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Understanding Mercury Transfer to the Sulfate-Reducing Bacteria *Desulfovibrio desulfuricans* G20

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ABSTRACT

Mercury (Hg) in the environment can exist in a multitude of forms. This study, however, involved tests on two mercury species, one that is bound to natural organic matter (i.e., Hg-NOM) and mercury chloride ($HgCl_2$). The purpose of the study was to determine the rates at which these two different forms of mercury are accumulated by a strain of sulfate-reducing bacteria (SRB), *Desulfovibrio desulfuricans* G20. Some SRB are known to accumulate mercury and produce methylmercury, one of the most hazardous forms of mercury. Since the focus of this study was on the uptake of mercury, which is the first part of the methylating process, a non-methylating bacterial strain (*D. desulfuricans* G20) was selected. For metabolism, SRB require an electron donor and an electron acceptor in their growth media, so for this study, two cultures of *D. desulfuricans* G20 were grown and evaluated with two different electron donors and acceptors for comparison. One culture was grown in Wall's Medium amended with lactate and sulfate as the electron donor and electron acceptor respectively, while the second culture was grown in Wall's Medium amended with pyruvate as the electron donor and fumarate as the electron acceptor. The cells in each culture were grown and then transferred to smaller, individual glass test tubes. The test tube cultures were then given some more time to grow and then washed with a bicarbonate buffer solution amended with the appropriate electron donor and electron acceptor. For each type of culture (either lactate/sulfate or pyruvate/fumarate), half of the washed cell solutions were spiked with mercury chloride and the other half were spiked with mercury bound to natural organic matter. Four different incubation times were then tested following the mercury spikes. The lactate/sulfate cultures were incubated for 10, 20, 30, and 40 minutes respectively, while the pyruvate/fumarate cultures were incubated for 40, 50, 80, and 90 minutes respectively. After incubating for the appropriate times, each washed cell solution was transferred from its glass test tube to a clean sterile 50 mL plastic tube and centrifuged. The supernatant of each solution was saved for analysis and will represent the mercury not accumulated by the bacterial cells. The cell pellet for each washed cell solution was frozen to be analyzed for mercury accumulated inside the cells and for mercury accumulated on the surfaces of the cells. The original test tubes that were incubated for different times were also saved to be analyzed for mercury that may have attached to the glass test tube walls and was unavailable for bacterial uptake. All samples derived from the experiments will be analyzed using a Direct Mercury Analyzer (DMA-80, Milestone Microwave Laboratory Systems) and the results ascertained before October 10, 2010.

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1. INTRODUCTION

In the environment, mercury (Hg) can exist in numerous forms and can bind to many different substances. Mercury can form weak bonds with some substances and very strong bonds with others, and this can have an effect on how different forms of mercury can be accumulated by bacteria and organisms. The focus of this research was to test the different rates at which a strain of sulfate-reducing bacteria (SRB), *Desulfovibrio desulfuricans* G20, is able to accumulate two different forms of mercury. The two forms of mercury tested were mercury bound to natural organic matter (Hg-NOM) and mercury chloride ($HgCl_2$).

Sulfate-reducing bacteria (SRB) live in anaerobic environments, including sediments in lakes and creeks, deep ocean floors, and in general places with little or no oxygen. As discussed by Voordouw (1995), "Sulfate reducers contribute to the reduction and immobilization of heavy metals, which is generally desirable except in the case of formation of the hazardous environmental pollutant methylmercury." Not all SRB, however, are capable of methylating mercury (producing methylmercury) and even within the same genus and species of bacteria, some strains are capable of methylating mercury and some are not. For example, *Desulfovibrio desulfuricans* G20 is not known to methylate mercury, but *Desulfovibrio desulfuricans* LS has been found to methylate mercury (Pak and Bartha 1998). Because the focus of this research is on mercury uptake, and not methylmercury production, *D. desulfuricans* G20, which is not known to produce methylmercury, was chosen for this research.

