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Research Article

Assessing the impact of coal ash exposure on soil microbes in the Dan River

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Abstract. In February of 2014, over 50,000 tons of coal ash was spilled from a retired power plant into the Dan River of North Carolina. Coal ash exposure can have either positive or negative effects on an ecosystem, largely depending on the concentration and species of the heavy metals it contains. The resulting alterations within an ecosystem can include both abiotic factors, such as the pH of contaminated soils or waterways, and biotic factors, including the viability and diversity of exposed organisms. Herein, we report that one year following the coal ash spill into the Dan River significant differences were observed in several abiotic factors of contaminated bank and channel soils, including pH and content of chromium, sulfur, and calcium. Furthermore, the density, diversity, and fitness of the microbes in soils exposed to coal ash were also altered when compared to reference samples. The implications of these variations are discussed.

Introduction

Burning coal produces a variety of waste products, including coal ash. Coal ash is comprised of the residues left behind within a furnace following coal combustion as well as the filtrate scrubbed from the flue gas. Coal ash contains a variety of heavy metals that were present within the coal itself or are byproducts of the combustion process. Although the chemical make-up of the ash varies with the type and source of coal burned, the most common pollutants of concern are heavy metals, such as chromium, lead, copper, zinc, and manganese (Sushil and Batra, 2006). In order to contain coal ash waste, it is typically stored in landfills (impoundments) either on site or in close proximity to the power plant where it was generated. In spite of the fact that it contains materials that can be considered toxic, coal ash is not classified as hazardous waste. As a result, individual states are allowed to establish their own regulations regarding storage and disposal practices, including whether or not impoundments should contain liners that might reduce groundwater contamination. Recent analysis by the US Environmental Protection Agency (EPA) has indicated that there may be as many as 735 surface impoundments of coal ash within the United States (US EPA, 2015). In spite of efforts to contain coal-derived waste by impounding, numerous coal ash spills

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into aquatic environments have taken place. Such spills have been correlated with decreases in soil pH due to increases in sulfate concentrations (Guthrie et al., 1982; Komonweeraket et al., 2015), increases in soil nitrate concentrations (Ciećko et al., 2015), and elevated Chromium (Cr) VI concentrations that have exceeded EPA drinking water safety levels (Kosson et al., 2009). Elevations in Cr are of particular importance due to the fact that it is known to have cytotoxic effects, disrupt gene expression, and promote tumor growth in humans (Chiu et al., 2010; Nigam et al., 2014). Importantly, the toxic effects of Cr not only hold true for humans, but a range of other cellular organisms, including freshwater fish and macroinvertebrates (Canivet et al., 2001; Velma et al., 2009).

In February 2014, a pipe originating from a retired coal plant located along the Dan River of North Carolina ruptured, releasing an estimated 52,000 to 82,000 tons of coal ash into the river. EPA analysis of sediment samples obtained from the Dan River revealed that a number of heavy metals exceeded ecological risk screening levels following the spill, including aluminum, arsenic, barium, iron, selenium, silver, and thallium (US EPA, 2014). By July of 2014, the concentration of many of these heavy metals had been reduced to acceptable levels as defined and assessed by the EPA. However, some heavy metals, including aluminum, arsenic, barium, and iron remained elevated in various locations along the river even after an additional five months (US EPA, 2014).

The effects of coal ash and its pollutants on aquatic and riparian populations are routinely determined by analyzing the viability of fish (Rowe et al., 2002), birds (Walls et al., 2015), plants (Nayak et al., 2015), insects (Nehring, 1976; Wesner et al., 2014) and macroinvertebrates (Cherry et al., 1979; Roig et al., 2016). However, given that many heavy metals, such as selenium, produce their most profound impact on contaminated organisms through teratogenic effects, assessing the long-term impact of coal ash exposure may take years to manifest with such large organisms. Consequently, having an early indicator of undesirable changes in an ecosystem would be of great value in assessing the overall impact of pollution spills. Due to their rapid proliferative rate and sensitivity to their environment, soil microbes, particularly bacteria, hold such promise. In fact, some reports have indicated that alterations in an ecosystem may first become apparent through modifications within the soil flora (Brookes, 1995; Giller et al., 1998).

Herein we report that one year following the Dan River spill, significant differences could be detected in soil samples exposed to coal ash when compared to upstream reference samples. These differences include changes to soil pH, nutrient content, and microbial density, enzymatic activity, and biodiversity.

Materials and Methods

Sample collection

Soil and water samples were taken from 8 locations along the Dan River. Three sample sites were located upstream from the plant, one site was located immediately downstream of the spill (within 10 m), and four sample sites were located downstream of the spill site (Fig. 1). At each location, soil samples were taken in triplicate from a deposition zone along the bank and in duplicate from the channel of the river. Water samples were taken in triplicate at each location as well. Samples were collected on January 13 and 20, 2015. Samples were not collected from Locations 6 and 7 on January 13 due to time and weather constraints, therefore additional samples were taken from these locations on January 20 so that all sample locations had an equivalent number of samples in total. Bank samples were taken from above the water line at a height of 8 inches. Channel samples were taken as close to the center of the channel as possible where sediment was present. Soil samples were collected in labeled Ziploc® bags and were stored unsealed at room temperature for 12 hours prior to analysis.

