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Short-Term Exposure to Coal Combustion Waste Has Little Impact on the Skin Microbiome of Adult Spring Peepers (*Pseudacris crucifer*)

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ABSTRACT

Disruptions to the microbiome can impact host health as can exposure to environmental contaminants. However, few studies have addressed how environmental contaminants impact the microbiome. We explored this question for frogs that breed in wetlands contaminated with fly ash, a by-product of coal combustion that is enriched in trace elements. We found differences in the bacterial communities among a fly ash-contaminated site and several reference wetlands. We then experimentally assessed the impacts of fly ash on the skin microbiome of adult spring peepers (*Pseudacris crucifer*). Frogs were exposed to fly ash in the laboratory for 12 h, the duration of a typical breeding event, and the skin microbiome was assessed after 5 days (experiment 1) or after 5 and 15 days (experiment 2). We examined bacterial community structure using 16S rRNA gene amplicon sequencing and metabolite profiles using high-pressure liquid chromatography-mass spectrometry (HPLC-MS). We found little impact as the result of acute exposure to fly ash on the bacterial communities or metabolite profiles in either experiment, suggesting that the bacterial symbiont communities of adults may be relatively resistant to brief contaminant exposure. However, housing frogs in the laboratory altered bacterial community structure in the two experiments, which supports prior research suggesting that environmental source pools are important for maintaining the amphibian skin microbiome. Therefore, for contaminants like fly ash that may alter the potential source pool of symbionts, we think it may be important to explore how contaminants affect the initial assembly of the amphibian skin microbiome in larval amphibians that develop within contaminated sites.

IMPORTANCE

Animals are hosts to many symbiotic microorganisms, collectively called the microbiome, that play critical roles in host health. Therefore, environmental contaminants that alter the microbiome may impact hosts. Some of the most widespread contaminants, produced worldwide, are derived from the mining, storage, and combustion of coal for energy. Fly ash, for example, is a by-product of coal combustion. It contains compounds such as arsenic, selenium, cadmium, and strontium and is a recognized source of ground and surface water contamination. Here, we experimentally investigated the impacts of short-term fly ash exposure on the skin microbiome of spring peepers, one of many species of amphibian that sometimes breed in open fly ash disposal ponds. This research provides a look into the potential impacts of fly ash on an animal's microbiome and suggests important future directions for research on the effects of environmental contaminants on the microbiome.

Vertebrates host a wide array of symbiotic microorganisms, mainly in the gut, but there are unique microbial communities spread throughout the body from the nasal cavity to the lungs to the skin (1). While we have long understood that the microbiota residing in the gut are diverse and are important in helping to digest food, only recently have we begun to appreciate the more diverse functions of these microbial symbionts. Our knowledge in this area is growing in large part because of advances in molecular microbiology and next-generation sequencing technologies that now allow us to study these complex microbial communities without culturing them. Studies performed by the Human Microbiome Project indicate complex interactions in these symbiotic microbial communities that influence their function (2), and disturbance and dysbiosis in these communities contribute to a variety of disease states (3, 4).

Most studies that examine the perturbation of symbiotic microbial communities in vertebrates have thus far focused on the impacts of antibiotics in humans and model lab systems (5–9). There are relatively few studies that examine the role of environmental contaminants on the human microbiome or in model systems (10–12) and even fewer that address this in wildlife. One such study indicates that arsenic exposure in drinking water can alter the composition of gut microbiota in mice and the subsequent suite of metabolites produced by the microbes, thereby potentially altering the function of this complex symbiotic community (11).

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With more than 82,000 chemicals listed as having been for sale or in use in the United States between 1979 and 2007 (13), humans and wildlife are inevitably exposed to a multitude of exogenous chemicals, and many of these have been linked to distinct health outcomes (14). The consequences of these exposures on the microbiome, and how they might impact the critical physiological functions of the host, are of increasing interest.

Some of the most widespread contaminants produced worldwide are derived from the mining, storage, and combustion of coal for energy. When coal is combusted, the predominant solid waste by-product is fly ash, a fine particulate material that is enriched in trace elements, including arsenic, selenium, cadmium, and strontium. In the United States, more than 130 million tons of fly ash is produced each year, making it the second largest solid waste stream in the country (15). Fly ash is a recognized source of ground and surface-water contamination from routine disposal procedures as well as from occasional unintentional releases. For example, technological failures at disposal facilities have repeatedly resulted in catastrophic releases of fly ash into surface waters in the United States, including recent high-profile spills in the Dan River in North Carolina (February 2014) and in the Emory River near Kingston, TN (December 2008). The Emory River case resulted in the single largest solid waste spill in U.S. history and was five times larger than the 2010 *Deepwater Horizon* BP oil spill in the Gulf of Mexico (16).

When fly ash is not recycled into other products, it is frequently disposed of in open aquatic surface impoundments that attract wildlife, including waterfowl and amphibians. When wildlife use these impoundments, they are exposed to trace elements, such as selenium, mercury, and arsenic, that can affect their health (17). Health impacts of fly ash exposure mediated through changes in the microbiome have not been investigated. However, of the relatively few studies that have investigated the potential effects of contaminants on the microbiome, several have focused on trace elements (11, 18), and it is known that microbiota can alter the metabolism of these elements (19, 20).

Amphibians have been model systems for understanding contaminant effects on vertebrates in the lab and in more natural settings (21). Some amphibians use fly ash ponds for breeding (22), and there is a large amount of literature documenting the impacts of fly ash on amphibian development, physiology, and survival (e.g., references 22, 23, 24). In the last decade, research on the amphibian microbiome has also increased, mainly in relation to the potential protective role of the skin microbiome in defense against the lethal skin pathogen *Batrachochytrium dendrobatidis* (25–27). From this research, it is clear that amphibians host a diverse group of symbiotic microbes on their skin (28, 29). Many of these microbial symbionts are found in amphibian habitats, suggesting an environmental origin (29, 30); furthermore, environmental source pools appear to be important for the maintenance of the amphibian skin microbiome (31). To begin to examine the potential impacts of fly ash on microbial communities in general and on the amphibian skin microbiome, we assessed bacterial communities in substrate and in water from reference and fly ash-contaminated wetlands where amphibians breed. We then conducted two laboratory experiments using the same design each time, but with differing sampling points, to determine how the symbiotic skin bacteria of adult spring peepers (*Pseudacris crucifer*) were impacted by short-term exposure to fly ash.

