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Effects of marine paints on microbial biofilm development on three materials

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The development of biofilms of *Pseudomonas aeruginosa* PAO-1 was studied using modified Robbins devices. Biofilm development was measured using viable counts, acridine orange direct counts (AODC), and a colorimetric method for exopolysaccharide (EPS). Biofilms reached their maximum population 24–72 h after inoculation on coupons with no paint or on coupons coated with marine paint VC-18 without additives. Biofilms on stainless steel contained higher numbers of total cells and of viable cells than biofilms on fiberglass or aluminum. Coating the surfaces with marine paint VC-18 resulted in decreased numbers of cells on stainless steel but had little effect on numbers of cells on fiberglass or aluminum. Addition to the paint of Cu or tributyltin (TBT), the active components in two types of antifouling paints, inhibited the initial development of biofilms. However, by 72–96 h, most biofilms contained the same number of cells as surfaces without additives as shown by both viable counts and AODC. Biofilms that formed on surfaces coated with Cu- or TBT-containing paint did not synthesize more EPS, suggesting that *P. aeruginosa* PAO-1 does not respond to these compounds by synthesizing more EPS, which could bind the metal and protect the cells. Rather, these biofilms may contain Cu- or TBT-resistant cells. TBT-resistant cells made up 1–10% of the viable counts in biofilms on uncoated stainless steel, but in biofilms on stainless steel coated with marine paint containing TBT, TBT-resistant cells made up as much as 50% of the population. For non-coated stainless steel surfaces, Cu-resistant cells initially made up the majority of the population, but after 48 h they made up less than 1% of the population. On Cu-coated stainless steel, Cu-resistant cells predominated through 48 h, but after 48 h they comprised less than 10% of the population. These results suggest that the growth of TBT-resistant and Cu-resistant cells contributes to biofilms of *P. aeruginosa* PAO-1 at early stages of development but not at later stages.

Keywords: microbial biofilms; modified Robbins device (MRD); antifouling paint; tributyltin (TBT); copper

Introduction

Many of the bacteria in natural environments grow on surfaces, including liquid–solid and liquid–air surfaces [31], forming biofilms. Biofilms can form on surfaces which range from surfaces in mountain streams to artificial organs, contact lenses and dental tubing. They can form on metals, including aluminum [22], stainless steel [5,17,21,25,30], and copper [14], some of which, such as Al or Cu, were formerly considered toxic to bacteria [2,3,18,19,24,28].

Biofilms can have negative effects on human activities in many ways, including: energy waste, heat transfer resistance, requirement for excess equipment capacity, decreased life of equipment, quality control problems, and safety problems [8]. Microbial biofilms can develop on surfaces placed on, in or attached to the human body [6,10]. Microbial biofilms themselves can increase boat hull roughness, thereby increasing fuel consumption, and they may contribute to the attachment of shellfish larvae to the hull [23,27], which leads to markedly increased fuel consumption [7,9]. To prevent fouling problems on surfaces immersed in water, antifouling paints can be applied to some surfaces. Both organotin- and copper-based antifouling paints effectively prevent fouling problems by macroorganisms, although organotin-based paints are effective for longer periods than copper-based paints [6]. However, both organotins and copper can be toxic to non-target marine species, such as the dog-whelk [15], oysters [1,4,26], and juvenile carp [11]. Tributyltin (TBT), in particular, causes imposex, a condition wherein females grow male sex organs, in the dog-whelk and other shellfish. Imposex interferes with reproduction [15] and has led to limitations on the use of paints containing TBT in several countries.

Little has been published on the effect of antifouling paints on microbial biofilms. Therefore, we examined the development of biofilms formed by a single organism, *Pseudomonas aeruginosa* PAO-1, on three materials without paint, on those materials coated with a marine paint, and on those surfaces coated with paint containing TBT or copper.

