CHARACTERIZATION OF BACTERIAL COMMUNITIES OF RIVERBANK SEDIMENTS CONTAMINATED WITH POLYCYCLIC AROMATIC HYDROCARBONS

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OVERVIEW OF DISSERTATION

Understanding the ecology of microorganisms in the natural environment is one of the most challenging tasks for microbiologists. Microbes are extensively distributed in a variety of terrestrial and aquatic environmental habitats and are important because of their abundance, diversity and variety of metabolic activities (reviewed in Ranjard et al. 2000). Microbial communities are of extreme importance in biogeochemical cycling; microbes catalyze many reactions including conversion of organic molecules to inorganic forms that can be further used throughout microbial loops and food webs (Rubin and Leff 2007; Olapade and Leff 2005). Indeed, it is widely recognized that microbes influence Earth surface and subsurface processes (Viles 2012; Su et al. 2012; Gao and Tao 2012). Microbial community interactions are important to ecological processes and to the surrounding physical and chemical environment; these interactions can alter environmental conditions and in turn the environment shapes the community. For instance, microbial communities directly affect oxidation-reduction processes in sediments resulting in a spatial organization of the associated communities as observed in a simple Winogradsky column experiment (Anderson and Hairston 1999; Atlas and Bartha 1998).

Microbial communities in soils and sediments respond directly to abiotic factors, such as temperature (Collins et al. 2008; Angel et al. 2010), nutrients (Hullar et al. 2006), moisture (Torskiv and Obreas 2002), and contamination (Wang and Tam 2012; Jiang et al. 2009). Microbes inhabiting contaminated environments respond to changes in environmental conditions by oxidizing, immobilizing and transforming pollutants and are directly linked to processes including environmental restoration (Lovley 2003) and bioremediation (Prosser et al. 2007) (Fig. 1). Sediment contamination impacts different attributes of microbial communities. For example, metals might change microbial biomass (Epelde et al., 2010), microbial activity (Mosher et al. 2003), and/or community structure (Jiang et al. 2010; Wang et al. 2010; Yang et al. 2006). Similarly, organic xenobiotics can have an effect on bacterial richness and diversity (Fragoso dos Santos et al. 2011).
In the last few decades, investigations on microbial diversity and ecosystem functions have increased since newer molecular techniques have become more powerful and available (but not necessarily more accessible). Culture-independent techniques have become of great importance since less than 1% of most microbial communities can be cultured (Amman et al. 1995). These techniques have allowed the identification of novel organisms and have given some answers to questions such as ‘What microbes are present?”, “What processes are they doing”?, and “How are they doing it? (Newman and Banfield 2002). In addition, in a number of environments considered “extreme”, where the majority or microbes are unknown and unculturable, the use of culture-dependent methods is essential (Su et al. 2012). Yet, despite this great ‘molecular revolution’, surprisingly relatively little is known about the relationship between bacterial community composition and ecosystem functioning in many ecosystems. Microbial processes are key elements for all biogeochemical cycles in all habitats of natural ecosystems but also for addressing questions about diversity and environmental changes (Deng et al. 2012). It is of great importance to elucidate how environmental changes and temporal variations influence changes in community composition (Peter et al. 2011). Combining geochemical approaches and microbial measures has been neglected (Viles 2012). There is a great need for multidisciplinary studies using molecular techniques and genomic-based microbiology and geochemistry to simultaneously: i) identify microbial communities and ii) to study their interaction with each other and with the environment (relating function to biotic and abiotic factors) (Andren et al. 1999; Gao and Tao 2012; Ranjard et al. 2000).

Although sediment contamination and environmental pollution is a current and a future problem worldwide, there is a gap of information at the interface between chemical and physical conditions and microbial community structure as well as microbial spatial-temporal variations in contaminated environments (Gao and Tao 2012). In particular, investigations on both geochemical composition and identification of microbial communities that can lead to more accurate predictions of natural attenuation and bioremediation are very limited (Lovley 2003). Even more limited is our knowledge of the geochemistry and microbial communities present in riverbank sediments impacted by long-term pollution. In urban settings where riparian vegetation is the only remnant native vegetation, riverbanks are main components for restoration programs (Fisher and Goldney 1997). Riparian areas are considered ‘keystone’
ecosystems in which surrounding ecological communities depend on the health of the riparian zone (Innis 2000). Riverbanks have an extensive integration with surrounding ecosystems where water, soil, sediment, nutrients and other chemical compounds interact closely with the water channel, not to mention the biogeochemical and hydrological interplay associated with the interface sediment:water (Amin and Jacobs 2013). Although riparian zones have become of more interest in landscape ecology and river restoration, as described below, there are few investigations on microbial communities and geochemistry in highly contaminated riverbank sediments.

Contaminated sediments by polycyclic aromatic hydrocarbons.- Sediments are contaminated by metals (Varol 2011), pesticides (Kumar et al. 2011), herbicides (Magbanua et al. 2013), polychlorinated biphenils (Szalinska et al. 2013) and dioxins (Josefsson et al. 2012) among other toxins. Yet, the most common type of pollutants in sediments and soils are polycyclic aromatic hydrocarbons (PAHs) (Husain 2008 and references therein). Their intrinsic nature, including hydrophobicity, low bioavailability and resistance to degradation, facilitate their accumulation in soils, sediment, aquatic biota and humans via direct or indirect exposure including intake and inhalation (Nandita et al. 2009). Thus, there is a need to characterize contaminated environments affected with PAHs. PAHs are ubiquitous and toxic; some PAHs are considered potential carcinogens and/or mutagens and are contaminants of concern in priority lists of environmental agencies in the U.S. and Europe. Indeed, even at low concentrations some PAHs pose a risk to wildlife and human populations.

Extensive research on geochemistry and microbial communities associated with PAH-contamination has been conducted in marine sediments (Mazias-Zamora et al. 2002; Tahir et al. 2014; Tsapaki et al. 2010), estuaries (Tian et al. 2008; Al-Thukair et al. 2007; Brito et al. 2006), rivers (Cao et al. 2010; Chen et al. 2013; Guo et al. 2009) and lakes (Barakat et al. 2011; Yan et al. 2013; Zhang et al. 2012). There is only one study that has investigated microbial communities in riverbank sediments (Pratt et al. 2012) but it did not include a comprehensive geochemical characterization of the sediments.

There is a paucity of information about the structure of indigenous communities of microorganisms in riverbank sediments with a long history of PAH contamination, such as the Mahoning
River (Northeast Ohio, USA). Characterizing the indigenous bacterial community in anaerobic contaminated sediments is critical because it might reveal that some species have adapted to pollution and that some taxa could use the xenobiotic as energy and/or carbon sources. In sediments, PAH distribution combined with the presence of metals and the age of contaminants has been shown to influence microbial community structure and diversity (Zhang et al. 2008). The distinctiveness of this system and the long-term contamination presented a unique opportunity to understand microbial ecology under conditions of extreme pollution. More specifically my dissertation research aimed to: i) assess the microbial communities present, ii) characterize the environmental conditions, iii) determine possible influence of the environmental conditions in bacterial community structure and diversity and iv) evaluate if microbial communities have the potential to anaerobically degrade PAHs. The work presented here used a combined approach that measured a suit of environmental conditions by using standard analytical chemistry methods and relatively modern molecular tools to identify the microbial communities by terminal restriction length polymorphisms (T-RFLP) and to construct clone libraries using 16S rDNA and dsrAB genes.

Study site and sampling strategies.- My study site, the Mahoning River in Northeastern Ohio, extends for 108 km of which 51 km are considered polluted (USACE 1999; more detailed information is provided in subsequent chapters). Three sites along the Mahoning River were chosen based on accessibility, previous reports on contamination, location, and budget constraints (Fig. 2). Sampling was conducted either manually using steel liner cores (l=15 cm, x= 5 cm) or a manual auger device as shown in Fig. 3 and 4 respectively.