Analysis of abiotic factors in soil and water

Water temperature, pH, dissolved oxygen, and nitrate levels were measured in the field

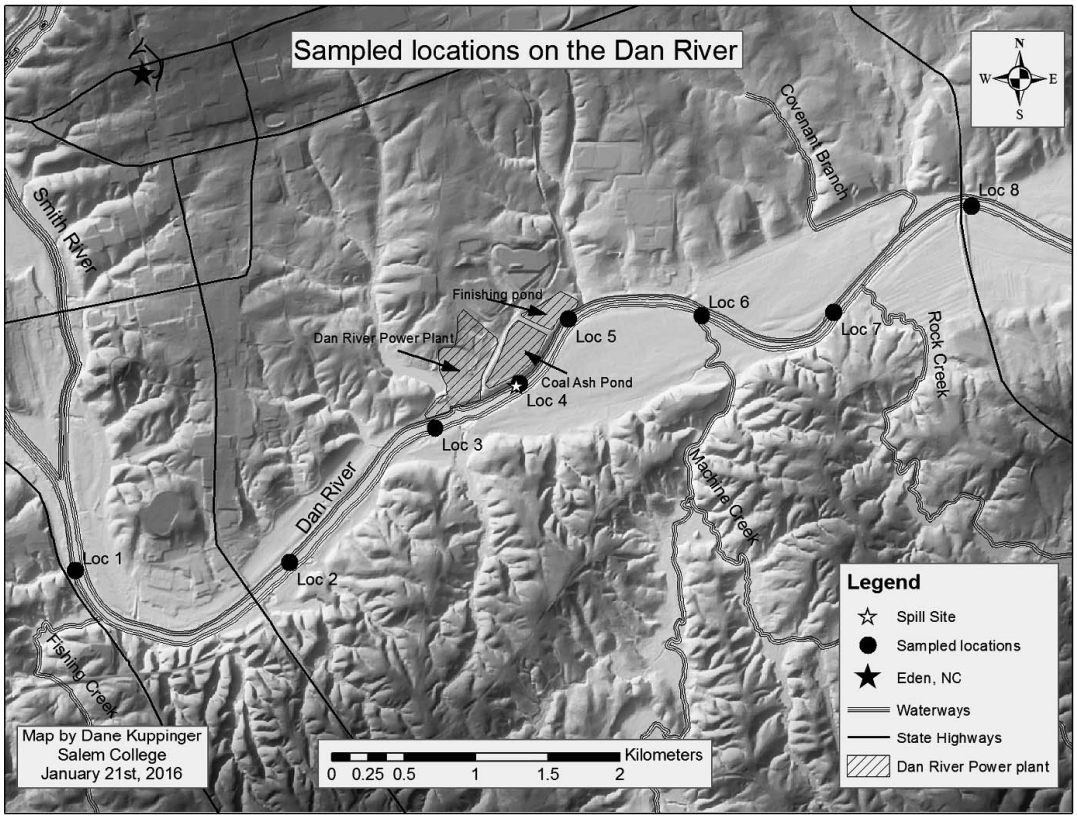

Figure 1. Map of sample locations along the Dan River. Water samples and soil samples were taken via canoe on the Dan River near Eden, NC, on January 13, 2015 and January 20, 2015. Samples were not collected from Locations 6 and 7 on January 13 due to weather constraints.

using the LabQuest Biopac sensors according to manufacturer's protocols (Vernier Software Protocol 2013a; 2016). Water turbidity was measured in the field using Secchi disks. Soil samples were air dried and sieved to 4000 microns. Soil nitrate concentrations were measured in the lab using LabQuest Biopac sensors (Vernier Software Protocol, 2013b). Soil samples from each position (bank and channel), location (1 through 8), and date were also sent to the NC Agricultural extension service for further analysis of soil nutrients: percent humic matter, weight:volume, cation exchange capacity (CEC), percent base saturation, exchangeable acidity, and the concentration of phosphorus, potassium, calcium, magnesium, manganese, zinc, copper, sulfur, and sodium (Mehlich, 1984).

Chromium analysis

To assess chromium VI levels in the soil, 1 g of soil was stirred into 380 mL of 8 M nitric acid for two hours. The filtrate containing the dissolved Cr was diluted to a 1 M solution and the Cr VI concentration was measured using atomic absorption spectroscopy. Distilled water and 1 M nitric acids were used as baselines and sample concentrations were calibrated against 5, 10, 20 and 40 mg/L standard solutions of Cr VI within 1 M nitric acid.

Culturing soil bacteria

50 g of soil were weighed and centrifuged at 2,000 RPM for 5 min to remove water. 1 g of this soil was then resuspended in 9 mL of 0.85% saline and vortexed for 5 min. All particulate matter was allowed to settle. 100 μL of the

supernatant was then plated or serial dilutions were prepared in Luria Bertani (LB) broth and plated. All samples were plated on soil-extract agar which was prepared as follows. 400 g of soil from a nonrelated site was added to 400 mL of H_2O. This solution was then autoclaved, centrifuged for 10 min at 3,400 RPM, and the supernatant was added to standard tryptic soy agar for a final concentration of 26%.

Upon plating, all samples were cultured at 30°C for 48 hours prior to counting. Although both bacteria and fungus are capable of growing on soil extract agar, this approach favors bacterial growth because culture conditions are generally insufficient for actively growing hyphae to form a colony.

16s rRNA analysis

Plates cultured as above were wrapped in Parafilm® and shipped overnight to GeneWiz, Inc (South Plainfield, NJ) for 16S rRNA gene amplification and sequencing. Sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis using the National Center for Biotechnology Information (NCBI) website. Sequences utilized for phyla determination averaged $>$ 90% query coverage and $>$ 95% sequence identity.

