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DEVELOPMENT OF STATIC SYSTEM PROCEDURES TO STUDY AQUATIC BIOFILMS AND THEIR RESPONSES TO DISINFECTION AND INVADING SPECIES

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The microbial ecology facility in the Analytical and Physical Chemistry Branch at Marshall Space Flight Center is tasked with anticipation of potential microbial problems (and opportunities to exploit microorganisms) which may occur in partially closed systems such as space stations/vehicles/habitats and in water reclamation systems therein, with particular emphasis on the degradation of materials. Within this context, procedures for microbial biofilm research are being developed. Reported here is the development of static system procedures to study aquatic biofilms and their responses to disinfection and invading species. Preliminary investigations have been completed. As procedures are refined, it will be possible to focus more closely on the elucidation of biofilm phenomena.

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INTRODUCTION

Biofilm phenomena have been studied since Zobell's discovery, almost 50 years ago, that bacteria grow preferentially on surfaces, rather than in aqueous phases around them.[1] However, interest in this topic has greatly accelerated during the past decade. As it was discovered that biofilms afford protection from biocides and antibiotics[2–6] and are frequently responsible for corrosion of materials,[7–10] investigators became more aware of their importance. A biofilm, representing the cooperative efforts of individuals, is much more effective than planktonic bacteria, representing merely the totaled efforts of individuals. A biofilm is sometimes a solution rather than a problem: in sewage treatment systems, biofilms remove organic and inorganic pollution. Characklis and Wilderer[11] offer the following definitions: a biofilm is the surface accumulation of microorganisms, frequently characterized by large amounts of organic polymers of microbial origin that bind cells and other organic and inorganic materials together and to the substratum; a biofilm community is a group of microorganisms living together within a biofilm whereby species selection, spatial distribution, and abundance of the individual species are the result of the prevailing environmental factors.

Many approaches are used by investigators who study biofilms. Analyses may be destructive or nondestructive, chemical or biological, quantitative or qualitative, online or offline, microscopic or macroscopic. The studied biofilms may be naturally occurring (in natural or engineered systems) or laboratory-developed. The particular work reported here involves laboratory-developed biofilms. When biofilms are developed in a laboratory, the system used falls into one of two major types—dynamic (where suspension or substratum is moving) or static (where suspension and substratum remain essentially stationary). Both types of system are used in the microbial ecology facility. This report describes the development of static system procedures.

The microbial ecology facility in the Analytical and Physical Chemistry Branch at Marshall Space Flight Center is tasked with anticipation of potential microbial problems (and opportunities to exploit microorganisms) which may occur in partially closed systems such as space stations/vehicles/habitats and water reclamation systems therein, with particular emphasis on the degration of materials. Toward this end, the laboratory has focused on the assessment of biofilms on candidate materials for Space Station *Freedom*. One objective is to predict the influence which disinfection will exert on biofilms within water reclamation and distribution systems. Another objective is to create scenarios of particular species invading particular types of biofilm and then to translate these into experiments. The aim is not to anticipate precisely actual conditions in operating space station systems, but to reveal trends that are likely.

Most of the literature on biofilm has involved monocultures or natural, undefined mixed cultures. This laboratory recognizes a need to examine biofilms formed in defined multispecies cultures. This approach addresses many of the basic unanswered questions about biofilm formation and activity and appropriately models relevant systems and subsystems.

Measurement or evaluation of a biofilm may be in terms of population or community density (viable cells), metabolic activity, or microbial amount (viable and nonviable cells). For each of these categories, numbers of different procedures have been employed by various investigators.[12–25 26–32 33–44]

EXPERIMENTS CONDUCTED

To develop useful static system procedures, it was necessary to select an appropriate sampling apparatus; inoculation, growth, and sampling timeframes which reveal activities of interest; techniques to assess the biofilm; and proper conditions (nutritional and otherwise) for attachment. This selection process is still evolving. The procedure in use at this time is as follows.