2. EXECUTIVE SUMMARY

This research work has been supported by the DOE-FIU Science & Technology Workforce Initiative, an innovative program developed by the US Department of Energy's Environmental Management (DOE-EM) and Florida International University's Applied Research Center (FIU-ARC). During the summer of 2010, a DOE Fellow intern (Mr. Amaury Betancourt) spent 10 weeks doing a summer internship at ORNL under the supervision and guidance of Dwayne Elias. This internship was organized and directed by the Higher Education Research Experience (HERE) and the Oak Ridge Institute for Science and Education (ORISE). The intern's project was initiated in June 5, 2010, and continued through August 14, 2010.

Mercury (Hg) in the environment can exist in various forms. This study, however, involved tests on two mercury species, one that is bound to natural organic matter (i.e., Hg-NOM) and mercury chloride ($HgCl_2$). The purpose of the study was to determine the rates at which these two different forms of mercury are accumulated by a strain of sulfate-reducing bacteria (SRB), *Desulfovibrio desulfuricans* G20.

For metabolism, SRB require an electron donor and an electron acceptor in their growth media, so for this study, two cultures of *D. desulfuricans* G20 were grown on Wall's Medium (see Appendix A) and evaluated with two different electron donors and acceptors for comparison. For the first set of experiments, the Wall's Medium was amended with lactate and sulfate as the electron donor and electron acceptor, respectively, and L-cysteine as a reductant that can also help remove oxygen gas ($O_2(g)$) from the growth medium. For the second set of experiments, the Wall's Medium was amended with pyruvate and fumarate as the electron donor and electron acceptor, respectively. Ascorbic acid was added as the reductant in this case, which was also able to help remove $O_2(g)$ as well as prevent the formation of thiols (compounds that contain hydrogen bound to sulfide, HS^-, in their chemical structure) in the growth medium, which can bind with mercury (interpreted from E-Mail communication between Wall and Elias, 2010).

Each culture was grown in a nitrogen gas ($N_2(g)$) atmosphere. The lactate/sulfate culture and the pyruvate/fumarate culture were gently shaken while being incubated at 33°C for approximately 24 hours, after which each culture was transferred into individual anaerobic test tubes containing the appropriate medium. The 16 lactate/sulfate test tube cultures were incubated at 33°C for approximately 27 hours, while the 16 pyruvate/fumarate test tube cultures were incubated at 33°C for approximately 12 hours, all being gently shaken. Ultimately there were two experimental sets, each with 16 test tube cultures, for a total of 32 test tube cultures.

After each set of test tube cultures were incubated for the appropriate time, the cells in each test tube culture were washed with a bicarbonate buffer solution with a pH of 6.8, which was amended with either lactate and sulfate (for the lactate/sulfate cultures) or pyruvate, fumarate, and ascorbic acid (for the pyruvate/fumarate cultures). According to Dr. Judy Wall, Professor of Biochemistry at The University of Missouri, ascorbic acid was added to the

bicarbonate buffer solution to prevent the formation of thiols (E-Mail communication between Wall and Elias, 2010). Once the cells in each of the 32 test tube cultures were washed, they were spiked with either one of the two types of mercury solutions to give a final concentration of approximately 10 μg/L of Hg. The washed cell solutions from each type of medium (either lactate/sulfate or pyruvate/fumarate) were divided in half. One half of each type of washed cell solution (8 test tubes) was spiked with $HgCl_2$ solution and the other half (8 test tubes) spiked with Hg-NOM solution. Four different incubation times were then tested following the mercury spikes. The lactate/sulfate cell solutions were incubated for 10, 20, 30, and 40 minutes respectively, while the pyruvate/fumarate cell solutions were incubated for 40, 50, 80, and 90 minutes respectively. Duplicate test samples of the washed cell solutions were run for each incubation time for each form of mercury.

Once the cultures were incubated for the appropriately designated times, each culture was transferred to a clean 50 mL plastic tube, centrifuged, and the supernatant decanted and stored in a plastic bottle. For each centrifuged test sample, the remaining cell pellet was saved in the 50 mL plastic tube and kept frozen at -80°C. The supernatant samples were acidified to preserve the samples. In addition, the test tubes which had originally contained the spiked test samples that were incubated for different periods of time, were treated with Milli-Q (nanopure deionized) water and 1% BrCl to remove any mercury that may have remained attached to the test tube walls.

