

Bacterial community composition and biogeochemical heterogeneity in PAH-contaminated riverbank sediments

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Abstract

Purpose Predicting response of microbial communities to pollution requires an underlying understanding of the linkage between microbial community structure and geochemical conditions. Yet, there is scarce information about microbial communities in polycyclic aromatic hydrocarbons (PAH)-contaminated riverbank sediments. The aim of this study was to characterize bacterial communities in highly PAH-contaminated sediments and establish correlations between bacterial communities and environmental geochemistry of the sediments.

Materials and methods Sediment core samples were collected from a highly PAH-contaminated site for (1) analysis of geochemical parameters including total nitrogen, total organic matter, moisture, total carbon, sulfate, pH, and PAH concentrations and (2) bacterial enumeration, 16S rDNA-based terminal restriction fragment length polymorphism analysis and sequencing.

Results and discussion Non-metric dimensional scaling analyses revealed that bacterial community composition was strongly influenced by PAH concentration. Sulfate, organic matter, pH, and moisture were also related to community composition. A diverse microbial community was identified by the large number of operational taxonomic units recovered and by phylogenetic analyses. δ -Proteobacteria, firmicutes, and bacterioidetes were the dominant groups recovered. We also observed a high number of phylotypes associated with sulfate-reducing bacteria, some of which have been previously described as important in PAH degradation.

Conclusions Our study suggests that, despite intense pollution, bacterial community composition did exhibit temporal and spatial variations and were influenced by sediment geochemistry. Significant relationships between bacterial community composition and PAHs suggest that, potentially, extant microbial communities may contribute to natural attenuation and/or bioremediation of PAHs.

Keywords Microbial communities · PAH-contamination · Riverbank sediments · T-RFLPs

1 Introduction

Sediments contaminated with polycyclic aromatic hydrocarbons (PAHs) are a common problem in freshwater and marine environments worldwide (Eggleton and Thomas 2004). Composition of bacterial communities has been described in a variety of polluted aquatic environments (Baniulyte et al. 2009), yet few studies have provided information about microbial community structure in riverbank sediments contaminated with PAHs (Pratt et al. 2012).

Riverbanks are important in riverine systems; they act like buffers, hold onto contamination, and are habitat for wildlife (Buckley et al. 2012; Christen and Dalgaard 2013). Microbial communities in river systems are impacted by complex interactions among periodic inundation, vegetation, and river hydrology that are spatially heterogeneous (Sponseller et al. 2013). Even under extreme conditions of contamination, bacteria are central to biogeochemical cycles, constitute a major portion of biomass in sediments, and can be very active. For example, Mosher et al. (2006) found higher levels of microbial activity (respiration measured as 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride reduction) in PAH-contaminated sediments than in less contaminated sediments. They also suggest that hydrocarbon and metal

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pollution can increase microbial biomass if pollutants are used as carbon sources or decrease microbial biomass if pollutants are toxic. Over a long period of contamination, environmental factors (e.g., carbon availability) may alter microbial community structure (Kadnikov et al. 2013). Some microbial populations might prevail over others under conditions of environmental stress caused by contaminants, leading to a shift in community structure (Bernhard et al. 2005) and function (Lors et al. 2010; Machado et al. 2012).

Because many bacteria are capable of transforming and mineralizing PAHs (Bamforth and Singleton 2005; Dell'Anno et al. 2009), bacterial degradation is a common approach for bioremediation (Johnston and Johnston 2012). Several strains of *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Burkholderia*, and *Sphingomonas*, isolated from PAH-contaminated soils, can use PAHs as a sole source of carbon and energy (Johnsen et al. 2005). Mineralization of naphthalene and phenanthrene can be achieved by some β - and γ -Proteobacteria and Flavobacteria (Rogers et al. 2007). PAH-degrading bacteria have also been isolated from extreme environments and are of particular importance for bioremediation of oil-contaminated desert soils (Zeinali et al. 2007). Extremophiles, like the thermophilic bacterium *Nocardia otitidiscaviarum* strain TSH, can grow on PAHs (pyrene, phenanthrene, anthracene, and naphthalene) as sole sources of carbon and energy. In addition to direct degradation, there are indirect effects of bacteria on PAHs degradation. For example, bacteria can increase PAH bioavailability by producing biosurfactants (Bastiaens et al. 2000; Ho et al. 2000) and by forming biofilms (Johnsen and Karlson 2004). Collectively, these direct and indirect effects suggest that PAHs have an important impact on bacterial community composition.

Obtaining data on microbial composition is critical for prediction of the fate of PAHs. Thus, understanding the microbial ecology of contaminated riverbank sediments lays the foundation for exploration of bioremediation potential. In this study, we combined chemical and microbial molecular techniques to describe bacterial community composition in highly PAH-contaminated riverbank sediments. These properties were related to concentrations and composition of PAHs and other environmental variables of the Mahoning River (North-eastern Ohio, USA), which was long exposed to contamination (United States Army Corps of Engineers USACE 2001).

2 Materials and methods

2.1 Study site

The Mahoning River drains 2,965 km², is 174 km long, and downstream with the Shenango River form the Beaver River in western Pennsylvania, a tributary of the Ohio River. The Mahoning River watershed consists of layered sedimentary

rocks (mainly sands and silts) and is covered by deposits of unconsolidated clay, sand, and gravel (United States Army Corps of Engineers USACE 2001). Between 1920 and 1970, the lower Mahoning River supported one of the highest productions of steel worldwide (United States Army Corps of Engineers USACE 1999). Industries along the river used it for disposal of wastes and for cooling. As a result, high concentrations of PAHs and other organic contaminants remain in the river bottom sediments. According to the USACE, one of the most contaminated sites is in Lowellville, Mahoning County, OH (41°2'23"N 80°32'25"W); thus, we collected samples as described below from this location. Lowellville riverbank sediments have been described as “oil-soaked banks”, and it was inferred that the riverbank sediments contained higher concentrations of PAHs than the river channel (United States Army Corps of Engineers USACE 1999).