MATERIALS AND METHODS

Field sampling of fly ash-contaminated and reference sites. We used sterile rayon swabs (MW113; Medical Wire Equipment & Co. Ltd., Corsham, United Kingdom) to collect environmental samples from the water and substrate of four reference ponds (three ephemeral ponds and one permanent pond) and one permanent fly ash-contaminated pond in South Carolina. The contaminated site that was sampled was the same site where we subsequently collected the fly ash used in experiments. To collect the samples at each site, three swabs were passed through the water for approximately 5 s/swab in different regions of the pond, and three swabs were run through the substrate for approximately 5 s/swab in different regions of the pond (as in references 29, 30, and 32).

Study species. Spring peepers are treefrogs (family Hylidae) that occur throughout the eastern United States (33). They are particularly noticeable in early spring when they emerge from hibernation to breed in ponds and wetlands. During the breeding season, males call at night from shallow water or the surrounding vegetation to attract females. During the day, the adults typically retreat to hiding places under logs and beneath the bark of trees in the surrounding woodlands. Eggs are deposited in water, and the larvae continue to develop there until they metamorphose into juvenile froglets. Juveniles and adults occupy similar habitats. Of importance here, adult spring peepers are typically only closely associated with water during nightly breeding bouts, although all of their development from egg to froglet also occurs in aquatic habitats.

Experimental design overview. Our experimental design consisted of two treatments, a contaminant treatment, in which adult frogs were exposed to fly ash, and a control treatment, in which frogs were exposed only to water. To evaluate the effects of fly ash, we swabbed the skin of the frogs prior to exposure—at the time when individuals were collected in the field—and then again at several time points after brief exposure to fly ash in the laboratory. We assessed changes in bacterial community composition and in secondary metabolite profiles. We ran two experiments, using the same methods each time, first in 2013 and then again in 2014. In 2014, we extended the duration of the experiment from 5 days to 15 days to examine the potential latent effects of acute exposure to fly ash on the microbiome. All work was approved by the Virginia Tech Institutional Animal Care and Use Committee with scientific collection permits from the Virginia Department of Game and Inland Fisheries.

Field collection of frogs. In spring 2013, we collected 14 adult male spring peepers from a single pond at Virginia Tech's Kentland Farm research property (Montgomery County, VA, USA). In spring 2014, we collected an additional 16 adult male spring peepers from the same pond. Given the location of the pond and the home range of this species, no individuals in this population had ever been exposed to fly ash previously. Individuals were caught by hand, using new nitrile gloves for each individual, and were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI, USA). All individuals were swabbed within ~30 min of capture in the field to describe the bacterial communities and associated metabolites in a natural state. Before swabbing, we rinsed each frog by pouring ~50 ml of sterile deionized water over its body to remove any dirt and transient bacteria. After swabbing, we returned each individual to its plastic bag for transport to the laboratory.

Each individual was swabbed twice: first, to sample the cutaneous bacterial community and, second, to sample secondary metabolites. We sampled the cutaneous bacterial community using sterile rayon swabs (MW113; Medical Wire Equipment & Co. Ltd., Corsham, United Kingdom) as in Walke et al. (29). We then used foam-tipped swabs to sample metabolite profiles as in Umile et al. (34). Prior to use, the polyurethane swabs were prerinsed twice in methanol to remove methanol-soluble impurities and were then allowed to dry under sterile conditions in the laboratory. To standardize swabbing across individuals, we swabbed the ventral surface 20 times, each thigh 5 times, and each hind foot 5 times for a total of 40 strokes/swab type/individual. We placed swabs in sterile, empty 1.5-ml microcentrifuge tubes on ice in the field and transferred them to a −80°C container in the laboratory prior to processing. Swabs

for metabolite profile analysis were shipped frozen to Villanova University.

Experimental exposure to fly ash. In the laboratory, frogs were housed at 21°C with a 12/12-h light cycle. We weighed and measured the snout-vent length of each frog and then immediately placed each frog into an individual treatment container for a 12-h exposure period. The duration of exposure was chosen to mimic natural exposure that occurs during a single evening of breeding. Individuals were randomly assigned to treatments. Treatment containers were sterile 415-ml plastic containers with lids containing 50 ml sterile reverse osmosis water. Control containers had only the 50 ml of sterile water. For the fly ash treatment, a 25-ml layer of fly ash was added to the bottom of each container in addition to the sterile water. The weathered fly ash that was used in the experiments was collected from the temporary settling basin at the D-area power generation facility on the Savannah River Site in South Carolina. The elemental composition of fly ash from this site has been well characterized and subjected to extensive studies, especially with regard to its effects on amphibians (22, 24, 35). Prior to use in the experiments, we collected samples of the bacterial communities in fly ash by passing swabs through the fly ash for approximately 5 s/swab ($n = 3$ in 2013 and 2014). While this swabbing may have missed some bacteria within the fly ash compared to that found with the bulk extraction method, we used swabs so that we could focus on sampling the bacteria that frogs were most likely to contact during exposures. We checked the frogs every hour during the exposures to ensure that they were continuously bathed in solution.

At the end of the 12-h exposure period, we rinsed all of the frogs by placing each one in a bath of 100 ml sterile reverse osmosis water. We then transferred each one to a larger sterile plastic enclosure with a lid (15 by 33 by 23 cm). Each of these enclosures contained a sterile paper towel to cover the bottom, a sterile crumpled paper towel for cover, and 100 ml sterile water to maintain appropriate moisture levels. Frogs were checked daily to assess condition. In 2013, frogs were swabbed 5 days postexposure using the same protocol described above for field sampling. In 2014, frogs were swabbed at 5 and 15 days postexposure. All animals were euthanized at the end of the experiment.

DNA extraction, amplification, and sequencing. DNA was extracted from all of the rayon swabs using the DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA, USA). We followed the manufacturer's quick-start protocol; however, for step 1, we added 180 μl lysis buffer solution (20 mg lysozyme/1 ml lysis buffer) to each tube and incubated at 37°C for 1 h, and for step 2, we added 25 μl proteinase K to each reaction mixture, in addition to 200 μl buffer AL, and incubated at 70°C for 30 min.