The supernatant samples and the samples of the test tube walls will be analyzed using the Direct Mercury Analyzer (DMA-80, Milestone Microwave Laboratory Systems) in the Soil and Groundwater Laboratory at the Applied Research Center (ARC) in Florida International University (FIU). The supernatant samples contain mercury that remained in solution during the mercury accumulation rate experiments and thus can be analyzed as the mercury that was not taken up by the cells. The test tube samples for mercury that may have been attached to the glass test tube walls will be considered as mercury that was not available to the bacterial cells. The cell pellets must be broken down in order to release any mercury contained within the cells. This mercury can be analyzed as mercury accumulated inside the cells. In addition, the remaining cell fragments can be dissolved and analyzed as mercury that attached to the cell membranes. Due to difficulty in locating an ultracentrifuge capable of spinning the cell pellet samples at a speed of approximately 100,000×gravity, it may only be possible to analyze the cell pellet samples for the mercury "accumulated" by the cells, which means that the mercury accumulated inside the cells may not be distinguishable from the mercury attached to the cell membranes. Therefore, if an ultracentrifuge is not found, the cell pellet samples can still be analyzed but the difference between mercury accumulated inside the cells and mercury accumulated on the cell surfaces will remain undetermined.

Dong et al. (2010) report that "even at low concentrations (~3 mg L^{-1}), natural dissolved organic matter (DOM) (which may be comparable to NOM) strongly complexes with ionic Hg^{2+} (mercury ion) and CH_3Hg^+ (methylmercury ion), thereby influencing biological uptake, and methylation of Hg in aquatic environments." Dong et al. (2010) also discuss that, in water from the East Fork Poplar Creek (EFPC) in Oak Ridge, Tennessee, "The estimated concentration of Hg-DOM complexes is at least six orders of magnitude higher than other inorganic Hg^{2+} complexes such as $Hg(OH)_2$, Hg(OH)Cl, and $HgCl_2$." Miller et al. (2009)

performed kinetics experiments with mercury bound to different organic compounds and NOM. In their research, Miller et al. (2009) found that "The decrease in reducible Hg and the increase in C_{18} extractable [hydrophobic] Hg throughout the experiment indicate that Hg is being transferred from inorganic complexes or reducible Hg-NOM complexes to stronger nonreducible/hydrophobic Hg-NOM complexes." However, Hg-NOM complexes that are hydrophobic may or may not enter the cell more easily than hydrophilic complexes. In lactate/sulfate cultures, the bacteria reduce sulfate (SO_4^{2-}) to sulfide (S^{2-}). Sulfide is expected to form a strong bond with mercury and form mercury sulfide compounds, which may be unavailable for uptake by bacteria.

The results of the mercury accumulation rate experiments will be obtained before October 10, 2010. A Direct Mercury Analyzer (DMA-80, Milestone Microwave Laboratory Systems) will be used to analyze the samples from the experiments. It is hypothesized that the bacterial cells in the pyruvate/fumarate cultures spiked with Hg-NOM will accumulate more mercury than the other cultures.

3. RESEARCH DESCRIPTIONS

The focus of this research was to determine the rates at which two different forms of mercury, mercury bound to natural organic matter (Hg-NOM) and mercury chloride ($HgCl_2$), are accumulated by a strain of sulfate-reducing bacteria (SRB), *Desulfovibrio desulfuricans* G20. The general experimental plan has six main steps as follows:

1. Grow an initial culture of the *D. desulfuricans* G20 for approximately 24 hours.
2. Transfer the culture of *D. desulfuricans* G20 to individual test tubes to grow the test tube cultures for 12 to 24 hours.
3. Wash the cells in the cultures with bicarbonate buffer solution.
4. Introduce mercury to each culture.
5. Conduct mercury accumulation rate experiments.
6. Prepare the samples for analysis.

This experimental plan is summarized in Figure 1.

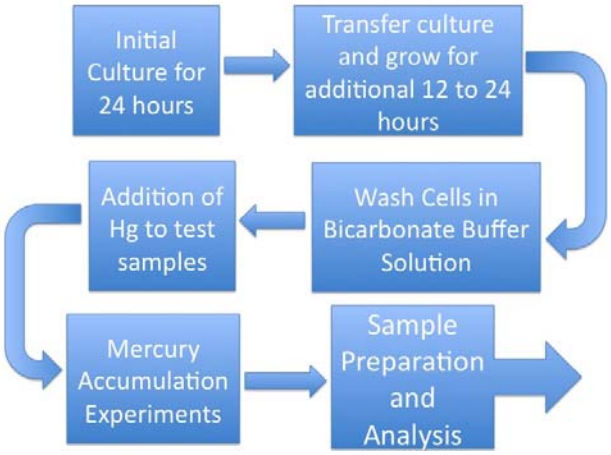

Figure 1: Experimental plan for mercury accumulation rate experiments.

