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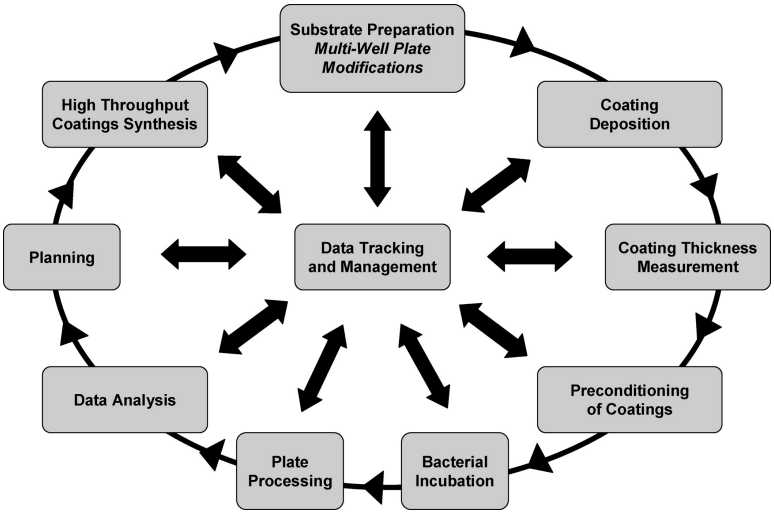

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Articles

Combinatorial Materials Research Applied to the Development of New Surface Coatings I: A Multiwell Plate Screening Method for the High-Throughput Assessment of Bacterial Biofilm Retention on Surfaces

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Combinatorial, high-throughput capabilities have been established to aid in the rapid development of new and effective antifouling marine coatings for naval applications. A biological screening process involving marine bacteria was developed that allows for rapid and effective quantification of bacterial biofilm growth and retention on large numbers of coating surfaces in parallel. The screening process involves (1) multiwell plate modifications for coating deposition, (2) deposition of combinatorial coating libraries via an automated liquid dispensing robot, (3) coating thickness measurements of cured coatings, (4) preconditioning of coatings via immersion in deionized water, (5) bacterial incubation, (6) plate processing, and (7) data analysis for identification of promising candidates. The details of the method developed are described in this document.

Introduction

Fouling of the hulls of ships by marine organisms results in increased frictional drag and corrosion, loss of maneuverability, frequent dry-docking, and introduction of invasive species.[1−4] Historically, the most effective marine coatings employed toxic metal compounds, such as tributyltin (TBT) or copper, to combat fouling.[5] Although effective in minimizing fouling, the release and accumulation of such compounds in ocean waters is adverse or harmful to the environment.[6−9] As a result, the International Maritime Organization (IMO) has recently instituted a ban on the use of TBT-containing marine paints, facilitating the need for the development of new environmentally friendly marine coatings.

In response to the need for new environmentally friendly marine coatings, the authors have applied a combinatorial, high-throughput approach to rapidly develop desirable new coatings.[10−14] The high-throughput capabilities include rapid synthesis and formulation of new polymers and coatings as well as parallel screening of coating surface and mechanical properties. Although fundamental material properties are important for understanding the materials produced, it was felt that a suite of high-throughput biological assays would be necessary to effectively down-select coating candidates and identify promising formulations that would warrant advanced testing at ocean test sites.

The compositional makeup of biofouling communities usually consists of numerous species and is influenced by several factors, including salinity, temperature, pH, and other important variables.[5] Several relevant fouling species have been utilized in laboratory assays to assess the performance of antifouling materials or compounds, including barnacles, tubeworms, mussels, oysters, algae, diatoms, and bacteria.[15−20]

The authors chose to develop a high-throughput screening workflow based on marine bacteria to screen coating performance. Marine bacteria are relatively easy to acquire, maintain, and utilize and can be continually cultured from cryo-preserved stocks to conserve genotype from experiment to experiment. It was anticipated that incorporation of other relevant fouling organisms into the existing workflow may be required to enhance the effectiveness of the screening of coatings. The development and implementation of a high-throughput biological screening workflow involving marine bacteria to rapidly assess the antifouling characteristics of coatings is described.