To characterize the taxonomic diversity of the bacterial skin community, we amplified the V4 region of the 16S rRNA gene following Caporaso et al. (36). PCRs were run in triplicate for each sample, and the three triplicates were combined after amplification. Each 25-μl reaction mixture contained 11.5 μl PCR water, 10 μl 5 Prime HotMasterMix, 0.5 μl 515f forward primer, 0.5 μl 806r reverse primer including a 12-base barcode sequence, and 2.5 μl genomic DNA. Thermocycler conditions were set as follows: a denaturation step of 94°C for 3 min, followed by 34 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and final extension at 72°C for 10 min.

Amplified DNA was run out on a gel and then quantitated using a Qubit 2.0 flourometer and a double-stranded DNA (dsDNA) high sensitivity (HS) assay kit according to the manufacturer's guidelines (Life Technologies, Carlsbad, CA, USA). Samples were pooled by combining equal concentrations of each amplicon into a single tube, and then this pooled sample was cleaned using the QIAquick PCR purification kit according to the manufacturer's guidelines (Qiagen, Inc., Valencia, CA, USA). The pooled sample (final elution volume = 50 μl) was sent to the Molecular Biology Core Facilities of the Dana Farber Cancer Institute at Harvard University (Cambridge, MA, USA) for 16S amplicon sequencing on an Illumina MiSeq instrument using a 250-bp paired-end strategy.

Metabolite isolation, detection, and data processing. For metabolite analysis, we used methods similar to Umile et al. (34). To extract metabolites from the foam swabs, 1.0 ml of methanol was added to each swab tip in its centrifuge tube. The tubes were capped and vortexed for 5 s, allowed to sit for 10 min, and then vortexed for a second time. The swab tip was then removed using forceps, taking care to squeeze out any methanol adsorbed into the porous swab on the inside wall of the centrifuge tube. This methanolic extract was slowly filtered into another centrifuge tube using 13-mm-diameter syringe filters (0.2-μm-pore-size polytetrafluoroethylene [PTFE] membrane; VWR) to remove any insoluble environmental material. Before use, syringes (1-ml HSW Norm-Ject disposable syringes) and filters were prewashed by taking up 1 ml of methanol into the syringe and slowly passing it through the filter. Filtered extracts were evaporated *in vacuo* using a DNA120 SpeedVac with the heating function turned off.

Dried metabolite extracts were reconstituted in 100 μl of methanol that contained 1 ppm naphthalene as an internal standard. The reconstituted extracts were analyzed using reversed-phase high-performance liquid chromatography (HPLC; 25-μl injection) using a Shimadzu LC-20 liquid chromatograph equipped with an ACE C_{18} column (3-μm pore size; 150 by 4.6 mm), a Shimadzu SPD-M20A diode array detector, and an Applied Biosystems SCIEX API 2000 triple quadrupole mass spectrometer (operating in positive electrospray ionization mode). Compounds were separated with a binary mobile phase flowing at 0.5 ml/min and consisting of acidified water (0.1% formic acid, vol/vol; solvent A) and acidified acetonitrile (0.1% formic acid, vol/vol; solvent B). The gradient was as follows: 10% B (2-min hold) ramped to a final mobile phase concentration of 100% B over 18 min (5-min hold). Total wavelength chromatograms (TWCs) of field samples were compared with the TWCs of extracted, unused, and washed swabs (controls) and also blank methanol injections.

The retention times of all detected compounds (peaks) were normalized to that of the naphthalene internal standard (20.69 min). The retention time and relative abundance of each chromatographic feature were determined by integrating each peak in the TWC using Applied Biosystems Analyst software v. 1.5.1. This data set was further manually revised to account for slight variations in retention time across multiple samples and to focus on major chemical components. First, compounds that were eluted with retention times of ±0.03 min across all of the samples were investigated for UV-visible (UV-Vis) chromophores (λ_{max}) and positively charged ions. Those compounds with similar retention times and identical spectroscopic features were "consolidated" and assigned as a single compound. Next, unique compounds that were only detected in a single sample were disregarded as noise. Finally, all features that had a peak area of less than 3,000 milli-absorbance units (mAU) were disregarded as minor components (32).

Microbial community data processing. We used two data sets in our analyses that were produced separately. The first data set was composed of the field sampling of the reference and contaminated sites. The second data set was composed of the experimental data from both years (2013 and 2014). We kept these data sets separate so that potentially important 16S rRNA sequences in our experiment were not swamped out by sequences from environmental samples during the clustering and filtering of the operational taxonomic units (OTUs) (approximately representing bacterial species). For each data set, forward and reverse reads from the raw Illumina files were joined, demultiplexed, and filtered using the Quantitative Insights Into Microbial Ecology pipeline (MacQIIME v. 1.8.0 [35]) using the default settings, except that we allowed for no errors in the barcode, the maximum number of low-quality reads allowed before read truncation was set at 10, and the minimum fraction of consecutive high-quality base calls required to include a read was set at half of the total read length. Sequences were then uploaded to Geneious v. 8.0.4, any remaining PhiX sequence (used to increase base diversity in the sequencing run) was filtered out, and the sequences from 250 to 255 bp in length were extracted. Using QIIME, sequences were assigned to OTUs based on 97% sequence similarity with the UCLUST method (37). To represent each OTU, we used the most abundant sequence from each cluster. Represen-

tative sequences were aligned to the Greengenes v. 13.8 reference database (38) using PyNAST (39). Taxonomy was assigned using the RDP classifier (40).

Prior to statistical analyses, we removed all chloroplast and mitochondria sequences and all OTUs with fewer than 0.01% of the total number of reads. This 0.01% cutoff was established for these data sets by plotting the number of retained OTUs (overall richness) versus filtering cutoffs between 0.001% and 0.015%. From those figures, we identified the filtering cutoff (in this case, 0.01%) at which OTU richness leveled off, indicating that this most likely represented the real community of OTUs present as discussed in Bokulich et al. (41). To minimize the effects of variable sequencing depth on OTU relative abundances, the two data sets were rarefied to 30,000 reads/sample. The final field survey data set consisted of 900,000 reads that were clustered into 1,110 OTUs, while the experimental data set consisted of 34,200,000 reads that were clustered into 342 OTUs. All statistical analyses were conducted in R v. 3.1.3 (42) using the vegan package (v. 2.3-0 [43]) unless specified otherwise.

Statistical analysis: field sampling of sites. During the field survey, we were only able to access a single fly ash-contaminated site, which limited our ability to conduct statistical analyses on the field survey data set. However, we still thought it worthwhile to explore general patterns in alpha and beta diversity of fly ash-contaminated and reference sites. We calculated the alpha diversity of the bacterial communities as richness (number of OTUs/sample) and Faith's phylogenetic diversity and then determined the mean and standard deviations for the water and substrate samples from each site. For beta diversity, we visualized general patterns based on Bray-Curtis dissimilarities using nonmetric multidimensional scaling.

