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Characterization of Biofilm in 200W Fluidized Bed Reactors

September 2014

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U.S. DEPARTMENT OF ENERGY
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Abstract

Contaminated groundwater beneath the 200 West Area at the Hanford Site in Southeast Washington is currently being treated using a pump and treat system to remove organics, inorganics, radionuclides, and metals. A granular activated carbon-based fluidized bed reactor (FBR) has been added to remove nitrate, hexavalent chromium and carbon tetrachloride. Initial analytical results indicated the microorganisms effectively reduced many of the contaminants to less than cleanup levels. However shortly thereafter operational upsets of the FBR include carbon carry over, over production of microbial extracellular polymeric substance (biofilm) materials, and over production of hydrogen sulfide. As a result detailed investigations were undertaken to understand the functional diversity and activity of the microbial community present in the FBR over time. Molecular analyses including terminal restriction fragment length polymorphism analysis, quantitative polymerase chain reaction and fluorescent in situ hybridization analyses were performed on the microbial community extracted from the biofilm within the bed and from the inoculum, to determine functional dynamics of the FBR bed over time and following operational changes. Findings from these analyses indicated: 1) the microbial community within the bed was completely different than community used for inoculation, and was likely from the groundwater; 2) analyses early in the testing showed an FBR community dominated by a few *Curvibacter* and *Flavobacterium* species; 3) the final sample taken indicated that the microbial community in the FBR bed had become more diverse; and 4) qPCR analyses indicated that bacteria involved in nitrogen cycling, including denitrifiers and anaerobic ammonia oxidizing bacteria, were dominant in the bed. These results indicate that molecular tools can be powerful for determining functional diversity within FBR type reactors. Coupled with micronutrient, influent and effluent chemistry evaluations, a more complete understanding of the balance between system additions (nutrients, groundwater) and biology can be achieved, thus increasing long-term predictions of performance. These analyses uniquely provide information that can be used in optimizing the overall performance, efficiency, and stability of the system both in real time as well as over the long-term, as the system design is altered or improved and/or new streams are added.

Acknowledgments

The scientists and staff would like to thank CHPRC field personnel who collected and preserved the samples for these analyses. Funding for this work was provided by CH2M Hill Plateau Remediation Contract. The Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the Department of Energy (DOE) under Contract DE-AC05-76RL01830.

Acronyms and Abbreviations

aps	adenosine 5'-phosphosulfate reductase
CHPRC	CH2M Hill Plateau Remediation Company
DOE	U.S. Department of Energy
dsrA	dissimilatory sulfate reductase
EPS	extracellular polymeric substances
FBR	fluidized bed reactor
FISH	fluorescent in situ hybridization
H	hydrogen
H_2SO_4	sulfuric acid
ICP	inductively coupled plasma
mcrA	methyl coenzyme M reductase
mL	milliliter
Na	sodium
NaOH	sodium hydroxide
nir	nitrite reductase
NO_2^-	nitrite
O_2	oxygen
OES	optical emission spectroscopy
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNNL	Pacific Northwest National Laboratory
qPCR	quantitative polymerase chain reaction
RCF	relative centrifugal force
SO_4	sulfate
SRB	sulfate reducing bacteria
TKN	total Kjeldahl nitrogen
tRFLP	terminal restriction fragment length polymorphism
TVS	total volatile solids

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1.0 Purpose

1.1.1 General

This report details the microbial and chemical analysis of biomass (biofilm) that has accumulated on granular activated carbon (GAC) media in the 200 West Fluidized Bed Reactors (FBRs). The primary purpose of this work was to aid in CH2M Hill Plateau Remediation Company's (CHPRC's) efforts to characterize the FBR and its influent and effluent stream to complete a thorough system mass balance. Scientists at PNNL assisted in this effort through the characterization of the FBR microbial biofilm and evaluated sampled materials for nutrient content, biofilm structure, bacterial activity, and community functional types present in the biofilm. This information was provided to CHPRC and subsequently used to define various system inputs, determine/understand biofilm activity and function, biomass yield and minimize carry-over of the biofilm materials that caused issues downstream. In addition to helping to optimize the operational metrics for the FBR, the data gathered herein is of great importance to the microbial community at large as there are no other documented large volume FBR inoculated with GAC operating in the world (most FBRs are seeded with sand).

Evaluating and measuring the biofilm cultivated on the GAC in the FBR over depth and time is critical for several reasons, most importantly: (a) to identify if biomass accumulation on GAC is consistent with conversion of substrates in the influent groundwater stream and (b) to understand the individual activities AND the community structure that is most beneficial to the continued operation of the FBR as part of a treatment train for removing contaminants from groundwater.

1.1.2 Pump and Treat System

The Department of Energy's 200 West Pump and Treat system is designed to capture and treat contaminated groundwater to reduce the mass of carbon tetrachloride, trichloroethene, nitrate, chromium, technetium-99, and iodine-129. The system is designed as a treatment train for the diverse contaminants present in groundwater across the central plateau. Briefly, groundwater from wells containing radioactive contaminants is pumped through the ion exchange system, which removes technetium-99 and iodine-129 using a Purolite A530E resin, and then on to the equalization tank where it is blended with groundwater from wells without radioactive contaminants. The water passes through the recycle tank and up through the FBRs. The media in the fluidized bed biological reactors is granulated activated carbon; these materials were inoculated with a bioaugmentation culture acquired from a corporation which enriched the culture from a natural environment and specifically enriched for denitrifying microorganisms. MicroCg™ (i.e., organic carbon substrate used as the electron donor in biological denitrification), phosphoric acid, and a micronutrient solution are fed immediately upstream of the FBRs to facilitate biological growth. The treated FBR effluent is discharged into carbon separators before the groundwater flows into a splitter structure to divide the stream amongst four MBRs for removal of solids by membrane filtration, residual carbon substrate by aerobic biodegradation, and carbon tetrachloride, chloroform, and trichloroethene by air stripping. Vapor emissions are collected for treatment with granular activated carbon. The final treated water is then transferred to the injection wells.

2.0 Introduction

The following report details the analysis of biomass collected from the 200 West Pump and Treat FBR. The primary objective of the work is to characterize the biofilm communities present (and active) in the system as a function of time. Samples consisting of process water and biofilm covered granular activated carbon (GAC) were collected from within the FBR at various time points throughout the year. Biofilm on the GAC was extracted and analyzed for nutrient content, biofilm structure, and to determine the physiological types of bacteria present in the biofilm (e.g. denitrifiers, sulfate reducers, methanogens). The following tasks/analyses were completed:

- Extracellular polymeric substances (EPS) content
- Quantitative polymerase chain reaction (qPCR)
- Total volatile solids
- Total sulfides
- Total Kjeldahl nitrogen
- Extraction of biofilm from granular carbon
- Microtox
- Fluorescent in situ hybridization (FISH)

3.0 Methods

3.1 Sample Receipt

(CHPRC) collected samples from the FBR. Samples were shipped and received on ice; samples remained on ice and in the dark until processed. A total of nine samples have been collected by CHPRC and delivered to PNNL and are listed in Table 3.1. Copies of the chain-of-custody forms are shown in Appendix A.

Table 3.1. Summary of Received Samples

Sample ID	Date Received	Sampling Location
B2P0C6	March 14, 2013	289-T, FBR-A, Top of Bed 17.5 ft
B2P0C7	March 14, 2013	289-T, FBR-A, Middle of Bed 22 ft
B2P0C8	March 14, 2013	289-T, FBR-A, Bottom of Bed 27 ft
B2P0M9	April 2, 2013	289-T, FBR-A, Top of Bed 20 ft
B2P0N0	April 2, 2013	289-T, FBR-A, Middle of Bed 23 ft
B2P0N1	April 2, 2013	289-T, FBR-A, Bottom of Bed 27 ft
B2P100	April 16, 2013	289-T, FBR-A, Top of Bed 29 ft
B2P101	April 16, 2013	289-T, FBR-A, Middle of Bed 35 ft
B2P102	April 16, 2013	289-T, FBR-A, Bottom of Bed 37 ft
B2RKF4	September 10, 2013	289-T, FBR-A, Middle of Bed XX ft.
B2RKF5	September 10, 2013	289-T, FBR-B, Middle of Bed XX ft.

Samples received on March 14, 2013 are shown in Figure 3.1. Figure 3.2 shows the samples received on April 2, 2013. The samples representing the top and bottom (20’ and 27’) were archived. Only the sample representing the middle (23’) depth of the FBR was processed for analysis. A third set of three samples was received on April 16, 2013 and are shown in Figure 3.3. Again, only the sample representing the middle depth of the FBR (35’) was processed for analysis and the samples representing the top and bottom (29’ and 37’) were archived. Figure 3.4 shows samples taken from the middle of both FBR-A and FBR-B.

Figure 3.1. Photos of sample bottles received from CHPRC on March 14, 2013.

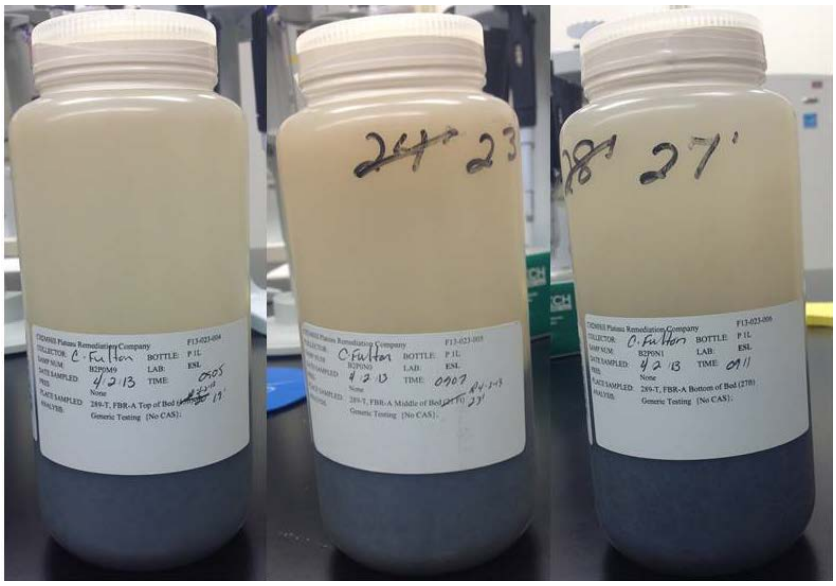

Figure 3.2. Photos of sample bottles received from CHPRC on April 2, 2013.

Figure 3.3. Photos of sample bottles received from CHPRC on April 2, 2013.

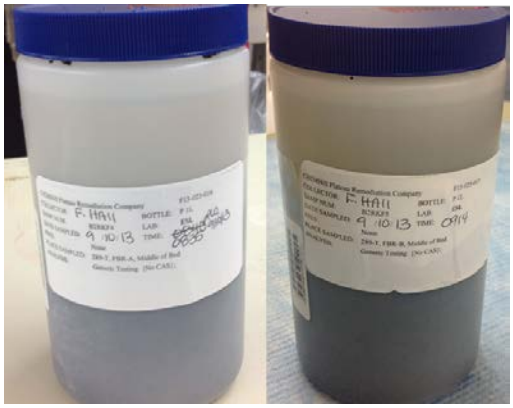

Figure 3.4. Photos of sample bottles received from CHPRC on September 10, 2013.

3.2 Analytical Methods

3.2.1 Sample Observation

Samples were shipped and received on ice; samples remained on ice and in the dark until processed. Initial sample processing included photographing the samples as soon as they were received, photographing both the solid and liquid materials, and recording general observations for each sample (Table 3.2).

Table 3.2. Summary Observation of Received Samples

Sample ID	Color of Liquid	Amount of Carbon (assume 1 ml = 1 g)	Ease of Pipetting	Other Comments
B2P0C6	Clear with high flocculated material	Less than half of container (~400g/L)	Low to moderate difficulty	Low biofilm content on carbon
B2P0C7	Clear with high flocculated material	Less than half of container (~300g/L)	Low to moderate difficulty	Moderate biofilm content on carbon
B2P0C8	Clear with high flocculated material	Half of container (~500g/L)	Low to moderate difficulty	Moderate biofilm content on carbon
B2P0M9	Clear with high flocculated material	~300-400 g/L	Moderate difficulty	High biofilm content on carbon
B2P0N0	Clear with high flocculated material	~300-400 g/L	Moderate difficulty	High biofilm content on carbon
B2P0N1	Clear with high flocculated material	~400 g/L	Moderate difficulty	High biofilm content on carbon
B2P100	Clear with high flocculated material	~400 g/L	Moderate to High difficulty	High biofilm content on carbon
B2P101	Clear with high flocculated material	~400-500 g/L	High Difficulty	High biofilm content on carbon, carbon is finer
B2P102	Black	Minimal, less than 20 g/L	Low difficulty	Large amount of fine carbon in solution, minimal solids
B2RKF4	Clear with moderate flocculated material	~500-600 g/L	Moderate difficulty	High biofilm content on carbon
B2RKF5	Clear with moderate flocculated material	~500-600 g/L	Moderate difficulty	High biofilm content on carbon

3.2.2 Sample Preparation and Stabilization

Samples from the FBR were prepared by adding 20 mL of FBR water, containing cellular material, to approximately 20 grams (assumed 1g = 1 mL) of solid carbon/biomass sample in sterile 50-mL falcon tubes. Sample volumes were measured using sterile serological pipettes. Five replicates were prepared for each sample, for a total of 100 g of solid material and 100 mL of overlying water processed for analysis. The samples were sonicated for 2 hours at the highest setting. The liquid was removed immediately from the solid and placed into new sterile 50-mL falcon tubes (approximately 25 mL). Once the supernatant was removed, 10 mL of phosphate buffered saline (PBS) was added to the solid material. The sample was then vortexed for 1 to 2 minutes to separate the biological materials from the solids.

After vortexing, the removal of solution from the solid using a pipette was repeated (this solution was combined with previous one). An additional 10 mL of PBS was added and the sample was vortexed for an additional 1 to 2 minutes followed by removal of the solution using a pipette. This solution was also combined with the previous one for an approximate volume of 45 mL in 5 separate falcon tubes. These samples were weighed, balanced and then centrifuged at 10,000 relative centrifugal force (RCF) for 10 minutes. An easily visible pellet was produced for all of the sample replicates. The supernatants were poured off into a collection bottle, leaving the pellet undisturbed. In order to wash the pellet of interfering materials such as organics and/or other chemical contaminants, the pellet was re-suspended in 10 mL of PBS, and centrifuged (details above), decanted, and re-suspended 2 more times. Following the second wash, each sample replicate (total of 5) was re-suspended in 15 mL of sterile 50% glycerol solution (50% glycerin, in nanopure water). These samples were then aliquotted into 1-mL fractions in sterile pre-labeled cyrovials and stored at -80 °C. This process generated approximately 75 sample tubes.

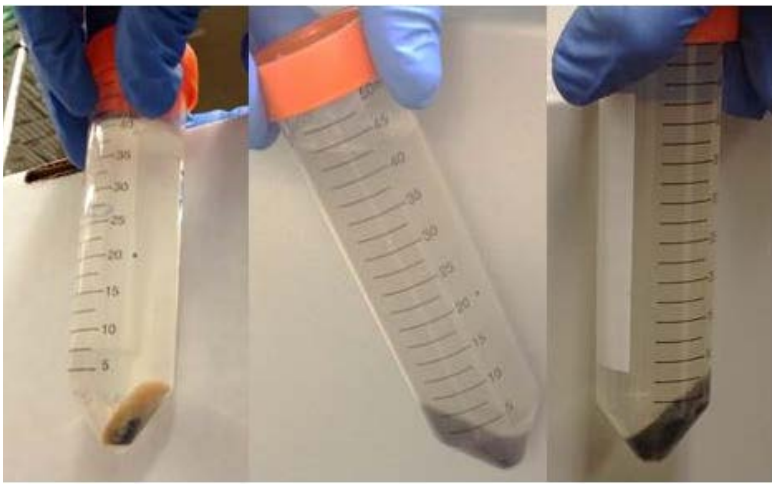

Figure 3.5. Typical pellets resulting from spinning samples (40-45 mL). From the first sampling, March 14, 2013 (left); from the second sampling, April 02, 2013 (middle); and from the third sampling, April 16, 2013 (right).

3.2.3 Analyses of EPS

Biofilms are primarily composed of microorganisms and a conglomerate of polysaccharides, proteins, lipids and nucleic acids called extracellular polymeric substances (EPS) (Fleming and Wingender 2010). EPS possess many of the functions including anchoring of bacterial cells, retention of water, sorption of organic and inorganic compounds, and ion exchange. Chemical analysis of EPS provides the necessary information to determine the overall physiological condition of the biofilm (activity, function, etc.).

To date there is no standardized methodology for extracting EPS. One of the more common methods is the use of cation exchange resins (Jahn and Nielsen, 1995; Frolund et al., 1996), followed by centrifugation and filtering prior to analysis the constituents of the EPS. Additional methods include treatment with formaldehyde, followed by centrifugation and filtering prior to analysis or no treatment with only filtration prior to analysis. Different extraction methods can greatly impact the amount and composition of EPS. Chemical methods over physical methods often result in higher extraction efficiency of the EPS components; however, some methods can contaminant downgradient processing. For example, Comte et al, 2007 found that heat induced cell hydrolysis, and Jahn and Nielsen (1995) found that Na

resins bind to EPS, potentially increasing osmotic pressure. Thus, a comparison of methods was completed in order to best provide a broad range of data values for different conditions. Both of these methods were shown to decrease the total amount of EPS. Figure 3.6 outlines the procedures that were used to process the frozen biofilm samples.

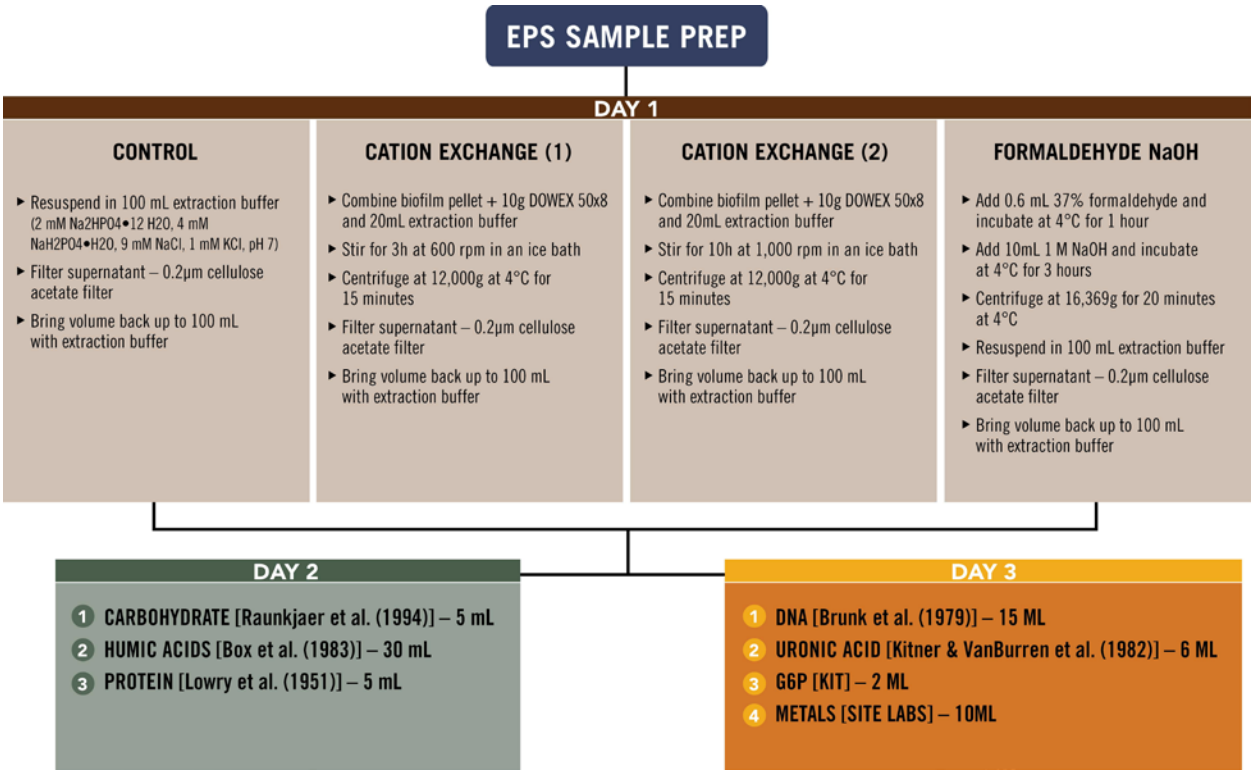

Figure 3.6. Summary of procedures to process samples for EPS measurement.

The amount of starting material (carbon covered with biomass) for each sample must be accounted for in the analysis. For the first six samples received, 100 g of solid material had been prepared. This was ultimately divided into three samples representing 33.33 g of solid starting material each. Samples B2P100, B2P101 and B2P102, had 80 g of solid material divided into three samples representing 26.67 g of solid starting material.

Following extraction, the composition of EPS was analyzed using standard methodologies as follows: DNA (Brunk et al., 1979), carbohydrates (modified from the Manual of Methods for General Bacteriology 1981), glucose-6-phosphate (Sigma-Aldrich assay kit) and total organic carbon (TOC; ASTM E1915-01), humic acids with the Folin-Ciocalteau Phenol reagent (Box,1983), protein (Lowry, 1951), and uronic acid was determined by the m-hydroxydiphenyl sulphuric acid method described by Kintner and van Burren (1982). Inductively coupled plasma optical emission spectrometry (ICP- OES) was used for cation analyses following PNNL-AGG-ICP-AES, a procedure similar to EPA Method 6010B. Detailed procedures are provided in Appendix B.

3.2.4 Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR was completed on the samples in order to quantify specific target genes present in the FBR. Functionally relevant, primarily functional genes were targeted, included nitrate reducing, sulfate and iron reducing microbes and methanogens. In addition, qPCR primers for 16S rRNA were targeted to quantify total Eubacteria and Archaea. Following processing, stabilization and storage of samples, DNA was extracted and qPCR reactions were completed with DNA from positive control organisms. Table 3.3 provides a consensus list of the organisms and functional genes that were targeted using qPCR. Total Bacteria and Archaea were evaluated using non-quantitative PCR while the remaining targets were evaluated using qPCR analyses.

Table 3.3. List of the Organisms to be Targeted Using qPCR

Target	Primers	Details/Notes
Total Bacteria	341 F, 907R	Total Bacteria. *PCR to be completed; not qPCR.*
Total Archaea	4F, 915R	Total Archaea. *PCR to be completed; not qPCR.*
Sulfate-Reducing Bacteria	dsrA apsA	The dsrAB genes, which encode the dissimilatory sulfate reductase, the key enzyme in dissimilatory sulfate reduction, can be used as a phylogenetic marker for identification of SRBs. These genes can be found in all known SRB prokaryotes. Microbes possess adenosine-50-phosphosulfate (APS) reductase. The genes for APS reductase, apsBA from Bacteria and aprBA from Archaea.
Methanogenic Bacteria	mcrA *Methanosarcina*	During the final step of methanogenesis, a methyl moiety attached to coenzyme M is reduced to methane. This reaction is catalyzed by methyl coenzyme M reductase (mcrA), an enzyme that appears to be conserved in all methanogens. Targets Methanosarcina and other commonly found methanogen targets.
Iron-Reducing Bacteria	Geo564, 840R	*Geobacter* type organisms
Denitrifying Bacteria	nir K nir S	Respiratory nitrate reduction, as well as the dissimilatory reduction of nitrate to ammonia, is catalyzed by a nitrate reductase encoded by the narGHJI operon. The reduction of nitrite (NO^{2-}) to nitric oxide distinguishes denitrifiers from other nitrate-respiring bacteria. This reaction is catalyzed by two different types of nitrite reductases (Nir), either a cytochrome cd1 encoded by nirS or a Cu-containing enzyme encoded by nirK. The reduction of nitrous oxide is the last step in the denitrification pathway and is catalyzed by nitrous oxide reductase encoded by the nosZ gene.
Anaerobic Ammonia-Oxidizing Bacteria	anammox	Anaerobic ammonium oxidation process, in which ammonium and nitrite are directly oxidized to nitrogen gas under anoxic conditions.

3.2.5 Total Volatile Solids

Total volatile solids were measured using EPA Method 160.4 *"Residue, Volatile (Gravimetric, Ignition at 550°C)"*.

3.2.5.1 Sample Preparation and Stabilization

The total volatile solids (TVS) extractions were prepared in a manner similar to those described in the Sample Preparation and Stabilization section. After washing of the pellets, the sample was re-suspended in 45 mL of sterile nanopure water and the 4 replicates for each sample were combined to give a total sample volume of approximately 180 mL. Once these samples were combined, additional sterile nanopure water was added to the sample to bring the total volume up to 1300 mL. The 1300 mL sample was then divided into two 500-mL aliquots and one 300-mL aliquot. The 300-mL aliquot was adjusted to a minimum pH of 9 with the addition of 5M sodium hydroxide (NaOH). The sample was stored at 4°C until analyzed.