Experimental Section

Materials, Reagents, and Media Preparation. All media and buffers were prepared with deionized water. Marine broth (MB) and marine agar (MA) (Catalogue no. 279110, Catalogue no. 212185, respectively; Becton Dickinson La-

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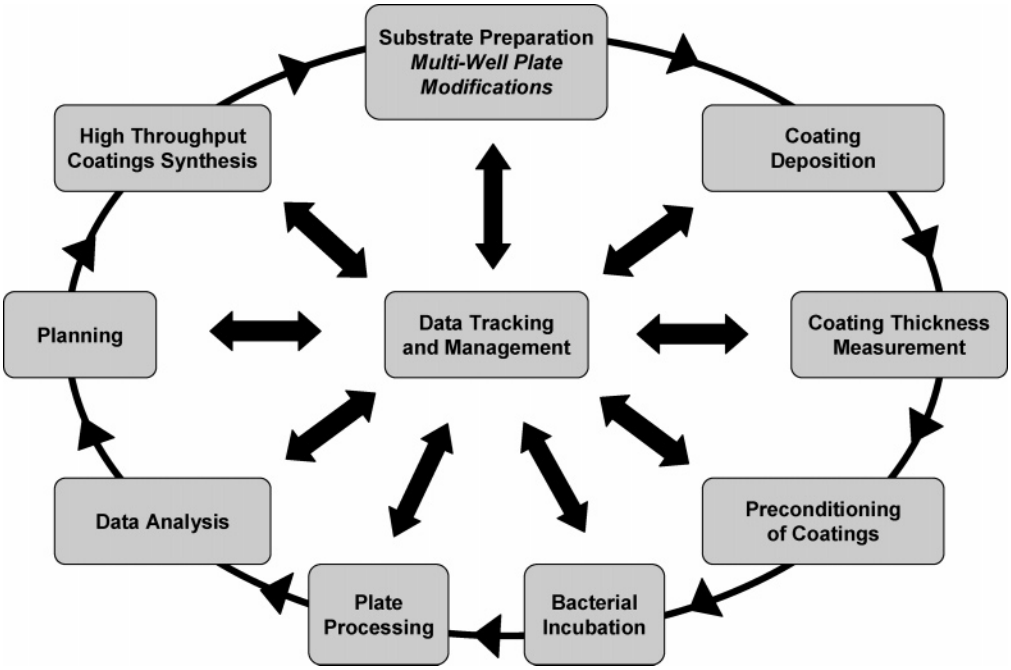

Figure 1. Schematic of the high-throughput biological workflow developed.

bware, Franklin Lakes, NJ) were prepared according to the manufacturer's specifications. MB was vacuum-filtered to remove residual particulate material. MB and MA preparations were autoclaved at 121 °C and 15 psi for 15 min. Artificial seawater (ASW) was prepared by dissolving 38.5 g of sea salts (Catalogue no. S9883, Sigma-Aldrich, St. Louis, MO) in a liter of deionized water. Biofilm growth media (BGM) was prepared by supplementing 1 L of ASW with 500 mg of dextrose or peptone and 100 mg of yeast extract (Catalogue no. DX0152-1, EMD Chemicals, Gibbstown, NJ; Catalogue no. 211693, Becton Dickinson Labware, Franklin Lakes, NJ, Catalogue no. 1-03753-0500, EMD Chemicals). ASW and BGM were filter-sterilized via vacuum filtration utilizing 0.2-μm VacuCap bottle-top filters (Pall Life Sciences, East Hills, NY). Falcon 24-well polystyrene plates (Product no. BD 351147, Becton Dickinson Labware, Franklin Lakes, NJ), 15-mm glass coverslips (Catalogue no. 72195-15, Electron Microscopy Sciences, Fort Washington, PA), and a two-component potting epoxy (Catalogue no. 7548A11, McMaster-Carr, Chicago, IL) were used for substrate preparation. Harleco crystal violet solution (0.3%) (Product no. 65092A, EMD Chemicals) was used for staining biofilms. Folding skirt septa (3BC-78317, Lab Safety Supply Inc., Janesville, WI) and hold-down clamps (Product no. 5126A29, McMaster-Carr) were utilized for fabrication of the extraction template and clamping device. Glacial acetic acid (33%) (Product no. 9507-05, J. T. Baker, Phillipsburg, NJ) and 96-well microtiter plates (Product no. 269620, Nalge Nunc Intl., Rochester, NY) were used as received for crystal violet quantification.