Contact slide analysis

150 g of soil from each site were weighed and placed in glass beakers. 10 mL of sterile H_2O was added to each beaker. A glass slide and a glass coverslip were inserted vertically into the soil, leaving ~2 cm of each slide projecting from above the soil surface. The beakers were then covered with ventilated Parafilm® and incubated for 7 days at room temperature. Microbes that adhered to the slides or cover slips were fixed with 40% (v/v) acetic acid and stained with phenolic Rose Bengal. Slides were analyzed by microscopy at either 400X or 1000X magnification. For absorbance readings, Rose Bengal bound to the cover slips was removed with 1 mL 20% acetone/80% ethanol. The dissolved dye was transferred to a cuvette and analyzed by spectroscopy (λ = 550 nm).

Dehydrogenase analysis

1 mL of 3% TTC (2,3,5-triphenyltetrazolium chloride; Sigma, St. Louis, MO) and 2.5 mL of H_2O were added to 6 g of soil in a 50 mL conical tube. The samples were then mixed and the tubes were sealed to exclude oxygen. Following a one week incubation at room temperature, 10 mL of methanol were added to each sample and mixed. The supernatant was filtered using Whatman #42 filter paper. The filter was subsequently rinsed with methanol (20 mL) until the filtrate ran clear. Absorbance was then assessed by spectrophotometry (λ = 485 nm).

Results

Abiotic factors differ between reference and coal ash impacted sites

Although EPA analysis indicated that the majority of heavy metals had been reduced to acceptable levels within 5 months of the spill, we wanted to determine if Cr VI could still be detected at our sampling locations (Fig. 1). On the banks, we found that Cr concentrations were highest downstream of the spill site at Location 5 (112.5 $\pm$ 51.92; Fig. 2, n = 3). Channel Location 8 (33.17 $\pm$ 60.57) had Cr levels significantly different from Cr levels of nearly all other locations (Fig. 2, n = 3; t-test). On the bank, there was a significant difference between Cr content upstream of the spill site (9.615 $\pm$ 1.773), at the spill site (21.154 $\pm$ 0.962), and downstream of the spill site (54.021 $\pm$ 13.631; Table 1, n = 20; t-test, $p < 0.05$). In the channel, there was no significant difference in the Cr content at the spill site when compared to that found in the upstream samples. However, there was a significant difference when comparing Cr levels in bank samples obtained upstream (1.923 $\pm$ 0.608) vs. downstream of the spill site (37.139 $\pm$ 8.347) and at the spill site (9.615 $\pm$ 8.215) vs. downstream of it (Table 1, n = 17; t-test, $p < 0.05$).

Given that the addition of coal ash has been shown to modify the acidity of amended soils, we next sought to determine if there were differences in the pH at our sample locations (Cox et al., 2001). Soil pH from all locations

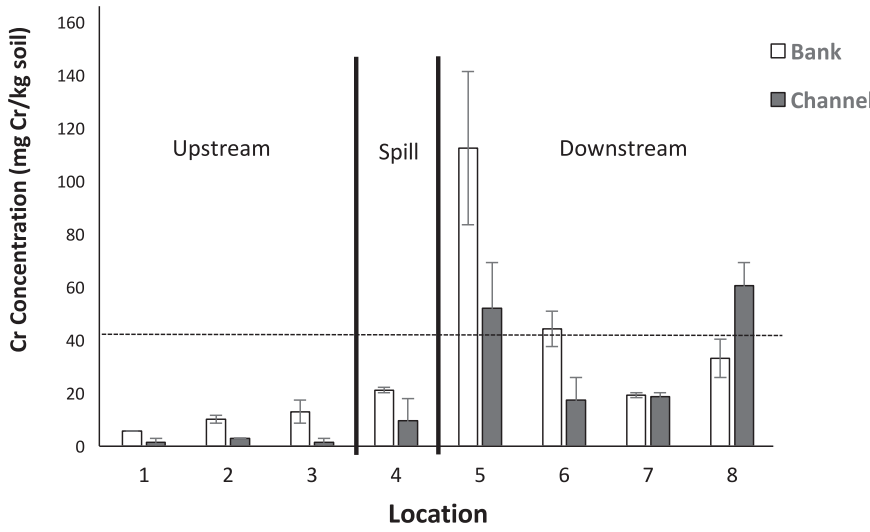

Figure 2. Mean (+/- 1 s.e.) soil chromium concentration at each sampling location (3 samples per location) along the Dan River near Eden, NC on 1/13 and 1/20. Horizontal line indicates the EPA Ecological Risk Screening Levels (ERSLs) for Chromium concentrations (43.4 mg/kg soil).

was primarily acidic. However, there was a significant difference between the pH of the soil in the channel upstream (6.22 ± 0.339) vs. downstream (5.24 ± 0.186) of the spill site (Table 1, n = 10; t-test, $p < 0.05$) and between the pH at the spill site (5.9 ± 0.1) vs. downstream of the spill site (5.24 ± 0.186, n = 7; t-test, $p < 0.05$). Conversely, bank soil pH

Table 1. Summary of p values from t-test comparisons of abiotic data collected upstream of the spill site, at the spill site, and downstream from the spill site.

