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On-line monitoring of antifouling and fouling-release surfaces using bioluminescence and fluorescence measurements during laminar flow

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A laminar flow biofilm-monitoring system was used to determine the efficacies of three antifouling (AF) coatings and five fouling-release (FR) coatings against *Vibrio harveyi* attachment. On-line measurements of tryptophan fluorescence and bioluminescence from each coating, normalized to an upstream stainless steel coupon, were used to determine the effects of AF and FR surfaces on biofilm formation. The AF coatings consisted of 5, 10, and 35 wt% Sea Nine 211 (C9211) incorporated into a vinyl copolymer. Both the 10 and 35 wt% coatings significantly inhibited biofilm biomass development measured by tryptophan fluorescence compared to the stainless steel control. *V. harveyi* bioluminescence was significantly greater than tryptophan fluorescence in cells attached to these coatings, suggesting that bioluminescence expression may be a marker for cellular stress or toxicity in biofilms. Five different polydimethylsiloxane (PDMS) FR coatings did not inhibit biofilm formation under low flow conditions. However, four PDMS coatings demonstrated decreased biomass levels compared to stainless steel after exposure to a shear stress of 330 dynes cm^{-2}. There was no toxic additive in these coatings; bioluminescence and tryptophan fluorescence were proportional.

Keywords: antifouling; biofilms; bioluminescence; Sea nine 211; fluorescence; polydimethylsiloxane; *Vibrio harveyi*; on-line monitoring

Introduction

Submerged surfaces in marine environments are rapidly colonized by bacteria and eukaryotic microorganisms. Biofilms composed of microfouling organisms can significantly alter substratum properties; from inducing and maintaining the corrosion of metal surfaces [9], to causing increased drag and consequently fuel consumption of ships [4]. The presence of a primary microfouling biofilm can also influence the subsequent attachment of macrofouling organisms to surfaces. Mitchell and Maki [12] demonstrated enhanced larval settlement of the bryozoan *Bugula neritina* on *Vibrio vulnificus* biofilms compared to *Deleya marina* biofilms. Holmstrom *et al* [8] isolated a toxin from a Gram-negative marine bacterium D2 which inhibited the settlement of *Balanus amphitrite* larvae.

The adhesion of microorganisms to surfaces is dependent on both physicochemical and biological factors. For example, bacteria may act as colloidal particles, demonstrating adsorption kinetics directed by short- and long-range forces between the cell and substratum [16], as well as being influenced by hydrodynamic parameters of the bulk phase such as fluid viscosity and shear stress [13]. Bacteria can also produce exopolymeric substances which serve to neutralize repulsive forces and allow for permanent attachment to a surface [11].

Traditional antifouling (AF) paints aimed at inhibiting or controlling biofouling contain copper, tributyltin or other organometal compounds, and have proven to be effective against a wide range of fouling organisms. Unfortunately, the environmental persistence and toxicity to non-target organisms make metal-based AF coatings undesirable for continued use [1]. Current approaches towards the production of alternative coatings include the incorporation of natural, degradable AF components into paints, and the use of non-toxic, fouling-release (FR) coatings designed to inhibit irreversible attachment of fouling organisms [6]. The surface energy characteristics of FR coatings fall into a narrow range with critical surface tensions of 20–30 dynes cm^{-2} [3].

Many systems utilized for investigating biofilm formation and development involve the removal or destruction of attached cells for enumeration and for determination of cellular activity, thus limiting the ability to accurately measure biofilm processes spatially in real time [15]. A non-destructive, laminar flow biofilm monitoring system [14] has been used to detect biofilm biomass and to evaluate the efficacy of AF coatings using tryptophan fluorescence (TF) and bioluminescence (BL) of *Vibrio harveyi* biofilms. Tryptophan fluorescence is a reliable indicator of bacterial biomass and correlates with plate counts and with acridine orange direct counts of biofilm samples removed from stainless steel coupons [2]. Bioluminescence from *V. harveyi* cells can be used to determine biomass during the initial

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stages of biofilm development but the method loses sensitivity as the biofilm matures [2]. However, BL may be used as an indicator of the overall metabolic state of the cell. The expression of BL by *V. harveyi* depends on the nutritional status of the cell, the oxygen concentration of the bulk-phase liquid, temperature, and the level of catabolites and cAMP [7]. Perturbations in a cell's environment that interfere with cellular metabolism may result in a measurable change (usually but not always a decrease) in the light intensity emitted by a population.