My dissertation thesis.- Here, I briefly introduce each chapter of my dissertation and summarize major findings. The thesis is structured according to manuscripts for future publication; one chapter was published, some chapters are under peer review while other chapters are under preparation.

In chapter I, an extensive review of PAHs, sources, exposure, contamination in aquatic systems, remediation strategies insights of microbial degradation of PAHs, as well as background information of the study site is presented. Chapter I includes excerpts from a published book chapter, which I co-authored and
have copyright permission for using as part of my dissertation. The first author Dr. Carl Johnston was invited to write a book chapter in bioremediation of PAHs in 2011, which could include additional authors. Since his expertise is mainly fungal degradation of PAHs he extended the invitation to me so I could contribute by writing other sections required based on the guidelines for authors. At the end of 2010, I wrote my prospectus and a large portion of that document extensively covered the literature of PAHs including, sources, exposure pathways, description, remediation strategies, bacterial degradation and bioremediation. I was able to use aspects of that document as the foundation for my contributions to the book chapter.

In chapter II, the characterization, origins, distribution and ecological risk assessment of PAHs in three sites along the Mahoning River (Lowellville, Girard and Newton Falls) is reported. In Chapter II, we quantified 12 PAHs and identified sources using distribution patterns, PAH ratios, and principal component analysis (PCA). High PAH concentrations (94,300 to 560,000 µg/kg) were found in riverbank sediments. Different analyses using ratios of particular PAHs used to determine origins of PAHs revealed that pyrolytic and petrogenic sources of PAHs mingled in riverbank sediments. This finding correlated with the history of the site; the Mahoning River supported one of the biggest steel industries for decades specifically steel mills and related industries used the river as a coolant source. This was reflected in the combined PAH sources found in the riverbank exposed to decades of pollution. High concentrations of PAHs ranked this sediment as one of the most polluted aquatic ecosystems in the world. Sediment guidelines used to assess ecological risk of PAHs overwhelmingly indicated that the riverbank sediments of the Mahoning posed a very high ecological risk to aquatic organisms, even at what it was previously considered an unpolluted site.

In chapter III, the first molecular assessment of the microbial community structure in a heavy long-term PAH contaminated riverbank site (Lowellville) is reported. 16S rDNA-based T-RLFP analysis and sequencing were used to characterize bacterial communities in highly PAH-contaminated sediments. Selected environmental parameters were measured to establish possible correlations between bacterial communities and environmental geochemistry. Non-metric dimensional scaling analyses (based on T-RFLPs) revealed that bacterial communities followed a temporal pattern similarly found in other aquatic
environments. In addition, PAH concentration, sulfate, pH and moisture appeared to be associated with bacterial communities (significant Pearson correlations). A small phylogenetic analysis (110 sequences) derived from three selected core samples collected in August revealed a number of groups of bacteria present in the sediments including δ-proteobacteria, firmicutes, actinobacteria and caldiserica. Some members of these groups (phylotypes) were related to sulfate reducing bacteria, some of which have been previously described as important in PAH degradation. Highly significant Spearman correlations between NMS components (representing bacterial communities) and PAH concentrations (2, 3 and 4 rings) might indicated an association of bacteria and PAHs. This finding suggests that, potentially, extant microbial communities might contribute to natural attenuation and/or bioremediation of PAHs.

Study of dissimilatory sulfite reductase genes (dsrAB) has led to the discovery of new sulfate reducing bacteria (SRB); these genes are found consistently in SRB, making them excellent markers for phylogenetic studies (Rampinelli et al 2008). In Chapter IV, dsrAB genes were used to investigate the SRB community impacted by metals and PAHs in three sites along the Mahoning River: Lowellville, Girard and Newton Falls. In this chapter, non-metric dimensional scaling analysis (based on T-RFLPs) revealed a spatial segregation between bacterial communities found at each sampling site. Principal component analysis of environmental sediment parameters including PAHs and metal concentrations revealed a strong geochemical gradient that segregated the geochemical data according to sampling site. This environmental gradient likely influenced the bacterial community composition. SRB and T-RFLP diversity based indexes (based on a small number of operational taxonomic units using the dsrAB gene, and terminal restriction fragments using 16S rDNA gene) indicated that highly contaminated sediments showed lower diversity than the low-contamination site. Yet, these results need to be further corroborated by including more sequences in the clone libraries. Interestingly, abundances of total bacteria and SRB were higher in the highly contaminated sites compared to the low-contamination site. Phylogenetic analyses included a small set of recovered sequences (~110) revealed that the majority of dsrAB sequences recovered were closely affiliated with unknown, uncultured SRB.

The long history of PAH contamination in the Mahoning River (almost a century) suggests that microbial communities may include members able to use these organic compounds as carbon sources. As
much of the organic matter metabolism in river sediments is anaerobic, it is likely that indigenous communities play an important role in anaerobic PAH degradation. In chapter V, based on this information and the fact that bacteria can degrade PAHs under various redox conditions, a bioremediation experiment was implemented to determine if microbial communities had the potential to degrade PAHs under anaerobic conditions. Based on the information provided in Chapter III and IV, two PAHs were used as models to measure their disappearance over time under sulfate reducing conditions and when a humic acid analogue (anthraquinone-2,6-disulfonate [AQDS]) was added to sediments. Only two PAHs were selected because of their use in previous studies, time constraints and budget limitations. After 40-days of anaerobic incubation phenanthrene and fluorene degradation was greatly increased under AQDS and AQDS + sulfate amendments in comparison to sediments undergoing natural attenuation. The relatively high numbers of PAH-degrading and sulfate reducing bacteria, and the fact that other mechanisms of PAH disappearance (photooxidation, volatilization and leaching) were ruled out, degradation was likely a biotic mediated process. The final chapter VI is a synthesis in which I summarize my observations and findings in addition to provide insights of future research. My dissertation followed a multidisciplinary approach that combined geochemical methods with molecular tools to investigate the microbial communities in long-term impacted riverbank sediments by PAHs and metals. This work is the first of its kind and addressed knowledge gaps related to highly impacted riverbank sediments, their microbial communities and biogeochemistry; it also provided some evidence that bioremediation of the riverbanks by extant microbes is feasible. This information implies that the restoration of the Mahoning riverbanks, as well as other similar sites worldwide, could be successfully implemented.

References


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CHAPTER I: POLYCYCLIC AROMATIC HYDROCARBONS: SOURCES AND CONTAMINATION

Abstract

PAHs are common toxic pollutants that persist in the environment. PAHs have low solubility in water and tend to attach to organic matter and particles in water, soils and sediments. High concentrations of PAHs are found in a variety of ecosystems, especially where anthropogenic pollution is increased. PAHs pose risks to aquatic biota and are linked to deformities and cancer in humans, fish, and benthic organisms.

Aerobic bioremediation of PAHs is a common approach successfully implemented in soils, waters and sediments. Yet other organisms including algae, fungi and plants are also suitable for bioremediation. On the other hand, anaerobic biodegradation of PAHs is limited and less research has been dedicated to this type of degradation. Nevertheless, anaerobic biodegradation of PAHs can occur under nitrate or sulfate reducing conditions. Bioremediation of contaminated soils and sediments with PAHs can be simple, cost-effective and ex situ and in situ. More research is needed to fully understand mechanisms of bacterial metabolism in degradation of PAHs.

