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Bioaugmentation of an Engineered Metal Treatment Wetland with *Cupriavidus SRS*

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Cupriavidus SRS is a metal resistant, rod shaped, gram negative bacteria that was isolated from a location with heavy metal contamination at the Department of Energy's Savannah River Site in Aiken, South Carolina. This isolate has also been able to demonstrate antibiotic resistance and predatory behavior to other microorganisms, giving it a competitive advantage. The effect of bioaugmentation with *Cupriavidus SRS* and copper (Cu) uptake is not well understood, although multiple studies have analyzed this effect with other metal resistant bacteria. In this study, sediment from the AL1 outfall area will be inoculated with *Cupriavidus SRS* to analyze the effectiveness of the degradation of Cu.

Introduction

Historic use of copper (Cu) has resulted in legacy contamination and persistence in the environment. Cu has been known to humans for at least 6000 years and is used for a variety of purposes: electrical applications, motors, plumbing, marine equipment, brake pads, cooking utensils, and trace nutrients in livestock feeds (Agency…,2015). Cu can enter the environment through improper disposal, leakage from factories, and waste dumps. In 2000, about 1,400,000,000 pounds of Cu were released into the environment by industries (Agency…,2015). Although, Cu is essential for life the element can become toxic at low concentrations to microorganisms in sediments and to aquatic organisms (Maneva et al. 2009). When Cu is released into the sediment it strongly attaches to the organic layer and has a low bioavailability. Whereas, when Cu enters surface water the metal can dissolve and bind to sediment particles. Cu does not break down in the environment and the Environmental Protection Agency has set limits for copper in drinking water at 1.3ppm and in sediment 2-100 ppm (Agency…,2015).

There are many chemical methods to remediate copper (Cu) from the environment (Cornu et al. 2017). However, the use of microorganisms has been identified to be cheaper and more efficient in remediating toxic compounds in a method known as bioremediation. (Hamdi et al. 2007). A microorganism, *Cupriavidus SRS*, is a metal resistant, rod shaped, gram negative bacteria that was isolated from a location with heavy metal contamination. As a predator this isolate has the ability to attack gram-negative and gram-positive bacteria. It is highly tolerant to Cu, and the isolates growth initiation is stimulated by Cu (Makkar et al. 1987). Wang et al. (2015) determined that efflux pump activity is one of the most important mechanisms of heavy metal tolerance and the *Cupriavidus* genus demonstrates this behavior. In this study, *Cupriavidus SRS* will be inoculated to sediment and water from an area with high concentrations of Cu and zinc, to determine the ability of the isolate to grow and survive in a high level Cu wetland as well as the bioremoval efficiency.

Study Area

The H-02 wetland in the AL1 outfall area is an engineered wetland that was constructed to utilize natural processes associated with sediments and microbial communities to remove contaminants from waste waters at the Savannah River Site (Figure 1). Specifically, this wetland

treats the building processes and runoff form the Tritium Processing Facility in H-area to prevent Cu and Zn from entering the water before the contaminants go in the Savannah River(Mills, 2016). Distributions of Cu and Zn follow organic matter levels with higher densities along the shoreline showing that metal concentrations correlate with organic matter content (Mills, 2016).