Materials and methods

The organism used was *Pseudomonas aeruginosa* PAO-1, a biofilm former. It was maintained on tryptic soy agar (TSA, Difco, Detroit, MI, USA). For inoculum preparation, *P. aeruginosa* PAO-1 was shaken at 37°C in tryptic soy broth (TSB, Difco) for 18 h, reaching stationary phase.

Biofilms were formed in modified Robbins devices (MRD) made of transparent acrylate [20] (AIN Plastics, Mt Vernon, NY, USA), on coupons made of aluminum, epoxy fiberglass phenolic (G-10), or stainless steel (No. 316). The materials were tested with four different surface treatments:

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without a coating, after coating them with the marine paint VC-18 (Courtaulds Coating Co, Union, NJ, USA), and after coating them with VC-18 paint containing copper or tributyltin fluoride (TBTF) used at the concentrations used in a commercial paint.

The MRD set-up (Figure 1) has been used in our laboratory and is described in Doolittle *et al* [12]. MRDs and coupons were made in our university machine shop. In each experiment, four MRD systems with the desired coupons were sterilized by filling them with 2.5%(v/v) hypochlorous acid (commercial bleach); they were allowed to stand for 2 h and then rinsed with sterile deionized water. The MRD was dried by placing it in a sterilized container, removing several studs and allowing air to circulate through the MRD. After the MRDs were dry, the coupons in four MRDs were treated with: no paint, marine paint, marine paint with 19.5% (w/v) copper (a concentration used in paints available commercially), or marine paint with 13.8% (w/v) TBTF (a concentration used in antifouling paints). Paint with copper was not applied to aluminum coupons because paints with copper are not used on aluminum boats due to problems with electrolysis. The coupons with paints were allowed to dry overnight and inserted in the MRD. The medium (1/10 strength TSB) was pumped through four MRDs operated in parallel and maintained in an environmental chamber at 37°C. Preliminary experiments indicated that a flow rate of 150 ml h^{-1} supported good biofilm development without turbulence, and without shear forces that would give uneven biofilm development in different portions of the MRD or strip the biofilm from the surfaces. Medium flow was started 20 min prior to inoculation to precondition the surface and the rate was maintained throughout the experiment except during sampling when the pump was turned off. About 1 ml of broth, containing 3×10^9 CFU of *P. aeruginosa* PAO-1, was injected into the biofilm system upstream from the MRD. Throughout the experimental period, the MRDs were inverted (coupon-side face down) to avoid bubbles at the coupon surfaces, except 20 min before and during sampling when the coupon side was up. The coupon side was turned up before sampling to avoid deposition of planktonic cells on the coupons.

Samples were taken at 6, 12, 24 h and daily thereafter up to a week. Although preliminary experiments indicated that all coupons contained an approximately equal number of cells, at each time point three coupons were sampled, one each from the upper, middle, and lower sections of the MRD (Figure 1), and the biofilms from them were combined to yield a single sample. Studs were removed from the MRD and rinsed by swirling them in phosphate-buffered saline (PBS) three times to remove loosely attached cells. Each coupon was removed and placed in an Eppendorf tube with 1.0 ml PBS. Tubes were kept in an ice bath. The coupons were then sonicated using an ultrasonic cleaner (Model 1210, Bransonic Co, Danbury, CT, USA) for 8 min and vortexed for 10 s to remove biofilms from the coupons. Suspensions from the three coupons were combined.

To quantify biofilm formation the suspensions were assayed for total viable count on tryptic soy agar; total cell counts using acridine orange (AODC) [16]; and extracellular polysaccharide (EPS) by the phenol-sulfuric acid method [13]. EPS was not measured on copper-painted coupons because copper interfered with the phenol-sulfuric acid method. To measure copper-resistant cells, viable counts were determined on plates of TSA containing

