further details on the design of the electrochemical measurement and data acquisition system are given in Mittelman et al. [14].

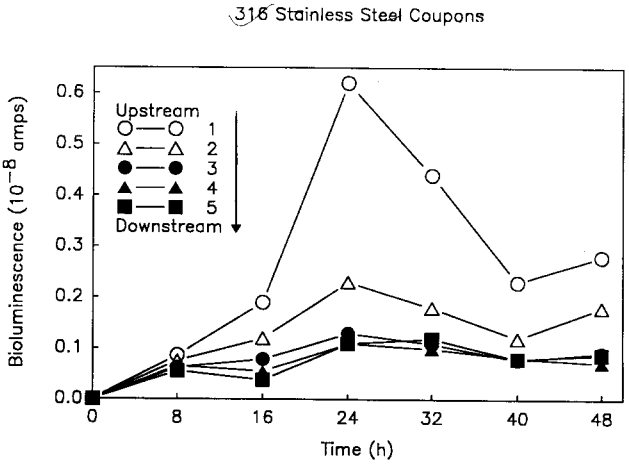

Fig. 4. Bioluminescence of 316 stainless steel flow cell coupons colonized with *V. harveyi*.

Biofilm analyses

Reproducibility of colonization was determined by direct counting of acridine orange stained bacteria (AODC) and by viable counts on marine agar. Cells were quantitatively extracted from coupon surfaces via a sonication procedure employing 1.131 cm^2 glass O-ring extractors (Kontes Glass, Vineland, NJ).

Results and Discussion

Replica experiments with *Vibrio harveyi* biofilms demonstrated reproducible colonization on coupons 3–5 within the laminar-flow adhesion cells (Fig. 4). The first two coupons typically showed greater numbers of cells and higher bioluminescent readings than did coupons 3–5. These differences may have been due to differential substrate availability. Bioluminescence, AODC, and viable counts were reproducible for the 5 ml min^{-1} flow rates. A significant positive correlation was established between bioluminescence and viable/direct bacteria counts (Fig. 5).

Resistance to colonization of *V. harveyi* was noted in the order of F-121 (Navy) > BRA 640 (IP) > 15% DNP (Fig. 6, Table 1). There was good agreement between bioluminescence, viable count, and direct count data for AF coated surfaces as with the uncoated SS controls. The effect of AF compound release on non-AF containing coatings is shown in Table 1. There was decreased attachment from AF coatings release in the order of F-121 > 15% DNP ≈ BRA 640 (IP). Bioluminescent biofilm bacteria were shown to be useful indicators of AF coating efficacy under dynamic-flow conditions.

The SPEX system enabled detection of fluorescence emissions from tryptophane. Tryptophane was detected in bulk-phase cultures and in situ biofilms of *V. harveyi* associated with 316 SS surfaces (Fig. 7). Biomass and metabolic activity can be monitored on AF coated and control surfaces in situ on a real-time basis. Several

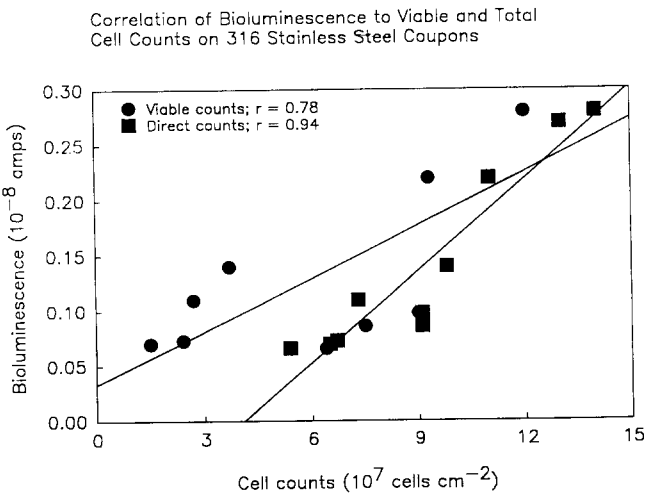

Fig. 5. Relationship between bioluminescence and viable and total bacteria counts on uncoated 316 SS coupons.

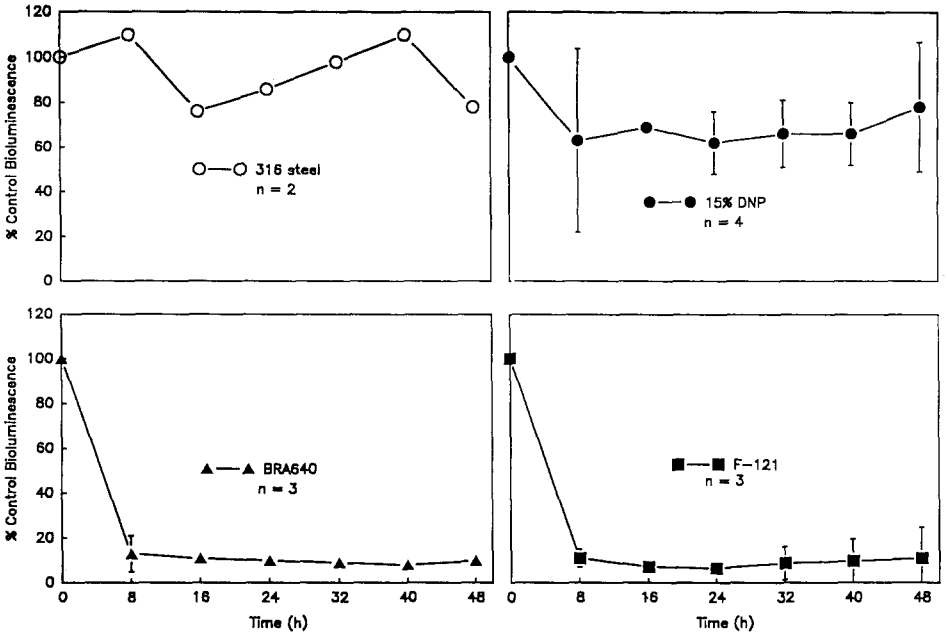

Fig. 6. Treatment efficacy of coatings expressed as a percentage of control bioluminescence vs. time.

compounds show promise as biomass/metabolic activity markers within biofilms (Table 2). Bioluminescence detection via the Spex instrument was, however, inferior to that of the Oriel system. Studies are in-progress to validate the application of fluorometry to AF coating efficacy studies.

