APPENDIX A: EFFECTS OF BLACK CARBON ON CU AVAILABILITY, MICROBIAL UPTAKE AND DENITRIFICATION

BACKGROUND

Because of my research on stormwater management, and interest in fossil fuel combustion pollution, I began to wonder if soot (or black carbon) deposition in the urban environment could be collecting in stormwater basins. Black carbon (BC), like biochar, is a strong adsorbent of organic and metal pollutants. I began to wonder if black carbon (BC) accumulation in stormwater basins was leading to unintended consequences, mainly impacting biogeochemical cycles. Specifically, I hypothesized that BC could be adsorbing copper (Cu) and making it unavailable to microorganisms needing it for the denitrification process. Consequently, I began a series of experiments to explore the interactions of BC, Cu adsorption and denitrification.

INTRODUCTION

Because of the global climate change impacts associated with N_2O emissions, the last step of denitrification, which reduces N2O to N2, is a critical area of research. When considering the bioenergetics of denitrification, complete reduction to N_2 is not highly favored (Richardson et al., 2009). The Nos enzyme, which reduces N2O to N2, is particularly sensitive to environmental conditions, like pH and O_2 (Firestone et al., 1980; Liu et al., 2010). Nos is a Cu-rich enzyme, with two Cu sites (Richardson et al., 2009). Because this enzyme relies heavily on Cu and is often the weakest link in the denitrification pathway, recent research has begun to investigate the role of Cu limitation on Nos activity (Bartacek et al., 2010; Felgate et al., 2012; Wang et al., 2013).

Cu complexation and limitation is a function of the bioavailable Cu in the media (as the bioavailable Cu decreases so does the activity) (Moffett et al., 2012). Some work has examined

the effects of Cu on denitrification within pure cultures(Cervantes et al., 1998; Iwaski and Terai, 1982).Felgate et al. (2012) studied the effects of Cu, NO_3^-, and carbon limitation on denitrification with *Paracoccus denitrificans* (NirS species) and *Achromobacter xylosoxidans* (NirK species; dependent on Cu). They reported high Cu media resulted in a higher N_2:N_2O production in both species when compared to the low Cu media. This suggests Nos was enhanced in both species with Cu additions. However, the *A. xylosoxidan*s N_2:N_2O was higher than within *P. denitrificans,* which relies on a non-Cu dependent enzyme, NirS, to reduce NO_2. Thus, Nos productivity may be dependent on the ability of preceding reactions to reduce NO_2^-.

Granger and Ward (2003) investigated the effects of Cu concentrations on cultures of *P. denitrificans* in artificial seawater medium. Ethylenediaminetetraacetic acid (EDTA) was used to chelate Cu and reduce the availability to the bacteria. They reported that Cu-limited medium resulted in accumulation of N_2O, and the activity of Nos in *P. denitrificans* was below detection. They suggested the complexation of Cu with ligands (similar to EDTA) within oceans could have negative effects on the global N cycle. Moffett, Tuit, and Ward, "Chelator-Induced Inhibition of Copper Metalloenzymes in Denitrifying Bacteria." reported the inhibition of Nos within pure cultures amended with Cu chelators, Triethylenetetramine (TETA) and tetrathiomolybdate (TTMo). They determined that the threshold where limitation occurs was between 10^{-16} mol L^{-1} and 10^{-17} mol L^{-1} free Cu^{2+}. They noted that these levels were 100-1000 times lower than those observed with EDTA (Granger and Ward, 2003), which suggested EDTA may be affecting Cu uptake.

In addition to natural ligands and organic matter, another carbon material, black carbon (BC) may affect Cu uptake in the environment. Black carbon, or soot, is the product of incomplete combustion of fossil fuels and is a growing area of atmospheric research (Bond and Sun, 2005).

Because fossil fuel burning is ubiquitous, and atmospheric currents distribute BC throughout the environment, BC is distributed globally in ecosystems and soils (Ming et al., 2009; Nehls and Shaw, 2010).

Organic matter is a highly effective sorbent for metals and organic pollutants. Various forms of carbon have been exploited in filtering and pollutant removal systems for centuries (Reungoat et al., 2010; Scott and Ollis, 1995). For example, activated carbon (AC), which is charcoal treated to produce high surface areas, has long been used in the wastewater treatment industry to remove metals and organics (Corapcioglu and Huang, 1987; Snyder et al., 2007; Yin et al., 2007). Research has focused on the ability of bio-char, the incomplete combustion of biomass (e.g., wood, crop residue), to sequester soil carbon and act as a soil enrichment (Lehmann et al., 2006). Unlike bio-char, BC, is usually considered a nuisance particulate matter (PM) linked to climate change via reduced albedo (Bond and Sun, 2005).

Because BC is formed during poor combustion processes like coal-fired power plants, fuel oil-fired power plants, and vehicles, particularly diesel vehicles (Laschober et al., 2004; Schauer, 2003), it is comingled with harmful pollutants and not used as a soil amendment. According to Brandli et al. (2008), "AC is a manufactured, clean type of BC" and is a strong sorbent. Black carbon derived from vehicle soot is discussed here, because to our knowledge, no previous work has examined the role of this "dirty" form of carbon in biogeochemical cycling.

Researchers have noted places with elevated BC deposition, such as roadways, have correspondingly high metal concentrations. (Turer et al., 2001) noted metals in highway soils were strongly correlated with refractory carbon (likely BC). (Chen et al., 2010) noted that Cu and other metals were positively correlated with BC in urban roadside soils. Morse et al., "Roadside Soils Show Low Plant Available Zinc and Copper Concentrations." also noted that Cu was positively

correlated with soil carbon (although BC was not measured) in highway soils. These observations, and those from others noting biochar and activated carbon's affinity for Cu (Ahmad et al., 2014; Corapcioglu and Huang, 1987; Uchimiya et al., 2010, 2011), led me to hypothesize that BC may hinder denitrification by binding with Cu and making it less bioavailable for necessary enzyme. Black carbon, like biochar, is expected to sequester Cu by (1) forming surface complexes between Cu^{+2} and functional groups (*e.g.*, -COOH and -OH), (2) providing microsites and pores where Cu can become embedded, and (3) graphitic-like pi bonds creating pi-cation bonds (Machida et al., 2006; Park et al., 2011).