	Upstream vs. downstream		Upstream vs. site		Site vs. downstream	
	Bank[a]	Channel[b]	Bank	Channel	Bank	Channel
humic_mat (%)	+	+	NS	NS	+	+
weight:volume (g/cm^3)	-	-	NS	NS	- - -	NS
CEC (meq/cm^3)	NS	NS	NS	NS	+	NS
Base_Sat (%)	NS	- - -	NS	-	NS	-
Exchangeable acidity	NS	+ + +	NS	NS	NS	+ + +
pH	NS	- - -	NS	NS	NS	- - -
P (mg/dm^3)	NS	NS	NS	NS	NS	NS
K (mg/dm^3)	NS	+	NS	NS	NS	NS
Ca (mg/dm^3)	NS	- - -	NS	NS	NS	NS
Mg (mg/dm^3)	NS	- - -	NS	NS	NS	NS
Mn (mg/dm^3)	+	NS	+	- - -	+	NS
Zn (mg/dm^3)	NS	+	NS	NS	+ + +	+
Cu (mg/dm^3)	NS	+ + +	NS	NS	NS	+ + +
S (mg/dm^3)	NS	+	-	NS	NS	+ + +
Na (mg/dm^3)	NS	NS	N/A[c]	N/A	NS	NS
Chromium (mg/g)	+ + +	+ + +	+ + +	NS	+ + +	+ + +
Nitrate (mg/kg)	NS	NS	N/A	N/A	N/A	N/A

[a] Bank samples analyzed against other bank samples
[b] Channel samples analyzed against other channel samples
+, -, = Variables significantly increased or decreased (respectively) at $p < 0.01$
+++, --- = Variables significantly increased or decreased (respectively) at $p < 0.05$
NS = Variable did not significantly change
[c] N/A = No analysis due to lack of variance

did not vary significantly between sample locations.

When grouped by location relative to the spill, significant differences in the other measured abiotic soil properties were also found for all variables except phosphorus and nitrate (Table 1). Percent humic matter, cation exchange capacity (CEC), exchangeable acidity, potassium, zinc, copper, sulfur, and sodium were all significantly higher below the spill site and the weight to volume ratio, percent base saturation, pH, calcium, and magnesium were all significantly lower downstream of the spill site (Table 1). Bank and channel sample values changed in the same direction (positively or negatively) for all variables except manganese.

Soil samples from downstream and spill site locations exhibit a reduction in microbial density

Coal waste products and heavy metals have been shown to impact the size, fitness, and diversity of microbial populations in soil (Giller et al., 1998; Nayak et al., 2015; Oliveira et al., 2006). In order to determine if exposure to coal ash had an impact on the abundance of soil microbes along the Dan River, contact slide analysis was performed. Microscopic analysis of the slides revealed the presence of fungi, protists (specifically diatoms), and bacteria at all locations (Fig. 3A). In order to quantify the density of microbes that adhered to each slide, spectrophotometric analysis was performed. A significant reduction in the density of microbial populations was observed when comparing upstream bank and downstream bank samples ($p \leq 0.05$) and spill site bank and downstream bank samples ($p \leq 0.05$; Fig. 3). A significant reduction was also noted between upstream channel and downstream channel samples ($p \leq 0.01$). No difference was detected between upstream bank and spill site bank samples, while channel samples from these locations exhibited a decrease in microbial density that approached statistical significance ($p = 0.055$). Taken together, these results demonstrate an overall trend in the reduction of microbial density between reference site samples and those impacted by coal ash.

Dehydrogenase activity is increased in coal ash impacted sites

Enzyme activities are commonly used as indicators of soil quality and microbial fitness. Enzymes analyzed frequently included ureases, acid phosphatases, and dehydrogenases. Dehydrogenases are nonspecific enzymes found in all living organisms that transfer hydrogen anions to electron acceptors, particularly NAD^+ or $NADP^+$. Numerous reports have demonstrated that heavy metal pollutants in soil can greatly diminish dehydrogenase activity (Oliveira et al., 2006). In order to assess if samples obtained from the various locations along the Dan River exhibited a difference in the activity of these enzymes, soil from each site was cultured with the NAD^+ inhibitor TTC. Reduction of TTC was then assessed by spectrophotometry. Given that the overall density of soil microbes appeared to have been reduced when subjected to contact slide analysis as well as the fact that heavy metals are known to impede the enzymatic activity of soil microbes, we anticipated that the dehydrogenase activity of samples collected both at the spill site and downstream of the spill site would also be reduced. However, this did not prove to be the case for every sample (Fig. 4). In bank samples collected from the spill site, dehydrogenase activity was significantly reduced by over 90% ($p \leq 0.01$). Conversely, bank samples collected downstream of the spill site exhibited more activity than upstream samples ($\sim$35%) and significantly greater than the spill site samples ($p \leq 0.001$). Channel samples collected from the spill site were comparable to upstream samples, while those taken from downstream sites exhibited over twice the activity of reference or spill site samples ($p \leq 0.01$). These data indicate that coal ash contaminant may have negatively impacted the microbial fitness along the bank at the spill site itself. However, in both bank and channel soils collected downstream of the spill, the activity of microbial dehydrogenases appears to have been increased.