Bacterial Strains. *Halomonas pacifica* ATCC 27122 was received as a lyophilized powder and revived in MB. *Cytophaga lytica* was generously provided by Dr. Michael Hadfield of the Kewalo Marine Laboratory, University of Hawaii.[21] Revived stocks were subcultured twice and stored in frozen 1.0-mL aliquots at −80 °C in MB containing 20% glycerol. Experimental cultures were maintained weekly on MA at 4 °C from cryopreserved stocks. Bacteria were precultured from MA plates in MB at 28 °C for ∼20 to 24 h with shaking prior to bacterial inoculation. Bacterial cells were harvested by centrifugation at 4000*g* for 10 min, washed three times in ASW, and resuspended in BGM to a final density of 10^6-10^7 bacteria/mL.

Preparation of Control Coatings. Silicone resins Dow Corning 3140 (DC 3140), General Electric RTV-11 (RTV), Dow Corning T2 Silastic (T2) (Ellsworth Adhesives, Bloomington, MN), and methyl methacrylate Paraloid B-44S (ChemCentral, Lakeville, MN) were purchased and prepared via the manufacturer's specifications. Suspensions for deposition were prepared in Reducer No. 15 (Product no. R7K15, Sherwin Williams, Cleveland, OH) to a final solids content of 30−40% by weight.

Results and Discussion

Overview of the High-Throughput Biological Workflow. The high-throughput biological screening workflow developed and implemented is shown schematically in Figure 1. Combinatorially produced arrays of liquid coatings are deposited into custom modified multiwell plates. Plates are modified with solvent-resistant liners to minimize contact and degradation of the plate material, enabling uniform and uncontaminated films to be obtained. Plates are then tagged with a barcode label, corresponding to the coating library identification, to track each coating of the array as it transverses through the workflow. Once tagged, plates are placed in temperature-controlled vacuum ovens to facilitate an adequate curing process for subsequent testing. Once fully cured, each coating material is then measured for film thickness via a standard depth displacement gauge mounted on a machined block. To remove any residual solvent, catalyst, or monomers that may be present in the coating matrix upon curing, plates are then placed into a custom-made, high-capacity, circulating water tank. Upon completion of the preconditioning treatment, plates are then removed,

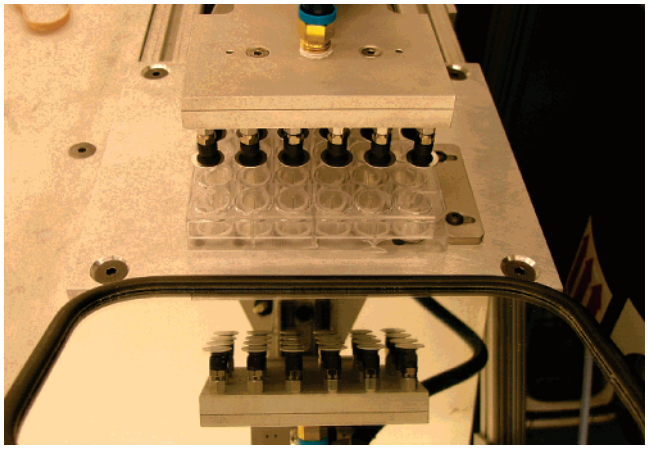

Figure 2. Image of the semiautomated substrate modification apparatus.

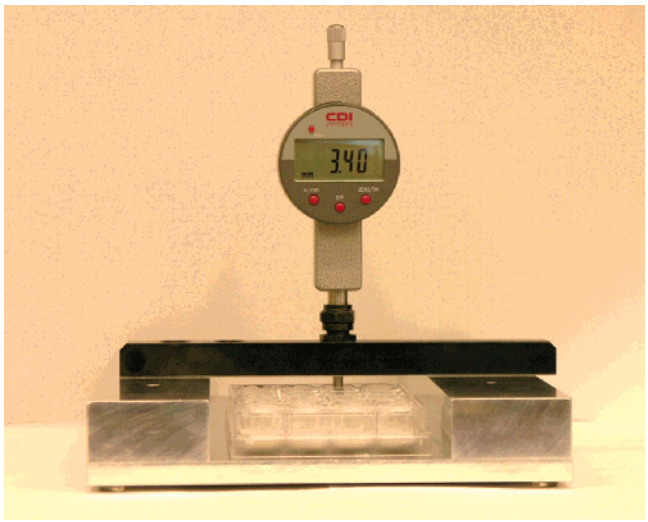

Figure 3. Image of the depth displacement gauge utilized to measure coating thickness in multiwell plates.