In the present study, a laminar flow system was used to evaluate several types of AF and FR coatings for their ability to inhibit cellular attachment and/or to control biofilm growth. The AF coatings were polymers designed to release a model biocide, C9211, at different flux rates which varied with time. The FR coatings included five different non-toxic polydimethylsiloxane (PDMS) polymers that possessed surface energy characteristics compatible with minimal attachment of fouling organisms. *V. harveyi* cells were allowed to attach and grow on the coating surfaces for 4 days under low flow conditions while biofilm biomass and activity were measured non-destructively as TF and BL respectively. Fouling-release efficacy of the PDMS coatings was determined by subjecting the coatings to a high shear stress (330 dynes cm^{-2}) and calculating the percentage of biomass stripped from the surface.

Materials and methods

Organism and culture conditions

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 [14].

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 L 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 over a period of 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.

Flow cell system

The laminar flow cells (Figure 1, [14]) had flow channels measuring 76 mm × 405 mm × 2 mm. The bottom half of the cell was constructed of high molecular weight plastic into which a series of five coupons (35 × 70 × 3 mm) were inserted flush with the flow channel. The top half consisted of translucent Lexan® (General Electric, Mt Vernon, IN, USA) which could accommodate 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 via fiber optic light cables. 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. The C9211 coatings were sterilized by exposure to germicidal UV light.

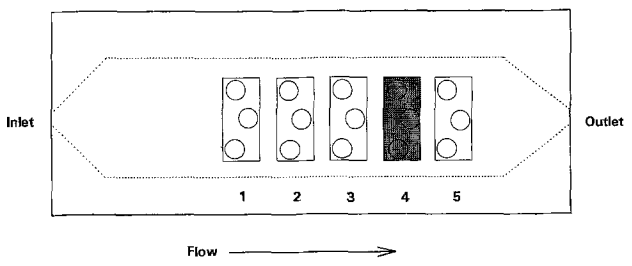

Figure 1 Overhead schematic of the laminar flow cell indicating flow direction and relative positions of the upstream stainless steel control coupon (#3), the test coating or surface (#4), and the downstream stainless steel coupon (#5). The dotted line indicates the flow channel and the circles represent viewing ports above each coupon

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}.

Fluorescence and bioluminescence measurements

Biofilm fluorescence was measured using an F212 Fluorolog II fluorometer (Spex Industries Inc, Edison, NJ, USA) equipped with a bifurcated fiber optic light cable and containing dual gratings for both excitation and emission wavelengths [2]. Tryptophan fluorescence 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, USA) in units of namps as described by Mittelman *et al* [14].

Determination of antifouling coating efficacy

As reported previously [14], stainless steel coupons in positions #3–5 (Figure 1) of the laminar flow cells were colonized by similar numbers of bacteria, determined by TF, BL, direct and viable cell counts. The AF efficacy of each coating was determined by the ratio of measurements from the coating inserted at position #4 of the flow cell (Figure 1, Table 1) to those of the stainless steel control coupon inserted at position #3 (upstream of position #4). This design allowed for a direct comparison of results between experiments and minimized error due to differences in inoculum density.

In order to detect the effect of coating components released downstream, the ratio of TF and BL measurements obtained from a stainless steel coupon in position #5 (directly downstream of the coating) to the upstream stainless steel coupon was determined.

Fouling-release coating properties

To determine the strength of biofilm adhesion to the PDMS coatings, 4-day-old biofilms were subjected to a flow rate of 1 L 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.