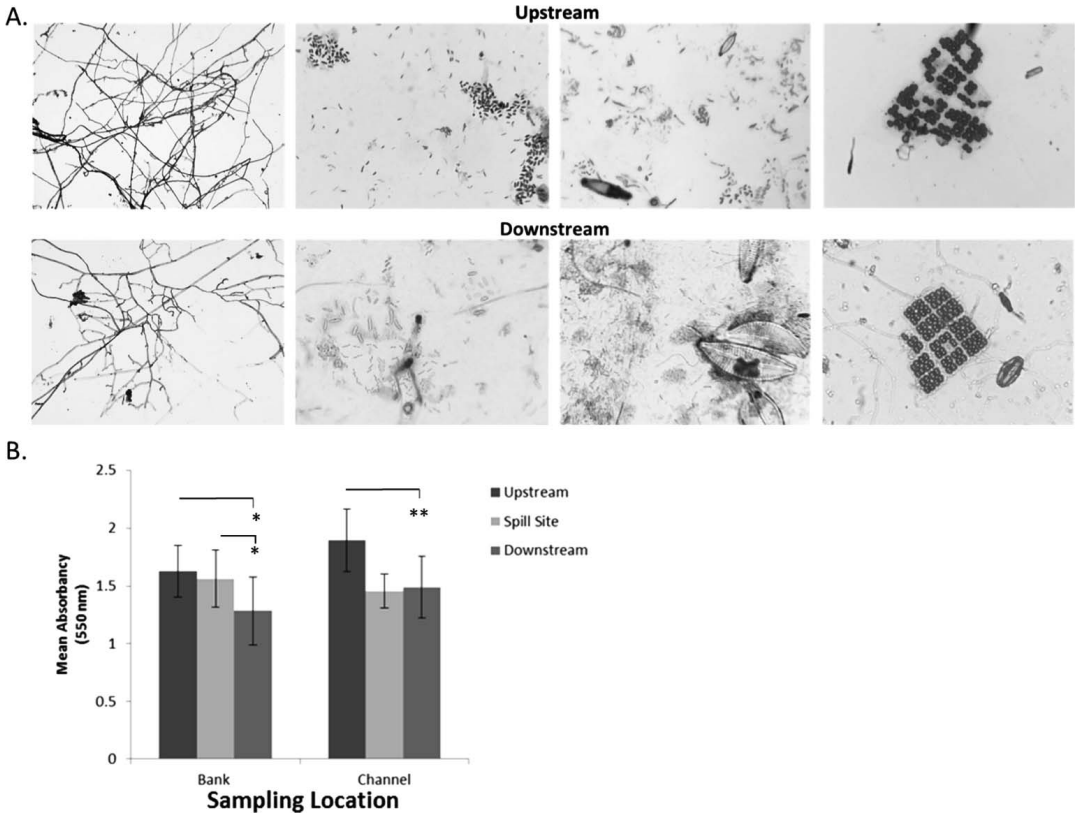

Figure 3. Soil samples downstream of the coal ash spill site exhibit a decrease in overall soil microbe density. A. 150 g of soil from the indicated sites were aliquoted into glass beakers. A glass slide and coverslip were inserted vertically into the soil, leaving ~2 cm of each slide projecting from above the soil surface. The cups were covered and incubated for 7 days at room temperature. Microbes adherent to the slides or cover slips were then fixed with acetic acid and stained with phenolic Rose Bengal. Slides were analyzed by microscopy at 1000X magnification. B. Rose Bengal bound to the cover slips was removed with 1 mL 20% acetone/80% ethanol. The dissolved dye was then measured at a wavelength of 550 nm. Results are representative of two assays with triplicate samples for each location. * $p \leq 0.05$ compared to spill site bank; ** $p \leq 0.01$

Soil downstream of the spill site exhibits an increase in culturable bacteria

We next sought to determine if there was a reduction in mesophilic bacterial populations upstream, downstream, or at the spill site itself. Having observed a decrease in soil microbe density when comparing downstream location to upstream samples, we hypothesized that the overall quantity of bacteria would be similarly reduced. However, this was not found (Fig. 5). When comparing upstream bank and channel to spill site bank and channel samples, we did observe a significant decrease in the number of colony forming units (CFU) per gram of soil ($p \leq 0.05$). However, downstream bank samples exhibited an average of twice the number of CFU/g of soil ($p \leq 0.05$) when compared to upstream bank samples, and almost four times as many CFU/g when compared to spill site bank samples ($p \leq 0.001$).

Coal ash exposed soil samples exhibit a unique taxonomical distribution for certain phyla

Scientists have estimated that a single gram of soil may contain as many as 13,000 species of bacteria (Torsvik et al., 1990). This diversity is important to the health of an ecosystem since different bacterial populations play distinct metabolic roles in the soil. For example, some genera of bacteria are capable of nitrification

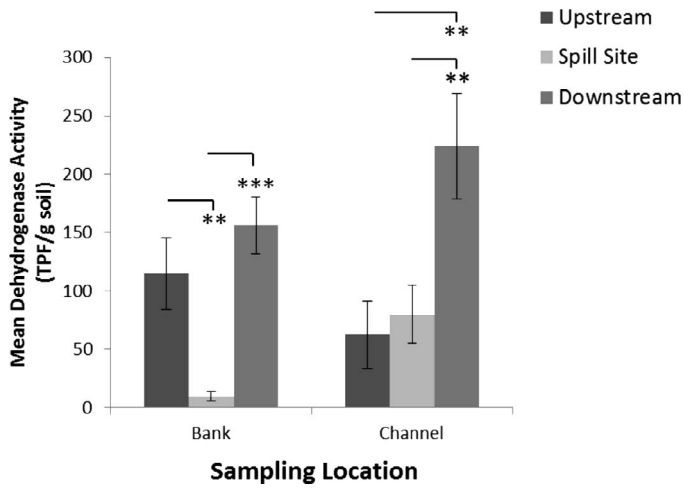

Figure 4. Soil microbes downstream and at the spill site exhibit distinct levels of dehydrogenase activity when compared to upstream samples. 3% 2, 3, 4-triphenyltetrazolium chloride (TTC) was added to equivalent volumes of soil from the indicated locations. The soil was then incubated at room temperature in air-tight containers for one week. Methanol was mixed with the sample and filtered to remove particulate matter. TTC reduction to trephenyl formazan (TPF) was then assessed by spectrophotometry at 485 nm. Data represent the mean TPF/g of soil. ** $p \leq 0.01$; *** $p \leq 0.001$