Specific research on combustion derived BC is rare, however fullerene (*e.g.*, Buckminster balls) research is quite broad (Astefanei et al., 2015; Petersen and Henry, 2012). Because BC is comprised of fullerene-like particulates and other amorphous carbon (Bond et al., 2013), extrapolations from fullerene research helped inform this proposed work. Huang et al., "A Fullerene Colloidal Suspension Stimulates the Growth and Denitrification Ability of Wastewater Treatment Sludge-Derived Bacteria." reported that fullerenes (5 mg L^{-1}) increased denitrification in *Bacillus* isolates grown in medium with 5mM $CuSO_4$ and 0.5mM $NaNO_3$. However, (Su et al., 2015) reported that single walled carbon nano-tubes (fullerenes rolled up in a tube) oxidized to contain OH functional groups reduced denitrification in *P. denitrificans*. Because fullerenes complex with organic matter and aggregate in soils, they are reported to have little impact on soil microbial biomass (Berry et al., 2016; Nyberg et al., 2008; Tong et al., 2007), although impacts from its ability to bind to metals and organic matter deserve further investigation.

Because BC may bind metals, like Cu, I hypothesize that this could be limiting Cu availability and subsequent NirK and Nos activity. As urbanization and BC emissions are expected to increase in the future (United Nations, 2015), it is important to better understand these mechanisms controlling

denitrification to help determine potential water quality and greenhouse gas impacts. Thus, this research aims to quantify how BC affects Cu availability, and denitrification.

EXPERIMENT 1: COLLECTED SOOT AND CU ADSORPTION

In an effort to understand how additional soot-BC in the environment alters the adsorption of Cu to soils, we conducted lab-scale batch adsorption and column flow-through experiments.

EXPERIMENT 1 METHODS

Soot and Soil Collection and Preparation

Jonker and Koelmans (2002) reported that NIST diesel soot was substantially different than vehicle soot collected in the environment, and consequently this study used soot collected from the environment. Because vehicle exhaust and particulate matter condense on adjacent snow, this street snow serves as a representative source for soot in the average traffic environment. Soot covered snow adjacent to a street and bus stop on Cornell's campus was collected (Figure 1). The snow was scooped into glass jars and stored at room temperature. The soot was treated similarly to methods in Jonker and Koelmans (2002), however they were interested in aqueous sediment soot and therefore isolated the smallest soot particle fraction most likely to become airborne and transported to the aqueous environment. Our study hopes to simulate the terrestrial urban environment, and the entire soot sample was used. The soot and melted snow solution was centrifuged at 2,600 g for 5 minutes. The supernant was discarded, and the remaining soot fraction was dried at 60°C, sieved at 2mm and homogenized for use in this experiment.

Figure 1. Location of soot-BC collection on street snow of Cornell University campus.

The goal of this experiment was to test how soils with relatively low levels of BC responded to soot additions. Consequently, soil was collected from McGowen Wood near Cornell University's campus (42.43° N, -76.45°W). Soil was oven dried at 40°C and sieved at 2mm for experiments herein. Soil pH was determined in water (Robertson et al., 1999), and texture was determined via the hydrometer method (Gee and Bauder, 1986).

Carbon Analyses

The soil and soil amended with soot were analyzed for BC and TOC as described in Gustafsson et al. 2001. The chemothermal oxidation method (CTO) (1) removes inorganic carbon (IC), (2) removes natural organic carbon (OC), and (3) quantifies remaining carbon in CHN elemental analyzer as BC. Briefly, the same sample is sub-divided in half and analyzed without pretreatment for total carbon, and the second half is treated with 1M HCL (to remove inorganic C), and then heated at 375°C for 24-hours (to remove natural organic C), and analyzed for BC. Samples were analyzed on a CHN Elemental Analyzer (ConFlo III).

Batch Sorption Experiment

Sorption experiments were conducted in 50-ml conical polypropylene tubes with 1 g soil (or soil and BC mix) and 25 ml of Cu solution. The Cu solution was prepared with analytical grade $CuNO_3$ and $CaCl_2$ to be approximately 0.5 mg L^{-1} Cu, and 0.1 M $CaCl_2$. The Cu solution concentration was chosen to resemble representative stormwater runoff concentrations, likely to reach urban soils. Calcium chloride was used to saturate the exchange sites within the soils, and any additional adsorption with the background presence of $CaCl_2$ was judged representative of specific adsorption and/or complexation. The Cu solution was buffered to pH 6.3 prior to starting the experiment. The solution pH was measured immediately after equilibrating with the soils, and then after the 24-hour period.

Black carbon was amended in various concentrations (w/w). While all of the collected soot is not BC, for ease of use this paper will refer to the soot amendments as BC. For example, the treatment with 5% soot was created by adding 0.05 g soot to 0.95 g soil, and is referred to as "soil + 5% BC". Five experimental treatments were examined: soil, soil + 5% BC (BC5), soil + 10% BC (BC10), soil + 15% (BC15), BC only (BC), and two controls, soil + water (soil cont.) and BC + water (BC cont.). Treatments were run in triplicate. The tubes were shaken end-over-end for 24-hours. The tubes were then centrifuged and the supernant filtered through 0.45 um filters and analyzed for dissolved Cu via inductively coupled plasma atomic emission spectroscopy (ICP-AES). The percentage adsorbed was calculated as the difference between the initial and final Cu concentrations, divided by the initial Cu concentration. The amount of Cu adsorbed per unit mass of sorbent (q_e, mg Cu g^{-1}), was calculated as the difference between the initial and final concentration divided by the soil (or soil + BC) mass.

Cu Isotherm Experiment

Similar to the batch experiments, the isotherm experiments used 50-ml polypropylene tubes with 1 g soil (soil treatment) or 0.95 g soil and 0.05 g BC (soil + BC treatment) and 25 ml of Cu solution. The Cu solution was prepared at the following concentrations: 0.01, 0.05, 0.31, 0.61, 0.68, and mg L^{-1}. This Cu solution did not contain $CaCl_2$, and was buffered to a pH of 6.5. After shaking for 24-hours, the dissolved Cu concentrations were measured via ICP. The percent adsorbed was calculated from the difference between initial and final dissolved Cu concentrations.

EXPERIMENT 1 METHODS

Soil and Soot Characteristics

The soil and soot characteristics are given in Table 1. The soil had 0% BC, according to the CTO method commonly used for BC determination, and soot had approximately 2.27%. The soil was classified as a Sandy Loam based upon USDA classification. The soil pH (2:1 in water) was 5.5.

Table 1. Soil and collected soot characteristics

	TC %	BC %	TC:BC	Sand %	Silt %	Clay %
Soil	6.4	0	0	62.9	33.1	4.0
Soot	7.98	2.27	3.52	NA	NA	NA

Batch Adsorption

The soil only and BC only controls Cu concentrations were below the detection limit. This shows that the soil and BC were not leaching Cu, and any Cu measured within experimental treatments was from the added Cu solution. The final equilibrium concentration (c_e), mass adsorbed, and mass adsorbed per kg adsorbent (q_e) for each treatment are shown in Table 2. Analysis of Variance (ANOVA) revealed significant differences between treatments for c_e, %

adsorbed, and q_e (all p-values <0.001). Tukey HSD differences between treatments are also shown in Table 2.

