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Project Title

Impact of Landfill Leachate on Iron Release from Northest Florida Iron Rich Soils

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Fourth Progress Report

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1. Introduction

For this part of our research, we enumerated and identified the soil microbial species. We also quantified the soil iron content of the samples. For microbial enumeration, we used the plate count method. For microbial identification, we used Polymerase Chain Reaction (PCR) analysis. We report here the results of above experiments and analysis.

2. Objectives

Our objective for this part of research is to quantify and identify the microbial species in the sample soil. We also would like to provide evidence of the presence of possible iron reducing bacteria.

3. Methods

3.1 Soil Iron Content

Following the extraction and oxidation of iron from the soil, soil iron content was then determined using spectrophotometric analysis techniques. For the metal of iron, highly colored complexes are formed when reacting with the thiocyanate ion:

$$Fe^{3+}(aq) + 6SCN^{-}(aq) = [Fe(SCN)_6]^{3-}(aq)$$

Because the thiocyanate complex is colored red, it absorbs at 447nm on the absorption spectrum.

3.2 Plate Counts

For enumeration of aerobic bacterial growth present in the soil samples, the plate count method was employed. A general nutrient agar was used as the growth medium. Sterile techniques were used throughout the entire process. For each soil sample collected, one gram of soil was diluted in sterilized tap water to obtain a concentration of bacteria that was countable on the plates (Figure 1). Samples were vigorously mixed during dilution to assist in dislodging the bacteria from the soil particles. A total of 100 µl of diluted soil suspension was plated on three plates per soil sample. Sterilized water was spread on an agar plate to serve as the control.

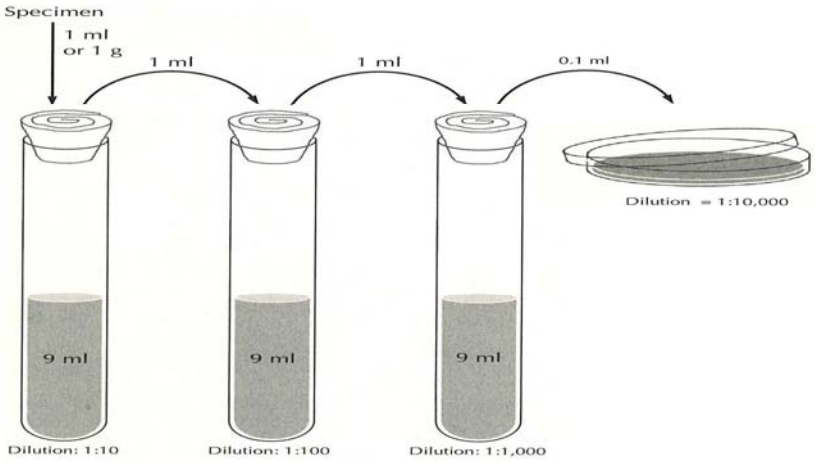

Figure 1. Plate Count Dilution

3.3 Aerobic Microbial Culture Cultivation and Identification

Aerobic microbial species were cultured using the following media and apparatus described earlier. The growth bottles received constant air supply for approximately two weeks. From these growth chambers, material was withdrawn for identification both through morphology and PCR analysis. For the morphology identification, both mixed cultures and pure cultures were fixed on slides by heating and viewed under a bright field microscope. For the mixed culture, a small amount of soil/water material was withdrawn from the chamber and fixed onto the slide by heating.

3.4 Anaerobic Species Cultivation and Identifiaction

The same culturing preparation used in the aerobic culturing was also used in the anaerobic culturing, with a few alterations. For anaerobic growth, the soil and media mixture were placed in a 750 ml flask and sealed. Approximately 5 ml of potassium hydroxide was placed in the arm tube to serve as the CO_2 entrapping device. This method helps to alleviate pressure in the system and closely mimic real world conditions. The system was allowed approximately one month of incubation before further analysis. For the morphological identification, a small amount of water, soil mixture was removed from the system. To avoid introduction of oxygen to the system, a needle was inserted through the rubber stopper. The same steps were then followed as were done with the aerobic species.

3.5 Polymerase Chain Reaction (PCR) Analysis

One of the more reliable and most cost-effective methodologies for identification of bacterial species is Polymerase Chain Reaction (PCR) analysis. The current literature regards PCR analysis as one of the most reliable means of bacterial species identification. Also, Dr. Reeves from Biology Department at Florida State University was consulted on this manner and he also suggested employing the PCR analysis for identification of bacterial species. We used the method developed by Brenda Bennison at Florida State University in the PCR analysis.

4. Results

4.1 Soil Iron Content

The soil iron content for all the samples is listed in Table 1. Among the samples, Okaloosa has the most iron content, while Jackson County has the least.

