Final report:
The effects of Fe- and S-oxidizing microorganisms on post-biostimulation permeability reduction and oxidative processes at the Rifle IFRC site

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1. Summary

Fe oxidation and biomineral formation is important in aquifers because the highly-reactive oxides can control the mobility of nutrients (e.g. phosphate, C) and metals (e.g. arsenic, uranium). Mineral formation also has the potential to affect hydrology, depending on the volume and distribution in pore spaces. In this exploratory study, we sought to understand how microbial Fe-oxidizers and their biominerals affect, and are affected by groundwater flow. As part of work at the Rifle aquifer in Colorado, we initially hypothesized that Fe-oxidizers were contributing to aquifer clogging problems associated with enhanced bioremediation. To demonstrate the presence of Fe-oxidizers in the Rifle aquifer, we enriched FeOM from groundwater samples, and isolated two novel chemolithotrophic, microaerophilic Fe-oxidizing Betaproteobacteria, *Hydrogenophaga sp.* P101 and *Curvibacter sp.* CD03. To image cells and biominerals in the context of pores, we developed a "micro-aquifer," a sand-filled flow-through culture chamber that allows for imaging of sediment pore space with multiphoton confocal microscopy. Fe oxide biofilms formed on sand grains, demonstrating that FeOM produce Fe oxide sand coatings. Fe coatings are common on aquifer sands, and tend to sequester contaminants; however, it has never previously been shown that microbes are responsible for their formation. In contrast to our original hypothesis, the biominerals did not clog the mini-aquifer. Instead, Fe biofilm distribution was dynamic: they grew as coatings, then periodically sloughed off sand grains, with some flocs later caught in pore throats. This has implications for physical hydrology, including pore scale architecture, and element transport. The sloughing of coatings likely prevents the biominerals from clogging wells and aquifers, at least initially. Although attached biomineral coatings sequester Fe-associated elements (e.g. P, As, C, U), when biominerals detach, these elements are transported as particles through the aquifer. Our work shows that microbial mineralization impacts in aquifers are dynamic, and that the fate and transport of biomineral-associated elements depend not only on geochemical conditions, but also physical pore-scale processes.

2. Project goals

The overall project goal was to understand the contribution of microbial biomineralization to biomineral production in the Rifle aquifer. The initial motivation was to understand the contributions to well clogging, though our work gives insight into how microbes contribute to Fe biogeochemical cycles in the subsurface. The originally proposed questions were: Are microbial Fe- and S-oxidation processes significant at Rifle? Do these oxidative processes increase or decrease the volume of biominerals and organic biomass? We ended up focusing on Fe oxidation, in particular determining how Fe-oxidizing microbes (FeOM) produce Fe oxyhydroxides in porous media, and the fate of these oxides in an experimental microaquifer.

3. Accomplishments

The major project activities and accomplishments included:

1. Culturing and isolation of Fe-oxidizing microbes from the Rifle aquifer, demonstrating the presence of novel, previously unrecognized facultative FeOM.
2. Developing a flow through micro-aquifer system for growing and imaging Fe biominerals in porous media. This new system can accommodate sand or other sediment types, and allows for visualization by confocal microscopy.
3. Confocal microscopy of cells and Fe biominerals on porous media, demonstrating microbial involvement in producing Fe coatings on sand grains, as well as the dynamics of these coatings under variable flow conditions.

3.1. Fe-oxidizing microbial cultures

In order to demonstrate the presence of FeOM in the Rifle wells and aquifer, we used a culturing approach. This is in contrast to other microbial work at Rifle, much of which is DNA and RNA-based. We chose the culturing approach in order to demonstrate the metabolism because there are no isotope or genetic markers of Fe oxidation; therefore culturing is the most direct link between the microbes and biogeochemical effects. Using FeS gradient tubes and a freshwater minimal medium, we were able to enrich FeOM from Rifle groundwater. Our original intention was to quantify aerobic FeOM over the oxygen addition experiment of 2012 using a culture-based most probable number approach. During this field experiment, oxygenated groundwater was injected into Plot C of the Rifle aquifer. We sampled from one upgradient well and two downgradient wells. We did indeed detect aerobic FeOM, but at relatively low concentrations (up to $10^{2.5}$ cells/mL). The aquifer was highly redox-buffered by reduced mineral phases and organics, as evidenced by the very slow oxygenation—much slower than expected. During the ~4 months of monitoring in the wells we sampled, O_2 levels never reached above ~2 μM; the experiment was terminated in December due to freezing conditions at the site. Beller et al. found evidence for anaerobic Fe oxidation, which may have been more important during this field experiment, since O2 would have reacted with reduced N (e.g. ammonium) to form nitrate. This might explain the low levels of culturable aerobic FeOM.

Given the unexpected outcome of the field experiment, we focused the rest of our work on lab-oriented experimentation. We did isolate two novel FeOM, a *Curvibacter spp.* CD03 and *Hydrogenophaga spp.* P101, demonstrating the presence of FeOM in the Rifle aquifer. Culture purity was confirmed by unambiguous 16S rRNA gene sequences (phylogeny shown in Fig. 1). Strain P101 is 97% similar to *Hydrogenophaga taeniospiralis*, known as a hydrogen oxidizer and heterotroph. Isolate CD03 is 98% similar to *Curvibacter delicatus*, known to be an organotroph. Neither of the Rifle isolates was closely related to known iron-oxidizing microbes, but similar microbes have been identified in other studies of Rifle aquifer sediments. In sediment column experiments which tested effects of oxidizing conditions on microbial communities, *Hydrogenophaga sp.* accounted for ~25-98% of the total microbial population (N'Guessan et al., 2010), suggesting that the related isolate is representative of the aquifer community during

oxidation. Note that FeOM presence at Rifle is also supported by work in the Beller and Banfield labs using 16S iTag, metagenome, and transcriptome data, so we did not need to pursue our originally proposed sequencing efforts.

Both isolates oxidize Fe (Fig. 2) and produce iron oxyhydroxide biominerals, identified as lepidocrocite based on electron diffraction (Fig. 3). Confocal microscopy shows a close association between cells and mineral clusters (Fig. 4). Unlike many known FeOM, the isolates were able to grow on other substrates, including organics and possibly hydrogen (Table 1). C-13 uptake experiments suggest that the strains can take up organic carbon, so we conclude that they function as either organotrophs or mixotrophs. This metabolic flexibility may reflect the organic-rich and Fe-rich aquifer with fluctuating redox conditions.