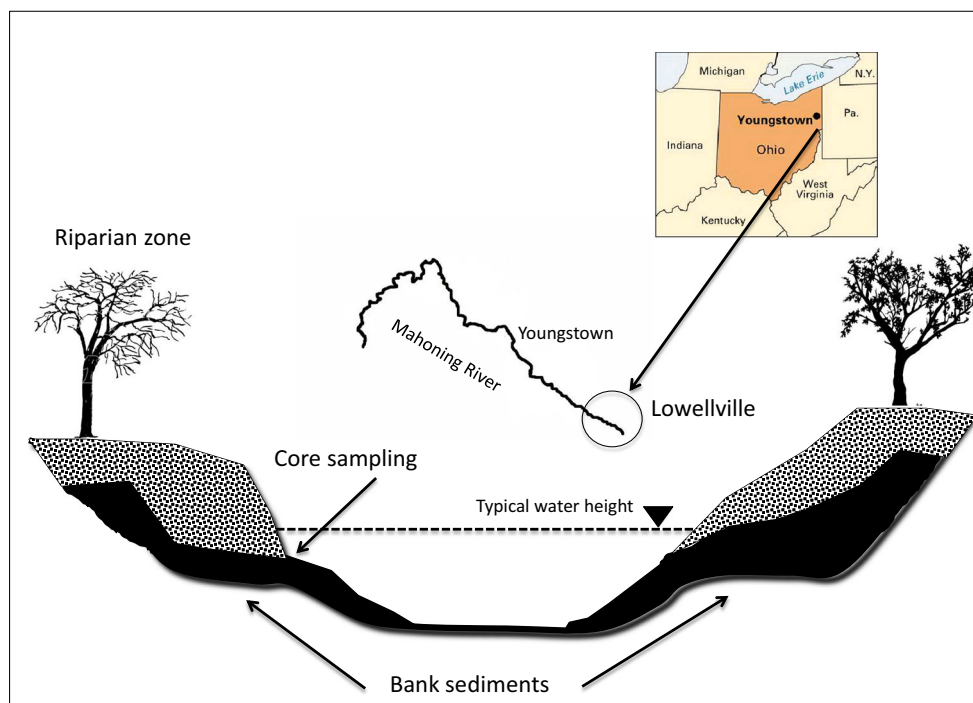
2.2 Sample collection and processing

Sediment cores were collected on January 21st, August 24th, and October 29th in 2011 using stainless steel tubes (length=15 cm; diameter=5 cm) from the riverbank (edge of the channel) as shown in Fig. 1. Preliminary analyses of triplicate core sediments (data not shown) revealed high heterogeneity in biogeochemical parameters. For this reason, nine cores (20 cm apart from each other) were collected to minimize statistical variation of the parameters measured between cores. To maintain anaerobic conditions of the sediments, immediately after sampling, cores were capped (using stainless steel liners and plastic caps), sealed in plastic bags, and transported on ice to the laboratory. In the laboratory, bagged cores were placed in another sealed plastic bag, filled with nitrogen gas, and kept at -20 °C until analysis. Before analysis, cores were thawed and both ends discarded. Inner core sediments were homogenized under nitrogen (to maintain anaerobic conditions) using aseptic technique inside an anaerobic bag (Sigma, St. Louis, Missouri). Once homogenized, sediment was partitioned for geochemical analysis and DNA extraction.

2.3 PAH extraction and analyses

PAHs were extracted by Soxhlet extraction Method 3540C (United States Environmental Protection Agency USEPA 1996). We chose this method because Soxhlet has been considered as the “industry standard” in extraction techniques in the environmental field (United States Environmental Protection Agency USEPA 2012); it is relatively simple and inexpensive. When performed correctly, it is assumed to be an exhaustive technique (Shen and Shao 2005). Approximately 8 g of thawed sediment were placed in Whatman cellulose extraction thimbles (Sigma, St. Louis, Missouri). Anhydrous granular sodium sulfate was added to the thimbles until

Fig. 1 Location and scheme of sampling of riverbank sediments



sediments reached a “sand-like” consistency to remove water. Ten microliters of surrogate standard mix (Restek, Bellefonte, Pennsylvania) was added directly to the sample, followed by 200 ml of dichloromethane. The solvent was then heated and refluxed for 18 h. Sample extracts were concentrated to a final volume of 30 mL and were analyzed according to the guidelines set forth in USEPA Method 8270D (United States Environmental Protection Agency USEPA 2007), using internal standard quantitation (acenaphthene-d10, chrysene-d12, 1,4-dichlorobenzene-d4, naphthalene-d8, perylene-d12, phenanthrene-d10 [SV Internal standard mixes] (Restek, Bellefonte, Pennsylvania)). We used a Gas Chromatograph Hewlett-Packard 5890, a Mass Spectrometry Hewlett-Packard 5970B, and a Restek XTI-5, 30 m×0.25 mm ID×0.25 μm df column. The injector port was held at 250 °C, with an initial oven temperature of 40 °C. The oven was held at 40 °C for 8 min, then ramped at 10 °C/min to a final temperature of 310 °C, and held for 10 min. One microliter of sample was injected using a splitless mode in a CTC-103 autosampler (Zwingen, Switzerland). The precision and accuracy of the analytical procedure were determined by calculating the percent recovery of surrogates, as well as the relative percent standard deviations (%RSD). Surrogate recoveries in sediment samples were high, ranging from 78 to 89 %, and % RSD values were low (ranging from 5 to 25 %).

2.4 Geochemical analysis

Total nitrogen (TN) and total carbon (TC) were determined, from sediments dried at 60 °C, by combustion at 900 °C and

measured on an ECS-4010 elemental analyzer (Costech Analytical, Valencia, California). Sulfate was measured by extracting 2 g of sediment with 0.016 M $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ for 1 h (Li et al. 2009). The extract was filtered through Whatman no. 42 filter paper, and eluted sulfate was quantified using ion chromatography (Dionex Ion Chromatograph Model ICS-1100, Dionex Corp., Sunnyvale, California). Total organic matter was determined in 2 g of dried sediment by loss of mass following ignition at 550 °C for 1 h. Sediment pH was measured in a 1:5 (w/w) aqueous solution (5 g of air-dried sediments and ultrapure water [18.2 MΩ cm]) using a commercial electrode (Accumet, Fisher Scientific, Waltham, Massachusetts). Moisture content determination followed standard methods for soil analysis (Carter 2000).

2.5 Bacterial enumeration

To determine the total number of bacteria, a sediment subsample was preserved with equal parts of paraformaldehyde (8 % final concentration) and phosphate buffered saline. Samples were vortexed, sonicated for 10 min at low frequency (Ultrasonics Cleaner, VWR, Radnor, Pennsylvania), and centrifuged for 30 s at 1,000 rpm. Approximately 25–50 μl of sample was evenly dispersed using 0.2 μm filtered autoclaved water and then concentrated by vacuum onto 0.2-μm (25 mm) pore black polycarbonate filters (Poretics, Livermore, California), stained with 4,6-diamidino-2-phenylindole (1 mg/ml final concentration) for 5 min, and rinsed with filtered autoclaved water (Lemke and Leff 2006). An epifluorescence

microscope (Olympus DP71, Center Valley, Pennsylvania) was used to count bacteria in ten randomly selected fields.

2.6 Molecular analyses of bacterial communities

Total DNA was extracted from sediment using the PowerSoil DNA Isolation Kit (Mo-Bio Laboratories, Carlsbad, California) and according to the manufacturer's instructions with minor modifications. Total DNA includes DNA from all organisms, so it is not a bacteria-specific measurement. Although other microorganisms have been reported for these sediments (e.g., fungi) (Pratt et al. 2012), we only focused on and amplified DNA from bacterial communities because degradation of PAHs in sediments and soils is mainly performed by bacteria (Johnston and Johnston 2012). Triplicate DNA extractions per core were performed. DNA extracts were stored at -20°C until analysis.

