

**Scanning Electron Microscopy
Techniques For Preservation and
Observation Of Microbe-Mineral
Assemblages and Biominerals**

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Abstract

Scanning Electron Microscopy (SEM) of microbe-mineral assemblages can be challenging, especially when the objective is to characterize biominerals associated with cellular surfaces and exopolysaccharides (EPS). Biominerals are often loosely associated with the organic substrate on which they form making it difficult to determine the origin of minerals as biogenic or abiotic oxidation products. There are a variety of techniques that can be used for preservation of specimens ensuring that observation of desired relationships can be preserved. Here we examine various fixation, dehydration, and staining techniques using a suite of specimens in an effort to determine which technique works best with a particular type of biofilm. Types of biofilms used for this study included, biofilms attached to glass slides, flocculent non-cohesive biofilms, and cohesive highly mineralized biofilms. Microscopy was done to characterize the integrity of microorganisms, biominerals, microbe-mineral assemblages, alteration of specimens, and artifacts introduced during fixation and dehydration.

Results indicate that vapor fixation of biofilms is the ideal methods of preservation for enumeration and characterization of biofilm architecture. Microbe-mineral assemblages remained intact when collected and immediately preserved in soft agar containing 2% glutaraldehyde.

Introduction

Biofilms and microbial mats are typically characterized as organized consortiums of prokaryotic micro-colonies forming three-dimensional architectural structures attached to a substratum or held together by semi-solid hydrated EPS matrices (Dolan, 2002; Crang et al., 1988). These biofilm communities form at an inert solid/liquid interface deriving dissolved gases and nutrients from their surrounding fluid environment (Dolan, 2002; Ramsing et al., 1993). Inertial forces that drive the transport of nutrients through convection and eddy diffusion on a microscopic scale govern bulk-flow of fluids. For example, on a macroscopic scale internal friction of water exhibits little effect, but on a microscopic scale internal friction is the dominating force changing the physical environment where water viscosity increases (Ramsing et al., 1993).

EPS matrices serve a variety of important functions within biofilms by providing a mechanism for surface attachment, diffusion gradients, channels for nutrient/waste products, active sites for nucleation of minerals and temporary protection from dehydration and predation (Krümbein et al., 2003; Dolan 2002; Dykstra, 1993). In addition EPS traps and binds nanoparticulate minerals and other inorganic substances incorporating them into the biofilm

architecture (Krümbein et al., 2003; Hugenholtz et al., 1998; Ramsing, 1993; Crang et al., 1988). The EPS possesses various polyanionic molecules with specific chemical and physical properties attributed to the surrounding environment and provides more surface area for mineral nucleation.

Preparation of biofilms for electron microscopy (EM) results in dehydration and collapse of the biofilm, cellular structures, and EPS impeding visualization and characterization of microbe-mineral assemblages, biofilm architecture, and the presence of EPS. Image contrast for SEM can be enhanced by chemically preparing biological specimens using a fixative technique optimal for the desired data to be collected. Fixation stabilizes the structural organization of biofilms, allowing for microbe-mineral assemblages to be preserved in satisfactory condition, and enhancing image contrast. It is imperative to know the objective of visualization as to use the proper fixation technique. The effects of fixation and dehydration protocols were characterized using artificial substratum deployed in silica- and manganese-depositing hot springs to a iron depositing microbial biofilms, where various fixative techniques were tested (Fig. 1).

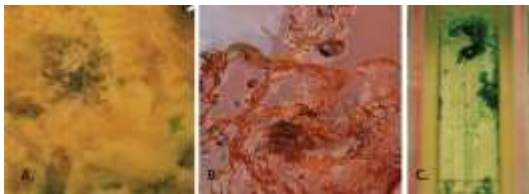


Figure 1. Biofilms illustrating various types of biofilms morphologies, (a) flocculent, (b) cohesive, and (c) mineral laden. Fixation and dehydration techniques were tested on each type of biofilm.

Fixation Techniques

Fixation preserves the integrity of cellular and biofilm structures with the knowledge that there are always artifacts of fixation and dehydration to contend with. The ideal fixative should halt cellular processes and stabilize cell walls allowing for characterization. Fixatives typically used are glutaraldehyde, a combination of glutaraldehyde and formaldehyde in a 4:1 ratio (Trump's fixative), ethanol (EtOH), or osmium tetroxide each can be an ideal fixative given for what is the target to be characterized (Dykstra, 1993).