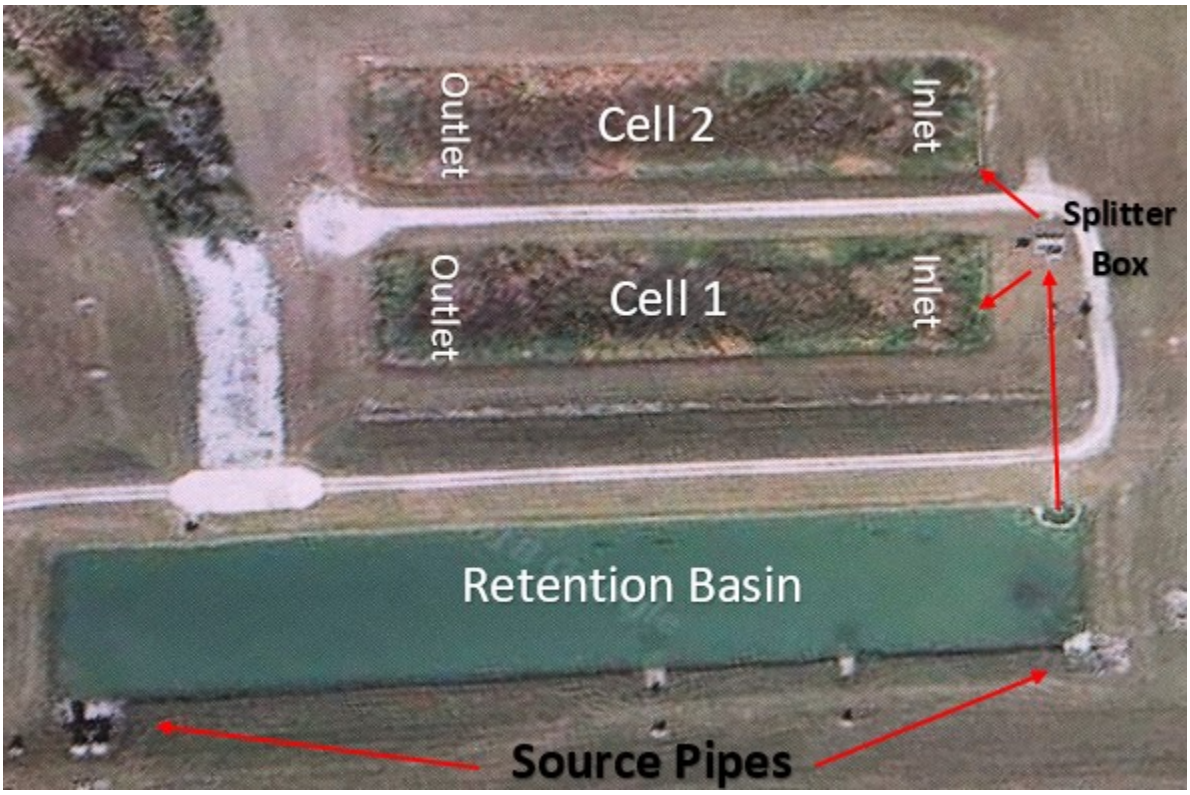

Figure 1) Water exits the retention basin through a culvert where it flows to a splitter box equally into two separate cells. Image from GoogleMaps.

Materials & Methods

Sediment and Water Collection: All samples were collected from the AL1 Outfall area within the Department of Energy's Savannah River Site. Sediment cores, organic layer samples, and water samples were collected from the Inlet and Outlet cells in triplicate. Only water samples were collected from the basin.

***Cupriavidus SRS* Growth**: *Cupriavidus SRS* was isolated at the Savannah River Site from an area with heavy metal contamination. Cultures were grown in R2A medium at 25°C and shaken at 80 rpm for liquid cultures for 5 days.

Cell Pellet: After centrifugation at 10,000 RPM for 10 minutes the supernatant was removed with a micropipette. 1mL of phosphate buffer saline was added to the tube, vortexed, and recentrifuged. The supernatant was removed again and 1mL of phosphate buffer saline solution was added to the tube. The cell pellets were combined in a 50mL centrifuge tube and brought up in an additional 20ml of

Millique water to have a total of 40ml of the isolate. To determine the concentration of the isolate added to the samples 1ml was micropippetted onto an R2A plate and a plate count was done. (Figure 2)

Figure 2: *Cupriavidus SRS* cell pellet

Inoculation of Sediment and Water: All samples were done in triplicate, vortexed after inoculation, and put on the shaker for 48 hours. Day 0 begins when the samples are removed from the shaker. The Cell 2 Inlet and Cell 2 Outlet water samples were autoclaved and 29ml of each water sample was put in 50ml centrifuge tubes with 1ml of cupriavidus SRS. Also, 29 ml of the organic layer samples from Cell 1 and 2 and retention basin water samples were put in 50ml centrifuge tubes with 1ml of the isolate. The water basin has the highest concentration of Cu, so it was used as the control with no cupriavidus srs; 30ml of the basin water was put in a 50ml centrifuge tube. A negative control of 0.1mm NaCl solution was used with 29ml salt solution with 1ml cupriavidus srs.(Figure 3)

Experimental Set-Up: All samples were done in triplicate. All sediment samples were centrifuged at 7500 RPM for 10 minutes on Day 0 and Day 5. All the sediment and water samples were filtered with a 0.2µm nylon filter with a 10ml syringe to obtain approximately 10ml of supernatant into a 15ml centrifuge tube on Day 0 and Day 5. 2% nitric acid was added to the final solution for ICP-MS analysis on Day 0 and Day 5. The samples were put back on the shaker after analysis on Day 0. pH was taken 24 hours after the samples were put on the shaker as well as 48 hours after the shaker, and Days 0-4.(Figure 3)

Plate Count: Plate counts were done on Day 0 and Day 5 to determine the isolates growth. A micropipette was used to put 9.9mL of sterile water in a 15mL tube in triplicate. 0.1mL of the inoculated medium was added to the first tube. Then 0.1mL was removed from the first tube, put in the second tube, and mixed. Lastly, 0.1mL from the second tube was put in the third tube, and mixed to have a final dilution factor of 10^{-6}. From each of the 3 tubes add 1mL on R2A plates. Incubate the plates for *5* days and count the number of colonies. To calculate the number of bacteria per mL multiply the number of colonies on the plate by the dilution factor.