Overall differences in bacterial community composition were examined using terminal restriction fragment length polymorphisms [T-RFLP] (Liu et al. 1997). 16S rRNA genes were amplified using the primers 8 F (5'-AGAGTTTGATCA TGGCTCAG-3') and 1492R (5'-GGCTACCTTGCCACGA CTTC-3') (Zhang et al. 2008). The 8 F primer was 5' labeled with 6-carboxy-fluorescein phosphoramidite (FAM). Each polymerase chain reaction (PCR) (25 μl) contained 0.5 μl of each primer (10 mM), GoTaq Green Master Mix 1X, 2 μl of DNA, and 9.5 μl of molecular-grade water. PCR product sizes were checked on 1 % agarose gels and purified with the Wizard PCR preps DNA purification system (Promega, Madison, Wisconsin) according to the manufacturer's protocol. The following conditions were used for PCR amplification: initial denaturation at 94°C for 3 min; 32 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s), extension (72°C for 90 s), and a final extension step of 7 min at 72°C . PCR products were visualized by electrophoresis to verify amplification and size of the fragment amplified.

For T-RFLPs analysis, PCR products were digested using HaeIII at 37°C for 16 h. After digestion, products were purified using a Wizard[®] SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin). Samples were analyzed at the Ohio State University Plant Microbe Genomics Facility, Columbus, Ohio, using a 3730 DNA Analyzer (Applied Biosystems, Inc., Carlsbad, California). Data were processed, sorted by peak intensity, and formatted using Gel ComparII (Applied Maths, Austin, Texas) before statistical analysis.

We chose to obtain 16S rRNA gene sequences from summer samples by combining PCR products of three selected summer cores. Bacterial 16S rRNA genes were amplified using the same primers, 8F and 1492R (Zhang et al. 2008), both unlabeled, and PCR was performed as above. PCR products were purified using the Wizard[®] SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin) according to the manufacturer's instructions. PCR products (from three

DNA extractions per core) were combined for cloning procedure. Products were cloned into competent *Escherichia coli* cells using the StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, California) following the manufacturer's recommendations. Two sets of dilutions were plated for each sample. Transformed cells were plated on LB medium containing 0.1 mg/ml of ampicillin and 4 μl of 2 % of X-gal and incubated overnight at 37°C . Control plasmids and competent cell controls were also included. White recombinants were transferred into 96-well plates containing LB medium, 0.1 mg/ml of ampicillin and 10 % glycerol, and shaken for 24 h at 37°C . The insert sizes of selected colonies were determined by PCR as above; 160 clones from summer were sequenced at the University of Kentucky Advanced Genetic Technologies Center, Lexington, Kentucky.

Taxonomic affiliations were assigned to bacterial operational taxonomic units (OTUs) using the Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov>). Nucleotide sequences were checked using Vector NTI Software (Life Technologies Corp., Carlsbad, California) and manually curated. Sequences were aligned using the multiple sequence comparison by log-expectation (MUSCLE) software. The phylogenetic tree was constructed with the MEGA software version 5.0 (Kumar et al. 2004) using the neighbor-joining method; the distance was calculated on the basis of Kimura's two-parameter algorithm; 100 bootstrap resamplings were performed to estimate the reproducibility of the tree.

2.7 Statistical analysis

One-factor multivariate analysis of variance (MANOVA) was used to determine if there were significant differences among sampling dates in TC, TN, sulfate, organic matter, moisture, pH, bacteria, and PAHs. We also calculated Pearson correlation coefficients for the relationships between those variables. Statistical analyses were performed using IBM SPSS Statistics 20 for Windows (IBM Corp., Armonk, New York).

Nonmetric multidimensional scaling (NMDS) was used to compare T-RFLP fragment composition among sampling dates. T-RFs community composition NMDS dimension scores were assessed as variables and correlated (using Spearman correlation coefficients) with total bacteria, sulfate, total PAHs, individual PAHs, TN, TC, moisture, pH, and organic matter to determine their influence on microbial community composition. NMDS was chosen because it is an ordination technique in which variables do not have to be linear (as in principle-component analysis); there is no need for specific distance measures (covariance), and it makes few assumptions of the data (Holland 2008). Completeness of the summer clone library was calculated by using coverage (C) values as follows $C=1-(n/N)\times 100$ (Jiang et al. 2009), where n is the number of clones present only once and N is the total number of clones recovered.

Table 1 Average values and standard deviations (in brackets, $n=9$) of environmental variables measured from riverbank sediments during sampling dates

Sampling date	pH	OM %	TN%	TC%	MC %	SO ₄ ⁻² (μg g ⁻¹)	Total PAHs (μg dry weight g ⁻¹)	Total Bacteria (cells dry weight g ⁻¹)
January	7.4 (0.2)	16.3 (3.8)	0.2 (0.0)	12.4 (1.8)	56.1 (12.1)	83.6 (11.8)	35251.1 (11234.8)	7.5×10 ⁶ (3.5×10 ⁶)
August	7.8 (0.2)	14.6 (4.4)	0.2 (0.0)	12.6 (1.2)	44.9 (5.6)	9.1 (3.4)	65314.8 (18879.1)	3.2×10 ⁷ (1.8×10 ⁷)
October	7.6 (0.2)	14.3 (1.7)	0.3 (0.0)	14.5 (1.5)	46.8 (6.9)	327.5 (390.1)	62207.4 (29107.8)	5.6×10 ⁷ (5×10 ⁷)

2.8 Nucleotide sequence accession numbers

The sequences determined in this study were submitted to the GenBank database and assigned Accession Nos. KF906488 to KF906499 and KF906500 to KF906509.

3 Results

3.1 Sediment characteristics

Contaminated sediments samples were black, viscous, and had a strong petroleum odor when sampled. There was high variability among cores for most environmental parameters and in total bacterial abundance (Table 1). Lowellville sediments had relatively high amounts of total organic carbon (ranging from 12 % in January to 15 % in October) which corresponded to the high amount of organic matter (14 % to 16 %); pH of sediments did not vary substantially (7.4 to 7.8) while moisture content did exhibit greater variability over time (45 to 56 %). Sulfate concentrations were highest in October sampling, ranging from 57 to 1,008 μg g⁻¹; in contrast, lowest sulfate concentrations were found in August (2 to 13 μg g⁻¹). TN was low on all dates. Total bacterial abundance ranged from 4.17×10⁶ to 1.16×10⁸ cells dry weight g⁻¹.

3.2 PAH concentrations

Fourteen PAHs were detected, quantified, and classified according to the number of benzene rings (Fig. 2) as follows: naphthalene, acenaphthylene, acenaphthene, and fluorene (two-ring PAHs); phenanthrene, anthracene, and fluoranthene (three-ring PAHs); pyrene, benzo[a]anthracene, chrysene, and benzo[b,k]fluoranthene (four-ring PAHs); benzo[a]pyrene and dibenzo[a,h]anthracene (five-ring PAH) and benzo[ghi]perylene (six-ring PAHs). Dibenzo[a,h]anthracene and benzo[ghi]perylene were only detected in January 2011.

Total PAH concentration ranged from 19,700 to 102,000 μg/kg dry weight (Table 2). High concentrations of total PAHs were measured in October and August, while lowest PAHs concentrations were detected in January (Table 1). In general, four-ring PAHs were the most

abundant across sampling dates (Table 2 and Fig. 2). Individually, the most abundant PAH across dates was pyrene, followed by fluoranthene, phenanthrene, chrysene, and benzo[a]pyrene (Table 2). On average, high-molecular-weight PAHs (four-, five-, and six-ring) accounted for 67 % of the total PAHs in January, 41 % in August and October, while low-molecular-weight PAHs (two- and three-ring) contributed 33 % in January and 59 % in August and October.