Glutaraldehyde - stabilizes cellular structures as the aldehyde groups primarily react with the lysine in proteins, and to a lesser extent with lipids, carbohydrates and nucleic acids. The penetration rate of the fixative is slow due to its relatively large molecule size, with a penetration rate of less than 1 mm per hr (Bozzola and Russell, 1999).

Trump's fixative - increases the rate of fixative penetration into biofilms of more than a few mm thick (Dykstra, 1993).

Ethanol- halts cellular activity and facilitates the removal of low molecular weight molecules and lipids from microbial cells.

Osmium tetroxide - a post-fixative that further stabilizes cells by cross-linking lipid moieties. Once reduced, the heavy metal component of the molecule adds contrast and density to electron transparent objects. The penetration rate of osmium is slow, with 0.5 mm penetration per hr. Specimens should not

be exposed more than 2 hr. In addition biofilms possessing reduced metals should not be post-fixed due to abiotic oxidation of metals producing artifacts (Dykstra, 1993; Glauert, 1975).

Dehydration Techniques

Dehydration techniques include air-drying (AD) from solvent evaporation, using hexamethyldisilazane (HMDS), chloroform, and propylene oxide, and critical point drying (CPD) (Dykstra, 1993). Upon dehydration biofilm associated EPS structures and biominerals, were observed to characterize structural collapse and deformation of soft materials from dislocation and mobilization of low-molecular-weight substances outside of the cells, resulting in the formation of holes in cell walls due to differences between soft and firm structures (Bozzola and Russell, 1999; Crang, 1998). SEM was done to determine which dehydration technique resulted in the least amount of cellular and EPS collapse.

Methods

Fixation Techniques

Five fixation techniques were characterized in this study. Fixative solutions were prepared in either 0.2- μ m filtered water from the environment (FWE), or in filtered double distilled water (DDW).

Fixation Techniques Used:

1. 25% glutaraldehyde vapor,
2. 3% glutaraldehyde in FWE,
3. 4% glutaraldehyde in DDW,
4. Trump's Fixative in DDW,
5. 70% Ethanol in DDW.

Fixation of Metalliferous Biofilms

Biofilms display a variety of morphologies ranging from cohesive semisolid structures, gelatinous aggregates, to flocculent microbe-mineral assemblages loosely bound together (Fig. 1). In order to characterize the architecture of biofilms, microbe-mineral assemblages, and biominerals, It is imperative to understand the type of biofilm being sampled. Biofilms produced by iron-oxidizing microorganisms (FeOB) are comprised of flocculent microbe-mineral assemblages. Careful sampling must be done to preserve biofilm architecture and microbe-mineral assemblages, by subjecting the biofilm to as little fluid motion as possible thereby, reducing the dislocation of microbes from biogenic oxides.

Microbe-mineral assemblages can be preserved using one of two techniques; i) vapor fixation using a 25% glutaraldehyde saturated cotton plug in the bottom of the collection tube, and store at 5°C, or ii) fixation in a soft agar plug prepared with glutaraldehyde. Agar plug fixation is done by preparing 0.5% agar with 2% glutaraldehyde added after melting agar, the specimen is placed or injected into the agar solution and stored at 5°C, analysis can be carried out by cutting small pieces of mat out of agar.

Measuring Biofilm Degradation

Degradation of the biofilm begins upon collection, with the sloughing of material off of solid surfaces, such as glass slides. Sloughed material can be measured by weighing fixative tubes before and after, as it settles to the bottom of collection tubes.

Dry weight of sloughed material was measured by collecting material with a transfer pipet and depositing it into a pre-weighted 5-ml eppendorf tube. Material was centrifuged for 30-min at 8092-rcf, the supernatant was removed and the pellet was air-dried in a desiccator over night and then weighed.

Biofilms on glass slides were imaged and analyzed every three months to determine particle counts. Particle analysis was done on Optical Light Microscopy (OLM) images collected for each fixative.

Analysis was done using ImageJ 1.49v, NIH. Particles were defined as microbial cells, mineral grains, and mineralized EPS; this was done to illustrate the degree of sloughing induced by each fixative technique. Background noise was removed from the image by setting a standard threshold for and producing a binary image used for classification (Blackburn et. al., 1998). Objects smaller than 10 pixels were removed.

