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Effect of Mineral Composition on Bacterial Attachment to Submerged Rock Surfaces

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Abstract. A direct microscopic count technique employing fluorescein isothiocyanate stain was used to compare microbial colonization on the exposed surfaces of rocks and minerals suspended in several ponds for various time intervals. Hematitic sandstone was never colonized at a rate greater than limestone, but quartz was always colonized more rapidly than calcite. The use of single-crystal minerals (quartz and calcite) in a nested factor experiment showed that the effect of the minerals on colonization was statistically significant, but that differences among the immersion sites were also significant. Sandstone samples placed in a pond outflow accumulated microbial colonizers more rapidly than those placed in the still waters of the same pond. The results indicate that the composition of the mineral substrate, in concert with the immersion environment, controls the formation of primary slime layers in aquatic systems.

Introduction

The importance of microbial attachment to solid surfaces is recognized in a wide variety of environments. Although many different materials have been used as substrates for the study of microbial attachment, most have been manmade. The use of glass, plastics, and various metals, either untreated or treated with experimental coatings, is appropriate for questions related to biofouling. However, when studying the role of attached bacteria in a natural ecosystem, it is more relevant to use local, native substrates (e.g., rocks) found in that environment. Bacteria attached to rocks are termed *epilithic* [1, 9], and the epiliths have been shown to be abundant and active in shallow water systems such as streams [1, 5, 6, 13].

Most techniques for enumerating epilithic populations involve scraping or scrubbing of the microbes from the substrate surfaces, followed by direct or indirect counting [1, 5, 6, 13]. However, Ladd et al. [13] used epifluorescence microscopy to directly enumerate bacteria attached to quartzite and shale coupons exposed in a mountain stream for periods of 1–52 weeks. The study compared the results of enumerations made using disturbed (scraped) and undisturbed (directly examined) epilithic communities; differences between the rock types were not compared. Higher numbers of organisms were obtained by counting the microbes on the coupons directly as opposed to scraping the rock surface and counting the scrapings.

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We have also used a method of direct counting to examine the effects of differences in substrate minerals and environmental factors on colonization of native substrates. The results indicate that the type of mineral substrate may influence the rate and extent of microbial colonization, but environmental factors must also be considered, since they may exert a significant effect, independent of the rock type being colonized.

Materials and Methods

Sample Collection and Preparation

Pieces of the rocks and minerals to be tested were cut into small sections of about 1 × 1 × 0.5 cm using diamond saws, and were polished with 30 strokes against 400 grit emery cloth. Three chips of each rock type were glued to a nylon line of 0.5 mm diameter with Duco cement. All of the rock samples to be withdrawn at a single time were randomly positioned on a 10 cm segment of the line. The strings were then tied to a horizontal support rod suspended just below the surface of the water, and the entire apparatus was anchored to the bottom. Prior to immersion, all surfaces were cleaned by swabbing with acetone to remove any traces of cutting oil or other contaminants.

Samples were collected by removing a single segment of string and placing it in a vial with a solution of 2% formaldehyde that had been prefiltered through a 0.2 μm membrane filter. The formaldehyde solution is referred to as FDW. The samples were stained and counted in the laboratory. Determinations of numbers of bacteria free in water collected at the time the chips were removed were made using the acridine orange direct count method (AODC) [10].

Staining and Microscopic Examination

In the laboratory, the rock segments were carefully removed from the strings, the rocks were placed into the bottom of a Petri dish lined with nylon mesh, and gently covered with FDW to prevent desiccation until the stain could be added. The fluorescein isothiocyanate (FITC) solutions of Fliermans and Schmidt [7] were used for the staining. The FDW was replaced with FITC, which was left in place for 15 min. The stain was removed with a pipet and the samples were rinsed twice for 5 min with 0.5M Na_2CO_3. Finally, a 2 min rinse with a 5% $Na_4P_2O_7$ solution was used to remove any remaining FITC. All solutions had been prefiltered through a 0.2 μm filter. The chips were allowed to dry, and the surfaces were examined under oil immersion (1000 × magnification) using an epifluorescence microscope.