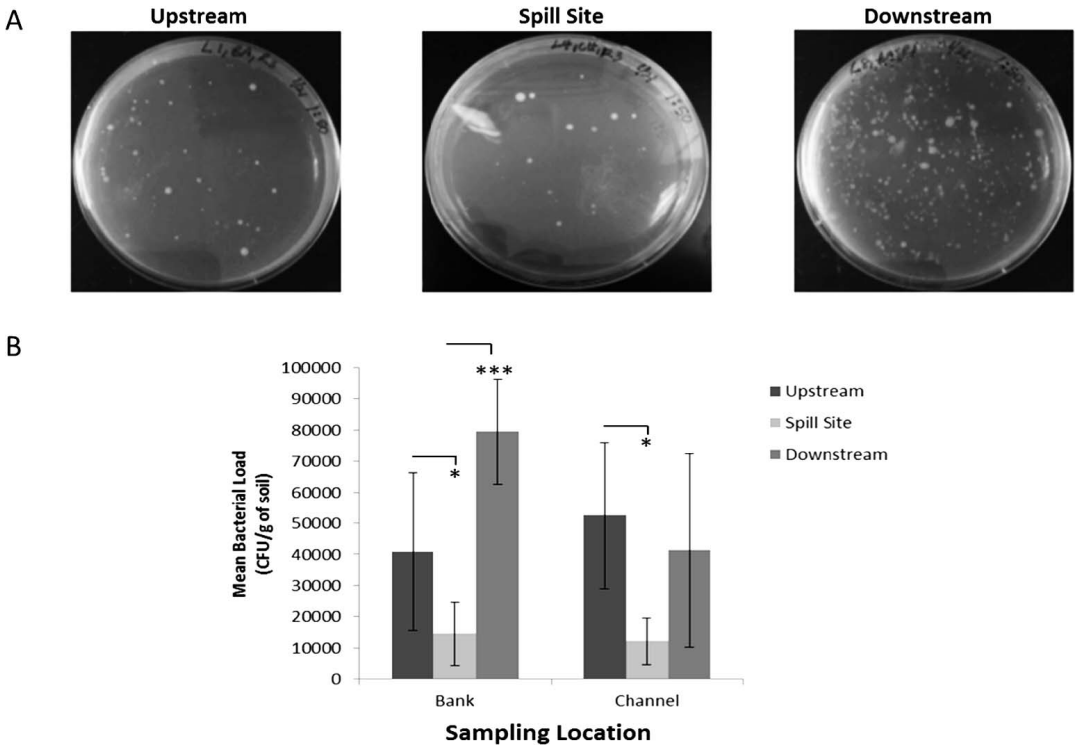

Figure 5. Soil samples upstream, at, and downstream of the coal ash spill site exhibit distinct loads in culturable bacteria. A. Soil microbes from the indicated sampling locations were plated onto soil extract agar. Plates were incubated at 30°C for 48 hours. Images are representative of plates from each location. B. The total number of colonies on each plate was counted and the average bacterial load was calculated as colony forming units (CFU) per gram of soil. Graph represents two independent assays with all samples in triplicate (bank) or duplicate (channel). * $p \leq 0.05$; *** $p \leq 0.001$

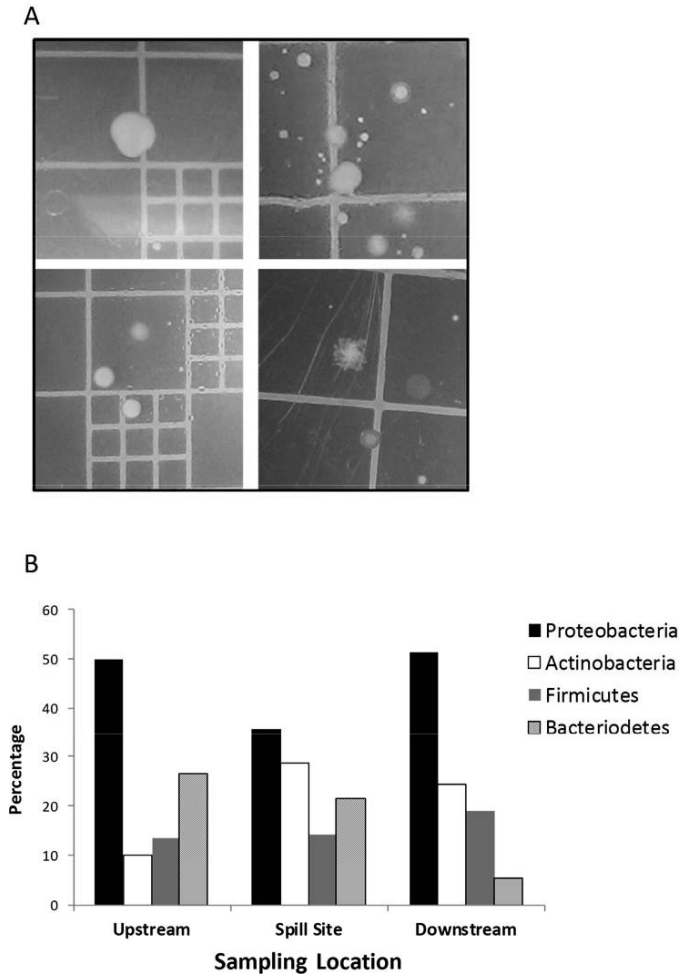

Figure 6. Bank soil samples obtained from coal ash exposed sites exhibit a distinct distribution for certain bacterial phyla. A. Soil samples taken from either the bank or channel of the various locations were centrifuged to remove excess water, resuspended in saline, and vortexed. Soil supernatant was then cultured on soil extract agar at 30°C for 48 hours. The images reflect the typical form of bacterial colonies isolated from all locations. B. Bacterial colonies cultured from the bank of the indicated locations were subjected to 16S rRNA analysis. Graph represents the percentage of each phyla in comparison to the total number of samples submitted.