3.2. Development of a flow through mini-aquifer for confocal imaging

In order to connect the Fe-oxidizing microbial metabolism with pore-scale effects, we aimed to image Fe oxide biominerals/biofilms in a simulated aquifer. The design and testing of this setup occupied much of our efforts, and is the basis for our main results (section 3.3). We designed the setup so that we could image a 3-dimensional pore, rather than a flat, simulated pore typical of micromodels. The set up includes the ability to include any porous medium, up to sand-size, and has ports for injection and outflow of media (Fig. 5). Near the injection point, we included a reservoir of FeS, which served as a slow-release Fe(II) substrate (similar to the Rifle aquifer). We initally tried to use Nafion as the porous medium because it has a refractive index similar to water, and therefore we could image through a grain into a pore. We were successful in imaging microbes on Nafion but even after much effort, we could not get the microbes to well enough in the presence of Nafion to produce much mineral. We therefore switched to using quartzose sand (Cape Cod, MA), which produced much better results. By using sand, we gave up the ability to image past the first layer of sand, restricting the visible area to the void between several sand grains and the cover glass (~a half-pore). However, this is still more realistic than a flat 2D-like pore, and we can also compare colonization of the glass coverslip with the sand.

The mini-aquifer was inoculated with culture and allowed to sit without flow overnight in order to promote colonization; then freshwater medium was pumped through at a rate of 0.2 µL/min, to simulate Rifle aquifer flow rates. We were able to get the FeOM strains to grow over 5-7 days in the miniaquifer (w/sand), using both FeS and organic (R2A) substrates (details in section 3.3 below). We used both Rifle strains P101 and CD03, as well as a groundwater seep isolate, Gallionellaceae *Ferriphaselus sp.* R-1 (Krepski et al., 2012), though we have the most complete dataset for strain P101. We tried to correlate growth and biomineralization with impacts on groundwater flow, by measuring changes in pressure (with the aim of calculating hydraulic conductivity). However, any pressure changes were below the detection limit of the pressure transducer.

3.3 Microscopy of cells and Fe biominerals on porous media

Once we had a working flow through mini-aquifer, we were able to conduct experiments to see how FeOM produce biominerals in porous medium, including the spatial distribution and the fate during groundwater flow events. Although we know that the FeOM produce Fe biominerals, it was not clear if these would form as coatings or they would be separate from the sand, in the interstitial pore space. Another way of thinking of this is: do groundwater FeOM grow as biofilms, or planktonically?

To address this, we grew the Rifle FeOM on either FeS (Fe(II) substrate) or R2A (low organic oligotroph medium). After 7 days, we injected a nucleic acid stain for cells (either DAPI or SYTO) and a rhodamine-conjugated RCA lectin, which bound the biogenic oxides. In some

cases, fluorescein was added to the medium so that we could image the remaining pore space. Here we highlight confocal imaging results from strain P101, though results from CD03 and R-1 are similar. When grown on R2A, cells formed a webby network across pores (Fig. 6). In contrast, when grown on FeS, cells preferentially grew as coatings on sand surfaces, in association with Fe oxide minerals (Fig. 7, 8). Sand coatings were patchy, with some grains covered more than others (Fig. 7). Although we do not know exactly why this is the case, we surmise that while the sand mineralogy is relatively homogeneous, the surfaces vary in roughness and composition depending on the history of weathering and exposure. In any case, this highlights the need to use real aquifer materials, since colonization is surface-dependent. The patchiness is fortuitous, since it allows us to more easily examine cell-mineral spatial relationships. The close association between cells and minerals in confocal images, in combination with TEM results, suggests that the microbes are responsible for formation of Fe oxide coatings on sand.

Some oxides were present as granular aggregates, in some cases bridging pore throats (Fig. 9). We hypothesized that these aggregates were coatings that had detached and become caught in narrow pore throats. To test this, we prepared miniaquifers with biogenic Fe oxide sand coatings, and then imaged them using high speed confocal microscopy while increasing the flow rate stepwise up to 100 μL/min, mimicking higher flow events such as infiltration of precipitation, spring snowmelt, or local pumping. Indeed, as we increased flow rate, subjecting coatings to increased shear stress, they eroded resulting in sloughed particles (Figs. 10, 11). Some of these particles did become caught in pore throats. These results show that biogenic iron coatings are dynamic under changing hydrologic conditions. The sloughing of coatings likely prevents the biominerals from clogging wells and aquifers, at least initially. Although attached biomineral coatings sequester Fe-associated elements (e.g. P, As, C, U), when biominerals detach, these elements are transported as particles through the aquifer. Our work shows that microbial mineralization impacts in aquifers are dynamic, and that the fate and transport of biomineral-associated elements depend not only on geochemical conditions, but also physical pore-scale processes.

4. Training and professional development

This grant funded a masters student, Kevin Cabaniss, for ~2.5 years. Kevin came from a geology background, so this project gave him the opportunity to learn about geomicrobiology, and integrate this with mineralogy and hydrology. The PI Chan trained him in field sampling, microbiology, geochemistry, and microscopy techniques, in addition to research, writing, and presentation skills. Kevin is now a high school science teacher in Virginia.

The grant also partially funded a postdoc, Chaofeng Lin, who contributed the molecular analyses and some culturing help. (Lin was mainly paid on other grants.) Lin is now a science writer/editor and environmental consultant in China.

An undergraduate researcher, Kara Hoppes, has been helping to wrap up this work. Kara is a junior (rising senior) in Environmental Sciences and Geological Sciences. I am training her in microbiology, geochemistry, and microscopy using this system, as well as helping her to prepare for graduate school. Kara is characterizing the mineralogy of the iron sand coatings, and rigorously testing biotic versus abiotic effects, in preparation for our publication.

4. Dissemination of results (public outreach)

We have used the ideas and results in designing 2-3 day workshop, at the Serviam Academy in Wilmington, DE to introduce 5th-8th grade girls to geo/environmental microbiology using a combination of soil sampling, culturing, and microscopy. We participated in our college's annual

Coast Day in October, which attracted thousands of community members. My lab hosts tours as part of the Delaware Biotechnology Institute's outreach efforts to ~200 students and ~20 teachers per year.