Statistical analysis: experiments. Bacterial community diversity and metabolite profiles of initial field-collected frog skin samples differed between the two years (permutational multivariate analysis of variance [PERMANOVA] using Bray-Curtis dissimilarities for OTUs and Jaccard dissimilarities for metabolites; OTUs: pseudoF = 149.1, $P = 0.001$; metabolites: pseudoF = 38.6, $P = 0.001$). We thought that this might influence the responses to treatment; therefore, we analyzed the 2013 and 2014 experimental data sets separately.

We calculated the alpha diversity of bacterial communities as richness (number of OTUs/sample) and Faith's phylogenetic diversity. We assessed the differences in alpha diversity among treatments over the course of the experiment and their interaction using generalized linear mixed models, including "individual" as a random effect to account for repeated measures of the same individual's pretreatment and posttreatment. Alpha diversity data were normally distributed (Lilliefors [Kolmogorov-Smirnov] test for normality, $P > 0.05$); thus, we fitted all models using an underlying Gaussian distribution and identity function (package lme4 [44]).

We assessed the beta diversity of bacterial communities using Bray-Curtis and UniFrac distances. Prior to computing distance matrices, rarefied sequence data were converted to relative abundance values for each sample by dividing the number of sequence reads for each OTU by the total number of reads in the sample (set at 30,000 reads for rarefaction). Variations in the beta diversity of bacterial communities across treatment and time point and their interaction were analyzed using PERMANOVA (function adonis [45]) based on 999 permutations. Following significant results, OTUs that were associated with specific treatments or time points were identified using indicator species analysis (package indicspecies [46]). We focused on the robust associations (IndVal ≥ 0.9). Patterns of beta diversity in microbial communities were visualized using nonmetric multidimensional scaling.

For metabolite profiles, the HPLC method yielded information about the concentration and light-absorbing ability of the metabolites in our experiments. As such, a more intense peak may indicate a greater abundance of a metabolite, an increased capacity to absorb light, or some combination of the two, which precludes comparing the relative abundance of different metabolites within a sample. Thus, metabolite community profiles were transformed to presence/absence data prior to statistical analysis. Analyses were conducted similarly to those for the bacterial community data, except that analyses of alpha diversity were based only on richness and were log-transformed prior to analyses to achieve normality and analyses of beta diversity were based on Jaccard distances. For the 2014 data set, we excluded one data point from our analyses of richness because the point appeared to significantly influence the outcome of the analyses. Compared to all of the other individuals, this individual had low metabolite richness at that particular time point (2 metabolites on day 5; all others had a metabolite richness of ≥ 5).

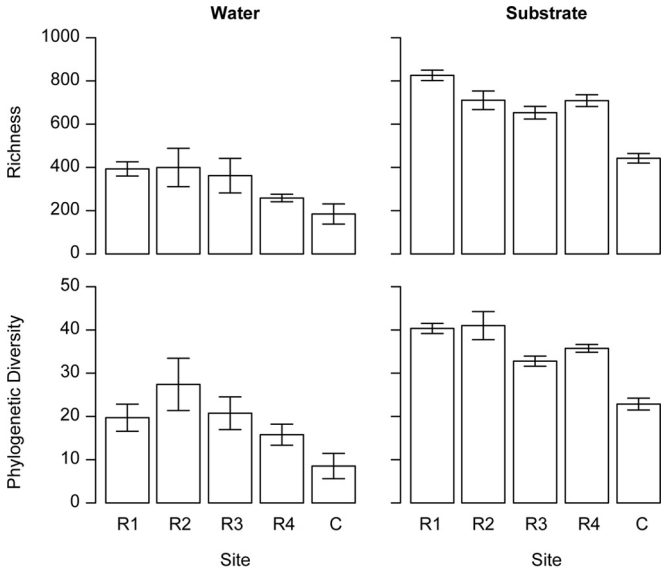

FIG 1 Alpha diversity of water and substrate samples collected during a field survey that compare the diversity of the bacterial communities in water (left) and substrate (right) samples from sites contaminated with coal combustion waste and from uncontaminated reference sites ($n = 3$ swabs/habitat/site). Alpha diversity was assessed as richness (the total number of unique OTUs) (top panels) and Faith's phylogenetic diversity (bottom panels). Data are presented as mean ± standard deviation. Site C was contaminated while all other sites (R1 to R4) were not. Reference site R1 is a permanent pond and references sites R2 to R4 are ephemeral ponds. The contaminated site C is a permanent fly ash disposal wetland.

Nucleotide sequence accession numbers. Sequence data are available from the NCBI database under SRA accession numbers SRP070506 (water and substrate at contaminated and reference sites), SRP070161 (frog skin), and SRP070504 (fly ash used in the experiment).

RESULTS

Field sampling of fly ash-contaminated and reference sites. Amphibian breeding habitats contained a high diversity of bacteria. Substrate samples were particularly species-rich, with as many as 848 unique OTUs recovered from a single swab (minimum richness, 422) (Fig. 1). Compared to substrate samples, water samples were somewhat less rich, although by no means deficient in OTUs (richness range, 139 to 481) (Fig. 1). Phylogenetic diversity was also relatively high (phylogenetic diversity range, water [7 to 33] and substrate [22 to 43]) (Fig. 1). At the phylum level, the OTU diversity of water and substrate samples encompassed 18 and 19 phyla, respectively. *Proteobacteria* accounted for approximately half of all of the OTUs in the two habitats (water 50% and substrate 48%). Other prominent phyla (accounting for at least 1% of OTUs) in water samples included the *Verrucomicrobia* (14%), *Bacteroidetes* (9%), *Acidobacteria* (7%), *Planctomycetes* (4%), *Spi-*