while others carry out nitrogen fixation or denitrification. As a result, a soil may contain a great deal of microbial biomass, but still be relatively unfit if this biomass is of limited taxonomical and functional diversity (Giller et al., 1989). Having observed a quantitative difference in the number of culturable bacteria and the enzymatic activity of the soil microbes obtained from each location, we next questioned if there were also differences in the community structure of the soil bacteria from these sites. To assess this, we subjected bacterial colonies isolated from the banks of each location to 16S rRNA analysis. An attempt was made to select an equivalent number of colonies with similar colony characteristics (i.e. colony morphology, color, texture, etc.) from each location. The vast majority of colonies on the plates had smooth margins, were opaque, and white in color (Fig. 6A). The second most common colony type had smooth margins, a translucent border, and an opaque, white center. Few colonies exhibited undulating edges, uneven margins, or a distinct color (either yellow or

green). A total of 32 samples were submitted from upstream locations, 16 from the spill site, and 37 from downstream locations. Two of the samples from both the upstream and spill site locations produced mixed colony results, and were thus excluded from the analysis. All samples submitted were classified under the domain Bacteria and belonged to one of four phyla, namely *Proteobacteria*, *Actinobacteria*, *Firmicutes*, or *Bacteroidetes*. In accordance with published reports, *Proteobacteria* was the most common phyla isolated from all three locations, representing 50%, 35%, and 51% of the submitted samples from upstream, spill site, and downstream locations, respectively (Fig. 6B; Janssen, 2006). Conversely, the percentage of *Actinobacteria* more than doubled in soil samples taken from coal ash exposed sites when compared to the upstream reference site. Finally, while the ratio of *Firmicutes* was relatively constant among the various sites, the percentage of *Bacteroidetes* was considerably reduced in downstream samples (upstream = 26%, spill site = 21%, downstream = 5%). Log-linear analysis of the data set demonstrated that the differences observed in the phyla were statistically significant in a manner that was dependent upon the location from which the samples were isolated ($p \leq 0.001$).

Discussion

Decades of research have established the fact that heavy metal contamination of soils can have deleterious effects on soil microbes (Bååth, 1989; Giller et al., 1998; Kuperman et al., 1997; Nayak et al., 2015; Oliveira et al., 2006). However, coal-derived waste, particularly fly ash, is routinely used as a soil amendment for agricultural lands. Fly ash has been shown to improve the quality of soil by serving as a source for essential macronutrients, such as Calcium, Nitrogen, and Potassium, increasing the soil's carbon content and thus its ability to retain nutrients, altering the bulk density of soil, improving its water retention capacity, and buffering soil against changes in pH (Basu et al., 2009). Furthermore, studies have demonstrated that the addition of limited quantities of ash waste can actually improve the enzymatic activity of soil microbes (Nayak et al., 2015; Sharada and Sahu, 2004). Herein, we report that channel and bank soils obtained from the Dan River approximately one year following a coal ash spill exhibited significant differences in abiotic factors, namely the concentration of chromium, sulfur, calcium, and soil pH, as well as the density, diversity, and fitness of the microbes contained therein. The changes in almost all abiotic soil variables upstream of the spill vs. downstream of the spill strongly suggests that the coal ash has had a significant and lingering impact upon the microbial environment. While increases in humic matter concentrations, zinc, copper, sulfur, and chromium and decreases in soil pH are consistent with the expected impacts of coal ash deposition, the observed decrease in soil nitrate and calcium is contrary to the expected impacts of ash deposition.

In terms of heavy metal concentrations, we found that Cr VI levels increased significantly at the spill site when compared to upstream channel and bank samples. Downstream samples also had significantly increased levels of Cr, even when compared to those taken from the spill site. Notably, the most contaminated sample, located immediately downstream of the spill had Cr concentrations above 43.4 mg/kg, the amount that the EPA deems an ecological risk for aquatic organisms (US EPA, 2014). The levels of Cr from other sample locations, namely Location 6, corresponded with values determined by the EPA, which also analyzed samples in this approximate area (US EPA, 2014). Overall, this indicates that soil microbes at the spill site and downstream of the spill were undoubtedly exposed to Cr following the spill and likely other heavy metals as well. Furthermore, it suggest that pockets of heavy metals may persist, thus increasing the potential for ongoing and future impacts to the soil ecosystem. Ultimately, it stands to reason that the presence of Cr in our sampling locations had an impact on the microbes therein, however, it cannot be excluded that other factors, including the presence of heavy metals that were not

tested for, may also have contributed to our findings.

The pH of coal ash varies with the acidity of the coal from which it was derived (Basu et al., 2009; Openshaw, 1992). The measured significant decrease in soil pH is consistent with either direct exposure to coal ash or to other combustion waste products, such as the nitrogen oxide and sulfur oxide. Regardless of the cause, variations in soil pH can directly impact the behavior of heavy metals within a soil. Alkaline conditions have been demonstrated to alter the mobility and bioavailability of heavy metals in a soil (Chaudhuri et al., 2003; de Matos et al., 2001; Wong et al., 1986). Furthermore, the speciation of a heavy metal can also be influenced by the pH of the local environment (DiPalma et al., 2012; Nederlof et al., 1993). The lower pH downstream of the spill site may have made the heavy metals within these soils more accessible to the soil microbes. Studies investigating the mobility of Cr found that it is more readily extracted from soils at a lower pH (DiPalma et al., 2012). This may explain why an overall decrease in soil microbe density was observed.