Table 2. Equilibration concentration (C_e), mass adsorbed, and mass adsorbed per adsorbent (q_e).

Treatment	Equilibrium concentration (C_e)		Mass adsorbed		Mass Cu adsorbed per mass adsorbant	
	mg Cu L^{-1}	HSD	*ug*	HSD	*mg kg^{-1}*	HSD
Soil	0.31 (0.02)	a	4.1 (0.47)	b	4.1 (0.47)	c
BC5	0.09 (0.09)	b	9.6 (2.26)	a	9.6 (2.26)	b
BC10	0.03 (<0.01)	b	11.1 (0.12)	a	11.1 (0.12)	b
BC15	0.03 (<0.01)	b	11.1 (0.02)	a	11.1 (0.02)	b
BC	<0.001 (<0.01)	b	11.8 (0.02)	a	117.5 (0.18)	a

The initial solution concentration of 0.47 mg Cu L^{-1} was reduced in all treatments. However, the treatments with BC had significantly lower final concentrations (Tukey HSD $p<0.05$). Likewise, the soil only treatment had the lowest % adsorbed for all treatments. This indicates the BC enhanced adsorption of Cu within the soil. On a mass adsorbed basis, the BC only treatment had the highest (117.5 mg kg^{-1}), and the soil only had the lowest (4.1 mg kg^{-1}). The BC5, BC10, and BC15 treatments all had higher adsorption per mass than the soil, however the amount of BC amended did not significantly alter the mass adsorbed.

The BC only treatment used the same mass of BC as the BC10 treatment (0.1 g BC in 25 ml Cu solution). Consequently, comparing the BC only and BC10 treatments allows a direct comparison of the effects of soil and BC interaction. When soil and BC were mixed, the extremely high ability of BC to adsorb Cu is slightly reduced (99% compared to 94%). If we assume the average mass adsorbed per kg BC found in the BC only treatment is applicable to the BC10

treatment, the calculated mass removal of Cu would be 11.8 ug ($\frac{117.5\ mg\ Cu}{kg\ BC}\ x\ 0.1\ x\ 10^{-6}\ kg\ BC$). This is approximately 6.6% lower than the observed removal in BC10 (11.1 ug). This slight dampening of adsorption is consistent with other literature and indicates soil may be clogging micropores within the BC, therefore diminishing Cu adsorption (Brändli et al., 2008).

Although the initial Cu solution pH was 6.3, when the solution was mixed with the slightly acidic soil (pH=5.5), the solution pH immediately decreased to about 5.1. However, in the soils amended with BC, the solution pH increased and was sustained for the 24-hour period. Figure 4 illustrates the average initial and final pH of the Cu solution within the batch experiment. The average Cu adsorbed (%) and final solution pH are shown in Figure 5. Clearly the treatments with higher pH had higher Cu adsorption. Similar increases in soil pH with bio-char additions are reported in the literature (Cayuela et al., 2013; Lehmann et al., 2006; Uchimiya et al., 2010).

Isotherm Results

The isotherm experiment was conducted without a background concentration of $CaCl_2$ to determine the affinity of the soil, and soil and BC mixture for Cu. The Freundlich isotherm performed slightly better (higher R^2 values) than the linear isotherm; and was consequently selected as the appropriate model herein. Table 3 shows the K_F values, empirical n constants, and R^2 values for both treatments, and Figure 2 illustrates the isotherms for both treatments. The high R^2 values (both near 0.8), imply this model accurately represents Cu adsorption for these treatments. The soil+BC treatment (5% BC w/w) had a K_F value roughly 14 times higher than the soil only treatment, which indicates the 5% addition of BC significantly enhanced Cu adsorption. As a limitation of this study, we did not analyze the soil directly for Cu adsorption. Consequently, what we term adsorption here, could in fact include Cu precipitation. However, most adsorption

studies rely on subtraction rather than direct quantification of adsorbent within the matrix to describe adsorption. We admit this as a limitation of this study.

Table 3. Freundlich isotherm coefficients for Cu adsorption to soil or soil+BC treatments.

Treatment	K_f	n	R^2
Soil	0.19	1.28	0.79
Soil+BC	2.65	0.84	0.88

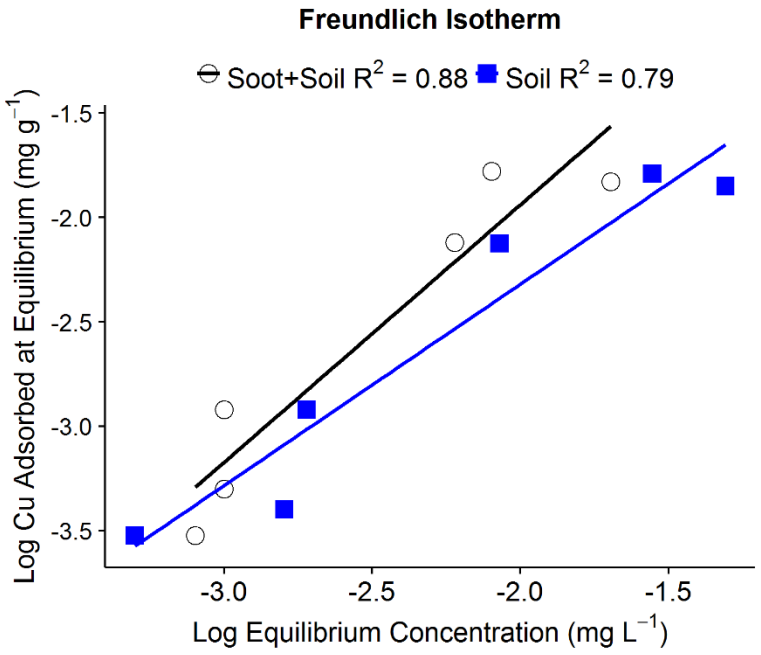


Figure 2. Freundlich isotherm results for soil only, and soil+5% soot-BC.

FURTHER WORK

After these initial trials, we hypothesized that Cu could be strongly adsorbed to BC in the environment and possibly affecting microbial access to this nutrient. Consequently, we decided to explore the direct microbe and BC interactions. We were concerned that these results could be due to something else in the collected soot, and not solely due to the BC component. Therefore, instead

of using this collected soot-BC, we decided to switch the BC source to a uniform and readily available fullerene-BC.

EXPERIMENT 2: FULLERENE-BC CU ADSORPTION AND DEA EFFECTS

In an effort to establish what, if any, relationship exists between BC and denitrification, lab-scale analyses were employed for this project. Because the environmental conditions and soil characteristics of the stormwater basins were well characterized (Morse et al., 2017), soils from Wet Basin1 and Dry Basin2 (Wet Basin and Dry Basin, respectively) from these basins were used for this project instead of the soils from McGowan woods used previously.