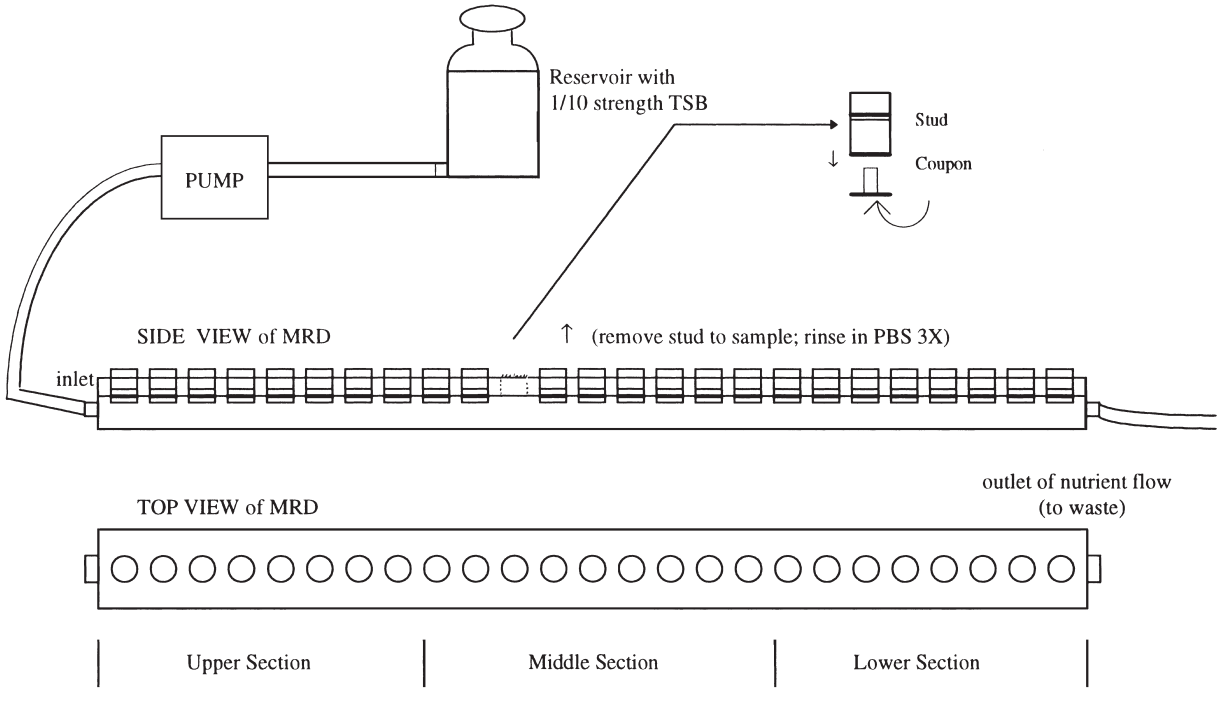

Figure 1 Modified Robbins Device (MRD). The nutrient, 1/10 strength tryptic soy broth (TSB), was pumped in by a peristaltic pump at 150 ml h^{-1}. At each sampling time, one stud was taken from each of three sections and the biofilms from them were combined. PBS: phosphate-buffered saline.

20 mM copper (II) ion, which we established was the MIC_{90} for *P. aeruginosa* PAO-1. To measure TBT-resistant cells, viable counts were determined on TSA plates with 84 μM TBT added to the medium, which we established was the MIC_{90} for *P. aeruginosa* PAO-1. All data were processed using the SAS statistics package. Differences were considered significant at the alpha = 0.05 level.

Results and discussion

The development of biofilm on different materials

Viable counts (Figure 2) and total counts (Figure 3) on all three materials without a coating reached a maximum in 24–96 h and the number remained stable for the remainder of the 6-plus-day incubation period. Viable counts were higher on uncoated stainless steel than on uncoated aluminum or fiberglass (Figure 2). The viable counts of biofilms on stainless steel were almost 10-fold higher than those on aluminum and fiberglass. Cell numbers were 10- to 100-fold higher for total counts than viable counts, suggesting that a large number of cells in the film were dead or viable-but-non-culturable on the coupon surfaces. For EPS, typical values are shown; each involved pooling samples so that a single value was obtained and standard deviations were not calculated. EPS varied throughout the incubation period. More EPS was produced in biofilms on uncoated fiberglass than on uncoated aluminum or stainless steel (Figure 4). Numbers of viable cells and total cell counts remained stable after reaching their maximum, but values for EPS fluctuated.

