N85-32747

CHAPTER V

MICROBIAL COLONIZATION AND GROWTH ON METAL SULFIDES AND OTHER MINERAL SURFACES

Prof. D.E. Caldwell
A.R. Sundquist
J. Lawrence
A.P. Doyle

Introduction

Sulfur-containing minerals present in soils, sediments, and other environments are often coated with a film of actively metabolizing microorganisms. Because these films are difficult to study, cell suspensions are frequently used. Laboratory studies have formed the basis of knowledge concerning microbial activity and the chemical development of Earth. Because there could be important differences between the activities of microbes in uniform cell suspensions and those in nature, this research aimed to document the presence and formation of mineral films. It was found that microbial films formed rapidly on all of the (SbS_2), barite ($BaSO_4$), selenite ($CaSO_4$), amorphous elemental sulfur, and hematite (Fe_2O_3). Gradients of soluble sulfur compounds (cysteine, glutathione, thioglycolate, sulfite, and thiosulfate) did not increase the rate of attachment or growth of bacterial populations colonizing these surfaces. Microbial activities, including metabolism, growth, behavior varied substantially depending upon whether the cells were in suspension or in surface films.

Materials and Methods

In Situ Incubation of Membrane Enrichments

A membrane filter (polycarbonate capillary membrane, 0.05 um pores, 25 mm diameter, Nucleopore, Pleasanton, CA) was cemented to the ground flat surface of one tube of a Bellco Parabiotic Chamber (catalog no. 1945) (Fig. V-1). This was done by coating the surface with an uninterrupted ring of silicone rubber and applying the filter with the shiny side out. The assembled "J-tube" was autoclaved to sterilize the apparatus and to solidify the silicone rubber. Care was taken to protect the outer surface of the filter from pore-clogging debris.

50 mM solutions of the following five sulfur compounds were prepared: L-cysteine, reduced glutathione, and the sodium salts of sulfite, thiosulfate, and thioglycolate. The compounds were dissolved in water and the pH adjusted to neutrality.

Acridine orange, a fluorescent stain, was used to stain the incubated filters. A 0.1 percent stock solution was first prepared by dissolving acridine orange in 10 mM potassium phosphate buffer at pH 7.2. A 1:10 dilution of the stock into

Surface	N (cells/field)	A (cells/field-hr)	$N=A/\mu(e^{\mu t}-1)$ μ solved(hr^{-1})	$\mu=\ln(N/c_1+1)/t$ μ calculated(hr^{-1})
pyrite (natural)	70.0	3.1	0.051	0.23
	67.6	2.8	0.060	0.19
pyrite polished	99.6	3.9	.066	0.22
	43.4	1.8	0.062	0.20
pyrite polished	73.7	2.7	0.074	0.20
oblique	44.9	2.0	0.054	0.22
average	66.5±21	2.7±0.78	0.061±0.008	0.21±0.02
pyrrhotite natural	73.9	2.9	0.066	0.20
	98.7	4.3	0.052	0.23
pyrrhotite polished	120	4.0	0.085	0.19
top	124	3.8	0.094	0.19
" " side	79.3	3.4	0.057	0.18
average	99.2±22.8	3.7±0.6	0.071±0.016	0.20±0.02
galena	71.2	2.5	0.076	0.28
	47.6	1.9	0.04	0.22
stibnite	80.2	2.9	0.076	0.19
	50.3	2.4	0.044	0.24
sulfur	57.4	2.4	0.061	0.21
	41.6	1.7	0.064	0.23
glass	55.7	2.4	0.056	0.22
	45.8	2.3	0.040	0.21
average	56.2±13.3	2.3±0.4	0.060±0.013	0.23±0.03
calcite	32.2	1.2	0.071	0.21
	69.4	2.2	0.083	0.21

Table V-1. Bacterial colonization of sulfide mineral surfaces.

	cells per field	colonies per field	attachment rate (cells field^{-1}h^{-1})	specific growth rate (h^{-1})
cystine	18.0	13.0	1.9	0.29
glutathione	8.9	5.5	0.78	0.21
thioglycollate	7.5	6.0	0.85	0.18
sulfite	11.0	7.2	1.0	0.23
thiosulfate	14.0	10.0	1.4	0.20
control	15.0	9.2	1.3	0.23

note: 4,900 μm^2 per field

Table V-2. Effect of sulfur compounds on bacterial attachment to mineral surfaces.