5. Products

Seminars *(all presented by Chan)*

1. Microbial Fe oxidation and mineral formation at groundwater-surface water interfaces. Stroud Water Research Center, PA, June 5, 2012.
2. Rusty waters: iron-oxidizing bacteria in coastal and alluvial aquifers. Bigelow Laboratory for Ocean Sciences, July 11, 2013.
3. Rusty waters: iron-oxidizing bacteria in coastal and alluvial aquifers. Dept. of Civil and Environmental Engineering, University of Connecticut, November 8, 2013.

Conference presentations *(*=presenter, ^=Chan group member)*

4. **Chan, C. S.***, Cabaniss, K. A.^, Lin, C.^, Williams, K. H. Isolation of Fe-oxidizing microorganisms from the Rifle IFRC site: toward understanding post-biostimulation permeability reduction and oxidative processes DOE SBR PI meeting, May 1, 2012, *poster*.
5. **Chan, C. S.*** Microbial Fe oxidation and mineral formation in modern and ancient environments, Telluride Iron Biogeochemistry Workshop August 2012, *invited talk*.
6. **Chan, C. S.** Microbial Fe oxidation and biomineralization: Visualizing at the nanomineral, pore, and biofilm scales, Soil Science Society of America Conference, Cincinnati, 2012, *invited talk*.
7. Cabaniss, K. A.^, **Chan, C. S.***, Moore, M., Lin, C.^, Williams, K. H. Fe-oxidizing microorganisms in microscopic model aquifer systems: toward understanding post-biostimulation permeability reduction and oxidative processes at the Rifle IFRC site, DOE TES/SBR PI meeting, May 14, 2013, *poster*.
8. **Chan, C. S.*,** Cabaniss, K. A.^, Williams, K. H., Moore, M., Michael, H. A., Caplan, J., Lin, C.^, Fe-oxidizing microorganisms in microscopic model aquifer systems: feedbacks between flow and biomineralization, Ninth International Symposium on Subsurface Microbiology, October 2014, *talk*.

Publication in preparation

Chan, C. S., Cabaniss, K. A., Hoppes, K., Williams, K. H., Moore, M., Michael, H. A., Caplan, J., Lin, C., Microbial iron oxidation and contribution to Fe oxide coatings in aquifer sediment.

Funding was or will be acknowledged in all products.

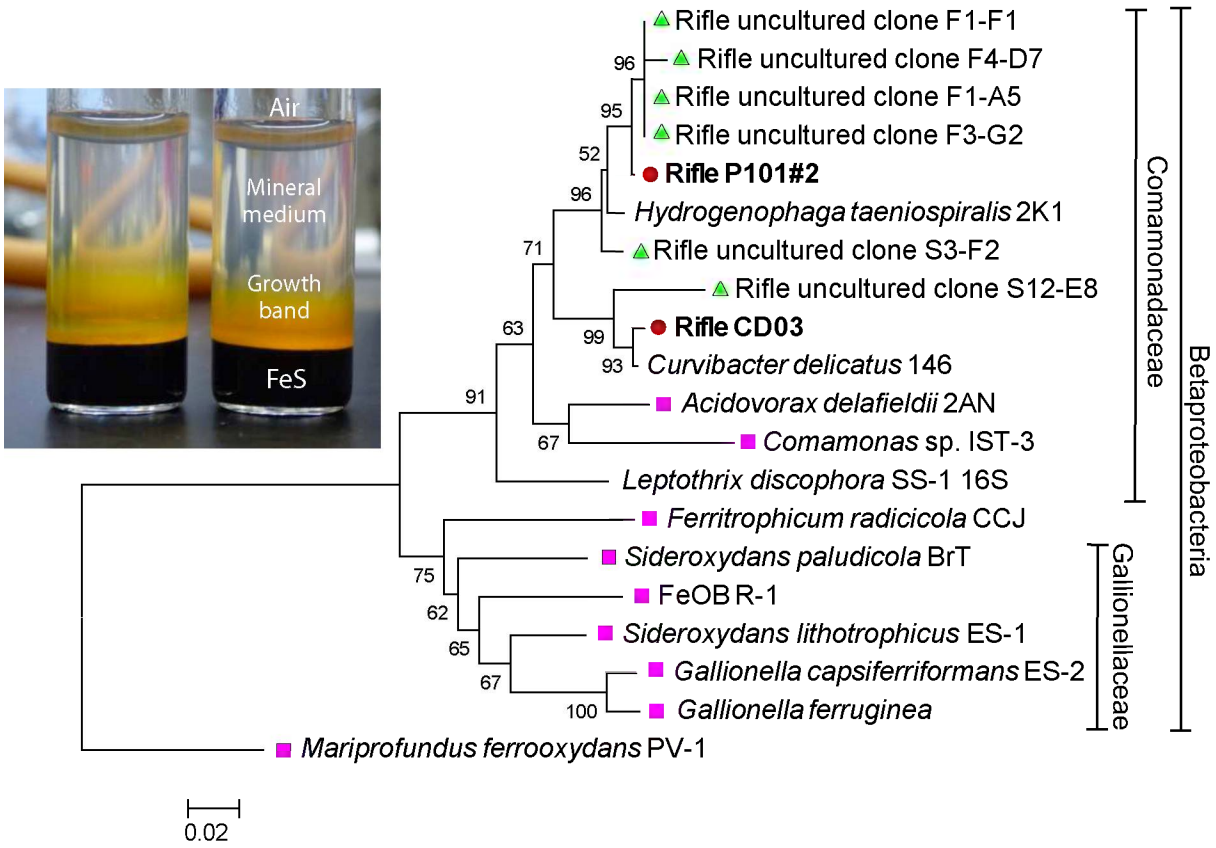


Figure 1. 16S rRNA gene phylogenetic tree of FeOM isolates (in bold and denoted by red circles). Sediment clones from N'Guessan et al., 2010 are shown with green triangles. Known FeOM are denoted by pink squares. Inset: gradient tube cultures with FeS in bottom plug, and Fe oxide growth band.