3.3 Temporal trends in environmental variables

MANOVA of pH, sulfate, TN, TC, moisture, organic matter, total PAHs, and total bacteria number revealed that there were significant differences among sampling dates (Wilks' Lambda=0.003, $F=20.241$, $p=0.000$). In addition, significant Pearson correlation coefficients between TN, TC, sulfate, moisture,

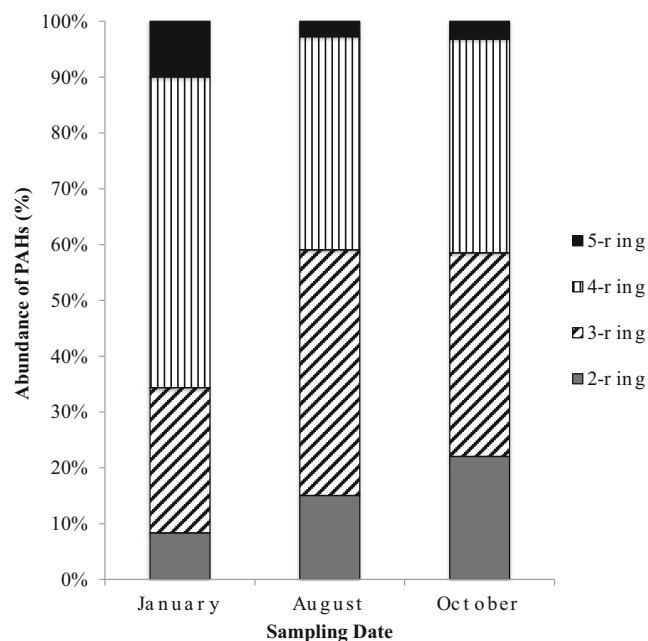


Fig. 2 Composition pattern of PAHs by ring size in riverbank sediment samples (two-ring: naphthalene, acenaphthylene, acenaphthene, fluorene; three-ring: phenanthrene, anthracene, fluoranthene; four-ring: pyrene, benzo[a]anthracene, chrysene, benzo[b,k]fluoranthene; five-ring: benzo[a]pyrene, dibenzo[a,h]anthracene. Data presented in figure do not include six-ring: benzo[ghi]perylene for all sampling dates. Data averaged per sampling date are presented

Table 2 Range concentrations of PAHs (expressed in $\mu\text{g}/\text{kg}$ dry weight) per sampling date in riverbank sediments from Lowellville, Mahoning River

Compound	No benzene rings	January	August	October
Naphthalene	2	976–1928	1708–4886	1855–8650
Acenaphthylene	2	239–355	146–438	221–512
Acenaphthene	2	174–648	1141–3598	371–7745
Fluorene	2	392–1261	1695–4648	724–8939
Phenanthrene	3	916–5806	6228–14759	2793–18399
Anthracene	3	642–2095	2498–7086	1160–6798
Fluoranthene	3	2723–8079	5417–17291	4891–16987
Pyrene	4	5076–10711	4322–13016	5443–14238
Benzo(a)anthracene	4	3469–5287	2655–9089	3658–8862
Chrysene	4	3341–6000	2761–9829	3869–9543
Benz(b,k)fluoranthene	4	1433–5312	1128–4280	1938–3824
Benzo(a)pyrene	5	1427–4388	417–3786	1277–2938
Dibenzo(a,h)anthracene	5	1033–2534	n.d.	n.d.
Benzo(ghi)perylene	6	937–2123	n.d.	n.d.
Σ PAHs		19712–55277	30046–86344	30057–102,346

organic matter, total PAHs, and total bacteria were found (Table 3). Interestingly, there were strong positive statistically significant correlation between total bacteria abundance and sulfate ($r=0.806$, $p<0.05$), between total bacteria and total PAHs ($r=0.706$, $p<0.05$), and more specifically between total bacteria and two-, four-, and four-ring PAHs (see Table 3).

3.4 Bacterial community composition

A total of 64 bacterial terminal restriction fragments [T-RFs] (ranging from 64 to 568 bp) were detected in core samples during the study (Fig. 3). In general, profiles were dominated by a limited subset of T-RFs on each date and in each core. On average, 33 T-RFs were recovered from the cores during January sampling, 37 T-RFs during August sampling, and 35 during October sampling. In general, most T-RFs contributed less than 2 % to the total relative abundance, and some T-

RFs showed significant correlations with environmental variables (Table 4).

Differences in T-RFs between sampling dates were analyzed by two-dimensional ordination via NMDS (stress <0.001 ; Fig. 4). The low stress value indicated that this ordination was a very good representation of the data. One-factor MANOVA comparing NMDS dimension scores from all T-RFs was significant (Wilks' Lambda=0.08, $F=6.326$, $p=0.008$). NMS1 separated most of the T-RFs by sampling date (Fig. 4) where August communities aggregated in ordination space more tightly than January and October communities. October communities were the most spread out in ordination space. T-RFs found in August were explained by the NMS2 axis while October and January were more related to axis 1.

Spearman correlation coefficient values from NMDS dimension scores of T-RFs and environmental variables (pH,

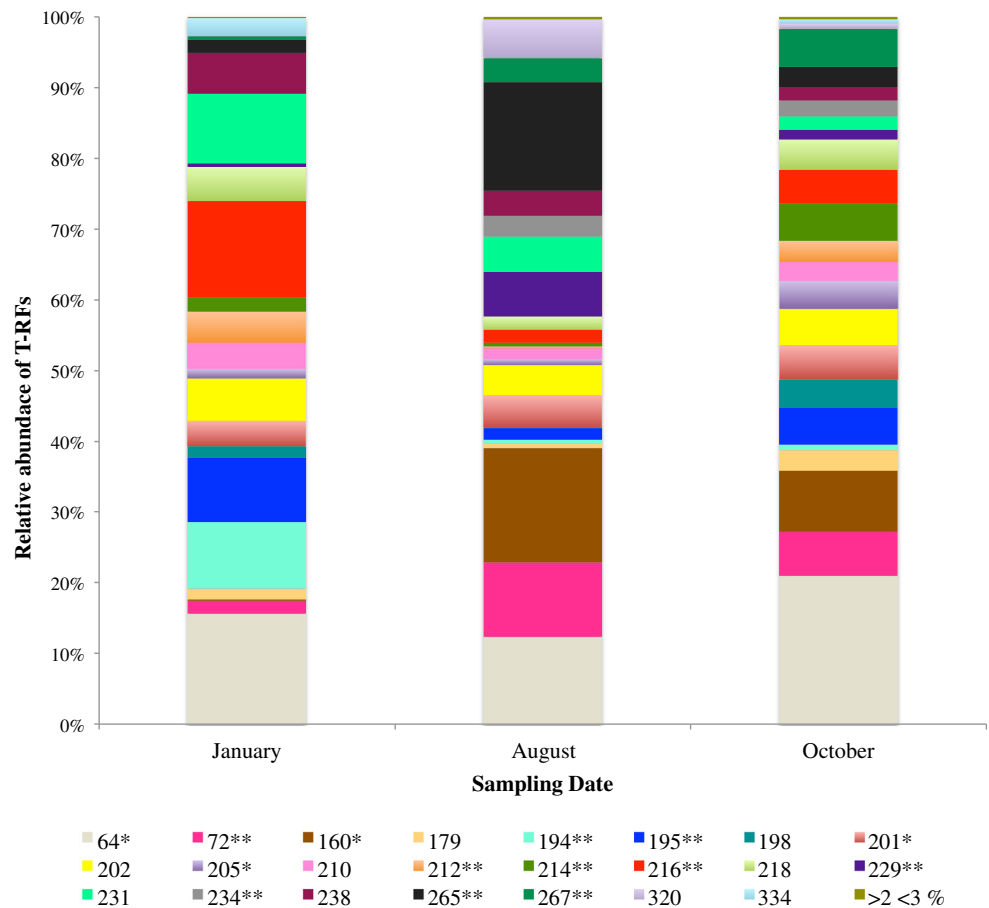
Table 3 Correlation coefficients of environmental variables: pH, organic matter (OM), sulfate, total nitrogen (TN), total carbon (TC), PAHs grouped by benzene rings, and total PAHs