Figure V-1. "J-tube" with filter.

the above phosphate buffer yielded a 0.01 percent working solution. Both solutions were kept refrigerated between uses.

A 4 percent solution of formalin was used as fixative.

Six assembled J-tubes were autoclaved and cooled. Into five separate J-tubes were added approximately 25 ml of the five sulfur-compound solutions. The sixth J-tube, the control, was left empty. The J-tubes were capped and fastened to a test tube rack with the membranes facing down. This positioning ensured that solution was always in contact with the filter and that no debris settled on the filter surface. The rack and J-tubes were submerged in the sulfur spring-fed pool at Alum Rock Park (Fig. V-2, site 1). After a seven hour incubation period (7:30 AM to 2:30 PM) the J-tubes were removed from the creek and the remaining solutions were poured out. The attached filters were rinsed with distilled water and the J-tubes were placed into a beaker of 10 percent formalin. The J-tubes were kept refrigerated in the formalin until the filters were to be examined.

The filters were stained while attached to the J-tubes. After rinsing away the formalin with water the filters were flooded with 0.01 percent acridine orange for 30 seconds and rinsed with water. The J-tubes were then placed into a beaker of water for 5 minutes. The latter ensured that the staining of the background was minimized. The cells of interest were those irreversibly attached to the filters so that loss of cells from a filter surface during rinsing, staining, or storage was insignificant. The filters were then peeled away from the J-tubes and placed onto glass slides with the colonized side up. After trimming the filters, cover slips were placed on the filters and sealed with vaspar, an equal mixture of petroleum jelly and paraffin.

A Zeiss Photomicroscope III with epifluorescence was used to view the filters at 1000X magnification. Twenty fields were selected randomly from each filter. Fields containg debris were discarded. All bacteria and microcolonies that fell within or intruded upon a 65x65 um grid were counted as being in that field. A microcolony was defined as an accumulation of morphologically similar cells that were no further than a cell-length away from a neighboring cell. The number of microcolonies containing 1, 2, 3-6, 7-12, 13-24, or more cells as well as the total cell count were noted for each filter. From the distribution of cells within these microcolonies the growth and attachment rates for *in situ* colonization of the filters were determined.

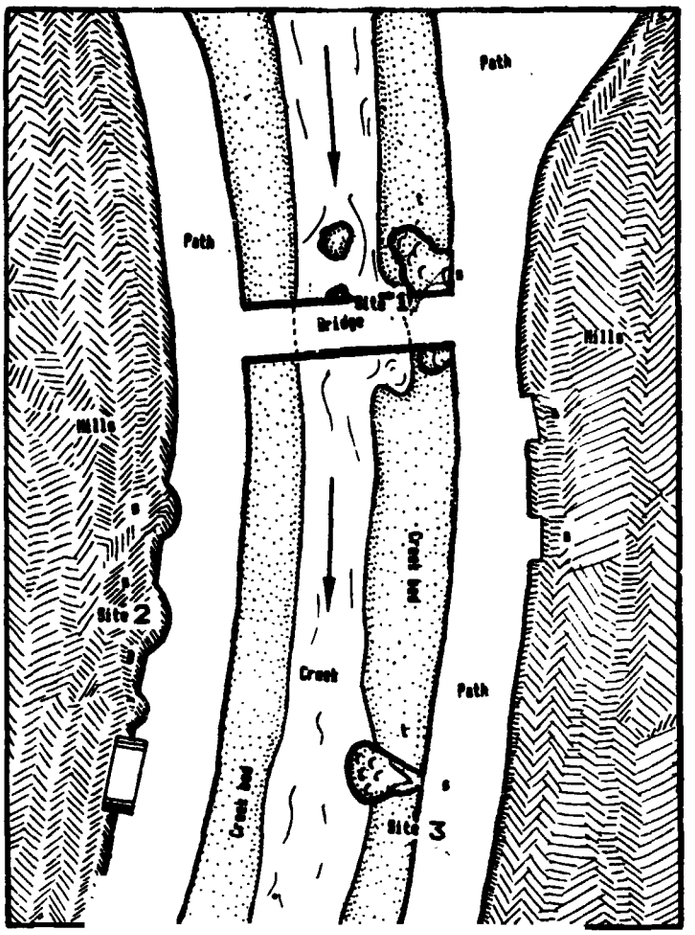


Figure V-2. Close-up of Penitencia Creek inset, Alum Rock Park Map 3.