Table 1. Substrate usage by Rifle FeOM isolates

Substrate	CD03	P101 #2
FeS	Yes	Yes
$FeCO_3$	Yes	Yes
$FeCl_2$	Yes	Yes
R2a (low organic)	Yes	Yes
Yeast Extract (0.5g/L; low organic)	Yes	Yes
LB (rich organic*)	No	No
Glucose	No	No
Acetate	No	No
H_2	Yes?	Yes

*For comparison, LB has 5 g/L yeast extract, among other organics

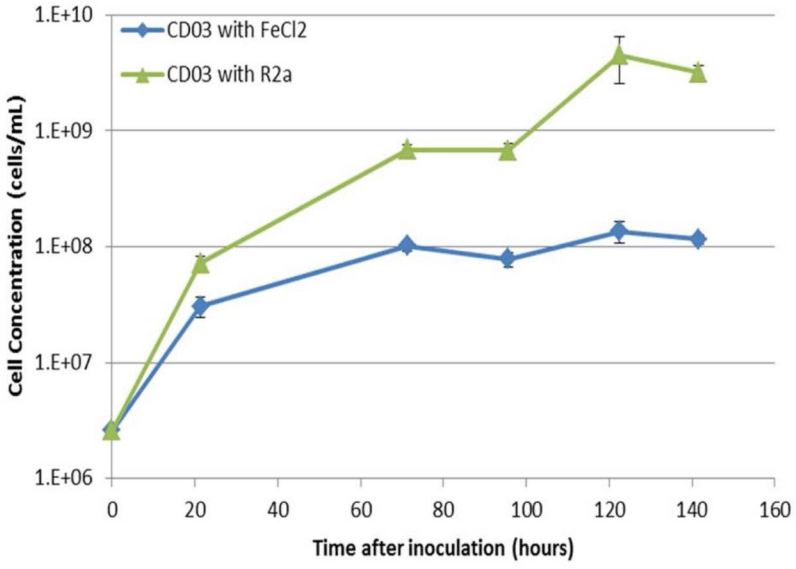


Figure 2. Growth curve of isolate CD03 grown on Fe(II) chloride and R2a oligotrophic medium.

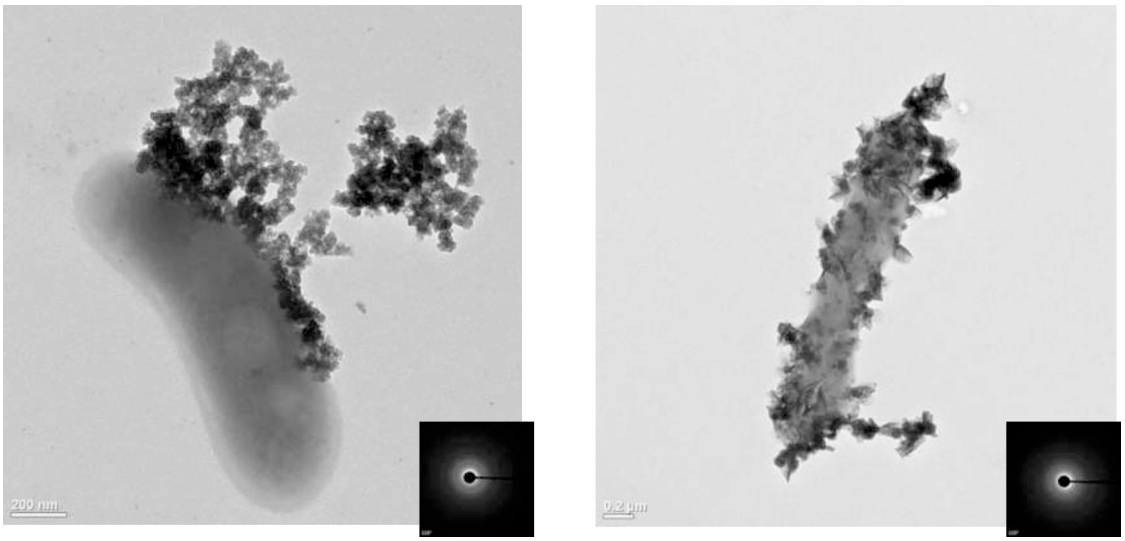


Figure 3. TEM images of FeOM isolate cells and biominerals. Left: *Curvibacter sp.* CD03; Right: *Hydrogenophaga sp.* P101. Insets: electron diffraction patterns.