	pH	OM	TN	TC	Sulfate	Bacteria	Total PAHs
MC		0.514**					
TC			0.957**				
Sulfate						0.806**	0.552**
Total PAH				0.202		0.706**	
2-ringPAH					0.686**	0.834**	0.879**
3-ringPAH	0.420*					0.698**	0.965**
4-ringPAH						0.515**	0.928**

Five- and six-ring PAHs were not included in the analysis since they were not detected in all cores

* $p<0.01$, ** $p<0.05$ show significant correlations

Fig. 3 Bacterial community composition in riverbank sediments. The figure shows relative abundance of terminal restriction fragments (T-RFs) that contribute to the community with more than 2 %; data are means per sampling date ($n=9$ cores). Groups of bacteria that highly correlated with NMDS 1 (*) and with NMDS 2 (**) from the ordination analyses are also shown



sulfate, TN, TC, total PAHs, moisture, organic matter, and total bacteria) were used to create ordination distances (Fig. 4) and represent the influence of these variables on bacterial community composition. As shown in Fig. 4, pH, three-ring PAH, and sulfate are highly significantly correlated ($p < 0.01$) with NMS2 while two-ring PAHs, four-ring PAHs, total PAHs, and bacteria number also correlated ($p < 0.05$) with NMS2. Nitrogen, moisture, total carbon, and organic matter were not significantly correlated with neither NMS1 nor NMS2 ($p > 0.05$), indicating that perhaps these environmental conditions were not as related to the bacterial communities in Lowellville. However, in the ordination plot, PAHs and pH appeared to be more associated with bacterial communities sampled in August, while moisture, organic matter, and sulfate were more associated with bacterial communities in January. NMDS components were also correlated with individual PAHs (see Table 5).

3.5 Bacterial taxa

A total of 160 bacterial clones were randomly selected for sequencing (summer sampling date only). Sequences were manually revised for quality, and only 153 were used for

analyses. Bacterial sequences were binned into OTUs using a 97 % cutoff using the CD-Hit package (Li and Godzik 2006), and the longest sequence was selected as a representative sequence for that OTU (Table 6). The CD-Hit analyses resulted in 62 OTUs represented in eight clusters, which at the same time represented 61 % of the sequences (Table 6). The homologous coverage from our study was 71 %, and the rarefaction curve did not reach a plateau, indicating that the microbial diversity in these sediments is greater than revealed by our analyses (data not shown). The largest cluster contained 33 clones (22 % of all clones); these were most closely related to sequences from δ -Proteobacteria mainly *Syntrophus*. Other sequences were grouped in other seven clusters (Table 6), with highest sequence identity to that of bacterioidetes-like strains, *Smithella*, *Desulfobacca* strains, *Syntrophorhabdus* strains, firmicutes including *Thermincola* strains and *Desulfobacterium* strains, Caldiserica strains, and actinobacteria-like strains. The other 39 % were represented by singleton and doubleton sequences in the clone library (data not shown).

Recovered sequences were matched, where possible, to sequences from the most closely related uncultured and cultured relatives; however, the majority of phylotypes detected

were not closely related to any cultivated representatives. At the phylum level (Fig. 5), the clone library was dominated by organisms related to the proteobacteria (50 %), firmicutes (27 %), bacterioidetes (11 %), acidobacteria (3 %), actinobacteria (3 %), and caldiserica (3 %). Other groups of bacteria with fewer clones were phylogenetically related to chloroflexi (2 %), nitrospirae (1 %), and elusimicrobia. Among the proteobacteria, the vast majority of sequences were related to the δ -Proteobacteria (89 %) while the α -, β -, and γ -Proteobacteria were only represented by 1 %, 4 %, and 6 % respectively. At the order level, the δ -Proteobacteria was highly represented by organisms related to the syntrophobacterales (66 %), desulfomonadales (11 %), desulfobacterales (11 %), and burkholderiales (4 %). Other important groups in the proteobacteria included xanthomonadales, chromatiales, legionellales, desulfurellales, and sphingomonadales. Among the non-proteobacteria, at the order level, the majority of retrieved sequences were represented by bacteroidales (17 %), clostridiales (17 %), thermoanaerobacterales (15 %), halanaerobiales (10 %), negativicutes (7 %), and caldisericales (5 %). Other sequences were related to anaerolineales, solirubrobacterales, and uncultured bacteria.

4 Discussion

River sediments, in general, are sinks for pollutants in aquatic systems (Machado et al. 2012). Metal and xenobiotic concentrations, vegetation, and other sediment chemistry characteristics influence microbial community composition (Almeida et al. 2013). Relatively few studies have investigated bacterial communities associated with PAH-contaminated riverbank sediments (Pies et al. 2008; Pratt et al. 2012). To our knowledge, this is one of the first molecular characterizations of an anaerobic microbial community in riverbank sediments affected by a long history of contamination. The potential influence of environmental variables on bacterial community composition in the highly contaminated study site was also evaluated via multivariate statistical analyses. In general, our results suggest that bacterial community composition in PAH-contaminated sediments changes concurrently with temporal changes in environmental conditions despite their long-term exposure to PAHs. Bacterial communities appeared to be strongly influenced by heterogeneity in biogeochemical parameters.