OCP values, which provide an indication of surface potential, were perturbed by the addition of *V. harveyi*; however, neither the magnitude nor the onset of the observed perturbations were diagnostic for biomass quantity or community structure (Fig. 8). Changes in potential preceded visible biofilm formation and bioluminescence production. The OCP is a net potential, describing the sum of cathodic and anodic reactions. The cathodic reaction, which predominates in stainless steels

TABLE 1

Treatment and release effects on *V. harveyi* colonization

Treatment	n	Treatment efficacy[1]		Effect of release[1]	
		Viable	AODC	Viable	AODC
DNP	4	38 (41)[2]	[3]*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)

[1]Expressed as a percent of control value.

[2]Standard deviation.

[3]Significantly different from the control (non-treatment) at $p < 0.05$.

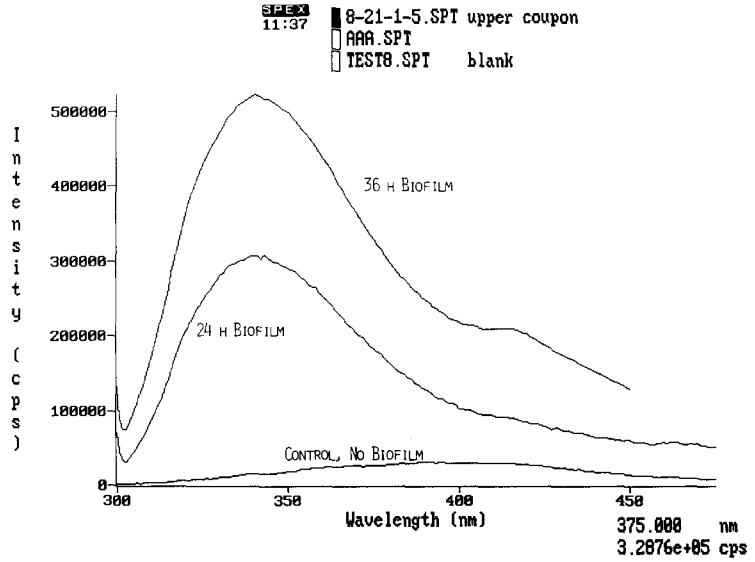

Fig. 7. Detection of the aromatic amino acid tryptophane in *V. harveyi* biofilms associated with 316 SS.

exposed to aqueous environments (contrasted with mild or carbon steels, in which the anodic reaction predominates) is described by

$$O_2 + 2H_2O + 4e^- \rightarrow\rightarrow 4OH^-$$

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

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

While OCP measurements may prove useful for monitoring the onset of fouling on

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

*Potential interferences exist from protein emissions.

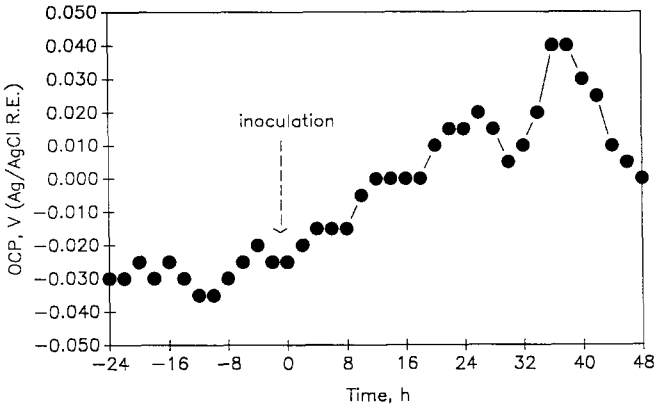

Fig. 8. OCP of *V. harveyi* biofilms associated with 316 SS.

uncoated, metallic surfaces, electrochemical surface potentials cannot be measured on non-metallic coatings; i.e., AF or fouling release epoxy combinations. However, these measurements might prove useful in evaluations of coating integrity. If mechanical and/or biological degradation created holidays in the coatings, changes in OCP would result upon contact of seawater with the underlying metal surfaces.

A new test system and analytical regime for assessing the effectiveness of AF coatings against microfouling organisms has been developed. The utility of bioluminescent bacteria in performing these studies was demonstrated. The significance of this work may be seen in the light of evidence that AF compounds targeted towards particular macrofoulants may be subject to rapid biodegradation by the in situ microbial population. In previous work performed in this laboratory (unpublished data), benzoic acid contained within an AF coating was rapidly degraded by *Alteromonas atlantica* biofilms. While benzoic acid may inhibit larval settlement, it is clear that this compound is rendered ineffective by a naturally occurring marine bacterium, which utilized this compound as a sole carbon source.

Future research will utilize this test system for studies of sublethal toxicity effects on microbial monocultures and consortia in order to better understand the effects of AF compounds on the ecology of microfouling organisms.

Acknowledgements

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