After biofilms reached equilibrium, the differences for viable counts and total counts between stainless steel and the other two surfaces were significant at the $\alpha = 0.01$ level (Tables 1 and 2). Thus, a more extensive biofilm developed on non-coated stainless steel than on aluminum or fiberglass.

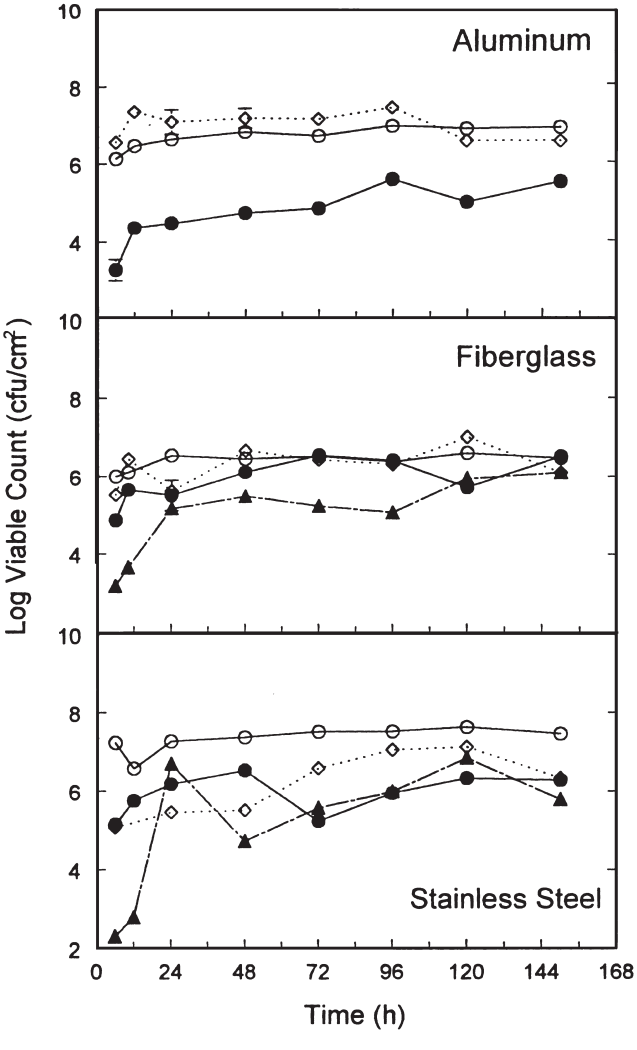

Figure 2 Viable counts from biofilms grown on the surface of aluminum, fiberglass, or stainless steel. ○: No paint; ◇: painted with VC-18; ▲: VC-18 paint with copper added; ●: VC-18 paint with TBTF added. Paint with copper was not tested on aluminum. In this figure and subsequent figures, error bars indicate one standard deviation. When no error bar is shown, the error bar fell within the symbol.

