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Abstract

Microbial biofilms, composed of microorganisms originally obtained from estuarine harbor-exposed commercial organotin-painted panels, accumulated tributyltin (TBT) spiked into estuarine water. Both algal or bacterial-dominated biofilms were grown by varying nutrient conditions and were found to accumulate TBT in excess of 300 ng/mg biomass (dry weight basis) from estuarine water solutions containing 50 μg/L TBT, corresponding to bioconcentration factors of over 7000. No degradation of TBT to dibutyltin (DBT) species was detected, either in the biofilm material or in the surrounding solution. Thus, the microbial biofilms concentrated but did not degrade TBT. These results suggest that microbial films on TBT-painted structures in marine environments concentrate TBT at materials surfaces, thereby acting as "capacitors". Such effects might make practical the use of paints with extremely low TBT release rates delivering antifouling service with reduced environmental hazard.

Keywords: antifouling coatings, bioaccumulation, biodegradation, biofilms, biofouling, organotins, tributyltin

1.0 Introduction

Organotin-based antifouling coatings are in world-wide use to prevent attachment of marine life to ship hulls, saving time, fuel, and maintenance costs [1]. The U.S. Navy has an interest in painting its fleet with advanced controlled release (CR) polymer coatings that provide antifouling protection for six years or longer [2]. However, tributyltin (TBT), the most widely used organotin compound for CR antifouling activity, is extremely toxic to non-target marine life [3,4]. A number of countries including France and the United Kingdom, and more recently individual states and the Environmental Protection Agency in the United States, have imposed or are considering restrictions on the use of these materials on recreational vessels [5,6].

The actual mechanism by which TBT repels fouling organisms remains to be elucidated. Microbial biofilms rapidly develop on TBT-painted hull surfaces, but higher forms of life (i.e. barnacles) are effectively repelled [7]. The role of the microbial biofilm in concentrating or affecting the release rate and form of the TBT toxicant has received very little attention. Loeb and colleagues (unpublished data) found that the presence of microbial biofilms on TBT-painted panels affected the release rate of the TBT into surrounding solutions [2]. Laughlin and co-workers [7] found that microbial biofilms growing on TBT painted panels accumulated TBT. This suggests the microbial biofilm may contribute to paint performance by reducing organotin loss, in effect acting as a secondary controlled release medium, as well as having protective functions toward non-target marine life [2,7]. The extent of bioaccumulation of TBT in microbial biofilms and its possible transformations remain uncertain, because it is difficult to measure and speciate organotins within the biofilm matrix. The problem is further complicated by the

difficulty of removing biofilm from painted surfaces without also removing some of the paint coating below.

Our earlier studies showed that estuarine heterotrophic bacteria can rapidly accumulate TBT from solution without TBT molecular transformation [8]. The purpose of the current research was to determine the extent of TBT accumulation and transformation in microbial biofilms. Methodology developed for speciation of TBT in waters and sediments [9,10] was applied to the analysis of butyltins in the biofilm matrix. We grew biofilms on glass slides and exposed them to TBT in solution. While this does not precisely mimic the leaching of TBT from a painted surface, it does offer a reliable system in which we could be certain that none of the TBT observed in the biofilm had been physically removed from paint. We assumed that films capable of accumulating TBT leached from paint would also be able to accumulate TBT from surrounding free solutions, if grown on a surface not painted with TBT. In addition, we investigated using organotin-fluorescent ligand combinations and epifluorescence microscopy to directly detect organotins on biofilms [11,12]. We did not find a fluorescent probe for TBT but epifluorescence microscopy of monobutyltin in a microbial biofilm was demonstrated.

2.0 Experimental

2.1 Biofilm Growth

All biofilm experiments employed a mixed culture of estuarine microorganisms originally obtained from the surface of a steel panel painted with a commercial ablative copper and TBT-containing antifouling paint (International Hysol BFA-254 copolymer). The panel had been immersed in the Severn River at Annapolis, MD for 6.5 months at an average salinity of