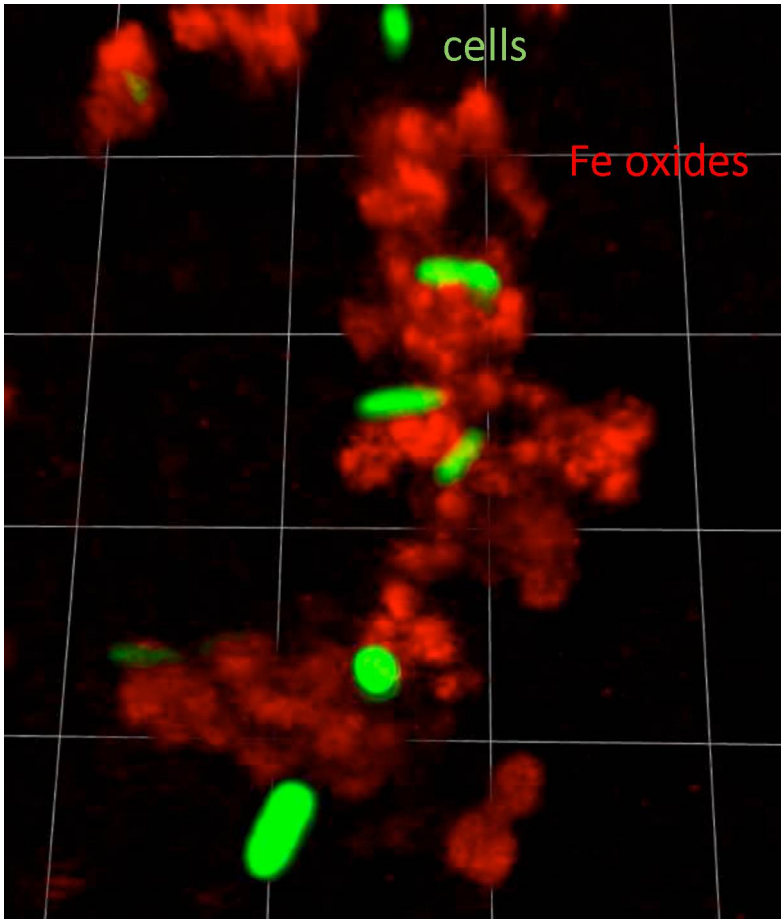
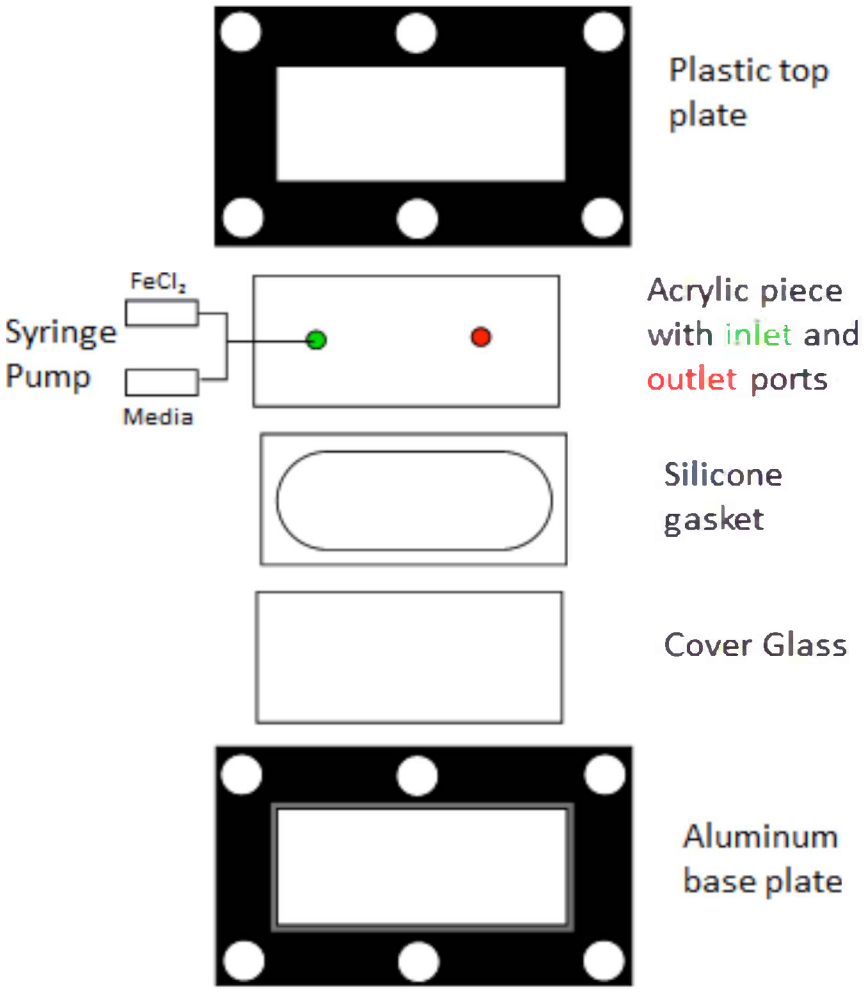


Figure 4. 3D rendering of confocal imaging of FeOM isolate P101 cells and biominerals.



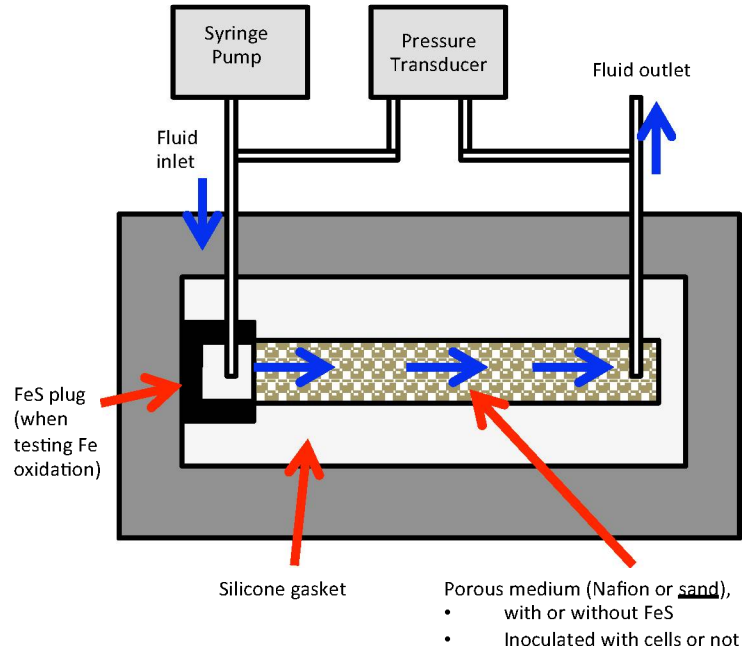


Figure 5. Flow through miniaquifer culture chamber design. Top: individual pieces; Bottom: assembled. It was designed so that Fe(II) could be introduced in the dissolved ($FeCl_2$) or solid (FeS) phase for slow release.