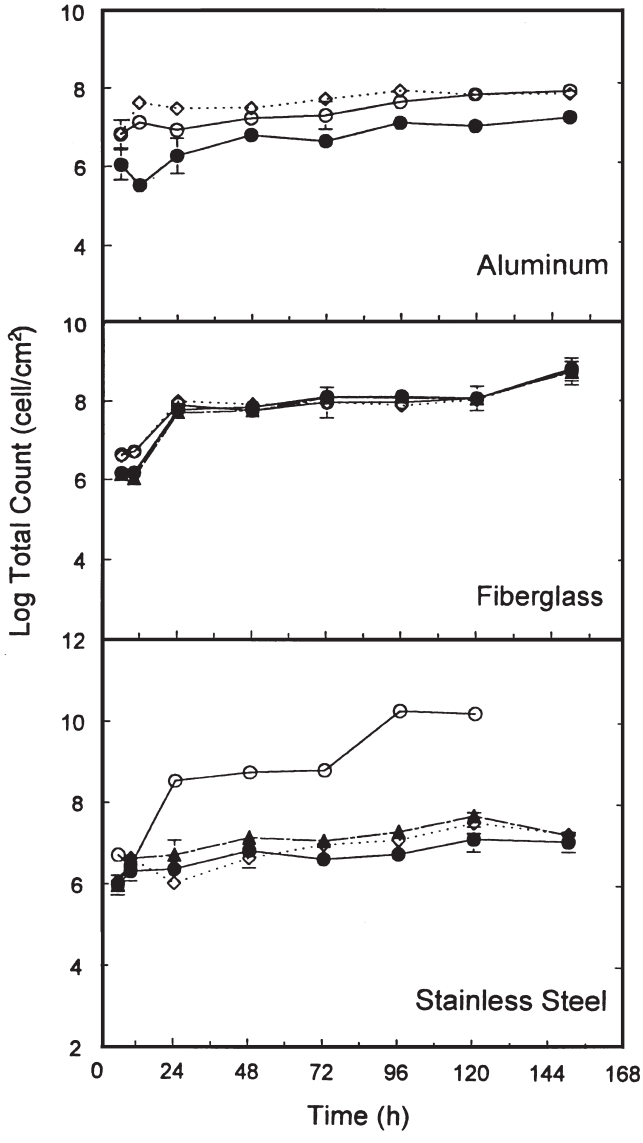

Figure 3 Direct counts from biofilms grown on the surface of aluminum, fiberglass, or stainless steel. ○: No paint; ◇: painted with VC-18; ▲: VC-18 paint with copper added; ●: VC-18 paint with TBTF added.

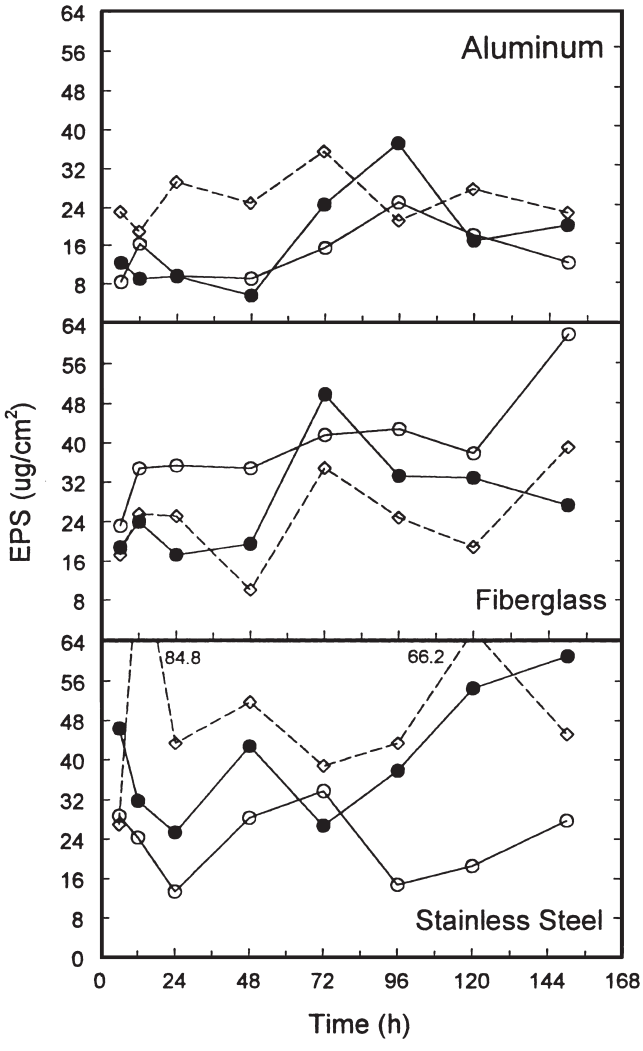

Figure 4 Exopolysaccharide content of biofilms grown on the surface of aluminum, fiberglass, or stainless steel. ○: No paint; ◇: painted with VC-18; ●: VC-18 paint with TBTF added. Error bars are not shown because each point represents a single determination from three coupons which were combined to constitute one sample, as described in the text.