TVS analysis for samples B2RKF4 and B2RKF5 was performed using the pellet generated from 100 g of carbon. Rather than resuspension in nanopure water, the sample was resuspended in a small amount of water and then processed using EPA Method 160.4 as a guide.

3.2.6 Total Sulfides

Total sulfides were measured using EPA Method 376.2 *"Sulfide (Colorimetric, Methylene Blue)"*. Analysis of total sulfides was not performed for samples B2RKF4 and B2RKF5.

3.2.6.1 Sample Preparation and Stabilization

The total sulfides (TS) extractions were prepared as described in the sample preparation and stabilization section for total volatile solids (Section 3.2.5.1). One of the 500-mL aliquots was placed in stored at 4°C until analyzed. No preservation was required.

3.2.7 Total Kjeldahl Nitrogen

Total Kjeldahl nitrogen were measured using EPA Method 351.2 "*Determination of Total Kjeldahl Nitrogen by Semi-Automated Colorimetry*". Total Kjeldahl nitrogen analysis was not performed for samples B2RKF4 and B2RKF5.

3.2.7.1 Sample Preparation and Stabilization

The total Kjeldahl nitrogen (TKN) extractions were prepared as described in the sample preparation and stabilization section for total volatile solids (Section 3.2.5.1). The pH of the -500-mL aliquot samples was adjusted to less than 2 with the addition of 0.01M sulfuric acid (H_2SO_4). The sample was stored at 4°C until analyzed.

3.2.8 Extraction of Biofilm from Granular Carbon

In general, the biofilm community was gently separated from the granular carbon through sonication and vortexing. After subsequent washes of the carbon material, the biomass were spun down into a pellet and provided back to CHPRC. If the supernatant is required or needed, it will also be provided to CHPRC.

3.2.8.1 Sample Preparation and Stabilization

The biomass extractions were prepared by combining approximately 25 grams (assumed 1g = 1mL) of solid carbon/biomass sample in sterile 50-mL falcon tubes with 15 mL of FBR water. Samples were measured using serological pipettes. Four replicates were prepared for each sample, for a total of 100 g of solid material and 60 mL of overlying water processed for analysis. The samples were sonicated for 2 hours at the highest setting. The liquid was removed immediately from the solid and placed into new sterile 50-mL falcon tubes (approximately 20 mL). Once the supernatant was removed, 10 mL of PBS was added to the solid material. The sample was then vortexed for 1 to 2 minutes to separate the biological materials from the solids. After vortexing, the removal of solution from the solid using a pipette was repeated (this solution was combined with previous one). An additional 10 mL of PBS was added to the solid material. The sample was vortexed for an additional 1 to 2 minutes followed by removal of the solution using a pipette. This solution was also combined with the previous one for an approximate volume of 40 mL in 4 separate falcon tubes. The solution from all 4 replicates was combined into one sample and stored at 4°C until returned to CHPRC.

3.2.9 Microtox

Microtox is a standardized toxicity test system which employs the bioluminescent marine bacterium (*Vibrio fischeri*) as the test organism. The toxicity of the samples was determined with a ModernWater DeltaTox II analyzer using the "81.9% B-Tox Test Procedure for Low Toxicity Samples" procedure. The test exposes Vibrio fischeri to samples, and then the increase or decrease in light output by the organisms is measured. A difference in light output (between the sample and the control) is attributed to the effect of the sample on the organisms and can be related to toxicity. The percent of light lost is equal to the percent of inhibition or the toxicity of the sample. The percent of light gained is equal to the percent of stimulation and bioreactivity. For this procedure, duplicate sample tubes for the control and each sample were prepared. One millimeter of control solution or sample was placed in one of the duplicate tubes and the luminescent organisms were placed in the remaining tubes. After measuring the amount of light being emitted by the organisms in each tube, the sample and organisms were mixed. After standing at room temperature for five minutes, the amount of light being emitted was measured again.

3.2.9.1 Sample Preparation and Stabilization

Samples for Microtox analysis were prepared by adding 150 mL of FBR water to approximately 100 grams of solid carbon/biomass sample in a 250-mL sterile bottle. Each bottle was closed tightly and sealed with parafilm. The samples were then stored in pre-labeled Whirlpak bags and stored at 4° C until further processed and analyzed.

3.2.10 Fluorescent in situ Hybridization (FISH)

Fluorescent in situ hybridization is a staining technique which allows specific groups of organisms to be targeted using small oligonucleotide sequences tagged with a fluorescent probe which can be visualized using an epifluorescent microscope. FISH is a powerful technique which can be used to determine relationships present in the FBR and more importantly determine the activity of specific functional groups present including denitrifiers, methanogens, and sulfate and iron reducers.

FISH analysis was conducted on biomass generated from the Sample Preparation and Stabilization process described above. The samples were re-suspended in 10 mL of PBS, 30 mL of 4% paraformaldehyde and incubated for 1 hour at room temperature. Following the incubation, the samples were centrifuged at 10,000 RCF for 10 minutes. The supernatants were poured off into a paraformaldehyde collection bottle. The pellets were washed two additional times using PBS: the pellet was re-suspended in 10 mL of PBS and centrifuged (details above), decanted, and re-suspended again. Following the second wash, the pellets were re-suspended in 10 mL of a solution composed of 50% ethyl alcohol (EtOH) and 50% PBS. One mL fractions of the sample were pipetted into pre-labeled cryovials and the samples stored at -20 °C. This process generated approximately 50 samples to be analyzed for FISH.

3.2.10.1 Targets for FISH

Table 3.4 provides a consensus list of the targets for FISH. Each group of organisms were targeted with more than one oligonucleotide probe; however, each of the probes for any given group will have the same fluorescent dye attached. For example, four methanogen oligonucleotide probes were synthesized and applied to the sample, but all of them will have the Cy3 probe attached. When the image is captured, all of the probes that have hybridized to the sample were 'colored' a yellow-green color, representing the targeted group, methanogens. The advantage of this approach is that multiple targets provide significantly more data without the added costs.

Table 3.4. List of the Targets for FISH Analysis

Target	Name	Probe	Details/Notes
Bacteria	Eub 338	Cy3	Total Bacteria
Archaea	Arch 915	Fluorescein	Total Archaea
SO4	SRB385Db	Cy5	Many but not all deltaproteobacterial SRPs, *Aerothermobacter* spp.,*Thermomonospora* spp., *Actinobispora* spp., *Actinomadura* spp., *Thermoanaerobacte*r spp., *Frankia* spp., *Clostridium* spp., *Streptosporangium* spp., *Nitrospira* spp., *Geodermatophilus* spp., *Nocardiopsis* spp., and many more
SO4/FE	SRB385	Cy5	Many but not all deltaproteobacterialSRPs, *Geobacter* spp., *Pelobacter* spp., *Campylobacter* spp., *Saccharopolyspora* spp., *Acetivibrio* spp., *Syntrophus* spp., *Clostridium* spp., *Nitrospina* spp., *Chlorobium* spp., and many more
FE	SRB804	Cy3	*Desulfobacter*, *Desulfobacterium*, *Desulfococcus*, *Desulfosarcina*, and *Desulfobotulus* spp.
FE	SRB687	Cy3	*Desulfovibrio* spp. plus members of the genera *Geobacter*, *Desulfuromonas*, *Desulfomicrobium*, *Bilophila*, and *Pelobacter*
CH4	MC1109	Cy3	*Methanococcales*
CH4	MB1174	Cy3	*Methanobacteriaceae*
CH4	MG1200	Cy3	*Methanomicrobiaceae*
CH4	MSMX860	Cy3	*Methanosarcinaceae* +
N	Curvi997	Cy3	*Curvibacter* species
N	ACl208	Cy3	*Acidovorax* species
N	CF319aS	Cy3	*Cytophaga-Flavobacter-Bacteroides* species

3.2.11 Additional Analyses

Additional analyses were supported by DOE Richland Operations Office, to look at the dominant organisms present in the inoculant in the summer and fall of 2012. These techniques and approaches were validated in the laboratory during this time. Because the techniques, supplies, expertise were available, the samples collected for the CHPRC contract, were also analyzed using these approaches.

3.2.11.1 DNA Extractions

DNA extractions were performed using both FastDNA soil kit (formerly Bio 101) and the MoBio UltraClean Soil DNA kit, as described by the manufacturers. Two kits were used to ensure that biases associated with one kit or another did not provide a false positive or negative result for the presence of the genes or organisms of interest. The concentration of DNA was measured using a Thermo Scientific Nanodrop 8000 spectrophotometer. Quality and quantity of DNA was recorded for each sample and for negative control samples (water). The samples consistently yielded high-quality bacterial DNA, based on Bioanalyzer Labchip analysis.

3.2.11.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) amplification reactions were performed in 50 µL (total volume) reaction mixtures in 0.2-mL thin-walled tubes. The PCR experiments were performed using the *Taq* PCR Core kit from Qiagen. The PCR conditions for the 16S rRNA are as described previously (Amann et al., 1995; 1996). The primers used for Bacteria were 8F and 907R and for Archaea, 4F and 915R. Briefly, each PCR was performed in triplicate and consisted of 38.75 µL of molecular water, 5 µL of 10x buffer, 1 µL dNTP, 0.25 µL *Taq* polymerase, 1 µL BSA, 1 µL each of the forward and reverse primer, and 2 µL of DNA template. All molecular work and PCR reactions were performed in a biological safety/laminar flow hood. The PCR cycle used was 5 minutes 95°C, [1 minute 94°C, 1 minute 55°C, 2 minutes 72°C] repeated 30 cycles, and 10 min 72°C. Samples were then held at 4°C or stored at -20°C.

Following PCR, the amplified products were separated and visualized using an Agilent 2100 Bioanalyzer and DNA 1000 LabChip. Each LabChip has 16 wells, twelve of which are used for experimental samples, one well is used for a molecular weight DNA ladder, and three are used for loading the gel-dye mix. All experiments were performed using Agilent Biosizing software.

3.2.11.3 Terminal Restriction Fragment Length Polymorphism (tRFLP)

Restriction enzyme digests were performed in triplicate using the cleaned PCR product. Enzymes used were MspI for reactions amplified with the 8F FAM primer, and RsaI for reactions amplified with the 4F FAM primer. Each reaction consisted of 1 µL of enzyme, 1.5 µL of the buffer accompanying the enzyme, the volume of PCR product needed to add 350 ng of DNA based on the measured concentration, and enough molecular water to bring the final reaction volume to 15 µL. Reactions were incubated at 37 °C for 6 hours followed by 65 °C for 10 minutes, after the reaction was complete samples were stored at 4 °C. Samples were sent to the ISU Molecular Research Core Facility for fragment analysis.

tRFLP Data Analysis

Since the samples were cleaned prior to fragment analysis, no fragment lengths were excluded from the analysis. Fragments with less than relative fluorescence value of 50 (recorded as peak height) were discarded. Each tRFLP sample was provided as a true triplicate; the replicates for each sample were then aligned (fragment lengths) based on band size. Bands were considered equal if they differed by < 0.75bp. If a given fragment was not present in all three samples, it was discarded. The consensus fragments lengths and peak heights (relative abundance) were averaged to produce a single profile for each sample Species richness was determined and statistics evaluating the overall diversity and evenness were also determined.

3.2.11.4 Clone Libraries

PCR fragments were cloned into competent cells, using the TOPO TA cloning kit, as described by the manufacturer. Briefly, PCR products were mixed with TOPO vectors and incubated with One Shot *E.coli* cells. Cell dilutions were plated on antibiotic resistant plates and within 8-10 hours, individual colonies, or clones, were visible on the surface of the plate. Each colony was streaked onto a secondary selection plate. Colonies which show rapid growth were inoculated into a small volume of liquid media (~5 mL). Turbid cultures were extracted using Invitrogen's PureLink Quick Plasmid Miniprep Kit, according to the manufacturer's instructions. The plasmid DNA was eluted in TE Buffer and stored in the -20 freezer until shipped out for analysis at ISU Molecular Research Core Facility in Pocatello, ID.

Samples were analyzed through either the Applied Biosystems 3130XL Capillary DNA Sequencer or the Ion Personal Genome Machine. Within one week, sequences were uploaded to a secure server and then downloaded by PNNL staff (minimum of one forward and one reverse for each sample). The sequences were aligned using the freeware program, BioEdit. The contig is then matched, or BLASTed against NCBIs nucleotide database (http://www.ncbi.nlm.nih.gov/). The highest 'hits' or sequence matches were recorded. Identifications greater than 95% are considered significant; those than fall below 95% are re-BLASTed against the unknown/environmental sample database on the NCBI website. In most cases, these sequences are recorded as the closest match.

4.0 Results

4.1 Total Volatile Solids, Total Sulfides, and Total Kjeldahl Nitrogen

Table 4.1 presents the analytical results for total volatile solids, total sulfides, and total Kjeldahl nitrogen for the first five samples, for which these analyses were obtained through ALS Laboratories. All measurements were conducted in triplicate. Complete reports from ALS can be found in Appendix C. Analysis of the results of the samples taken on March 14, 2013 indicates the concentration of total volatile solids and total Kjeldahl nitrogen were higher in the samples collected within the bottom of the bed (27'); however, there was no statistical difference in the concentration of total sulfides within the bed. The results from B2P0N0, taken on April 2, 2013, show lower concentrations for all three analytical measurements when compared to the initial sample set. Comparison of the results from B2P101, taken on April 16, 2013, to the initial sample, B2P0C7, show higher concentration for total volatile solids and total Kjeldahl nitrogen and lower concentration for total sulfides. The results from B2P101 show higher concentrations for all three analyses when compared to sample B2P0N0. See Table 4.2 for the percent differences.

Total volatile solids results for samples B24KF4 and B2RKF5 were 0.295 g and 0.319 g, respectively. Only one replicate from each sample was used to determine total volatile solids. Total sulfide and total Kjeldahl nitrogen were not determined for these samples.

Figure 4.1 shows differences in the amount of total volatile solids as a function of the mass of granular activated carbon from which the solids were extracted. With the exception of the sample taken on April 16, 2013, the total volatile solids were between 3 and 5 mg/g carbon. These results show that the amount of total volatile solids, more than doubled between April 2, 2013 and April 16, 2013. When samples were taken in September, total volatile solids dropped to a level near 3 mg/g carbon. In general, total volatile solids are thought to be equivalent to the biological component in the system.

Table 4.1. Analytical Results from ALS Laboratories

Sample ID	Analysis Repetition	Total Volatile Solids (mg/L)	Total Sulfides (mg/L)	Total Kjeldahl Nitrogen (mg/L)
B2P0C6	1	320	6.6	50
	2	290	10	47
	3	360	8.7	48
	Average	323	8.4	48
	Std. Dev.	35	1.7	1.5
B2P0C7	1	340	8.7	47
	2	390	8.0	50
	3	340	11	43
	Average	357	9.2	47
	Std. Dev.	29	1.6	3.5
B2P0C8	1	400	7.1	56
	2	410	6.9	53
	3	380	8.7	56
	Average	397	7.6	55
	Std. Dev.	15	1.0	1.7
B2P0N0	1	270	4.8	27
	2	270	4.8	35
	3	280	1.2	31
	Average	273	3.6	31
	Std. Dev.	6	2.1	4.0
B2P101	1	610	9.3	59.0
	2	640	7.5	51.0
	3	610	8.4	56.0
	Average	620	8.4	55
	Std. Dev.	17	0.9	4.0
MDL	-	n/a	n/a	0.093
RL	-	50	1	5
Dilution Factor	-	1	1	10

n/a = not available
MDL = Method detection limit
RL = Reporting limit

Table 4.2. Percent Difference in Measured Average Values Compared Between Samples

Sample ID	Total Volatile Solids (mg/L)	Total Sulfides (mg/L)	Total Kjeldahl Nitrogen (mg/L)
Percent difference compared to sample B2P0C7			
B2P0N0	23%	61%	34%
B2P101	74%	9%	19%
Percent difference compared to sample B2P0N0			
B2P101	127%	133%	78%

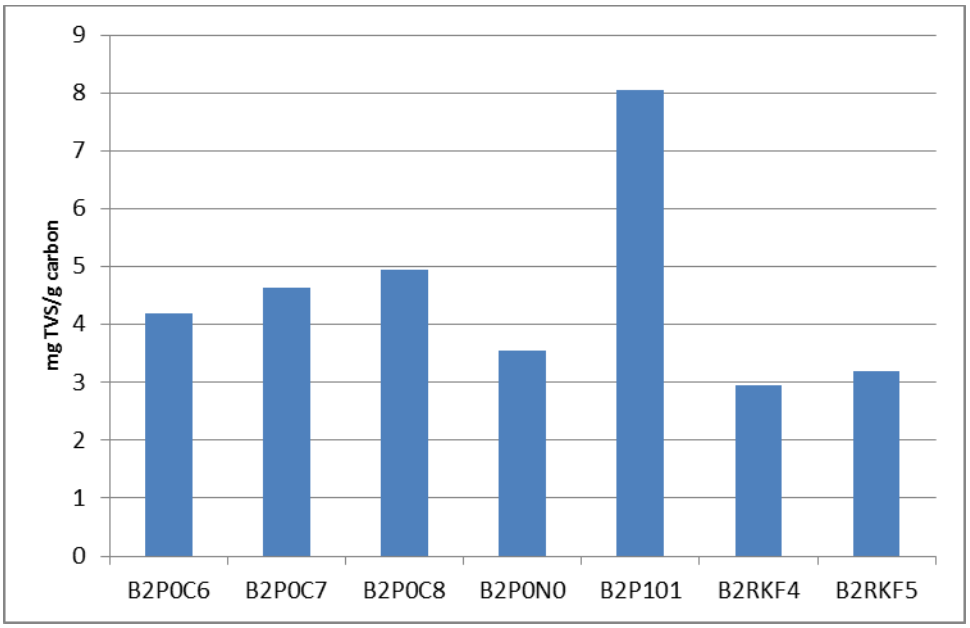

Figure 4.1. Comparison of the mass of total volatile solids (TVS) to estimated mass of carbon processed for analysis. TVS is equivalent to the mass of organic material, which likely represents biofilm components, including bacteria cells and EPS.

4.2 Extracellular Polymeric Substances (EPS) Content

EPS samples were processed in two batches with the suite of analyses for each batch completed within one week. The first set included samples B2P0C6, B2P0C7 and B2P0C8 received March 14, 2013. The second set of samples included samples B2P0N0 and B2P101 received on April 2, 2013 and April 16, 2013, respectively. New standard calibration curves were generated for each batch of samples analyzed.

4.2.1 Carbohydrate Analysis

Figures showing the carbohydrate standards used for each analysis and the calculated carbohydrate concentrations extracted from the samples are located in Appendix C. Figure C.1, Figure C.2 and Figure C.3 show the calibration curves generated from the carbohydrate standards and the calculated carbohydrate concentrations extracted from samples taken on March 14, 2013 (B2P0C6, B2P0C7 and B2P0C8), respectively. A summary of the carbohydrate results is given in Table 4.3. Figure 4.2 shows the amount of carbohydrate per gram of activated carbon from which it was extracted. Data in Table 4.3 and Figure 4.2 show that in general, the formaldehyde treatment appears to have extracted more carbohydrate than treatment with the cation exchange resin.

Table 4.3. Summary of Carbohydrate Analysis Results

		Duplicate 1 Concentration (mg/L)		Duplicate 2 Concentration (mg/L)		Average Concentration (mg/L)	Std. Dev. (mg/L)
		1	2	1	2		
B2P0C6	Control	8	—	10	—	9	1.5
	Cation Exchange	21	7	3	14	11	8.1
	Formaldehyde	112	—	125	—	119	8.8
B2P0C7	Control	8	—	17	—	12	6.5
	Cation Exchange	24	—	16	—	20	5.7
	Formaldehyde	226	266	155	176	206	49.9
B2P0C8	Control	26	—	12	—	19	10.1
	Cation Exchange	8	—	5	—	6	2.1
	Formaldehyde	124	—	82	—	103	29.0
B2P0N0	Control	0	—	10	—	5	6.9
	Cation Exchange	7	19	0	0	7	9.0
	Formaldehyde	256	—	256	—	256	0.0
B2P101*	Control	2	—	0	—	1	0.9
	Cation Exchange	102	51	41	43	59	28.8
	Formaldehyde	467	477	414	411	442	34.6
B2RKF4	Control	7.11		6.67		6.89	0.31
	Cation Exchange	8.67		8.44		8.56	0.16
	Formaldehyde	12.22		11.11		11.67	0.79
B2RKF5	Control	7.56		4.89		6.22	1.89
	Cation Exchange	4.44		5.78		5.11	0.94
	Formaldehyde	11.33		14		12.67	1.89

Sample B2P101 had 80 g of starting material. All other samples had 100 g of starting material.

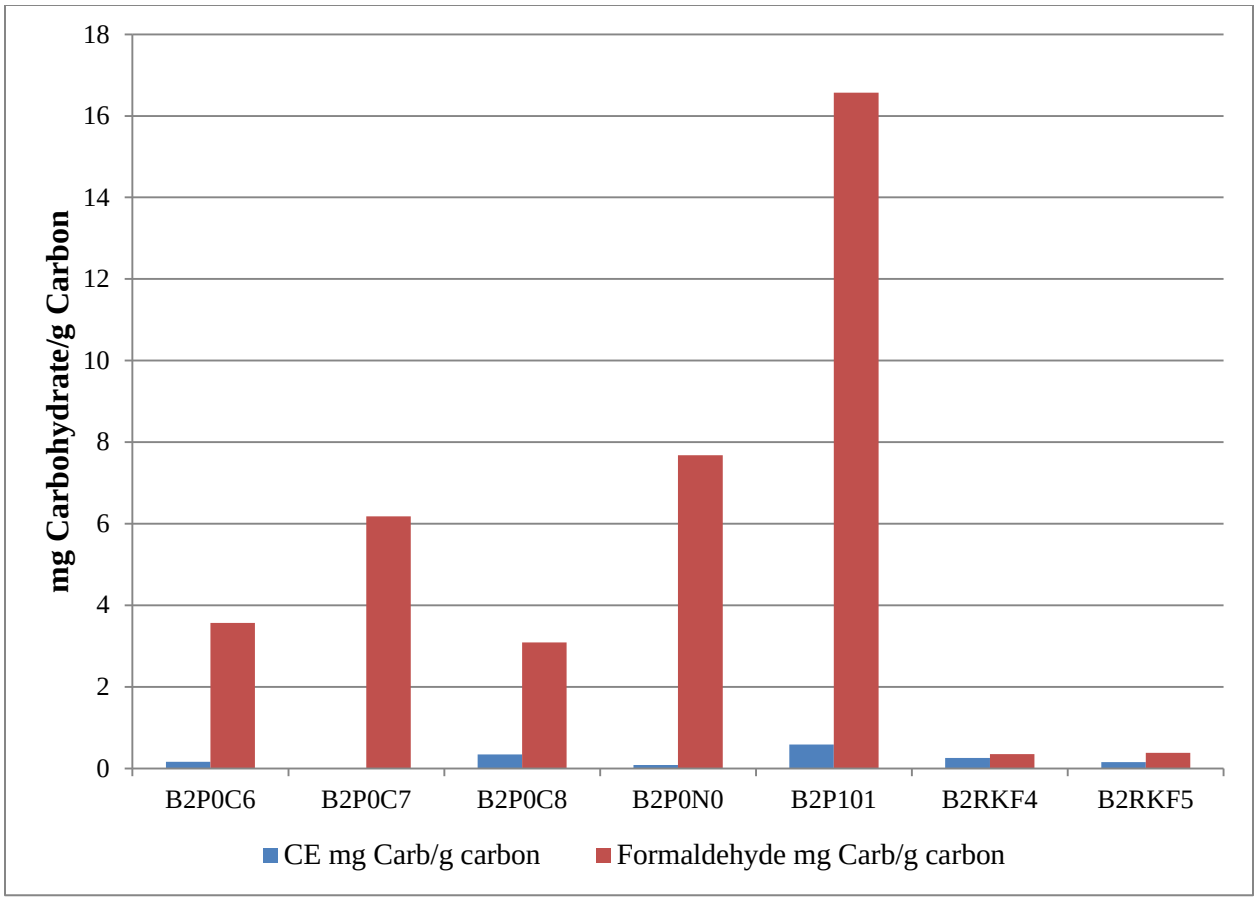

Figure 4.2. Comparison of the mass of carbohydrate per gram of carbon from which the EPS was extracted.

4.2.2 Humic Acid Analysis

Figure C.3, Figure C.4 and Figure C.5 show the calibration curves generated from the humic acid standards and the calculated humic acid concentrations from the FBR samples that were processed on April 2, 2013 (B2P101) and April 16, 2013 (B2P0N0). A summary of the results is given in Table 4.4. Figure 4.3 shows the amount of humic acid per gram of activated carbon from which it was extracted. In general, more humic material was extracted by the cation exchange method than the formaldehyde extraction.