Calculations of total cell count, N, and colony count, Ct, or attachment rate, A, and growth rate, u, were done for each field. These field values were totaled and averaged to give the mean field value for each filter. All results were repeated as average counts and rates for a filter.

u_1 and u_2 were also corelated by first averaging the cell count for the filters and then using these averages in the growth rate equation of Caldwell.

Field values of A were correlated by counting the attachment events (i.e., all microcolonies) and dividing by the incubation period. From these values $\bar{A}$ (the average of the field values) was determined. (Three different values for A and $\bar{A}$ were calculated depending on the value of Ct used, which depended in turn on the value of C chosen - see A_1, A_2, and A_3).

Individual growth rates, u, were calculated with the following equation:

$$u = 1\ (N/Ct + 1)/t$$

Four values, $u1$-$u4$ were calculated. A second equation was used to caluculate u; three values were determined, u-$u7$, for each field.

In Situ Incubation of Minerals

Either natural crystal faces or cleavage faces were used as colonization surfaces. In some cases the crystals were polished using 0.2 micron diamond abrasive. In the case of pyrite and pyrrhotite, oblique faces (with respect to cleavage and crystal planes) were obtained by grinding and the colonization of these faces compared to that of the natural faces (Fig V-3). Crystal faces were cleaned before use by wiping with petroleum ether. Crystals were submerged at Site 1, Penitencia Creek Alum Rock State Park.

Study of Pure Cultures and Natural Communities in Continuous Slide Culture

Perfilev capillaries (Perfilev and Gabe, 1969) were used to construct continuous slide cultures in which a laminar flow velocity of 10 cm per second was maintained during microbial colonization and growth. The capillaries were two mm in depth and 1 mm in width (internal dimensions). The small size of these capillaries made it difficult to properly focus the condenser on the cells for phase microscopy. In addition the capillaries contained stretch marks that resulted in optical distortions. As a result, capillaries were produced by bonding cover slips between glass slides using silicone seal. The dimensions of this culture system were 2 by 2 mm. Liquid was supplied via 22 gauge hypodermic needles inserted through the silicone. Sterile medium or sterile filtered stream water was supplied continuously and the inoculum was injected through a silicone septum. The inoculum was pulsed repeatedly or continuously until the

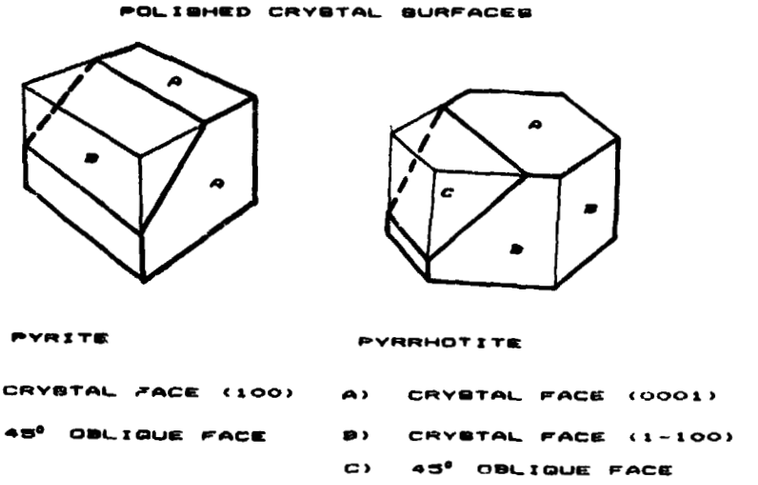
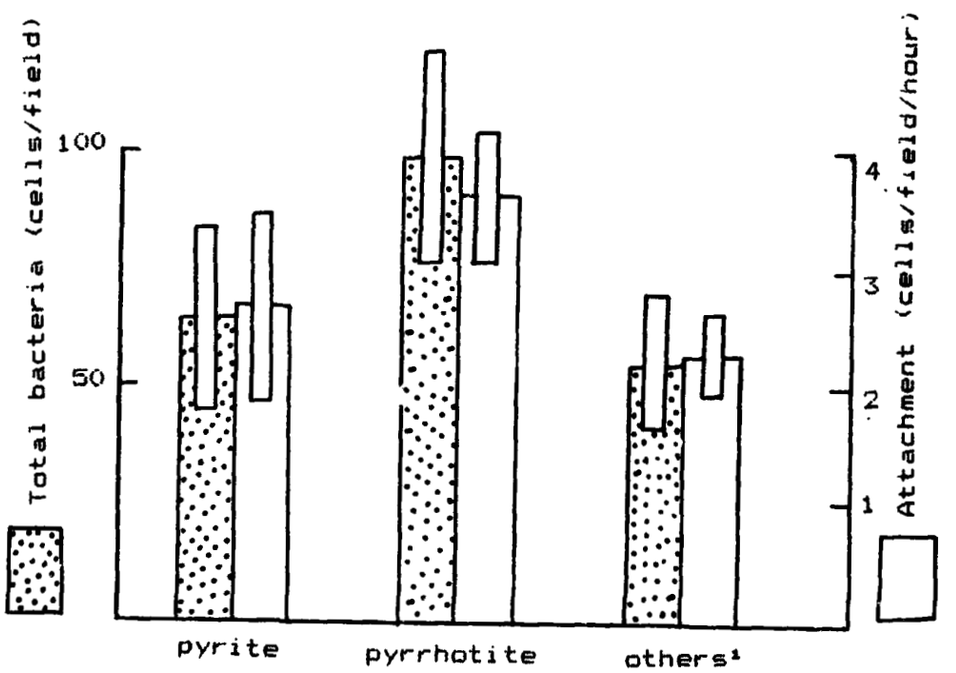