A minimum of 5 fields were counted for each chip. Beyond that point, counting was stopped at 10 fields or 200 individual cells, whichever was reached first.

Substrate Types

Samples of limestone and hematitic sandstone (a Fe_2O_3 cemented quartz sand) were used to determine if rocks differing in mineral composition were colonized at different rates. Chips were suspended for 24 days in Riopel Pond and in Mountain Lake at the Mountain Lake Biological Station of the University of Virginia. During that time, samples were periodically removed, and the adherent bacteria were counted.

In other experiments, chips were prepared from single crystals of pure calcite ($CaCO_3$) and quartz (SiO_2) in order to determine if differences in colonization rates could be consistently demonstrated to be a function of specific minerals. Furthermore, to ensure that any differences observed were associated with the mineral types, i.e., quartz and calcite, and not the particular crystal chosen, 3 chips from each of 3 specimens of each mineral were used for each sampling time, and each crystal was collected from a different location. To determine if the effects were universal, i.e., independent of site effects, experiments were conducted simultaneously in three separate ponds near Charlottesville, VA. The variance at each level of replication was calculated using a nested

analysis of variance (ANOVA) [12, 17] to determine if effects observed were due to differences in mineral composition, immersion site, or analytical factors.

Water Flow and Colonization

Chips of hematitic sandstone were suspended in three areas of a pond near the grounds of the University of Virginia. The areas were ones in which there was no flow (< 1 cm sec^{-1}), moderate flow (near the outlet, 1–2 cm sec^{-1}), and rapid flow (in the outlet, 10–15 cm sec^{-1}). Flow rates were estimated by placing vermiculite chips in the water and measuring the amount of time needed for the chips to travel a horizontal distance of 1 m. Statistical analysis for all experiments was performed using the MANOVA program included as part of the SPSS package [17].

Results

Effect of Mineral Composition

Attached bacterial cells appeared as brightly stained rods or spheres against a dull green or black background. Cell dimensions for the rods were usually around 1.0–1.5 μm in length by 0.3–0.5 μm in diameter. Spherical cells were generally 0.5 μm in diameter. Samples withdrawn early in the incubation period were charaterized by single bacteria distributed randomly around the surface of the chip. In later samples, some cells were observed in clumps or microcolonies, containing from 3 to 20 cells. The number of cells in microcolonies tended to increase with incubation time, but never reached high densities in any of the experiments. Thus results are reported as total cells averaged on a uniform area basis.

When freshly prepared specimens of hematitic sandstone and limestone were immersed in Mountain Lake, the overall colonization of the limestone was greater during the 24-day incubation period (Fig. 1B). Analysis of variance showed that the observed differences for the entire period were significant at $\alpha = 0.001$. Samples of the rocks immersed in Riopel Pond for the same period failed to show a significant overall difference in colonization between the rock types (Fig. 1A). The shape of the curves shown in Fig. 1—viz., an initial increase in the numbers of adherent bacteria followed by a decrease, then a gradual increase—was also observed in several other colonization experiments conducted but not reported here, and may be a common pattern for microbial attachment to mineral surfaces.

When pure mineral specimens (quartz and calcite) were used as substrates, marked differences in attachment to the surfaces were noted between the 2 materials. The nested ANOVA design allowed comparison of differences among 3 chips of the same mineral specimen, among 3 different specimens of each mineral, between the 2 minerals, and among the 3 sites used for the experiment. Each site was sampled twice, after 10 days and again after 20 days of exposure.