Table 4.4. Summary of Humic Acid Analysis Results

		Concentration Measurement 1 (mg/L)	Concentration Measurement 2 (mg/L)	Average Concentration (mg/L)	Std. Dev. (mg/L)
B2P0C6	Control	76	74	75	1.8
	Cation Exchange	42	41	41	0.8
	Formaldehyde	34	36	35	1.0
B2P0C7	Control	104	105	104	0.8
	Cation Exchange	117	123	120	3.9
	Formaldehyde	59	59	59	0.5
B2P0C8	Control	92	96	94	2.9
	Cation Exchange	54	54	54	0.3
	Formaldehyde	31	33	32	1.3
B2P0N0	Control	141	160	147	9.0
	Cation Exchange	95	103	99	4.3
	Formaldehyde	64	69	66	2.5
B2P101*	Control	93	92	92	0.8
	Cation Exchange	158	163	160	3.3
	Formaldehyde	93	97	95	2.8
B2RKF4	Control	43	41	42	1.2
	Cation Exchange	41	48	48	0.2
	Formaldehyde	19	19	19	0.4
B2RKF5	Control	17	19	18	1.2
	Cation Exchange	17	17	17	0.2
	Formaldehyde	14	12	13	1.0

*Sample B2P101 had 80 g of starting material. All other samples had 100 g of starting material.

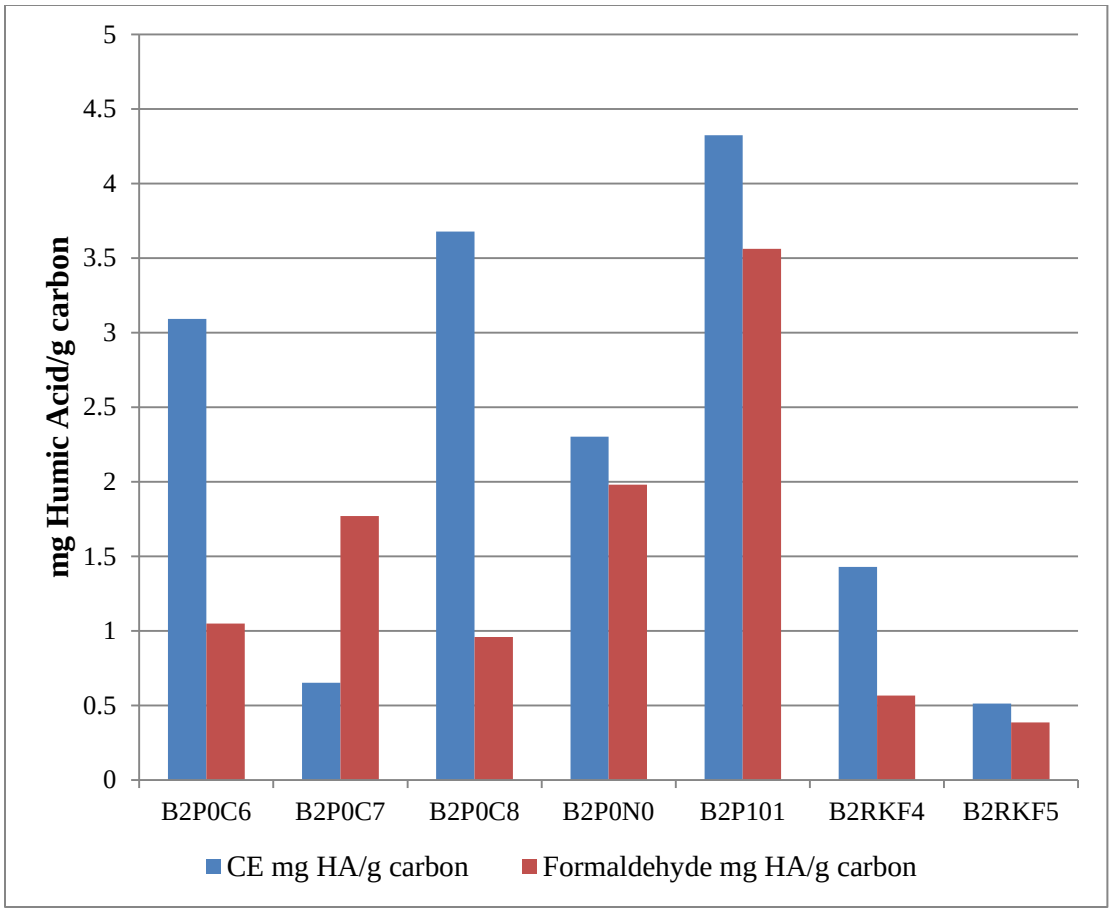

Figure 4.3. Comparison of the mass of humic acid per gram of carbon from which the EPS was extracted.

4.2.3 Protein Analysis

Figure C.7, Figure C.8 and Figure C.9 show the calibration curves generated from the protein standards and the calculated protein concentrations from samples processed on 9/10/2013. A summary of the results is given in Table 4.5. A comparison of the mass of protein per gram of carbon for all of the samples is shown in Figure 4.4. The ratio of carbohydrate to protein is shown in Table 4.6, these ratios indicate that there was not substantial lysis of cells from the extraction releasing additional protein into the mixture, during extraction using the cation exchange method. Carbohydrate to protein ratios were higher for formaldehyde extractions performed in May and April, indicating that there may have been cell lysis increasing protein in fraction analyzed.

Table 4.5. Summary of Protein Analysis Results

		Concentration Measurement 1 (mg/L)	Concentration Measurement 2 (mg/L)	Average Concentration (mg/L)	Std. Dev. (mg/L)
B2P0C6	Control	55	57	56	1.0
	Cation Exchange	36	36	36	0.3
	Formaldehyde	34	32	33	1.3
B2P0C7	Control	77	79	78	1.6
	Cation Exchange	98	94	96	3.2
	Formaldehyde	68	70	69	1.9
B2P0C8	Control	77	74	75	2.2
	Cation Exchange	51	50	51	0.6
	Formaldehyde	31	31	31	0.0
B2P0N0	Control	88	86	87	1.5
	Cation Exchange	70	71	71	0.3
	Formaldehyde	56	66	61	7.1
B2P101*	Control	66	70	68	2.7
	Cation Exchange	98	101	99	2.1
	Formaldehyde	82	83	83	0.6
B2RKF4	Control	41	44	43	1.9
	Cation Exchange	70	66	68	2.3
	Formaldehyde	24	26	25	1.3
B2RKF5	Control	26	30	28	2.9
	Cation Exchange	28	26	27	1.3
	Formaldehyde	16	17	16	0.6

*Sample B2P101 had 80 g of starting material. All other samples had 100 g of starting material.

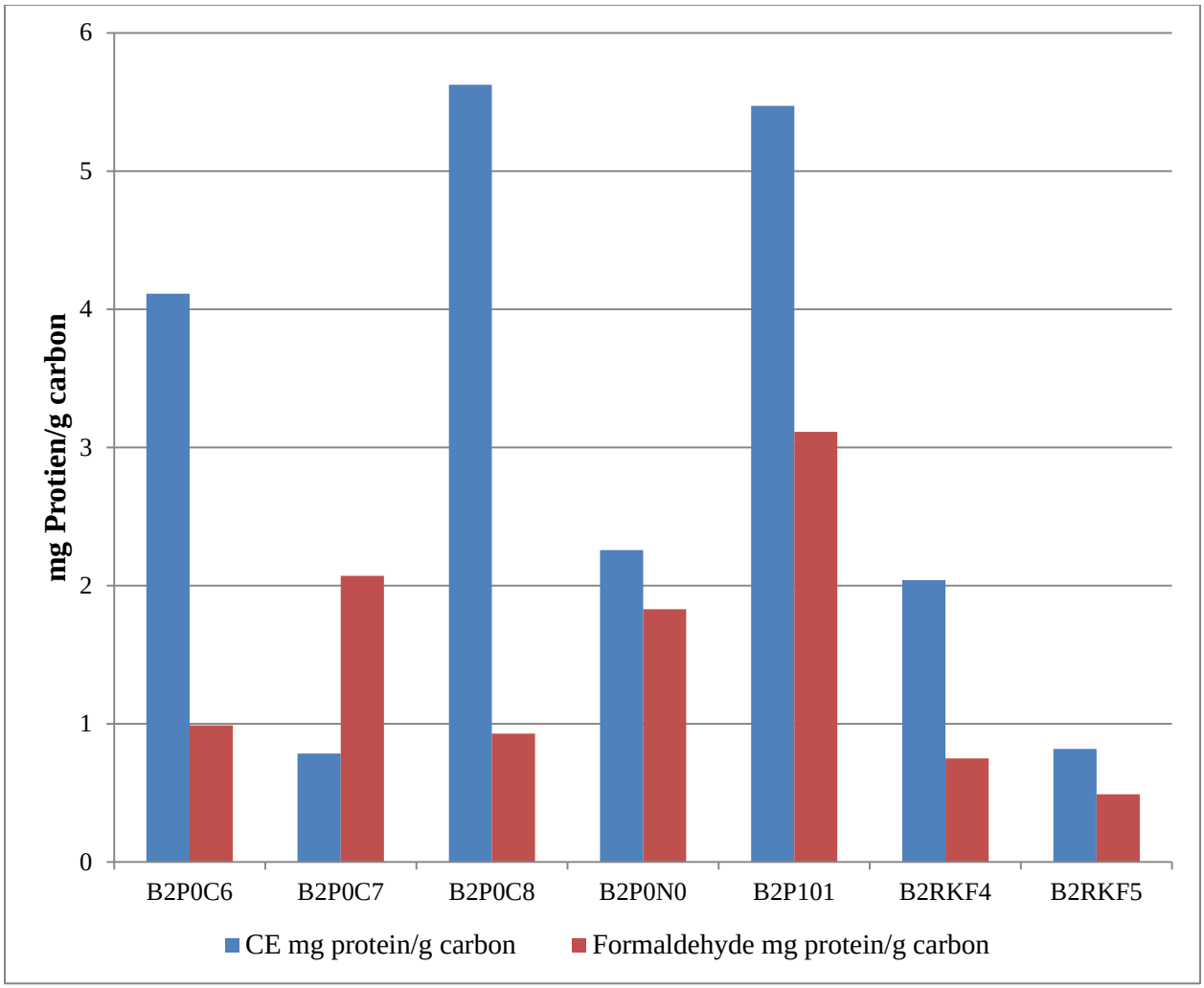

Figure 4.4. Comparison of the mass of protein per gram of carbon from which the EPS was extracted.

Table 4.6. Carbohydrate to Protein Ratios using Average Values

	Control	Cation Exchange	Formaldehyde
B2P0C6	0.16	0.31	3.61
B2P0C7	0.15	0.21	2.99
B2P0C8	0.25	0.12	3.32
B2P0N0	0.06	0.10	4.20
B2P101	0.01	0.60	5.33
B2RKF4	0.16	0.13	0.46
B2RKF5	0.22	0.19	0.67

4.2.4 DNA Analysis

Results from the DNA analysis are shown in Figure 4.5 through Figure 4.19. The plots show the fluorescence observed after incremental additions of sample followed by additions of the DNA standard.

A summary of the DNA concentrations is given in Table 4.7. DNA analysis was not performed for samples taken on September 10, 2013 (B2RKF4 and B2RKF5).

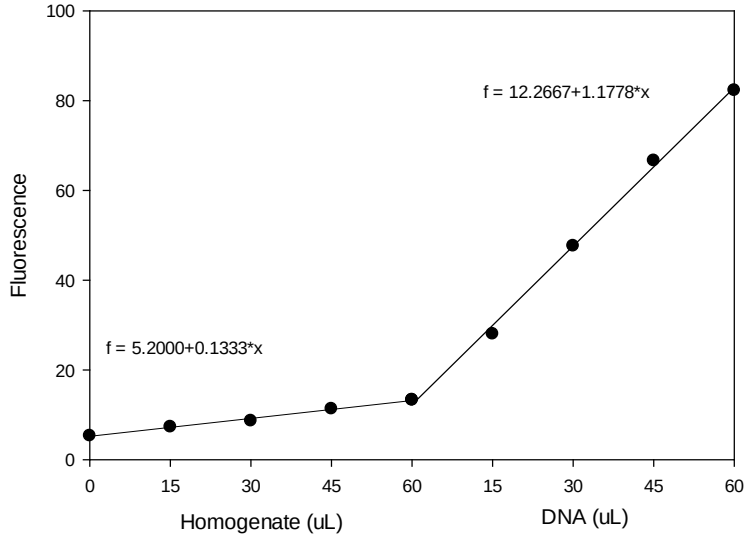

Figure 4.5. DNA analysis results for sample B2P0C6 control sample preparation.

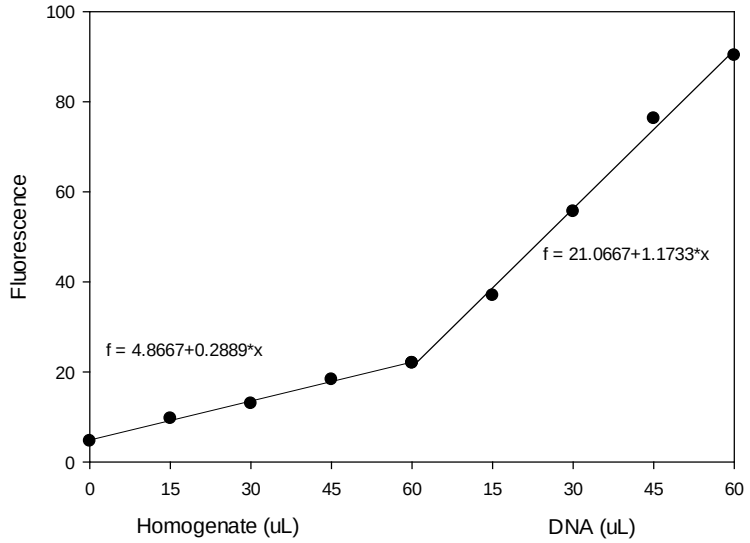

Figure 4.6. DNA analysis results for sample B2P0C6 cation exchange preparation.

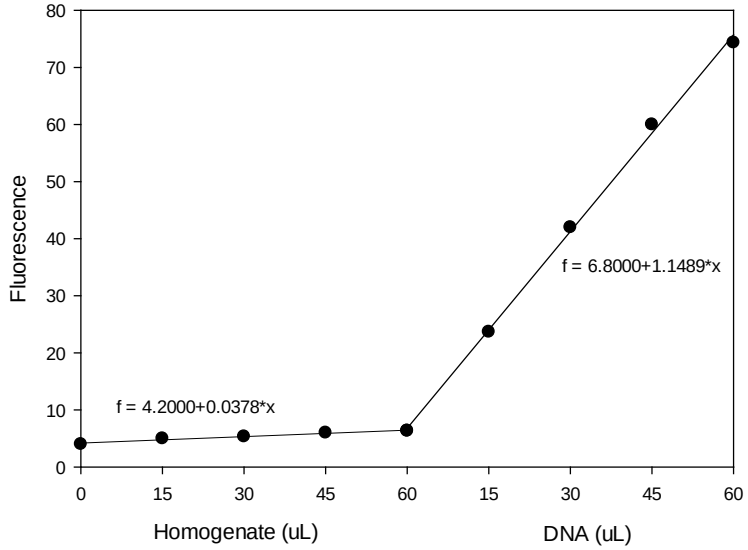

Figure 4.7. DNA analysis results for sample B2P0C6 formaldehyde sample preparation.

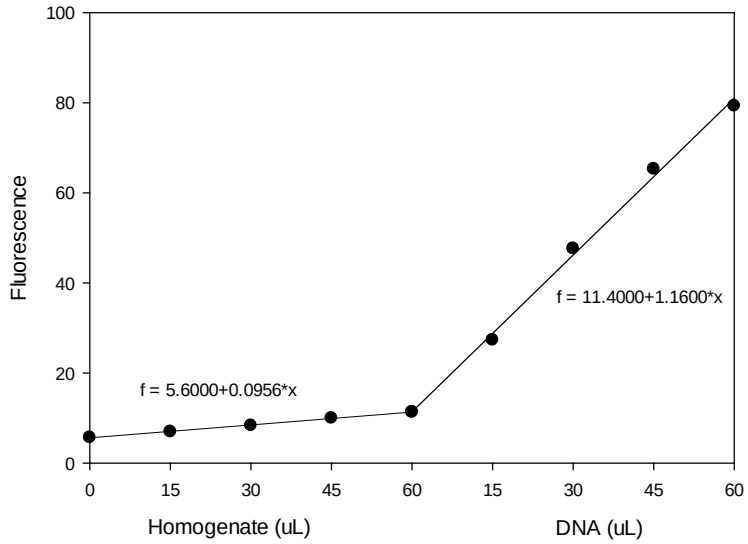

Figure 4.8. DNA analysis results for sample B2P0C7 control sample preparation.

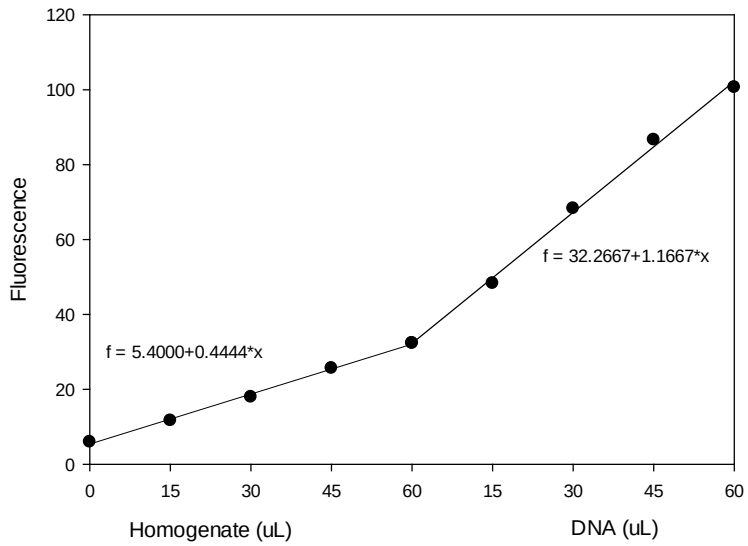

Figure 4.9. DNA analysis results for sample B2P0C7 cation exchange sample preparation.

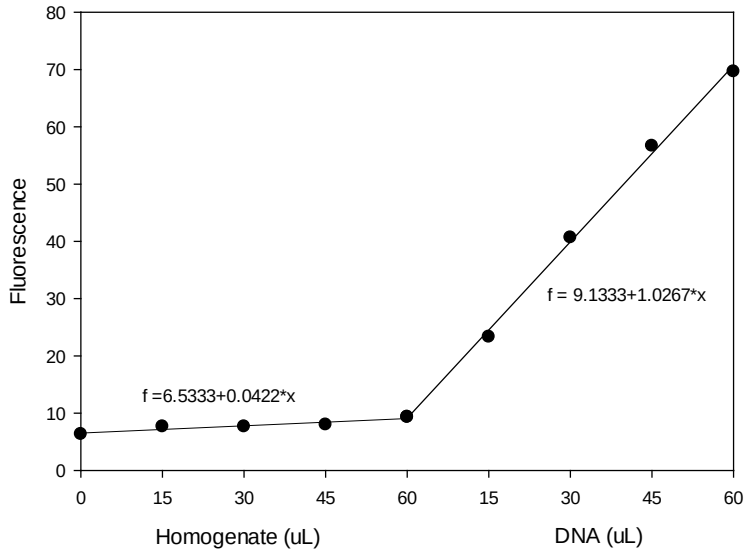

Figure 4.10. DNA analysis results for sample B2P0C7 formaldehyde sample preparation.

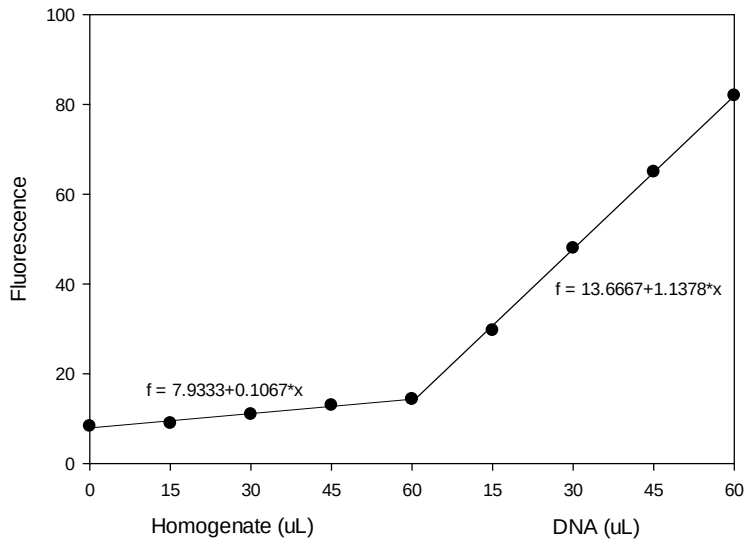

Figure 4.11. DNA analysis results for sample B2P0C8 control sample preparation.

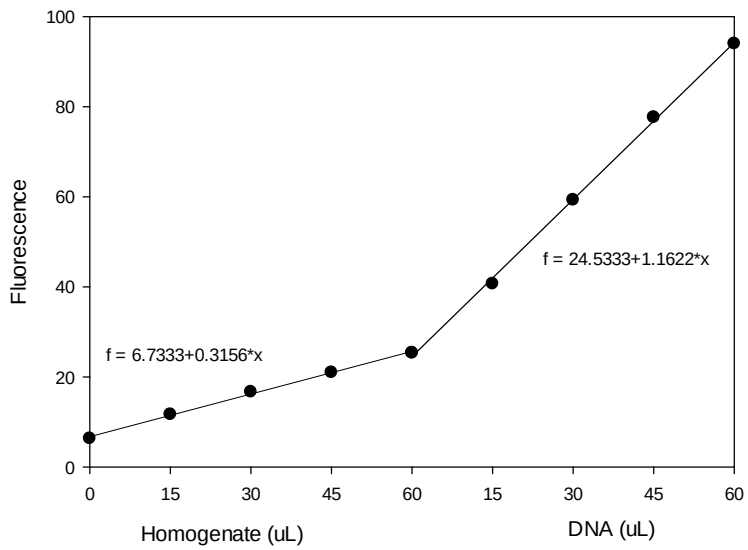

Figure 4.12. DNA analysis results for sample B2P0C8 cation exchange sample preparation.

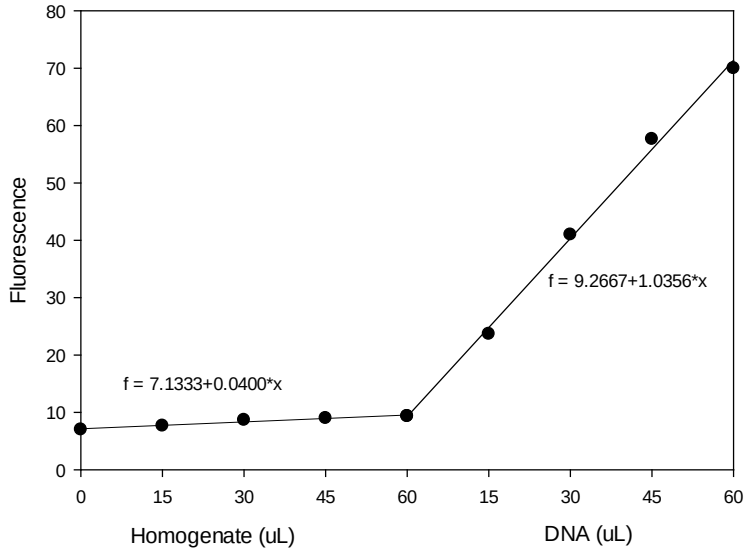

Figure 4.13. DNA analysis results for sample B2P0C8 formaldehyde sample preparation.

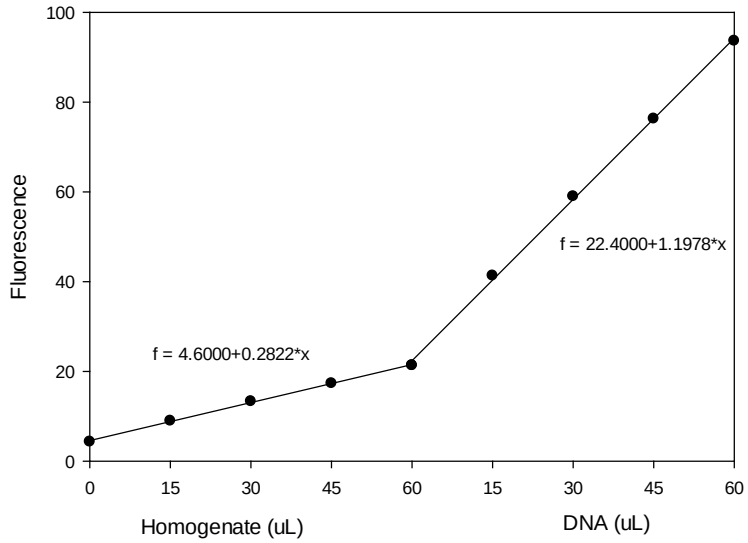

Figure 4.14. DNA analysis results for sample B2P0N0 control sample preparation.