and screening via the high-throughput biological challenge is initiated. Coating materials cast in plate wells are inoculated with the appropriate bacterial suspension and placed into a custom built, circulating, temperature-controlled water bath for incubation. Once the incubation is complete, plates are removed from the temperature-controlled tanks and rinsed with deionized water to remove any planktonic or loosely attached biofilm. Once dry, a biomass indicator dye, crystal violet, is dispensed into each well to stain the retained biofilm. The crystal violet methodology, utilized to measure the total amount of bacterial biomass, has been routinely employed in the past to quickly and effectively determine the amount of bacterial attachment and colonization on surfaces.[22−25] Custom templates fabricated to exclusively extract the crystal violet retained in the biofilms on the coating surfaces are attached to plates, and the crystal violet is extracted with an acetic acid solution. A small aliquot of the eluate from each sample is then transferred to a 96-well plate for absorbance measurements at 600 nm utilizing a multiwell plate reader. Replicates are then averaged, and the control value (material subjected to BGM without bacteria) is subtracted to account for any potential uptake or retention of crystal violet dye by the coating material itself. Absorbance values obtained are then proportional to the amount

Figure 4. Image of the high-capacity circulating water preconditioning tank. Tank can accommodate up 360 plates simultaneously.

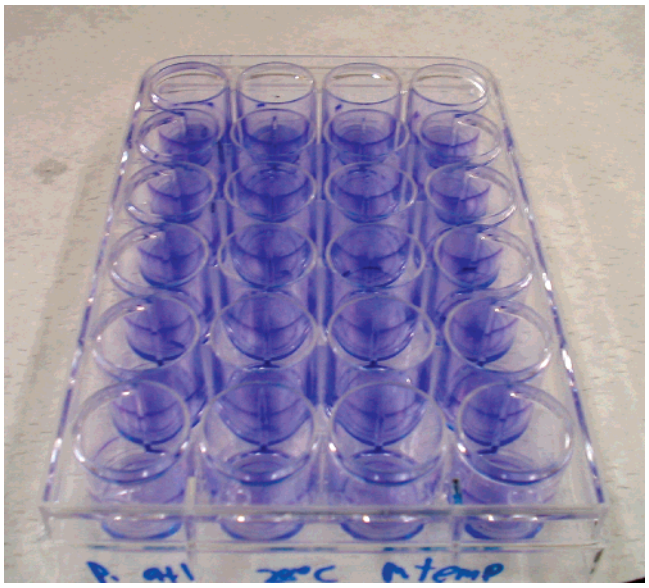

Figure 5. Image of a crystal-violet-stained 24-well polystyrene plate. Biofilm retention on the sides of the wells can be visualized.

of biofilm retained on the coating surface. Reference materials are included within each plate as a means to gauge performance of experimental materials and allow results from separate experiments to be compared to one another for the identification of promising material candidates. The text to follow describes each component of the workflow in detail and provides a brief example of its application to the analysis of coating materials.

Substrate Preparation for Coating Deposition. Polystyrene plates (24-well) were selected as the most suitable format for coating deposition and biological analysis. The modification of the well bottoms with an inert glass liner served as a means to dispense and cast coating materials with an organic component. Other materials, such as plastics

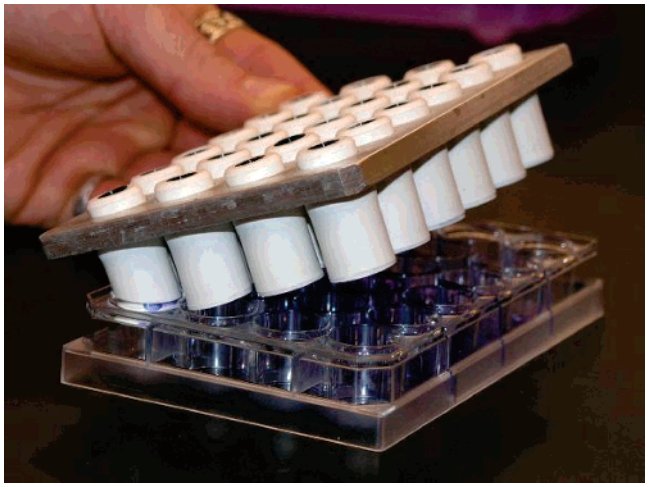

Figure 6. Image of a custom extraction template being applied to a standard 24-well polystyrene plate.