Figure V-3. Diagrams of polished crystal surfaces of pyrite and pyrrhotite. Numbers in parentheses refer to mineralogists' code for either crystal face or cleavage plane.



¹ glass, galena, stibnite and sulfur

Figure V-4. Distribution of colony occurrence on mineral surfaces: bacterial attachment to pyrite, pyrrhotite, and other minerals.

density of cells was 5 to 50 per field (4,900 square microns per field).

Fluorescent Staining

Bacterial cells on mineral and filter surfaces were visualized using epifluorescent microscopy to observe cells stained with a 0.01 percent (w/v) solution of acridine orange in a 10 mM phosphate buffer (pH 7.2).

A novel stain, MBBR, was also evaluated as a potential staining technique. It gives a blue fluorescence upon reaction with thiols (Kosower et al., 1978; Newton et al., 1981).

A 50 mM stock of MBBR (Calbiochem-Behring - now called Behring Diagnostic) in acetonitrile was made. Acetonitrile (CH_3CN) is used because it will dissolve MBBR and it is only a poorly nucleophilic solvent. This solution can be kept refrigerated for months. 3 mM solutions are made by diluting the stock into Tris buffer (50 mM Tris, pH 8.0, 3 mM EDTA). This working solution should be made fresh daily since the MBBR will react slowly with water. Also, since uv radiation speeds up the nucleophilic attack on MBBR, hydrolysis can be greatly reduced by shielding the 3 mM solution from light. This will prolong the "lifetime" of the working solution.

Results and Discussion

Incubation of Minerals Under *In Situ* Conditions

To determine whether a bacterial film forms on sulfur minerals *in situ*, various sulfur-containing and other minerals were incubated at Site 1 in Penitencia Creek. The rate of cell growth and attachment within the surface microenvironment of mineral surfaces was also determined (Caldwell et al., 1981, 1983). Minerals studied included pyrite, pyrrhotite, galena, stibnite, barite, selenite (calcite), amorphous ingots of elemental sulfur, and hematite. A film rapidly formed on each of the minerals, however, no differences between the rate of colonization of various minerals were observed (Table V-1) with the exception of pyrite and pyrrhotite which were more rapidly colonized than other minerals. This confirms results previously obtained in continuous culture but which evaded repetition under *in situ* conditions (Kieft and Caldwell, 1984).

Data for bacterial colonization of each mineral are presented in Table V-1. The average number of cells in each field (N) and the hourly attachment rate (A) are given in the first two columns. No outstanding differences between minerals in final population could be seen there. Because the number of replicates for each surface type was small, pyrite, pyrrhotite and the remaining surfaces were compared as groups as shown in the histogram in Figure V-9. Pyrrhotite had fifty percent more bacteria than other surfaces, but this difference may be due to errors in the estimates. The error bars represent one standard deviation of the five to ten surfaces compiled in each group.