Microscopy

Optical Light Microscopy

Biofilms attached to glass slides were characterized by visualization on a Leica DMRX OLM, digital images were collected using a Leica CCD camera (Wetzlar, Germany). Prior to analysis slides were rinsed in DDW (Barnstead Nanopure Diamond Water Purifier) three times to remove fixative and reduce vapor exposure during imaging. Specimens were observed at 3-month intervals post fixation, imaging the same transects each time to document alterations occurring as a function of time and fixation technique. Once imaging was complete, slides were returned to their fixative and stored in their respective fixatives in falcon tubes

that were wrapped in aluminum foil and at 4°C.

Fluorescent Microscopy- Enumeration of microorganisms in biofilms was done using the fluorescent stain 4', 6-diamidino-2- phenylindole (DAPI). Cells were counted and data was compared to enumeration done using phase contrast microscopy where all particulates, cell and mineral grains were counted. Fluorescent microscopy allowed for the quantification of the relative number of cells remaining in the biofilm as a function of fixation technique used and through time in storage. Enumeration was done by counting cells in 10 fields of view, at 200X magnification.

Cation Staining- Visualization of EPS can be difficult using traditional microscopic techniques due to the instability of the three-dimensional structure during dehydration and the lack of electron interaction during EM analysis. Polycationic stains allows for characterization of phenotypic structures in the EPS structure particularly structures responsible for cellular attachment to substrata (Erlandsen et al., 2004). Cationic stains used included alcian blue, ruthenium red, safranin O, and L-lysine.

Cationic Stains

Alcian Blue- is a large (~4 nm) planar, water-soluble polyvalent basic dye. The molecule stains sulfated and carboxylated acid mucopolysaccharides and/or sulfated and carboxylated glycoproteins.

Safranin O- a large (~3 nm) planar molecule comprised of a mixture of two compounds and counter stains nuclei red.

Ruthenium red- is a small (~1 nm) spherical molecule and stains mucopolysaccharides and capsules red.

Lysine- is a small (~1 nm) planar molecule that forms a colorless solution that polymerizes slowly relative to the other cationic stains.

Calothrix biofilms were used to test cationic stains. Samples were pre-fixed for 1 hr and then submerged in a 0.15% cationic solution for short-term (4 hr) and long-term (45 hr) time points. Specimens were rinsed with a 0.15M-cacodylate buffer to remove unbound stain. Biofilms were examined on the OLM and then submerged in 1 ml HMDS and allowed to air-dry overnight for SEM analysis. Dehydrated samples were sputter coated with 100 Å gold-palladium (Au-Pd) and examined. *Calothrix* biofilm preparation using cationic stains was modified from Erlandsen protocol (Erlandsen et al., 2004).

Protocol

1. Fix samples in 3% glutaraldehyde in 0.1M-cacodylate buffer for 3 hr.
2. Incubate for 4 and 45 hr in one of each stain: ruthenium red, alcian blue, lysine, and safranin.
3. Rinse in 0.15M-cacodylate buffer. 2x 15 min to remove unbound stain.
4. Postfix 90-120 min in 1% osmium tetroxide in 0.1M-cacodylate buffer and 1.5% Potassium ferricyanide.
5. Rinse in 0.15M cacodylate buffer 2x 15 min each.
6. Postfix 90-120 min in 1% osmium tetroxide in 0.1M-cacodylate buffer and 1.5% Potassium ferricyanide.
7. Rinse in 0.15M cacodylate buffer 2x 15 min each.
8. Dehydrate graded ethanol series: (50, 70, 80, 95, and 2x-100%)

9. HMDS submersion 2x 20 min each, air-dry in desiccator.
10. Postfix 90-120 min in 1% osmium tetroxide in 0.1M-cacodylate buffer and 1.5% Potassium ferricyanide.
11. Rinse in 0.15M cacodylate buffer 2x 15 min each.
12. Dehydrate graded ethanol series: (50, 70, 80, 95, and 2x-100%)
13. HMDS submersion 2x 20 min each, air-dry in desiccator.

Enumeration

Glass slides were submerged in the hot spring for 24 hr allowing for the formation of thin biofilms. These specimens were used for enumeration of microorganisms to identify which fixation technique produced the best results. Cell counts were conducted using three fields of view at 100X magnification, in which digital images were captured. Image analysis was done using ImageJ as previously described.

Dehydrating Techniques

Dehydration the final step in preparing specimens for SEM analysis is also an important step for visualization of biofilms. Once fixation techniques were characterized, one method was selected to test dehydration techniques. A glass with a thin biofilm was sectioned into sixths, with each section undergoing various dehydration techniques.