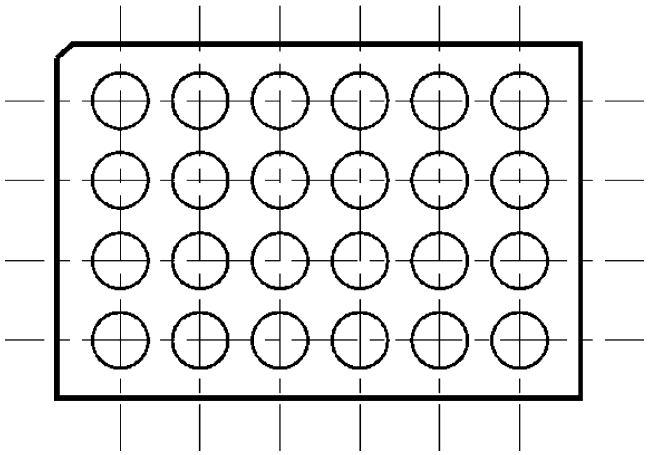

Figure 7. Illustration of the aluminum block utilized to mount the inverted folding skirt septa. Material, 6061 aluminum; hole diameter, 13.4 mm; hole spacing, 19.4 mm; L × W × H = 127 × 86 × 6.4 mm.

or metals, may be utilized as protective liners if they are compatible with the coating formulation. In the absence of this modification, organic-based resins and solvents may chemically attack the polystyrene plate, resulting in contaminated and nonuniform films. By dispensing small volumes of coating formulations directly onto the glass liner (typically 250 uL), uniform and uncontaminated films are obtained. A slight interaction does occur at the periphery of the well between the polystyrene and coating material, but this area has minimal or no effect on subsequent analysis, since the custom septa extraction template covers or masks this area, excluding it from analysis. In the case in which an extremely aggressive solvent is utilized, such as toluene, coating formulations are dispensed onto nonreactive liners outside the plate and cured. Once cured, coated disks are then adhered to the bottom of the wells, eliminating the potential for polystyrene diffusion and contamination of cast films. Alternative, commercially available well-plates were considered, but they were determined to be too expensive. The conventional 24-well format, as opposed to 48- or 96-, was chosen because a larger well area allows for higher assay sensitivity.

To modify the plates, 15-mm glass coverslips are adhered to the bottom of each well with a 30−50-μL drop of a two-component epoxy. Plates are then left at room temperature overnight to allow the epoxy to cure. To generate sufficient numbers of the modified multiwell plates, an apparatus was constructed to speed-up the disk gluing process. The apparatus is a 24-slide pick-and-place machine (Figure 2) that uses 24 vacuum-assisted suction cups mounted to a slide rail. The suction cups are first loaded with 15-mm glass coverslips from the disk storage base that has been preloaded with 2400 slides. The loaded suction cup array is then raised, allowing the operator to place a multiwell plate underneath. The array of disks is then brought down into the multiwell plate, at which point vibration and pressure are used to set the disks into the adhesive.

Coating Deposition. Coatings are dispensed into wells of the array using an automated robotic liquid handling system. The coatings are configured within the array such that each row of a 24-well plate contains one unique coating composition for a total of six replicates of four unique compositions per plate. Coating arrays are then placed in a vented enclosure at room temperature to cure. Curing conditions may vary, depending upon the composition of each coating library; however, typically, curing is done at room temperature for 1 week.

Coating Thickness Measurement. Thickness measurements of cured coatings are made with a depth displacement gauge mounted on a precision-machined gauge block (Figure 3) and have an accuracy of ±12 μm. Typically, coating materials are cast to achieve a dry film thickness of ∼200 μm.

Preconditioning of Coatings. Plates of cured coating are preconditioned in a recirculating deionized water bath to remove any residual solvent, monomers, or catalyst that may remain in the coating (Figure 4). The preconditioning tank or "preleach tank" was fabricated to accommodate large numbers of coatings substrates at one time (up to 360 well plates) in a recirculation bath. This unit uses submicrometer filtration, UV sterilization, and absorbent charcoal to maintain a clean water environment by minimizing unwanted biological and coating-to-coating leachate contamination.