4.2 Plate Counts

Plate counts were performed for all sixteen landfill locations from the fifteen counties in Northwest Florida (Table 1). Plates were allowed 48 hours of incubation before counting the colonies. The morphology of the colonies that formed was in general consistent across all samples. There were a few samples that developed a mold or fungus in addition to the bacterial colonies. The morphology of the colonies was circular in shape with variations in size, and color was typically a pale yellow to tan. Some plates would develop a light green pigmentation, which after consulting Dr. Reeves from Biology Department at Florida State University, was determined to be a reaction of *pseudomonas* bacterial species.

The plate counts show that Gadsden County had the highest CFU of 1.97×10^6. Santa Rosa Central showed the lowest bacterial CFU of 3.00×10^4. The average CFU for all samples was 4.96×10^5. The CFU counts are an average of three replications for each soil sample. The average CFU for the soils falls within the range suggested for a soil with a healthy biological community.

Table 1. Iron Content and Plate Count CFU of the Soil Samples

County	Landfill Name	Iron Content (mg/g)	CFU
Bay	Steelfield	49.6	7.00×10^5
Calhoun	Calhoun County	84.1	6.20×10^5
Franklin	Franklin County	39.4	4.00×10^4
Gadsden	Quincy-Byrd	65.8	1.97×10^6
Gulf	Five Points	46.4	9.00×10^4
Holmes	Holmes County	91.2	4.20×10^5
Jackson	Springhill	34.0	3.10×10^5
Leon	US 27 South	43.8	9.20×10^5
Liberty	Liberty County	68.8	9.50×10^5
Okaloosa	Baker	119.9	1.20×10^5
Santa Rosa	Santa Rosa Central	83.2	3.00×10^4
Santa Rosa	Santa Rosa Holley	94.0	7.50×10^5
Wakulla	Lower Bridge	67.3	4.60×10^5
Walton	Walton County Central	90.0	1.40×10^5
Washington	Mudhill	84.3	3.20×10^5

4.3 Aerobic Species Characterization

The aerobic bacterial species were characterized based on morphology. Only a select representative soil samples were used in the identification using morphology. For the aerobic species, Okaloosa County and Liberty County were selected based on visual appearance of the soil. Okaloosa County (Figure 2) had a very dark red coloration indicating high iron content. Although the red coloration does not definitively indicate high iron content, it is usually indicative of a soil with a high iron and clay content. Liberty County was selected based on its dark brown and rich organic appearance.

Figure 2. Okaloosa County Soil Sample

From Okaloosa County, two aerobic strains of *Bacillus* were positively identified. The gram positive bacteria *Bacillus cereus* (Figure 3) and *Bacillus subtilis* (Figure 4) were identified from pure cultures. These two *Bacillus* bacteria are well documented soil bacteria and are common in the depth zone where the samples were taken. Also, a culture from Franklin County was identified as *Bacillus thuringiensis*.

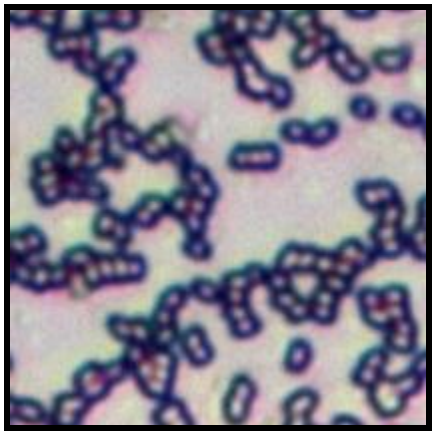

Figure 3. Okaloosa Co. Aerobic Sample
Bacillus cereus

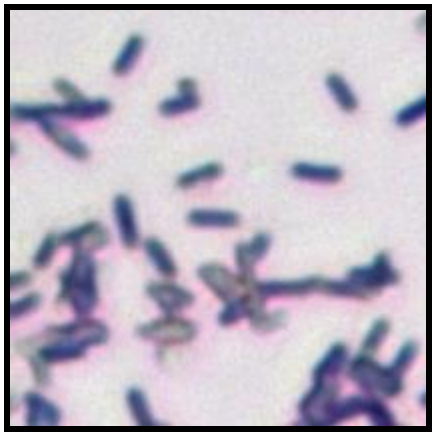

Figure 4. Okaloosa Co. Aerobic Sample
Bacillus subtilis

The remainder of the bacteria that were identified cannot be 100% accurately identified to genus and species name without DNA identification. Therefore, based on morphology, bacteria can be categorized in terms of class of species. The mixed culture bacteria were obtained from the growth chambers and placed on the slide and stained with Gram's stain. Two dominant bacteria observed in the Okaloosa sample are identified in Figures in 5 and 6. Figure 5 was a dominant species throughout the sample and had a head and tail morphology. Figure 6 showed a long rod-like bacteria that were also a dominant species within the Okaloosa samples.