Critical point dried- specimens were dehydrated by first rinsing them in a graded ethanol series (50, 70, 90, 100-2x) followed by CPD.

Evaporation from solution- sections of glass slides were dehydrated by submerging specimens in enough dehydrating solution to cover the surface

of the slide and allowed to dry in a desiccator over night. Dehydrating solutions used were HMDS, cacodylate buffer, propylene oxide, and chloroform.

Protocol

Specimens were dehydrated using the following techniques (Table 4):

- PD: graded ethanol series (50, 70, 90, 100-2x) followed by CPD.
- MDS: 5 min submersion in HMDS, air-dried on filter paper over night in desiccator.
- glutaraldehyde, osmium, and air-dried (GOAD): air-dried after second buffer rinse. Steps 1-4 of 3% glutaraldehyde in FWE fixation technique.
- glutaraldehyde air-dried (GAD): air-dried after first buffer rinse, no post fixation. Steps 1-2 of 3% glutaraldehyde in FWE fixation technique.
- propylene oxide air-dried (PAD): 5 min submersion in propylene oxide, air-dried on filter paper in desiccator.
- Chloroform air-dried (CAD): 5 min submersion in chloroform, air-dried on filter paper in desiccator.

Scanning Electron Microscopy

Biofilms attached to glass slides and natural biofilms were processed using various fixation and dehydration techniques. Specimens were mounted on aluminum pins and were sputter coated with gold-palladium using a Pelco 91000 sputter coater (Ted Pella, Redding, CA). Specimens were examined using a FEI Siron high-resolution scanning electron microscope (FEI, Hillsboro, OR) at 5kV with a working distance of 5 mm. Analysis was conducted at the CEMN at Portland State University, Portland, OR.

Results

Characterization of cellular integrity, EPS, minerals, loss of biofilm material from sloughing, and overall biofilm architecture using OLM and SEM allowed to determine which fixation and dehydration technique(s) yielded optimal biofilm integrity.

Enumeration of cells in biofilms was done to identify the fixation technique that yielded the highest cell density (Fig. 2, Table 1). Cell counts were highest when biofilms were vapor fixed (11,041 cells), or fixed with 70% EtOH in DDW (9,797), cell counts decreased rapidly from critical point dried (4,216) to air-dried in Trumps (1,384).

Table 1: DAPI enumeration for each type of primary fixative.

Fixation Technique	Cells Enumerated
25% Glutaraldehyde Vapor	11,041
70% EtOH	9,797
3% Glutaraldehyde FWE	4,216
4% AD	4,135
4% Glutaraldehyde - CPD	2,602
Trumps	1,384

Vapor fixation- was the best overall method for enumerating microorganisms using OLM; this technique yielded 11,041 cells for a 24 hr period.

3% glutaraldehyde in FWE- yielded optimal results for enumeration and SEM visualization. Enumeration yielded 4,216 cells. SEM analysis allowed for characterization of cellular surface features such as texture, and resulted in little collapse of the overall biofilm. Microorganisms appeared to experience the least amount of shrinkage, due to the commonality in pH, and osmolarity (Fig. 3A).

70% ethanol- this technique yielded the second best results for enumeration with 9,797 cells counted. This technique does not produce ideal results for SEM observation due to excessive shrinkage and collapse of cells and EPS (Fig. 3B).

4% glutaraldehyde in DDW- yielded results slightly better than Trumps fixative. Enumeration yielded 2,602 cells. SEM analysis produced adequate results for characterizing the biofilm, however cell walls and EPS experienced an increase in collapse when compared to those fixed in FEW (Fig. 3C).

4% glutaraldehyde and air-dried- produced results similar to those fixed in 3%-FWE for enumeration using OLM. However, this method of fixation did not yield adequate results for SEM analysis of thin biofilms due to excessive collapse of cells and EPS (Fig. 3D).

Trumps fixative- this technique worked best for biofilms more than a few millimeters in thickness. In contrast, thin biofilms attached to glass slides experienced a significant loss of material enumeration and yielded 1,384 cells. This technique produced precipitates evident in thin biofilms, identified using both OLM and SEM analysis (Fig. 3E).