Fossil-fuel derived BC is difficult to obtain. As we hope to mimic the substance commonly deposited in the environment, we used fullerene soot BC. Landa et al., "Nanoparticle-Specific Changes in Arabidopsis Thaliana Gene Expression after Exposure to ZnO, TiO2, and Fullerene Soot." used this fullerene BC soot as a surrogate for fossil-fuel derived BC because it's morphology is similar to environmental fossil fuel combustion BC, and is easily obtainable and uniform. Fullerene soot, produced by the Kratschmer-Huffman graphite arc method, was purchased from Alfa Aesar, and reported to contain 7% fullerenes, 93% amorphous carbon, and a particle size range of 0.02 to 10 microns (μm). All amendments were made on a weight/weight (w/w) basis.

EXPERIMENT 2 METHODS

Copper adsorption

A batch Cu adsorption study with the fullerene-BC soot was conducted. Fullerene-BC was mixed with Wet Basin soils at varying concentrations, 0, 0.5, 1, 2, 5, and 10% (w/w), in triplicate. Then 1 gram of soil and 25-ml of Cu solution (1 mg L^{-1}) were mixed and shaken end-over-end for 24-hours. The tubes were then centrifuged and the supernant filtered through 0.45 um filters and

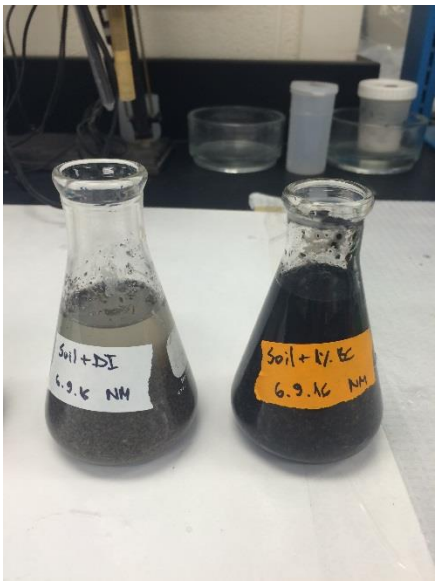
analyzed for dissolved Cu via inductively coupled plasma atomic emission spectroscopy (ICP-AES). Absorption was calculated as the difference between the initial and final Cu concentrations.

Effects of BC and Cu on Potential denitrification enzyme assays (DEA)

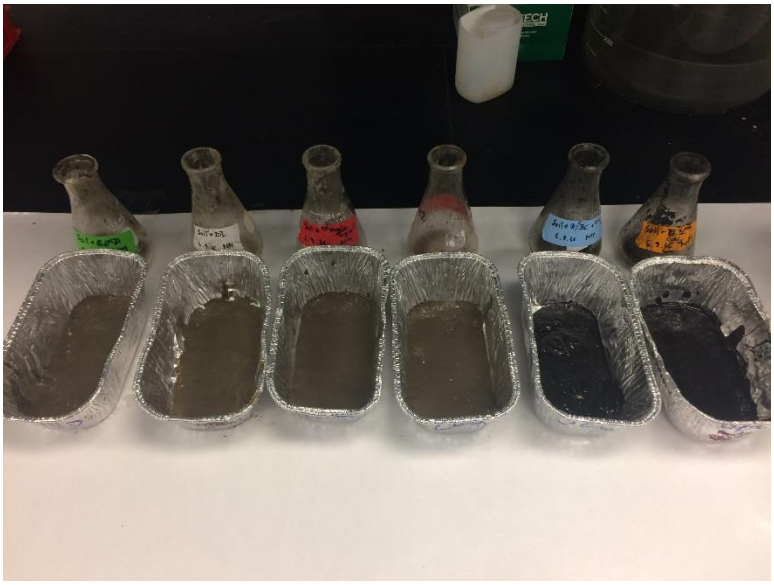
Soils were collected from the Wet Basin, air-dried and sieved (<2mm), then 70 grams were placed in 6 individual flasks. Next, 80 ml of treatment solution, adjusted to achieve desired concentration, was added to each flask: (1) Soil Only, (2) 100 mg kg^{-1} EDTA, (3) Cu Low (25 mg Cu kg^{-1}), (4) Cu High (100 mg Cu kg^{-1}), (5) Cu Low + 0.5% BC (w/w), and (6) 0.5% BC. Saturated soils were left to equilibrate for 7 days at room temperature (25°C) covered with perforated paraffin wax paper. On the seventh and fourteenth day (t=7, and t=14), sub-samples from each treatment were removed from the flask, and the DEA assay (described in Section 4.3) was performed. Each treatment was run in triplicate (n=3) with acetylene (C_2H_2) and in duplicate (n=2) without C_2H_2. Without the acetylene block full denitrification to N_2 is possible. The N_2 produced via denitrification was calculated as the difference between the N_2O produced in the samples receiving C_2H_2, and those without (Magalhães et al., 2011). In an effort to monitor treatment effects on overall soil microbial health, soil C mineralization rates were monitored at each treatment (n=1). Ten grams of soil were placed in incubation jars, then sealed and left at room temperature for 7 days. On the 7^{th} day, the jars were flushed with humidified are, re-sealed, and headspace samples were collected over a 2-hour period (G. P. Robertson et al., 1999). The headspace samples were analyzed via GC for CO_2, and reported on a g CO_2 g $soil^{-1}$ basis.

At t=7 and 14, pH was measured in the bulk soils, rather than individual vials, and bioavailable Cu, soil NO_3^- and NH_4+ were also measured at the end of the experiment (t=14). Bioavailable Cu was measured as dissolved Cu following a 2-hour equilibration with 0.01M $CaCl_2$. Pictures of the general lab procedure are shown below.