Quartz surfaces were more heavily colonized than the calcite at all locations examined, and bacterial attachment at site B was always greater than at the other locations for both minerals, even though the abundance of bacteria in the water column was not very different among the sites (Table 1). Furthermore, the numbers of attached organisms increased between days 10 and 20 at all of the sites, while the number of planktonic bacteria decreased slightly over the same period. The ANOVA results

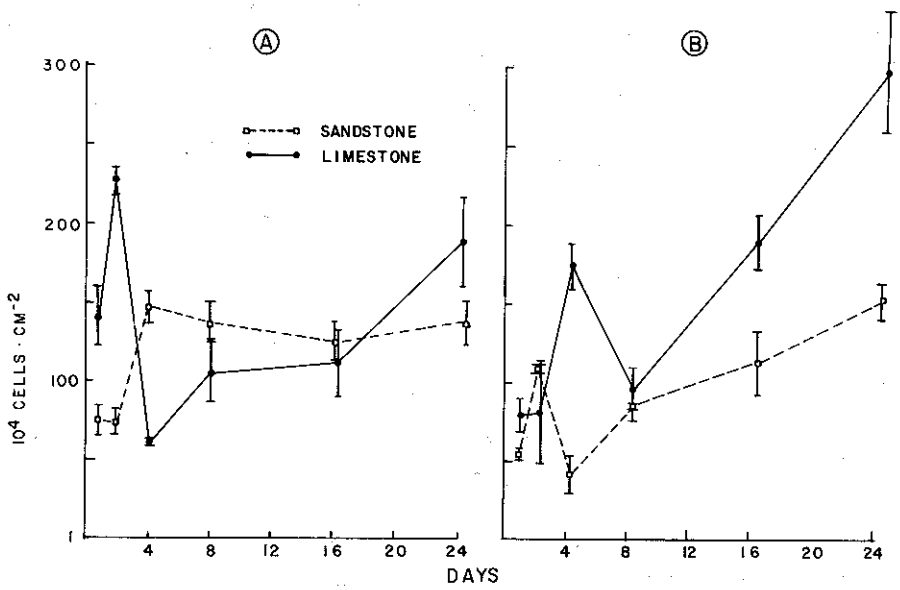

Fig. 1. Colonization of hematitic sandstone and limestone chips immersed in Riopel Pond (**A**) and Mountain Lake (**B**) for 24 days. The error bars represent ±1 SEM. Analysis of variance demonstrated a significant difference between the treatments in **B**, but not in **A**.

Table 1. Numbers of bacteria counted on the surface of quartz and calcite chips submerged and incubated in 3 ponds in the Charlottesville, Va. area

	Quartz	Calcite	Water	pH
Site	10^4 cells cm^{-2}		10^4 cells ml^{-1}	
		10 Days		
A	87 ± 11.5	60 ± 10.6	102	8.5
B	150 ± 8.9	149 ± 16.5	93	8.1
C	88 ± 9.0	44 ± 3.4	66	7.9
		20 Days		
A	143 ± 14.1	48 ± 11.6	65	8.4
B	269 ± 23.8	261 ± 31.0	77	8.1
C	141 ± 8.9	62 ± 6.9	57	8.0

Values presented are averages ±1 SEM of 3 chips of each of 3 specimens of each mineral. Bacterial counts for water are the average of 2 replicate samples. SEMs for the water counts were usually about 20% of the mean given.

indicate that the differences between minerals and among sites were significant at all times sampled (Table 2).

Effect of Streamflow on Colonization of Sandstone

When hematitic sandstone chips were submerged in 3 different water flow regimes, a significant effect on colonization by microorganisms was observed (Fig. 2). Initially,

Table 2. Results of nested ANOVA to test differences in colonization due to the effects of mineral type, mineral specimen, and immersion site

Source of variation	Sum of squares	D.F.	Mean square	*F*	Signif. of *F*
		10 Days			
Within specimen	32,148	36	893	—	—
Spec. within quartz	4,373	2	2,186	2.44	0.101
Spec. within calcite	3,235	2	1,617	1.81	0.178
Between mineral	8,830	1	8,830	9.88	0.003
Between site	77,072	2	38,536	43.15	0.001
		20 Days			
Within specimen	33,883	36	941	—	—
Spec. within quartz	8,223	2	4,111	4.36	0.20
Spec. within calcite	2,646	2	1,323	1.41	0.258
Between mineral	49,564	1	49,564	52.66	0.001
Between site	334,583	2	167,291	177.74	0.001
		Overall			
Within specimen	243,275	90	2,703	—	—
Spec. within quartz	560	2	280	0.10	0.902
Spec. within calcite	36	2	18	0.01	0.993
Between mineral	52.647	1	52,647	19.48	0.001
Between site	363,975	2	181,987	67.32	0.001

Results are associated with the values in Table 1.