Metalliferous Biofilms

Stabilizing metalliferous biofilms as soon as possible is imperative for preserving microbe-mineral assemblages for characterization of the relationship biominerals have with the microorganisms from which they formed. It also allows for better understanding of biofilm architecture, diversity of morphotypes, and identification of biogenic oxidation products. SEM observation of specimens stored in fixative solution showed few microbial cells associated with Fe-oxides due to dislocation from fluid motion, where as those immediately fixed in a stabilizing agar exhibited Fe-oxides with microbial cells still attached (Fig. 2).

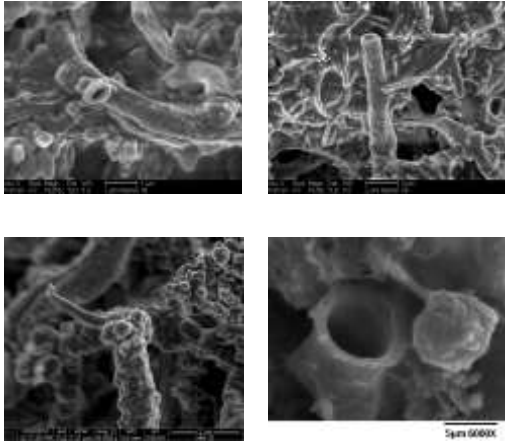


Figure 2. SEM of metalliferous biofilms comprised of Fe-oxides and FeOB. Top row SEM of Fe-oxide stalks made by FeOB, biofilms were preserved and stored in 2.5% glutaraldehyde solution. Microbial cells were dislodged during transport and while in storage. Bottom row SEM of intact microbe-mineral assemblages.

Measuring Biofilm Degradation

Fixation Techniques

Results indicate relative weights of material loss from the slides as a result of fixation technique. Visualization of collection tubes revealed that there was significant loss of material from the samples fixed using the Trumps technique, and less loss of material from other fixation techniques, this observation was supported from resulting pellet weights (Table 2). Biofilm material sloughed from glass slides during storage occurred due to a variety of reasons; i) time, ii) changes in cellular integrity, swelling and shrinkage of cells, iii) overall quantity and integrity of the EPS matrices affecting the three-dimensional structure of the biofilm, iv) dislodging of microbial cells from biominerals.

Table 2: Summary of dry pellet weights

Fixative	Pellet weight (g)
Vapor	N/A
Trumps	0.05
70% EtOH	0.001
4% GDW	0.002
3% FWE	0.013

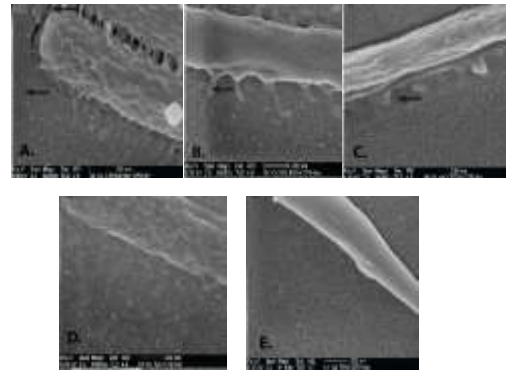


Figure 3: HR-SEM 64,000 mag images illustrating differences in biofilm integrity due to primary fixation.

A) 3% glutaraldehyde in 0.2- μ m FWE and CPD. The surface texture of the cell is rugose, the EPS has collapsed around the cells perimeter onto the slide; fine EPS strands can be seen between adjacent cells (arrow). B) 70% EtOH in DDW and CPD. The cell has lost its surface detail due to shrinkage and collapse; the EPS is visible around the perimeter of the cell. EPS strands appear broader in diameter than those seen in Figure A, 3% g/FWE sample (arrow). C) 4% glutaraldehyde in DDW and dehydrated with CPD. The cell has little surface detail and has decreased in diameter due to excessive shrinkage. The EPS is highly collapsed with little to no detail (arrow). D) 4% glutaraldehyde in DDW air-dried sample. The cell experienced less shrinkage however the EPS is highly collapsed and difficult to observe. E) Trump's in DDW and CPD. The cell has not retained any cellular features due to excessive shrinkage and collapse of the cell and EPS.

Dehydration Techniques

Choosing the optimal dehydration technique is just equally as important as choosing the fixation technique. Dehydration results in deformation and loss of cells and EPS within biofilms, which may lead to misinterpretation of biofilms (Fig. 4).

3%-FWE and CPD- Biofilms fixed with a 3% glutaraldehyde in FWE solution. Dehydration using the CPD technique resulted in a significant difference in the biofilm appearance with little to no visibility of the EPS within the biofilm. Specimens dehydrated using the HMDS technique preserved the EPS structure and orientation within the biofilm (Fig. 4A).