approximately 8-10 parts per thousand (ppt) (J. Mihm, David Taylor Research Center, Annapolis, personal communication). The panel displayed some green growth on the surface, suggesting the presence of algae. Upon removal from th river, the panel was gently rinsed with 0.5 L of Chesapeake Bay water (salinit 10 ppt), made sterile by autoclaving at 121°C for 15 min. The panel was then gently scraped with a sterile teflon spatula over a 10 x 10 cm area, and the scraped material was washed into a sterile polycarbonate bottle with 100 mL of sterile Chesapeake Bay water. The wash water was immediately transported to the laboratory, where 30 mL of the suspension was inoculated into a conical flask (250 mL) containing 170 mL of sterilized Bay water having 10 mM ammonium chloride and 2 mM dibasic potassium phosphate as nutrients. This flask was placed under fluorescent lamps (intensity 350-400 foot candles) at 22 °C. Another 30 mL of wash water was inoculated into a flask containing 170 mL of water containing 0.03% yeast extract and 0.03% polypeptone (Difco). These samples were incubated at 28 °C on a gyratory shaker at 200 r.p.m. (New Brunswick model G76). After one week of incubation, the contents of both flasks were mixed and centrifuged (6000 x g, 20 min). The supernatant was poured off and the pellet containing cells was resuspended in 70 mL of sterile Bay water. Sterile glycerol (7.7 ml) was added, the cell suspension was continuously stirred to mix the contents, and 1.0 mL aliquots were removed and added to each of 60 sterile 1.5 mL plastic screw-capped vials designed for cryogenic sample preservation. The vials were transported to the American Typ Culture Collection (ATCC, Rockville, MD), where they were frozen and stored under liquid nitrogen. The cell suspensions contained 2.0×10^8 cells/mL (Petroff-Hauser counting chamber) and 4.3×10^7 colony forming units/mL when

spread on to peptone-yeast extract (0.03% concentration of each) agar and incubated at 28 °C for one week.

Microbial biofilms were grown on glass microscope slides (2.5 x 7.5 cm) immersed vertically in a small glass tank (Figure 1) containing 500 mL of sterile Chesapeake Bay water amended with peptone and yeast extract (0.05% of each), or ammonium chloride and dibasic potassium phosphate (10 and 2 mM, respectively) . The Bay water had been collected in a 50 L polyethylene carboy from 1.0 m depth at the mouth of the Severn River. The sample was passed through a 35 um filter during collection. It was stored in the dark at room temperature in the laboratory, and aliquots were removed and autoclaved as needed for biofilm experiments. The inoculum was prepared from frozen cells in storage at ATCC. The cells were quickly thawed in warm water (30 °C) and added to the biofilm growth tank. A sterile teflon stir bar was added to the tank and the tank was placed on a magnetic stirrer in the dark or under fluorescent lamps (intensity 350-400 foot candles), depending on the experiment.

2.2 Tributyltin Uptake on Biofilms

After several weeks, glass slides containing biofilms were removed from the growth tank and immersed in smaller growth cylinders of pyrex glass containing sterile Bay water amended with 50 μg/L TBT (as the cation). Additionally, two groups of control slides were included, which consisted of clean glass slides placed into TBT solutions and biofilm slides placed into sterile Bay water minus the TBT spike. Small teflon stir bars were added to the cylinders and the solutions were held in the dark or under fluorescent lamps at room temperature. After incubation, the slides were dipped twice into Bay water free of TBT and scraped with a clean razor blade. A small volume of

water was used to rinse the scraped material into 15-mL glass centrifuge tubes. Scrapings from the duplicate or triplicate biofilm slides incubated with TBT were pooled in one centrifuge tube. Aliquots were removed to separate tubes for spiking with butyltin compounds (method of additions quantitation) and for dry weight determinations.

2.3 Butyltin Extraction and Analysis

The procedure is a modification of a method for extraction of TBT from sediment [10]. All concentrations of organotins are listed on a weight basis in terms of the cations. Approximately 200 μL of the pooled biofilm suspension was added to a 150-mL round bottom flask along with an internal standard consisting of 50 μL of tripropyltin chloride (1.5 μg/μL in methanol). Concentrated HCl (0.5 mL) followed by methanol (25 mL) was added and the mixture was refluxed for 30 min at 80-85°C. The sample was cooled to room temperature and was extracted twice with 5-mL aliquots of cyclohexane in a 125 mL separatory funnel with shaking (10 min) on a wrist action shaker (Burrell). The combined cyclohexane layers were evaporated to about 2 mL under a gentle stream of air and then shaken for 45 min with 1.0 mL of 4% (w/v) aqueous $NaBH_4$. A small aliquot of the cyclohexane layer was injected into a gas chromatograph equipped with a flame photometric detector operated in a tin-selective mode as described previously for detection of DBT and TBT [9,10].