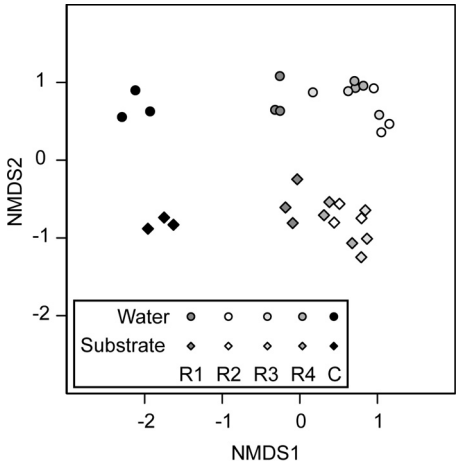

FIG 2 Beta diversity of water (circles) and substrate (diamonds) samples collected during a field survey comparing the diversity of the bacterial communities from sites contaminated with coal combustion waste and from uncontaminated reference sites ($n = 3$ swabs/habitat/site). Site C was contaminated while all other sites (R1 to R4) were not. Reference site R1 is a permanent pond and references sites R2 to R4 are ephemeral ponds. The contaminated site C is a permanent fly ash disposal wetland. The ordination was creating using nonmetric multidimensional scaling with Bray-Curtis dissimilarities.

rochaetes (2%), *Actinobacteria* (1%), *Armatimonadetes* (1%), and *Chlorobi* (1%). In substrate samples, prominent phyla were similar: *Verrucomicrobia* (14%), *Acidobacteria* (9%), *Bacteroidetes* (9%), *Planctomycetes* (4%), *Spirochaetes* (2%), *Actinobacteria* (2%), and *Chlorobi* (2%). An additional 7% and 6% of OTUs were unclassified at the phylum level in water and substrate samples, respectively.

Alpha and beta diversity varied among the sites we sampled. On average, richness and phylogenetic diversity were lowest at the contaminated site compared to that at all of the other sites (Fig. 1). In visualizing patterns of beta diversity, we found that water and substrate samples from the contaminated site clustered separately from the reference sites, which all clustered together (Fig. 2).

Experimental exposure to fly ash. Spring peepers harbored a diverse community of bacteria on their skin, with a total of 305 and 309 unique OTUs from six phyla present in the frog population in 2013 and 2014, respectively. Prominent phyla in the two years included the *Proteobacteria* (proportion of OTUs in 2013 was 75% and in 2014 was 76%), *Bacteriodetes* (2013, 12%; 2014, 13%), *Actinobacteria* (2013, 9%; 2014, 8%), and *Firmicutes* (2013, 2%; 2014, 1%). Less than 1% of OTUs were unclassified at the phylum level in 2013 and 2014.

At the time of capture, individual frogs harbored from 145 to 230 OTUs in 2013 and from 118 to 216 OTUs in 2014. Although diverse, the cutaneous bacterial communities were fairly consistent among individuals (proportion of OTUs shared by any two individuals as mean ± standard deviation [SD], 57% ± 12% in 2013 and 48% ± 10% in 2014), and communities were typically dominated by one or two bacteria. In 2013, the communities of all of the individuals were dominated by an actinobacterium (*Cellulomonas* sp., OTU X235695), which accounted for approximately one-half (mean ± SD, 0.45 ± 0.06) of all sequences in each sample (range, 0.28 to 0.52 sequences/sample). A proteobacterium (OTU X4451011) in the family *Pseudomonadaceae* accounted for roughly another one-tenth (mean ± SD, 0.12 ± 0.04) of all sequences in 13 out of 14 of these samples (range, 0.11 to 0.17 sequences/sample). In 2014, the communities of all of the individuals were dominated by a different *Cellulomonas* sp. (OTU X4473756), which accounted for about one-third (mean ± SD, 0.32 ± 0.07) of all sequences in each sample (range, 0.18 to 0.44 sequences/sample). Another actinobacterium (OTU X4378239) in the *Cellulomonadaceae* accounted for approximately one-fifth (mean ± SD, 0.23 ± 0.06) of all sequences in 13 out of 16 of these samples (range, 0.13 to 0.31 sequences/sample).

The bacterial community present in the fly ash that we used in the lab experiments differed in composition from that of spring peepers and was less diverse in general (overall diversity, 128 OTUs [2013] and 173 OTUs [2014]; per sample diversity, 88 to 102 OTUs [2013] and 103 to 145 OTUs [2014]). Fly ash samples in both 2013 and 2014 contained a high abundance of an actinobacterium (OTU X160333) in the order *Acidimicrobiales* (range, 0.15 to 0.41 sequences/sample). In 2013, a proteobacterium (OTU X157064) in the order *Methylophilales* was also abundant across all three samples (0.29 to 0.31 sequences/sample), whereas in 2014, there was a proteobacterium (OTU X394796) in the order *Pseudomonadales* (0.14 to 0.33 sequences/sample).

In our laboratory experiments, in 2013 and 2014, we found no differences in the richness of the frog skin bacterial communities between exposure treatments (generalized linear mixed model [GLMM], 2013: $\chi^2 = 3.0$, $P = 0.08$; 2014: $\chi^2 = 2.4$, $P = 0.1$) (Fig. 3A). In 2013, richness decreased in captivity from day 0 to day 5 ($\chi^2 = 5.6$, $P = 0.02$) to similar degrees across exposure treatments (interaction: $\chi^2 = 2.5$, $P = 0.1$). In 2014, richness declined but then recovered over the course of the experiment ($\chi^2 = 9.3$, $P = 0.002$) (Fig. 3A). The interaction between exposure treatment and sample day was not significant ($\chi^2 = 0.005$, $P = 0.9$).

In terms of phylogenetic diversity, we found no differences between treatments in 2013 and those in 2014 (GLMM, 2013: $\chi^2 = 0.8$, $P = 0.4$; 2014: $\chi^2 = 0.2$, $P = 0.7$) (Fig. 3B). In 2013, phylogenetic diversities were similar at day 0 and day 5 in the two exposure treatments (sample day: $\chi^2 = 1.4$, $P = 0.2$; interaction: $\chi^2 = 2.3$, $P = 0.1$). In 2014, phylogenetic diversity changed during the experiment, increasing by day 15 in the two exposure treatments (sample day: $\chi^2 = 21.7$, $P < 0.0001$; interaction: $\chi^2 = 0.1$, $P = 0.8$) (Fig. 3B).