Table 1 List of ratios for on-line analysis of AF and FR coatings

Description	Ratio[a]	Measurement
Biomass (AF efficacy)	#4/#3	Tryptophan fluorescence
Cellular activity (AF efficacy)	#4/#3	Bioluminescence
Leaching	#5/#3	Tryptophan fluorescence Bioluminescence
Biomass (FR efficacy)	#4/#3 (post-exposure to shear stress)	Tryptophan fluorescence
Percent biomass loss	1–(#4 post-exposure/ #4 pre-exposure)	Tryptophan fluorescence
Endpoint cell count	#4/#3	Acridine orange direct counts

[a]Numbers preceded by # indicate coupon positions in Figure 1

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 to sterilize it. Glass clamp-on extractors with an extraction area of 1.131 cm^2 (Kontes Glass, Vineland, NJ, USA) were fastened with an o-ring onto the coupon surface. The biofilm material was pulse-sonicated (Heat Systems sonicator, Plainview, NJ, USA) into 1 ml of ASW with three 1-s pulses at 20% power. The sonication suspension was transferred to a sterile screw-capped tube containing glass beads. The procedure was repeated and the extractor rinsed with 1 ml of ASW, resulting in a total of 3 ml of sonicated suspension. Vortexing the suspension in the presence of the glass beads served to break up the biofilm material to make it suitable for microscopic examination. Three samples (the area viewed by each port) were extracted from each coupon.

For direct microscopic evaluation, the sonicated suspension was diluted 1 : 10 in ASW, and 1 ml was filtered (0.2-μm pore size black polycarbonate membrane; Costar, Cambridge, MA, USA) and stained for 3 min with an acridine orange solution (1% w/v acridine orange in 100 mM phosphate buffer, pH 7.5). Air-dried filters were fixed onto glass slides using immersion oil and examined under epifluorescence illumination.

Test coatings

The descriptions of the test coatings are listed in Table 2. The C9211 coatings consisted of the biocide dispersed into a vinyl co-polymer. Further characterization of the coating process and flux studies are described by Weisman *et al* [19]. Three concentrations of C9211 were tested for AF efficacy: 5, 10, and 35 wt%, yielding flux rates of 0.9, 2.1, and 20 $\mu g\ cm^{-2}\ day^{-1}$, respectively at the time of the tests.

Five polydimethylsiloxane (PDMS) coatings were also tested in this study. TD1 and TD2 differed in the proportion of difunctional PDMS chains and filler material. The smaller proportion of PDMS in TD2 conferred a higher modulus and lower degree of molecular rearrangement compared to TD1. TD3 was similar to TD1 except that 15% of the PDMS was monofunctional; one end of the chain was tethered to the coating and the other end extended into the bulk phase. TD4 and TD5 contained greater proportions of difunctional PDMS than the previous three coatings, and TD5 contained an additive which hydrolyzed upon exposure to seawater, increasing the concentration of silanol groups at the surface.

Statistical analysis

Three measurements (one from each port) were recorded for each coupon. Ratios were calculated at four time points over a 96-h period from three independent replicates for a sample size of 36 for each coating. Statistical significance of the results was determined by the Student's *t*-test at a 95% probability level. The normal distribution of measurements was confirmed by the Shapiro–Wilke's test.

Table 2 Coating designations and descriptions

Coating	Type	Description
STL	Control	316 stainless steel with 600 grit finish
C05	AF	5 wt% C9211; 0.9 $\mu g\ cm^{-2}\ day^{-1}$ flux rate
C10	AF	10 wt% C9211; 2.1 $\mu g\ cm^{-2}\ day^{-1}$ flux rate
C35	AF	35 wt% C9211; 20 $\mu g\ cm^{-2}\ day^{-1}$ flux rate
TD1	FR	70 : 30 difunctional PDMS : filler
TD2	FR	25 : 75 difunctional PDMS : filler
TD3	FR	70 : 30 PDMS : filler; PDMS composed of 85% difunctional and 15% monofunctional chains
TD4	FR	90 : 10 difunctional PDMS : filler
TD5	FR	90 : 10 difunctional PDMS : filler, with hydrolyzable additive

Results

Antifouling efficacy

The AF efficacy of each coating was determined by comparing the TF measured from the coating inserted into position #4 of the flow cell, to that of the stainless steel coupon upstream from the coating in position #3 (Figure 1, Table 1). When stainless steel coupons were inserted into both positions as a control (STL), the TF ratio was 0.92 ± 0.24 over 4 days (Figure 2), which indicated a similar rate of biofilm development on both surfaces during that time period. Based on these results, coatings that demonstrated a TF ratio of approximately 0.65 or less within one standard deviation were considered effective at inhibiting biofilm biomass.