Bacterial Incubation. A 1.0-mL aliquot of the appropriate bacterial suspension is delivered to each well of a coating array. Once inoculated with bacterial suspension, lids are applied, and plates are placed in a custom-fabricated, temperature-controlled, circulating, water bath incubator which has the capacity to hold 35−40 plates simultaneously. Plates are then allowed to incubate statically for 18−48 h with the incubator top closed to prevent evaporation of media. Plates are then removed and rinsed three times with 1.0 mL of deionized water to remove any residual planktonic and loosely attached bacteria. Plates are then inverted and tapped firmly against a paper towel several times and then dried at room temperature for 1 h. Upon drying, 0.5 mL of crystal violet solution is added to each well for 15 min to stain the retained biofilms. Excess stain is removed by rinsing three times with 1.0 mL of deionized water. Plates are then inverted and tapped firmly against a paper towel to help remove any residual unbound stain from wells. Plates are

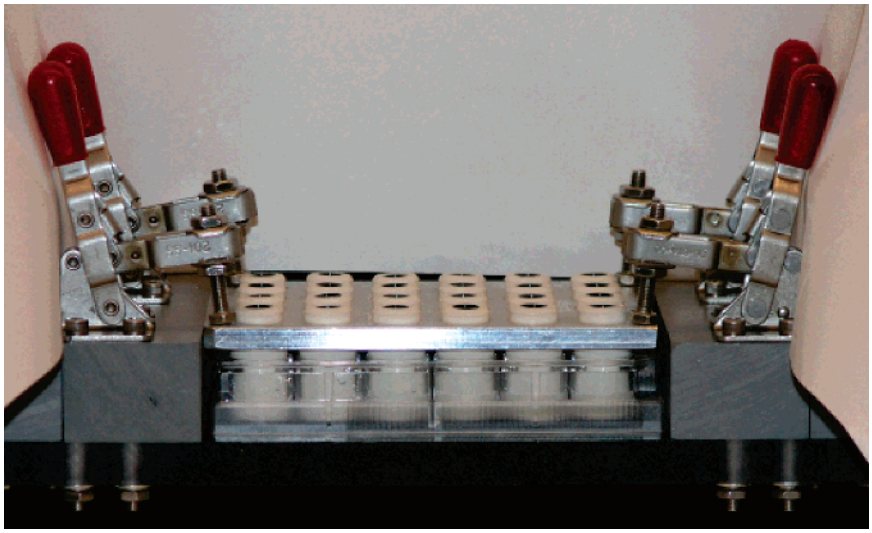

Figure 8. Image of the clamping device being utilized to apply the appropriate pressure on extraction template for crystal violet extraction.