In 2013, the composition of skin microbial communities changed over time from day 0 to day 5 (PERMANOVA, Bray-Curtis: pseudoF = 11.8, $P = 0.001$; UniFrac: pseudoF = 3.7, $P = 0.002$) (Fig. 4A). There was neither a main effect of exposure treatment on diversity (Bray-Curtis: pseudoF = 2.0, $P = 0.1$; UniFrac: pseudoF = 1.5, $P = 0.2$) nor an interaction between exposure treatment and sample day (Bray-Curtis: pseudoF = 1.7, $P = 0.1$; UniFrac: pseudoF = 0.2, $P = 0.97$). In 2014, results were similar. Microbial community composition changed over the course of the experiment (PERMANOVA, Bray-Curtis: pseudoF = 11.8, $P = 0.001$; UniFrac: pseudoF = 7.9, $P = 0.001$) (Fig. 4B) and did not differ between exposure treatments (Bray-Curtis: pseudoF = 1.5, $P = 0.1$; UniFrac: pseudoF = 1.4, $P = 0.2$), and there was no interaction between treatment and sample day (Bray-Curtis: pseudoF = 0.4, $P = 0.97$; UniFrac: pseudoF = 0.5, $P = 0.9$).

Two and 15 OTUs (in 2013 and 2014, respectively) were strongly associated only with field-collected frog samples (IndVal ≥ 0.9), indicating that the presence of these OTUs on the skin decreased dramatically during the laboratory experiment (Fig. 5). In 2013, the two field-associated OTUs were in the families *Coma-*

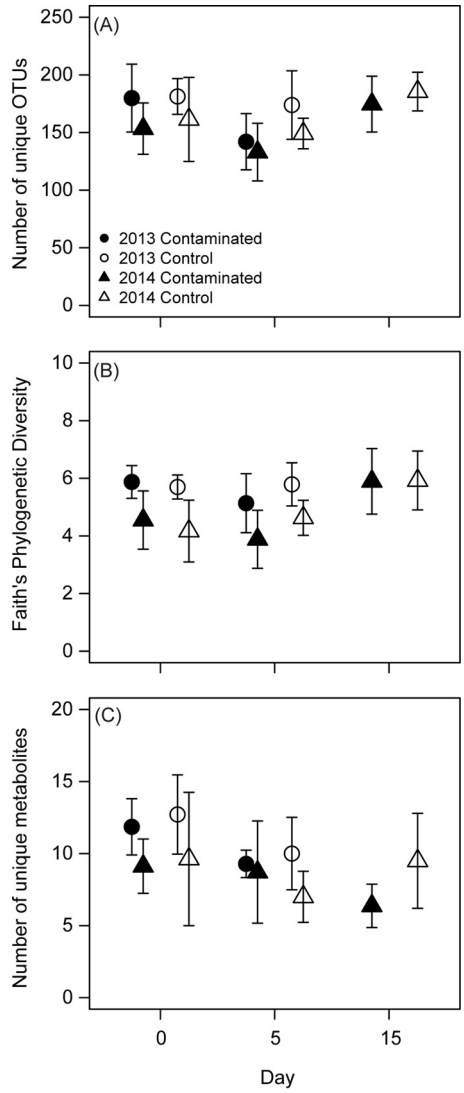

FIG 3 Changes in the alpha diversity of spring peeper skin bacterial communities ([A] OTU richness and [B] phylogenetic diversity) and metabolite profiles ([C] metabolite richness) prior to and following experimental exposure to fly ash (Contaminated) relative to those of controls exposed to sterile water (Controls). We completed experiments in 2 years: 2013 and 2014 ($n = 7$ and 8 replicates, respectively).

monadaceae and *Rubrobacteraceae*. In 2014, the *Comamonadaceae* family (33%) again accounted for a large proportion of the OTUs that disappeared in captivity as did the *Caulobacteraceae* and *Xanthomonadaceae* families (13% each). One and 26 OTUs (in 2013 and 2014, respectively) were strongly associated with laboratory sample days (day 5 samples in 2013 and day 5 and/or day 15 samples in 2014). In 2013, the one OTU associated with laboratory samples was part of the *Enterobacteriaceae* family. In 2014, the *Pseudomonadaceae* (42%) family accounted for the largest proportion of the OTUs that increased in captivity, followed by the *Brucellaceae* and *Comamonadaceae* families (11% each).

High-pressure liquid chromatography-mass spectrometry (HPLC-MS) analysis identified 64 unique metabolites that were associated with the skin of spring peepers. Initially (i.e., for the field-collected samples), the numbers of metabolites associated with any given individual ranged from 9 to 17 and 5 to 17 in 2013 and 2014, respectively.

In 2013, metabolite profile richness decreased slightly over the course of the experiment from day 0 to day 5 in a similar manner for animals exposed to fly ash and controls (GLMM, sample day: $\chi^2 = 12.6$, $P = 0.0004$; exposure treatment: $\chi^2 = 0.6$, $P = 0.4$; interaction: $\chi^2 = 0.007$, $P = 0.9$) (Fig. 3C). In 2014 (the 15-day experiment), there were no main effects of exposure treatment or sample day, but there was a significant interaction term (GLMM, sample day: $\chi^2 = 2.0$, $P = 0.2$; exposure treatment: $\chi^2 = 0.3$, $P = 0.6$; interaction: $\chi^2 = 5.4$, $P = 0.02$). Metabolite profile richness decreased throughout the experiment in the fly ash treatment, whereas it appeared to decrease slightly and then recover in the control treatment (Fig. 3C).

In 2013 and 2014, the compositions of the metabolite profiles changed over sample day (PERMANOVA, 2013: pseudoF = 3.6, $P = 0.002$; 2014: pseudoF = 4.0, $P = 0.001$) (Fig. 5C and D), similarly for control frogs and those exposed to fly ash (2013 exposure treatment: pseudoF = 1.1, $P = 0.3$; interaction: pseudoF = 0.8, $P = 0.6$; 2014 exposure treatment: pseudoF = 0.6, $P = 0.8$; interaction: pseudoF = 0.8, $P = 0.6$).

DISCUSSION

We found that adult spring peepers harbor a diverse community of symbiotic bacteria on their skin that is comparable to the levels of diversity and the dominant taxa that have been documented for other temperate frogs (28–30). However, we detected little impact as the result of short-term fly ash exposure on the structure of the bacterial skin community or on the baseline function (diversity of metabolite production) of these bacteria. The one exception to this was a small change in metabolite diversity in 2014 in individuals exposed to fly ash; however, this change was not seen in 2013. Thus, our data suggest that during the course of a single 12-h breeding event in fly ash settling basins, it is unlikely that the skin microbiome of adult frogs is severely disrupted. Given that fly ash contains a complex mixture of potentially toxic elements, including mercury, arsenic, and selenium, the lack of clear impact on the adult skin bacterial microbiome following exposure was somewhat surprising. We expected that we would see substantial changes in the structure and function of the bacterial communities through disruption of host regulation of the microbiome, direct impacts on the skin symbionts, or indirect competition and colonization of microbes from the fly ash. Similar resistance has been seen in other host-symbiont systems. For example, when exposed to thermal, nutrient, and food shortage stressors, no clear changes in sponge symbiont communities have been observed, even though these stressors can lead to host death in some instances (47, 48).