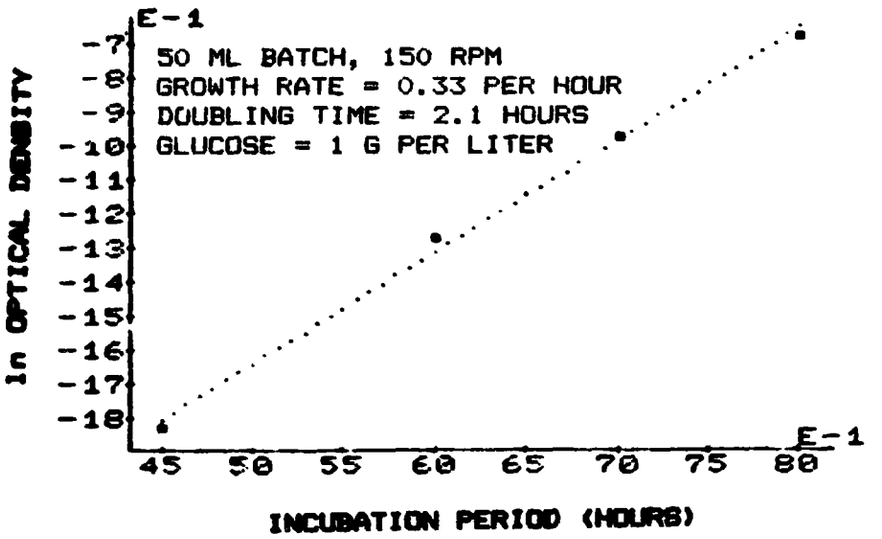


Figure V-5. Exponential growth of *Pseudomonas fluorescens* batch culture (50 ml volume, 150 rpm shaking) μ =0.331 hr^{-1}. Ta =2.09 h.

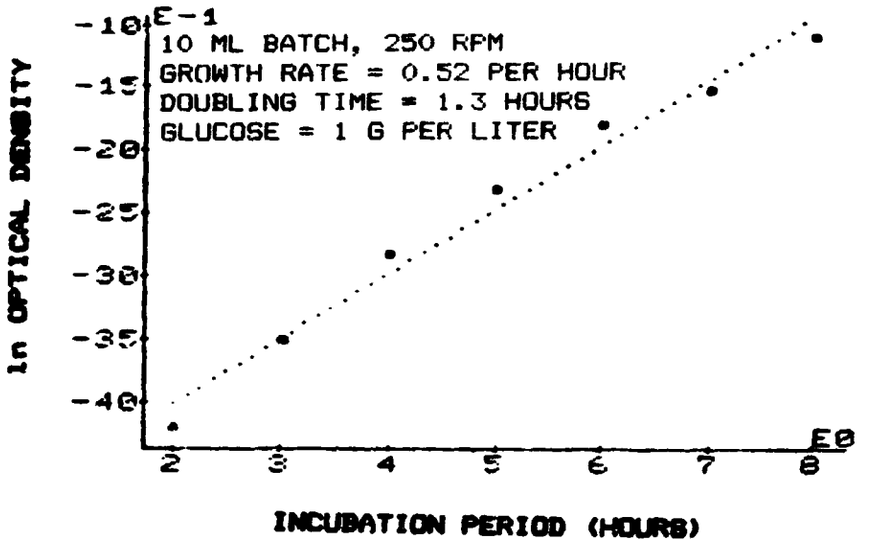


Figure V-6. Exponential growth of *Pseudomonas fluorescens* batch culture (10 ml volume, 250 rpm shaking) μ =0.515 hr^{-1}. Ta =1.346 h.