Table 1 Viable counts in equilibrium phase (24–96 h) biofilms on different surfaces and with different treatments

Treatment	Log CFU*, mean ± 95% confidence interval on:		
	Aluminum	Fiberglass	Stainless steel
No paint	$^{b}6.89 \pm 0.04^{z}$	$^{a}6.50 \pm 0.04^{y}$	$^{c}7.49 \pm 0.06^{z}$
Paint	$^{b}7.24 \pm 0.11^{y}$	$^{a}6.43 \pm 0.10^{y}$	$^{a}6.70 \pm 0.17^{y}$
Paint + Cu	–	$^{a}5.50 \pm 0.17^{x}$	$^{b}5.94 \pm 0.21^{x}$
Paint + TBT	$^{a}5.24 \pm 0.17^{x}$	$^{b}6.32 \pm 0.19^{y}$	$^{b}6.07 \pm 0.20^{x}$

*Means with different superscripts are significantly different at the $\alpha = 0.05$ level; letters a–c indicate results of statistical analysis of differences between materials (aluminum, fiberglass, and stainless steel); letters x–z indicate results of statistical analysis of differences between treatments.

Table 2 Total counts in equilibrium phase (24–96 h) biofilms on different surfaces and with different treatments

Treatment	Log CFU*, mean ± 95% confidence interval on:		
	Aluminum	Fiberglass	Stainless steel
No paint	$^{a}7.71 \pm 0.16^{y}$	$^{a}7.93 \pm 0.18^{x}$	$^{b}8.72 \pm 0.22^{z}$
Paint	$^{ab}7.75 \pm 0.13^{y}$	$^{b}7.96 \pm 0.23^{x}$	$^{a}7.29 \pm 0.27^{y}$
Paint + Cu	–	$^{b}8.07 \pm 0.14^{x}$	$^{a}7.41 \pm 0.26^{y}$
Paint + TBT	$^{a}7.04 \pm 0.13^{x}$	$^{b}8.09 \pm 0.19^{x}$	$^{a}6.88 \pm 0.24^{x}$

*Means with different superscripts are significantly different at the $\alpha = 0.05$ level; letters a–c indicate results of statistical analysis of differences between materials (aluminum, fiberglass, and stainless steel); letters x–z indicate results of statistical analysis of differences between treatments.

Coupons with marine paint

Coating the coupons with a layer of VC-18 paint had little effect on the rate of biofilm development or the numbers of viable cells in films on aluminum or fiberglass, but viable counts were markedly lower on painted stainless steel than on non-painted stainless steel (Figure 2). The differences were less pronounced on painted surfaces than on non-coated surfaces (Figure 2, Table 1). Total cell numbers (Figure 3) showed similar results, but were 10- to 100-fold higher than viable counts, similar to the results on non-coated surfaces. The polysaccharide content of biofilms varied during the 150-h period, but EPS values tended to be higher on painted aluminum and stainless steel than on non-coated ones. On fiberglass, biofilms contained less EPS on painted than unpainted surfaces but the differences are not significant (Figure 4, Table 3).

Paint + copper

When copper was added to the paint, biofilms developed more slowly and contained fewer viable cells than surfaces covered with paint only (Figure 2). In the first few hours, the number of viable cells was 1000-fold lower on coupons with copper in the paint, but after 24–48 h, the biofilm reached equilibrium, the viable cell number increased to only 10-fold less in biofilms on surfaces without copper in

Table 3 EPS in equilibrium phase (24–96 h) biofilms on different surfaces and with different treatments

