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FIELD AND LABORATORY METHODS

Biofilm and Stromatolite Collection

We refer to the biofilm and Fe-rich biolaminates as stromatolites based on the definition of stromatolites by Awramik et al. (1976, p. 149), who state "…stromatolites are organosedimentary structures produced by sediment trapping, binding, and/or precipitation resulting from metabolic activity and growth of organisms…". Samples of stromatolites collected from the Green Valley site included large hand samples containing both biofilm and underlying Fe-rich biolaminates, pipette-dissected biofilm samples, and biofilm grown on glass slides. These samples were used to identify type and distribution of microorganisms in the biofilm and underlying biolaminates and to characterize textural features of the stromatolites.

The large hand samples were collected with a shovel, trowel, and spatula, and placed in glass containers or plastic bags containing acid mine drainage. Samples were transported to the laboratory and immediately dissected with a straight probe and scalpel. Layers were swabbed or scraped to obtain microorganisms for microscopic examination. Pasteur pipettes were also used in the field to collect samples from the surface, middle, and base of the biofilm, depending on biofilm thickness. Samples were placed in 5 mL sterile plastic tubes for transport to the laboratory, and were analyzed immediately or stored at 4^{o}C for evaluation within 24 h. In addition, samples of biofilm were obtained by placing a set of four glass slides (5 x 7.6 cm each) on the substrate at six locations in the channel where *Euglena*-dominated communities covered over 60% of the substrate. The biofilm was allowed to grow on the slides for 6 weeks. The slides were then collected and placed in glass containers with acid mine drainage. Containers were sealed with tape and stored at 4 ^{o}C for 24 h prior to examination.

The distribution and type of microorganisms in the collected stromatolites were determined microscopically by preparing wet mounts of the samples and viewing the prepared slides with transmitted light, dark field, and phase contrast microscopy using 10x, 40x, and 100x objectives. Population size was recorded as very abundant (>75% field of view), abundant (~50% field of view), common (~25% field of view), rare (~10% field of view), and very rare (<5% field of view). Larger microorganisms were identified based on morphology, using identification guides for freshwater microbial communities. Fungal hyphae and yeasts were distinguished either in wet mount or after Gram stain (see Lim, 1998, for details on Gram stain procedure). Internal structures in *E. mutabilis* were enhanced by staining fixed smears with methylene blue. Bacteria residing in the biofilm were cultured by inoculating samples on Tryptic soy agar plates and broth and incubating at room temperature (20-22 ^{o}C) for 72 h. Broth cultures were then serially diluted and plated onto Tryptic soy agar plates. Bacterial differentiation was based on colony morphology and Gram reaction. Anaerobic bacteria were identified by plating samples onto Tryptic soy agar (pH 7.0) and artificial acid mine drainage agar (pH 5.5 and 3.5) and incubating the bacteria in an anaerobic chamber at room temperature (20-22 ^{o}C). The artificial acid mine drainage media consisted of KH_2PO_4 (1.0 g), $MgSO_4$ (0.25 g), $(NH_4)_2SO_4$ (0.75 g), and $FeSO_4$ (3.859 g) in 500 mL distilled H_2O plus 15 g of argarose, adjusted to pH 5.5 and 3.5 with H_2SO_4. Prior to plating, the artificial media was stirred while heating to <100 ^{o}C, and then autoclaved for 15 min to sterilize. All plates were observed at 24, 48, and 72 h for growth. Biofilm generally did not contain a distinct stratification of microbial communities, such as that commonly observed in prokaryote-dominated biofilm. In addition, there was a notable absence of undermat communities composed of anaerobic bacteria.

Samples of the stromatolites selected for petrographic study were air dried and impregnated with epoxy using a vacuum oven. Ten thin sections were prepared and

polished to <0.1 mm using a Bueler Petro-Thin Thin-Sectioning System and a Bueler Minimet Polisher. The sections were examined with a polarizing petrographic microscope to characterize biofilm and laminae thickness, to describe the textural characteristics of the laminae, and to identify microorganisms in each layer. In addition, over 50 Fe-rich stromatolitic samples were examined macroscopically to describe the textural characteristics and thickness of the various laminae.