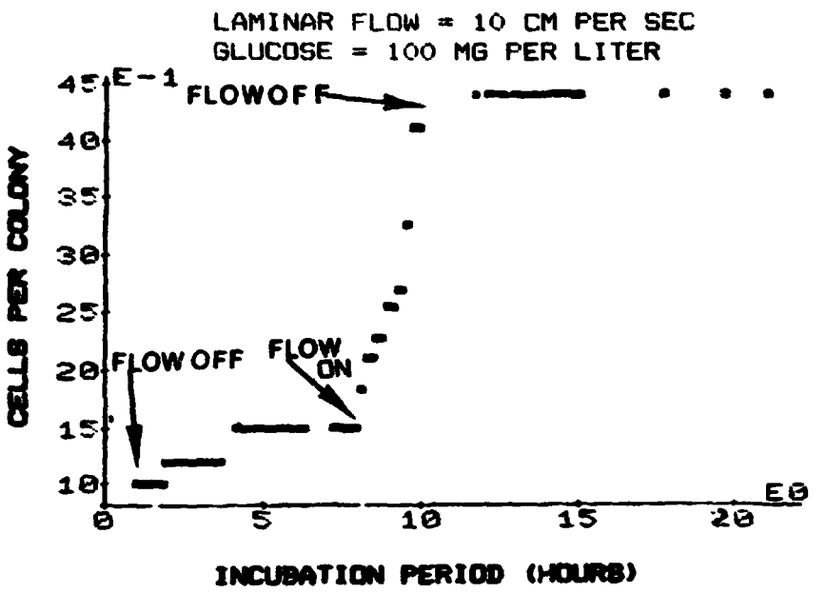


Figure V-7. Growth of *Pseudomonas fluorescens* colonies in a Perfil'ev capillary with and without laminar flow at a glucose concentration of 100 g/liter.

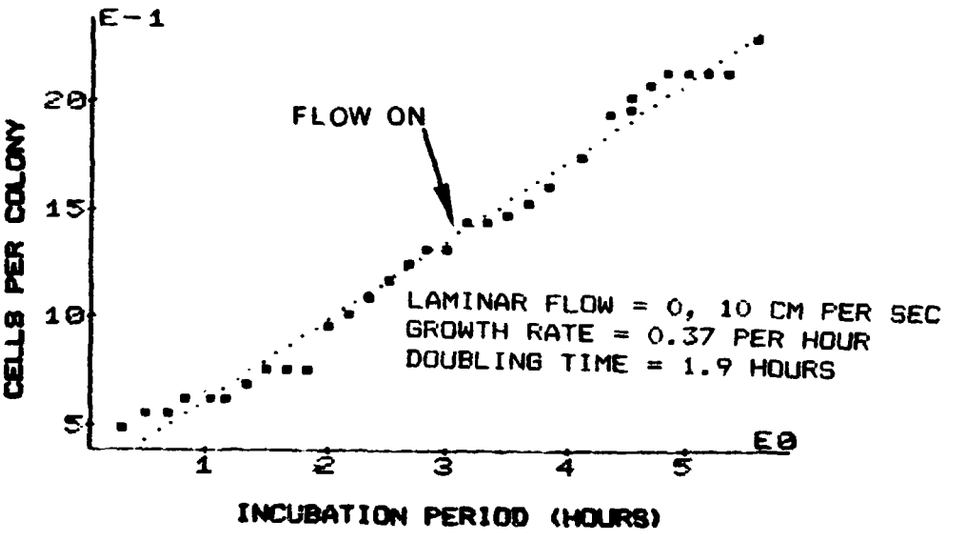


Figure V-8. Growth of attached colonies of *Pseudomonas fluorescens* without and with laminar flow at a glucose concentration of 1 g/liter

Two growth rates were calculated for bacteria on each surface. Although these values differed by about a factor of four depending on the method of calculation, they were constant from sample to sample within experimental error. The lack of a significant enhancement of growth by one mineral type indicates that either the substrates had a negligible effect on growth or that the bacterial community was too heterogenous to reveal enhanced growth. Sulfide oxidizers were probably not the predominant bacteria in the incubation site.

The growth equation developed by Caldwell et al. (1981) was used to obtain growth rates from the observed bacterial populations. With values for total cells (N) and attachment (A), a looping, trial-and-error computer program was used to solve the equation for growth (u).

$$N = A/u\ (\exp(ut) - 1) \qquad (1)$$

This gave the first growth rate listed in Table V-1. With the assumption that the colonies reached steady growth and attachment rates, a second equation was derived by Caldwell et al. (1983). In this equation growth could be directly calculated.

$$u = \ln(N/Ci + 1)/t \qquad (2)$$

Ci was the average number of occurrences that a colony size class (1-, 2-, 4-, 8-, 16-cells, etc.) was seen on a surface and t was the incubation time. This growth rate is the second value given in Table V-1. Direct observation of two actively growing colonies described earlier in this chapter gave an average growth rate of 0.58/h. The second growth rate was closer to this value, but this may have been an artifact.

The surfaces of the selenite crystals dissolved markedly, so the results from their colonization were not included here. One polished side and both polished oblique surfaces of the pyrrhotite were microscopically streaked or blemished, so their counts were not included either. A visual comparison of the polished pyrrhotite and pyrite indicated that there was more shallow pitting on the pyrrhotite, which may have been due to its faster weathering. Quantitative examination with such techniques as scanning electron microscopy or computer-aided image enhancement of these surfaces before and after colonization are needed to confirm this observation.