1. Add Fullerene soot to soil

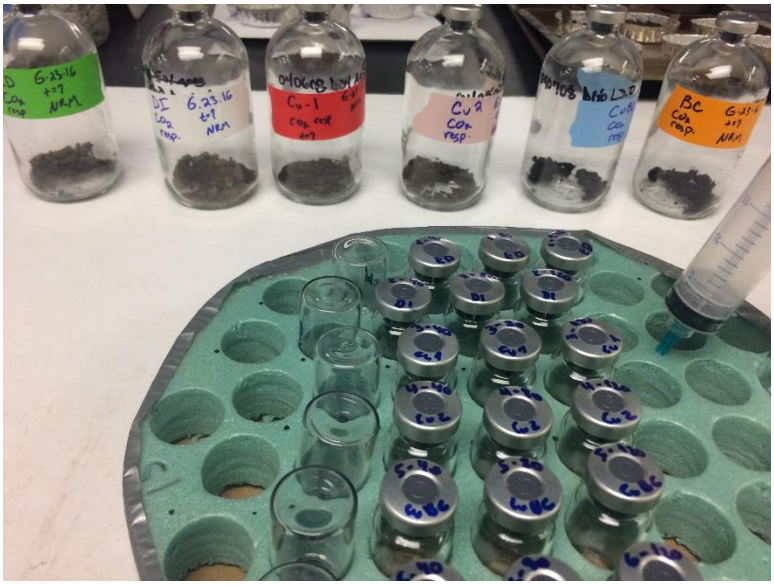


2. Add Cu solution (or DI, or EDTA solution) to soil, mix, and let equilibrate for 7-days.

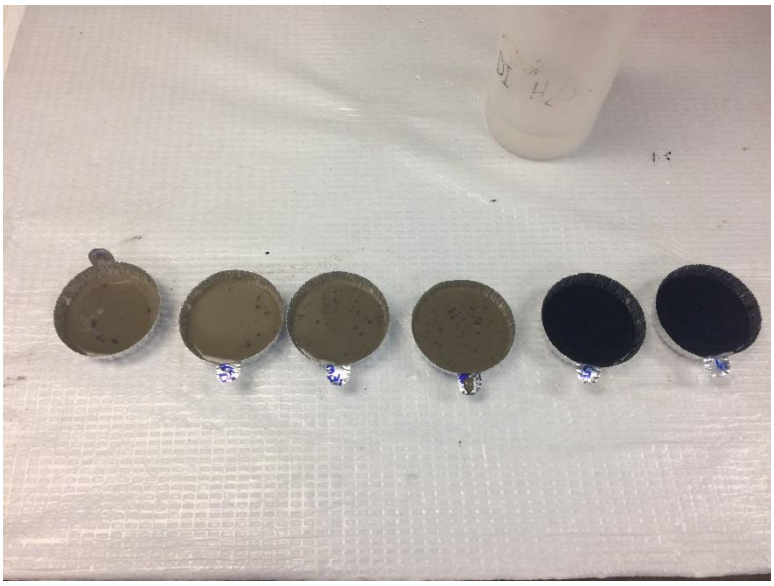


3. Remove half of soil from flask to air-dry overnight prior to denitrification enzyme assay (DEA) experiment for two timeponts (t=0 and t=7 days)

4. Remove sub-sample (5 g each) for denitrification enzyme assay (DEA); 3 replicates and 2 replicates without acetylene.



5. Remove sub-sample (5 g each) for CO2 respiration (1 vial/treatment).



6. At end of experiment (t=7), analyze sub-sample for total and bioavailable (0.01M KCL ext) Cu, and soil NO_3 and NH_4 (1M KCL ext).

EXPERIMENT 2 RESULTS

Copper adsorption to fullerene-BC

The BC did improve Cu adsorption, although only slightly (Figure 3). The addition of 1% BC increased the adsorption of Cu from 96.64% in the Soil only control, to 98.18%. The highest BC treatment (10%) did increase Cu adsorption the most. However, because the soil only control had very high Cu adsorption (96.64%), it was difficult to see large increases in adsorption due to BC. This is likely because the Cu solution (1 mg L^{-1}) was relatively low.

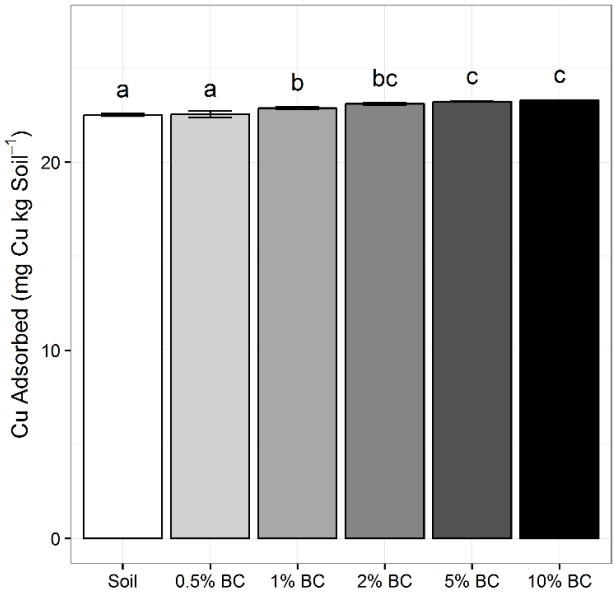


Figure 3. Cu adsorbed at each treatment after 24-hour equilibration. Different letters denote significant Tukey HSD ($p<0.05$) differences between the treatments.

Fullerene-BC effects on DEA

The high Cu treatment (100 mg Cu kg^{-1}) significantly reduced the DEA rates, as expected. However, the low Cu treatment (25 mg Cu kg^{-1}) did not reduce DEA and indicates that this Cu exposure level was not harmful to the bacteria. The addition of BC significantly decreased DEA rates (Figure 4). I hypothesized that the BC could be limiting Cu uptake to the bacteria, but the mixed Cu Low + BC treatment did not cause a rebound in DEA. This implies that the extra Cu was either still inaccessible (bound to BC), or that a different mechanism was depressing DEA within the BC treatment.

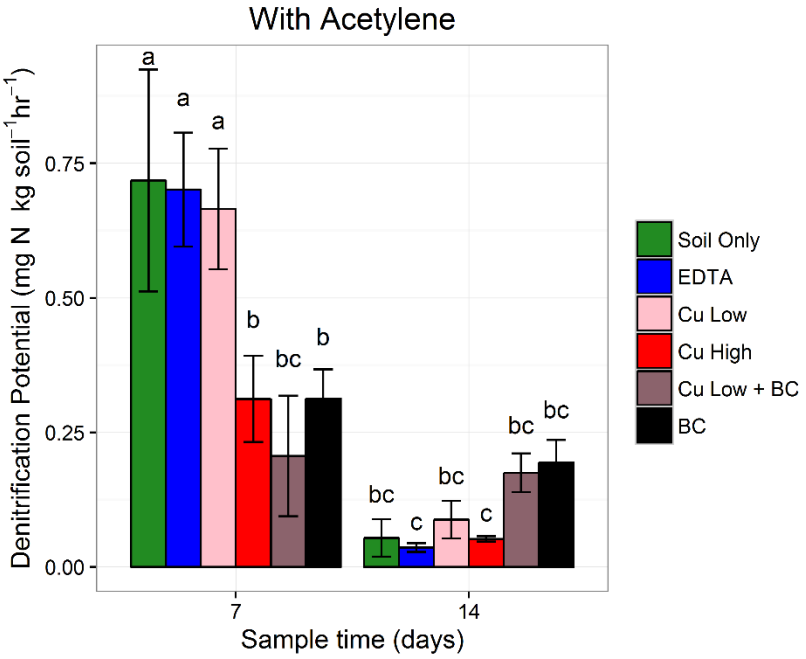


Figure 4. Denitrification potential (DEA at each treatment (n=3). Different letters denote significant Tukey HSD ($p<0.05$) differences between the 12 groups

The soil characteristics were sampled at the 7 and 14 day timepoints. The total dissolved Cu was highest in the EDTA treatment, and lowest in the BC treatment (Table 4). This indicates that BC likely adsorbed Cu and made it inaccessible to microorganisms. The soil NO_3^{-1} levels were highest in the soil only control, and Cu treatments, and this could be because the bacteria reduced NO_3^{-1} during denitrification.