Table 4 Correlation coefficients of selected T-RF (bp) and environmental variables measured in riverbank sediments

T-RF size (bp)	MC	TC	Bacteria	Sulfate	Total PAHs	Two-ring PAH	Three-ring PAH	Four-ring PAH	Five-ring PAH
171							-0.531		
194	0.725								
198				0.552					
200				0.534					
205				0.619					
214			0.720	0.873		0.600			
216	0.599								0.593
218				0.563					
221			0.516	0.557	0.515	0.507	0.527		
227	0.533								0.599
231		-0.620			-0.607	-0.563	-0.573	-0.545	
238			-0.515		-0.508	-0.673	-0.523		
241	-0.526								
254	-0.759								-0.559
257			0.736	0.741	0.508	0.552			
270		-0.540							
287					0.556		0.597	0.530	
290	-0.558								
295				0.502					
300			0.577	0.575					
304				0.593					
334	0.653								0.730
360			0.628	0.686		0.501			
420					-0.660				

Only T-RFs with significant correlation ($p < 0.01$) are shown

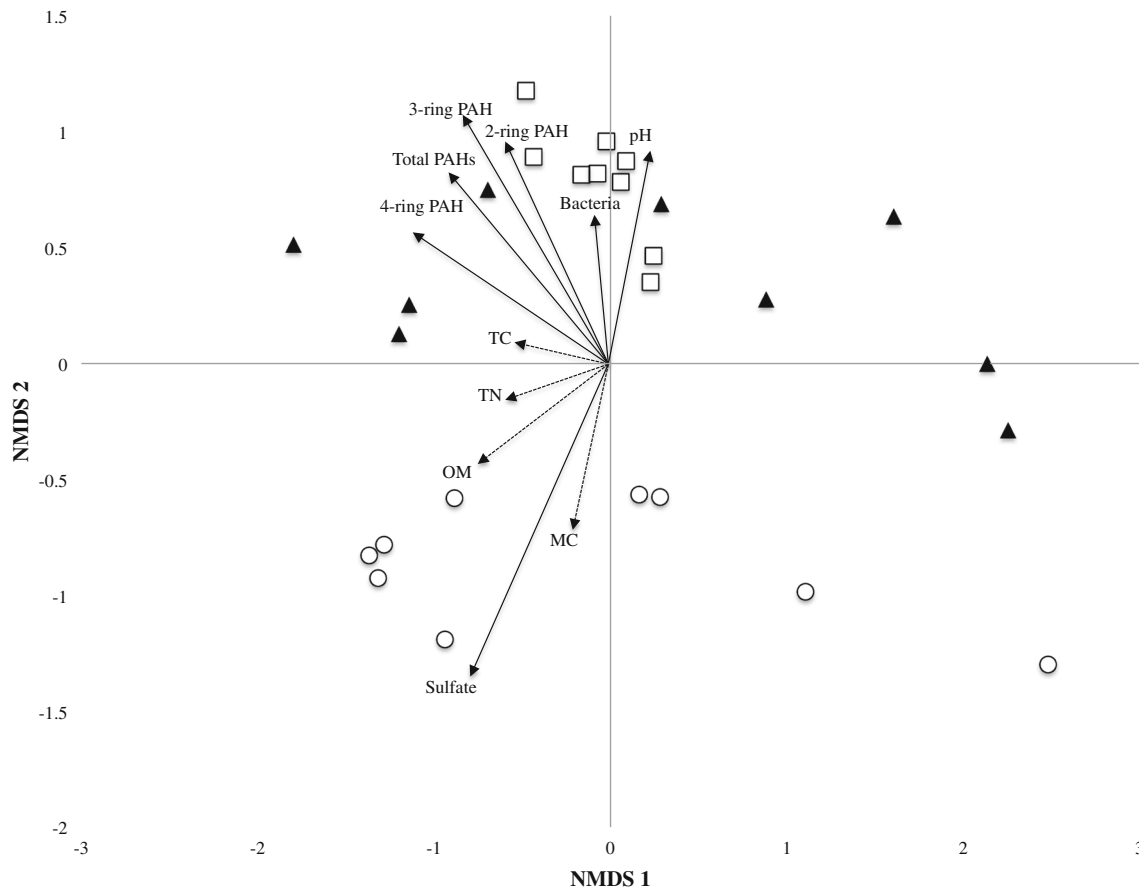


Fig. 4 NMDS ordination showing the similarity and distribution over time of microbial communities (T-RFs of the 16S rRNA gene) in riverbank sediments. Each symbol represents the microbial community from one sample (January circles, August squares, and October triangles); the graph also shows ordination distances created using Spearman

correlations values from NMDS dimension scores of T-RFs and environmental variables: pH, sulfate, TN, TC, total PAHs, moisture, organic matter, and total bacteria. *Solid arrows* indicate statistically significant correlations, and *broken arrows* indicate non-significant correlations

Table 5 Correlation coefficients between T-RF derived communities expressed as NMDS components and individual PAH concentrations measured in riverbank sediments

Compound	NMDS 1	NMDS 2
Naphthalene	-0.090	0.458*
Acenaphthylene	-0.079	0.291
Acenaphthene	-0.150	0.408*
Fluorene	-0.045	0.479*
Phenanthrene	-0.256	0.520**
Anthracene	-0.189	0.604**
Fluoranthene	-0.185	0.564**
Pyrene	-0.451*	0.263
Benzo(a)anthracene	-0.240	0.485*
Chrysene	-0.274	0.405*
Benzo(b,k)fluoranthene	-0.360	-0.034
Benzo(a)pyrene	-0.200	-0.084
∑ PAHs	-0.330	0.443*

* $p < 0.01$, ** $p < 0.05$ show significant Spearman's coefficients

In aquatic systems, temporal changes have a major impact on microbial community structure (Wang and Tam 2012). However, environmental factors, including moisture and nutrients, are also important drivers of variability in community composition (Bernhard et al. 2005). We measured selected environmental variables and found that some were related to bacterial community composition to varying extents. TC, TN, moisture, and organic matter did not appear to play an important role in shaping the bacterial community as reported also for other contaminated sediments with PAHs, where organic matter had very little effect in microbial communities (Wang and Tam 2012). Total carbon in our sediments was much higher compared with other similar contaminated sites (Guo et al. 2007) and was perhaps explained by the high amount of total petroleum hydrocarbons also present in these sediments (not measured in this study).

Microbial community composition was strongly related to pH, PAHs, and bacteria abundance during August, while sulfate was the predominant factor in January. The pattern observed in temporality respect to PAHs was unusual, since PAHs tend to sorb to sediments and do not change much in

Table 6 Phylogenetic affiliation of clones amplified from Mahoning river bank sediments

Clusters	No of clones in library	Phylogenetic affiliation	Representative clone (bp)	Closest environmental sequences GenBank ID	Closest culture relative	
					Habitat	GenBank ID
Cluster 1	33	δ -Proteobacteria	MRS80 (542 bp)			<i>Syntrophus aciditrophicus</i> (94 %)
Cluster 2	12	Bacteroidetes	MRS92 (1485 bp)			<i>Alistipes shahii</i> WAL 8301 (85 %)
Cluster 3	11	Firmicutes	MRS154 (1495 bp)	<i>Thermincola potens</i> JR (84 %)	Sludge reactor	
Cluster 4	10	Caldiserica	MRS33 (1,509 bp)	<i>Caldisericum exile</i> (82 %)	Hot springs	
Cluster 5	7	δ -Proteobacteria	MRS133 (567 bp)	<i>Syntrophorhabdus aromaticivorans</i> (93 %)	Methanogenic degrading enrichment culture	
Cluster 6	6	δ -Proteobacteria	MRS124 (1,525 bp)	<i>Desulfobacterium anilini</i> (94 %)	n.a.	
Cluster 7	5	δ -Proteobacteria	MRS156 (1,528 bp)	<i>Smithella propionica</i> LYP (90 %)	Anaerobic digester	
Cluster 8	4	δ -Proteobacteria	MRS90 (1,526 bp)	<i>Desulfobacca acetoxidans</i> (90 %)	Fresh water	

Clustered sequences represent 61 % of the total number of retrieved sequences. Closest environmental or culture relative sequences indicating percent similarity in brackets

such short time. However, a more recent analysis of the hydraulic conductivity in this site (Lowellville) indicated hydraulic interconnection between shallow aquifers (present in the riverbank) and the water in the main river channel (Amin and Jacobs 2013). This may explain temporal changes in PAH distribution. Our results indicate that even small changes observed from core to core (spatial heterogeneity over a relatively modest scale) in the physical environment of sediments affected structure of bacterial communities. This trend was particularly noticeable for sulfate, which had a strong correlation with differences in community composition. Although most freshwater systems are characterized by low sulfate concentrations (Miletto et al. 2008), the Mahoning River exhibited high sulfate concentrations that fluctuated spatially and temporarily (Table 1). High sulfate concentrations were also reported from streams contaminated with heavy metals and mercury (Vishnivetskaya et al. 2011) and from sediments located in the vicinity of Oak Ridge (Porat et al. 2010), where both studies showed an effect on microbial communities.