In Situ Incubation of Membrane Enrichments

To determine whether surfaces enriched with soluble sulfur substrates (cysteine, glutathione, thioglycolate, sulfite, and thiosulfate) increased the rate of growth or attachment of natural communities, membrane enrichments were incubated at Site 1. These rates were determined as described by Caldwell et al. (1981, 1983). The enrichments were incubated slightly downstream from sulfur springs which released sulfur-oxidizing bacteria. This ensured that the *in situ* concentration of sulfur substrates would be below that supplied by the membrane enrichments and that appropriate communities would be

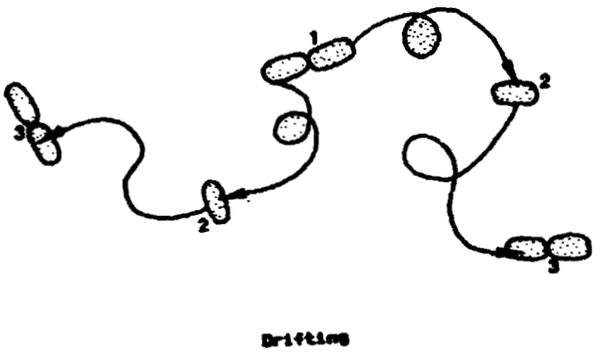


Figure V-9. Drifting mode of surface colonization.

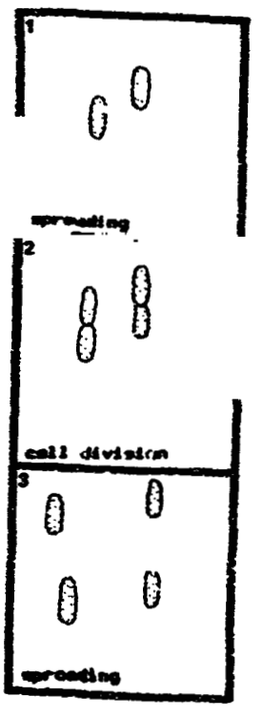


Figure V-10. Spreading mode of surface colonization.

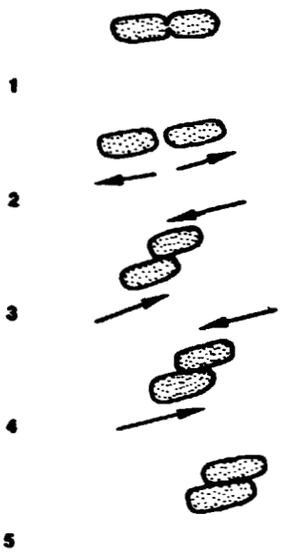


Figure V-11. Static mode of surface colonization.