Table 4. Average soil characteristics from DEA experiment

Treatment	**Cu Bioavailable**			**Cu Total**	**Bioavailable/Total**			**Cu Dissolved**	**soil NO_3**	**soil NH_4**
	mg kg^{-1}			mg kg^{-1}	%			mg L^{-1}	mg kg^{-1}	mg kg^{-1}
Day	7	14	avg	avg	7	14	avg	14	14	14
Soil Only	0.005	0.005	0.005	30.18	0.02	0.02	0.02	0.008	19.67	0.15
EDTA	0.401	0.078	0.240	25.53	1.57	0.31	0.94	0.112	3.59	0.16
Cu Low	0.005	0.005	0.005	77.51	0.01	0.01	0.01	0.043	13.09	0.16
Cu High	0.127	0.225	0.176	191.75	0.07	0.12	0.09	0.120	15.82	0.15
Cu Low + BC	0.005	0.126	0.066	76.94	0.01	0.16	0.09	0.016	8.79	0.18
BC	0.005	0.005	0.005	28.37	0.02	0.02	0.02	0.001	7.42	0.16

Day indicates the day the experiment was performed, 'avg' is the average of day 7 and 14. All NH_4 results were below the detection limit; half of the detection limit (0.005 mg L^{-1}) was used as the concentration to calculate mg kg^{-1}

FURTHER WORK

After these trials, we decided to explore the direct effects of BC and Cu on microbial growth. We hoped to gain a more mechanistic understanding of these interactions, instead of relying on DEA as a proxy.

EXPERIMENT 3: FULLERENE-BC AND CU EFFECTS ON MICROBIAL GROWTH

I hypothesized that BC could be altering Cu availability and influencing denitrification. Therefore, experiments on bacteria growth response to different BC and Cu treatments were explored to determine if bacterial access to Cu was altered with BC additions. If bacteria were more or less affected by Cu in the presence of BC this could indicate that BC adsorption of Cu is a possible mechanism for removing Cu from bacteria and reducing denitrification. *Bacillus subtilis*, a bacteria with known Cu uptake (*ycnJ*) and Cu efflux (*copZA*) genes, was used as a model organism (Gaballa and Helmann, 2003).

These experiments were conducted with the help of Dr. Helmann's lab in Microbiology. Bacteria growth was observed with plating trials and also optical density (OD) measurements. The plating trials allowed bacteria growth directly in contact with the fullerene-BC material. In contrast, OD trials had to expose the BC to the growth media and then filter the BC out prior to bacteria addition, so OD measurements could be completed. Optical density measurements rely on the ability of light to pass through a clear tube, and the more the light is obscured, presumably due to microorganism growth, the higher the recorded microorganism growth. But, in this experiment the black BC could obscurificate the OD measurements and make it seem like there was more bacteria growth, than there really was. Thus, we had to filter out the BC prior to adding the bacteria, so all growth could be accurately quantified.

EXPERIMENT 3 METHODS

BC and Cu effects on microbial growth

Bacillus Plating Experiment

First, we tested if Bacillus growth would be hindered by lack of Cu by using a Cu-chelator, bathocuproine disulfonate (BCS). Bathocuproine disulfonate is a Cu+ chelator where the ligand-Cu complex cannot cross the cell membrane. Plating trials with Bacillus subtilis were done on minimal media, where Cu was not prevalent. The minimal media used was prepared as:

100 mM KH_2PO_4 (potassium phosphate) buffer pH 7
3 mM $Na_3C_6H_5O_7$ (trisodium citrate)
3 mM $MgSO_4$ (magnesium sulfate)
2% $C_6H_{12}O_6$ (glucose)
0.2% $C_6H_{11}KO_7$ (potassium glutamate)
0.1% casein hydrolysate (phosphoproteins used to cultivate bacteria)
22 ug/ml $(NH_4)_5[Fe(C_6H_4O_7)_2]$ ferric ammonium citrate
50 ug/ml tryptophan (amino acid)

The minimal media was then amended with the following treatments:

1. Control
2. Bathocuproine disulfonate (chelator) 0.5mM
3. Black Carbon (BC) 0.5 g L-1
4. Bathocuproine disulfonate + BC
5. BC previously treated (BC_{PreT}) 0.5 g L^{-1} filtered <0.2um prior to bacteria addition
6. Bathocuproine disulfonate + BC_{PreT}
7. 1 mM Cu
8. 0. 5mM Cu
9. 1 mM Cu + BC
10. 0.5 mM Cu +BC
11. 1 mM Cu + BC_{PreT}
12. 0.5 mM Cu + BC_{PreT}

After the treatments were prepared with the minimal media, the bacteria (previously grown for 24-hours) was added, 4ml of the treatment media was mixed with 0.4 ml of bacteria. After an 4-hour equilibrium with the treatment, the bacteria were then plated on minimal growth media agar, covered with the plate top, and left to grow in a dark environment at room temperature. Colony plate counts were conducted 24-hours later.

Optical density growth experiment

Bacteria growth, as assessed through (OD) measurements was conducted on the wild type Bacillus, and also the Bacillus mutants without the abilities for Cu-uptake (ycnJ deficient) and Cu-efflux (copZA deficient). The same treatments and media as the plating experiment were conducted. However, these trials could not allow the direct addition of BC, as it would make OD measurements impossible. Therefore, all BC treatments in the OD experiment are those done with the BC filtered out prior to bacteria addition (BC_{preT}). The bacteria and treatment media were put into a 96-count well tray and grown in the microplate spectrophotometer for 24-hours. OD measurements were taken every 15 minutes, and all treatments were done in triplicate. Four trials were completed on 6/13/17, 6/20/17, 6/27/17, and 7/6/17.

EXPERIMENT 3 RESULTS

Plating Bacteria Growth Results

The colony forming units for each treatment are shown in Table 5. These were not done in triplicate, as this was an initial experiment to test how Cu and BC may impact bacteria growth. These results suggest that BC did not hinder Bacillus growth, and 1mM Cu hindered growth. If BC were making Cu less bioavailable, we would expect that the 1mM Cu + BC treatment would have higher growth than the 1mM Cu treatment, but this was not the case, and we actually saw a

slight decrease in the 1mM Cu + BC treatment (log CFU = 6.48) compared to the 1 mM Cu treatment (log CFU = 7.60). Figure 5 shows photos of the plates.

Table 5. Colony forming units for the plating experiment done with the wild-type Bacillus.