Figure 2 shows that C35 and C10 inhibited *V. harveyi* biofilm growth as demonstrated by TF ratios of 0.18 and 0.42 respectively. C05 was somewhat effective although this result was not statistically different from STL. The coatings were designed to release the biocide at variable flux rates (Table 2) but the TF ratios did not vary significantly over the 4-day test period.

The PDMS coatings were not as effective as the C9211 coatings at inhibiting biofilm growth under low flow conditions (Figure 3) and were not significantly different from STL. Furthermore, there was no significant difference in the TF ratios between the PDMS coatings (0.67–0.83).

Bioluminescence measurements

Generally, the BL ratios for the test coatings were less than 1.0, exhibiting a lower overall light intensity from the biofilm cells on the coatings compared to cells attached to the upstream stainless steel coupon (Figures 2 and 3). There

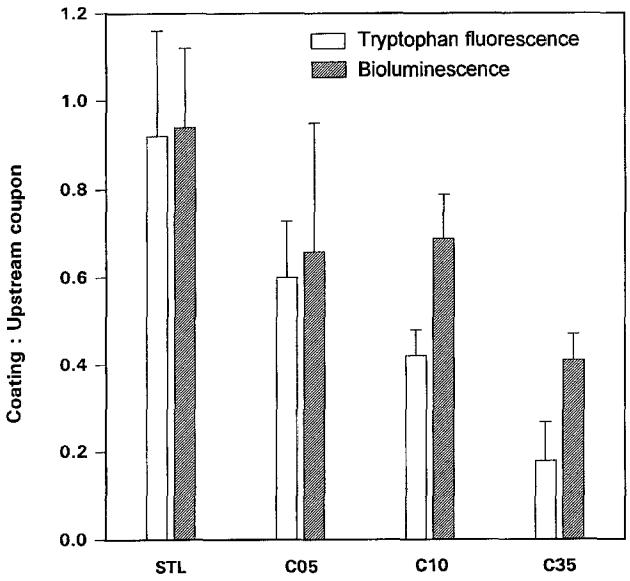

Figure 2 Antifouling efficacies of three concentrations of C9211 determined by TF and BL ratios of the coating to upstream stainless steel coupon. Coatings demonstrating a TF ratio of 0.65 or less were considered effective at inhibiting biofilm biomass accumulation. Values represent the mean of readings taken over a 4-day period (in triplicate) during low flow conditions. STL was the same ratio with a stainless steel coupon inserted in the coating position #4 (see Figure 1). Tryptophan fluorescence was measured as relative fluorescence; BL units were in namps

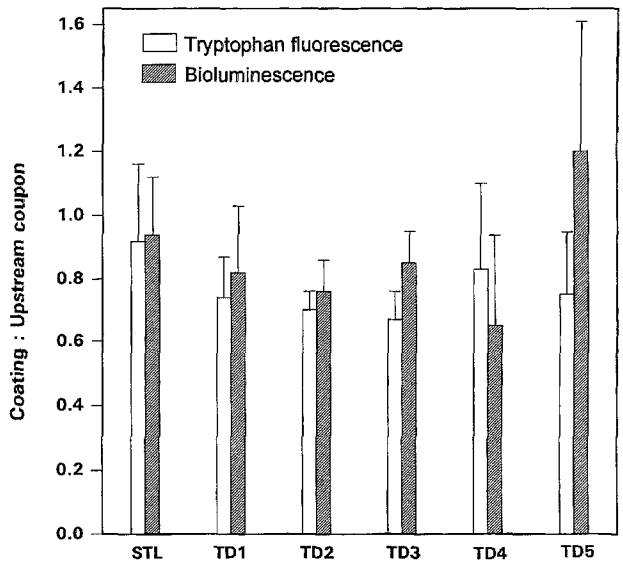

Figure 3 Antifouling efficacies of five PDMS coatings determined by TF and BL ratios of the coating to upstream stainless steel coupon. Coatings which demonstrated a TF ratio of 0.65 or less were considered effective at inhibiting biofilm biomass accumulation. Values represent the mean of readings taken over a 4-day period (in triplicate) during low flow conditions. STL was the same ratio with a stainless steel coupon inserted in the coating position #4 (see Figure 1). Tryptophan fluorescence was measured as relative fluorescence, BL units were in namps

was no significant difference between the BL and TF ratios for the PDMS coatings; however, the BL ratios for all C9211 concentrations were higher than the TF ratios. This difference was significant for the C10 and C35 coatings, suggesting that the BL yield was greater from cells attached to these coatings compared to cells attached to stainless steel. This result has also been observed with AF coatings containing furan compounds and zosteric acid (data not shown).