Treatment	EPS concentration (μg cm^{-2})*, mean ± 95% confidence interval on:		
	Aluminum	Fiberglass	Stainless steel
No paint	$^{a}7.91 \pm 2.7^{x}$	$^{b}21.9 \pm 4.6^{x}$	$^{a}12.3 \pm 3.4^{x}$
Paint	$^{a}13.1 \pm 2.5^{x}$	$^{a}12.7 \pm 5.1^{x}$	$^{b}24.6 \pm 4.7^{y}$
Paint + TBT	$^{a}10.3 \pm 5.0^{x}$	$^{ab}18.3 \pm 5.4^{x}$	$^{b}21.3 \pm 5.8^{xy}$

*Means with different superscripts are significantly different at the $\alpha = 0.05$ level; letters a–c indicate results of statistical analysis of differences between materials (aluminum, fiberglass, and stainless steel); letters x–z indicate results of statistical analysis of differences between treatments.

the paint. However, total cell numbers were not affected (Figure 3). This suggests that the numbers of cells which attached were the same between the biofilms that grew on coupons coated with or without copper but cells were later inhibited or killed. After 48–72 h, copper-resistant cells may have increased in number, or their exopolysaccharide product protected them from the toxicity of copper. However, EPS was not measured for biofilms grown on copper-coated surfaces because copper interfered with the phenol-sulfuric acid method.

Paint + TBT

Aluminum coupons with marine paint with TBT had significantly lower numbers of viable bacteria (Figure 2) and of total cells (Figure 3) than coupons with paint alone. However, TBT had relatively little effect on viable counts on fiberglass or stainless steel. A comparison of viable counts and total counts indicates that on all three surfaces about 1–10% of the cells present were able to form colonies.

When aluminum coupons were coated with paint with TBT added, the number of viable cells in biofilms was decreased around 1000-fold at early stages. When the film reached a stable stage, numbers were 10- to 100-fold lower than in the films on coupons coated with paint lacking TBT. On fiberglass and stainless steel, the differences were not as great. Although TBT paint decreased viable counts in early biofilm development, it had a lesser effect after the biofilm reached stationary phase (Figure 2, Table 1). Values for EPS varied (Figure 4). It seems that TBT inhibited EPS formation during early biofilm development.

Selection of copper- or TBT-resistant cells

Biofilm development on surfaces containing copper or TBT may involve growth of resistant cells. Biofilms of *P. aeruginosa* PAO-1 were allowed to develop on uncoated stainless steel coupons or on stainless steel coupons coated with paint containing TBT. The total number of viable cells and the number of TBT-resistant viable cells were determined in each case. On non-painted stainless steel, TBT-resistant cells made up approximately 10% of the viable population early in biofilm development and less than 1% late in development (Figure 5). On a surface coated with TBT-containing paint, TBT-resistant cells made up more than 50% of the population early in the development and 10% or less late in development.

Biofilms developed from *P. aeruginosa* PAO-1 were also tested for copper resistance. The results are shown in Figure 5. As with TBT-resistant cells, there was a higher percentage of Cu-resistant cells in biofilms grown on stainless steel coated with Cu, but on non-Cu-coated stainless steel, Cu-resistant cells made up approximately 85% of the viable population in early biofilm development and less than 10% later in development. On Cu-coated stainless steel, Cu-resistant cells made up the entire population in the first 48 h and then decreased to less than 10% of the population by the end of the experiment.

Significance of the results

Addition of TBT to a marine paint decreased the numbers of viable cells and total cells in biofilms of *P. aeruginosa* PAO-1 which developed on aluminum, but not on the other