	CFU	**log CFU**
Control	3.00E+07	7.48
BCS Chelator	1.00E+08	8.00
BC	3.00E+08	8.48
BCS + BC	2.00E+08	8.30
BC_{PreT}	1.00E+08	8.00
BCS + BC_{PreT}	NA	NA
1mM Cu	4.00E+07	7.60
1Cu + BCS	1.00E+08	8.00
0.5 mM Cu	2.00E+08	8.30
1Cu + BC	3.00E+06	6.48
0.5Cu + BC	4.00E+07	7.60
1Cu + BC_{PreT}	2.00E+07	7.30
0.5Cu +BC_{PreT}	3.00E+07	7.48

Notes: BCS_{reT} indicates the BC was mixed with the media and filtered prior to bacteria addition. ‘BCS’ is the bathocuproine disulfonate chelator.

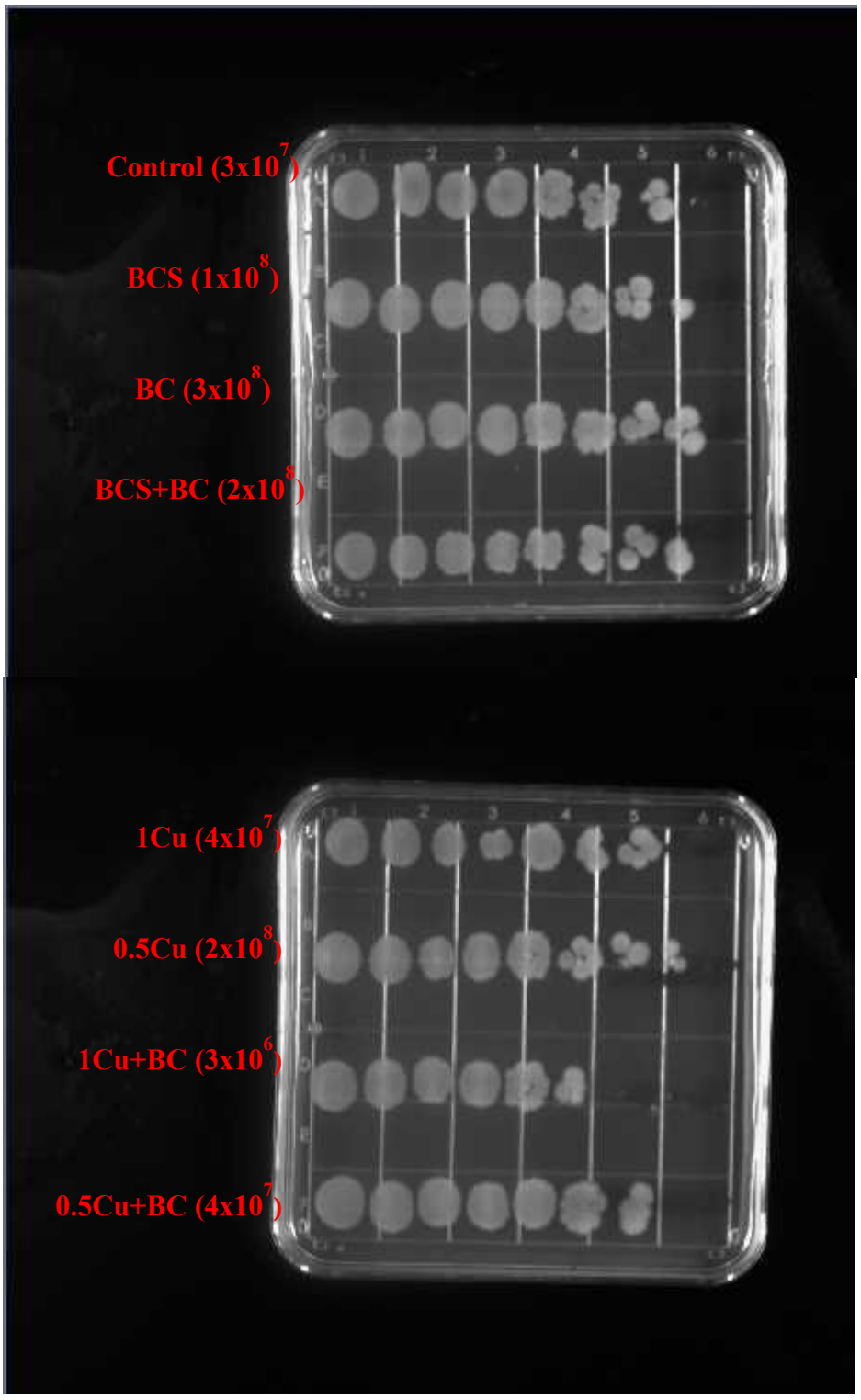


Figure 5. Plate results for BC and Cu treatments (BCPreT not shown). 'BCS' is the Cu chelator, bathocuproine disulfonate.

CONCLUSIONS

After these experiments, it was not clear if BC was affecting Cu uptake and ultimately denitrification. With the collected soot-BC, we saw a very clear adsorption affinity for Cu. With the fullerene-BC Cu adsorption was still observed, but it was not as pronounced. This indicates that the environmental soot may have slightly different characteristics than the fullerene-BC. Additions of fullerene-BC to soils did reduce potential denitrification (DEA) rates. However, adding Cu to the fullerene-BC did not ameliorate this negative effect on DEA. This indicates that either (1) we did not add enough Cu and BC was still binding it all, or (2) a different mechanism was affecting DEA rates. With the microbial growth studies, the fullerene-BC did not ameliorate the negative effects of Cu addition to the plating experiment or OD growth curves. Thus, while fullerene-BC was shown to bind Cu, it was not clear if this translated into a loss of bacteria growth and/or denitrification.

Optical Density Results

The results of the OD trials were mixed. A general summary is given below:

- BC had minimal effect on bacteria growth (6/27/17, 7/6/17 trial)
- BC slightly reduced bacteria growth (6/13/17, 6/20/17 trial)

- Chelator BCS reduced bacteria growth
    - Adding BC to chelator did ameliorate this negative effect (6/27/17 trial; 7/6/17 trial inconclusive)
        - BC did improve Cu availability
    - Adding BC to chelator did **not** ameliorate this negative affect (6/20/17 trial; 6/13/17 BC+BCS treatment not included)
        - BC did **not** improve Cu availability

- Copper additions reduced bacteria growth
    - Adding BC to Cu mix did ameliorate this negative effect slightly (6/20/17, and **7/6/17** trials; 6/13/17 trial inconclusive)
        - BC did reduce Cu availability and/or toxicity
    - Adding BC to Cu mix did **not** ameliorate this negative effect (6/27/17 trial)
        - BC did **not** reduce Cu availability and/or toxicity

Growth curves for the 6/27/17 trial are shown for reference in Figure 6, and Figure 7 displays a subset of these treatments for ease of viewing.