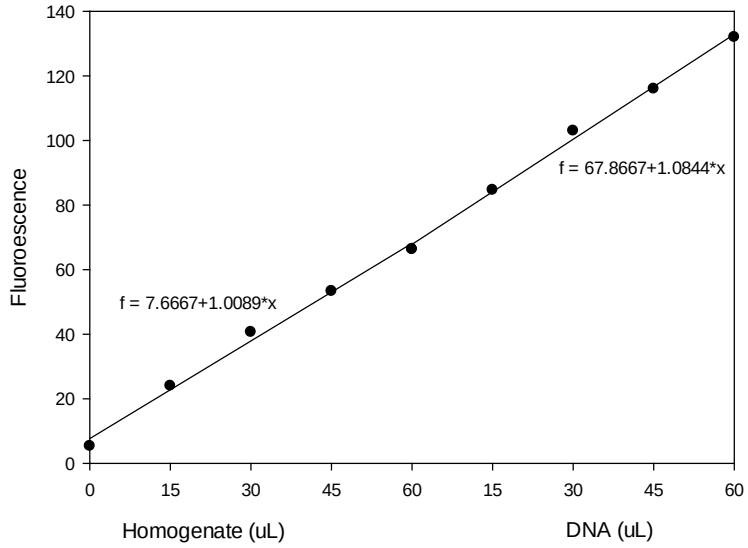

Figure 4.15. DNA analysis results for sample B2P0N0 cation exchange sample preparation.

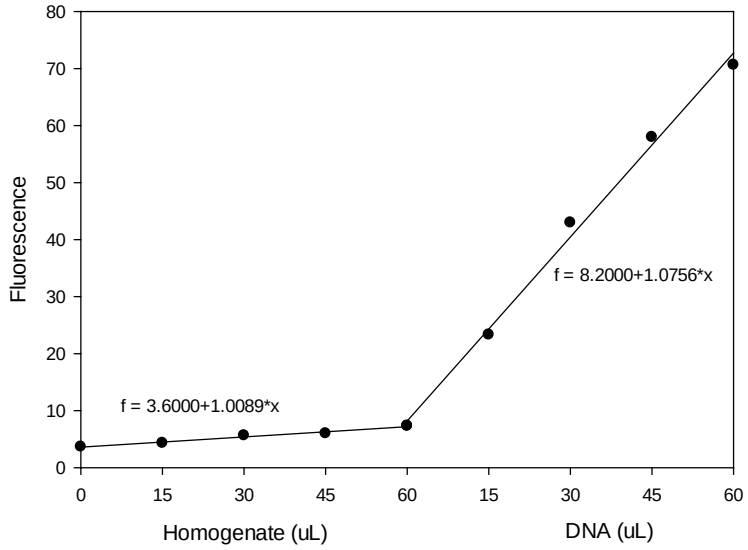

Figure 4.16. DNA analysis results for sample B2P0N0 formaldehyde sample preparation.

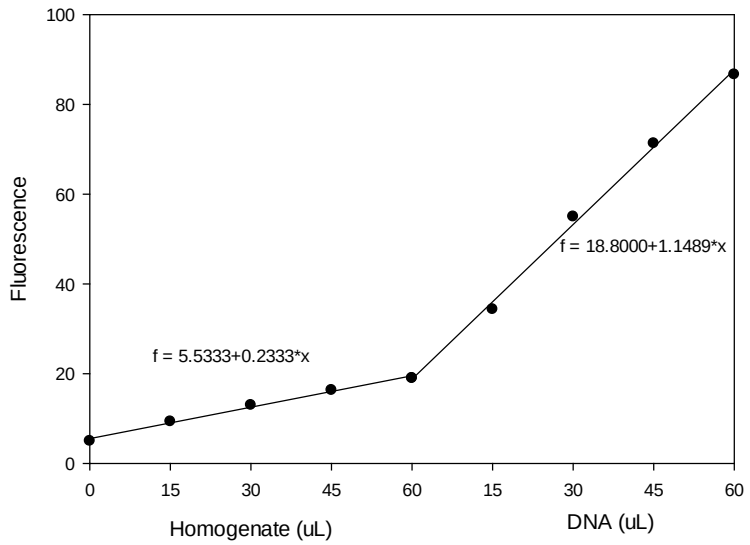

Figure 4.17. DNA analysis results for sample B2P101 control sample preparation.

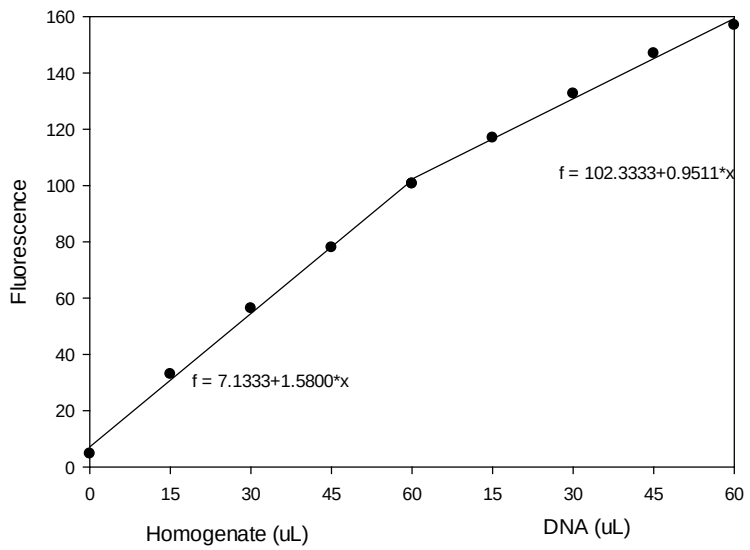

Figure 4.18. DNA analysis results for sample B2P101 cation exchange sample preparation.

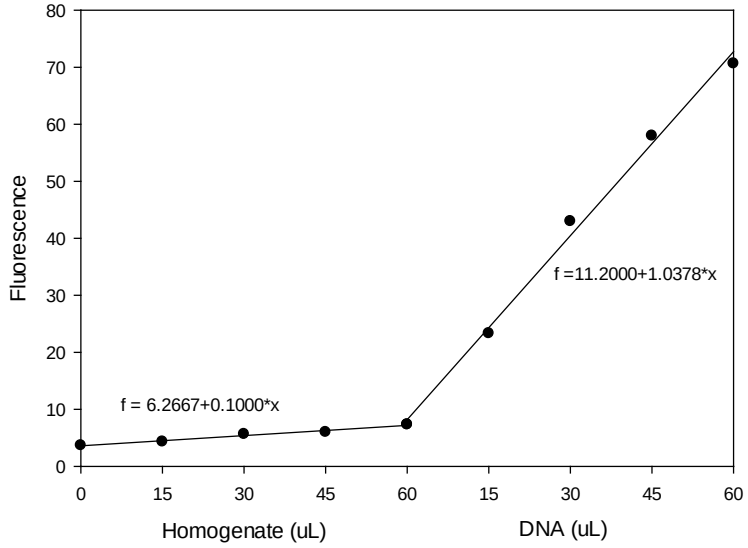

Figure 4.19. DNA analysis results for sample B2P101 formaldehyde sample preparation.

Table 4.7. Summary of DNA Concentrations

DNA (mg/L)	Control	Cation Exchange	Formaldehyde
B2P0C6	2.26	4.92	0.66
B2P0C7	1.65	7.62	0.82
B2P0C8	1.88	5.43	0.77
B2P0N0	4.71	18.61	18.76
B2P101*	4.06	33.22	1.93

*Sample B2P101 had 80 g of starting material. All other samples had 100 g of starting material.

4.2.5 Uronic Acid Analysis

Figure C.10, Figure C.11 and Figure C.12 show the calibration curves generated from the uronic acid standards and the calculated uronic acid concentrations from the processed samples. A summary of the results is given in Table 4.8.

Table 4.8. Summary of Uronic Acid Results

		Duplicate 1 Concentration (mg/L)		Duplicate 2 Concentration (mg/L)		Average Concentration (mg/L)	Std. Dev. (mg/L)
		1	2	1	2		
B2P0C6	Control	23	—	5	—	14	12.5
	Cation Exchange	8	—	8	—	8	0.0
	Formaldehyde	37	—	47		42	7.1
B2P0C7	Control	25	50	29	12	29	15.7
	Cation Exchange	20	20	7	8	14	7.4
	Formaldehyde	95	113	115	120	110	10.9
B2P0C8	Control	20	—	45	23	29	13.6
	Cation Exchange	15	—	18	—	17	2.7
	Formaldehyde	90	98	63	67	80	17.4
B2P0N0	Control	10	—	7	—	9	2.2
	Cation Exchange	7	—	6	—	7	0.8
	Formaldehyde	40	42	26	29	35	8.0
B2P101	Control	5	6	8	7	6	1.3
	Cation Exchange	7	—	11	12	10	2.7
	Formaldehyde	42	55	39	40	44	7.6
B2RKF4	Control	3.9		2.9		3	0.7
	Cation Exchange	3.3		3.3		3	0
	Formaldehyde	11.6		11.3		11	0.2
B2RKF5	Control	0.8		1.1		1	0.2
	Cation Exchange	0.9		1.4		1	0.3
	Formaldehyde	9.9		10.2		10	0.2

*Sample B2P101 had 80 g of starting material. All other samples had 100 g of starting material.

4.2.6 G6P Kit

The results from the G6P kit indicated no G6P activity, which is an indication that the EPS extraction methods did not disrupt cells in the biofilm.

4.2.7 Total Organic Carbon (TOC) and Metals Analysis

TOC results for the seven samples are found in Table 4.9. Metals analysis results are summarized in Table 4.10 for samples B2P0C6, B2P0C7 and B2P0C8. Results for the metals analysis for samples B2P0N0 and B2P101 are shown in Table 4.11. Metals analysis results for samples B2RKF4 and B2RKF5 are shown Table 4.12. All ICP-OES samples were run with a 2X dilution.

Table 4.9. Total Organic Carbon Results

TOC (mg/L)	Control	Cation Exchange 1	Cation Exchange 2	Formaldehyde
B2P0C6	206	200	*	186
B2P0C7	257	249	*	185
B2P0C8	61.3	291	*	172
B2P0N0	144	124	*	300
B2P101	68.9	285	*	343
B2RKF4	57.4	108	336	239
B2RKF5	ND	63.6	85.5	125

ND – Not detected
* - Not determined

Table 4.10. ICP-OES Metals Analysis Results for Samples B2P0C6, B2P0C7, and B2P0C8

	EQL (μg/L)	B2P0C6 (μg/L)			B2P0C7 (μg/L)			B2P0C8 (μg/L)		
		Control	Cation Exchange	Formaldehyde	Control	Cation Exchange	Formaldehyde	Control	Cation Exchange	Formaldehyde
Aluminum	32.9	ND	ND	116	ND	ND	142	ND	ND	129
Antimony	7.26	ND	ND	ND	ND	ND	ND	ND	ND	ND
Arsenic	119	ND	ND	ND	ND	ND	ND	ND	ND	ND
Barium	98.1	ND	ND	ND	ND	ND	ND	ND	ND	ND
Beryllium	11.6	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bismuth	17.4	ND	ND	ND	ND	ND	ND	ND	ND	ND
Boron	20	209	284	275	185	218	287	155	254	244
Calcium	6.86	3740	2570	1090	4560	2190	977	1490	2780	1100
Chromium	45.4	ND	ND	ND	13.4	24.3	15.1	ND	ND	ND
Cobalt	143	ND	ND	ND	ND	ND	ND	ND	ND	ND
Copper	592	ND	28.6	22.2	ND	ND	38.1	ND	ND	ND
Iron	435	85.5	38.3	32.1	150	110	76.8	157	61.3	55.2
Lead	262	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lithium	89.4	ND	ND	ND	ND	ND	ND	ND	ND	ND
Magnesium	28.5	1020	541	387	1230	530	322	534	613	545
Manganese	665	ND	ND	ND	ND	ND	ND	ND	ND	ND
Molybdenum	59.8	ND	ND	ND	ND	ND	ND	ND	ND	ND
Nickel	143	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phosphorus	131	192000	187000	181000	193000	190000	187000	189000	185000	181000
Potassium	4.74	43600	19200	40900	45800	17100	41200	44400	20600	40000
Rhenium	170	ND	ND	ND	ND	ND	ND	ND	ND	ND
Selenium	36.6	ND	ND	ND	ND	ND	ND	ND	ND	ND
Silicon	51.8	2920	2650	6490	3250	1550	7160	3380	1650	8710
Silver	53.4	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sodium	18.8	448000	425000	568000	454000	436000	636000	429000	421000	582000
Strontium	38	32.3	ND	ND	39.4	ND	ND	ND	ND	ND
Sulfur	69.9	1060	1180	1200	1320	1600	912	862	1480	1160
Thallium	35.8	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tin	163	ND	ND	ND	ND	ND	ND	ND	ND	ND
Titanium	84.9	ND	ND	ND	ND	ND	ND	ND	ND	ND

	EQL (µg/L)	B2P0C6 (µg/L)			B2P0C7 (µg/L)			B2P0C8 (µg/L)		
		Control	Cation Exchange	Formaldehyde	Control	Cation Exchange	Formaldehyde	Control	Cation Exchange	Formaldehyde
Uranium	31.8	ND	ND	ND	ND	ND	ND	ND	ND	ND
Vanadium	87.8	ND	ND	ND	ND	ND	ND	ND	ND	ND
Zinc	39.6	ND	ND	ND	ND	ND	ND	ND	ND	ND
Zirconium	41.5	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND = non detect

Table 4.11. ICP-OES Metals Analysis Results for Samples B2P0N0 and B2P101

	EQL (μg/L)	B2P0N0 (μg/L)			B2P101 (μg/L)		
		Control	Cation Exchange	Formaldehyde	Control	Cation Exchange	Formaldehyde
Aluminum	32.9	ND	ND	129	ND	ND	181
Antimony	7.26	ND	ND	ND	ND	ND	ND
Arsenic	119	ND	ND	ND	ND	ND	ND
Barium	98.1	10.4	ND	ND	ND	ND	ND
Beryllium	11.6	ND	ND	ND	ND	ND	ND
Bismuth	17.4	ND	ND	ND	ND	ND	ND
Boron	20	172	176	142	141	148	120
Calcium	6.86	3870	526	807	3390	2070	996
Chromium	45.4	29.2	39.4	17.6	14.7	46.2	22
Cobalt	143	ND	ND	ND	ND	ND	ND
Copper	592	24.7	45.1	89.3	ND	72.5	243
Iron	435	297	115	52.7	130	167	115
Lead	262	ND	ND	ND	ND	ND	ND
Lithium	89.4	ND	ND	ND	ND	ND	ND
Magnesium	28.5	1250	175	199	1260	513	224
Manganese	665	ND	ND	ND	ND	ND	ND
Molybdenum	59.8	ND	ND	ND	ND	ND	ND
Nickel	143	ND	ND	ND	ND	ND	ND
Phosphorus	131	192000	187000	183000	189000	190000	186000
Potassium	4.74	44200	15500	40400	44800	12700	41100
Rhenium	170	ND	ND	ND	ND	ND	ND
Selenium	36.6	ND	ND	ND	ND	ND	ND
Silicon	51.8	1530	905	10400	1770	1250	8170
Silver	53.4	—	—	—	—	—	—
Sodium	18.8	441000	417000	657000	457000	444000	687000
Strontium	38	29.9	ND	ND	ND	ND	ND
Sulfur	69.9	1700	1530	1230	1470	2110	1690
Thallium	35.8	ND	ND	ND	ND	ND	ND
Tin	163	ND	ND	ND	ND	ND	ND
Titanium	84.9	ND	ND	ND	ND	ND	ND
Uranium	31.8	ND	ND	ND	ND	ND	ND
Vanadium	87.8	ND	ND	ND	ND	ND	ND
Zinc	39.6	ND	ND	ND	ND	ND	ND
Zirconium	41.5	—	—	—	—	—	—

ND = non detect; — = not reportable

Table 4.12. ICP-OES Metals Analysis Results for Samples B2RKF4 and B2RKF5

	EQL (μg/L)	B2RKF4 (μg/L)				B2RKF5 (μg/L)			
		Control	Cation Exchange 1	Cation Exchange 2	Formaldehyde	Control	Cation Exchange 1	Cation Exchange 2	Formaldehyde
Aluminum	32.9	ND	ND	51.7	41.8	ND	ND	ND	86.1
Antimony	143	ND	ND	ND	ND	ND	ND	ND	ND
Arsenic	131	ND	ND	ND	ND	ND	ND	ND	ND
Barium	7.26	10.8	ND	16.4	ND	ND	ND	ND	ND
Beryllium	4.74	ND	ND	ND	ND	ND	ND	ND	ND
Bismuth	170	ND	ND	ND	ND	ND	ND	ND	ND
Calcium	98.1	3330	400	903	678	2170	358	482	663
Chromium	11.6	22.5	30.6	112	13.5	13.5	15.5	34.3	14.6
Cobalt	36.6	ND	ND	ND	ND	ND	ND	ND	ND
Copper	17.4	20.1	57.6	505	28.7	23.4	ND	141	27.3
Iron	20	197	262	1200	27.9	42.1	54.9	296	35
Lead	51.8	ND	ND	ND	ND	ND	ND	ND	ND
Lithium	53.4	ND	ND	ND	ND	ND	ND	ND	ND
Magnesium	6.86	669	48.4	181	266	501	65.1	65.9	173
Manganese	18.8	ND	ND	ND	ND	ND	ND	ND	ND
Molybdenum	38	ND	ND	ND	ND	ND	ND	ND	ND
Nickel	45.4	ND	ND	ND	ND	ND	ND	ND	ND
Phosphorus	143	188000	179000	199000	185000	177000	176000	185000	176000
Potassium	592	47100	28200	31500	41100	42000	28400	33700	39600
Rhenium	69.9	ND	ND	ND	ND	ND	ND	ND	ND
Selenium	435	ND	ND	ND	ND	ND	ND	ND	ND
Silicon	262	867	2270	1970	6520	2520	1460	1630	7100
Silver	35.8	ND	ND	ND	ND	ND	ND	ND	ND
Sodium	89.4	458000	428000	491000	569000	405000	415000	449000	554000
Strontium	28.5	34.2	ND	ND	ND	ND	ND	ND	ND
Sulfur	665	1500	802	4980	849	961	ND	1330	902
Thallium	163	ND	ND	ND	ND	ND	ND	ND	ND
Tin	84.9	ND	ND	ND	ND	ND	ND	ND	ND
Titanium	31.8	ND	ND	ND	ND	ND	ND	ND	ND
Uranium									
Vanadium	39.6	ND	ND	ND	ND	ND	ND	ND	ND
Zinc	59.8	157	ND	ND	ND	ND	ND	ND	ND
Zirconium	41.5	ND	ND	ND	ND	ND	ND	ND	ND

ND = non detect; —

4.3 PCR/qPCR

Table 4.13 summarizes the initial results for specified targets using qPCR primers. DNA was extracted from samples which were used as FBR inoculum (Keg1) and from samples taken directly from the FBR during various sampling events. Values shown represent total Eubacteria and Archaea based on amplification of the 16S rRNA gene, while functional types are targeted by genes encoding nitrite reductase (*nirS* and *nirK*), adenosine-5′-phosphosulfate (*apsA*), sulfite reductase (*dsrA*), and methyl-coenzyme M reductase (*mcrA*). In addition, qPCR primers that targeted a region of the 16S rRNA gene specific to iron reducing bacteria (Geobacter) and for anaerobic ammonia oxidizers (Anammox) were also tested. Results are presented as copies of each gene per mass of sample used for extraction. Archaea were the dominant prokaryote in the FBR inoculum samples (>77% of the total population), while Eubacteria were the dominant prokaryote in the FBR bed. Percentage of physiological types for each sample is shown in Figure 4.20. In the inoculum (Keg1), there is a much higher percentage of what are most likely obligate anaerobes (i.e., *apsA* and *mcrA*), and facultative anaerobes (i.e., *nirK* and *nirS*). As time proceeds, the overall community in the FBR bed shifts to a population dominated by nitrate reducing bacteria as indicated by the number of *nirK* and *nirS* genes. In March, nitrate reducing bacteria represent less than 10% of the population, one month later, the number climbed to nearly 50% of the population. When the FBR was sampled in September, nitrate reducers dropped to ~30% of the total population, but were still dominant. Anaerobic ammonia oxidizers also appear to be dominant within the FBR bed accounting for nearly 15% of the total Eubacterial population in samples taken during winter and spring. While there were some anaerobic ammonia oxidizers in samples taken in September, numbers were substantially lower. Aerobic heterotrophs are hypothesized to account for the balance of the microbial population in the FBR bed. DNA amplification has been problematic using the *dsrA* primer set, and PCR has yielded non-specific product from FBR samples. Subsequent electrophoretic analysis of initial results from amplification of *mcrA* genes indicates that fluorescence generated during amplification was from a product slightly larger than the actual amplicon. The Keg sample had a product of the proper size but not samples from within the FBR bed. These current findings support results generated with the Archaeal primers. FISH analyses are in progress using optimized hybridization protocols for the different probes. FISH probes for total Eubacteria are in progress. Additional FISH results will be included in updates that will follow during the week of September 30.All of the data together indicated that the microbial population present within the FBR bed showed different members and physiological traits than the microbial population in the inoculum.

Table 4.13. Quantitative/Qualitative Results for qPCR Targets.

Target	Keg1 Inoc (copies/g sample)		B2P0C6 (copies/g sample)		B2P0C7 (copies/g sample)		B2P0C8 (copies/g sample)		B2P101 (copies/g sample)		B2P0N0 (copies/g sample)		B2RKF4 (copies/g Sample)	
Eubacterial 16S	$2.09x10^6$	$6.20x10^5$	$3.64x10^6$	$2.25x10^5$	$5.43x10^6$	$1.85x10^6$	5.86×10^6	$1.73x10^6$	2.36×10^6	$8.68x10^4$	4.18×10^6	$1.07x10^5$	2.7×10^6	$4.78x10^5$
Archaeal 16S	$7.17x10^6$	$5.42x10^5$	BDL		BDL		BDL		BDL		BDL		BDL	
nirS	$6.93x10^5$	$8.00x10^4$	$1.46x10^5$	$1.24x10^4$	3.84×10^5	$3.17x10^4$	3.28×10^5	$5.32x10^4$	4.45×10^5	$5.84x10^4$	8.55×10^5	$7.43x10^4$	2.33×10^5	$2.34x10^4$
nirK	5.46×10^4	$2.52x10^4$	9.25×10^4	$3.31x10^3$	1.70×10^5	$1.23x10^4$	1.10×10^5	$8.53x10^3$	$2.76x10^5$	$7.94x10^4$	1.28×10^6	$2.50x10^5$	5.11×10^5	$2.38x10^5$
Geobacter	7.64×10^2	32	1.95×10^3	$1.69x10^2$	3.11×10^3	$4.09x10^2$	2.89×10^3	$2.47x10^2$	1.08×10^4	$1.06x10^3$	6.26×10^3	$1.17x10^3$	9.96×10^2	$1.10x10^2$
apsA	8.18×10^5	$3.97x10^4$	1.52×10^4	$2.06x10^3$	2.27×10^4	$6.00x10^3$	$1.87x10^4$	$2.57x10^4$	2.04×10^4	$3.03x10^3$	3.85×10^4	$1.12x10^4$	BDL	
dsrA	+++		+/-		+		+		-		-		NA	
mcrA	6.68×10^5	$7.07x10^4$	BDL		BDL		BDL		BDL		BDL		BDL	
Anammox	9.63×10^4	$1.98x10^3$	BDL		7.33×10^5	$2.68x10^4$	5.30×10^5	$1.28x10^4$	3.09×10^5	$1.44x10^4$	3.22×10^5	$3.31x10^4$	Trace	

dsrA +++ = present at high levels, +/- = barely distinguishable, + = present at low levels, - = not present
BDL = Below detection limit for analysis
NA = Not analyzed
Trace = There was a light band of the proper size, but the concentration was outside of the quantifiable range.