1 Selected portions of this chapter were included (granted copyright permission) in the following publication: Johnston C, Johnston GP. Rajesh Arora (Ed.). 2012; Bioremediation of polycyclic aromatic hydrocarbons, in Microbial Biotechnology: Energy and Environment, CAB International, Wallingford, UK), 279-296 pp. Dr. Carl Johnston was responsible for fungal degradation and phytoremediation sections, as well as final editorial changes and final revision. I wrote the following sections: general description of PAHs, sources of PAHs, exposure of PAHs, PAHs in aquatic environments, aerobic remediation (composting and landfarming), microbial degradation (algal and bacterial) and anaerobic degradation.
General Description of polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are among the most common organic pollutants in soil, water and sediments worldwide. They are a large, diverse class of persistent hydrophobic organic compounds. Some PAHs are considered potential carcinogens, mutagens and teratogens by the Environmental Protection Agency (USEPA 2011) and the Agency for Toxic Substances and Disease Registry (ATSDR 2010) and are listed in the National Waste Minimization Program of the U.S. Environmental Protection Agency (http://www.epa.gov/osw/hazard/wastemin/priority.htm) and the European Union as priority pollutants (PAHs’ main physicochemical properties and hazard assessment are shown in Table 1).

PAHs are composed of carbon, hydrogen, nitrogen, sulfur and oxygen atoms. PAHs can be colorless or pale yellow with low solubility in water, high melting points, and low vapor pressure (Haritash, et al. 2009). PAHs are classified as semivolatile organic compounds (SVOC) because of their high boiling points (greater than 200 °C; USEPA 2011). The simplest PAH is naphthalene (C\textsubscript{10}H\textsubscript{8}), consisting of two fused benzene rings. The most complex PAH is coronene (C\textsubscript{24}H\textsubscript{12}) with 7 fused benzene rings. In between, there are at least one hundred identified species of PAHs, all of which possess different physical and chemical properties and biodegradability (Manoli and Samara 1999). The recalcitrance of PAHs increases with mass. Low molecular weight (LMW) PAHs usually have high solubility, low water-octanol partition coefficients (K\textsubscript{ow}), (Alexander 1994) and are more likely to be biodegraded. High molecular weight (HMW) PAHs, which have low solubility, high K\textsubscript{ow}, tend to sorb into organic matter and soils (Manoli and Samara 1999; Abbondanzi et al. 2005) and persist for longer times in the environment.

Sources of PAHs

Research on the origins and geochemistry of PAHs revealed that anthropogenic PAHs can be categorized as pyrolytic and petrogenic. Pyrolytic PAHs result from incomplete fuel combustion, wood and coal burning, car emissions, tobacco related activities and grilling meat (Farhadian et al. 2010). This type of PAHs is also found in coal tar, creosote, roofing tar and parking lot seal coats (ATSDR 2010). Petrogenic PAHs are mainly derived from crude oil, unburned fuel and refinery products (Elias et al. 2007).
Most terrestrial natural sources of PAHs come from wildfires. Emissions from volcanic eruptions and thermal geological reactions are considered negligible sources of PAHs (reviewed in Maliszewska-Kordybach 1999). PAHs are also components of surface waxes of leaves, plant oils and cuticles of insects (Millero and Sohn 1991) and are naturally produced by some organisms. For instance naphthalene is produced by Magnolia flowers (Azuma et al. 2010), flowers from Annonaceae species (Jurgens et al. 2000), the endophytic fungus *Mucor vitigenus* (Daisy et al. 2002), and the subtropical North American termite *Coptotermes formosanus* (Chen et al. 1998).

Both anthropogenic and natural PAHs that are released into the atmosphere enter aquatic and terrestrial ecosystems via deposition. In both urban and rural areas, PAHs can be transported as gases or aerosols over great distances and can be found far from their sources in relatively pristine areas (Manoli and Samara 1999). PAH deposition in sediments and water bodies are the result of point sources in industrialized and urbanized zones, such as harbors, railroads, steel mills, and shipping ports. High PAH deposition into soils, sediments and water leads to elevated concentrations that affect coastal and riverine systems and present a hazard for humans, fish and wildlife (Wang et al. 2002).

**Human and Wildlife Exposure to PAHs**

According to the U.S. EPA, the majority of PAHs are harmful toxins, and some are considered potential carcinogens. In laboratory studies, PAHs cause tumors in animals exposed through contaminated food, breathing contaminated air, and by skin contact with contaminated soils or sediments (ATSDR 2010). PAHs and their actual and potential health effects are summarized in Table 2.

PAHs in air, soils, water, and groundwater are usually present as mixtures rather than as single compounds (Gan et al. 2009). Exposure through air is mainly due to PAH vapors that are carried along with dust and particles. Carcinogenic PAHs in air are produced by everyday activities such as cigarette smoking, car exhaust, asphalt roads, agricultural and household wood burning, cooking meat or other foods at high temperatures (ATSDR 2010) and waste incineration (Menzie et al. 1992). Soil exposure occurs via contact with previously contaminated soils (e.g. after wood, coal or gasoline were burned at a particular site). Coal tar production sites, petroleum facilities, asphalt and aluminum production plants, coal-gasification, and
wood-preserving facilities usually have elevated levels of PAHs in soils (Wehrer and Totsche 2009). Industrialized areas and urban zones close to industries mentioned above contain higher concentrations of PAHs than rural soils (Van Metre and Mahler 2005). Contaminated sediments in aquatic ecosystems also contain elevated concentrations of PAHs, especially in harbors and shipping ports (discussed below) ref. PAHs are widespread and their occurrence in groundwater is also of concern. In groundwater, PAHs usually occur in combination with other monoaromatic hydrocarbons (benzene, toluene, ethyl benzene and xylenes) most commonly known as BTEX (Bianchi et al. 2012). BTEX and PAHs are associated with gasoline and other petroleum derived fuels in underground storage tanks. It has been estimated that 65% of these tanks in the U.S. leak (Kane et al. 2001), and thus they are the main source of groundwater contamination. This situation is no different in other countries. For instance, groundwater monitoring wells of gasoline stations of Rio de Janeiro City in Brazil, exceeded PAHs and BTEX maximum concentrations limits for drinking water (Pires do Rego and Pereira 2007).

While human contact bans can be issued to prevent contact with contaminated sediments, wildlife are exposed regardless. Bottom dwelling fish can develop deformations, erosions, lesions and tumors (DELTs) in PAH-contaminated sediments as a result of direct and indirect exposure (USACE 1999). The U.S. Fish and Wildlife Service (www.fws.gov/chesapeakebay) found that 53% of brown bull heads (Ameirus nebulosus) in the South River, a tributary of the Chesapeake Bay, had skin lesions and carcinomas, and 20% had liver tumors. The study concluded that the tumors were likely a result of exposure to PAHs in sediments. A survey conducted from 1997 to 2000 in industrially contaminated Lake Erie tributaries (the Detroit, Ottawa, Ashtabula, and Niagara Rivers) revealed that concentrations of PAH metabolites in bile of A. nebulosus positively correlated with concentrations of PAHs in sediments (Yang and Baumann 2005). A similar situation was found in the Black, Buffalo, Cuyahoga, and Detroit Rivers where 27% to 60% of brown bullheads surveyed from 2002 to 2007 presented abnormal communication between the oral cavity and cutaneous surface (orocutaneous tumors) as a result of ingestion of contaminated sediments (Blazer et al. 2009). Also, reptiles, such turtles, can be exposed to contaminated sediments. A study from 2000 through 2003 conducted at the John Heinz National Wildlife Refuge, Philadelphia, PA, (Bell et al. 2006) showed a high incidence of deformities in embryos of snapping turtles,
Chelydra serpentine, and painted turtles, Chrysemys picta, (13 to 19% and 45 to 71% respectively). Snapping turtle adults and embryos had high levels of PAHs in their fat, indicating bioaccumulation of PAHs (lipophilic compounds are transferred from female to eggs). The study concluded that the high deformity rates were primarily caused by sediment pollution. Benthic organisms that inhabit sediments (e.g. macroinvertebrates) can also develop morphological deformities due to direct exposure of heavy metals and organic compounds, including PAHs, in sediments (Boonyatumanond et al. 2006). The presence of chironomid larvae with mouthpart deformities in Lake Piediluco, Central Italy, correlated with the exposure to contaminated sediments with PAHs, pesticides, organochlorine pesticides and heavy metals (Di Veroli et al. 2010).