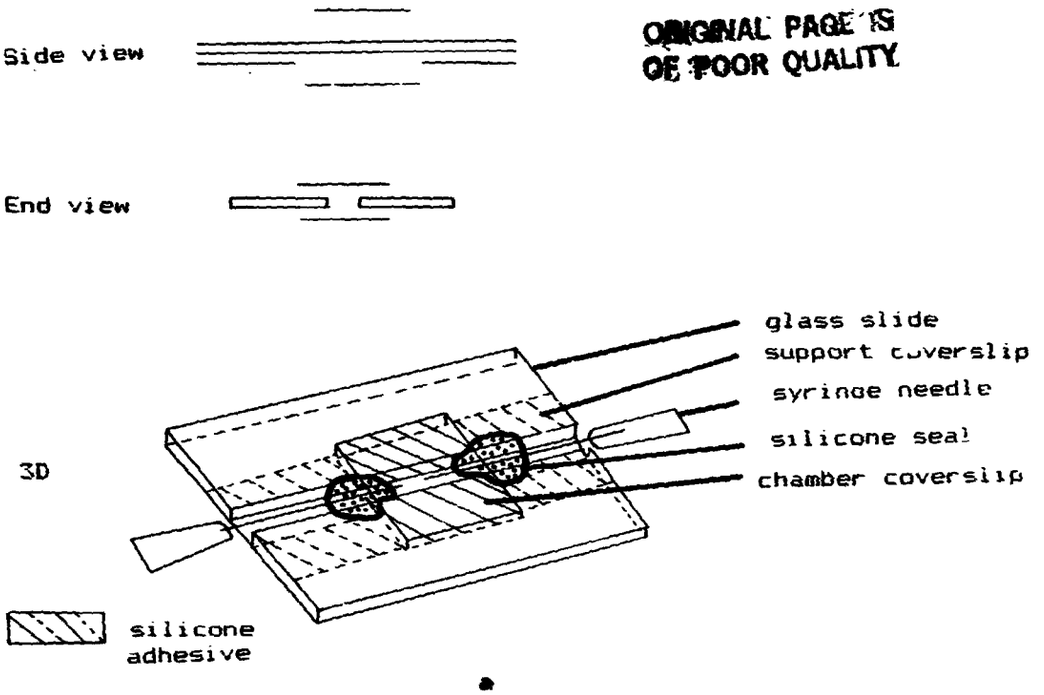


Figure V-12. (a) Schematic diagram of colonization and chamber culture apparatus; (b) diagrams of observation chamber.

present. The results of this study (Table V-2) showed no significant difference between the experimental treatments and the control membranes which lacked substrate.

Differences between Microbial Growth Kinetics in Cell Suspensions and in Films

The growth of *Pseudomonas fluorescens*, a heterotrophic sulfur oxidizer, was studied in batch cell suspensions and in continuous culture. In batch culture the cells were oxygen limited (growth rate 0.33 per hour under oxygen limitation and 0.52 per hour when vigorously aerated, Fig V-5 and 6). As shown in Fig V-7 and 8, growth within the film was glucose limited. Glucose limitation could be eliminated by increasing laminar flow and consequently decreasing the thickness of the hydrodynamic boundary layer. It can be seen that the attached cells are capable of growth rates equal to or faster than the rates achieved in liquid culture.

Differences between Microbial Behavior in Cell Suspension and in Biofilms

Several behavioral phenomena were observed for cells growing within the hydrodynamic boundary layer that have not previously reported. Despite a flow of 10 cm per second in the environment, the bacteria were able to move freely in both directions within the hydrodynamic boundary layer which apparently provides a calm zone within which turbulence is avoided and in which bacterial motility is remarkably effective. During the division of cells on surfaces unique mechanisms of remaining attached during cell division were observed as the colony formed. In the drifting mode the cells tumble and drift across the surface at a very slow rate as they divide. In the spreading mode of surface colonization the cells spread outward from the center of the colony as they divide. In the static mode of surface colonization the cells stay tightly grouped within the colony although they do undergo separation and reorientation. (Figures V-9-11.)

References

Baldensperger, J., Guarraia, L.J., and Humphreys, W.J., 1974. Scanning electron microscopy of thiobacilli grown on colloidal sulfur. Arch. Microbiol., 99:323-329.

Bennet, J.C., and Tributsch, H., 1978. Bacterial leaching patterns on pyrite crystal surfaces, J. Bacteriol., 134:310-317.

Caldwell, D.E., Brannan, D.K., Morris, M.E., and Betlach, M.R., 1981. Quantitiation of microbial growth on surfaces, Microbial Ecol., 7:1-11.

Caldwell, D.E., Malone, J.A., and Kieft, T.L., 1983. Derivation of a growth rate equation describing microbial surface colonization, Microbial Ecol., 9:1-6.

Kieft, T.L., and Caldwell, D.E., 1984. Weathering of calcite, pyrite, and sulfur by *Thermothrix thiopara* in a thermal spring., Geomicrobiol. J., 3:201-216.

Kieft, T.L., and Caldwell, D.E., 1984. Chemostat and *in situ* colonization kinetics of *Thermothrix thiopara* on calcite and pyrite surfaces, Geomicrobiol. J., 3:217-229.

Kosower, E. M., Pazhenchevsky, B., and Hershkowitz, E., 1978. 1,5-diazabicyclo(3.3.0)-octadienediones (9,10-dioxabimanes). Strongly fluorescent syn isomers, J. Am. Chem. Soc., 100:6516-6518.

Lord, C.J. and Church, T.M., 1984. The geochemistry of salt marshes: Sedimentary ion diffusion, sulfate reduction, and pyritization. Geochim. Cosmochim. Acta, 47:1381-1391.

Newton, G.L, Dorian, R., and Fahey, R.C., 1981. Analysis of biologcal thiols: Derivation with monobromobimane and separation by reverse-phase high-performance liquid chromatography, Anal. Biochem., 114:383-387.

Perfiliev, B.W. and Gabe, D.R., 1969. *Capillary Methods of Investigating Microorganisms*, (J. M. Shewan, Transl.), University of Toronto Press, 627 pp.