Direct impacts of fly ash on amphibians have been clearly demonstrated, but most of these studies have considered longer-term exposures, including comparing amphibians that are naturally developing in contaminated sites with those from reference sites. For instance, bullfrog (*Rana catesbeiana*) tadpoles developing in coal ash-contaminated sites have reduced swimming performance and predator avoidance compared to those from reference sites (49), and adult toads (*Bufo terrestris*) collected in coal ash-contaminated sites had elevated levels of arsenic, selenium, and vanadium in their bodies relative to those of toads from reference sites (50). When toads were relocated from reference to contaminated sites, their levels of these trace elements were elevated within 7 weeks of

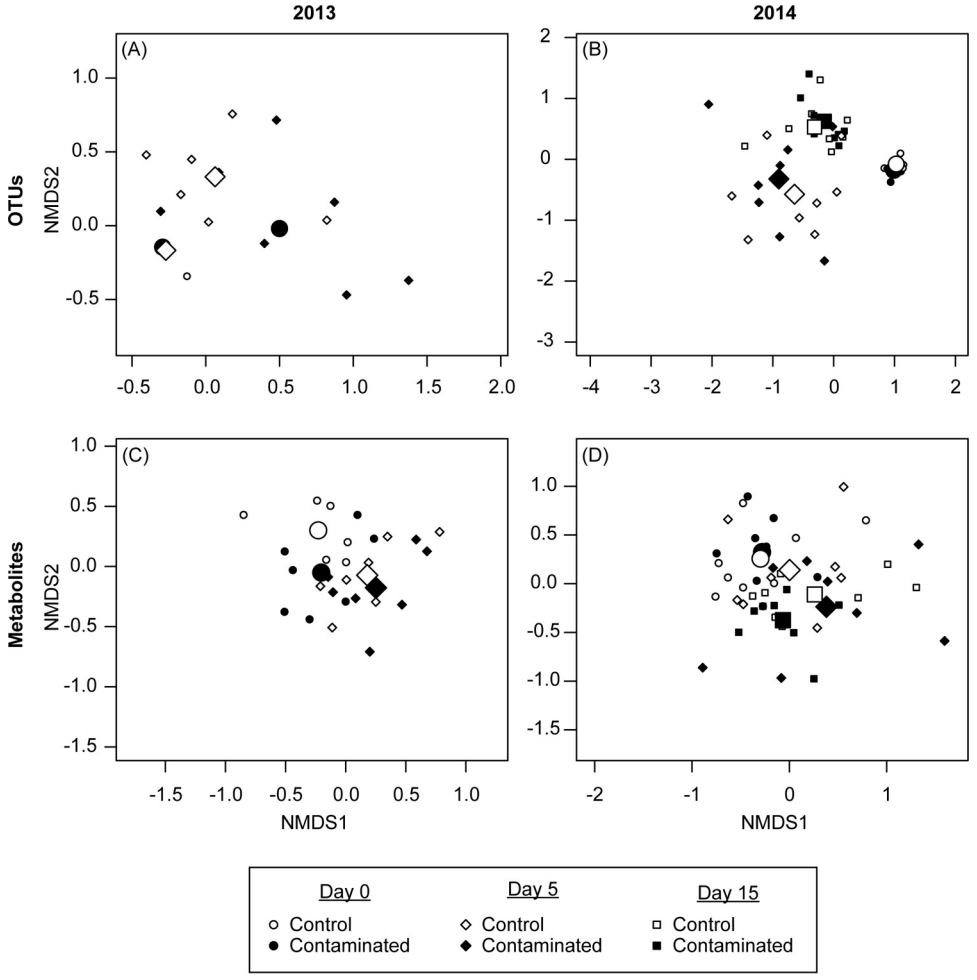

FIG 4 Changes in the beta diversity of spring peeper skin bacterial communities (A and B) and metabolite profiles (C and D) prior to and following experimental exposure to fly ash (Contaminated) relative to those of controls exposed to sterile water (Controls). Experiments were conducted in 2013 ([A and C] $n = 7$ replicates) and 2014 ([B and D] $n = 8$ replicates). Note that day 15 samples were not collected in 2013. Larger symbols represent the centroids of each group.

their transfer (50). Experimental laboratory exposures demonstrating impacts of coal ash on amphibians have also tended to be longer; a comparative study of tadpoles exposed individuals for the entire course of development, a range of approximately 5 to 30 weeks (51). Our experiment, with only an overnight exposure, was designed to focus on the direct impacts on the microbiome that might occur following a single bout of breeding relevant for this amphibian species. However, given all of the documented longer-term effects of fly ash exposure on amphibian hosts and that many amphibians spend considerably longer periods of time in contact with fly ash in settling basins, additional chronic exposure studies should be conducted as well as studies to determine whether host microbiomes can be altered indirectly through impacts on host health and performance.

Numerous studies have examined the direct impacts of metals, metalloids, and/or trace elements on microbial communities, including free-living microbial communities and host-associated communities. In free-living microbial communities, contamination can lead to many different outcomes in terms of the structure and function of the microbial community (52). For example, a recent study of free-living soil bacterial communities found that long-term metal pollution (zinc and lead) impacted the relative abundance of taxa, with an increase in the abundance of bacteria harboring metal resistance genes, but did not alter overall richness or composition (53). In host-symbiont systems, a recent study that examined arsenic effects on the mouse gut microbiome found impacts on bacterial community structure and metabolite production following 4 weeks of arsenic exposure in drinking water (11). However, not all studies examining microbiome responses to arsenic have seen pronounced effects (18).

Indeed, there is much more to learn about which types of perturbations might disrupt an established adult microbiome and what the mechanisms of resilience and resistance are in these complex ecological communities (54, 55). In amphibians, several studies that have tried to treat adult amphibians with single or mixed probiotics have failed to see establishment of the bacteria, presumably due to competition with the resident microbes and/or host filtering (56–58). The same is true in human studies, although treatments with more complex mixtures of probiotics (e.g., fecal transplants) are often more successful (55).