PAHs in aquatic environments

PAHs are ubiquitous pollutants in a variety of ecosystems around the globe. However, sediments are considered the major sinks of PAHs in subsurface environments (Stark et al. 2003; Mitra et al. 1999). Because of their hydrophobic nature, PAHs accumulate in bottom sediments and tend to sorb onto organic material.

Contaminated sediments, as illustrated in the examples below, are abundant. The Pearl River Delta, China, has been heavily impacted with anthropogenic PAHs (138 to 6,973 ng/g dry weight total PAHs) in the last three decades as a result of urbanization and development (Luo et al. 2008). Sediments of the Yellow River, the second largest river in China, are highly contaminated with PAHs (464 to 2621 ng/g dry weight total PAHs) because the river received wastes from oil refineries, paper mills and pharmaceutical companies (Xu et al. 2007). Sediments of the Oder River, Poland, situated in a heavy industrialized district (with numerous power stations, metallurgical and chemical industries), are heavily contaminated (633 to 146,400 ng/g dry weight total PAHs) with pyrogenic and petrogenic PAHs (Witt and Gründler 2005). In the same way, anthropogenic activities including coal mining and textile industries (Charriau et al. 2009) have resulted in sediment contamination of the Scheldt River, which flows through France, Belgium and the Netherlands. In the United States, sediment contamination is also a common environmental issue. For instance, sediments of the Hudson River, NY, exceeded concentrations of 47,000
ng/g dry weight (Lauenstein and Kimbrough 2007). Similarly, the Anacostia River, Washington, DC, presented high concentration of PAHs in sediments (211,300 µg/kg) which prompted health agencies to post advisories against fish consumption (Lu et al. 2006). Likewise, the Mahoning River, in Northeast Ohio and Western Pennsylvania has been heavily contaminated with polycyclic aromatic hydrocarbons (PAHs), metals, and polychlorinated biphenyls (PCBs) that remain in riverbank sediments (USACE 1999). Likewise, Fields Brook, a tributary stream of the Ashtabula River in northeast Ohio and a superfund site, contains elevated sediment concentrations of uranium, PAHs, Polychlorinated biphenyls (PCBs) and heavy metals (Li et al. 2001).

Oil spills, which include PAH-contamination, are common in intertidal estuarine wetlands, where contaminants tend to accumulate in mangrove sediments (Ke et al. 2002). In these ecosystems, concentrations of PAHs are usually elevated (>10,000 ng/g dry weight), and consequently impair benthic communities (Tian et al. 2008). Similarly, contamination of petroleum compounds associated with PAHs is commonly found in harbors due to intensity of shipping activities (Hayes et al. 1999). For instance, in Lake Erie, Ohio, U.S.A., the highest levels of PAHs were found in urbanized harbor cities: Detroit, Cleveland and Buffalo (DeBruyn et al. 2009). Similarly, these high concentrations appeared to have toxicological effects on fish and benthic communities of Lake Erie and its tributaries (Yang et al. 2005). The U.S. Geological Survey National Water-Quality Assessment (NAWQA) in a survey of 38 lakes from 1970 to 2001 (Van Metre and Mahler 2010) revealed that 42% of the lakes surveyed had increased PAH concentrations in sediments. These lakes were located in urbanized watersheds. The study concluded that rapid urbanization in the United States contributed to this trend. In support of this conclusion, analysis of the sediments in Lake Taihu, one of the five largest freshwater lakes in China, revealed high concentrations of total PAHs (1207 to 4754 ng/g dry weight) from high temperature pyrolytic origins (Qiao et al. 2006). Heavy industrialization and increased populations in the last decade have contributed to the sediment contamination and threatened water supply for nearby areas.

Recreational activities such as power boating, water and jet skiing have caused PAH contamination in bottom sediments of Brown Lake, Australia (Mosisch and Arthington 2001). Ten PAHs, including benzo(a)pyrene, fluoranthene, and chrysene (known indicators of fossil fuel combustion
processes) were found in high concentrations (1070 µg/kg dry weight) exceeding sediment quality guidelines, and posing a risk for aquatic organisms. The study concluded that the level of contamination was a consequence of four decades of unregulated motorized recreational activities. Garages, small-scale mechanics, and increased commercial activity have contributed to loading of used oil to Lake Victoria in the Winam Gulf, Kenya (Kwach and Lalah 2009). In this system, PAHs concentrations in sediments (0.4 to 31.95 µg/g dry weight) were higher in the rainy season and derived from non-point sources of pollution.

### Remediation of PAHs

Traditional remediation techniques of soils and sediments contaminated with PAHs involve excavation of soils or dredging of river sediments, followed by incineration or landfill storage and containment (USEPA 2011). These remediation approaches are usually expensive and sometimes only move the contamination from one place to another. Other technologies that can be applied in situ include physicochemical, chemical and thermal remediation (Wick et al. 2011). These forms of remediation are reviewed below; many of these methods have been evaluated in laboratory scale experiments but not in large-scale field remediation.

**Physicochemical.** In this type of remediation, PAHs are removed from soils or sediments by solvent extraction. Mixtures of solvents include ethanol, 2-propanol, acetone, dichloromethane and cyclohexane (Gan et al. 2009). Surfactant additives have also been included in the “washing” of PAHs from soils. Surfactants (e.g. Tween 40 and Brij 30) enhance PAH solubility in water, making them more available for extraction (Ahn et al. 2008). More recently, compounds formed during bacterial digestion of cellulose used as solubilizing agents to increase water solubility of lipophilic compounds (cyclodextrins) have replaced the use of organic solvents for increasing PAH solubility (Viglianti et al. 2006). PAH removal has also been achieved with vegetable oil as an inexpensive, biodegradable solvent. For instance sunflower and peanut oil have been used in orbital shakers and column percolation experiments for PAH removal (Gong et al. 2007).
Chemical. Chemical oxidation can be used to remove PAHs from sediments and soils. The most well studied and frequently used rely on Fenton’s reagent or ozone. In the Fenton’s reagent technique, hydrogen peroxide dissociates into hydroxyl radicals in the presence of ferrous iron (Flotron et al. 2005):

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \cdot \text{OH} + \text{OH}^- + \text{Fe}^{3+}
\]

Hydroxyl radicals are very unstable and can degrade organic molecules (RH/R) by hydrogen abstraction or by hydroxyl addition:

\[
\text{RH} + \cdot \text{OH} \rightarrow \cdot \text{R} + \text{H}_2\text{O}
\]

\[
\text{R} + \cdot \text{OH} \rightarrow \cdot \text{ROH}
\]

Fenton’s reagent has been used in the removal of fluoranthene, benzoantracene, benzopyrene, perylene, and benzo[ghi]perylene (Ferrarese et al. 2008; Bogan et al. 2003; Jonsson et al. 2007).

Ozone is also used as an oxidant for PAH removal. In the presence of ozone, PAHs degrade through direct reaction or radical reaction (Miller and Olejnik 2004). Ozone has been used in the remediation of spiked soils and contaminated sand and sediments (O’Mahony et al. 2006; Jonsson et al. 2006). Beyond these widely studied methods, other methods of chemical remediation have also been examined. Recently, titanium dioxide (TiO\textsubscript{2}) has been used as photocatalytic agent in remediation of PAHs. This process involves the degradation of organics by a series of oxidizing reactions under light radiation (Gan et al. 2009). TiO\textsubscript{2} has been used in photodegradation chambers and solar reactors to treat spiked soils with pyrene and phenanthrene (Zhang et al. 2008).