SRB must have an electron donor and an electron acceptor in their growth media for metabolism. Two cultures of *D. desulfuricans* G20 were grown for the purpose of this study. The first culture was grown in Wall's Medium amended with lactate and sulfate as the electron donor and electron acceptor respectively, as well as L-cysteine added as a reductant that can also help to remove oxygen gas ($O_2(g)$) from the growth medium. The second culture was also grown in Wall's Medium, but amended with pyruvate and fumarate as the electron donor and electron acceptor respectively. This time ascorbic acid was added to the growth medium as the reductant which was also able to remove $O_2(g)$ as well as prevent the formation of thiols (compounds containing a hydrogen bound to a sulfide, HS^-, in their chemical structure) (interpreted from E-Mail communication between Wall and Elias, 2010), which could react with mercury during the experiments. Thus, two different experiments, performed about one week apart, were carried out. The first experiment was carried out with the lactate/sulfate culture and the second with the pyruvate/fumarate culture.

The lactate/sulfate culture was incubated at 33 °C for approximately 24 hours while gently shaking. After the incubation period, individual 1 mL samples of the lactate/sulfate culture were transferred to 16 individual test tubes, each containing 9 mL of the fresh lactate/sulfate medium, for a final volume of 10 mL of lactate/sulfate culture in each test tube. The test tube cultures were then gently shaken while being incubated at 33 °C for approximately 27 hours. The same procedure was carried out for the pyruvate/fumarate culture, incubating it at 33 °C for approximately 24 hours while gently shaking, then transferring individual 1 mL samples to 16 individual test tubes, each containing 9 mL of the fresh pyruvate/fumarate medium, for a final volume of 10 mL in each pyruvate/fumarate test tube culture. The pyruvate/fumarate test tube cultures were then gently shaken and incubated at 33 °C for approximately 12 hours. Due to time constraints during the experiments, the pyruvate/fumarate test tube cultures were incubated for less time than the lactate/sulfate cultures.

More than 24 hours in advance of the mercury accumulation rate experiments (i.e., during the times that the lactate/sulfate test tube cultures and the original pyruvate/fumarate culture were incubating), the bicarbonate buffer solutions and the mercury stock solutions for each set of experiments were prepared. Two different bicarbonate buffer solutions were prepared. For the lactate/sulfate cultures, the bicarbonate buffer solution contained 30 millimolar (mM) sodium bicarbonate ($NaHCO_3$), 60mM lactate, and 30mM sulfate. For the pyruvate/fumarate cultures, the bicarbonate buffer solution contained 28.3mM $NaHCO_3$, 56.6mM pyruvate, 56.6mM fumarate, and 0.94mM ascorbic acid. As recommended by Dr. Judy Wall, Professor of Biochemistry at the University of Missouri, ascorbic acid was added to the bicarbonate buffer solution to prevent the formation of thiols (E-mail communication between Wall and Elias, 2010). The reason that the concentrations of components in the pyruvate/fumarate bicarbonate buffer solution were not round numbers was because fumarate was added to the pyruvate/fumarate bicarbonate buffer solution after adjusting the volume of the pyruvate/fumarate bicarbonate buffer solution.