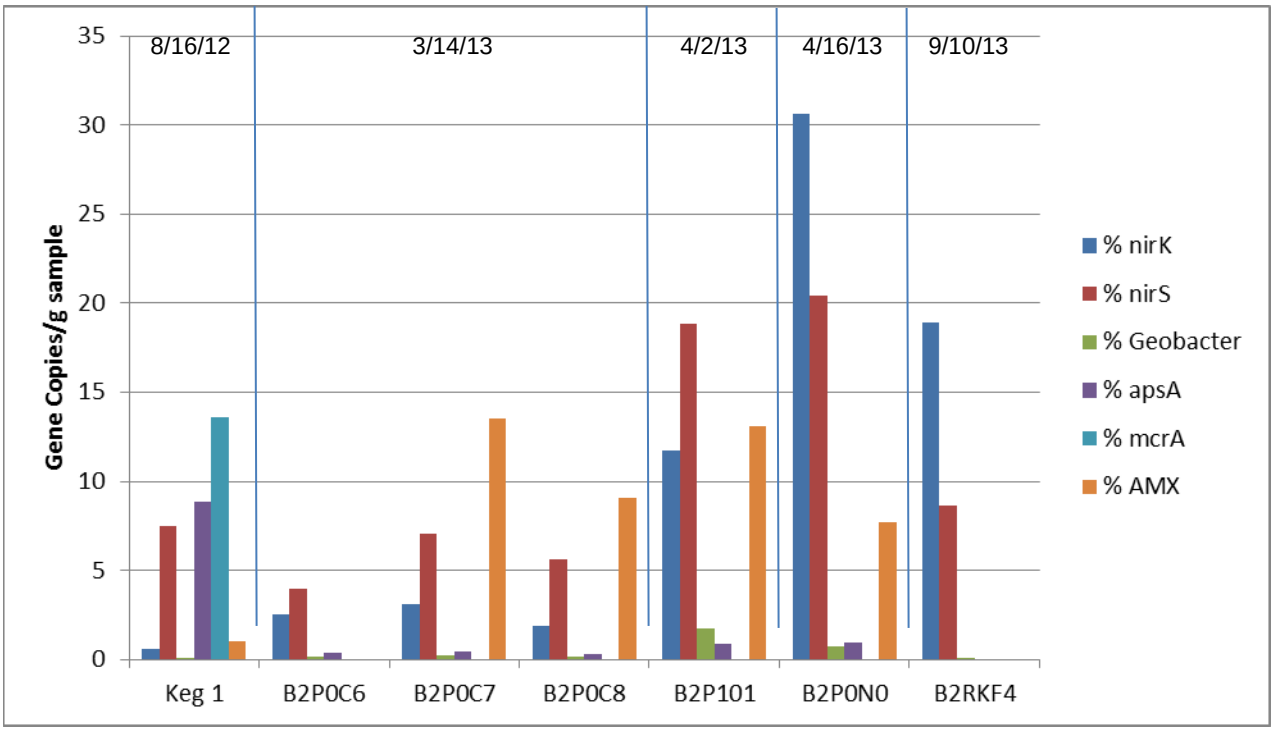

Figure 4.20. Bar chart showing percentage of functional genes copies compared to total copies of the 16S rRNA gene. Keg1 samples were compared to total which included both Eubacterial and Archaeal 16S rRNA, while FBR samples were only compared to Eubacterial gene copies since Archaea were not present.

4.4 FISH

Table 4.14 summarizes the results for specified targets using fluorescent in situ hybridization primers. Whole cell preparations were made from samples fixed in paraformaldehyde and frozen. Positive control cultures were grown in batch and cells were prepared identically to the FBR cell preps to be used as a reference (positive control) for each of the FISH probes. Each sample was also stained with DAPI, a total DNA stain, and used as a reference to determine fraction or percent of the total cells in a given sample were positive with any FISH probe. In addition (data not shown), negative control slides were made for each set of probes and included (a) filtered water with application of probes and (b) hybridization solutions with no sample. A number of targets were chosen for FISH in order to identify the diversity and activity of functional groups of organisms; these values were expected to complement the qPCR data and potentially provide additional information as the FISH method targets the 16S rRNA molecule which is indicative of activity in cells, not just presence of DNA. Values shown represent the average cells per mL and standard errors for each target for a minimum of 40 random fields of view, counted using epifluorescence microscopy. Targets for FISH are similar to those for qPCR and included total bacteria and archaea, anammox (anaerobic ammonia oxidizers), SO_4 (sulfate reducers), Fe (iron reducers), CH_4 (methanogens), and N (denitrifying bacteria). For many of the functional groups, multiple FISH probes were combined in order to increase the diversity of organisms targeted within the functional group and increase overall sensitivity of detection.

Table 4.14. Results for Specified Targets Using Fluorescent In Situ Hybridization Primers (cells/ml)

	B2P0C6	Standard Error	B2P0C7	Standard Error	BRP0C8	Standard Error	B2P0N0	Standard Error	B2P101	Standard Error	B2RKF4	Standard Error
Eubacteria	4.12E+09	2.97E+08	3.41E+09	2.64E+08	3.36E+09	3.07E+08	3.72E+09	3.89E+08	3.38E+09	3.84E+08	6.01E+09	4.72E+08
DAPI	7.16E+09	5.29E+08	4.68E+09	3.53E+08	4.41E+09	3.93E+08	5.51E+09	5.18E+08	6.57E+09	4.33E+08	7.23E+09	5.05E+08
Archaea	2.75E+07	1.25E+07	2.06E+07	7.22E+06	2.06E+07	1.13E+07	3.78E+07	1.53E+07	2.75E+07	1.96E+07	2.75E+07	1.43E+07
DAPI	9.08E+09	4.56E+08	8.06E+09	5.14E+08	6.24E+09	4.10E+08	6.06E+09	4.66E+08	6.60E+09	3.97E+08	7.50E+09	5.37E+08
Anammox	1.72E+07	1.22E+07	6.87E+06	6.87E+06	0.00E+00	0.00E+00	1.37E+07	9.46E+06	6.87E+06	4.73E+06	6.87E+06	4.73E+06
DAPI	8.13E+09	5.08E+08	7.17E+09	3.55E+08	6.56E+09	3.98E+08	6.52E+09	5.20E+08	7.47E+09	4.35E+08	7.47E+09	4.76E+08
SO4	2.75E+07	1.43E+07	2.75E+07	1.25E+07	3.43E+07	1.68E+07	7.21E+07	2.16E+07	1.37E+08	3.97E+07	7.56E+07	2.53E+07
DAPI	7.59E+09	4.13E+08	5.70E+09	3.93E+08	5.58E+09	2.96E+08	5.51E+09	3.64E+08	5.36E+09	2.95E+08	6.36E+09	4.04E+08
Fe	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.43E+06	3.43E+06	3.43E+06	3.43E+06	0.00E+00	0.00E+00	0.00E+00	0.00E+00
DAPI	6.70E+09	3.60E+08	6.49E+09	3.64E+08	4.77E+09	3.56E+08	4.87E+09	3.65E+08	5.56E+09	2.88E+08	5.47E+09	3.98E+08
CH4	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
DAPI	5.68E+09	3.48E+08	5.65E+09	4.45E+08	3.82E+09	3.52E+08	6.89E+09	3.67E+08	5.93E+09	3.34E+08	7.94E+09	4.25E+08
N	1.37E+07	1.37E+07	2.10E+09	2.60E+08	1.59E+09	2.54E+08	3.43E+06	3.43E+06	6.87E+06	6.87E+06	1.72E+07	1.15E+07
DAPI	5.32E+09	4.82E+08	5.52E+09	5.16E+08	5.43E+09	4.53E+08	7.43E+09	5.87E+08	8.59E+09	4.67E+08	7.95E+09	4.41E+08

In general FISH probes targeting Eubacteria and Archaea hybridized to ~70% of the cells that were stained with DAPI (Figure 4.21). As expected from qPCR results, Eubacteria dominated the community and were approximately two orders of magnitude higher than the numbers for Archaeal members. Results indicate that an annamox community was present within the bed over the nine months that the analyses were performed, but FISH probes developed for anammox bacteria hybridized to less than 1% of total bacteria in the community. Sulfate reducing bacteria (SRB) were present in the community in similar numbers to Archaea, which were present in the 107 range. Samples from mid-April showed 1.37 x 10^8 SRB. Probes developed to target bacteria known to denitrify indicated that in samples taken in March that nearly 25% of the community was composed of bacteria associated with denitrification. Interestingly, the FISH probes for nitrate reducing bacteria only hybridize to a small percentage of the bacteria in some of the samples, indicating that nitrate reducers shown from qPCR analyses may not have been represented in FISH probes chosen for these analyses. Selection of FISH probes was based on possible denitrifying genera found in the clone libraries (e.g., *Flavobacterium*, *Acidovorax*, *Curvibacter*, etc.). Since denitrifiers were higher in samples taken during March, much of the denitrifying community may be made up of *Curvibacter* species which drastically decreased between March and April and only represented less than 2% of the community when the FBR was sampled in September. Samples with few hybridized cells yielded low counts with a high standard error.

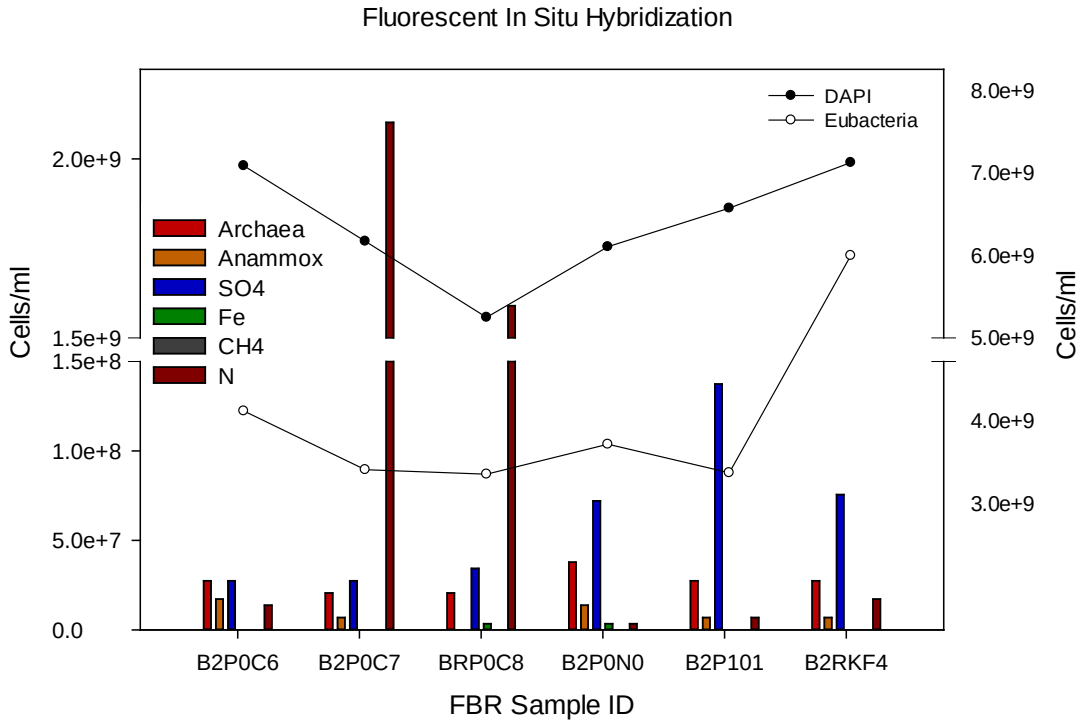

Figure 4.21. Bar and line chart detailing the activity of FBR samples as determined by FISH analysis. DAPI is a total DNA stain and was determined for each target as a metric for total microbes present in each sample/field of view; Eubacteria is included as a line graph because the majority of the community. Each value is determined based on 40 random field counts.

5.0 Conclusions

Six samples of biomass coated GAC representing four distinct time points were taken from an FBR treating nitrate contaminated groundwater as a component of a pump and treat system at the 200 West Area of the Hanford Site.

Whole cell staining preparations were completed and DNA was extracted from the biomass and used to perform a number of molecular analyses in an effort to define the microbial community in the bed from a phylogenetic and functional standpoint. In general, the FBR bed microbial community did not resemble the community used to inoculate the bed, and showed a significant shift in the microbial community. The seed inoculum, Keg 1, was primarily composed of obligate anaerobes including methanogens and fermentative prokaryotes. Over time the communities have shifted primarily to microaerophilic prokaryotes, or common groundwater microbes. The microbial community became dominated by a few members, primarily *Flavobacterium* and *Hydrogenophaga* species at the first three sampling points, but became more diverse when sampled in September of 2012. In contrast, from a functional perspective, denitrifying bacteria were the dominant functional type in the bed, and this trend continued even for the September sample, indicating that even though the community became phylogenetically diverse, that denitrifiers were the most abundant and likely most active members of the FBR community.

Analytical results indicate microorganisms in the FBRs are effectively reducing nitrate, carbon tetrachloride, and trichloroethylene to less than detection limits (< 0.038 mg/L, <1 µg/L for both chlorinated solvents).

Optimization of the microbial-enriched FBR component of the pump and treat system is not a trivial task. Microbial communities are dynamic and can exhibit phylogenetic and functional changes over days to weeks; thus, it is important to understand influent features including various groundwater chemistries, concentrations of add in nutrients at influent and effluent valves, and upstream treated-groundwater characteristics. A small change in any one component of this system can and likely will cause a change in the microbial community and potentially the overall function of the FBR and its contaminant removal efficiency. Some small changes that result in shifts in the microbial community may go unnoticed for months to years, however a monitoring program to look at baseline community structure and function could provide feedback metrics for performance independent of effluent chemistry and alert operators to a problem before a system failure or other observable failure that has the potential to increase costs, decrease performance and even delay in overall treatment of groundwater (temporary shutdowns or decreased process flow). A long-term program to provide quick turn-around information on the community could prove useful in keeping the system operating optimally and in balance.

6.0 References

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Appendix A

Chain of Custody

Appendix A

Chain of Custody

Copies of the chain of custody forms received are shown in the following pages.

CH2MHill Plateau Remediation Company	CHAIN OF CUSTODY/SAMPLE ANALYSIS REQUEST			F13-023-002	PAGE 1 OF 1
COLLECTOR C. Fulton 3/14/13	COMPANY CONTACT EVANS, RT	TELEPHONE NO. 373-7924	PROJECT COORDINATOR EVANS, RT	PRICE CODE 9N	DATA TURNAROUND 45 Days / 45 Days
SAMPLING LOCATION 289-T, FBR-A, Middle of Bed (~~21 ft~~) 22'	PROJECT DESIGNATION 200W Pump & Treat - Microbiological Special Studies - Other Solid		SAF NO. F13-023	AIR QUALITY ☐	
ICE CHEST NO. GWS-060	FIELD LOGBOOK NO. HNF-N-507-16	ACTUAL SAMPLE DEPTH N/A	COA 303110ES10	METHOD OF SHIPMENT GOVERNMENT VEHICLE	ORIGINAL
SHIPPED TO Environmental Sciences Laboratory	OFFSITE PROPERTY NO. SEE PTR N/A		BILL OF LADING/AIR BILL NO. N/A		

MATRIX*
A=Air
DL=Drum Liquids
DS=Drum Solids
L=Liquid
O=Oil
S=Soil
SE=Sediment
T=Tissue
V=Vegetation
W=Water
WI=Wipe
X=Other

POSSIBLE SAMPLE HAZARDS/ REMARKS
Contains Radioactive Material at concentrations that may or may not be regulated for transportation per 49 CFR/IATA Dangerous Goods Regulations but are not releasable per DOE Order 458.1.

SPECIAL HANDLING AND/OR STORAGE

PRESERVATION	None
HOLDING TIME	6 Months
TYPE OF CONTAINER	P
NO. OF CONTAINER(S)	2
VOLUME	1L
SAMPLE ANALYSIS	Generic Testing (No CAS);

SAMPLE NO.	MATRIX*	SAMPLE DATE	SAMPLE TIME	
B2P0C7	OTHER SOLID	MAR 14 2013	1151	✓

CHAIN OF POSSESSION		SIGN/ PRINT NAMES	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
C. Fulton	MAR 14 2013 1412	Danielle Saunders	MAR 14 2013 1412
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME

SPECIAL INSTRUCTIONS

** The 200 Area S&GRP Characterization and Monitoring Sampling and Analysis GKI applies to this SAF.
***Analysis of the GAC and associated biomass shall be performed by PNNL in accordance to the conditions and requirements set forth in CHPRC's contract with PNNL to perform this very analysis.

TRVL- 13- 036

LABORATORY SECTION	RECEIVED BY	TITLE	DATE/TIME
FINAL SAMPLE DISPOSITION	DISPOSAL METHOD	DISPOSED BY	DATE/TIME

PRINTED ON 3/13/2013

A-6003-618 (REV 2)

CH2MHill Plateau Remediation Company	CHAIN OF CUSTODY/SAMPLE ANALYSIS REQUEST			F13-023-003	PAGE 1 OF 1
COLLECTOR C. Fulton	COMPANY CONTACT EVANS, RT	TELEPHONE NO. 373-7924	PROJECT COORDINATOR EVANS, RT	PRICE CODE 9N	DATA TURNAROUND 45 Days / 45 Days
SAMPLING LOCATION 289-T, FBR-A, Bottom of Bed (27ft) KE 3/15/13	PROJECT DESIGNATION 200W Pump & Treat - Microbiological Special Studies - Other Solid		SAF NO. F13-023	AIR QUALITY ☐	
ICE CHEST NO. GWS-060	FIELD LOGBOOK NO. HNF-N-507-16	ACTUAL SAMPLE DEPTH N/A	COA 303110ES10	METHOD OF SHIPMENT GOVERNMENT VEHICLE	ORIGINAL
SHIPPED TO Environmental Sciences Laboratory	OFFSITE PROPERTY NO. SEE PTR N/A		BILL OF LADING/AIR BILL NO. N/A		

MATRIX*	POSSIBLE SAMPLE HAZARDS/ REMARKS		
A=Air DL=Drum Liquids DS=Drum Solids L=Liquid O=Oil S=Soil SE=Sediment T=Tissue V=Vegetation W=Water WI=Wipe X=Other	**Contains Radioactive Material at concentrations that may or may not be regulated for transportation per 49 CFR/IATA Dangerous Goods Regulations but are not releasable per DOE Order 458.1.**	PRESERVATION	None
		HOLDING TIME	6 Months
		TYPE OF CONTAINER	P
		NO. OF CONTAINER(S)	2
		VOLUME	1L
	SPECIAL HANDLING AND/OR STORAGE	SAMPLE ANALYSIS	Generic Testing (No CAS);

SAMPLE NO.	MATRIX*	SAMPLE DATE	SAMPLE TIME	
B2P0C8	OTHER SOLID	MAR 14 2013	1230	✓

CHAIN OF POSSESSION		SIGN/ PRINT NAMES		SPECIAL INSTRUCTIONS
RELINQUISHED BY/REMOVED FROM C. Fulton	DATE/TIME MAR 14 2013 1412	RECEIVED BY/STORED IN Danielle Saunders	DATE/TIME MAR 14 2013 1412	** The 200 Area S&GRP Characterization and Monitoring Sampling and Analysis GKI applies to this SAF. ***Analysis of the GAC and associated biomass shall be performed by PNNL in accordance to the conditions and requirements set forth in CHPRC's contract with PNNL to perform this very analysis. TRVL-13-036
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	

LABORATORY SECTION	RECEIVED BY	TITLE	DATE/TIME
FINAL SAMPLE DISPOSITION	DISPOSAL METHOD	DISPOSED BY	DATE/TIME

PRINTED ON 3/13/2013

A-6003-618 (REV 2)

CH2MHill Plateau Remediation Company	CHAIN OF CUSTODY/SAMPLE ANALYSIS REQUEST			F13-023-001	PAGE 1 OF 1
COLLECTOR C. Fulton	COMPANY CONTACT EVANS, RT	TELEPHONE NO. 373-7924	PROJECT COORDINATOR EVANS, RT	PRICE CODE 9N	DATA TURNAROUND 45 Days / 45 Days
SAMPLING LOCATION 289-T, FBR-A Top of Bed (18ft) 17.5'	PROJECT DESIGNATION 200W Pump & Treat - Microbiological Special Studies - Other Solid		SAF NO. F13-023	AIR QUALITY ☐	
ICE CHEST NO. GWS-060	FIELD LOGBOOK NO. HNF-N-507-16	ACTUAL SAMPLE DEPTH N/A	COA 303110ES10	METHOD OF SHIPMENT GOVERNMENT VEHICLE	ORIGINAL
SHIPPED TO Environmental Sciences Laboratory	OFFSITE PROPERTY NO. SEE PTR N/A		BILL OF LADING/AIR BILL NO. N/A		

MATRIX*	POSSIBLE SAMPLE HAZARDS/ REMARKS		
A=Air DL=Drum Liquids DS=Drum Solids L=Liquid O=Oil S=Soil SE=Sediment T=Tissue V=Vegetation W=Water WI=Wipe X=Other	**Contains Radioactive Material at concentrations that may or may not be regulated for transportation per 49 CFR/IATA Dangerous Goods Regulations but are not releasable per DOE Order 458.1.**	PRESERVATION	None
		HOLDING TIME	6 Months
		TYPE OF CONTAINER	P
		NO. OF CONTAINER(S)	2
		VOLUME	1L
	SPECIAL HANDLING AND/OR STORAGE	SAMPLE ANALYSIS	Generic Testing (No CAS);

SAMPLE NO.	MATRIX*	SAMPLE DATE	SAMPLE TIME	
B2P0C6	OTHER SOLID	MAR 14 2013	1110	✓

CHAIN OF POSSESSION		SIGN/ PRINT NAMES		SPECIAL INSTRUCTIONS
RELINQUISHED BY/REMOVED FROM C. Fulton	DATE/TIME MAR 14 2013 1412	RECEIVED BY/STORED IN Danielle Samuels	DATE/TIME MAR 14 2013 1412	** The 200 Area S&GRP Characterization and Monitoring Sampling and Analysis GKI applies to this SAF. ***Analysis of the GAC and associated biomass shall be performed by PNNL in accordance to the conditions and requirements set forth in CHPRC's contract with PNNL to perform this very analysis. TRVL- 13- 036
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	

LABORATORY SECTION	RECEIVED BY	TITLE	DATE/TIME
FINAL SAMPLE DISPOSITION	DISPOSAL METHOD	DISPOSED BY	DATE/TIME

PRINTED ON 3/12/2013

A-6003-618 (REV 2)

CH2MHill Plateau Remediation Company	CHAIN OF CUSTODY/SAMPLE ANALYSIS REQUEST			F13-023-005	PAGE 1 OF 1
COLLECTOR C. Fu Hon	COMPANY CONTACT EVANS, RT	TELEPHONE NO. 373-7924	PROJECT COORDINATOR EVANS, RT	PRICE CODE 9N	DATA TURNAROUND
SAMPLING LOCATION 289-T, FBR-A Middle of Bed (~~21 Ft~~) 23' 4/2/13	PROJECT DESIGNATION 200W Pump & Treat - Microbiological/Chemical Analysis - Other Solid		SAF NO. F13-023	AIR QUALITY ☐	45 Days / 45 Days
ICE CHEST NO. GWS-186-2	FIELD LOGBOOK NO. HNF-W-507-24	ACTUAL SAMPLE DEPTH 23'	COA 303110ES10	METHOD OF SHIPMENT GOVERNMENT VEHICLE	ORIGINAL
SHIPPED TO Environmental Sciences Laboratory	OFFSITE PROPERTY NO. SEE PTR		BILL OF LADING/AIR BILL NO.		

MATRIX*	POSSIBLE SAMPLE HAZARDS/ REMARKS		
A=Air DL=Drum Liquids DS=Drum Solids L=Liquid O=Oil S=Soil SE=Sediment T=Tissue V=Vegetation W=Water WI=Wipe X=Other	**Contains Radioactive Material at concentrations that may or may not be regulated for transportation per 49 CFR/IATA Dangerous Goods Regulations but are not releasable per DOE Order 458.1.**	PRESERVATION	None
		HOLDING TIME	6 Months
		TYPE OF CONTAINER	P
		NO. OF CONTAINER(S)	2
		VOLUME	1L
	SPECIAL HANDLING AND/OR STORAGE	SAMPLE ANALYSIS	Generic Testing (No CAS);

SAMPLE NO.	MATRIX*	SAMPLE DATE	SAMPLE TIME	
B2P0N0	OTHER SOLID	4-2-13	0507	✓

CHAIN OF POSSESSION		SIGN/ PRINT NAMES	
RELINQUISHED BY/REMOVED FROM C. Fu Hon	DATE/TIME 4-2-13 1020	RECEIVED BY/STORED IN	DATE/TIME 4-2-13 1020
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME

SPECIAL INSTRUCTIONS

** The 200 Area S&GRP Characterization and Monitoring Sampling and Analysis GKI applies to this SAF.
***Analysis of the GAC and associated biomass shall be performed by PNNL in accordance to the conditions and requirements set forth in CHPRC's contract with PNNL to perform this very analysis.
Analysis of the GAC and associated biofilm shall be performed by PNNL in accordance to the conditions and requirements set forth in CHPRC's contract with PNNL to perform this very analysis.

TRVL- 13- 040

LABORATORY SECTION	RECEIVED BY	TITLE	DATE/TIME
FINAL SAMPLE DISPOSITION	DISPOSAL METHOD	DISPOSED BY	DATE/TIME

PRINTED ON 3/26/2013

A-6003-618 (REV 2)

CH2MHill Plateau Remediation Company	CHAIN OF CUSTODY/SAMPLE ANALYSIS REQUEST			F13-023-004	PAGE 1 OF 1
COLLECTOR C. Fulton 4-2-13	COMPANY CONTACT EVANS, RT	TELEPHONE NO. 373-7924	PROJECT COORDINATOR EVANS, RT	PRICE CODE 9N	DATA TURNAROUND
SAMPLING LOCATION 289-T, FBR-A Top of Bed (15ft) 20'	PROJECT DESIGNATION 200W Pump & Treat - Microbiological/Chemical Analysis - Other Solid		SAF NO. F13-023	AIR QUALITY ☐	45 Days / 45 Days
ICE CHEST NO. GWS-186-2	FIELD LOGBOOK NO. HNF-N-507-24	ACTUAL SAMPLE DEPTH 19'	COA 303110ES10	METHOD OF SHIPMENT GOVERNMENT VEHICLE	ORIGINAL
SHIPPED TO Environmental Sciences Laboratory	OFFSITE PROPERTY NO. SEE PTR		BILL OF LADING/AIR BILL NO.		