2.4 Epifluorescence Imaging

Spectrophotometric detection of monobutyltin adsorbed to the biofilm was successfully demonstrated by epifluorescence microscopy [11]. Monobutyltin, which has a more intense fluorescence emission than tributyltin when complexed with flavonol [11], was chosen for this experiment to demonstrate that the concept of visual imaging of adsorbed organotins on the biofilm was practical

Glass slides covered with a well developed biofilm growth were exposed to a solution of monobutyltin in ethanol (4.5×10^{-4} M concentration) for approximately 1 hour. The slides were then rinsed with ethanol and exposed to an ethanol solution of flavonol (1.4×10^{-4} M concentration) for 15 min. Control slides, either exposed to monobutyltin but not to flavonol, or exposed to flavonol but not to monobutyltin, were also prepared.

Following the above treatment, the slides were examined by epifluorescence microscopy. The UV excitation frequency was set at 366 nm with a 28 nm bandpass. A barrier filter was used to eliminate any radiation at wavelengths over 400 nm.

The following compounds were tested for their abilities to chelate to butyltin species and generate luminescent emission: 3-hydroxyflavone, morin, apigenin, galangin, emodin, flavone, chrysin, 4',5,7-trihydroxyflavone. All of these compounds were purchased from a commercial source (Aldrich Chemical Co., Milwaukee, WI).

3.0 Results and Discussion

Preliminary experiments showed that microbial biofilms accumulated 331-784 ng TBT/mg (dry weight) on exposure of films to Chesapeake Bay water containing 0.344 mg TBT/L for 28 hr. Subsequent experiments were performed at lower (50 μg/L) TBT concentrations and comparably substantial quantities of TBT were also bound to biofilms in these experiments. The first experiment involved biofilms grown in the dark, then immersed in the spiked Bay water and held in the dark. After four days incubation the pooled biofilm sample contained 353 ng TBT/mg (dry weight; Table 1, Figure 2). This represents a bioconcentration factor of 7060. Each glass slide contained an average of

0.43 mg of biofilm material (dry weight) over an area of 25 cm^2. The concentration of TBT in the film was determined by standard additions of di- and tributyltin chlorides to aliquots of the pooled biofilm sample. These were then carried through the entire extraction procedure. Samples of the solution of Chesapeake Bay water were analyzed periodically and showed no biodegradation of TBT to DBT, nor was any DBT detected in the biofilm material. Control slides (clean slides exposed to TBT, or biofilm slides minus TBT) gave no butyltin peaks in the chromatograms.

Another experiment was run using biofilms that had been grown under fluorescent lamps. These biofilms contained a large proportion of algae, as evidenced by their green color and large numbers of red autofluorescing cells visible under epifluorescence microscopy using 365 nm excitation (owing to chlorophyll in the cells). These films were incubated under fluorescent lamps for 7 days and analyzed for TBT and DBT as above. This biofilm material contained 373 ng TBT/mg (dry weight; Table 1). This corresponds to a bioconcentration factor of 7460. Again, there was no evidence of light- or dark-induced degradation of TBT to DBT either in the biofilm material or in solution during the course of the experiment. Tetrabutyltin was detected in biofilms run in the first (dark incubated) experiment (Figure 2). This compound was detected as a contaminant in reagent TBT used as a spike and was evidently bioaccumulated. This peak was not seen in the light incubated experiments where a chromatographically purified source of TBT was employed which did not contain tetrabutyltin. Such bioconcentration of tetrabutyltin may be an important factor in its esturine mobility because we have also detected this compound in harbor waters [9].

The concentration of TBT used in these bioaccumulation studies (50 μg/L) greatly exceeds concentrations of TBT reported in bulk water in aquatic environments. However, bioaccumulation of TBT in more dilute samples is also likely, especially since biofilms accumulated nearly as much TBT from solution at 50 μg/L as at 0.344 mg/L (see above). In addition, it is likely that the concentration of TBT in the interstitial water of a microbial biofilm on an organotin-painted ship hull is considerably higher than in surrounding open waters. We therefore do not believe 50 μg TBT/L to be an unrealistic concentration to illustrate potential microbial biofilm effects.