Thermal. Removal of PAHs has also been achieved by treating soils using heat to volatilize PAHs. Incineration of PAHs is included in this type of technology. A 99.99% removal of PAHs can be accomplished when soils are incinerated at an average of 1000 °C (Harmon et al. 2001; Acharya and Ives 1993). Beyond this, fluid extraction techniques where pressure and temperature are raised have yielded high removal of PAHs. For instance, Kronholm et al. (2003) showed that removal of chrysene and perylene, both high molecular weight [HMW] PAHs, increased by 41% when water temperature reached 200 °C to 300 °C.
Although the removal of PAHs can be obtained by physical, chemical, and thermal technologies, it is important to recognize that only a combination of technologies can improve removal efficiencies and success of the treatment. These techniques rely on oxidation and extraction capabilities that only work if a) there is strong sorption of PAHs into other molecules, b) diffusion is fast and continue, and c) PAH availability is increased. PAHs in sediments and soils are usually found in mixtures, and present for many years. Aged PAHs (PAH sequestration) are the result of adsorption to soil particles and organic matter (Bogen and Sullivan 2003). With time, PAHs get trapped in soil micropores, which increases persistence and limits biodegradability, leachability, and volatility of PAHs (reviewed in Wick et al. 2011). The mechanism of organic matter-sorption is not clearly understood but is a limiting factor for most physico-chemical and oxidation techniques (reviewed in Wick et al. 2011). In contrast, bioremediation approaches (as discussed below) can greatly overcome these limitations since microorganisms can modify and interact with abiotic conditions in the environment such as nutrients, metals, and pH (Wick et al. 2011).

**Bioremediation of PAHs of soils and sediments**

The application of bioremediation strategies in the treatment of contaminated soils and sediments with PAHs has increased in recent decades because of public concern over threats that contaminants pose and the need to degrade pollutants to concentrations below limits established by regulatory agencies (Alexander 1994). Bioremediation is a process that relies on biodegradation, which is the partial or total transformation, detoxification or removal of pollutants by microorganisms, plants and enzymes.

Bioremediation technologies can be simple, cost-effective, reduce environmental risks, eliminate further contamination, and more important can be applied on site and in situ (Lovley 2003; Pazos et al. 2010; Abbondazi et al. 2005). However, limiting factors, such as length of exposure (Rathbone et al. 1998), existence of microbial populations with degrading capabilities (Lovley 2003), and lack of nutrients for optimal growth (Husain 2008), are not necessarily well understood and can obscure bioremediation potential.

Bioremediation of PAHs becomes a more complex process when other geochemical attributes of the sediments matrix (i.e. concentration and presence of metals in sediments, age of the contaminants,
organic matter and humic content) and environmental parameters (e.g. redox, pH, temperature) are considered. (Nguyen et al. 2005; Cerniglia et al. 1992; Xia et al. 2006). To overcome these limitations, two processes, biostimulation (adding nutrients or bulking agents) and bioaugmentation (addition of microorganisms with degrading capabilities) are usually included in most bioremediation efforts.

Strategies for PAH bioremediation

There are several approaches to bioremediate contaminated soils and sediments including composting, landfarming, and phytoremediation. The success of each strategy depends on microbial degradation, both aerobic and anaerobic, and is discussed in detail in a subsequent section.

Composting. Composting is a technique that mixes organic carbon compounds with contaminated soils or sediments in piles (Iranzo et al. 2004). Often, contaminated soils or sediments are also mixed with supplemental nutrients and aerobic microorganisms with degradative capabilities. Composting piles are monitored for moisture, temperature and aerated periodically. By products and wastes have been used as carbon sources in composting piles. A combination of poultry manure and wood chips (Atagana 2004) and mushroom compost with wheat straw, chicken manure and gypsum (Sasek et al. 2004) yielded high removal (~80%) of 3- and 4-rings PAH when treating contaminated soils. The length of the treatments can vary (100 days to 19 months) dependent upon the carbon source, initial concentration of PAHs, and temperature.

Landfarming. Landfarming is the incorporation of soil or contaminated sediments into surfaces of non-contaminated soil (Sylvia et al. 1999). Clean soils are mixed with contaminated soils or sediments, then tilled to improve aeration and promote microbial degradation by indigenous microbes. As in composting, waste products are usually utilized as bulking agents (e.g. rice hulls) and as nutrient sources (e.g. dried blood as a slow-release nitrogen source). Microbes including Pseudomonas aeruginosa strain 64 (Straube et al. 2003), P. citronellosis 222A and P. aeruginosa isolate 312A (Rodrigo et al. 2005) have been used in landfarming to treat highly contaminated sediments such as found at superfund sites. High removal
of PAHs (87%) can be achieved by landfarming in a very short time (11 months) (Mphekgo and Cloete 2004). Landfarming can be a relatively simple, low cost and low maintenance approach yet limited aeration, mobility of PAHs in soils, toxicity, and transformation of PAHs into more complex byproducts can restrict or prolong the remediation process (Oluseyi et al. 2011).

Phytoremediation The use of plants for degradation of contaminants in a variety of ecosystems is an inexpensive technology widely used (Lin and Mendelssohn 2009). The successful of phytoremediation depends on the amount of contamination and the type of plants selected. In general low molecular weight PAHs can be more effectively removed by phytoremediation. Disadvantages of phytoremediation include low uptake by plants, uneven distribution of roots in soils, soil heterogeneity, and contaminant heterogeneity (Conesa and Shulin 2010).

Microbial degradation of PAHs

In aquatic ecosystems, PAHs tend to sorb onto sediment and form complex organic particulates because of their hydrophobic nature (Lee et al. 1999). Microbial degradation in sediments or soils is believed to be dependent on the mass transfer of PAHs to the water phase; in other words bacteria can degrade PAHs only when they are dissolved in water (Johnsen et al. 2005). PAHs that are in crystalline forms or in a “non aqueous phase liquid-NAPL- state” are therefore, mostly unavailable to PAH-degraders (Johnsen and Karlson 2004). However, there are mechanisms, which bacteria have developed to promote PAH bioavailability. One of them is production of biosurfactants: compounds that reduce the surface tension between two liquids or between a liquid and a solid (e.g. detergents). The most studied PAH-degrading bacteria, *Pseudomonas aeruginosa*, when growing on phenanthrene and naphthalene, produce biosurfactants that increase PAH bioavailability (Déziel et al. 1996). Some Mycobacteria and Sphingomonads (Ho et al. 2000; Bastiaens et al. 2000) produce lipophilic dihydrosphingosine residues or heteropolysaccharides (“sphingans”) by cell membrane modification that allow them to accumulate PAHs in biofilms-like flow channels, after influxes of water bring soluble PAHs from soils or sediments (Johnsen and Karlson 2005).
In addition to biosurfactants, biofilm formation on crystalline PAHs has been observed in studies of *Mycobacterium frederiksbergense* LB501T as a strategy to increase bioavailability (Wick et al. 2001). Hydrophobic cell surfaces have a propensity to attach to PAH crystals (Bastiaens et al. 2000) following Fick’s first law of diffusion. According to Fick’s first law the shorter the distance between PAH crystals and biofilm cells, the higher the aqueous concentration gradient, and the higher the diffusive mass transfer of PAHs to the cells.

In sediments, PAHs occur in dense non-aqueous phase liquids (DNAPLs), and sink into lower layers where they accumulate. The general mechanism of PAH biodegradation in sediments is, simplified, as follows: when PAHs are sorbed onto sediments their concentrations increase due to desorption in the water-sediment interface where bacteria are present; therefore PAHs and bacteria cells have more interactions, resulting in an increased biodegradation rate (Xia et al. 2006). Several studies have shown that rates of biodegradation of PAHs are indeed higher where bacteria are associated with sediments (“solid-phase bacteria”) (Hwang et al. 2003) or associated with organic matter (Hinga et al. 2003). For instance, bacterial degradation rates of phenanthrene and fluoranthene were higher in experiments with sediment compared to those without sediments (Poeton et al. 1999). Hwang and Cutright (2002) showed that although the amount of clay in soils limited “microbial accessibility” to PAHs (by limiting mass transfer of PAHs), the presence of clays increased interaction of soil particles with bacteria resulting in higher biodegradation of pyrene (~65%). However, recently measurements of desorption and sorption rates estimate that since the major source of PAHs in the environment is coal (pyrolytic), desorption of pyrogenic PAHs is very slow and PAHs are more likely to be sorbed into organic matter fraction of sediments (Jonker 2008). Further research is needed to elucidate PAH biodegradation in sediments with high organic matter content and exposed to high loads of contaminants.