For all cultures, the mercury stock solutions, which were to be used for the mercury spikes in each test tube culture, were prepared at least 24 hours in advance of the mercury accumulation rate experiments. This was done to allow the mercury to equilibrate for at least 24 hours in each stock solution. For the lactate/sulfate cultures, the $HgCl_2$ stock solution was prepared by adding approximately 1.17 mL of 10% NaCl stock solution to approximately 18.8 mL of the lactate/sulfate bicarbonate buffer solution followed by 50 µL of Hg stock solution (40 µg Hg/mL). In addition, for the lactate/sulfate cultures, the Hg-NOM stock solution was prepared by adding approximately 1 mL of NOM stock solution to approximately 19 mL of the lactate/sulfate bicarbonate buffer solution followed by 50 µL of Hg stock solution (40 µg Hg/mL). For the pyruvate/fumarate cultures, the $HgCl_2$ stock solution was prepared by adding approximately 1 mL of 10% NaCl stock solution to approximately 19 mL of the bicarbonate buffer solution, which had been amended with pyruvate and ascorbic acid, but not yet with fumarate, then adding 1.2 mL of 1M fumarate followed by 50 µL of Hg stock solution (40 µg Hg/mL). In addition, for the pyruvate/fumarate cultures, the Hg-NOM stock solution was prepared by adding approximately 1 mL of NOM stock solution to approximately 19 mL of the bicarbonate buffer solution, which had been amended with pyruvate and ascorbic acid but not yet with

fumarate, then adding 1.2 mL of 1M fumarate followed by 50 µL of Hg stock solution (40 µg Hg/mL). ORNL Scientist Carrie Miller was very helpful in preparing the mercury stock solutions and in planning which mercury stock solutions to prepare. For the lactate/sulfate mercury stock solutions, the stock solutions had equilibrated for approximately 31 hours prior to use in the experiments. For the pyruvate/fumarate mercury stock solutions, the stock solutions had equilibrated for approximately 24 hours prior to use in the experiments.

After the test tube cultures for each experimental set were incubated for the appropriately designated times, the test tubes were placed in an airlock, which is a device that facilitates transfer of items into and out of an anaerobic glovebag. Once inside the anaerobic glovebag, the cells in each test tube culture were ready to be washed. Throughout the experiments the anaerobic glovebag had a gas composition of approximately 3.0% hydrogen gas ($H_2(g)$), 0 parts per million (ppm) $O_2(g)$ (both measured by the gas analyzer in the anaerobic glovebag) and 97% nitrogen gas ($N_2(g)$) (calculated from the gas mix tank used to fill the anaerobic glovebag). The following photograph shows an anaerobic glovebag similar to the one used for the experiments.

Figure 2: A photograph of an anaerobic glovebag similar to the one used in the experiments.

Once the test tube cultures were placed inside the glovebag, each test tube culture was opened by removing the aluminum crimp from the top of the test tube and then removing the rubber stopper from the top of the test tube. For each test tube, the culture inside each test tube was poured into an individual sterile plastic 50 mL tube and then each 50 mL tube was capped. Once eight test tube cultures were transferred to eight individual sterile plastic 50 mL tubes, the eight plastic 50 mL tubes were placed in the airlock, removed from the

glovebag, then centrifuged at approximately 12,000×gravity at a temperature of between 4 to 6 °C. Only eight tubes were centrifuged at a time due to the space limitation of the centrifuge. For the lactate/sulfate cultures, the centrifugation time for each test tube culture was about 5 minutes. For the pyruvate/fumarate cultures, the centrifugation time was approximately 7 minutes due to the cell pellet (the hard mass of cells formed after a culture is centrifuged) in each tube consistently dissolving back into solution after being centrifuged.

After the test tubes were centrifuged for the appropriate time, they were removed from the centrifuge, placed back into the airlock and then transferred into the anaerobic glovebag. The supernatant solution in each 50 mL plastic tube was then decanted into an Erlenmeyer flask for spent media (media that had contained bacteria and may still contain bacteria). In each 50 mL plastic tube, a cell pellet remained. A volume of 9 mL of the appropriate bicarbonate buffer solution was added to each 50 mL plastic tube and the tubes were capped. The tubes were then placed inside the airlock and removed from the glovebag. Each tube was shaken to dissolve the cell pellet back into solution, and then centrifuged again under the same conditions as described above. After centrifuging, the tubes were again transferred to the airlock and glovebag. The supernatant of each tube was decanted into the Erlenmeyer flask and 9 mL of the appropriate bicarbonate buffer solution was added to the cell pellet in each tube. The tubes were capped and then placed in the airlock and removed from the glovebag. Each tube was again shaken to dissolve the cell pellet back into solution.