70% EtOH in DDW - presented difficulties as the biofilm material on the slide was difficult to visualize using SEM due to excessive cellular and EPS shrinkage.

4% glutaraldehyde in DDW - solution yielded highly collapsed cells that appeared flattened in those dehydrated using the CPD technique. Specimens dehydrated using the HMDS technique provided satisfactory preservation of the biofilm matrix and community and was easily imaged with the SEM.

CPD - resulted in the loss of a substantial amount of biofilm material, particularly EPS and minerals, allowing for the examination and characterization of cell morphology, surface features, and the orientation of cells in the biofilm.

Characterizations of the microbial cells within biofilms were best demonstrated using the CPD technique for dehydration due to the removal of the overlying EPS from the cell surface. Substrata dehydrated using CPD in the final dehydration step had cells with narrow diameters as compared to other dehydration techniques.

Air-drying from hexamethyldisilazane – biofilm specimens were submersion is a desiccant for characterizing EPS and community distributions within biofilms when done carefully due to its rapid infiltration and evaporation rate and low surface tension (2.5 dynes/cm²). HMDS replaces water molecules in the biofilm allowing EPS to maintain its three-dimensional shape. Substrata dehydrated using HMDS rinses followed by air-drying allowed observation of a more intact biofilm. The attached cells and their surrounding EPS maintained their 3-dimensional structure with less collapse than those dehydrated using CPD dehydration technique (Fig. 4D). This technique was ideal for characterizing the EPS component of the biofilm structure. Slides dehydrated using the HMDS technique maintained visible biofilm material on the slide, although cellular collapse occurred the biofilm was better preserved, little EPS was removed and the cells did not collapse to the degree of the CPD removed and the cells did not collapse to the degree of the CPD dehydrated slide.

Air-drying from chloroform - produces less favorable results over air-drying due to its high surface tension (72.8 dynes/cm) resulting in significant collapse of microbial cells and associated EPS (Fig 4E).

Air-drying from propylene oxide - is also a good desiccant due to its low surface tension, (24.8 dynes/cm), reducing collapse of cellular structure, and lowering the chance of removing low molecular weight molecules. The chloroform and propylene oxide techniques produced the least desirable results with precipitation of artifacts onto the biofilm (Fig. 4F) .

Biofilms dehydrated using the GOAD technique resulted in preservation of the microbial cells, and associated EPS. The method allowed for characterizations of entire biofilm structure to be made, microbe, mineral and EPS interactions were preserved with this technique. Remaining EPS experienced little shrinkage and did not obstruct visualization of biofilm characteristics such as cell morphology and orientation, microbe-mineral associations.

Biofilms dehydrated using the GOAD technique resulted in preservation of microbial cells and associated EPS (Fig. 4B). This method allowed for the characterization of biofilm structure microbe-mineral and EPS associations. The remaining EPS experienced little shrinkage and did not obstruct visualization of biofilm characteristics such as cell morphology and orientation, microbe-mineral associations for SEM analysis.

Table 3: Effects of dehydration of biofilms attached to glass slides.

Technique	Effects of Dehydration
CPD	Dislodged cells, defined casts (outline of where cells were).
HMDS-Dykstra	EPS collapsed over cells giving a smooth texture, faint casts present.
GOAD	Best overall, minimal collapse of cells, several morphotypes present.
GAD	Minimal collapse of cells, several morphotypes present.
Chloroform	Precipitation around cell, obstructing observation.
Propylene Oxide	Deposition of precipitates, deformation of biofilm.

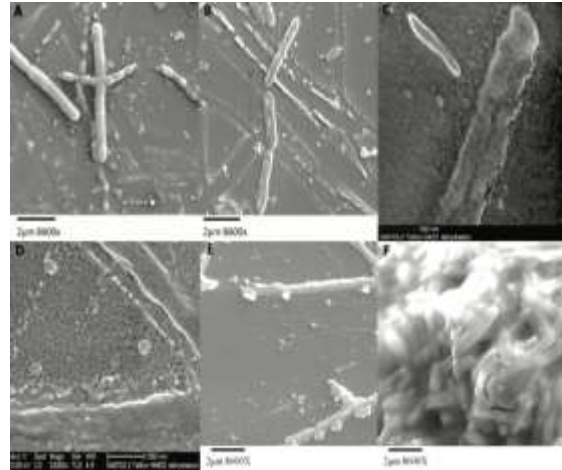


Figure 4: SEM of glass slides deployed in a silica-depositing hot spring. Each slide was fixed in 3% glutaraldehyde in 0.2- μm FWE and dehydrated with various desiccants. A) CPD slide. Cells retain their cellular integrity and can be seen overlaying EPS casts. B) GOAD dehydration. Cells are moderately collapsed while retaining their shape, whereas, EPS on the slide is not as collapsed.