1. *Aerobic bioremediation.* Aerobic bioremediation of PAHs is based on oxidation of benzene rings followed by breakdown into less complex metabolites and, sometimes, by complete mineralization into water and carbon dioxide (Bamforth and Singleton 2005; Haritash and Kaushik 2009). Algae, fungi and bacteria can degrade PAHs by mineralization or cometabolism.
1.1 Algal degradation of PAHs. Some algae, including cyanobacteria, green algae, and diatoms, are able to degrade PAHs (Abed and Koster 2005; Hong et al. 2008). Mechanisms of removal of PAHs by algae follow generally steps similar to that of other pollutants and heavy metals (fast removal initially, followed by slow absorption, accumulation and degradation; Hong et al. 2008). Algal degradation appears to be species-specific since size, cell wall composition, and cell morphology influence degradation (Lee 1999). Chan et al. (2006) demonstrated that cells of *Selenastrum capricornutum* were able to degrade a mixture of phenanthrene, fluoranthene and pyrene. *Chlorella vulgaris, Scenedesmus platydiscus, Scenedesmus quadricauda*, and *Selenastrum capricornutum* were also able to remove fluoranthene (47%, 48%, 56% and 81% respectively) and pyrene (41%, 49%, 35% and 74% respectively) after 7 days of incubation (Lei et al. 2007). *Nitzchia sp.* and *Skeletonema costatum* were able to degrade mixtures of fluoranthene and phenanthrene more efficiently than when provided with these same molecules as single compounds (Hong et al. 2008). Degradation by algae, unlike bacterial degradation, often requires consortia. Borde et al. (2003) reported 85% removal of phenanthrene when algae, *Chlorella sorokini-ana*, and bacteria, *Pseudomonas migulae* and *Sphingomonas yanoikuyae*, were combined in a microcosm experiment.

1.2 Fungal degradation of PAHs. Fungal degradation of PAHs is performed by non-ligninolytic fungi and wood degrading (lignolytic) fungi. Non-ligninolytic fungi including *Chrysosporium pannorum* and *Aspergillus niger* (Bamforth and Singleton 2005) cannot mineralize PAHs but can transform PAHs in to more soluble and less toxic metabolites (Pothuluri et al. 1999). On the other hand, wood degrading fungi, also called white rot fungi, accomplish the majority of PAH degradation (Chen et al. 2010; Acevedo et al. 2011), including mineralization to CO$_2$ (Silva et al. 2009; Potin et al. 2004), in the presence of oxygen.

1.3 Bacterial degradation of PAHs. Bacterial biodegradation of PAHs is mainly achieved by two mechanisms: mineralization and cometabolism. Bacterial taxonomic groups involved in mineralization of PAHs include Nocardiforms, Sphingomonads, Bulkholderia, Pseudomonas and Mycobacterium (Johnsen et al. 2002). The latter can mineralize sorbed phenanthrene and other PAHs with low solubility (Bastiaens
et al. 2000; Wells et al. 2005). In addition, some members of the β- and γ – Proteobacteria and Flavobacteria were also able to rapidly mineralize naphthalene and phenanthrene (~70%) within 40 days in aerobic microcosms (Rogers et al. 2007). Cometabolism, the process by which a pollutant is degraded in the presence of an analogous compound has been reported extensively. *Sphingomonas* LB126 can degrade phenanthrene, fluoranthene, and anthracene completely (without accumulation of metabolites) co-metabolically through multiple pathways when growing on glucose and pyruvate (van Herwijnen et al. 2003). *Sphingomonas* *spp* can also cometabolize pyrene, fluorene, anthracene, and benzo[a]pyrene after exposure to phenanthrene (Ho et al. 2000). *Sphingomonas* *paucimobilis* strain EPA 505 cometabolically can degrade high molecular weight PAHs (Ye et al. 1996) while *Rhodococcus* species can degrade fluoranthene in the presence of anthracene (Dean-Ross et al. 2002).

1.3.1. Aerobic bacterial degradation. Aerobic bacterial degradation of PAHs has been widely documented in a variety of environments, including marshes, marine sediments, brackish sediments, and contaminated soils (Lei et al. 2005; Lovley 2000; Meckenstock et al. 2004; Chang et al. 2005; Chang et al. 2008; Langenhoff et al. 1996; Amellal et al. 2001; Ambrosoli et al. 2005; Lovley 2000; Tian et al. 2008; Kim et al. 2008; Abbondanzi et al. 2005; Verrhiest et al. 2002). The extent of bacterial degradation of PAHs when oxygen is present has led to identification of bacteria able to degrade PAHs, more commonly known as PAH-degraders. PAH degrading bacteria are very diverse. Several strains of *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Burkholderia* and *Sphingomonas* have been isolated from PAH-contaminated soils and can use PAHs as a sole source of carbon and energy. Some PAH degrading bacteria have been isolated from extreme environments and are of special importance for bioremediation of, for example, oil-contaminated desert soils (Zeinali et al. 2007a). One example is the hydrocarbon degrading thermophilic bacterium *Nocardia oitidiscaviarum* strain TSH1, which can grow on straight chain aliphatic hydrocarbons and on PAHs (pyrene, phenanthrene, anthracene and naphthalene) as sole sources of carbon and energy (Zeinali et al. 2007b). Other extremophiles, such as *Halomonas aromativorans*, a moderate halophile (Garcia et al. 2004) and the thermophiles *Thermus brockii* (Feitkenhauer et al. 2003) and *Bacillus*
thermoleovorans (Annweiler et al. 2000), can also grow on pyrene and naphthalene as the sole source of carbon and energy.

Most bacterial degradation is accomplished by oxidation of benzene rings by dioxygenases enzymes, although some bacteria can use monooxygenases when degrading pyrene (Juhasz and Naidu 2000). Bacteria and fungi can use monooxygenases, including cytochrome P450s, to reduce dioxygen and incorporate one oxygen into a PAH and a second one into water by oxidizing NADPH (Husain 2008). Dioxygenases (aromatic ring hydroxylating dioxygenases and ring-cleaving dioxygenases) incorporate both oxygen atoms into PAHs. Generally, after the initial reaction of dioxygenases, cis-dihydrodiol compounds are formed and dehydrogenated to produce dihydroxylated intermediates or catechols. Catechols then can be further metabolized into carbon dioxide and water (Bamforth and Singleton 2005). Formation of different intermediates and enzymatic reactions depend on the degradation pathway used. Most PAH degrading bacteria follow either the ortho or meta pathways. Yet, other pathways such the upper and lower pathway, the Evans and Kiyohara pathway, the gentisate, the o-phthalate, and the beta keto adipate pathway (Husain, 2008) are also used by few bacterial species. For instance, the beta keto adipate pathway has been reported only for Mycobacterium vanbaleeni Strain PYIR-1 in pyrene degradation (Kim et al. 2007).

Genes that code for enzymes involved in aerobic bacterial degradation of PAHs belong to Gram negative and some Gram-positive bacteria (Habe and Omori 2003) and are generally on circular plasmids. The gene pcaD in Terrabacter sp. Strain DBF63 involved in fluorene degradation is a linear plasmid (Habe et al. 2003) and an exception. The most well studied gene is nahAc (in Pseudomonas putida NCIB 9816-4) which codes for the α subunit ring-hydroxylating-dioxygenase (RHDα). RHDs genes (composed of small (α) and large (β) subunits) are widespread in bacterial species from the α-Proteobacteria, β-Proteobacteria, and γ-Proteobacteria (Cébron et al. 2008). Other genes involved in the aerobic degradation of PAHs are the nidBA genes (in Mycobacterium sp. Strain PYR-1) (Kim et al. 2008).