The drastic decrease in concentrations of sulfate during August sampling may be attributed to increased microbial activity in summer months (Gudasz et al. 2012). Indeed, we detected higher number of T-RFs in August. In our sediments, perhaps SRB were using sulfate for respiration; higher numbers of SRB were detected in August compared with January and October, yet no statistically significant correlation between PAHs and SRBs was found (data not shown). We did find that, although bacteria were not particularly abundant

(ranged from 10^6 to 10^8 dry weight g^{-1}), numbers were highly correlated with PAH concentration, indicating that bacterial abundance was not limited by PAH contamination. Similar results from other studies have shown that biomass and bacterial cell counts in sediments contaminated with petroleum appeared to be unaffected by contamination (Feris et al. 2004; Shi et al. 2005). Total bacteria number was also highly correlated with sulfate, suggesting that sulfate was potentially used as terminal electron acceptor in the oxidation of PAHs; these results should be interpreted cautiously, since more studies are needed to understand carbon utilization in anaerobic contaminated environments.

T-RFLP analysis is a reproducible and sensitive method for comparing temporal changes in bacterial community

Fig. 5 Phylogenetic tree of selected bacterial 16S rRNA gene sequences retrieved from PAH-contaminated riverbank sediments. The tree was constructed using the neighbor-joining method with *Thermofilum pendens* as the outgroup. Numbers in parentheses indicate the number of sequences for selected representative sequences from each cluster (ReprSeq). Bootstrap values (in %) are based on 100 replicates each (distance and minimal evolution) and are shown at the nodes with >50 % bootstrap support. Accession numbers are MRSLW30 (KF906488), MRSLW112 (KF906489), MRSLW94 (KF906490), MRSLW58 (KF906491), MRSLW117 (KF906492), MRSLW136 (KF906493), MRSLW154 (KF906494), MRSLW153 (KF906495), MRSLW133 (KF906496), MRSLW124 (KF906497), MRSLW34 (KF906498), MRSLW90 (KF906499), MRSLW80 (KF906500), MRSLW56 (KF906501), MRSLW121 (KF906502), MRSLW33 (KF906503), MRSLW54 (KF906504), MRSLW129 (KF906505), MRSLW36 (KF906506), MRSLW79 (KF906507), MRSLW38 (KF906508), MRSLW92 (KF906509)

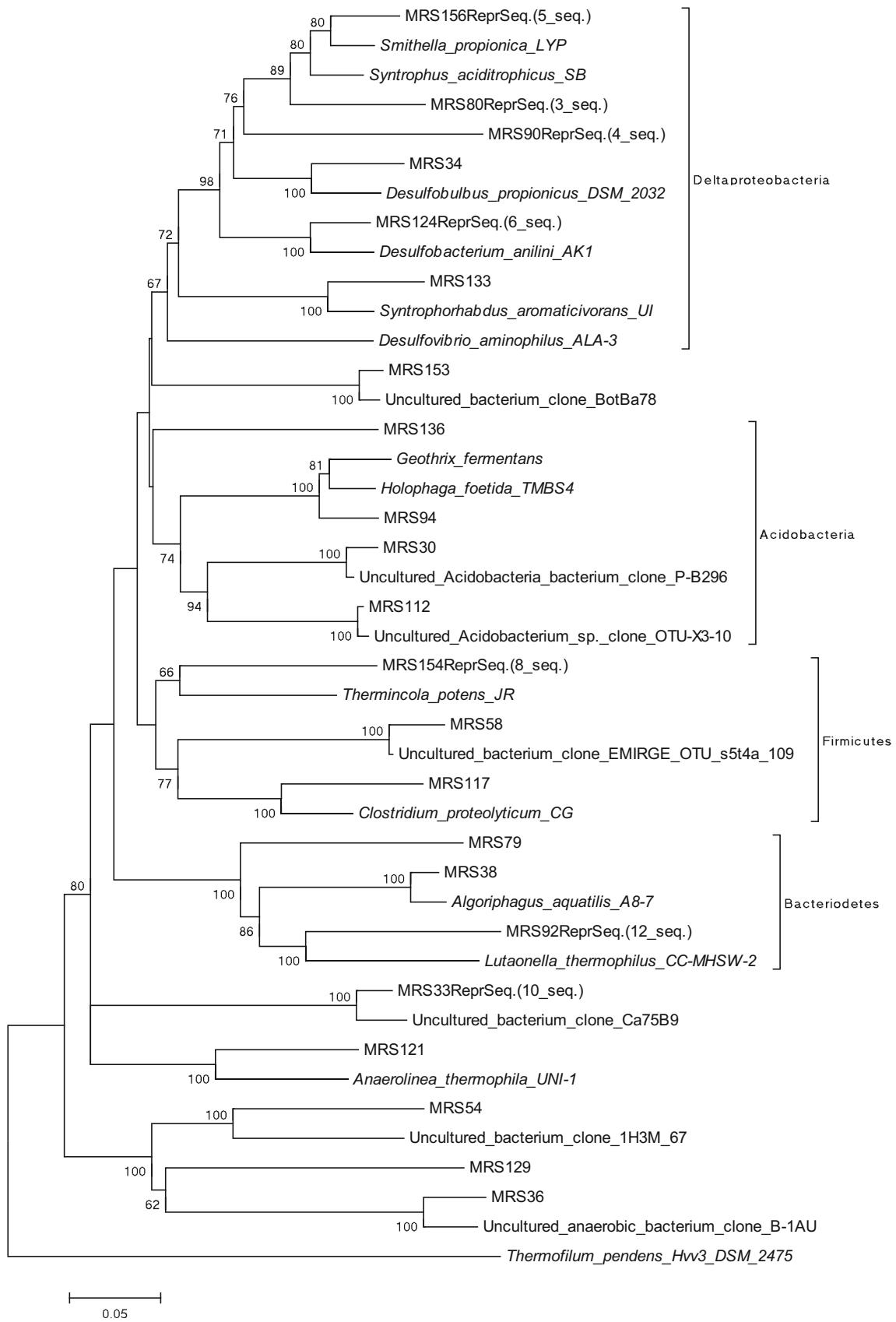
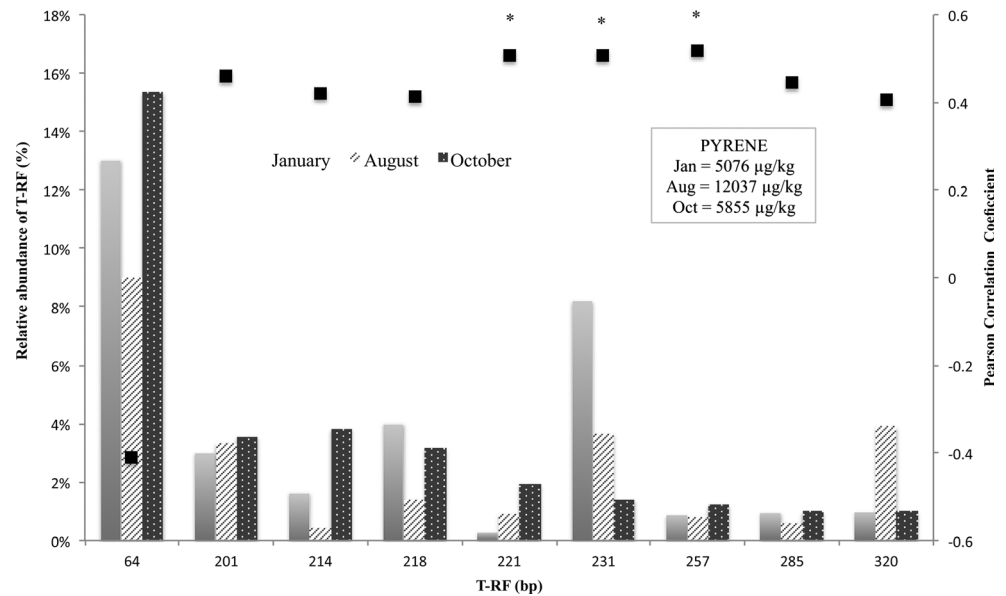