While fly ash did not alter the skin microbiome, there was a very strong effect of time spent in the laboratory. The number of OTUs tended to drop once frogs were transferred to the laboratory, and the structure of the bacterial community shifted. This

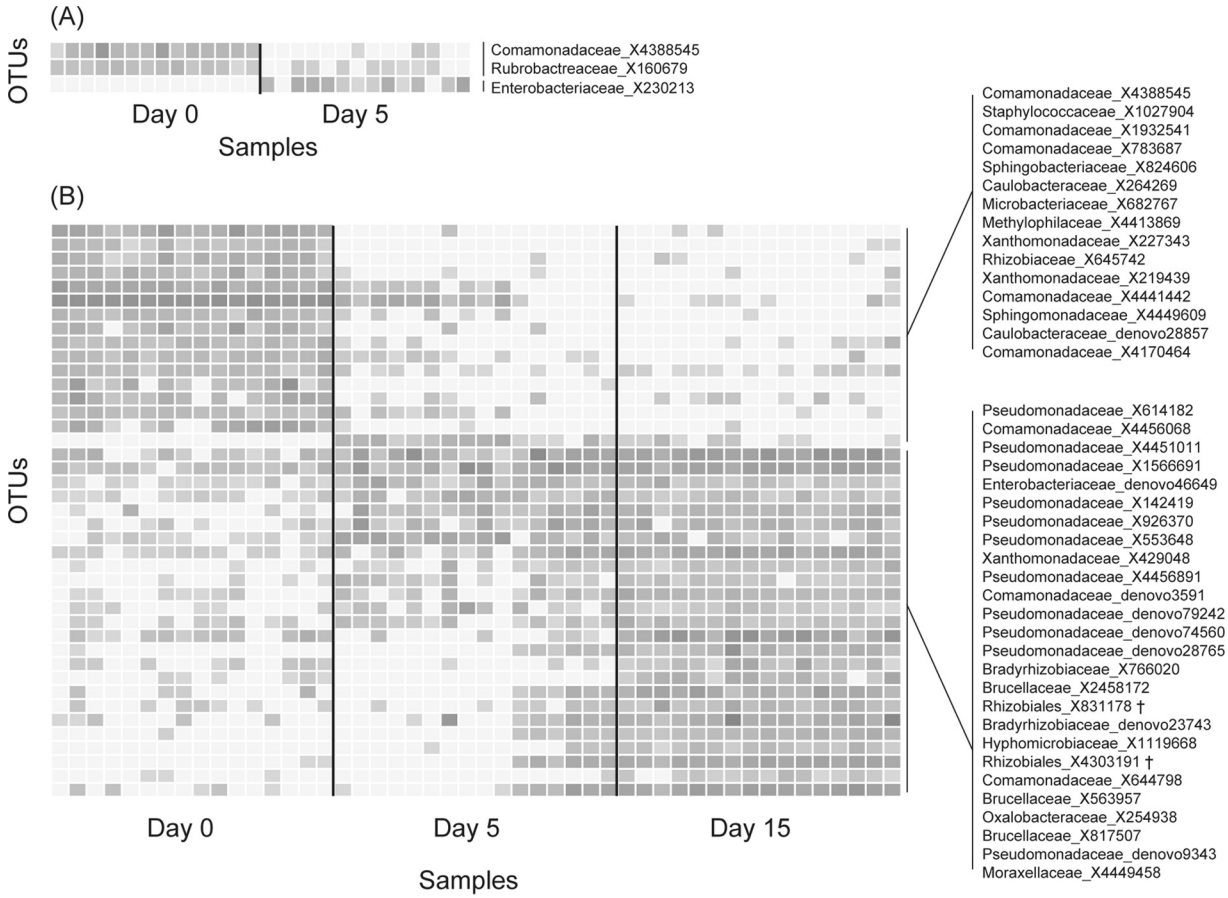

FIG 5 Heat maps of indicator operational taxonomic units (OTUs) that show changes in relative abundance that occurred over the course of each experiment, in 2013 (A) and 2014 (B). Samples are presented in the same order across time points. Lighter colors indicate lower relative abundances, and darker colors indicate higher relative abundances. All indicator OTUs shown had an indicator value of ≥0.9 and a *P* value of <0.03. Family level taxonomic classification is shown for each OTU. OTUs that were not classified at the family level are indicated by a cross (†). Order (*Rhizobiales*) classification has been provided for these OTUs.

has been seen previously in laboratory and mesocosm studies (58, 59). Changes in host physiology, including increases in the "stress" hormone corticosterone, may contribute to this effect, as amphibians can have elevated corticosterone levels for some time following capture and transfer to a laboratory environment (e.g., reference 60). However, recent research suggests that housing amphibians with an environmental source pool of microbes may also be important for maintaining the microbiome in laboratory environments (31), although even over multiple generations in captivity, some taxa in the skin microbiome appear to be maintained (61). This suggests that if we had maintained spring peepers with fly ash over the entire course of the laboratory experiment, instead of trying to maintain sterile conditions after a 12-h fly ash exposure, the frogs might have picked up more bacteria from the fly ash, and we might have seen more of an effect of the fly ash.

If the environmental source pool is critical for maintaining the amphibian skin microbiome, it might also be critical during initial microbiome community assembly during amphibian larval development. Arsenic contamination of soil can alter soil bacteria community composition, increase the abundance of *Acidobacteria*, and alter the presence of arsenic resistance genes (62). These types of impacts on the free-living microbial community may lead to different environmental source pools of potential symbiotic colonizers for amphibians developing in fly ash basins as opposed to those developing in noncontaminated sites. Our preliminary comparisons of bacterial communities in fly ash-contaminated and reference wetlands suggest that there may be distinct bacterial source pools present in contaminated environments. OTUs unique to contaminated sites may be interesting bacterial taxa to study in greater depth, as bacterioplankton associated specifically with fly ash basins are known to be more tolerant of metals and also to have elevated levels of antibiotic resistance (63). Given that disruption of the microbiome during initial colonization and assembly can influence the eventual function and composition of the adult microbiome (e.g., 64), it may be important to focus future work on understanding the role of variation in environmental source pools on establishment of these complex symbiont communities.

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K.P.C.M., A.A.I., C.N.S. and L.K.B. processed samples in the laboratory; M.C.H., L.K.B., T.P.U., E.A.B., A.A.I., and C.N.S. completed data processing and analysis; L.K.B. and M.C.H. produced the first draft of the manuscript; and all authors edited the manuscript.

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