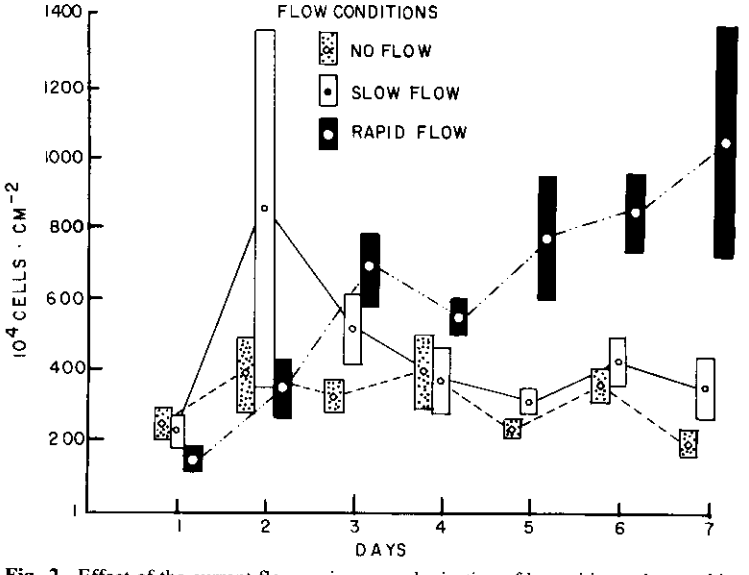

Fig. 2. Effect of the current flow regime on colonization of hematitic sandstone chips incubated in various locations in Mountain Lake. The spot in the center of the bars represents the mean and the entire bar represents ±1 SEM. Analysis of variance demonstrated a significant difference between rapid flow and each of the other conditions.

the colonization was retarded slightly in the fast flow environment, but by the third day of incubation, the samples there contained more organisms than the samples in the other locations, and the differences continued to increase thereafter throughout the 7-day incubation period. F-test comparisons from ANOVA showed overall significance between the slow and fast flow at $\alpha = 0.084$, between no flow and fast flow at $\alpha = 0.017$, and between no flow and slow flow at $\alpha = 0.116$. Although the latter comparison does not possess the low α values of the others, the value attained shows significance at the 88% confidence level, indicating that the differences might be meaningful though not statistically significant at the commonly accepted level of 95%. No significant differences among sites or sampling times were obtained by analysis of variance of AODC results of water column enumerations.

Discussion

The results of this study demonstrate the importance of the chemical composition of a mineral substrate on the initial microbial colonization process. Quartz and calcite were chosen as "pure chemical" analogs of the rocks sandstone and limestone, respectively, and in 2 of the 3 sites, quartz was colonized more rapidly than calcite. When limestone and hematitic sandstone were compared, either no difference was observed, or the quartz-based rock was colonized more slowly than the carbonate. Environmental factors not withstanding, this set of seemingly contradictory observations is consistant with the findings of Marszalek et al. [16] that surfaces which are biologically inert (such as glass) tend to be colonized more rapidly than those which are biologically active (i.e., that leach ions toxic to the colonizing community). The presence of potentially leachable iron in the hematitic sandstone may increase the "activity" of the rock surface and thereby inhibit microbial colonization.

The significant site effects observed in the comparisons of both rocks and minerals demonstrate the importance of the interplay of environmental factors with those of the substance material itself in the formation of primary films. Because of the lack of comprehensive data concerning differences in the environmental factors between Mountain Lake and Riopel Pond, and among the 3 ponds near Charlottesville, it is impossible to attribute the differences in colonization on the minerals from site to site to any specific variable or set of variables. Many factors may affect both the rate and extent of attachment to mineral surfaces. Although the variables might be separated by controlled experiments, in nature the elements act in concert, such that the quantitative contribution of each component to the process may be indeterminable.