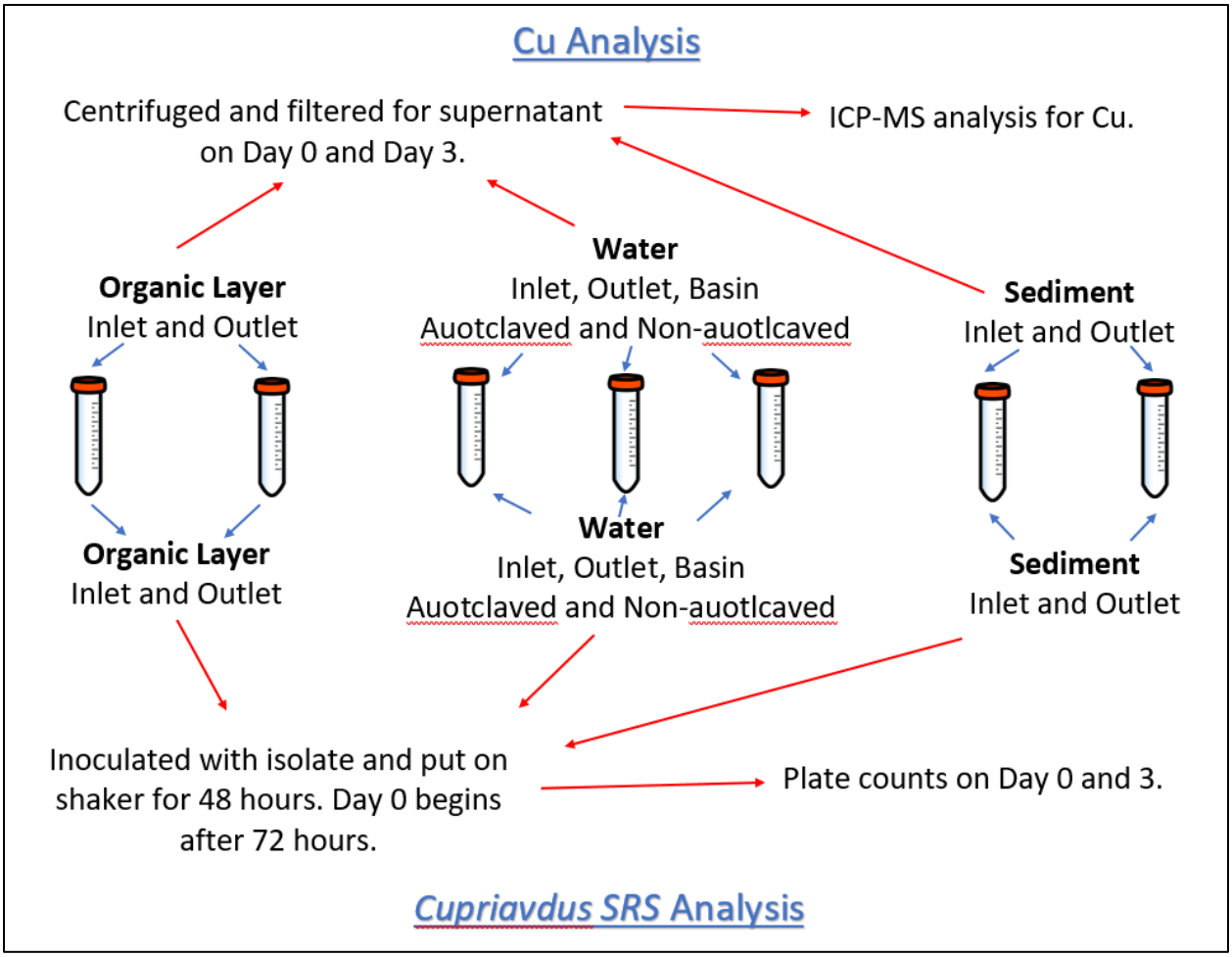

Figure 3: Flow diagram illustrating the experiments conducted to determine the amount of Cu in the samples and *Cupriavidus SRS* growth or death.

Results

This project is ongoing and the goal is to determine if *Cupriavidus SRS* has the ability to uptake excess Cu out of the environment. These could be some possible results for the experiment

- The isolate uptakes too much Cu, dies, and releases Cu back into the environment.
- The isolate is able to uptake Cu, continue to live, but chemically changes the environment introducing new problems.
- The isolate is able to uptake Cu, continue to live, and does not dirupt the environment with toxic chemical changes.

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