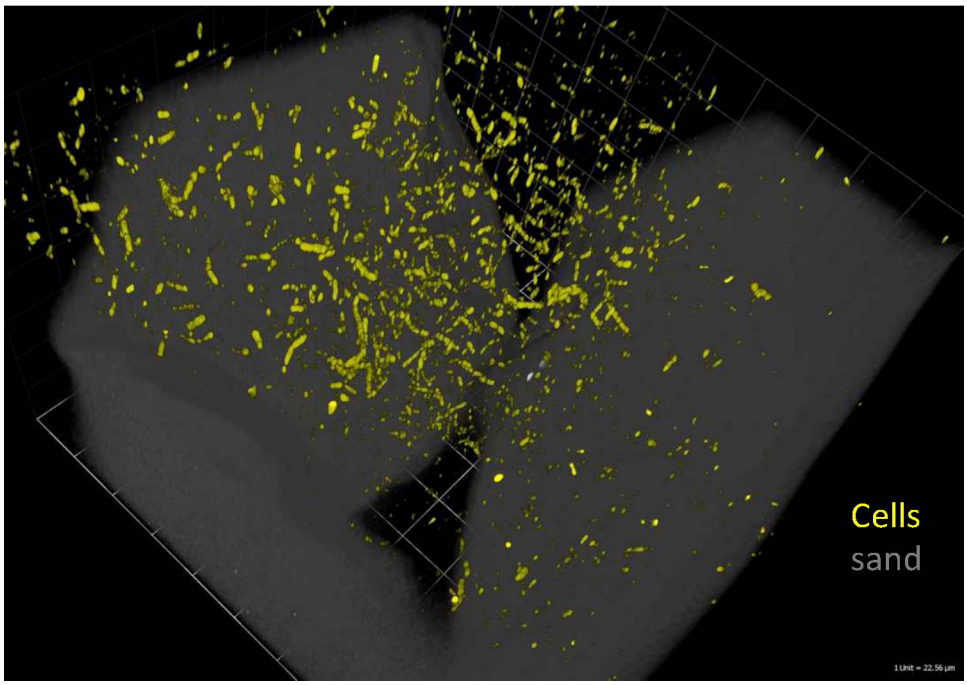
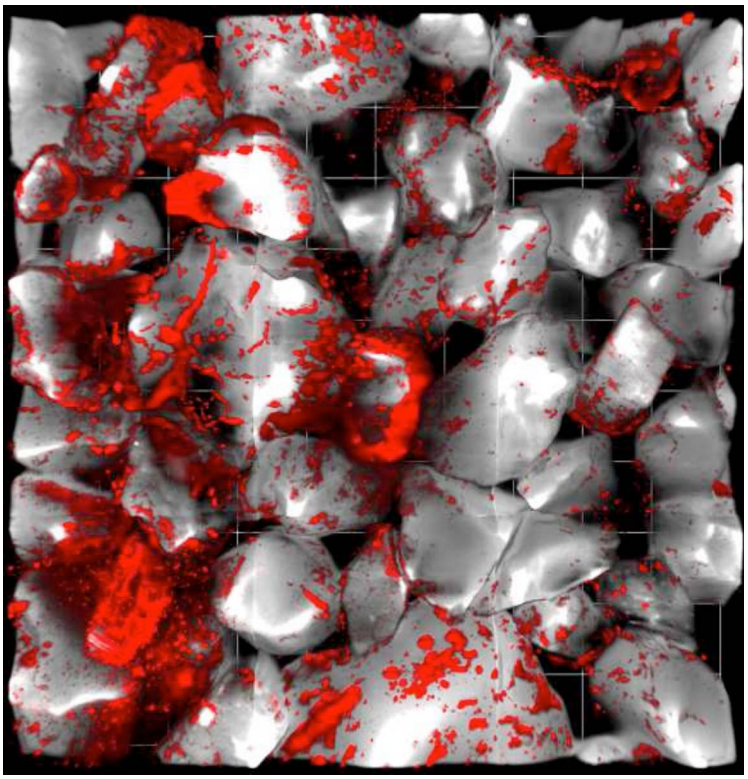


Figure 6. 3D rendering of confocal data of Hydrogenophaga strain P101 grown on yeast extract (organic oxidation). Cells (yellow) are present in a webby network across the pore space. Sand grains shown in grey.

Figure 7. 3D rendering of confocal data of Hydrogenophaga strain P101 grown on FeS. Fe oxides are shown in red. Sand grains are primarily quartz, ~200 µm diameter.