In contrast to the results from the C9211 coatings, there was no significant difference between the TF and BL ratios observed from STL or the five PDMS coatings.

Downstream effects

The effect of C9211 leachate from the coatings on biofilm cells downstream was determined by calculating the TF ratio of the stainless steel coupon downstream of the coating to that of the upstream stainless steel coating (leaching ratio). The leaching ratio was less than 1.0 at all three C9211 concentrations (data not shown). Only the C35 coating demonstrated a significant leaching effect (TF ratio, 0.43 ± 0.11). The leaching ratio for STL was 0.86 ± 0.16.

No significant leaching was detected from the 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.

The BL leaching ratio of the C35 coating was significantly greater than the TF leaching ratio. This difference was not observed with the C05 and C10 coatings. TD2 was the only PDMS coating which exhibited a similarly increased BL leaching ratio.

Table 3 The percent of biomass stripped from the coating after a 15-min exposure to a shear stress of 330 dynes cm^{-2}

Coating	% Biomass loss ± SD
STL	55 ± 12
TD1	70 ± 6.6
TD2	60 ± 14
TD3	68 ± 4.6
TD4	63 ± 9.6
TD5	69 ± 12

Fouling-release

To test the strength of biofilm adhesion after 4 days, the PDMS coatings were subjected to a shear stress of 330 dynes cm^{-2} for 15 min, mediated by an increased flow rate to 1 L min^{-1}. The amount of biomass stripped from each coating varied between 60–70% (Table 3). Biomass measurements from STL decreased by an average of 55%. The TF ratios (coating : upstream steel coupon) were generally decreased after high-flow exposure with the exception of TD4 (Figure 4). The most effective coatings were TD3 and TD5 with TF ratios of 0.55 and 0.45 respectively.

Direct microscopic counts

Microscopic count ratios generally correlated with the final TF ratios from all coatings tested (Table 4). C10 and C35 had ratios significantly lower than STL. TD4 had a significantly higher count ratio than the other PDMS coatings. Count ratios from TD1, TD3 and TD5 were lower than STL.

Discussion

The advantages of the on-line biofilm monitoring system used in this study were the ability to segregate spatially cell

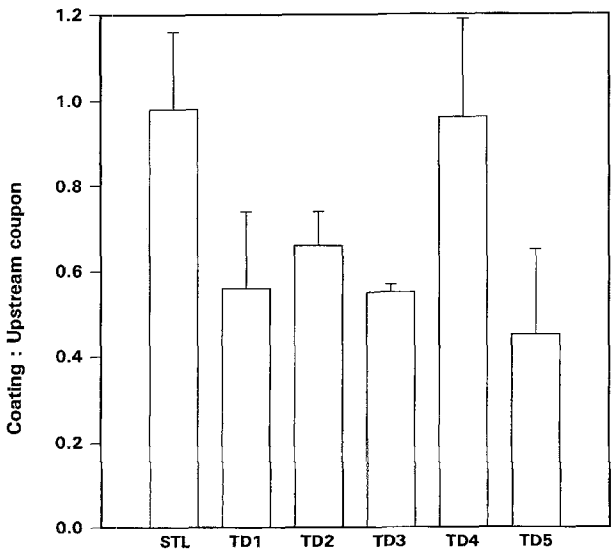

Figure 4 Fouling-release efficacies of the PDMS coatings determined by the ratio of TF measurements of the coating to the upstream steel coupon after 15 min of applied shear stress at 330 dynes cm^{-2}

Table 4 Ratio of endpoint microscopic counts from the coating to the upstream steel coupon

Coating	Ratio ± SD
STL	0.84 ± 0.10
C05	0.65 ± 0.09
C10	0.48 ± 0.06
C35	0.32 ± 0.12
TD1	0.55 ± 0.16
TD2	0.76 ± 0.12
TD3	0.48 ± 0.16
TD4	1.10 ± 0.24
TD5	0.52 ± 0.06

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.