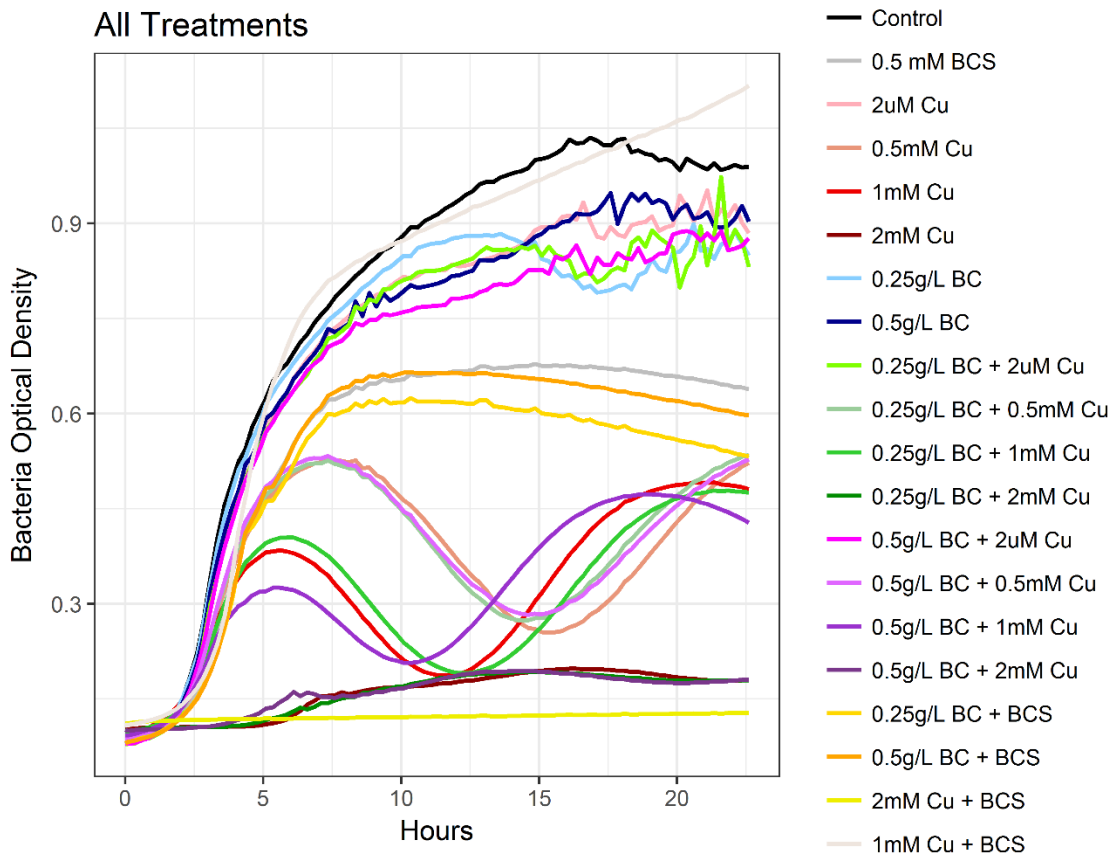


Figure 6. Wild-type Bacillus growth curve exposed to the different treatments on 6/27/17. We hypothesized that adding Cu would decrease bacteria growth, and this was shown with the Cu doses (except 2 uM which was too low). We also hypothesized that adding BC to the Cu solution would ameliorate this negative effect, but that was not supported with these trials. The medium treatment levels, shown in Figure 7 illustrate the negative effect of Cu, but fail to show a rebound with the addition of BC.

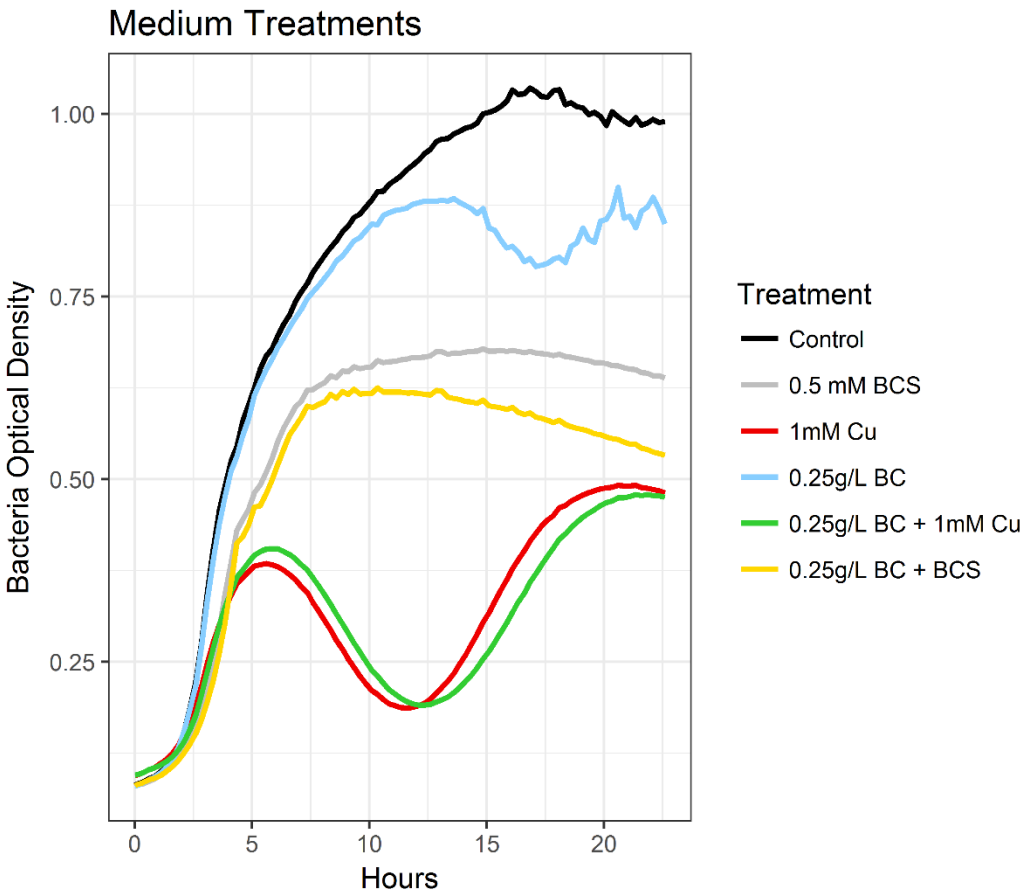


Figure 7. Wild-type Bacillus growth curve exposed to the 'medium' treatments on 6/27/17. Notice that adding BC to the 1mM Cu solution did not reduce the negative effects on bacteria growth.

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