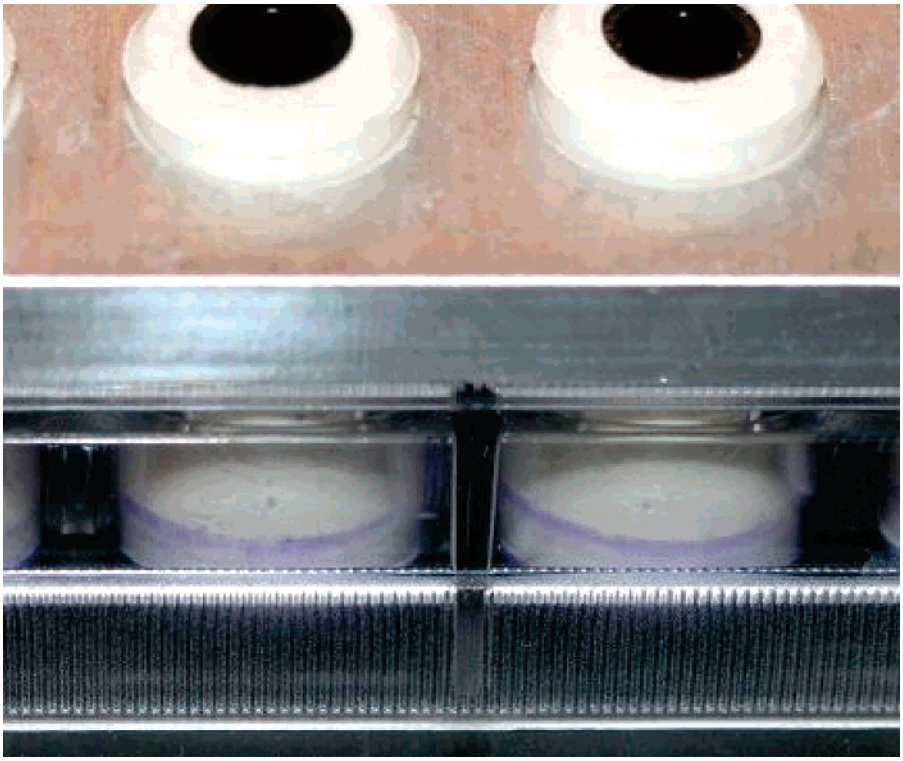

Figure 9. Image of the extraction template applied to a 24-well plate. Crystal-violet-stained biofilms retained on the sides of the wells can be visualized against the septa.

then air-dried at room temperature before extraction procedures are carried out.

Plate Processing. Since biofilms may also attach and retain to the sides of the wells (Figure 5) rather than the coating surface, a technique was developed to extract or elute crystal violet exclusively from the coating surface. To accomplish the task, custom extraction templates were fabricated to isolate extraction from the coating surface, as illustrated in Figure 6. Aluminum blocks were machined with holes to match the dimensions and configuration of the plates (Figure 7). Septa were inverted and modified with an opening for a pipet tip. The modified septa are then secured into an aluminum block. The extraction template is then immersed in deionized water briefly and then tapped on a paper towel to remove any remaining water drops before being applied to 24-well plates containing crystal-violet-stained biofilms. This additional step helps to lubricate the septa and facilitate insertion into the wells. Once the extraction template has been applied, the plate and template are placed in a custom-built clamping device to apply sufficient pressure to create a water-tight seal at the material−septa interface (Figure 8). In this configuration, the skirt of the septa masks or covers the side of the well while leaving the majority of the well bottom exposed for extraction of crystal violet on the coating surface. Due to the drying and staining procedures, bacterial films remain fixed to the sides of the well during application of the extraction template, effectively preventing this material from being extracted (Figure 9). A 500-μL portion of 33% glacial acetic acid is then added through the modified septa opening and allowed to sit for 10 min with occasional

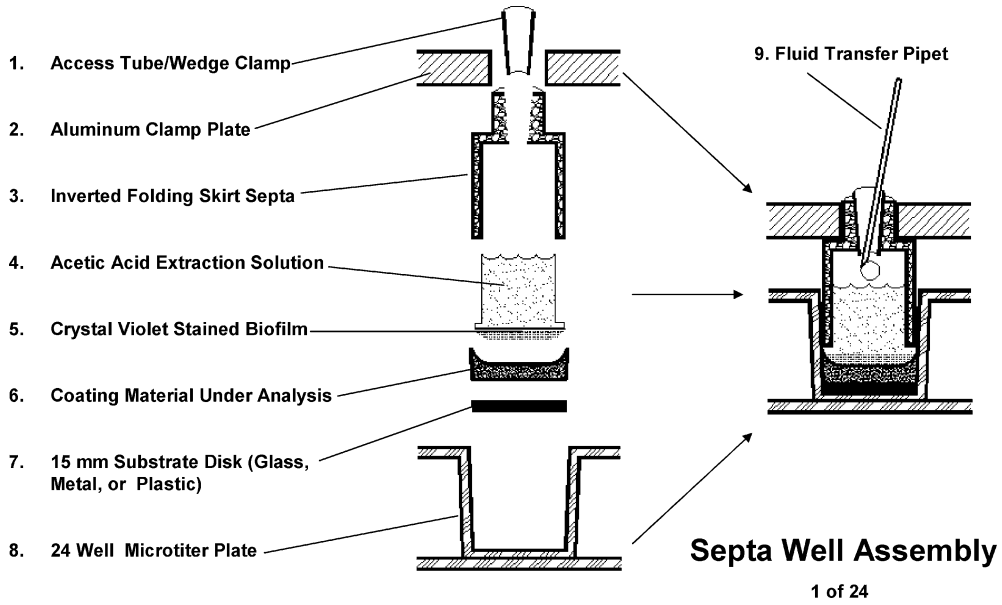

Figure 10. Exploded view of a single well assembly when the extraction template is applied.