The importance of substrate-environment interactions was pointed out by Gerchakov et al. [8] and Marszalek et al. [16]. In reporting the results of a study of biofouling associated with the Ocean Thermal Energy Conversion (OTEC) project, they showed that attachment (biofouling) is a function of the chemical composition of the substrate material, modified by the organisms at the immersion site, and the environmental factors at the site, such as water chemistry and temperature. Further evidence of environmental parameters acting in concert with substrate characteristics is offered by Fletcher and Loeb [4] and Fletcher [2], who reported that the degree of colonization of several substrates was related to the charge and hydrophobicity of the surfaces, but that the physiological state of the cells, the numbers of cells, and the temperature strongly affected the number of bacteria attaching to the surface of a single substrate, viz., polystyrene.

The relation of water flow rate to colonization observed in the present study is consistent with observations that epilithic organisms dominate the microbial community of flowing streams [1, 5, 6, 7, 13]. Presumably, the attached forms receive a continuously replenished supply of nutrients as the water passes by, and the presence of the surface may also help to concentrate dilute nutrients from the water [11, 14, 15, 18–21]. Based on heterotrophic activity determinations, Ladd et al. [13] showed that the epiliths removed a greater proportion of ^{14}C-labeled glutamate from the water than did the planktonic bacteria. Furthermore, the enhanced uptake was observed to be on a specific activity (per cell) basis as well as on a total activity basis (attributable to a larger epilithic than planktonic biomass), an observation consistent with that of Fletcher [3] in a study using plastic surfaces and microautoradiography. Higher heterotrophic activity was obtained from the disturbed as opposed to the undisturbed community, a fact explained by reasoning that the scraped samples possessed a lower diffusion resistance due to disruption of the natural biofilm. Even though the presence of a slime matrix appeared to retard substrate diffusion to the cells, uptake of the labeled glutamate was more rapid in the undisturbed than in the planktonic samples. In static (no flow) situations, adhesion may be of a lesser advantage, although the adsorptive forces at the surfaces still tend to increase the concentration of nutrients in the surface's vicinity.

The epifluorescence method described here for the direct examination of opaque surfaces may be quite valuable for studies on the initial colonization of those surfaces, but it is not without drawbacks. Although Ladd et al. [13] reported using a similar method for examining surfaces submerged for periods up to 52 weeks, obtaining a value of 4.3×10^6 cells cm^{-2}, in the present study about 3 weeks was the maximum time that could be used to successfully enumerate rock-attached microbes. After that time, the coating was so thick that many cells were covered by others. Attempts to count cells in a layer more than 1 cell deep are not advisable using this method.

The shallow depth of field at high magnification limits the applicability of the technique to smooth surfaces that can be fitted on the stage of the microscope. Marszalek et al. [16] successfully used an acridine orange staining and direct examination technique to observe the initial microfouling process on surfaces of metal and glass coupons immersed in seawater. Nonspecific staining of the substrate and natural fluorescence are also problems, with some minerals, but it is possible that use of more highly specific stains may overcome this problem. Within its limitations, the technique of direct epifluorescent examination of opaque surfaces should prove a valuable tool in the study of the initial processes surrounding colonization and biofouling of submerged surfaces.

Implications of the results of this work, although highly speculative, may be of great ecological significance, particularly when taken together with those of the OTEC studies [8, 16]. Several questions for future research arise. For example, do different rock types select for different populations of adherent microbes? (The OTEC studies demonstrated that not only do different substrate materials become colonized at different rates, but that the colonizing organisms are qualitatively different as well.) If so, does the difference in populations result in a difference in the functional abilities of the epilithic communities? Do the populations involved in the initial colonization influence the composition of the mature slime layer, and, if so, does that, in turn, influence the suitability of the habitat for organisms that gain nutrition by scraping and ingesting the slime layer from the rock's surfaces? Future research in these areas may indeed show that the physical-chemical controls on microbial colonization of rock surfaces may ultimately exert a strong influence on the chemistry and biology of the entire ecosystem in those locations dominated by epilithic forms.

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