A screw-capped test tube containing a 316L stainless steel coupon (2.5-cm long, 0.8-cm wide tapering to 0.5-cm wide, 0.2-cm thick) in 10 mL 1-percent brain heart infusion (BHI) is autoclaved. Tubes are inoculated with 0.1 mL of a 24-h culture (a Bactrol disk, lyophilized organisms, in 10-mL full-strength BHI, incubated at 35 °C). Inoculants are selected from a stock of 17 species, listed in table 1 (abbreviations given in this table will be used throughout the text). Lyophilized Bactrol disks, convenient to use and available in organism types frequently found in water reclamation systems, are used for 16 of the stock species. The 17th, a *Methylobacterium* species, was recovered from SLS-1 humidity condensate. After inoculation, tubes are incubated at 28 °C until biofilm evaluation. At evaluation, the coupon is rinsed (by aseptic passage through four tubes, each containing 10-mL sterile distilled deionized water (DDW)), then stained or sonicated. For staining, the coupon is placed in 0.01-percent acridine orange for 3 min, rinsed in sterile DDW, air dried, and observed with an epifluorescent microscope. For sonication (to remove biofilm), the coupon is placed in a tube containing 10-mL DDW and sonicated for 3 min in a Branson 3200 ultrasonic cleaner; 0.1 mL is then removed from the liquid and plated on agar. In order to distinguish biofilm activity from culture activity, it is necessary to plate some of the liquid surrounding the coupon before the coupon is removed for rinsing. Thus, there will be references in this report to plating of the liquid ("culture water") and plating of the coupon ("sonication water"). An "attachment ratio" (colony forming units (CFU's)/1-μL liquid:CFU/coupon) can then be calculated for each tube. With the exceptions noted, this procedure was used in the experiments described below.

Monocultures

An early experiment with five different monocultures indicated that attachment (number and possibly pattern) varied with species, nutrient concentration, and incubation time. However, in this experiment, only coupons were plated, and vortexing (an extremely poor substitute for sonication) was employed. Currently, a similar experiment is in progress, using sonication, both liquid and coupon plating, and an expanded range of nutrient concentrations.

Binary Populations

In the first of these experiments, there were three groups of tubes: group I received only SA, group II received only ST, and group III received SA and ST. In group III, the order and timing of inoculation were varied. For 4 weeks, at the end of each week, tubes were monitored (coupon stained and subjectively evaluated), reinoculated, or untouched. The schedule is detailed in table 2. Note that SA+ST2 and SA+ST3 evaluated at 4 weeks show higher attachment ratings than do SA/ST at any time, ST at any time, ST+SA2 or ST+SA3 at 4 weeks. Also, at 4 weeks, SA/ST is R-R-R but ST+SA3 is S-S-S.

The second experiment with binary populations was a repetition of the first experiment, substituting KP and PA for SA and ST. The results of this experiment are shown in table 3. It is interesting to compare KP+PA3 to PA+KP3. The order of introduction has apparently made a distinct difference in attachment.

Multispecies Biofilms

Survival of *Staphylococcus aureus*. Twenty-eight different biofilms were developed. On day 1, each of 28 tubes was inoculated with one to eight species; only eight of these tubes included SA. Two months later, liquids from the tubes containing SA were plated on Mannitol salt agar (MSA); SA had survived only when it was without competition (and then at only 3,000 CFU per mL). At this time, all 28 tubes were inoculated with SA. Nine days later all liquids and coupons were plated on MSA. This time, SA without competition showed confluency for the liquid and 63 CFU for the coupon, but no other coupon plated a single colony, and SA survived in the liquid in only a few tubes (where the number of competing species was three or fewer). The presence or absence of SA in the original inoculation had no clear-cut effect on later-inoculated SA survival, but in a group of four different biofilms (all involving three competitor species) SA survived only in the one case in which it was an initial inoculant; in a group of three different biofilms (all involving only one competitor species), SA survived only where it was not an initial inoculant.