Fig. 6 Correlations between pyrene concentrations and statistically significant groups of bacteria found in riverbank sediments. *X*-axis represents T-RFs. *Left Y*-axis indicates relative abundance of T-RFs measured at each sampling date. *Right Y*-axis shows Pearson's correlation coefficient. Statistically significant correlations are represented as black filled squares ($*p < 0.01$; no $*p < 0.05$)



composition (Liu et al. 1997). NMDS analysis of T-RFLPs suggests that microbial communities were subject to temporal effects despite the extent of contamination. Interestingly, our results are in agreement with others that have described clustering of bacterial communities temporally (Hullar et al. 2006) and transitional patterns marked by temporal variations (Smoot and Findlay 2001) in uncontaminated environments. Yet, in similar contaminated systems, hydrocarbon pollution has been shown as the main driving force for changes in the microbial community structure (Wentzel et al. 2007). More specifically, in “hot spots” of contaminated sediments (where high PAH levels are found), three- and five-ring PAHs appeared to be most important factor determining microbial community composition (Wang and Tam 2012). Concurrently, in our study, bacterial community composition was influenced strongly by concentrations of two-, three- and four-ring PAHs. Some of our sediment cores represented “pockets” of contamination, which exhibited 200X higher PAH concentrations than those reported for other river sediments around the world (Pies et al. 2008; Cao et al. 2010). Our analyses revealed that some of the most abundant PAHs, e.g., pyrene, had statistically significant correlations with some groups of bacteria (Fig. 6). Yet, because T-RFLP analyses represent an overall snapshot of community composition, it cannot be implied that only pyrene was associated with certain groups of bacteria; in fact, our NMDS components (based on T-RFs data) were highly correlated with most PAHs, except for acenaphthylene, benzo[b,k]fluoranthene, and benzo[a]pyrene (Table 5).

Clone library data revealed that bacterial communities included proteobacteria, firmicutes, acidobacteria, and bacteroidetes. As described for other contaminated sites (Edlund and Jansson 2006; Porat et al. 2010), the large

majority of sequences in our sediments were related to δ -Proteobacteria. This pattern has also been observed elsewhere in sediments contaminated with uranium where δ -Proteobacteria (Sun et al. 2013) and acidobacteria (Barns et al. 2007) were predominant groups of bacteria. In contrast, bacteroidetes, a phylogenetic group also associated with PAH contamination (Haller et al. 2011), was also found in Mahoning riverbank sediments, although in less abundance. In addition, some clones were closely related to uncultured bacteria clones retrieved from a very diverse array of environments including estuarine sediments (clone BotBa78), wetlands (clone P-B296), oil reservoirs (OTU-X3-10), lake sediments, and subsurface characterized by high sulfate (clone Ca75B9 and OTU-S5t4a-109, respectively), anaerobic lagoons (clone B-1 AU), and deep buried sediments (clone 1H3M-67).

A large number of clones were closely related to *Syntrophus*, bacteria commonly found in methanogenic hydrocarbon-degrading consortia that, under anaerobic conditions, can degrade hydrocarbons (Kadnikov et al. 2013). Syntrophic processes are essential for biodegradation of hydrocarbons to methane, yet recent studies suggest that not-yet-cultured relatives of *Syntrophus*-affiliated 16S rRNA gene sequences could perform hydrocarbon metabolism in the presence of electron acceptors such as nitrate or sulfate (Gieg et al. 2014).

We also observed phylotypes affiliated with desulfobacterales, desulfomonadales, and desulfurellales. Some members of these groups have been described as sulfate-reducing bacteria (SRB) involved in PAH degradation (Acosta-Gonzalez and Marques 2013; Gomes et al. 2013). For example, SRB that are members of the desulfobacterales are responsible for most anaerobic mineralization of hydrocarbons in marine sediments (Zhang et al. 2008). In agreement

with these results, we found culturable SRB ranging from 2 to 9×10^4 cells/g (data not shown) from contaminated sediments in Lowellville. In fact, it has been observed that hydrocarbon contamination (Suárez-Suárez et al. 2011), as well as anthropogenic activities such as pollution (Cleary et al. 2012), increases abundance of SRB. Cardenas and coworkers (2008) reported that the predominant species in sediment microbial communities in uranium contaminated sites were *Desulfovibrio* spp. and other SRB and iron-reducing bacteria including *Geothrix* spp. Therefore, the presence of SRB communities in sediments with high concentrations of sulfate such as the Mahoning River, and with a history of extensive PAH contamination, suggest they may have a role in anoxic degradation of hydrocarbons and could be used for in situ biodegradation strategies. Further studies and more clone library information are needed to verify this finding.

5 Conclusions

This is the first study using a molecular approach to report the bacterial community structure of highly contaminated PAH riverbank sediments. We demonstrated that, besides temporal changes, specific environmental variables (PAHs, sulfate, pH, and bacterial abundance) were strongly related to bacterial community composition. A diverse bacterial community was elucidated by the number of T-RFs recovered from the sediment and from phylogenetic analyses. The majority of recovered 16S rRNA gene sequences were related to δ -Proteobacteria while some sequences found were unaffiliated to known bacteria. In addition, we found abundant SRB sequences, some of which may be involved in hydrocarbon degradation. These bacterial taxa might have the potential to be used as functional markers to determine diversity and should be evaluated in future studies.

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