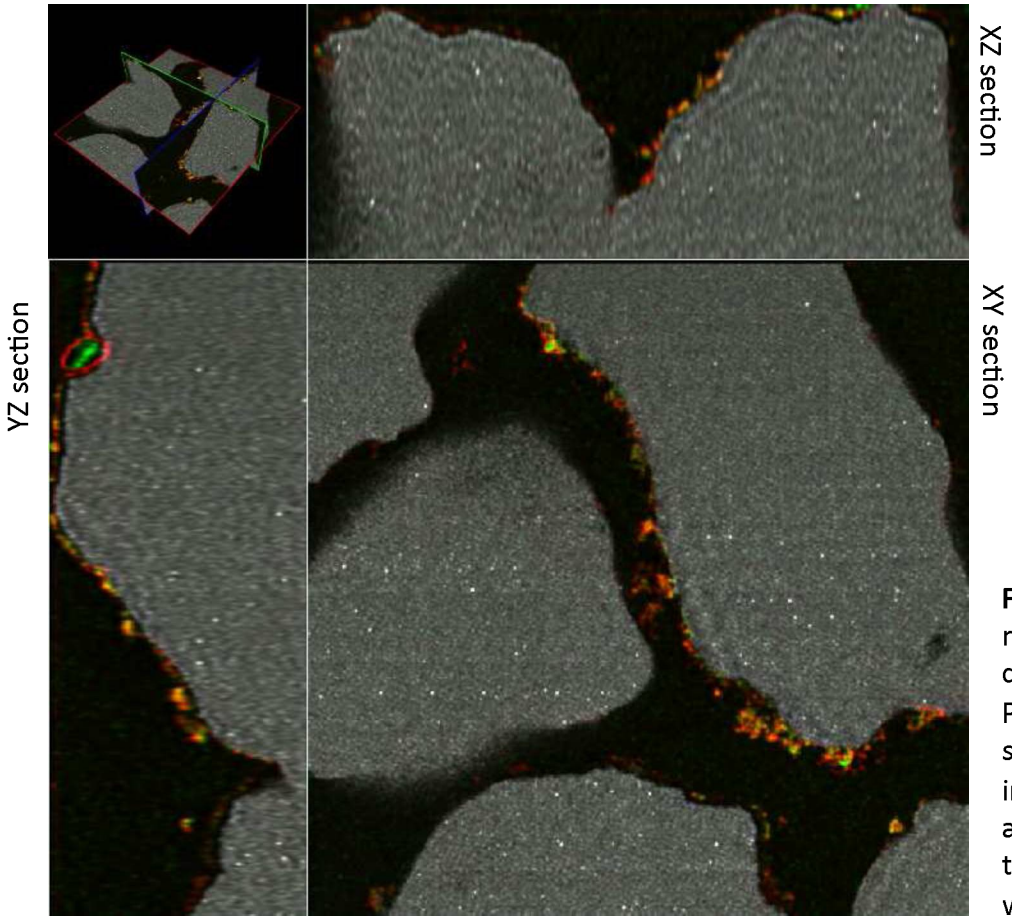
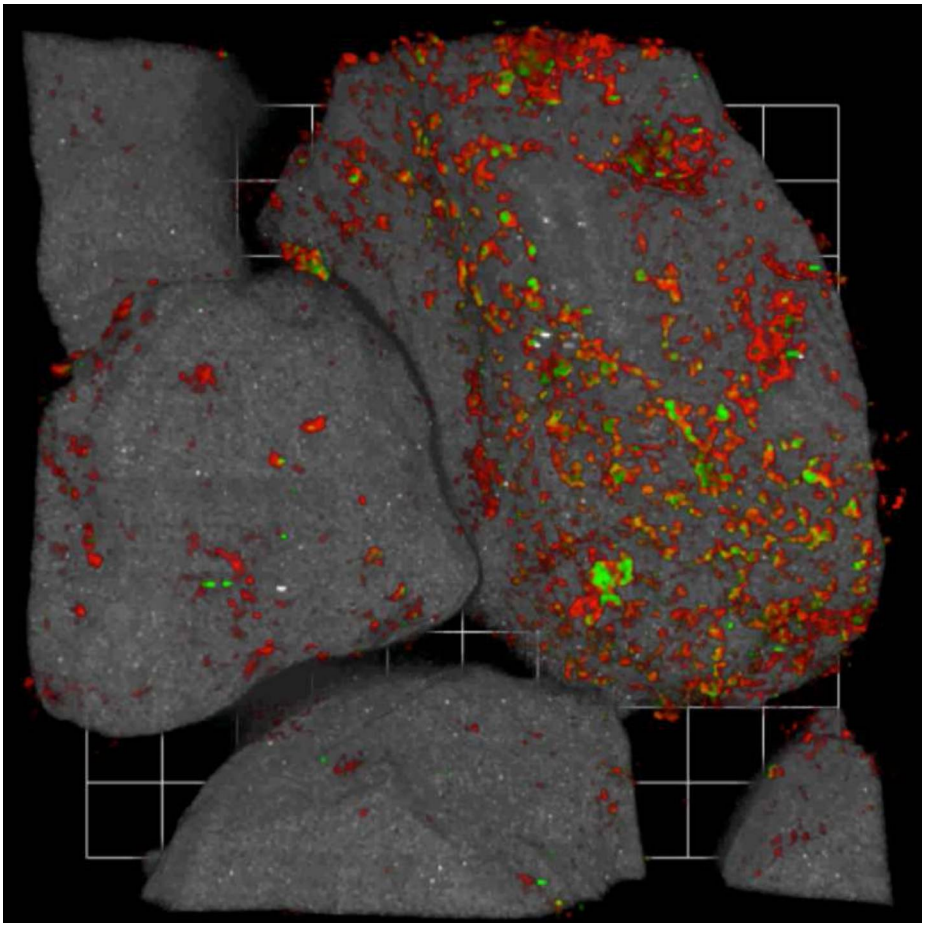


Figure 8. 2D slices (top) and 3D rendering (bottom) of confocal data of Hydrogenophaga strain P101 grown on FeS. Fe oxides are shown in red, cells in green, sand in grey. Fe-oxidizing biofilms are a few microns to ~10 microns thick. Cells are closely associated with Fe oxides.



Fe oxides
cells
sand

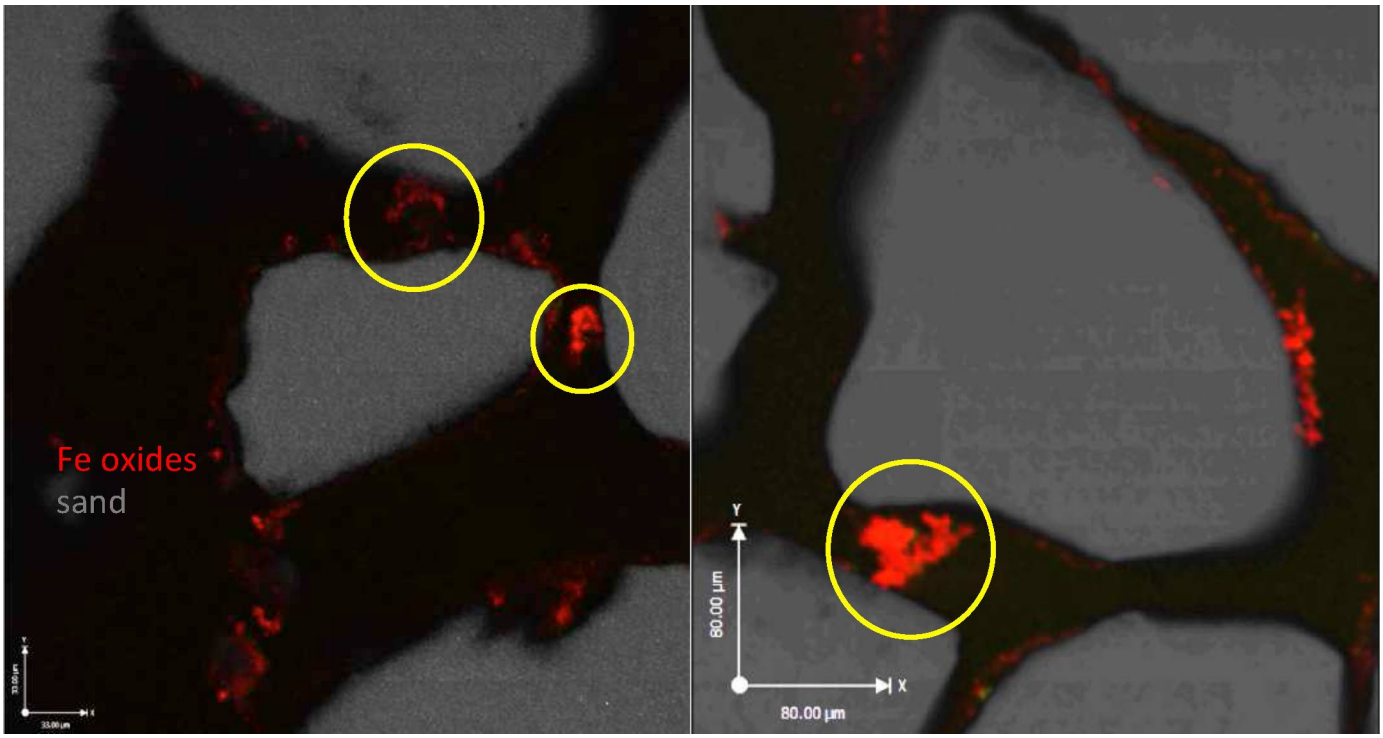


Figure 9. 2D confocal data showing Fe oxide aggregates bridging pore throats.