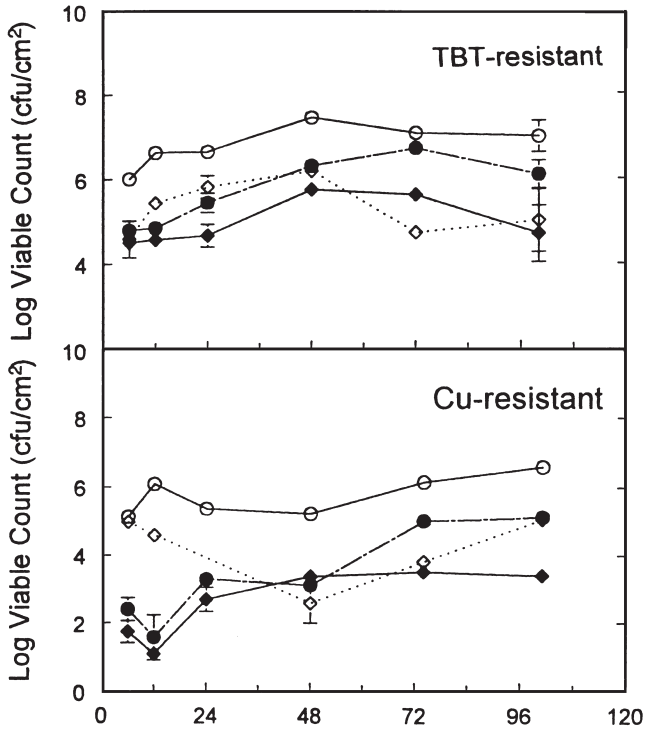

Figure 5 Effect of TBT or Cu on the number of TBT- or Cu-resistant cells in biofilms on stainless steel. ○: Total viable cells on stainless steel; ◇: TBT- or Cu-resistant cells on stainless steel; ●: total viable cells on TBT- or Cu-painted stainless steel; ◆: TBT- or Cu-resistant cells on TBT- or Cu-painted stainless steel.

two surfaces tested. Copper decreased viable counts initially, but after 6 days numbers were equivalent in surfaces either uncoated or with paint containing copper.

The purpose of antifouling paints is primarily to prevent development of macrobial organisms, particularly barnacles. Since microorganisms on a surface can increase attachment of shellfish larvae to a submerged surface [23,27], inhibition of a microbial biofilm might decrease development of barnacles on the surface. Our results with this model biofilm suggest that antifouling paints with copper or TBT are not likely to exert their effects primarily by inhibiting development of a microbial film. The studies are being extended to deal with mixed microbial films, including films developed from natural populations of microorganisms.

Our results suggest that a surface coated with paint can interact with materials in the paint in affecting microbial activities as indicated by the observations that: (1) paint with TBT was more effective on aluminum than on fiberglass or stainless steel; and (2) paint with copper decreased viable counts over the period from 48–96 h. The mechanism(s) of such interactions are not clear but may involve co-toxicity of aluminum with tin and of iron with copper.

Both copper and TBT inhibited development of a biofilm by *P. aeruginosa* PAO-1 in its early stages, but the inhibition was only at an early stage. On fiberglass and stainless steel, however, numbers of viable cells and total cell counts were equivalent by the end of the 150-h incubation period. After 5–6 days, viable counts were equivalent on all painted

surfaces. This could be due to inactivation of copper or TBT by the EPS—perhaps by sequestering the metal. It could also be due to growth of copper- or TBT-resistant cells; TBT-resistant cells can be isolated readily from aquatic ecosystems [29]. We have defined a TBT-resistant bacterium as one that grows on an agar-containing medium with 84 μM TBT, a concentration that kills approximately 90% of *P. aeruginosa* PAO-1 (data not shown). TBT-resistant cells made up a greater fraction of the viable population on a surface containing TBT than on surfaces without TBT. Thus, growth of TBT-resistant cells of *P. aeruginosa* PAO-1 contributes to biofilm development on TBT-coated surfaces but it does not account completely for mechanisms of biofilm formation on surfaces containing TBT. Perhaps the EPS contributes by binding TBT.

Using the same approach, we tested copper-resistant cells in the biofilms. TSA medium with 20 mM copper(II) that kills approximately 90% of *P. aeruginosa* PAO-1 (data not shown) was used to determine copper-resistant cells. Almost 100% of the viable cells harvested at 6 h from biofilms were copper-resistant. The growth of copper-sensitive cells on copper-painted surfaces after 48 h suggests that copper-resistant cells may form a layer on the surface of the biofilm that shields non-copper-resistant cells.

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