C) GAD dehydration. Cells and EPS are highly collapsed making characterization of cell and biofilm morphology difficult. D) HMDS dehydration. Cells and EPS show little evidence of shrinkage. EPS texture is evident on and around the cells. EPS obscures the visualization of cell morphology. E) Chloroform dehydration. Cells have decreased in diameter due to shrinkage, precipitates have formed around the cells, and EPS is highly collapsed. F) Propylene Oxide dehydration. EPS has retained its integrity and obstructs visualization of cell morphology.

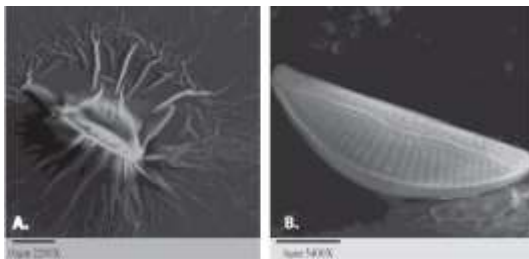


Figure 5: SEM images of diatom attached to glass slide. Samples were dehydrated with HMDS submersion (A) and CPD (B). A) HMDS dehydration: notice extensive EPS layer preserved with this technique, B) CPD dehydration, EPS layer was removed during the CPD process.

Cationic Stains

Stains were applied to *Calothrix*-dominated biofilms to enhance visualization of EPS associated with the microbes-mineral assemblages. Cationic stains bind to reactive sites in the EPS, staining specific moieties within the biofilm (Fig. 5).

Alcian Blue- Upon 4 hr incubation in the 0.15% alcian blue staining solution. The EPS had a smooth sheet texture spread between cells within the mat, the EPS sheets had begun to tear from dehydration, and there was minimal cell surface detail with some large surface structures visible. After 45 hr incubation in the staining solution there was significantly more surface detail with a fine detailed rugose texture, and large structures on the surface of filaments. The EPS between cells was fractured with a flaky texture (Fig. 6A). The EPS appeared to be porous, with a fine-grained compact texture around porous structures. Long-term exposure to the stain resulted in detailed EPS and cell surface features (Fig. 6B).

Safranin- Upon 4 hr incubation (Fig. 6D) in the staining solution. The EPS sheet was slightly torn between cells when pulled apart, and the EPS was hardly visible. After 45 hr incubation. There was some increase in surface detail, although not as detailed as with alcian blue stain. The large structures on the cell surface were visible, but the fine detail of the rugose cell surface was absent with the safranin stain (Fig. 6C). The EPS was not fractured and had a smooth, sheet appearance over the cells in the mat. Between cells the EPS collapsed into strands curling up together rather than fracturing or tearing. The surface of the cell has longitudinal fractures in the sheath and the EPS did not display a porous texture.

Ruthenium Red- Upon 4 hr incubation. (Fig. 6F) in the staining solution EPS sheets were visible although the filaments appeared highly collapsed. After 45 hr the EPS was highly collapsed and difficult to visualize and there was little to no surface detail or texture evident. The filaments in the mat sample demonstrated decreased diameter from collapse (Fig. 6E).

Lysine- Upon 4 hr incubation (Fig. 6H) in the staining solution. The EPS and cell surface demonstrated more detail. After 45 hr incubation in the staining solution. The EPS sheet shrunk, collapsing around filaments and encasing them. There is little surface detail on the filaments, with a few “hairs” seen around the edges of some filaments. The EPS appeared to be porous like that previously seen with the alcian blue staining technique (Fig. 6G).