The TBT bioaccumulation results are similar to our previous findings with isolated strains of estuarine bacteria (8). In that study we found that the organisms accumulated TBT to 3.7 to 7.7 μg/mg (dry weight; bioconcentration factors in the hundreds). There was no measurable degradation of TBT to DBT. Laughlin et al. [7] found that a microbial biofilm on a TBT painted panel contained 20-60 μg/mg (wet weight), substantially greater than the concentration we observed in our biofilms. This is not surprising, given the quite different experimental systems between that study and the present investigation. Laughlin et al. employed TBT painted panels immersed for four months in San Francisco Bay and removed biofilm by lightly scraping the film from the painted surface. However, both studies show that microbial biofilms bioaccumulate TBT to a significant extent.

Previous studies have shown that TBT is degraded to DBT in the environment, with half-lives on the order of 1-2 weeks [13,14,15]. In some cases, microalgae have been implicated in the biodegradation process [14]. However, the current experiments show that biodegradation of TBT did not occur in the microbial biofilm composed of organisms originally obtained from a panel

painted with TBT-containing paint. We believe that our past and current results indicate biodegradation is not a common mechanism of TBT resistance in estuarine bacteria.

Table 2 summarizes results employing EMI to detect monobutyltin in microbial biofilms. The biofilm slide exposed to both monobutyltin and flavonol had significantly greater fluorescence intensity than any of the control slides. Visual examination of this slide under the epifluorescence microscope revealed a dark blue-black background with localized areas of bright blue fluorescence, characteristic of the monobutyltin-flavonol complex. These images suggest that the monobutyltin moiety is not homogeneously retained in the biofilm, but additional studies will be required to determine whether the organotin is adsorbed chiefly by viable cells or by exocellular adhesive polymers.

Table 3 gives relative emission intensity measurements for the non-complexed compounds, as well as the $Bu_xSn^{(4-x)+}$ complexes of the compounds. All emission intensities were measured at the estimated wavelength of the emission maxima of the different compounds/complexes. An excess of metal species was used in each case to ensure complexation of all of the ligand and guarantee that the observed emission was due to a formed complex, not excess non-coordinated ligand. Low emission intensities of samples and multiplication by conversion factors limited the number of significant figures for certain species. Species which essentially did not emit gave "pseudo-emissions" at 405 nm, which actually are reflections from the 405 nm line of the mercury excitation source lamp.

Table 3 shows that only 3-hydroxyflavone, morin and galangin ligands form complexes which emit sufficiently for consideration as "fluorogenic ligands"

for tin species. In each of these cases, it is seen that Bu_3Sn^+ has a fluorescence of only about 1/100 of that of the other tin species, perhaps due to the UV degradation of TBT to more fluorescent species [12]. It therefore seems very unlikely that any of these ligands could be a sensitive, selective reagent for the detection of tributyltin species in mixtures of organotins. However, it seems reasonable to employ the EMI technique to survey biofilm communities for the presence of TBT degradation compounds. In conjunction with electron microscopy with element specific energy dispersive microanalysis, questions could be answered concerning the location of organotin species in the biofilm (cell mass or exocellular polymer).

It is interesting to note that reproducible results indicate that the relative intensity of emissions of either morin or 3-hydroxyflavone complexes of butyltin species in EtOH follow the trend:

$$BuSn^{3+} > Bu_2Sn^{2+} > Sn^{4+} > Bu_3Sn^+$$

This trend differs from the relative intensities reported for butyltin compounds in aqueous solution [11]. The $EtOH/H_2O$ ratio (v/v) greatly affects the relative emission intensities of these samples. There may be a solubility or miscibility problem in the case of organotin-flavanoid complexes in a highly aqueous environment. This may hinder analyses of "in-vivo" samples which require an aqueous environment. The best use of the EMI may be to detect tin species on a solid surface, after chromatography has been performed (i.e. paper chromatography, TLC).

4.0 Summary and Conclusions

Biofilms containing microorganisms obtained from organotin painted panels accumulated relatively large amounts of TBT from solution. There was no

detectable biodegradation of TBT to DBT in the films or in the surrounding solutions. The films may thus influence the controlled-release action of organotin coatings. Microbial biofilms may concentrate TBT on the surface of painted ship hulls. In this manner it is conceivable that the TBT charged microbial biofilms act as the primary repellent to settling larvae of hard fouling organisms. If so, then organotin paints with extremely low release rates may still provide antifouling protection by virtue of a naturally-occurring TBT-enriched surface biofilm, and may release very little TBT to surrounding waters. Such paints would minimize environmental impacts of the use of TBT-containing antifouling paints. Additional experiments involving exposure of TBT-enriched biofilms to macrofouling organisms thus are highly warranted. Epifluorescence microscope imaging of monobutyltin in microbial biofilms was demonstrated. The technique may be useful for the non-invasive detection of such TBT degradation products. Direct detection of TBT in solution or in biomass will require the development of a ligand which specifically chelates to TBT, yielding a complex which fluoresces with reasonable quantum efficiencies. Selected commercially available materials so far were found to be unsatisfactory for these requirements.