Dissolved Oxygen Study

E. mutabilis cells collected from *Euglena*-dominated biofilm at the Green Valley site were grown in artificial acid mine drainage media (described above), adjusted to pH 3.1 with H_2SO_4. The media was stirred while heating to <100 ^oC, and then autoclaved for 15 min to sterilize. *E. mutabilis* cells were added to the media and left to grow and reproduce for 4 months at room temperature (20-22 ^oC) under natural light conditions. At the end of this growth period, dissolved O_2 in *E. mutabilis*-bearing media was measured continuously for 1 week, using, a YSI model 5300 biological O_2 monitor, with an accuracy of $\pm$0.5%. Agitated distilled O_2 blanks were analyzed at the beginning and at the end of the week of O_2 monitoring.

Inductively Coupled Plasma Emission Spectrometric and Scanning Electron Microscopic Analyses

E. mutabilis cells collected from the Green Valley site were thoroughly washed with reverse osmosis water to remove extracellular material. Wash procedures consisted of resuspending cells in reverse osmosis water, centrifuging at 8000 x g for 4 min to pellet the cells, and decanting the water. This procedure was repeated 5 times to remove extracellular material. Washed samples were examined microscopically to ensure removal of solid residue. At the end of the last centrifuge cycle, the supernatant

was analyzed for Fe using colorimetry with a detection range of 0 to 10 mg Fe. Phosphate buffer (0.1 M KPO_4 at pH 7.2) plus Na Azide (0.02%) was added to the washed cells and vortexed to resuspend the pellets. The cells were centrifuged and the supernatant decanted. To break the cells, 1 mL of acetone was added, vortexed, and incubated at room temperature for 10 min. The samples were centrifuged at 27000 x g for 10 min, and the acetone decanted. Resultant pellets were resuspended in phosphate buffer (0.3 mL), and an equal volume of phenol/chloroform/isoamyl alcohol (ratio of 25:24:1) at pH 5.2 $\pm$ 0.2 was added to extract protein. The samples were then vortexed for 1 min, followed by centrifugation for 10 min; protein and other cellular material remained suspended in the supernatant while the granules formed a small pellet (~0.05 mL). The supernatant was decanted, and the pellets washed 3 times with reverse osmosis water (0.2 mL). Part of a pellet was Au coated and analyzed with a semi-quantitative JEOL scanning electron microscope with an energy dispersive system at the University of Nevada, Reno. The remaining pellet material underwent complete acid digestion for preliminary analysis of trace element composition using an inductively coupled plasma emission mass spectrometer at the University of Nevada, Reno.

Assessment of Phototactic/Aerotactic Behavior

The phototactic/aerotactic response of *E. mutabilis* was monitored in the field and laboratory. In the field, this behavior was observed after major rainfall events (i.e., >5 cm precipitation). During the rainfall event, dilution of the acidic effluent resulted in formation of aggregated Fe and Al colloidal sediments, which covered the biofilm. Movement of the microbial community through the biofilm was monitored 3 times over a 24 h period subsequent to rainfall events to visually determine the rate of microbial recovery. In the laboratory, the phototactic/aerotactic response of *E. mutabilis* was determined by placing 10 mL of acid mine drainage precipitates with *E. mutabilis*-

dominated biofilm in 10 test tubes. The tubes were agitated by hand to mix the cells with the unconsolidated precipitates. Half of the tubes were placed in a rack under normal light conditions for 24 h, and the remaining tubes were left for 24 h in the darkness of a refrigerator. Whether the behavior exhibited by *E. mutabilis* is in response to aerotactic or to phototactic stimuli has not yet been determined.

REFERENCES

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