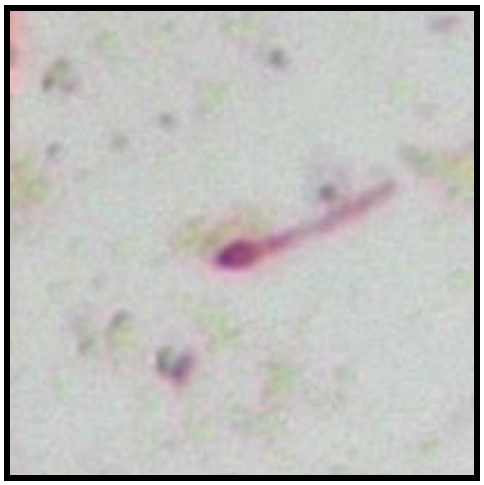

Figure 5. Okaloosa Co. Mixed Culture
Unknown *Pseudomonal*-like Bacteria

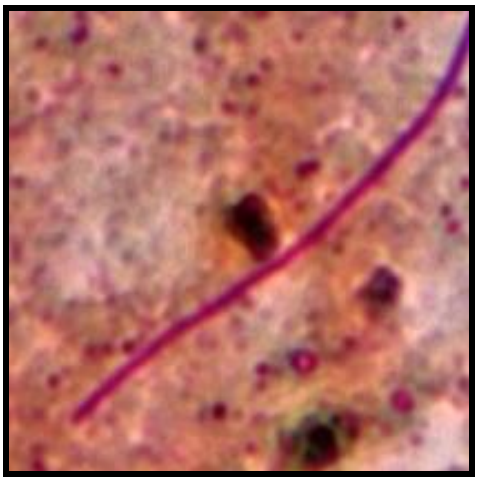

Figure 6. Okaloosa Co. Mixed Culture
Unknown *Bacilli* Bacteria

4.4 Anaerobic Species Characterization

The same methodology used for the aerobic identification was also used in the anaerobic identification. One pure culture was obtained for the anaerobic identification. From the Okaloosa sample, a strain of *Pseudomonas aeruginosa* (Figure 7) was identified through the PCR reaction.

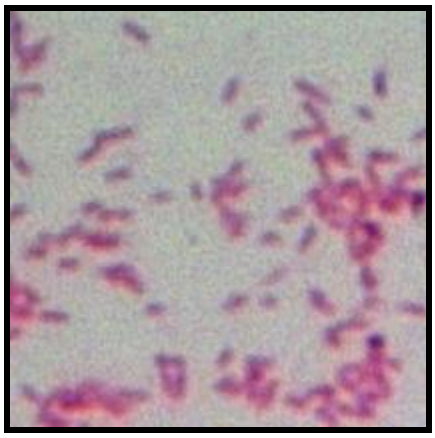

Figure 7. Okaloosa Co. Anaerobic
Pseudomonas aeruginosa

The remainder of the identification was obtained from other counties. In Figure 8 from Liberty County, a *Pseudomonal*-like bacteria similar to the one identified in the aerobic sample from Okaloosa County, was the dominant strain in that sample. From Walton County, a similar bacteria that had the head flagella morphology (Figure 9) was determined to be the dominant species in that sample. Gadsden County mixed culture samples (Figure 10) revealed a *bacilli*-like bacteria as the dominant species. For all samples, a total of three replications were made of the slides per sample and from those slides the dominant bacteria present was imaged. All images presented here followed this technique.

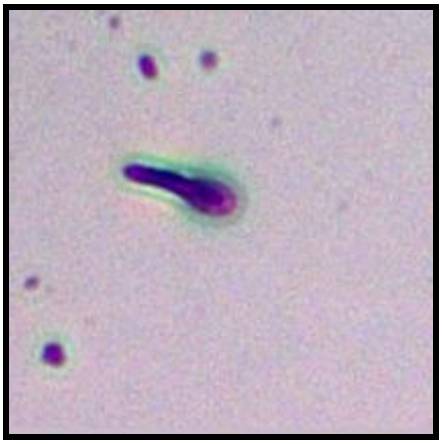

Figure 8. Liberty Co. Anaerobic Mixed Culture
Unknown *Pseudomonal*-like Bacteria