5.0 Acknowledgments

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included in the doctoral dissertation of TKT in partial fulfillment of the requirement of the Graduate School of the University of Maryland.

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TABLE 1

Bioaccumulation of TBT by Microbial Biofilms

Incubation Conditions	TBT Solution Conc	TBT Accumulated (dry wt)	Bioconc.Factor
Dark, 97 hr	50 μg/L	353 μg/g	7060
Light, 168 hr	50 μg/L	373 μg/g	7460

TABLE 2

Detection by EMI of Monobutyltin Bioaccumulation

SLIDE	EMISSION MAXIMUM	RELATIVE INTENSITY
No cells- no tin no ligand	435	8.58 +/- 0.25
Biofilm- no tin no ligand	435	8.54 +/- 0.27
Biofilm- with $BuSn^{3+}$ no ligand	436	9.03 +/- 0.28
Biofilm- with ligand no $BuSn^{3+}$	458	7.85 +/- 0.19
Biofilm- with $BuSn^{3+}$ with ligand	452	30.39 +/- 0.87

TABLE 3

Relative Emission Intensities Measured at Indicated Wavelengths (solvent was 95% EtOH)

Species	[M]:[L]	Relative Intensity	λem
3-hydroxyflavone	0	55.5	533
Sn^{4+}:3-hydroxyflavone	3	413	446
$BuSn^{3+}$:3-hydroxyflavone	3	7669	452
Bu_2Sn^{2+}:3-hydroxyflavone	3	1584	450
Bu_3Sn^{+}:3-hydroxyflavone	3	34.5	454
morin	0	5.17	559
Sn^{4+}:morin	3	310	497
$BuSn^{3+}$:morin	3	2007	496
Bu_2Sn^{2+}:morin	3	603.8	496
Bu_3Sn^{+}:morin	3	56.3	524
apigenin	0	41.6	465
Sn^{4+}:apigenin	3	3.8	531
$BuSn^{3+}$:apigenin	3	8	538
Bu_2Sn^{2+}:apigenin	3	12	461
Bu_3Sn^{+}:apigenin	3	72.2	476
galangin	0	1.66	405
Sn^{4+}:galangin	3	206	475
$BuSn^{3+}$:galangin	3	417	473
Bu_2Sn^{2+}:galangin	3	21	472
Bu_3Sn^{+}:galangin	3	2	468
emodin	0	36.8	532
Sn^{4+}:emodin	3	11.1	532
$BuSn^{3+}$:emodin	3	22	540
Bu_2Sn^{2+}:emodin	3	9.8	535
Bu_3Sn^{+}:emodin	3	25.3	535

TABLE 3, continued

Species	[M]:[L]	Relative Intensity	λem
flavone	0	2.07	405
Sn^{4+}:flavone	3	0.7	406
$BuSn^{3+}$:flavone	3	9.8	405
Bu_2Sn^{2+}:flavone	3	1	405
Bu_3Sn^{+}:flavone	3	0.37	405
chrysin	0	1.37	405
chrysin:Sn^{4+}	3	0.9	534
chrysin:$BuSn^{3+}$	3	2	545
chrysin:Bu_2Sn^{2+}	3	0	546
chrysin:Bu_3Sn^{+}	3	0.09	436
4',5,7-trihydroxyflavone	0	0.49	435
Sn^{4+}:4',5,7-trihydroxyflavone	3	0.9	497
$BuSn^{3+}$:4',5,7-trihydroxyflavone	3	9	495
Bu_2Sn^{2+}:4',5,7-trihydroxyflavone	3	3	483
Bu_3Sn^{+}:4',5,7-trihydroxyflavone	3	0.63	472