Using stainless steel (STL) as attachment substratum, no significant difference was detected between biomass measurements on coupons #3–5. However, C9211 at 10 and 35 wt% significantly inhibited biofilm biomass. Tryptophan fluorescence as a biomass measure was confirmed by direct epifluorescent microscopic counts. It was not apparent whether the biomass on these coatings decreased due to inhibition of initial cell attachment, or if growth and replication of attached cells was suppressed. Bioluminescence measurements did indicate that some portion of the attached cells were metabolically active.

The sensitivity of bacterial BL to toxic compounds (decreased light output as a function of increased toxicity) forms the basis of a number of commercial tests (eg, Microtox® Toxicity Test System, Microbics Corp, Carlsbad, CA, USA). The level of BL measured from cells attached to the C9211 coatings was indeed lower than levels obtained from cells on steel. However, for the effective concentrations of C9211, the BL ratios of coating to upstream steel coupon were significantly greater than the analogous ratios for biomass (TF; also correlated by endpoint microscopic counts) which suggests an increase in BL yield from the coating biofilm cells. Several factors could contribute to this result, including differences between coating and control biofilm architecture (ie channelling and thickness) that result in changes in oxygen and nutrient diffusivity [5,10,17]. Our data support the interpretation that the presence of C9211 increased BL yield. We propose that the biocide had a detrimental effect on cellular metabolism, which resulted in changes in luciferase activity similar to the effect of cyanide on *V. harveyi* as reported by Ulitzur *et al* [18] who suggested that inhibition of the electron transport system by cyanide and other agents created an increase in the intracellular pool of reduced coenzymes and aldehyde utilized by the luciferase system. Because the increase in BL yield was coupled to a significant decrease

in biomass, a similar disruption in metabolic function may have occurred in cells attached to the C9211 coatings. No increase in BL was observed in cells attached to STL or from cells attached to the non-toxic PDMS coatings; nor did any of these coatings demonstrate a significant decrease in biomass. Therefore, the increase in BL relative to biomass levels may reflect cellular toxicity or stress in cells attached to certain AF coatings.

Ideally, the active component in an AF coating should be effective at the surface of a coating, but be minimally toxic to non-target organisms. C9211, the AF compound used as a model in this study, demonstrated a significant decrease in biomass downstream at a concentration of 35 wt%. The toxic effect of this concentration was also reflected by an increased BL ratio relative to the TF ratio. C10 was also effective at the coating surface, but the downstream effect was not significant. Therefore, a C9211 concentration somewhere between 10 and 35 wt% may be optimal for surface effectiveness while not impacting the environment upon release.

The ineffectiveness of the PDMS coatings under low flow conditions was not surprising in the absence of a toxicant additive. Polydimethylsiloxane coatings have surface energy characteristics consistent with successful FR-type surfaces [3], which are designed particularly to minimize the strength of adhesion of hard-fouling organisms, resulting in a greater release of fouling biomass upon mechanical brushing or shear stress due to ship service. Four of the five PDMS coatings performed better than STL against soft fouling subsequent to high flow rate exposure. The exception, TD4, contained the highest PDMS : filler ratio and exhibited FR properties similar to STL. TD5 contained the same proportions of PDMS as TD4 (Table 2) yet performed significantly better. One possibility for the observed results is that sloughing of the hydrolyzable additive from the coating surface imparted an ablative characteristic to TD5. Also, the increased surface concentration of silanol resulting from the additive in TD5 may have resulted in an increased cation concentration at the surface which affected cellular attachment. Coatings TD1–3 all demonstrated similar efficacies, despite differences in PDMS : filler ratios (TD1 and TD2), and in TD3, that 15% of the PDMS was anchored to the coating at one end only, as opposed to being crosslinked. This tethered approach may be useful to attach active compounds to coatings to prevent excessive leaching into the surrounding environment.

The laminar flow cell system was utilized to evaluate the performance of both AF and FR type coatings against bacterial fouling. Using tryptophan fluorescence as a non-destructive measure of biomass, the efficacy of antifouling agents can be screened at the substratum level as well as their effect on downstream microbiota. *V. harveyi* activity was measured on-line via bioluminescence, which may provide insights into the mechanisms of toxicity of test compounds. By increasing the flow rate, the same techniques can be used to rank FR coatings for their relative ability to release bacterial fouling. Thus, this method can be used to compare different types of fouling control materials.

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