Separate Versus Group Culture. Twenty-eight different biofilms were developed. Tubes 1 to 12 each contained a different single species. Tubes 13 to 28 each contained three different species; no tube contained an identical assortment; all species used in these assortments were tested as single species in tubes 1 to 12. Almost 3 months after inoculation, liquids and coupons from all tubes were plated on R2A. A broad range of attachment ratios was observed. AC in monoculture exhibited conspicuously poor attachment. The following tubes exhibited relatively high attachment tendencies: EA, SM, SS, ST, KP, PA, AC-EA-ec, EA-ec-CF, SA-SF-EA, SS-ST-EA, SM-ST-SE, CF-KP-SS (as compared to AC, ec, SA, SE, SF, CF, SA-SE-SF, SM-SS-ST, CF-KP-PA, AC-EA-KP, AC-ec-SM, SA-SE-AC, SE-SF-PA, SM-SS-CF, KP-PA-SA, CF-PA-SF). The multispecies biofilms containing EA showed higher attachment tendencies than multispecies biofilms without EA. It was also interesting to note that the total number of attaching CFU when SM, SS, and ST were cultured separately was 1,400, but when these were cultured together, the number of attaching CFU was only 50. (This could be nutrient-related.)

Methylobacterium Survival. Twenty-seven different biofilms were developed, using one to seven species in each tube. Two days after initial inoculations, each tube was inoculated with *Methylobacterium* species subgroup B, isolated from Spacelab humidity condensate. Three days after initial inoculation, 0.02-g iodine-impregnated beads (derived from an Umpqua microbial check valve) were added to each of seven tubes. Four weeks later, liquids and coupons were plated on R2A agar and Sabouraud dextrose agar (SDA). All plates exhibited abundant bacterial growth within 24 h, but pink colonies (*Methylobacterium*) appeared (1 week later) on only three R2A coupon plates (and to a lesser extent, on two of the corresponding liquid plates) and never appeared on SDA. Of the 27 biofilms, only four incorporated only one species besides *Methylobacterium*—this was the group where *Methylobacterium* appeared. One of these four biofilms did not exhibit *Methylobacterium* when plated. This was most likely due to competition from the other species (*Pseudomonas aeruginosa*) or possibly to the addition of iodine beads (of the four biofilms, only this one received iodine). While *Methylobacterium* survival was the intended question in this experiment, perhaps the most interesting observation was the striking morphological difference between platings of removed biofilm and platings of the coupon's liquid environment. For R2A, in 14 of the 27 plate pairs, many minute white colonies appeared on the biofilm plates but none, or virtually none, on the liquid plates (all plates showed abundant average-sized

colonies). All but 2 of the 15 biofilms incorporating 5 or more species exhibited these minute colonies; only 1 of the 12 biofilms incorporating 4 or fewer species exhibited such colonies.

Pseudomonas aeruginosa/Salmonella typhimurium Competition—Liquid Versus Coupon. Each of 19 tubes was inoculated with 3 species to develop 19 different biofilms. Immediately afterwards, each tube was inoculated with PA. Ten weeks later, each tube was inoculated with ST. Two days after ST inoculation, 10 tubes (liquids and coupons) were sampled and plated on Pseudomonas isolation (PI) and SS agars. On the following day, the remaining nine tubes were sampled in like manner. The goal here was to discover whether the PI/SS ratios in the liquid and on the coupon were different, particularly in view of the fact that ST was the recent, invading species. For each tube, a PI/SS ratio was calculated for both the liquid and the coupon. While the PI/SS ratio for liquid and coupon usually exhibited a marked difference, for some biofilms PI colonies outnumbered SS colonies on the coupon but not in the liquid, but for other biofilms the opposite was true. Since all biofilms differed in composition, this was not surprising. Platings on SS frequently exhibited marked morphological difference between the liquid and the coupon; however, this (color difference) could possibly be correlated with CFU number.

Attachment of *Pseudomonas aeruginosa* to Conditioned Coupons. Each of 15 tubes was inoculated with 5 species to develop 15 different biofilms. Ten weeks later, the tubes were divided into three groups. In group I, coupons were rinsed and placed in a tube of DDW, which was then inoculated with PA. In group II, coupons were rinsed, treated for 1 min with 1.0-percent iodine, rinsed again and placed in a tube of DDW which was then inoculated with PA. In group III, coupons were treated the same as in group II except that 0.2-percent iodine was used. Group IV was created by placing clean coupons in DDW and inoculating with PA. Twenty-four hours later, one tube from each group was sampled. Both liquid and coupon were plated on PI agar. Remaining tubes were sampled 48 h after PA inoculation. Results indicate that attachment differs sharply between clean coupons and biofilm-covered coupons, and between biofilm-covered coupons and disinfected biofilm-covered coupons (table 4). These differences appear to be dynamic with time and to vary with the composition of the particular biofilm. This type of experiment promises to reveal some interesting phenomena and is currently being expanded from a screening stage to a more controlled and replicated stage.