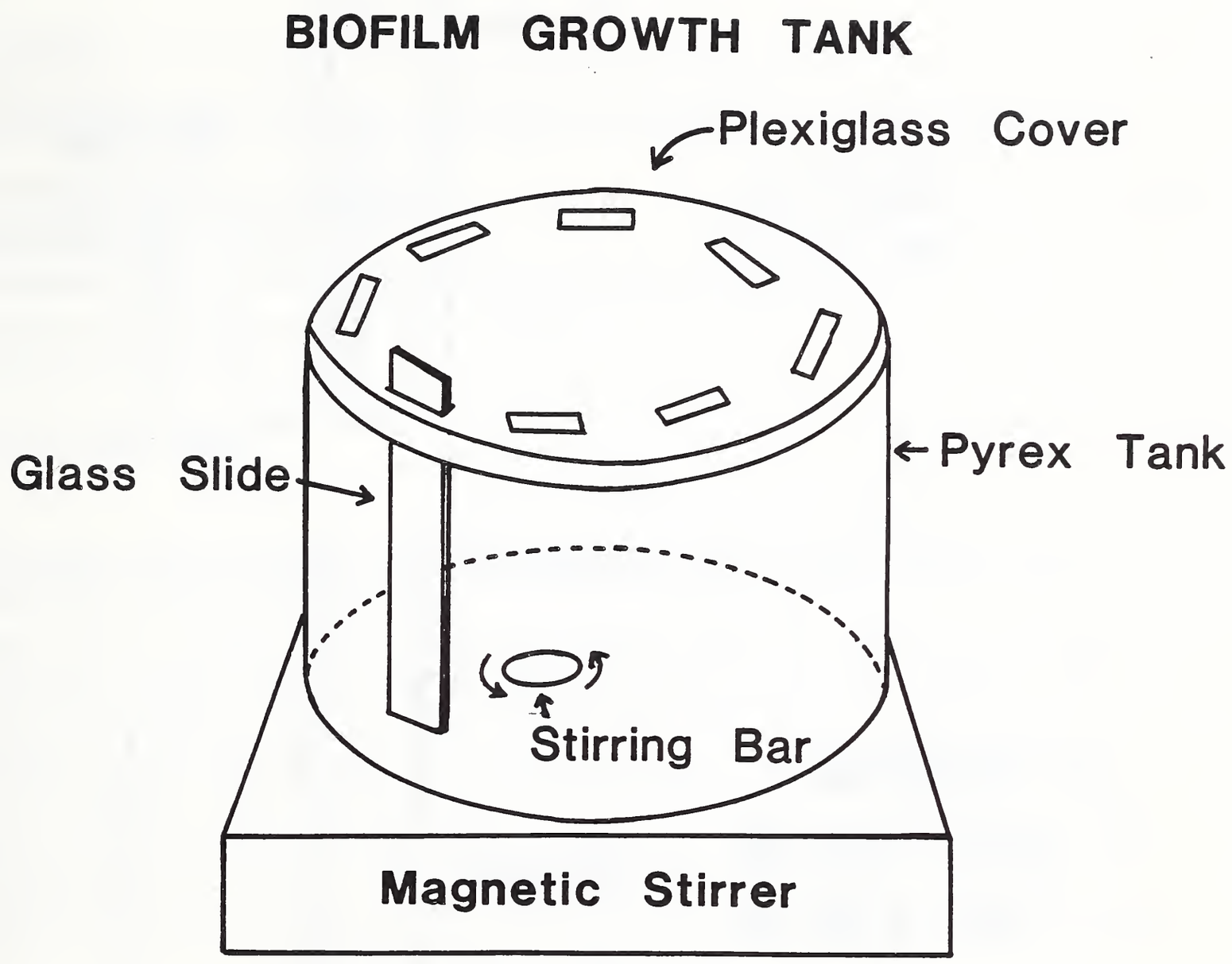

Figure 1. Apparatus used to grow microbial biofilms on glass slides.