The tubes were then centrifuged for a third time under the same conditions as described above. After centrifuging, the tubes were transferred to the airlock and glovebag. The supernatant of each tube was decanted into the Erlenmeyer flask. A 9 mL volume of the appropriate bicarbonate buffer solution was added to each tube, and then each tube was shaken to dissolve the cell pellet back into solution. For the pyruvate/fumarate cultures, a sample of 200 µL of the washed cell solution from each tube was extracted and added to a 1.5 mL tube to be used for counting cells in each washed cell solution. For the lactate/sulfate cultures, the cell counting samples were extracted after the entire washing procedure. ORNL Scientist Abir Biswas assisted in planning to prepare the samples for cell counting procedures. The washed cell solution in each 50 mL plastic tube was transferred to a clean glass test tube, which was then sealed with a lubricated rubber stopper. The entire washing procedure described above was repeated for all test tube cultures. It is important to note that, for the lactate/sulfate cultures, the clean glass test tubes that contained the washed cell solutions were crimped with an aluminum cap before being removed from the glovebag. However, for the pyruvate/fumarate cultures, the clean glass test tubes that contained the washed cell solutions were crimped with an aluminum cap after being removed from the glovebag. This was done accidentally, however the lubricated rubber stopper on each test tube appears to have provided a good seal for the test tubes despite them not being capped until after being removed from the glovebag.

For the lactate/sulfate cultures, once the entire washing procedure was completed, the washed cell solution test tubes were placed inside a biosafety cabinet and, using a sterile technique, a 200 µL sample was extracted from each tube and placed inside an individual clean 1.5 mL tube. Similar to the pyruvate/fumarate cultures, the 1.5 mL tubes prepared for the lactate/sulfate cultures were for counting the cells in each washed cell solution. For the

pyruvate/fumarate cultures, the cell counting samples were preserved in a 2% paraformaldehyde solution by adding 200 μL of 4% paraformaldehyde to each 1.5 mL tube, which were then placed in a refrigerator, maintained at a temperature of approximately 4 °C. For the lactate/sulfate cultures, the cell counting samples were saved in the refrigerator at approximately 4 °C and paraformaldehyde was added a day after the experiments. After the washing procedure and preparation of cell counting samples for each culture type, the test tubes of each culture type were placed in the mercury hood and were then ready for the mercury accumulation rate experiments.

For each type of culture (i.e., either pyruvate/fumarate or lactate/sulfate), half of the washed cell solutions in the test tubes (8 test tubes) were spiked with $HgCl_2$ solution while the other half (8 test tubes) were spiked with Hg-NOM solution. Four different incubation times were then tested after spiking the washed cell solutions with mercury. For the lactate/sulfate cultures, the incubation times were 10, 20, 30, and 40 minutes respectively, and for the pyruvate/fumarate cultures, the incubation times were 40, 50, 80, and 90 minutes respectively. Duplicates were prepared for all test samples.

After each washed cell solution was spiked with mercury and incubated for the appropriate time, each solution was transferred from its glass test tube to an individual clean sterile plastic 50 mL tube. Each glass test tube was saved to be analyzed for mercury that may have attached to the test tube walls and thus may have become unavailable for uptake to the bacteria. Then, each 50 mL tube was centrifuged at a speed of approximately 12,000×gravity at a temperature between 4 to 6 °C. For the lactate/sulfate cultures, the tubes were centrifuged for about 5 minutes. For the pyruvate/fumarate cultures, the tubes were centrifuged for about 7 minutes. Once each tube was centrifuged, the supernatant solution in each tube was decanted into an individual clean plastic bottle and then stored on ice. The supernatant samples were then later stored in a freezer at -20 °C. Each tube containing a cell pellet was also stored on ice and then later stored in a freezer at -80 °C. The test tubes that had been saved for analysis of the test tube walls were stored inside the mercury hood.

The day following the experiments, approximately four drops of 4N hydrochloric acid (HCl) were added to each supernatant sample to preserve the samples and prevent mercury from attaching to the plastic bottle walls in each sample. In order to help remove mercury that may have been present on the walls inside of the glass test tubes that were used in the mercury accumulation rate experiments, 10 mL of Milli-Q water and 100 μL of bromine chloride (BrCl) were added to each test tube that had been saved inside the mercury hood. This solution was allowed to equilibrate for approximately 24 hours before decanting the contents of each test tube into an individual clean plastic bottle. The plastic bottles containing solutions for analysis of mercury that may have attached to the test tube walls were stored in the refrigerator at a temperature of approximately 4 °C.