MATRIX*	POSSIBLE SAMPLE HAZARDS/ REMARKS		
A=Air DL=Drum Liquids DS=Drum Solids L=Liquid O=Oil S=Soil SE=Sediment T=Tissue V=Vegetation W=Water WI=Wipe X=Other	**Contains Radioactive Material at concentrations that may or may not be regulated for transportation per 49 CFR/IATA Dangerous Goods Regulations but are not releasable per DOE Order 458.1.**	PRESERVATION	None
		HOLDING TIME	6 Months
		TYPE OF CONTAINER	P
		NO. OF CONTAINER(S)	2
		VOLUME	1L
	SPECIAL HANDLING AND/OR STORAGE	SAMPLE ANALYSIS	Generic Testing (No CAS);

SAMPLE NO.	MATRIX*	SAMPLE DATE	SAMPLE TIME	
B2P0M9	OTHER SOLID	4-2-13	0505	✓

CHAIN OF POSSESSION		SIGN/ PRINT NAMES	
RELINQUISHED BY/REMOVED FROM C. Fulton	DATE/TIME 4/2/13 1020	RECEIVED BY/STORED IN	DATE/TIME 4/2/13 1020
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME

SPECIAL INSTRUCTIONS

** The 200 Area S&GRP Characterization and Monitoring Sampling and Analysis GKI applies to this SAF.
***Analysis of the GAC and associated biomass shall be performed by PNNL in accordance to the conditions and requirements set forth in CHPRC's contract with PNNL to perform this very analysis.
Analysis of the GAC and associated biofilm shall be performed by PNNL in accordance to the conditions and requirements set forth in CHPRC's contract with PNNL to perform this very analysis.

TRVL 13- 040

LABORATORY SECTION	RECEIVED BY	TITLE	DATE/TIME
FINAL SAMPLE DISPOSITION	DISPOSAL METHOD	DISPOSED BY	DATE/TIME

PRINTED ON 4/1/2013

A-6003-618 (REV 2)

CH2MHill Plateau Remediation Company	CHAIN OF CUSTODY/SAMPLE ANALYSIS REQUEST			F13-023-006	PAGE 1 OF 1
COLLECTOR C. Fulton	COMPANY CONTACT EVANS, RT	TELEPHONE NO. 373-7924	PROJECT COORDINATOR EVANS, RT	PRICE CODE 9N	DATA TURNAROUND
SAMPLING LOCATION 289-T, FBR-A Bottom of Bed (27ft)	PROJECT DESIGNATION 200W Pump & Treat - Microbiological/Chemical Analysis - Other Solid		SAF NO. F13-023	AIR QUALITY ☐	45 Days / 45 Days
ICE CHEST NO. GWS 186-2	FIELD LOGBOOK NO. HNF N: 507-24	ACTUAL SAMPLE DEPTH 27'	COA 303110ES10	METHOD OF SHIPMENT GOVERNMENT VEHICLE	ORIGINAL
SHIPPED TO Environmental Sciences Laboratory	OFFSITE PROPERTY NO. SEE PTR		BILL OF LADING/AIR BILL NO.		

MATRIX*	POSSIBLE SAMPLE HAZARDS/ REMARKS		
A=Air DL=Drum Liquids DS=Drum Solids L=Liquid O=Oil S=Soil SE=Sediment T=Tissue V=Vegetation W=Water WI=Wipe X=Other	**Contains Radioactive Material at concentrations that may or may not be regulated for transportation per 49 CFR/IATA Dangerous Goods Regulations but are not releasable per DOE Order 458.1.**	PRESERVATION	None
		HOLDING TIME	6 Months
		TYPE OF CONTAINER	P
		NO. OF CONTAINER(S)	2
		VOLUME	1L
	SPECIAL HANDLING AND/OR STORAGE	SAMPLE ANALYSIS	Generic Testing (No CAS);

SAMPLE NO.	MATRIX*	SAMPLE DATE	SAMPLE TIME	
B2P0N1	OTHER SOLID	4-2-13	0911	✓

4/2/13

CHAIN OF POSSESSION		SIGN/ PRINT NAMES		SPECIAL INSTRUCTIONS
RELINQUISHED BY/REMOVED FROM C. Fulton	DATE/TIME 4-2-13 1020	RECEIVED BY/STORED IN	DATE/TIME 4/2/13 1020	** The 200 Area S&GRP Characterization and Monitoring Sampling and Analysis GKI applies to this SAF. ***Analysis of the GAC and associated biomass shall be performed by PNNL in accordance to the conditions and requirements set forth in CHPRC's contract with PNNL to perform this very analysis. ****Analysis of the GAC and associated biofilm shall be performed by PNNL in accordance to the conditions and requirements set forth in CHPRC's contract with PNNL to perform this very analysis. TRVL- 13- 040
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME	

LABORATORY SECTION	RECEIVED BY	TITLE	DATE/TIME
FINAL SAMPLE DISPOSITION	DISPOSAL METHOD	DISPOSED BY	DATE/TIME

PRINTED ON 4/1/2013

A-6003-618 (REV 2)

CH2MHill Plateau Remediation Company	CHAIN OF CUSTODY/SAMPLE ANALYSIS REQUEST			F13-023-013	PAGE 1 OF 1
COLLECTOR C. Fulton	COMPANY CONTACT EVANS, RT	TELEPHONE NO. 373-7924	PROJECT COORDINATOR EVANS, RT	PRICE CODE 9N AIR QUALITY ☐	DATA TURNAROUND 45 Days / 45 Days
SAMPLING LOCATION 289-T, FBR-A, Top of Bed ~~(15-ft)~~ KS 4/10/13	PROJECT DESIGNATION 200W Pump & Treat - Microbiological/Chemical Analysis - Other Solid		SAF NO. F13-023		
ICE CHEST NO. GWS-103	FIELD LOGBOOK NO. HNF-N-507-21	ACTUAL SAMPLE DEPTH 29'	COA 303110ES10	METHOD OF SHIPMENT GOVERNMENT VEHICLE	ORIGINAL
SHIPPED TO Research Technology Laboratory ~~Environmental Sciences Laboratory~~ Bldg. 520 RE 4/10/13	OFFSITE PROPERTY NO. N/A		BILL OF LADING/AIR BILL NO. N/A		

MATRIX*	POSSIBLE SAMPLE HAZARDS/ REMARKS		
A=Air DL=Drum Liquids DS=Drum Solids L=Liquid O=Oil S=Soil SE=Sediment T=Tissue V=Vegetation W=Water WI=Wipe X=Other	**Contains Radioactive Material at concentrations that may or may not be regulated for transportation per 49 CFR/IATA Dangerous Goods Regulations but are not releasable per DOE Order 458.1.**	PRESERVATION	None
		HOLDING TIME	6 Months
		TYPE OF CONTAINER	P
		NO. OF CONTAINER(S)	2
		VOLUME	1L
	SPECIAL HANDLING AND/OR STORAGE	SAMPLE ANALYSIS	Generic Testing (No CAS);

SAMPLE NO.	MATRIX*	SAMPLE DATE	SAMPLE TIME	
B2P100	OTHER SOLID	4-16-13	0807	✓

CHAIN OF POSSESSION		SIGN/ PRINT NAMES	
RELINQUISHED BY/REMOVED FROM C. Fulton	DATE/TIME 4-16-13 1028	RECEIVED BY/STORED IN	DATE/TIME 4-16-13 1028
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME

SPECIAL INSTRUCTIONS

TRVL-13-050 ** The 200 Area S&GRP Characterization and Monitoring Sampling and Analysis GKI applies to this SAF.☐☐** WSCF requires a minimum of 100g of the biofilm to perform the analysis.☐** Separation of the biofilm from the carbon will be performed by PNNL.☐** WSCF requests that the biofilm be preserved in its natural state; and not centrifuged into a pellet.

TRVL- 13- 050

LABORATORY SECTION	RECEIVED BY	TITLE	DATE/TIME
FINAL SAMPLE DISPOSITION	DISPOSAL METHOD	DISPOSED BY	DATE/TIME

PRINTED ON 4/4/2013

A-6003-618 (REV 2)

CH2MHill Plateau Remediation Company	CHAIN OF CUSTODY/SAMPLE ANALYSIS REQUEST			F13-023-014	PAGE 1 OF 1
COLLECTOR C. Fulton	COMPANY CONTACT EVANS, RT	TELEPHONE NO. 373-7924	PROJECT COORDINATOR EVANS, RT	PRICE CODE 9N AIR QUALITY ☐	DATA TURNAROUND 45 Days / 45 Days
SAMPLING LOCATION 289-T, FBR-A, Middle of Bed ~~(21 ft)~~ KS 4/10/13	PROJECT DESIGNATION 200W Pump & Treat - Microbiological/Chemical Analysis - Other Solid		SAF NO. F13-023		
ICE CHEST NO. GWS-103	FIELD LOGBOOK NO. HNF-N-507-21	ACTUAL SAMPLE DEPTH 35'	COA 303110ES10	METHOD OF SHIPMENT GOVERNMENT VEHICLE	ORIGINAL
SHIPPED TO Research Technology ~~Environmental Sciences Laboratory~~ / Bldg. 520 RE 4/10/13	OFFSITE PROPERTY NO. N/A		BILL OF LADING/AIR BILL NO. N/A		

MATRIX*	POSSIBLE SAMPLE HAZARDS/ REMARKS		
A=Air DL=Drum Liquids DS=Drum Solids L=Liquid O=Oil S=Soil SE=Sediment T=Tissue V=Vegetation W=Water WI=Wipe X=Other	**Contains Radioactive Material at concentrations that may or may not be regulated for transportation per 49 CFR/IATA Dangerous Goods Regulations but are not releasable per DOE Order 458.1.**	PRESERVATION	None
		HOLDING TIME	6 Months
		TYPE OF CONTAINER	P
		NO. OF CONTAINER(S)	2
		VOLUME	1L
	SPECIAL HANDLING AND/OR STORAGE	SAMPLE ANALYSIS	Generic Testing (No CAS);

SAMPLE NO.	MATRIX*	SAMPLE DATE	SAMPLE TIME	
B2P101	OTHER SOLID	4-16-13	0815	✓

CHAIN OF POSSESSION		SIGN/ PRINT NAMES	
RELINQUISHED BY/REMOVED FROM C. Fulton	DATE/TIME 4-16-13 1028	RECEIVED BY/STORED IN Daniel Evans	DATE/TIME 4-16-13 1028
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME

SPECIAL INSTRUCTIONS

TRVL-13-050 ** The 200 Area S&GRP Characterization and Monitoring Sampling and Analysis GKI applies to this SAF.☐☐** WSCF requires a minimum of 100g of the biofilm to perform the analysis.☐** Separation of the biofilm from the carbon will be performed by PNNL.☐** WSCF requests that the biofilm be preserved in its natural state; and not centrifuged into a pellet.

TRVL- 13- 050

LABORATORY SECTION	RECEIVED BY	TITLE	DATE/TIME
FINAL SAMPLE DISPOSITION	DISPOSAL METHOD	DISPOSED BY	DATE/TIME

PRINTED ON 4/4/2013

A-6003-618 (REV 2)

CH2MHill Plateau Remediation Company	CHAIN OF CUSTODY/SAMPLE ANALYSIS REQUEST			F13-023-015	PAGE 1 OF 1
COLLECTOR C. Fulton	COMPANY CONTACT EVANS, RT	TELEPHONE NO. 373-7924	PROJECT COORDINATOR EVANS, RT	PRICE CODE 9N	DATA TURNAROUND
SAMPLING LOCATION 289-T, FBR-A, Bottom of Bed ~~(27 ft)~~ KS 4/16/13	PROJECT DESIGNATION 200W Pump & Treat - Microbiological/Chemical Analysis - Other Solid		SAF NO. F13-023	AIR QUALITY ☐	45 Days / 45 Days
ICE CHEST NO. GWS-103	FIELD LOGBOOK NO. HNF-N-507-21	ACTUAL SAMPLE DEPTH 37'	COA 303110ES10	METHOD OF SHIPMENT GOVERNMENT VEHICLE	ORIGINAL
SHIPPED TO ~~Environmental Sciences Laboratory~~ Research Technology / Bldg 5120 RE 4/9/13	OFFSITE PROPERTY NO. N/A		BILL OF LADING/AIR BILL NO. N/A		

MATRIX*	POSSIBLE SAMPLE HAZARDS/ REMARKS		
A=Air DL=Drum Liquids DS=Drum Solids L=Liquid O=Oil S=Soil SE=Sediment T=Tissue V=Vegetation W=Water WI=Wipe X=Other	**Contains Radioactive Material at concentrations that may or may not be regulated for transportation per 49 CFR/IATA Dangerous Goods Regulations but are not releasable per DOE Order 458.1.**	PRESERVATION	None
		HOLDING TIME	6 Months
		TYPE OF CONTAINER	P
		NO. OF CONTAINER(S)	2
		VOLUME	1L
	SPECIAL HANDLING AND/OR STORAGE	SAMPLE ANALYSIS	Generic Testing (No CAS);

SAMPLE NO.	MATRIX*	SAMPLE DATE	SAMPLE TIME	
B2P102	OTHER SOLID	4-16-13	0818	✓

CHAIN OF POSSESSION		SIGN/ PRINT NAMES	
RELINQUISHED BY/REMOVED FROM C. Fulton	DATE/TIME 4-16-13 1028	RECEIVED BY/STORED IN	DATE/TIME 4-16-13 1028
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME
RELINQUISHED BY/REMOVED FROM	DATE/TIME	RECEIVED BY/STORED IN	DATE/TIME

SPECIAL INSTRUCTIONS

TRVL-13-050 ** The 200 Area S&GRP Characterization and Monitoring Sampling and Analysis GKI applies to this SAF.☐☐** WSCF requires a minimum of 100g of the biofilm to perform the analysis.☐** Separation of the biofilm from the carbon will be performed by PNNL.☐** WSCF requests that the biofilm be preserved in its natural state; and not centrifuged into a pellet.

TRVL-13-050

LABORATORY SECTION	RECEIVED BY	TITLE	DATE/TIME
FINAL SAMPLE DISPOSITION	DISPOSAL METHOD	DISPOSED BY	DATE/TIME

PRINTED ON 4/4/2013

A-6003-618 (REV 2)

Appendix B

EPS Procedures

Appendix B

EPS Procedures

The step-by-step procedures used to make the EPS measurements are shown in this appendix.

B.1 Cation Exchange

A limited number of methods have been developed to extract Extracellular Polymeric Substances (EPS) from Biofilms. One method involves the use of cation exchange resins as described by Jahn and Nielsen (1995). In the procedure, the prepared resin is added to the biofilm, and stirred in an ice bath. The contents are transferred to a centrifuge and the supernate decanted. After washing and filtering, the EPS is ready for analysis.

B.1.1 Prepare Resin

NOTE: Wear gloves during the procedure.

1. Clean all glassware using alcohol or process it in an autoclave.
2. Clean all centrifuge tubes using alcohol.
3. Obtain ~10 g of Dowex Marathon C resin and add it to a 50 mL centrifuge tube.
4. Add ~25 mL of DIW and gently shake for 1 hour.
5. Gently centrifuge the resin and decant the DIW off the resin.
6. Repeat steps 2 and 3 three times.
7. For the last wash, add 25 mL of extraction buffer to the resin and shake overnight
8. Gently centrifuge the resin and decant the extraction buffer. The resin is ready to use.

B.1.2 Procedure

1. Clean all glassware using alcohol or run it through an autoclave.
2. Clean all centrifuge tubes with alcohol.
3. Clean stir bar with alcohol. Wear gloves, <u>do not touch with fingers!</u>
4. Combine the resin and biomaterial in a small bottle.
5. Add 20 mL of extraction buffer to the centrifuge tube that contained the resin.
6. Remove all the resin from the centrifuge tube.
7. Add a stir bar to bottle.
8. Place the bottle in an ice bath and stir for 3 hours.
9. Transfer the contents of the bottle to a centrifuge tube.

10. Centrifuge at 12,000g at 4°C for 15 minutes
11. Decant supernatant
12. Transfer the solids to a 100 mL graduated cylinder and bring to 100mL.
13. Set up a 0.2 µm filter cellulose acetate apparatus and pre-wet with extraction buffer.
14. Discard any extraction buffer that passed through the filter.
15. Filter the contents of the 100 mL graduated cylinder.
16. After filtering, transfer the filtrate to a clean 100 mL volumetric flask and bring to volume.
17. Transfer solution to a clean bottle and place in a refrigerator.

B.2 Carbohydrate Determination

Carbohydrate were determined by the anthrone method modified from the Manual of Methods for General Bacteriology (1981) with a glucose standard. Anthrone is a tricyclic aromatic ketone and is used in colorimetric determination of carbohydrates. The method consists of adding a solution of anthrone to the samples and standards. The samples are shaken and then placed in a heated water bath. After heating the samples are chilled and then measured spectrophotometrically at 625nm.

NOTE: Prior to making solutions, calibrate the necessary pipets and record the data

B.2.1 Reagents

The 0.1% anthrone solution in 75% (v/v) sulfuric acid must be prepared at least 2 hours before use and freshly prepared each day.

1. Prepare a 75% sulfuric acid (SpG=1.6692) solution by adding 193 mL of 95-98% Optima Sulfuric acid to a 250 mL volumetric flask.
2. Carefully and slowly bring the volume up 250 mL by adding DIW while stirring the solution.
3. After mixing the 75% sulfuric acid solution for 5 minutes, transfer approximately 40 mL to a 50mL volumetric flask.
4. While mixing the 40mL of 75% sulfuric acid, add 0.086 g of 97% anthrone to the flask.
5. After the anthrone dissolves, add additional 75% sulfuric acid to the flask and bring to volume.
6. Transfer to a clean, labeled glass bottle
7. Store in refrigerator until needed.

B.2.2 Stock Standard

New standards are prepared each day using a 1 g/L stock solution of glucose.

1. Add 0.1 grams of glucose, along with ~80 mL of DIW to a 100mL volumetric flask and mix until dissolved.
2. After the glucose has dissolved, bring the solution to volume.

3. Transfer the solution to a clean, labeled poly bottle. This is the stock solution.
4. Calculate the actual concentration for use in generating the standard solutions.
5. Store in a refrigerator until needed.

B.2.3 Standards

Prepare standards of 0, 5, 10, 20, 30 and 40 µg/mL glucose

NOTE: The actual volume of stock used to prepare the working stock in step 1 will on the concentration of the standard stock solution.

1. Prepare a working solution by adding approximately 2.5 mL of stock solution to a 25 mL volumetric flask. Mix. The concentration is 100 mg/L or 100 µg/mL.
2. Add DIW and bring to volume.
3. Transfer the solution to a clean, labeled poly bottle.
4. Pipet approximately 0, 0.5, 1.0, 2.0, 3.0, or 4.0 mL of the 100 mg/L stock solution into separate 10 mL volumetric flasks. Bring to volume with DIW.

B.2.4 Procedure

1. Turn on the spectrometer per manufacturer instructions.
2. Pipette a range of sample volumes (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0) into a series of boiling tubes. Adjust the final volumes to 1 mL using DIW.
3. Transfer 1 mL of each standard into Pyrex glass test tubes.
4. Chill all tubes in an ice water bath.
5. Add 5 mL of <u>cold</u> anthrone reagent to each test tube.
6. Close test tubes with stoppers or lids and <u>immediately</u> shake on vortex mixer. Return the tubes to the ice bath.
7. Transfer all test tubes into 100°C water bath for 10 exactly minutes.
8. After heating, the test tubes are placed into an ice water bath at until they are ready to be measured.
9. The samples and standards are measured spectrophotometrically at 625 nm.

B.3 Humic Substances Determination

Humic substances were determined by the Folin-Ciocalteau Phenol reagent using a method described by Box (1983) with a humic acid standard. The concentration of humic substances can be used to correct the total amount of protein measured using the method described by Lowry (1951). A detailed description of the procedure and corrective equations were provided by Frolund et al. (1995). The humic substances method involves successive additions of sodium carbonate and Folin-Ciocalteau phenol reagent to a sample. After each addition, the samples were kept at room temperature for 60 minutes and then analyzed at 750 nm using a spectrophotometer.

B.3.1 Reagents

- Sodium Carbonate

1. Prepare a 200 g/L solution of sodium carbonate by adding 10 g of Na_2CO_3 to a 50 mL volumetric flask.
2. Add ~40 mL of DIW to the flask and mix.
3. After the Na_2CO_3 has dissolved, bring to volume and transfer to a clean, labeled poly bottle.

- Folin-Ciocalteau Phenol reagent

1. Prepare 25 mL of 1 N Folin-Ciocalteu reagent by adding 12.5 mL of 2 N Sigma-Aldrich Folin-Ciocalteu phenol reagent to a volumetric flask.
2. Add 10 mL of DIW and mix.
3. Bring to volume and transfer to a clean, labeled dark glass bottle.

- Humic Acid Standard

1. Using humic acid from Alfa Aesar, a standard solution by adding 0.010g to a 100 mL volumetric flask.
2. Add approximately 90 mL of DIW and mix.
3. After the humic acid has dissolved, bring to volume and calculate the correct concentration.
4. Transfer to a labeled glass bottle. This is the working stock solution. Store at 3^oC.
5. Prepare 6 stock solutions with a concentration of 0, 5, 10, 20, 35, and 50 mL/L.
6. Add approximately 0, 0.575, 1.15, 2.30, 4.02 or 5.75 mL to a 10 mL volumetric flask. The exact amount were determined after the correct concentration of the working stock solution has been calculated.
7. Bring to volume, mix and transfer to clean labeled glass bottles.

B.3.2 Procedure

1. Prepare two duplicate 20 mL sample vials, five standard sample vials and a DIW blank.
2. Add 10 mL of sample, standard or DIW to separate vials.
3. Add 1.50 mL of sodium carbonate and 0.5 mL of Folin-Ciocalteau Phenol reagent to each tube.
4. Mix and let stand at room temperature for 60 minutes.
5. Analyze the samples, standards and a DIW blank at 750 nm.

B.4 Protein Determination

Protein was determined by the Lowry method (1951) using a bovine serum albumin standard and corrected for the humic substances. Protein and humic compounds can be determined by adding or omitting copper sulfate in the Lowry method. However, humic substances will be determined by a

method described by Box (1983) which is in another section. The Lowry method uses copper sulfate to develop a copper complex that is analyzed at 750 nm using a spectrophotometer.

B.4.1 Reagents

- Solution A

1. Prepare 2% sodium carbonate (w/w) in a 0.1M sodium hydroxide solution by adding 0.4 g of NaOH to a 100 mL volumetric flask.
2. Add ~75 mL to the flask and mix.
3. After the NaOH has dissolved, add 2 g of Na_2CO_3 to the flask and mix.
4. After the Na_2CO_3 has dissolved, bring to volume and transfer to a clean, labeled poly bottle.

- Solution B

1. Prepare 0.5% copper sulfate (w/w) in a 1% sodium tartrate by adding 0.593 g of sodium tartrate dihydrate ($C_4H_4Na_2O_6$-$2H_2O$ FW= 230.08) to a 50 mL volumetric flask
2. Add ~40 mL of DIW and mix.
3. After the $C_4H_4Na_2O_6$-$2H_2O$ has dissolved, add 0.39 g of copper sulfate pentahydrate ($CuSO_4$-$5H_2O$ FW=249.69)
4. After the $CuSO_4$-$5H_2O$ has dissolved, bring to volume and transfer to a clean, labeled poly bottle.

- Solution C

NOTE: Solution C is prepared just before use and is discarded at the end of the day.

1. Solution C is prepared by mixing 50 mL of solution A with 1 mL of solution B.
2. Solution D
3. NOTE: If humic substances are to be determined, prepare the Folin-Ciocalteau phenol reagent per the Humic Substance Determination procedure. In this procedure 4 mL of Solution D reagent are prepared.
4. Dilute the Folin-Ciocalteau phenol reagent with 2 N HCl 50% by transferring 2 mL of reagent into a labeled, dark glass bottle.
5. Add 2 mL of DIW to the bottle and mix.
6. Store in the dark.

B.4.2 Standards

1. Using bovine serum Albumin-Fraction V, also known as Cohn Fraction V, prepare a stock solution by adding 0.01 g to a 100 mL volumetric flask.
2. Calculate the actual concentration and record on storage bottle label.
3. Bring to volume and transfer to a clean, labeled dark glass bottle. This is your stock solution. (~0.1 mg/mL) Store at 3^oC.

4. Prepare five standard solutions containing 0, 5, 10, 25, 50 µg/mL of BSA by adding approximately 0, 0.26, 1.53, 1.33 or 2.66 mL stock to separate 10 mL volumetric flasks. Actual volume will be determined by stock solution concentration.
5. Add DIW, mix and bring to volume.
6. Transfer to a clean, labeled dark glass bottle. Store at 3°C.