Interestingly, despite the finding that the pH of all sampled soils was acidic, we did not identify any bacteria belonging to the phyla *Acidobacteria*, a phyla known to favor acidic soils (Jones et al., 2009). Failure to isolate members of this phylum is likely a reflection of the difficulty in culturing bacteria of this type, or may reflect a sensitivity to heavy metals (Jones et al., 2009; Ward et al., 2009). Although it has been hypothesized that a gram of soil may contain tens of thousands of bacterial species, the vast majority (90-99%) of these cannot be cultured using standard lab protocols (Torsvik et al., 1990, 1996). One significant factor that influences the growth of bacteria is the choice of media. Herein we utilized a standard tryptic soy agar amended with soil extract. However, studies have demonstrated that while soil extract agar may possess factors necessary for some bacterial strains to grow, alternative agars may bolster the growth of different species within the same soil. For example, Lutton et al. (2013) demonstrated that soil extract agar, 1% nutrient agar, and R2-A agar yielded distinct quantities of colony forming units when culturing bacteria from the same soil.

In addition to the choice of media, other culturing conditions that were utilized in this analysis, such as temperature (30°C), presence of oxygen, absence of light, and the duration of culture (48 hours), undoubtedly favored certain species over others. Consequently, it is likely that only a subset of bacteria were captured by these experiments, perhaps explaining the discrepancy we observed in the number of total microbes isolated from soil upstream of the spill compared to the number of culturable bacteria. Contact slide analysis revealed that the overall number of microbes was reduced downstream of the spill, while bacterial cultures suggested that the quantity of bacteria was increased. It stands to reason that the overall quantity of bacteria in soils exposed to coal ash may actually have been reduced. However, those bacteria that remained in the soil following coal ash exposure may have preferentially grown on the media utilized here, while alternative varieties of bacteria upstream of the spill may not have been able to grow as well. This notion ties into the observation that the phylogenetic profiles of the soil bacteria upstream, downstream, and at the spill site also differed. While the proportion of *Proteobacteria* and *Firmicutes* remained relatively constant between locations, *Actinobacteria* appears to have thrived in areas contaminated with coal ash. Conversely, *Bacteroidetes* populations suffered in downstream soils. These differences may be a reflection of the ability of species within these phyla to resist toxicity of heavy metal contaminants. In addition to expressing genes that encode resistance to heavy metals, *Firmicutes* species may have been unaffected since they frequently produce spores. The observation that the ratio of *Actinobacteria* increased in coal ash exposed soils is in accordance with studies demonstrating that members of this phyla are frequently resistant to a range of heavy metals, including Cr (Benimeli et al., 2011; El Baz et al., 2015). One potential mechanism for this resistance is the use of ATP-binding cassette (ABC) trans-

porters which can serve as heavy metal efflux pumps (Aldesuquy et al., 1998).

Microbial fitness measured by the dehydrogenase activity also exhibited distinct patterns between sampling locations. Unlike the overall microbial density in the soils, the dehydrogenase activity of soil microbes did not reveal a downward trend. Spill site samples had either a decrease in activity (in bank) or no difference (in channel) when compared to the reference site. However, the dehydrogenase activity in downstream locations was significantly higher than both reference and spill sites for bank and channel. Although contrary to our prediction, increases in the enzymatic activity of soil microbes have been observed in other studies following moderate heavy metal contamination (Fliessbach et al., 1994; Nayak et al., 2015; Sharada and Sahu, 2004). In terms of dehydrogenase activity, coal ash exposure seems to have had a positive impact on the fitness of microbes located along the bank. This may be due to a combination of factors, including heavy metal resistance of certain microbes as well as the fact that in downstream locations we observed increases in nutrients, such as humic matter, potassium, zinc, and sulfur. Since all four of these are classified as essential nutrients for microbes, their increased presence in downstream samples may account for the amplification in enzymatic activity. Alternatively, the increase in enzymatic activity downstream may be attributed specifically to the bacterial population since an increase in their presence was observed via plate counts.

This study examined the effect the coal ash spill had on Dan River in terms of Cr concentrations and other abiotic factors as well as microbial density, enzymatic activity and bacteria quantity and diversity. The collected data indicates some significant alterations in aforementioned aspects. However, this study was limited in its ability to make decisive conclusions about the impact of the spill upon the microbial community because of their variable responses and the complexity of the community in question. It is clear, however, that both biotic and abiotic properties of the Dan River ecosystem significantly changed below the site of the Dan River Steam Station. This study also demonstrates that the soil microbial community was impacted by coal ash exposure, and that impacts upon this community were still measurable one year following the spill; even when obvious impacts upon larger life forms are harder to quantify. What remains unclear is whether this change in the microbial community is a result of the impacts which have already worked their way through the macrobiota or is a precursor to impacts which are not yet apparent in longer-lived species. Thus, a more comprehensive and longer term study is needed to determine the enduring effects of the spill. By including an assessment of additional heavy metals and collecting data across multiple seasons and years, such a study could construct a clearer picture of the post-spill pattern of changes in abiotic factors and the impacts on soil microbes and the larger ecosystem.

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