1.3.2 Anaerobic bacterial degradation. Although much PAH degradation takes place in the presence of oxygen, sediments are mostly anaerobic. In anaerobic sediments, where oxygen is not available, electron acceptors are depleted in the following sequence: nitrate, manganese, iron, sulfate, and
labile organic compounds based on available free energy potential and thermodynamics (Stumm and Morgan 1981).

Research on anaerobic biodegradation of PAHs in sediments and soils have been performed mainly on marine sediments, examined low molecular weight PAH compounds, and were conducted using pure or enriched cultures in spiked soils or sediments. Few studies have focused on indigenous microbial communities associated with PAHs, and even fewer have assessed microbial community structure or diversity in PAH contaminated environments. In fact, information on anaerobic degradation of PAHs in freshwater systems is scarce and mechanisms of anaerobic degradation remain basically unknown.

Nevertheless, anaerobic biodegradation of PAHs has been demonstrated under denitrifying, sulfate-reducing, and methanogenic conditions (Coates et al. 1997; Rothermich et al. 2002; Hayes et al. 1999; Coates et al. 1996; Lovley et al. 1995). PAHs such as phenanthrene and fluorene (Ambrosoli et al. 2005), and pyrene and naphthalene (Hutchins et al. 1991; Nieman et al. 2001) can be anaerobically degraded when nitrate is used as terminal electron acceptor by native bacterial consortia. Denitrifying bacteria of the genera *Azoarcus* and *Thauera* have been isolated during anaerobic degradation of toluene (Leuthner et al. 1998) while *Clostridium pascui* strain MSA3 can use phenanthrene and pyrene as sole carbon sources for growth (Chang et al. 2008). Pure bacterial cultures (NAP-3-2 and NAP-4 closely related to *Vibrio pelagius*) showed degradation and mineralization of naphthalene anaerobically (~50%) during 57 days of incubation (Rockne et al. 2000). Rockne and Strand (2001) also showed mineralization of radiolabeled phenanthrene (96%) coupled with dissimilatory nitrate reduction using enriched cultures isolated from creosote-contaminated marine sediments.

Under sulfate-reducing conditions, anaerobic degradation of naphthalene in marine sediments has been reported (Hayes and Lovley 2002). Microbes related to the phylotype NaphS2 (from the δ-Proteobacteria), a sulfate reducer, appeared to be involved in anaerobic degradation of naphthalene. Indigenous bacteria from PAH-contaminated marine sediments mineralized fluorene, phenanthrene and fluoranthene when sulfate was used as terminal electron acceptor (Coates et al. 1997). Substantial degradation of phenanthrene was also achieved under sulfate reducing conditions by unidentified indigenous bacteria from aged contaminated river sediments (Lei et al. 2005).
Respiratory reduction of iron and manganese oxides has been also linked to oxidation of PAHs. Specifically, anaerobic degradation of pyrene from creosote-contaminated soils was demonstrated when iron oxyhydroxide and manganese oxide were used as electron acceptors (Nieman et al. 2001). Lovley (1994) showed mineralization of $^{14}$C labeled toluene and benzene, suggesting that iron-reducing bacteria were involved. Ferrous iron enhanced toluene degradation when sulfidogenic enrichment cultures were used to remediate contaminated subsurface soils from an aviation fuel storage facility (Beller and Reinhard 1995). Furthermore, microbial reduction of iron and humic substances, which are abundant in freshwater and marine environments, might be directly involved in anaerobic degradation of organic compounds. For example, phenolic compounds (Cervantes et al. 2000), toluene (Cervantes and Dijksma 2001), vinyl chloride and dichloroethene (Bradley et al. 1998) were degraded anaerobically when humic acids or humic model compounds, namely anthraquinone-2,6-disulfonate (AQDS), were used as terminal electron acceptors. Humic-reducing microorganisms can transfer electrons to AQDS, reducing it to anthrahydroquinone-2,6-disulfonate (AH$_{2}$QDS) (Lovley et al. 1996; Coates et al. 1998). Microbial reduction of iron and humic-reducing bacteria (Clostridium sp EDB2) accelerated bioremediation of cyclic organic explosives in the presence of AQDS (Bhushan et al. 2006). The reduction of humic compounds appears to be widely distributed in anaerobic microbial consortia from freshwater and marine sediments, as well as estuaries, groundwater, sludge and wastewater treatment facilities (Coates et al. 1998; Kappler et al. 2004). Identified humic-reducing microbes are functionally diverse and include iron and sulfate reducers, methanogens, and fermenters (Lovley et al. 1998; Cervantes et al. 2002; Cervantes et al. 2008).

Experiments have suggested that AQDS acts as an extracellular electron shuttle in anaerobic degradation of large molecules. Borch et al. (2005) showed biodegradation of trinitrotoluene (TNT) when AQDS was added to fermentative cultures. Kwon and Finneran (2008) and Bhushan et al. (2006) demonstrated that biodegradation of hexahydro-1,3,5-trinitro-1,3,5,-triazine (RDX) was mediated by AQDS. The role of AQDS as an electron shuttle is not well understood. However, electron shuttling via humic substances is likely a significant process for Fe(III) reduction in subsurface environments and anaerobic sediments with high humic content (Luu et al. 2003). Humic substances are the most abundant form of organic matter in riverine sediments and they can be used as electron acceptors for many bacteria.
and may be an important form of respiration under anaerobic conditions (Cervantes et al. 2001). These studies suggest that perhaps other complex molecules such as PAHs could be anaerobically degraded by reduction of humic compounds and iron or with the addition of AQDS.

**Background information and previous studies conducted in the Mahoning River**

The Mahoning River flows through Northeast Ohio and Western Pennsylvania and indirectly discharges into the Ohio River (Amin and Jacobs 2013). It extends for 208 km and for over 150 years, the Mahoning River supported the economic growth of the valley as an industrial sewer receiving high volumes of organic and inorganic waste from Youngstown’s coke, steel mills, railroads, and steel industries. Today, the riparian zone is habitat for aquatic and woodland organisms and is slowly recovering its biodiversity. However, high concentrations of PAHs, polychlorinated biphenols, and heavy metals, among other contaminants, remain in riverbank sediments along the riparian corridor (USACE 2001). These contaminated sediments are considered the main factor limiting aquatic life over a 51 km stretch and ranked the Mahoning River as the 5th most contaminated river in the U.S. (Eastgate Counsel of Governments 2009). The U.S. Army Corps of Engineers [USACE] (1999) indicated that the riverbanks are “grossly contaminated” and are in dire need of an effective remediation strategy.

Over the last 10 years, hydrology and microbiological data from riverbank sediments have been collected by ongoing graduate and undergraduate students at Youngstown State University. These findings are briefly summarized below.

**Hydrology.** Studies conducted by the Department of Geological and Environmental Sciences at Youngstown State University focused on geologic and hydrologic characterization of the Mahoning riverbanks and hydraulic conductivity along the Mahoning River between Lowellville and Leavittsburgh (Amin and Jacobs 2013). These studies revealed that distribution of contamination, including PAHs, is very heterogeneous (vertically and horizontally) and not systematic (ranging from high at Lowellville to low at Warren) as suggested by the USACE (1999).

**Geochemistry.** Several studies have focused on measuring particle size distribution, toxicity characteristics, metals, and PAH concentrations in riverbank sediments at Lowellville (Gacura et al. 2008,
Particle size distribution according to those studies revealed that sediments are mainly composed of silt. This characteristic along with the “oily” nature of the sediment represented a barrier to performance of standard soil methods.