After completing the mercury accumulation rate experiments, the bacterial cells in each washed cell solution were counted using a Petroff-Hausser Counter and a light microscope. The results of the experiments will depend on how many bacterial cells were present in each washed cell solution. The results of the cell counts are in Appendix B.

ORNL Scientists Meghan Drake and James Moberly assisted greatly throughout the entire course of the experiments. In addition, Peggy Shoffner and Angelique Lawrence from ARC-FIU assisted greatly in the technical editing of this report. A Direct Mercury Analyzer (DMA-80, Milestone Microwave Laboratory Systems) will be used to analyze the samples from the experiments. The results of the experiments will be ascertained before October 10, 2010.

4. RESULTS AND ANALYSIS

The results of the mercury accumulation rate experiments will be ascertained by October 10, 2010. The mercury concentrations in each sample will be normalized to the number of cells in the corresponding spiked washed cell solution. A supplemental report discussing the results and subsequent conclusions will be generated once the experiments are complete.

5. CONCLUSION

The conclusions of the experiment will be ascertained by October 10, 2010, when the results of the experiments will be analyzed. A supplemental report discussing the results and subsequent conclusions will be generated once the experiments are complete.

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APPENDIX A.

Table 1: Wall's Medium (or Wall Media) with either lactate/sulfate or pyruvate/fumarate

Wall Media by Judy Wall and Dwayne Elias. **Edited by Amaury Betancourt on 08/01/2010.**		
Total Volume of Media: 1000 mL		
Component	**Amount**	**Units**
Deionized Water	900.306	mL
$MgCl_2*6H_2O$ (1.35 M)	5.926	mL
NH_4Cl (3.74 M)	5.348	mL
$CaCl_2*2H_2O$ (0.95 M)	0.632	mL
K_2HPO_4 – NaH_2PO_4 (1 M)	2	mL
Trace Elements (Trace Minerals – No NTA, No $FeCl_2$)	1	mL
Tris-HCl (2 M), pH 7.4	15	mL
Yeast Extract	1	g
Thauers Vitamins (10 X)	1	mL
Resazurin (0.1 %)	0.8	mL
Add the following components post-autoclave:		
For media amended with Lactate and Sulfate as the electron donor and electron acceptor, respectively, add the following components:		
Lactate (2 M)	60	mL
Sulfate (2 M)	30	mL
L-Cysteine HCl (0.14 M)	7.1	mL
For media amended with Pyruvate and Fumarate as the electron donor and electron acceptor, respectively, add the following components:		
Pyruvate (2 M)	30	mL
Fumarate (1 M)	60	mL
Ascorbic Acid (0.14 M)	7.1	mL
Media should be labeled as Wall Medium and then designated with the electron donor/acceptor. For example, if you are using Lactate and Sulfate – the medium would be Wall Lac/Sulf.		

APPENDIX B

Table 2: Cell counts for washed cell solutions. To convert from average cell count per square to cells/mL, multiply the average cell count per square by $2*10^7$*Dilution Factor.

Date:	Medium	Sample	Average Cell Count Per square	Squares Counted	Dilution Factor	Cells/mL
8/3/10	Wall Lac/Sulf	0 min, HgCl2, #1	5.9	10	2	2.36E+08
			6.333333333	9	2	2.53E+08
8/4/10	Wall Lac/Sulf	0 min, HgCl2, #2	6.666666667	9	2	2.67E+08
			5.2	10	2	2.08E+08
8/5/10	Wall Lac/Sulf	0 min, Hg-NOM, #1	2.2	10	2	8.80E+07
			1.8	10	2	7.20E+07
8/5/10	Wall Lac/Sulf	0 min, Hg-NOM, #2	5.9	10	2	2.36E+08
			6	9	2	2.40E+08
8/5/10	Wall Lac/Sulf	30 min, HgCl2, #1	6.9	10	2	2.76E+08
			7.3	10	2	2.92E+08
8/5/10	Wall Lac/Sulf	30 min, HgCl2, #2	1.7	10	2	6.80E+07
			1	10	2	4.00E+07
8/5/10	Wall Lac/Sulf	30 min, Hg-NOM, #1	4.4	10	2	1.76E+08
			3.1	10	2	1.24E+08
8/5/10	Wall Lac/Sulf	30 min, Hg-NOM, #2	4	10	2	1.60E+08
			5.1	10	2	2.04E+08
8/6/10	Wall Lac/Sulf	10 min, HgCl2, #1	2.2	10	2	8.80E+07
			1.8	10	2	7.20E+07
8/6/10	Wall Lac/Sulf	10 min, HgCl2, #2	2.5	10	2	1.00E+08
			2.2	10	2	8.80E+07