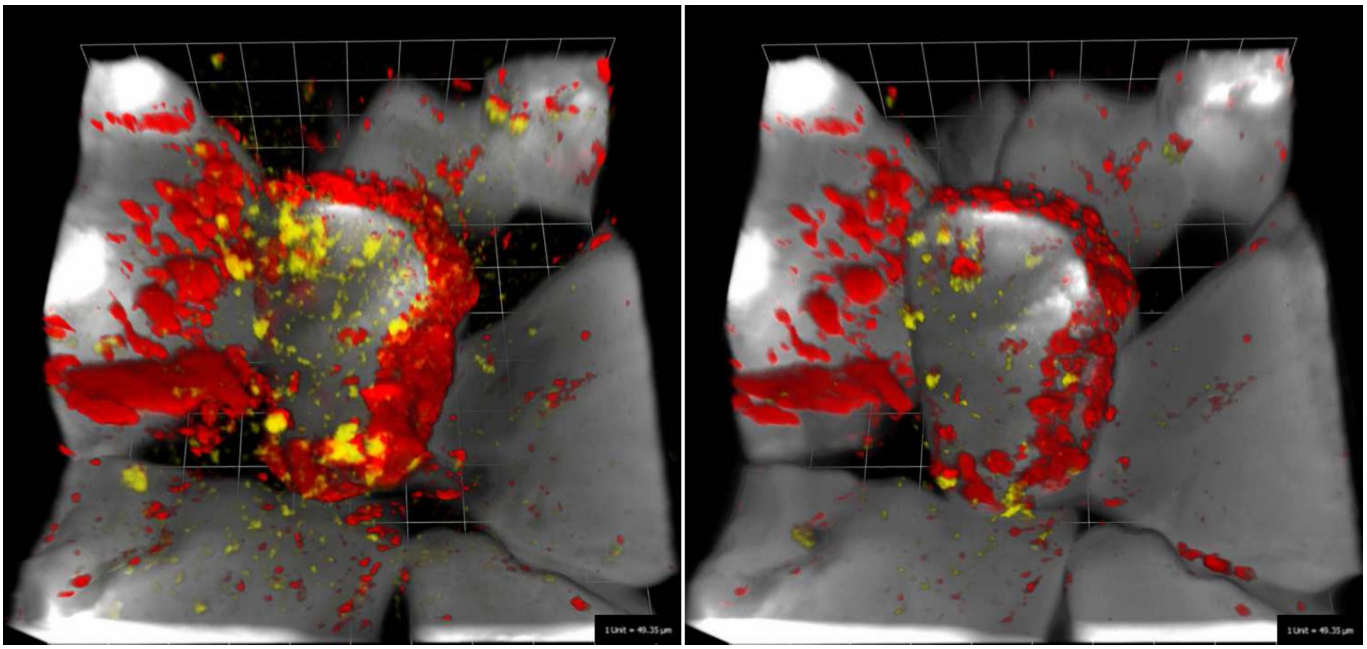


Figure 10. P101 cells (yellow) and iron oxides (red) before (left) and after (right) flow through experiment. A portion of the cells and oxides were sloughed from the system as a result of increasing shear stress.

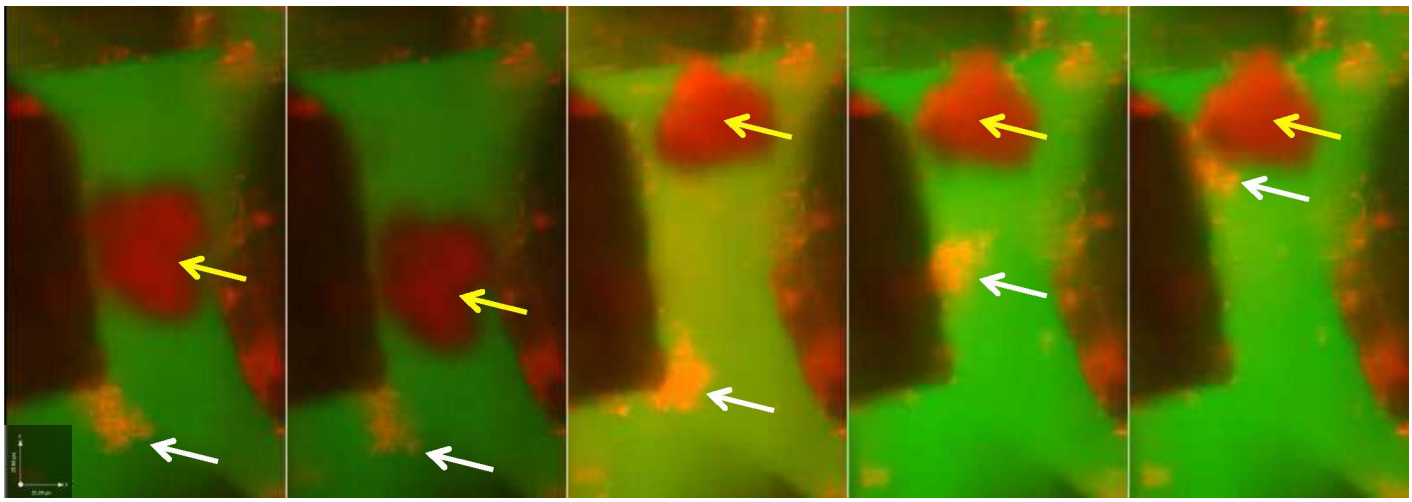


Figure 11. Confocal images from time series microscopy, showing the sloughing and reaccumulation of iron oxide biofilm. White arrows note the presence of sloughed particle. Yellow arrows indicate the movement of a small oxide sand grain in the pore space.