LESSONS LEARNED/FUTURE PLANS

Technique evaluation has been concurrent with analysis of results of experiments designed to elucidate biofilm phenomena.

Techniques

Biofilm Rinsing: The number of rinse tubes used in the static system experiments was determined from preliminary testing with 13 sequential rinses; the goal was to find the number of rinses that would ensure that the number of unattached organisms carried over in the rinse water would be negligible. While passage of the coupon through four rinse tubes is somewhat laborious, it was found to be a satisfactory technique.

Biofilm Harvesting: Investigators have used various methods to remove biofilm cells for colony counts: scraping and plating, swabbing and plating, the agar contact method, and sonication. Sonication, used to remove biofilm in the static systems, appears to be an effective method for biofilm harvesting, disposed to consistency. This technique will be further explored and evaluated.

Viable Cell Measurement: Colony counting on selective and nonselective agars has proven very useful. Future studies will also include vital dyes, molecular probes, isotopic-labeling, and tests for metabolic activity.

Viable + Nonviable Cell Measurement: Epifluorescent microscopic observation of coupons stained with acridine orange has proven to be a good technique, but in the future these measurements should be less subjective.

Biofilm Phenomena

Experiments with binary populations indicate that both sequence and timing of inoculation are influential in biofilm development. Future studies will include viable cell counts and consistent nutrient scheduling.

The *Methylobacterium* survival experiment supports the idea that, to recover a high percentage of the organisms present in any sample, investigators must design a recovery plan that anticipates masking problems and incorporates all available information about a sample's history. This experiment also indicated that dissimilarity between liquid and coupon platings is possibly enhanced when the number of competing species exceeds four. Finally, *Methylobacterium* recovery (and possibly survival) was precluded when the number of competing species exceeded one.

The experiment examining the attachment of *Pseudomonas aeruginosa* to conditioned coupons offers a promising experimental design. Varied conditioning of coupons appears to have a dramatic effect on subsequent attachment of *Pseudomonas aeruginosa*, within the parameters employed. This experiment will be expanded.

Many biofilm studies are planned for the future. These include experiments to examine desorption/detachment processes and motile versus nonmotile phenomena. When static system experiments result in interesting observations, similar experiments can be designed for dynamic systems, using real-time, nondestructive analysis. While measurement is the result of evaluating a single coupon, broader analysis is the result of correlations and deductions made when different biofilm experiments are examined as a group. The microbial ecology facility is building a body of data to examine as basic questions about biofilm communities are addressed.

Table 1. Stock organisms used in static systems experiments.

AC	*Acinetobacter calcoaceticus* ATCC 19606
CF	*Citrobacter freundii* ATCC 8090
EA	*Enterobacter aerogenes* ATCC 13048
EC	*Enterobacter cloacae* ATCC 23355
ec	*Escherichia coli* ATCC 25922
KP	*Klebsiella pneumoniae* ATCC 13883
PV	*Proteus vulgaris* ATCC 13315
PA	*Pseudomonas aeruginosa* ATCC 27853
ST	*Salmonella typhimurium* ATCC 14028
SM	*Serratia marcescens* ATCC 8100
SF	*Shigella flexneri* ATCC 12022
SS	*Shigella sonnei* ATCC 25931
SA	*Staphylococcus aureus* ATCC 25923
SE	*Staphylococcus epidermidis* ATCC 12228
sf	*Streptococcus faecalis* ATCC 19433
sp	*Streptococcus pyogenes* ATCC 19615
M	*Methylobacterium* recovered in SLS-1 humidity condensate

Table 2. Bacterial attachment to 316L stainless steel in an aqueous environment containing *Staphylococcus aureus* and *Salmonella typhimurium.*

	Triplicate Coupon Evaluation			
	1 week	2 weeks	3 weeks	4 weeks
Treatment				
SA	R R R	R	R	R
ST	R R S	S	S	R
SA/ST	*S *S R	S R R	S R S	R R R
SA+ST1		R *M R	R R *S	R S R
ST+SA1		R S R	S S S	R R R
SA+ST2			S R R	M M R
ST+SA2			R R *S	R *S R
SA + ST3				M S S
ST+SA3				*S *S S

Notes:

SA – tubes inoculated with SA on day 1.