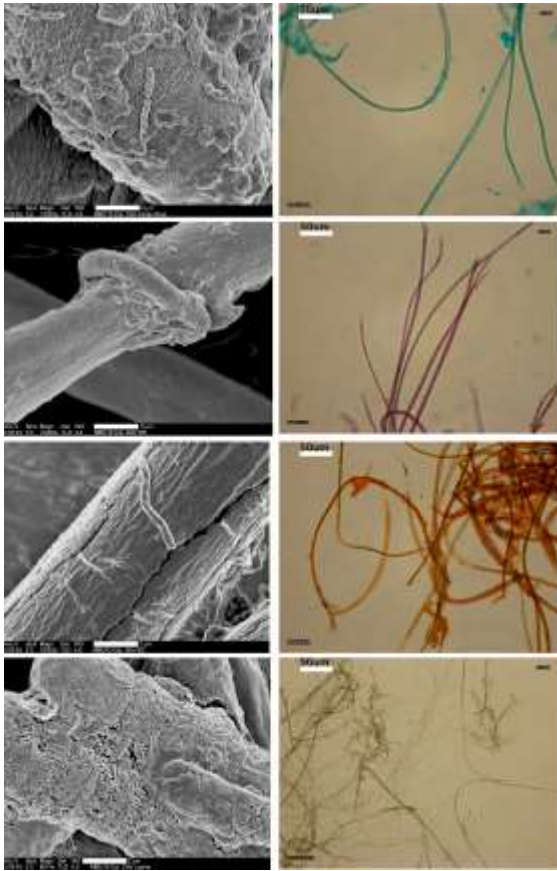


Figure 6: OLM and SEM images of *Calothrix* filaments that were treated with Ruthenium Red and L-Lysine cationic stains. (A-B) SEM and OLM images showing increased surface detail with EPS textural features of *Calothrix* filaments treated with Alcian Blue. (C-D) SEM and OLM images of an enrichment stained with Ruthenium Red. The OLM image show the stain bound to most of the sheathed filaments. The SEM image shows a decreased cell diameter with and a ring structure not seen without the use of staining. (E-F) SEM and OLM images showing relatively smooth surface features of filaments stained with safranin. (G-H) OLM & SEM images of enrichment stained with L-Lysine. The OLM image after 6 days incubation, show the stain bound to fine strands of EPS in the enrichment.

Discussion

Biofilms are ubiquitous in nature and the microbial communities they host are responsible for a host of biogeochemical processes that shape environments on local, regional and planetary scales. The microorganism in these biofilms are responsible for the recycling of elements and the production of redox reactive biominerals which they leave as proof of their existence in the rock record as microfossils and/or biominerals providing clues to Earth's early history and they are important drivers of biodiversity.

To fully characterize biofilm architecture, EPS structures, microbial morphology, and microbe-mineral associations it is important to first know what your objectives are for characterization in order to prepare the sample properly to meet those objectives. It may be necessary to use multiple fixation and dehydration techniques per sample. It is equally as important to know the features of the biofilm being collected; as this will allow for the proper preparation method to be used ensuring that the information you are seeking is obtainable. Finally, in order to avoid misinterpreting data, it is important to know what artifacts will be introduced during sample processing by doing control studies.

For this study the best technique to to characterize biofilm density (enumeration), characterization of EPS, cell morphology, microbe-mineral associations, and biominerals morphology the ideal technique was to vapor fix biofilms, and to use a mixed methods approach to dehydration (Fig. 7).

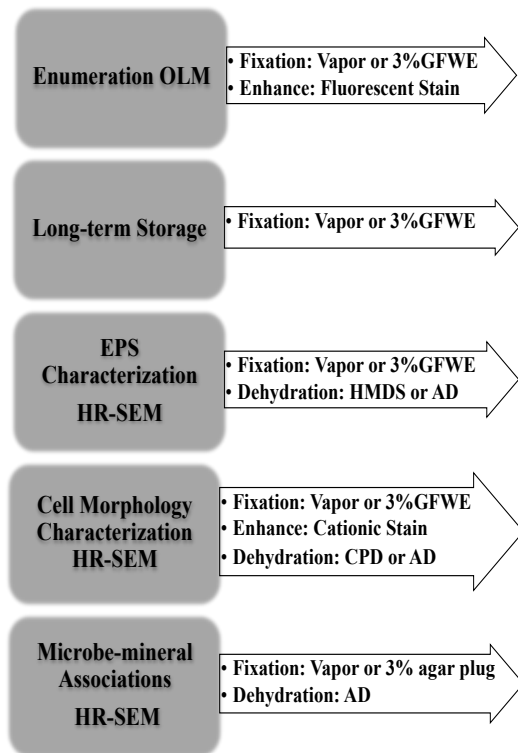


Figure 7. Summary of objectives (left) with best fixation, enhancement, and dehydration technique.

Acknowledgments

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