8/6/10	Wall Lac/Sulf	10 min, Hg-NOM, #1	4.5	10	2	1.80E+08
			4.4	10	2	1.76E+08
8/6/10	Wall Lac/Sulf	10 min, Hg-NOM, #2	0.6	10	2	2.40E+07
			0.3	10	2	1.20E+07
8/9/10	Wall Lac/Sulf	20 min, Hg-NOM, #2	5.2	10	2	2.08E+08
			3.5	10	2	1.40E+08
8/9/10	Wall Lac/Sulf	20 min, Hg-NOM, #1	1.5	10	2	6.00E+07
			1.3	10	2	5.20E+07
8/9/10	Wall Lac/Sulf	20 min, HgCl2, #2	1.8	10	2	7.20E+07
			2.2	10	2	8.80E+07
8/9/10	Wall Lac/Sulf	20 min, HgCl2, #1	6.7	10	2	2.68E+08
			5.6	10	2	2.24E+08
8/9/10	Wall Pyruv/Fum	II, 0 min, HgCl2, #1	2.8	10	2	1.12E+08
			2.9	10	2	1.16E+08
8/9/10	Wall Pyruv/Fum	II, 0 min, HgCl2, #2	1.8	10	2	7.20E+07
			1.1	10	2	4.40E+07
8/9/10	Wall Pyruv/Fum	II, 0 min, Hg-NOM, #1	4.3	10	2	1.72E+08
			4.2	10	2	1.68E+08
8/9/10	Wall Pyruv/Fum	II, 0 min, Hg-NOM, #2	1.2	10	2	4.80E+07
			1.9	10	2	7.60E+07
8/10/10	Wall Pyruv/Fum	II, 10 min, HgCl2, #1	3	10	2	1.20E+08
			3.5	10	2	1.40E+08
8/10/10	Wall Pyruv/Fum	II, 10 min, HgCl2, #2	1.5	10	2	6.00E+07
			1.8	10	2	7.20E+07
8/10/10	Wall Pyruv/Fum	II, 10 min, Hg-NOM, #1	3.7	10	2	1.48E+08
			2.2	10	2	8.80E+07
8/10/10	Wall Pyruv/Fum	II, 10 min, Hg-NOM, #2	2.2	10	2	8.80E+07
			1.6	10	2	6.40E+07
8/10/10	Wall Pyruv/Fum	II, 20 min, HgCl2, #1	3.1	10	2	1.24E+08
			1.4	10	2	5.60E+07

8/10/10	Wall Pyruv/Fum	II, 20 min, HgCl2, #2	6.7	10	2	2.68E+08
			4.7	10	2	1.88E+08
8/10/10	Wall Pyruv/Fum	II, 20 min, Hg-NOM, #1	4.4	10	2	1.76E+08
			5.2	10	2	2.08E+08
8/10/10	Wall Pyruv/Fum	II, 20 min, Hg-NOM, #2	6.5	10	2	2.60E+08
			6.6	10	2	2.64E+08
8/10/10	Wall Pyruv/Fum	II, 30 min, HgCl2, #1	4.4	10	2	1.76E+08
			4.5	10	2	1.80E+08
8/10/10	Wall Pyruv/Fum	II, 30 min, HgCl2, #2	3.2	10	2	1.28E+08
			3.5	10	2	1.40E+08
8/10/10	Wall Pyruv/Fum	II, 30 min, Hg-NOM, #1	1.4	10	2	5.60E+07
			2	10	2	8.00E+07
8/10/10	Wall Pyruv/Fum	II, 30 min, Hg-NOM, #2	5.2	10	2	2.08E+08
			2.8	10	2	1.12E+08