B.4.3 Procedure

1. Add 0.2 mL of sample, standard or blank to separate glass test tubes. Prepare duplicate test tubes for sample analysis.
2. Add 1 mL of Solution C to the glass tube.
3. Mix well and let stand for 10 minutes or longer at room temperature.
4. Add 0.1 mL of Solution D to the test tube and immediately mix using a vortex mixer.
5. Allow the test tube to stand for 30 minutes or longer to develop full color.
6. Analyze the samples at 750 nm.

B.5 DNA Determination

DNA will be determined by the DAPI method described by Brunk et al. (1979) with a salmon testes standard. DAPI or 4', 6-diamidino-2-phenylindole (CAS # 28718-90-3) is a fluorescent stain that binds strongly to A-T rich regions in DNA. It can pass through an intact cell membrane; therefore, it can be used to stain both live and fixed cells. Fluorescent quenching of heavy metal cations by adding magnesium or calcium chloride. Solutions of low ionic strength affect the fluorescence so a buffer of sodium chloride, EDTA and TRIs is added as needed. The samples are analyzed spectrophotometrically at 360 nm (excitation) and 450 nm (emission).

B.5.1 Reagents:

- Buffer

1. Prepare 100 mL 100 mM sodium chloride, 10 mM EDTA and 10 mM Tris buffer by adding 0.584g of NaCl to a 100 mL volumetric flask.
2. Add 80 mL of DIW and mix.
3. Add 0.292 g of EDTA (FW=292.24) to the volumetric flask.
4. Add 0.121 g of Tris (FW=121.14) to the volumetric flask.
5. Bring to volume and transfer to a clean labeled poly bottle.
6. Verify the pH is 7.0.

- DAPI solution

1. Prepare a solution of DAPI (FW= 350.25) by adding 0.001 g to a 25 mL volumetric flask.
2. Heat the solution as necessary. After the DAPI dissolves, bring to volume.

3. Transfer to a clean, labeled glass bottle.
4. Determine the exact concentration of the solution. This becomes the stock solution. Wrap the bottle in foil and store in refrigerator.

- Salmon Testes

1. Prepare a working solution of salmon testes by transferring the 1 mL standard (10 mg/mL) to a 10 mL volumetric flask.
2. Bring to volume and transfer to a clean, labeled glass bottle.
3. Store in -20C freezer.
4. Prepare a 20 µg/mL standard by adding 0.2 mL of working solution to a 10 mL separate volumetric flask.
5. Bring to volume and transfer to a clean, labeled glass bottle. Store in refrigerator.

B.5.2 Procedure

1. Pipette 200 µL of buffer into enough wells for triplicate of each sample in a sterile black polystyrene 96 well assay plate.
2. Add 7.5 µL of DAPI to each well.
3. Replace lid and gently stir the plate by moving it back and forth on the bench top. Do not allow the liquid to go over the lip of each well.
4. Cover with aluminum foil.
5. Wait 5 minutes.
6. Analyze the standard spectrophotometrically at 360 nm (excitation) and 450 nm (emission).

   Note: The first analysis is the blank.
7. Add 15 µL of sample to the sample well. Repeat steps 3 to 6.
8. Repeat step 7 three more times for a total addition of 60 µL of sample to each well.
9. Add 15 µL of 20 µg/mL standard to each well containing sample. Repeat steps 3-6.
10. Repeat step 9 three more times for a total addition of 60 µL of 20 µg/mL standard to the wells already containing 60 µL of sample.

B.5.3 Uronic Acid Determination

Uronic acids will be determined by the m-hydroxydiphenyl sulphuric acid method described by Kintner and van Burren (1982). m-hydroxydiphenyl is also known as 3-phenylphenol (CAS# 580-51-8). D-glucuronic acid will be used as a standard. This method consists of adding a sulfuric acid/sodium tetraborate solution to a cold sample or standard. After mixing, the samples are heated to 100C and then cooled. Next, the m-hydroxydiphenyl was added and mixed. After pouring the samples into glass cuvettes, and setting at room temperature, the red chromagen forms. The samples are analyzed at 520 nm on a spectrophotometer. Since carbohydrates also produce a pink chromagen, blanks have to be prepared

but the m-hydroxydiphenyl is replaced with sodium hydroxide. The sample blank absorbance is subtracted from the total absorbance to obtain absorbance due to m-hydroxydiphenyl.

B.5.4 Reagents:

- 0.15% m-hydroxydiphenyl solution

1. Prepare a 0.5% sodium hydroxide solution by adding 0.5 g of NaOH to ~ 90 mL of DIW in a 100 mL volumetric flask. Bring to volume and save in a clean, labeled poly bottle.
2. Add approximately 8 mL of the 0.5% sodium hydroxide solution to a 10 mL volumetric flask.
3. Add 0.0176 g of 85% 3-phenylphenol to the flask. Mix until dissolved.
4. Bring to volume using the 0.5% sodium hydroxide solution.
5. Transfer the m-hydroxydiphenyl solution to a clean, labeled glass bottle.
6. Cover with foil and store in a refrigerator at 3°C.

- Sulfuric acid/tetraborate solution

1. Prepare a 0.0125 M sodium tetraborate by adding 0.251 g of $Na_2B_4O_7$ (FW=201.22) to a 100 mL volumetric flask.
2. Add ~90 mL of concentrated Optima grade sulfuric acid. Stir until dissolved.
3. Bring to volume with concentrated sulfuric acid.
4. Transfer to a clean, labeled glass bottle.

B.5.5 Standards

- In the method section of Jahn and Nielsen (1995) they used D-glucuronic acid as a standard rather than the pectin standards used by Kintner and van Buren (1982). Standards between 5 and 75 mg/L will be prepared.

1. Prepare a 100 mg/L working standard solution by adding 0.01 g of D-glucuronic acid to 90 mL of DIW in a 100 mL volumetric flask. Stir until dissolved.
2. Bring to volume.
3. Transfer the solution to a clean, labeled glass bottle. This is the stock solution.

- Prepare 10 mL of standard solutions containing of 5, 10, 25, 50 and 75 mg/L of D-glucuronic acid. Calculate the exact amount of stock solution to add based on the concentration of the stock solution.

1. Add approximately 0.5, 1, 2.5, 5 or 7.5 mL of stock solution to separate 10 mL volumetric flasks. Mix.
2. Bring to volume.
3. Transfer the solution to clean, labeled glass bottles.

B.5.6 Procedure

1. Turn on the spectrometer per manufacturer instructions.

2. Transfer 1mL of sample into duplicate Pyrex glass test tubes (16 x 150 mm).
3. Transfer 1mL of sample into duplicate Pyrex glass test tubes (16 x 150 mm). These are the blanks.
4. Transfer 1 mL of each standard into Pyrex glass test tubes.
5. Place all tubes into an ice water bath to cool.
6. After cooling for 10 minutes, add 6 mL of the H_2SO_4/ $Na_2B_4O_2$ solution while the tubes are in the ice water bath.

NOTE: Proper sample mixing after the addition of reagents is critical. Stopping and starting several times during each mixing is recommended.

7. Using a vortex mixer, carefully mix each tube.
8. After mixing, heat each tube in a water bath set at 100C for exactly 5 minutes.
9. Immediately cool in an ice water bath.
10. Set the blanks aside.
11. Add 0.1 mL of the 0.15% m-hydroxydiphenyl solution to each tube (*except the blank tubes*) and mix using a vortex mixer per the above note.
12. Add 0.1 mL of 0.5% NaOH solution to the blank tubes and mix using a vortex mixer per the above note.
13. Allow the tubes to stand for 20 minutes at room temperature. A haze of tiny bubbles may form when the m-hydroxydiphenyl or NaOH solutions are added.
14. Analyze the standards, blanks and samples on the spectrophotometer at 520 nm.

NOTE: If the concentration of uronic acid is greater than the standard curve, the new samples will have to be diluted, prepared and analyzed.

15. Subtract the blank absorption value from the standards and sample absorption values.

B.6 Glucose-6-phosphate Dehydrogenase Determination

Glucose-6-phosphate dehydrogenase (G6PDH) activity will be determined by a Glucose-6-Phosphate Dehydrogenase activity Assay Kit from Sigma Aldrich. G6PDH is an enzyme in the pentose phosphate pathway that supplies reducing energy to cells by maintaining the level of NADPH. G6PDH reduces nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate reduced ($NADPH_2$) while oxidizing glucose-6-phosphate (G6P). In this analysis method, G6P is oxidized to a compound that is designed to be spectrophotometrically measured at 450 nm. The limit of detection is 0.4 mU.

B.7 Total Organic Carbon

Total Organic Carbon (TOC) is determined on a Shimadzu TOC-Vcsn Carbon Analyzer. A sample is inserted into the instrument and depending on the pH, acid sparged to remove inorganic carbon. The instrument then injects the solution into the analyzer where it is heated and the carbon converts to carbon

dioxide. A carrier gas, containing the CO_2 flows into a gas analyzer where a peak is produced which is proportional to the amount of carbon in the sample.

B.8 Metals Analysis

Metals were analyzed for using inductively coupled plasma optical emission spectrometry (ICP-OES) by Environmental Sciences Laboratory (the on-site analytical lab) following a procedure similar to EPA Method 6010B.

Appendix C

Chemical Data Reports from ALS

Appendix C

Chemical Data Reports from ALS

Copies of the formal reports from ALS Group for the analysis for total volatile solids, total Kjeldahl nitrogen, and sulfide are included in the following pages.

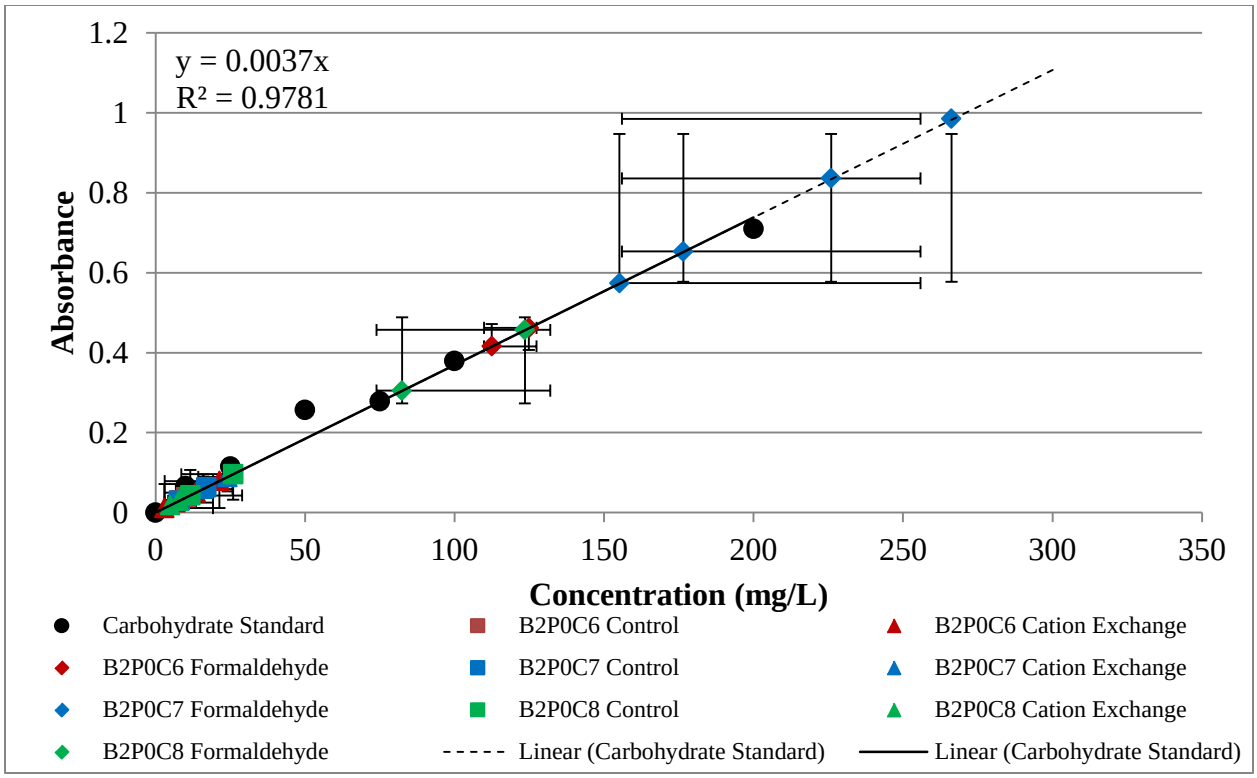

Figure C.1. Carbohydrate results from the standard calibration curve and samples B2P0C6, B2P0C7, and B2P0C8.

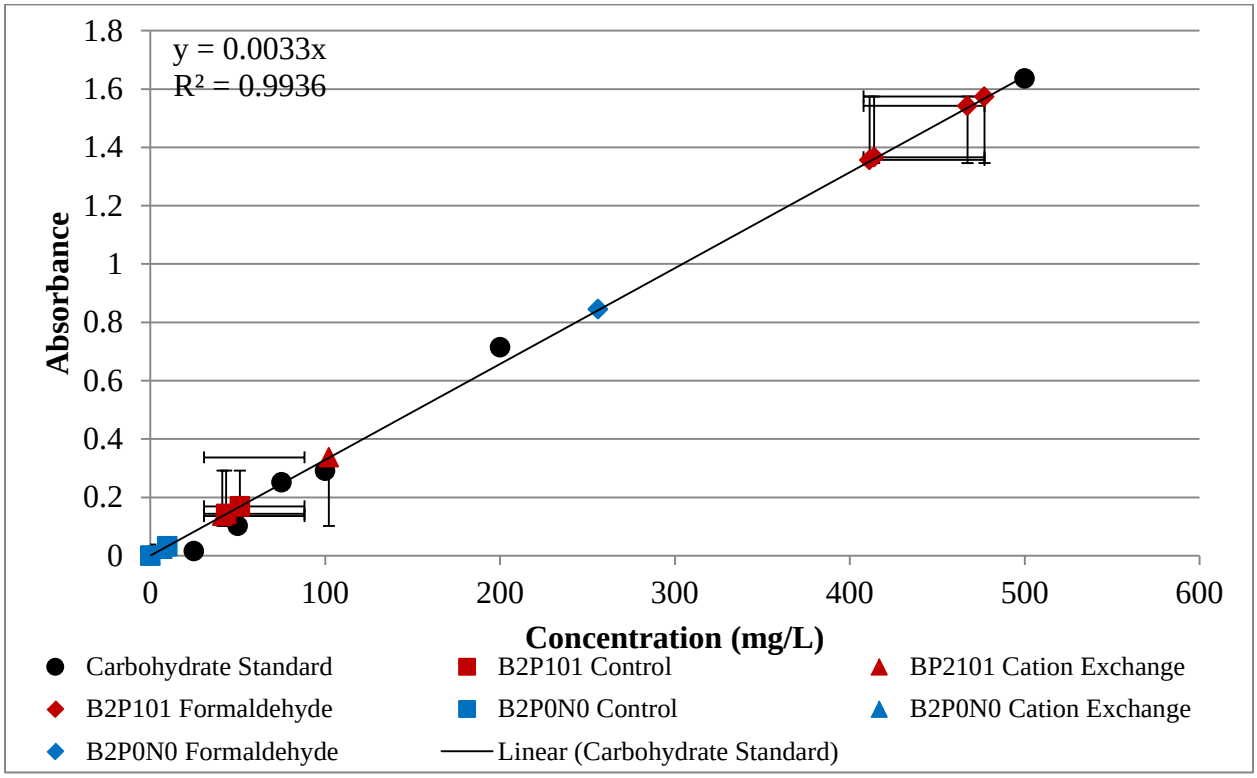

Figure C.2. Carbohydrate results from the standard calibration curve and samples B2P101 and B2P0N0.

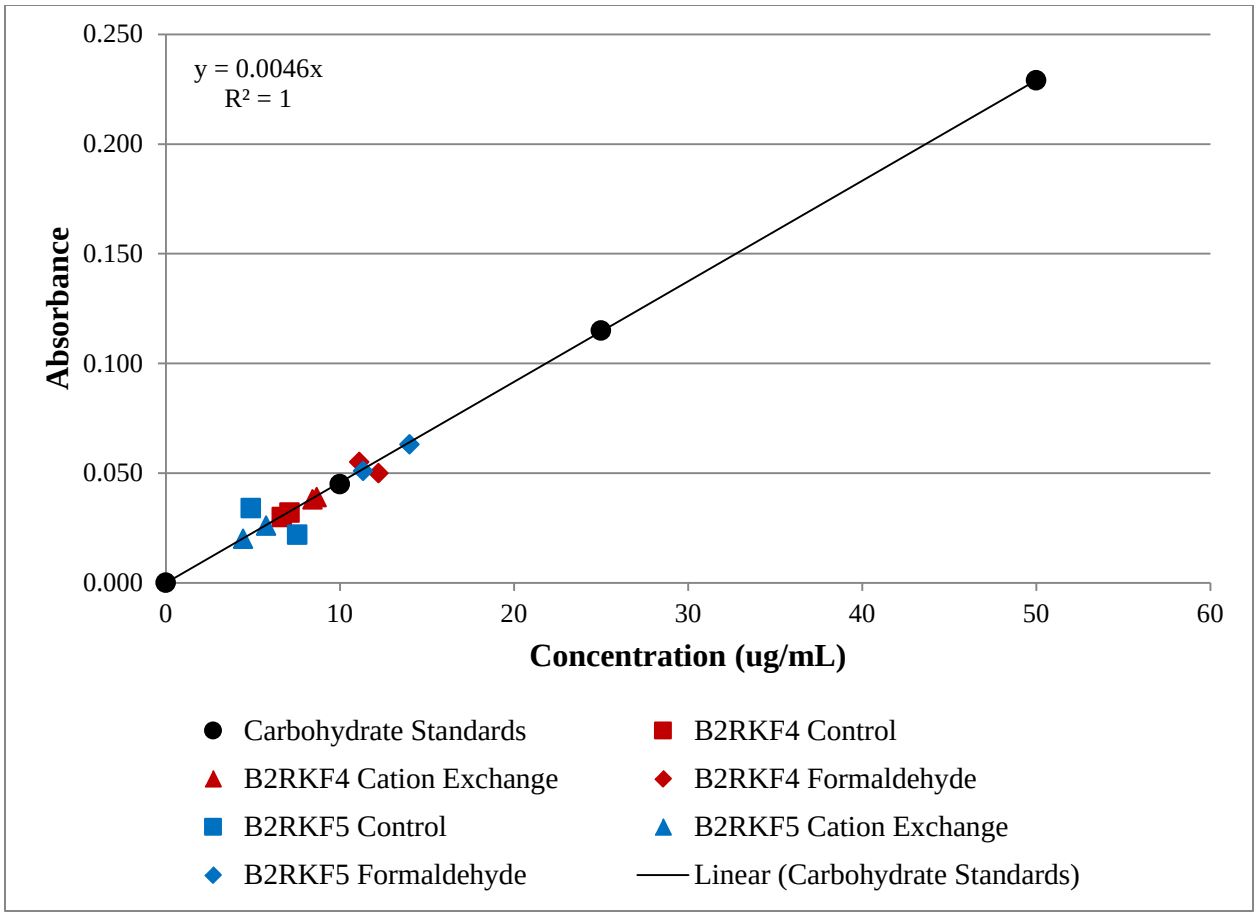

Figure C.3. Carbohydrate results from the standard calibration curve and samples B2RKF4 and B2RKF5.

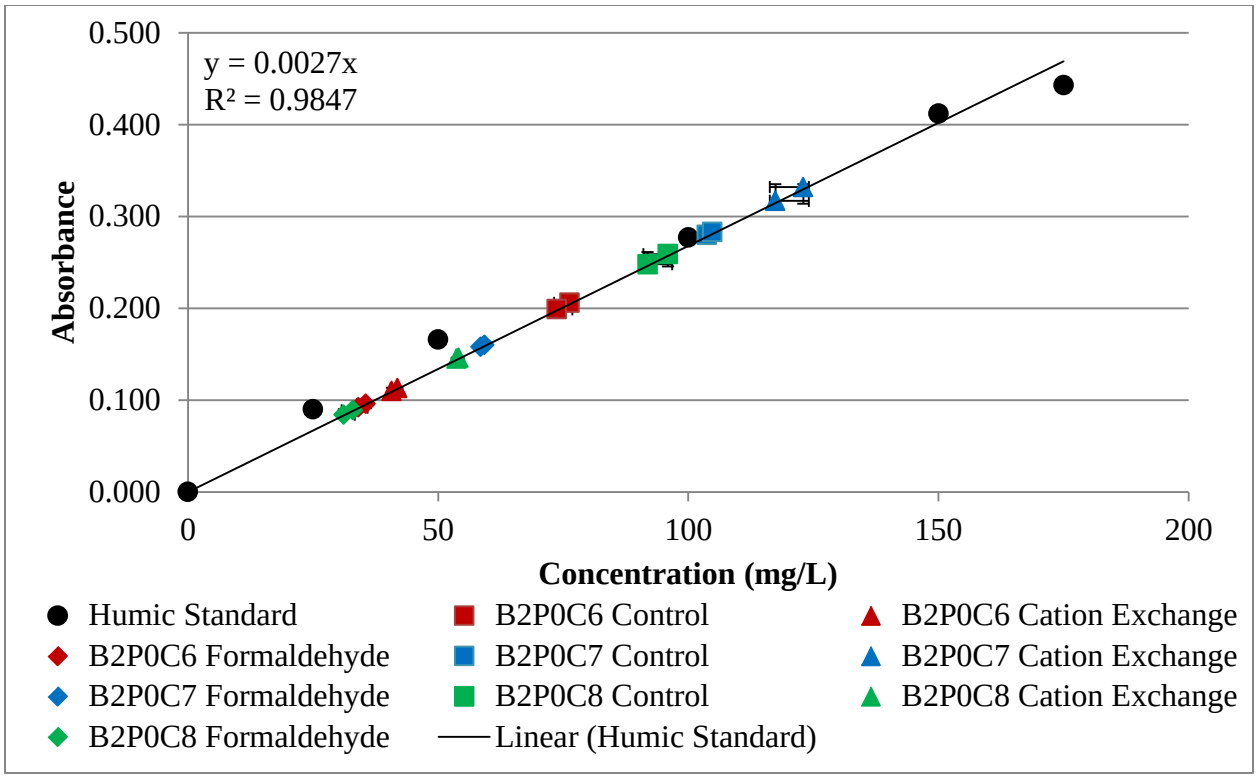

Figure C.4. Humic acid results from the standard calibration curve and samples B2P0C6, B2P0C7, and B2P0C8.

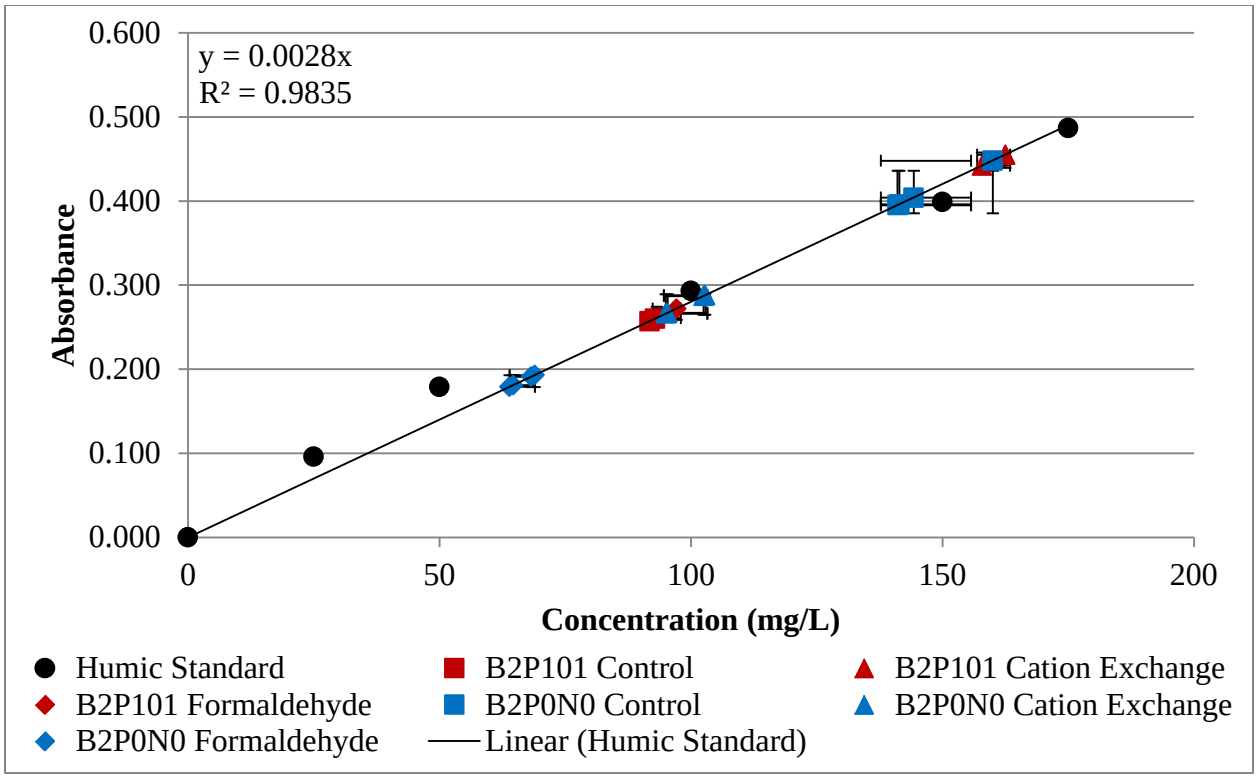

Figure C.5. Humic acid results from the standard calibration curve and samples B2P101 and B2P0N0.