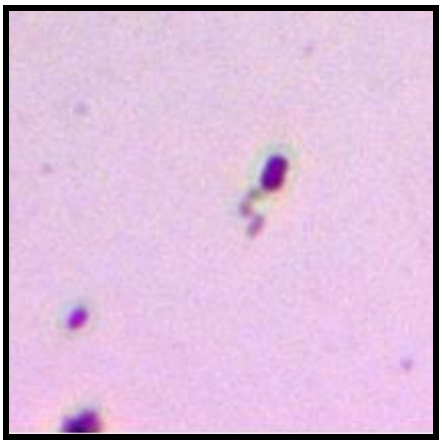

Figure 9. Walton Co. Anaerobic Mixed Culture
Unknown *Pseudomonal*-like Bacteria

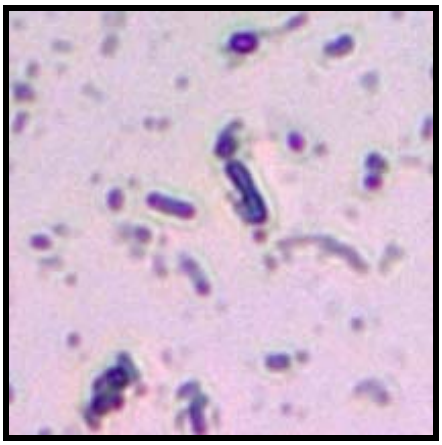

Figure 10. Gadsden Co. Anaerobic Mixed
Culture-Unknown *Bacilli* Bacteria

4.5 Polymerase Chain Reaction (PCR) Analysis

PCR analysis provided more accurate and reliable results. After going through the initial process as described in the Materials and Methods section, a 1% agarose gel was poured and the DNA samples were pipette into the wells within the gel. Voltage was then applied to the gel and allowed to run for approximately one hour and fifteen minutes. The resulting bands from the samples (Figure 11) were determined to be at the 1400 mark as indicated by the stepped band on the left side of the gel. This process was performed in Dr. Robert H. Reeves' laboratory in the Biology Department at Florida State University. Following the confirmation of the gel, the DNA samples were sent to the FSU DNA Sequencing Laboratory. The samples sent for identification were labeled and their subsequent identification are as follows:

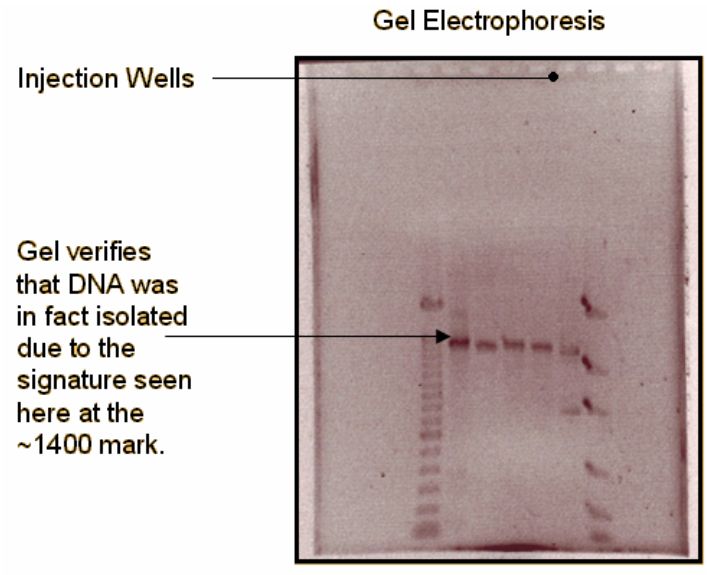

Figure 11. Gel Electrophoresis of DNA Samples

DNA Samples Analyzed by PCR	**Species Identified**
A. Okaloosa County Aerobic Sample	*Bacillus subtilis*
B. Okaloosa County Anaerobic Sample	*Pseudomonas aeruginosa*
C. Okaloosa County Aerobic Sample	*Bacillus cereus, Bacillus thuringiensis*
D. Franklin County Aerobic Sample	*Bacillus thuringiensis, Bacillus cereus*

Only four samples were chosen for the PCR analysis due to inability to culture a pure strain from other samples and also due to scheduling conflicts with the DNA Sequencing Laboratory. The DNA code obtained from the DNA laboratory were Blasted in the NCBI database (http://www.ncbi.nlm.nih.gov/) and the results present the top strain whose DNA code matches the samples code with the highest certainty. Samples C and D list the top strain match first, followed by the second closest match. The other samples results are a 100% match with the strain listed above.

5. Miscellaneous

We have set up a website (www.eng.fsu.edu/~gchen) for this project to facilitate the dissemination of our research discovery. We had the second TAG meeting on May 14, 2007. We presented part of our ongoing research at IRON AT LANDFIILS convened in Destin on October 18, 2006 and at the 83rd ACS Annual Florida Meeting and Exposition convened in Orlando on May 10, 2007. Currently, we have one paper published in Colloids and Surfaces A: Physicochemical and Engineering Aspects (doi:10.1016/j.colsurfa.2007.02.063). We acknowledged the work was support in part by Florida Center for Solid and Hazardous Waste Management. Detailed information of the project is available at www.eng.fsu.edu/~gchen.