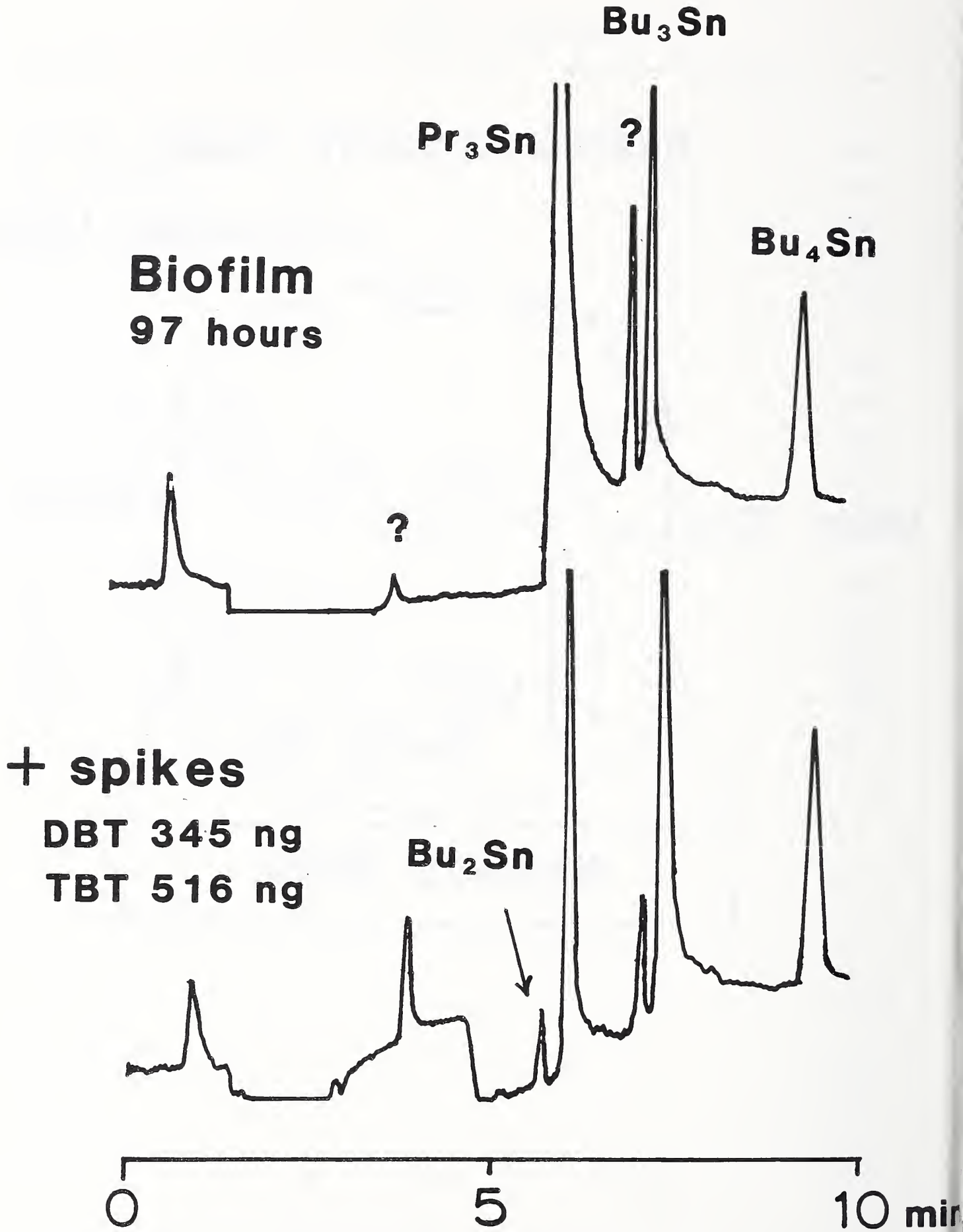

Figure 2. Chromatograms showing the bioaccumulation of TBT in microbial biofilms after 97 hours incubation (top). Spikes of tributyltin and dibutyltin species into biofilm matrix are shown at bottom.

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11. ABSTRACT (A 200-word or less factual summary of most significant information. If document includes a significant bibliography or literature survey, mention it here)

Microbial biofilms composed of microorganisms originally obtained from harbor exposed organotin painted panels accumulated tributyltin (TBT) spiked into estuarine water. Algal and bacterial dominated biofilms were grown by varying nutrient conditions. Both types of microbial communities accumulated TBT in excess of 300 ng/mg biomass dry weight from solutions containing 50 ug/L TBT, corresponding to bioconcentration factors of over 7000. No degradation of TBT to dibutyltin (DBT) species, either in the biofilm material of in the surrounding solution was detected in any of the experiments. Thus, the microbial biofilms concentrated but did not degrade TBT, suggesting that microbial films on TBT-painted structures in marine environments may act to concentrate TBT at materials surfaces, thereby acting as capacitors delivering extended antifouling service.

12. KEY WORDS (Six to twelve entries; alphabetical order; capitalize only proper names; and separate key words by semicolons)

antifouling coatings, biofilms,biofouling, organotins, tributyltin

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