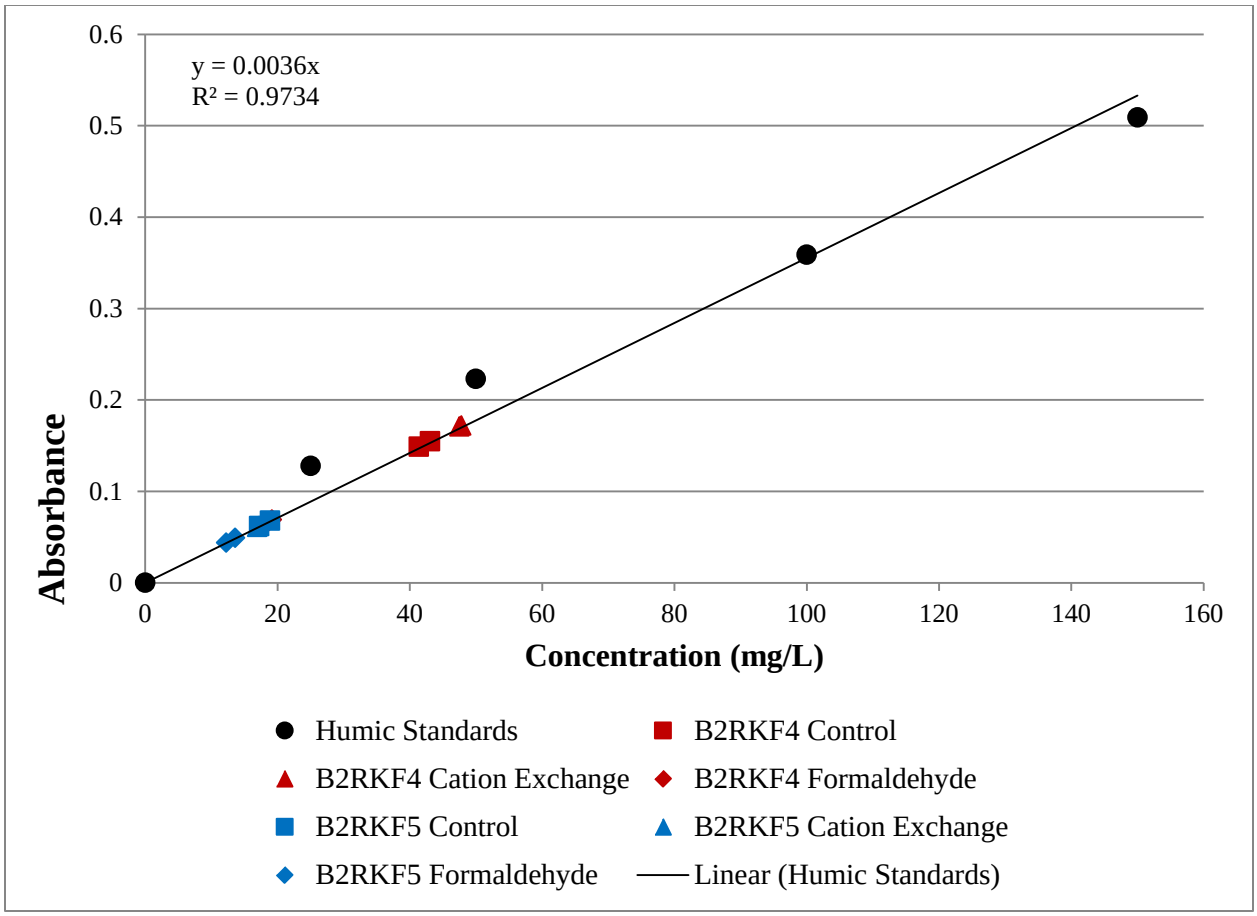

Figure C.6. Humic acid results from the standard calibration curve and samples B2RKF4 and B2RKF5.

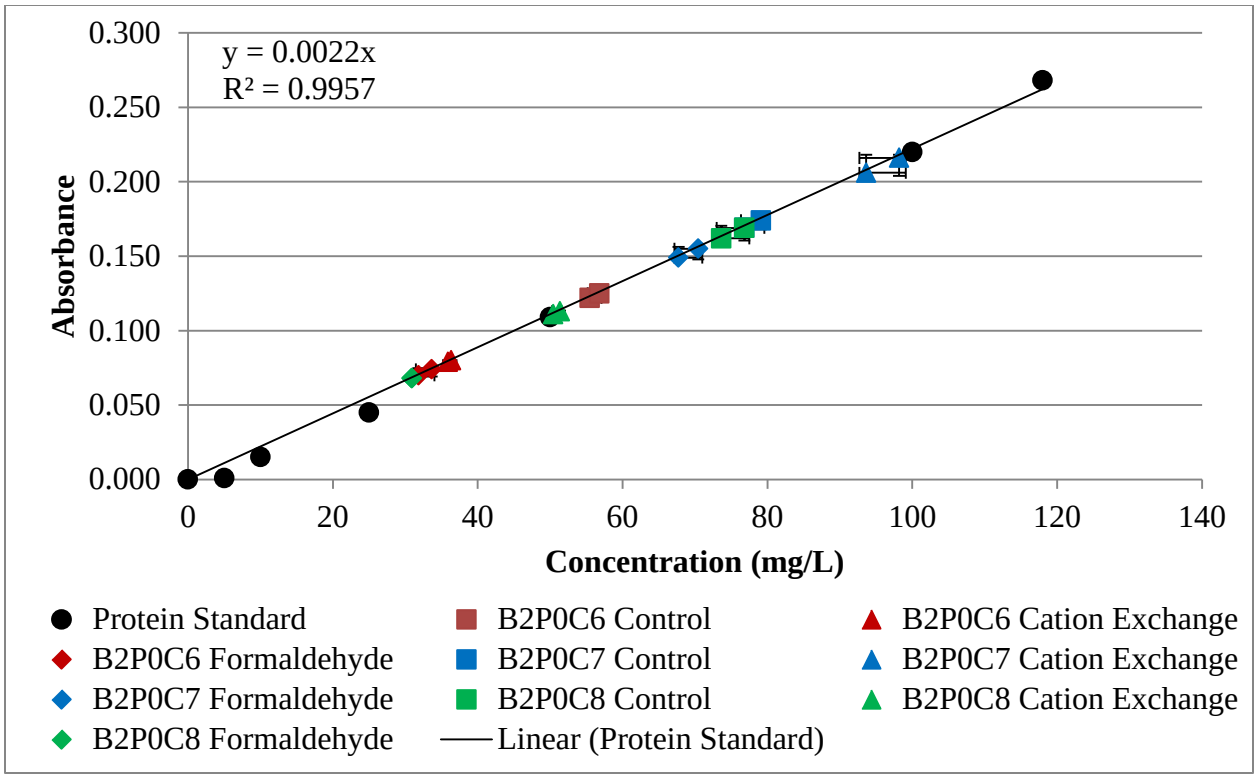

Figure C.7. Protein analysis results from the standard calibration curve and samples B2P0C6, B2P0C7, and B2P0C8.

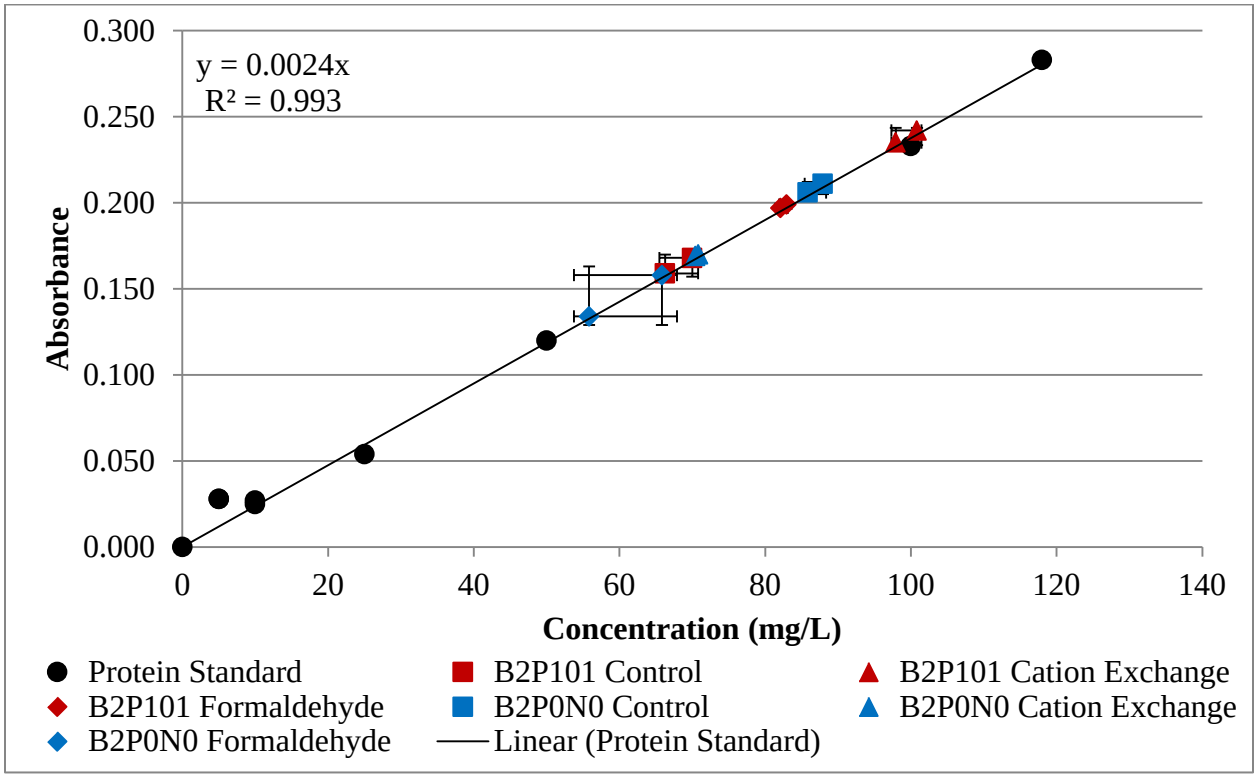

Figure C.8. Protein results from the standard calibration curve and samples B2P101 and B2P0N0.

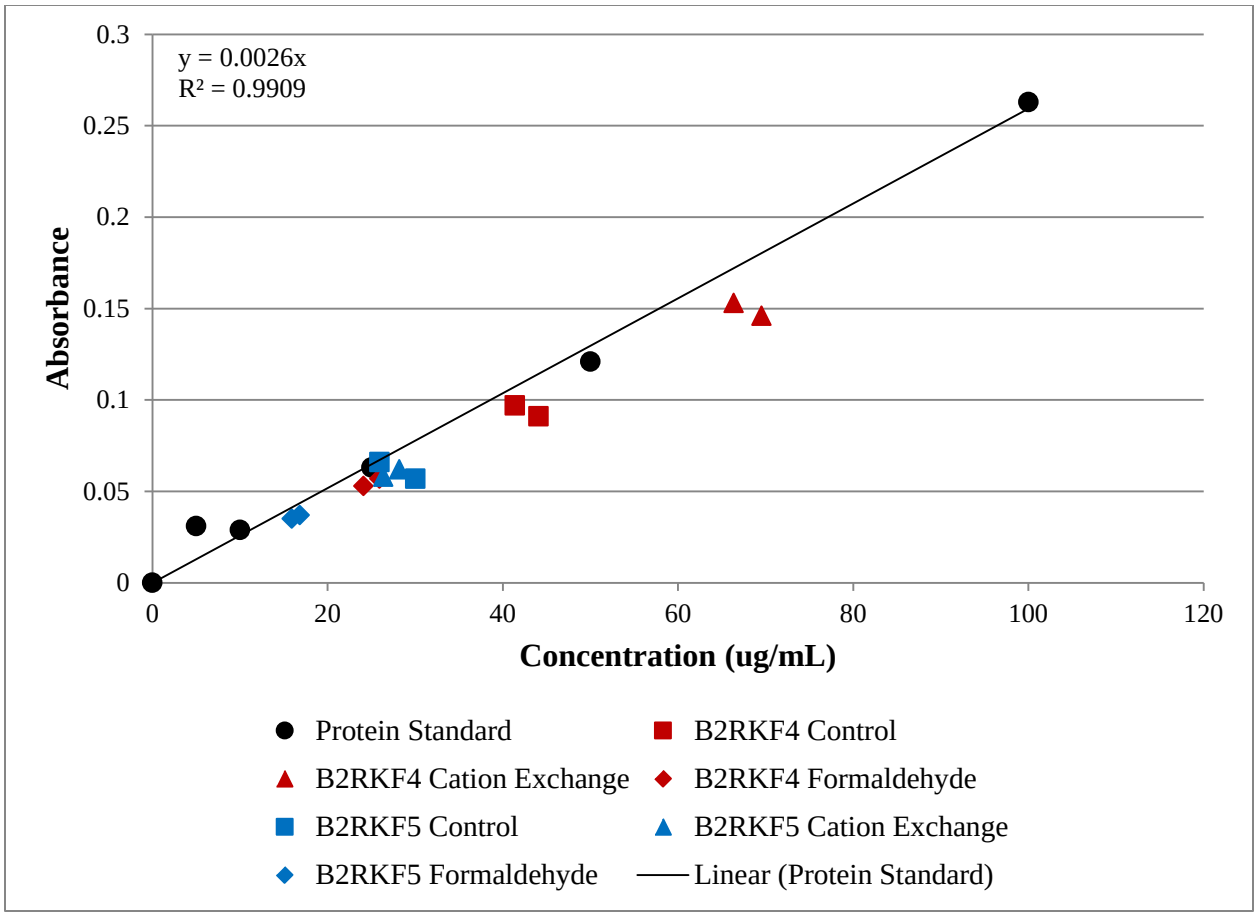

Figure C.9. Protein results from the standard calibration curve and samples B2RKF4 and B2RKF5.

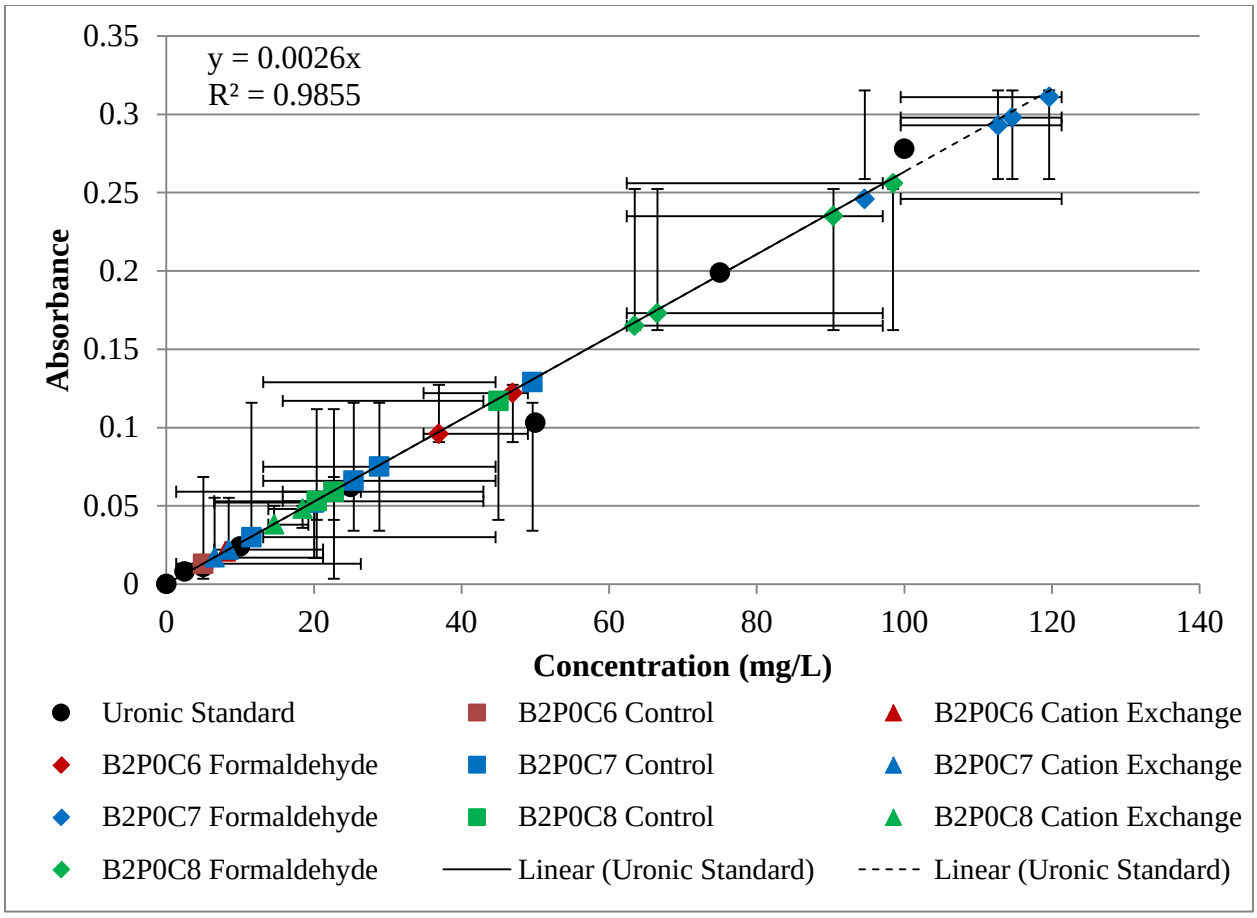

Figure C.10. Uronic acid analysis results from the standard calibration curve and samples B2P0C6, B2P0C7, and B2P0C8.

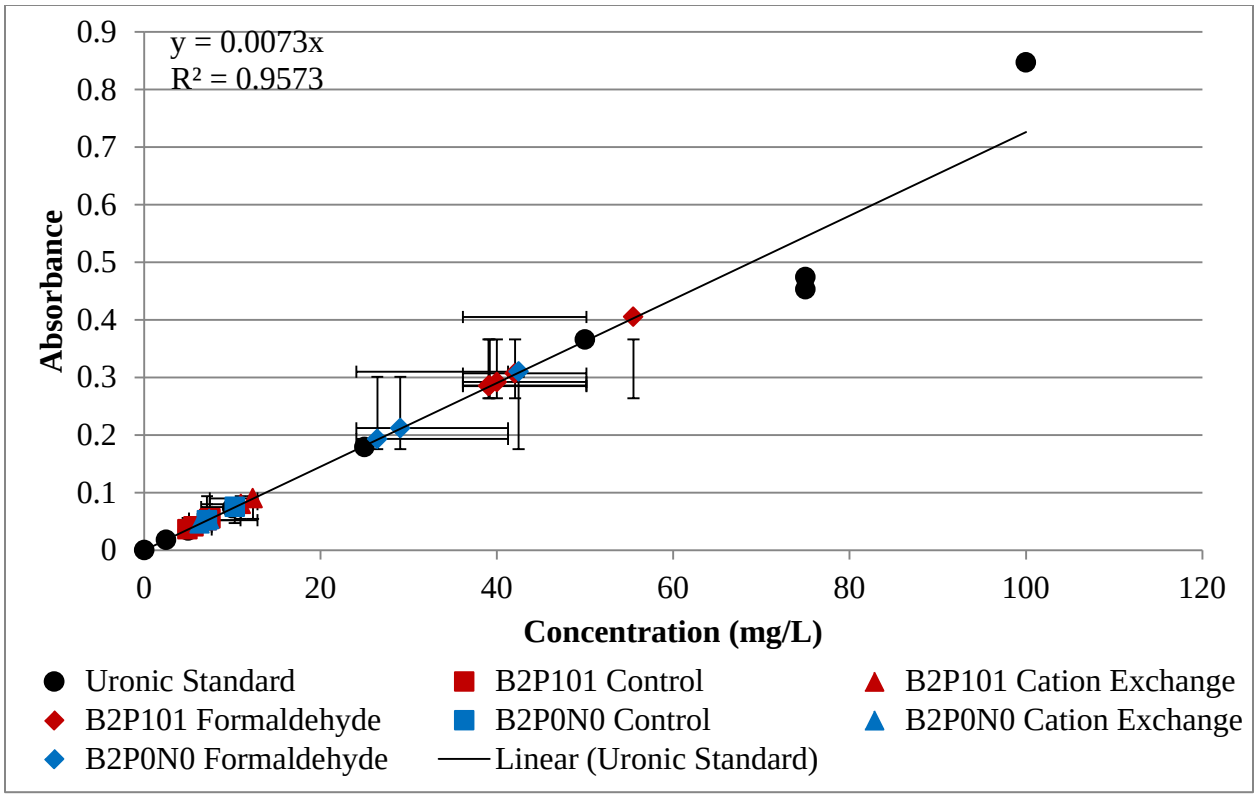

Figure C.11. Uronic acid analysis results from the standard calibration curve and samples B2P0N0 and B2P101.

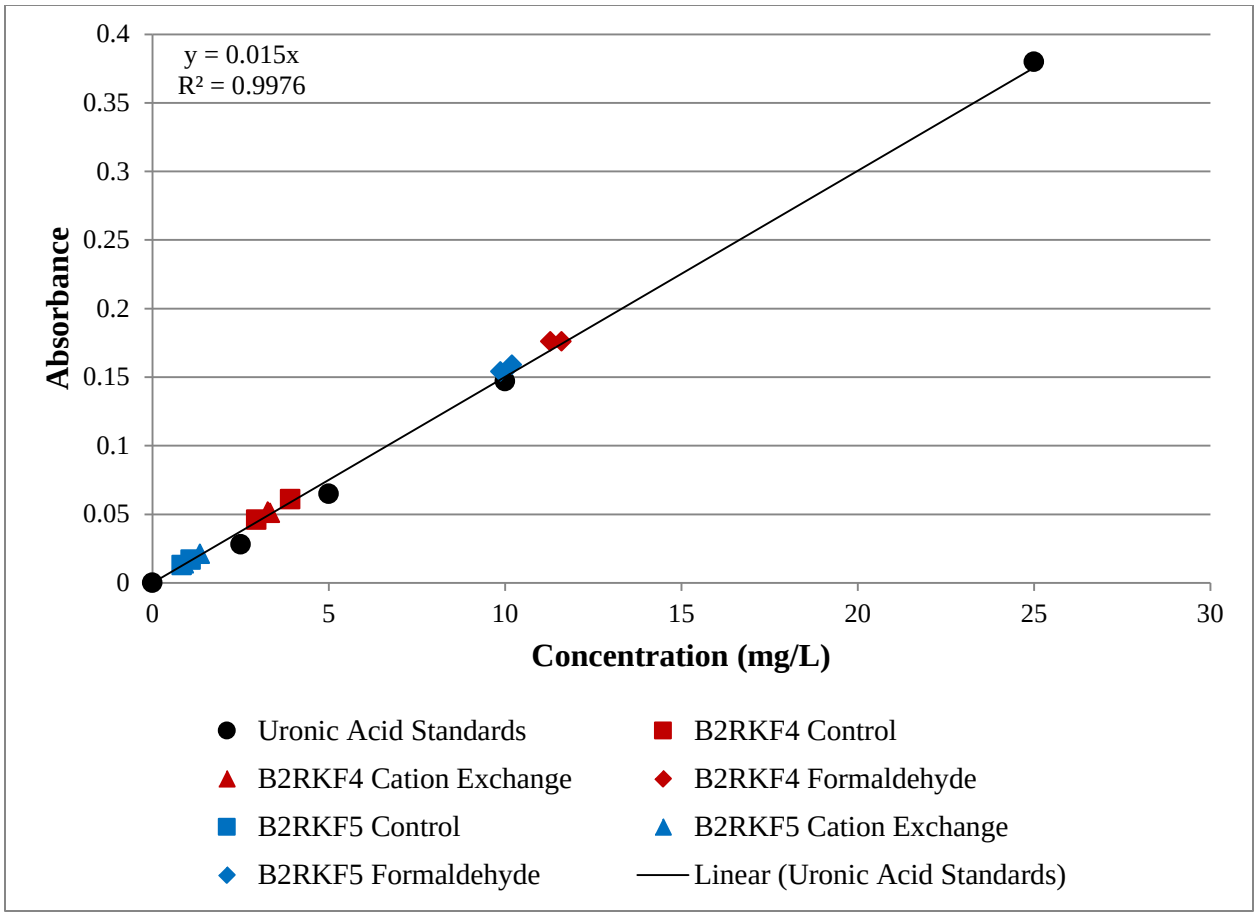

Figure C.12. Uronic acid analysis results from the standard calibration curve and samples B2RKF4 and B2RKF5.

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