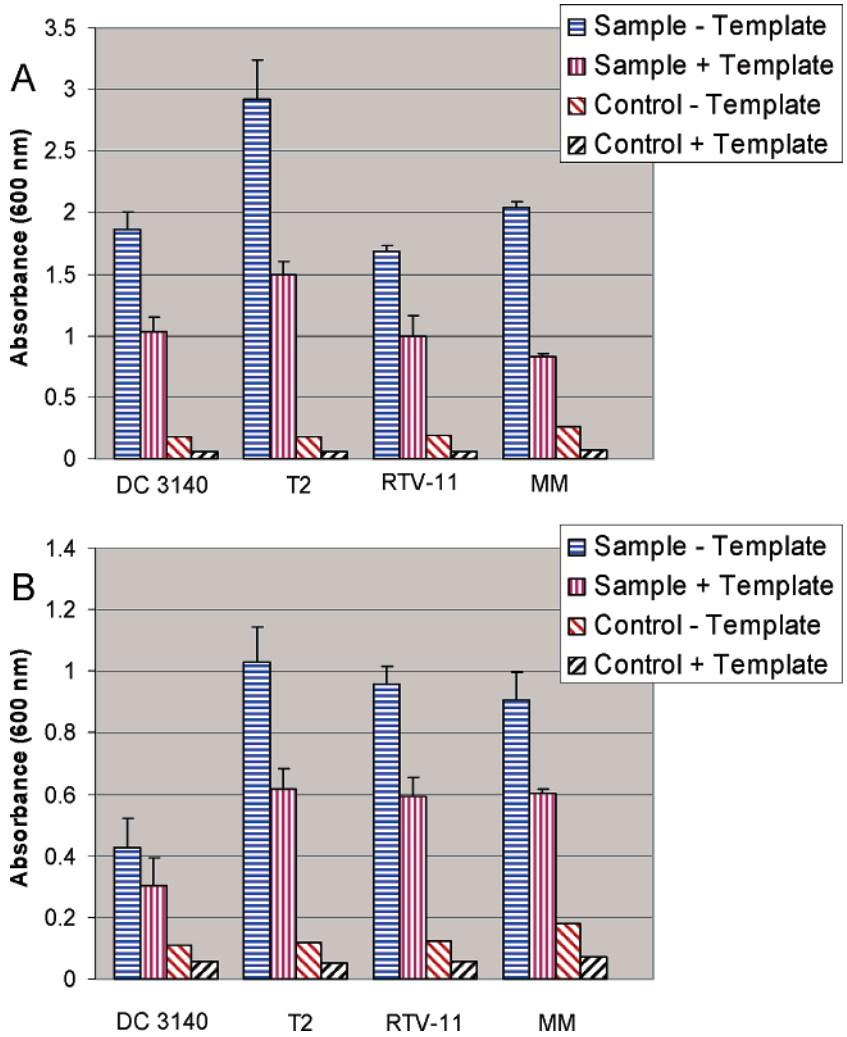

Figure 11. Crystal violet absorbance measurements with and without the extraction template. Retention of (A) *C. lytica* biofilms after 18 h of incubation at 28 °C and (B) *H. pacifica* biofilms after 48 h of incubation at 28 °C. Error bars represent one standard deviation for the average of five replicate samples. Control values are obtained from a single sample analysis. Sample = bacteria + growth media; control = growth media only.

shaking to extract the crystal violet from the coating surface. An exploded view of a single well assembly is shown in Figure 10. Aliquots (150 μL) of the eluate are then transferred to 96-well microtiter plates for absorbance measurements at

600 nm with a multiwell plate reader. In certain instances, it was necessary to make a dilution series of the eluate to obtain an absorbance value for those samples that exceed the dynamic range of the multiwell plate reader.

Analysis of Control Coatings. Results of control coatings incubated with the two marine bacteria and analyzed for biofilm retention via crystal violet quantification are illustrated in Figure 11. It is clear that when the custom extraction template is applied, release of crystal violet from the biofilms retained on the sides of wells is prevented, as indicated by the considerable reduction (30−70%) in absorbance when the template is applied. The ability of the custom extraction template to prevent side well inclusion in absorbance measurements enhances the ability of the assay to accurately discriminate between differences in coating performance.

Conclusions

A high-throughput, biological screening workflow for the discovery of new antifouling marine coatings has been developed and implemented. The basic elements of the workflow consist of processes for preparing substrates for coating deposition, measurement of dry film thickness, preconditioning via immersion in deionized water, bacterial incubation, plate processing, and data analysis for identification of promising materials. This workflow enables the production and analysis of ∼1000 coating samples/week.

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