ST – tubes inoculated with ST on day 1.

SA/ST – tubes received simultaneous inoculations of SA and ST on day 1.

SA+ST# – tubes inoculated with SA on day 1 and later with ST (# = time of addition, in weeks)

ST+SA# – tubes inoculated with ST on day 1 and later with SA (# = time of addition, in weeks).

R (rare) – hard to find any organisms.
M (many) – large number of organisms.
S (some) – a broad range in between rare and many.

* When an asterisk appears by the evaluation, spherical as well as rod-shaped bacteria were observed; when both were present, rods always – and greatly – outnumbered spheres. An evaluation without an asterisk ("R" excluded) indicates only rod-shaped bacteria.

Table 3. Bacterial attachment to 316L stainless steel in an aqueous environment containing *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

	Triplicate Coupon Evaluation			
	1 week	2 weeks	3 weeks	4 weeks
Treatment				
KP	S R S	R R R	R R R	R M R
PA	R S R	R *M R	R R R	R R R
KP/PA	*M *M R	*S *S *S	R R R	R * M R
KP+PA1		S S M	R R S	R R R
PA+KP1		R R *S	R *M R	*S R *R
KP+PA2			S S R	S M S
PA+KP2			R *S *S	R *S *M
KP+PA3				M S M
PA+KP3				R R R

Notes:

KP – tubes inoculated with KP on day 1.

PA – tubes inoculated with PA on day 1.

KP/PA – tubes received simultaneous inoculations of KP and PA on day 1.

KP+PA# – tubes inoculated with KP on day 1 and later with PA (# = time of addition, in weeks)

PA+KP# – tubes inoculated with PA on day 1 and later with KP (# = time of addition, in weeks).

R (rare) – hard to find any organisms.
M (many) – large number of organisms.
S (some) – a broad range in between rare and many.

* When an asterisk appears by the evaluation, the "organisms" observed were distinctly different (much smaller, less distinct, and shrunken in appearance) than the rods which are indicated by an evaluation without an asterisk. Possibly these "organisms" are one of the following: PA (notice they appear only where PA was inoculated on day 1); debris from disintegrating organisms; organisms in an "ultramicrobe" state; desorption or detachment marks.

Table 4. Attachment of *Pseudomonas aeruginosa* to coupons conditioned variously.

	CFU			
	24 hour		48 hour	
Group No.	Liquid (per 1 µL)	Coupon	Liquid (per 1 µL)	Coupon
I	1,000+	1,000+		
			1,000+	156
			1,000+	31
			1,000+	400
			1,000+	52
II	1,000+	300		
			1,000+	2
			1,000+	250
			1,000+	300
			1,000+	122
III	1,000+	200		
			1,000+	400
			1,000+	250
			1,000+	200
			1,000+	200
IV	1,000+	126		
			1,000+	1,000+
			1,000+	1,000+
			1,000+	1,000+
			1,000+	1,000+

Group I = biofilm-covered coupons.
Group II = 1.0-percent iodine-disinfected biofilm-covered coupons.
Group III = 0.2-percent iodine-disinfected biofilm-covered coupons.
Group IV = clean coupons.

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APPROVAL

DEVELOPMENT OF STATIC SYSTEM PROCEDURES TO STUDY AQUATIC BIOFILMS AND THEIR RESPONSES TO DISINFECTION AND INVADING SPECIES

By G.A. Smithers

The information in this report has been reviewed for technical content. Review of any information concerning Department of Defense or nuclear energy activities or programs has been made by the MSFC Security Classification Officer. This report, in its entirety, has been determined to be unclassified.

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