Microbiological data- Pratt et al. (2012) characterized the microbial community of two sites (Lowellville-high PAH contamination and Levittsburgh-low PAH contamination) along the Mahoning River using Fatty Acid Methyl Ester (FAME) analysis, revealing that 39% of the microbial community in Lowellville sediments corresponded to SRB and other anaerobic bacteria. Mosher et al. (2003) indicated that microbial activity, measured by dehydrogenase enzyme activity via the reduction of the 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride INT into the red colored formazan product, INTF in Mahoning River sediments was much higher (several orders or magnitude) than other contaminated and non-contaminated sites. Mosher et al. (2006) indicated that microbial activity in the Mahoning River sediments correlated with temperature and water holding capacity and higher microbial activity correlated with higher PAH concentrations. Johnston et al. (2009) also reported very high microbial activity measurements when riverbank sediments were exposed to high temperatures. They also reported that microbial activity correlated with PAH degradation indicating that indigenous microbes were most likely involved in aerobic PAH degradation.

References


Acharya L. 2008. Chemical and physical characteristics of Mahoning River sediment before and after fungal bioremediation. Master of Science, Chemistry Program, Chemistry Department at Youngstown State University.


Ferrarese E, Andreottola G, Oprea I. 2008. Remediation of PAH- contaminated sediments by chemical
Hong Y, Dong-Xing Y, Lin Q, Yang T. 2008. Accumulation and biodegradation of phenanthrene and
fluoranthene by the algae enriched from a mangrove aquatic ecosystem. Mar Pollut Bull 56, 1400–1405.


Menzie C, Potocki B, Santodonato J. 1992. Exposure to carcinogenic PAHs in the environment. Environ...


Pazos M, Rosales E, Gomez J, Sanroman M. 2010. Decontamination of soils containing PAHs by
electroremediation: Rev J Hazard Mat 177, 1–11.


Table 1 Physical and chemical characteristics of polycyclic aromatic hydrocarbons detected in this study.

Data compiled from the Agency for Toxic Substances and Disease Registry (ATSDR), Occupational Safety and Health Administration (OSHA) and Integrated Risk Information System (IRIS)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mol. weight</th>
<th>Benzene rings</th>
<th>Physical state</th>
<th>Color</th>
<th>Solubility</th>
<th>Organic solvents</th>
<th>$K_{ow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>128.19</td>
<td>2</td>
<td>Solid</td>
<td>White</td>
<td>31.69</td>
<td>Benzene, acetone</td>
<td>3.37</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>152.19</td>
<td>2</td>
<td>Solid</td>
<td>Yellow –colorless crystalline</td>
<td>3.93</td>
<td>Chloroform, ether</td>
<td>4.07</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>154.21</td>
<td>2</td>
<td>Solid</td>
<td>Colorless</td>
<td>3.93</td>
<td>Chloroform, ether</td>
<td>3.98</td>
</tr>
<tr>
<td>Fluorene</td>
<td>166.22</td>
<td>2</td>
<td>Solid</td>
<td>Colorless</td>
<td>1.7</td>
<td>Diethyl ether</td>
<td>4.18</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>178.22</td>
<td>3</td>
<td>Solid</td>
<td>Colorless crystals</td>
<td>1.4</td>
<td>Various</td>
<td>4.45</td>
</tr>
<tr>
<td>Anthracene</td>
<td>178.24</td>
<td>3</td>
<td>Solid</td>
<td>Colorless to yellow crystals</td>
<td>1.29</td>
<td>Chloroform, benzene</td>
<td>4.45</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>202.25</td>
<td>3</td>
<td>Solid</td>
<td>Colorless to pale yellow crystals</td>
<td>0.2</td>
<td>Benzene, ethyl ether</td>
<td>5.16</td>
</tr>
<tr>
<td>Pyrene</td>
<td>202.25</td>
<td>4</td>
<td>Solid</td>
<td>Colorless</td>
<td>0.135</td>
<td>Benzene, ethyl ether</td>
<td>4.88</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>228.28</td>
<td>4</td>
<td>Solid</td>
<td>Colorless</td>
<td>9.4X10$^{-3}$</td>
<td>Various</td>
<td>5.79</td>
</tr>
<tr>
<td>Chrysene</td>
<td>228.28</td>
<td>4</td>
<td>Solid</td>
<td>Colorless to white</td>
<td>1.89X10$^{-7}$</td>
<td>Ethyl ether, acetone</td>
<td>5.73</td>
</tr>
<tr>
<td>Benzo(b,k)fluoranthene</td>
<td>252.3</td>
<td>4</td>
<td>Solid</td>
<td>Yellow</td>
<td>0.0012</td>
<td>Benzene, acetone</td>
<td>6.04</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>252.3</td>
<td>5</td>
<td>Solid</td>
<td>Pale yellow</td>
<td>1.62X10$^{-4}$</td>
<td>Benzene, ether</td>
<td>6.06</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>278.35</td>
<td>5</td>
<td>Solid</td>
<td>Colorless</td>
<td>2.49X10$^{-3}$</td>
<td>Benzene, acetone</td>
<td>6.75</td>
</tr>
<tr>
<td>Benzo(ghi)perylene</td>
<td>276.33</td>
<td>6</td>
<td>Solid</td>
<td>Pale yellow-green</td>
<td>0.00026</td>
<td>Benzene, dichloromethane</td>
<td>6.63</td>
</tr>
</tbody>
</table>
Table 2 Health hazard assessment of PAHs measure in this study

<table>
<thead>
<tr>
<th>PAH</th>
<th>Health hazard assessment</th>
<th>Cancer assessment (IRIS)</th>
<th>Exposure standard: health hazard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>Destroy red blood cells: hemolytic anemia. Exposure to large amounts cause nausea, vomiting, diarrhea, blood in the urine, and a yellow color to the skin.</td>
<td>Possible human carcinogen</td>
<td>&gt;250 ppm</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>Respiratory toxicant</td>
<td>Not classifiable as human carcinogen</td>
<td>n.a.</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>Liver toxicant</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Fluorene</td>
<td>Decreased red blood cells, packed cell volume and hemoglobin</td>
<td>Not classifiable as human carcinogen</td>
<td>1.25 x 10^7 mg/kg-day</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>Respiratory toxicant</td>
<td>Not classifiable as human carcinogen</td>
<td>n.a.</td>
</tr>
<tr>
<td>Anthracene</td>
<td>Endocrine toxicant</td>
<td>Not classifiable as human carcinogen</td>
<td>n.a.</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>Nephropathy, increased liver weights, hematological alterations, and clinical effects</td>
<td>Not classifiable as human carcinogen</td>
<td>125 mg/kg/day</td>
</tr>
<tr>
<td>Pyrene</td>
<td>Kidney effects (renal tubular pathology, decreased kidney weights)</td>
<td>Not classifiable as human carcinogen</td>
<td>125 mg/kg/day</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td></td>
<td>Carcinogen</td>
<td>n.a.</td>
</tr>
<tr>
<td>Chrysene</td>
<td></td>
<td>Carcinogen</td>
<td>n.a.</td>
</tr>
<tr>
<td>Benzo(b,k)fluoranthene</td>
<td></td>
<td>Carcinogen</td>
<td>n.a.</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td></td>
<td>Carcinogen</td>
<td>n.a.</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td></td>
<td>Carcinogen</td>
<td>4.5 to 11.7 per (mg/kg)/day</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td></td>
<td>Carcinogen</td>
<td>n.a.</td>
</tr>
<tr>
<td>Indeno(1,2,3-cd)pyrene</td>
<td></td>
<td>Carcinogen</td>
<td>n.a.</td>
</tr>
<tr>
<td>Benzo(ghi)perylene</td>
<td></td>
<td>Mutagen/Carcinogen</td>
<td>n.a.</td>
</tr>
</tbody>
</table>
Table 3. Average and standard deviation in brackets (n=3) of particle size distribution expressed in percentage measured in surface sediments (5 cm below the surface of riverbanks) and subsurface sediments (4 m below the surface of riverbanks). Data compiled from Pratt et al. 2012 and Acharya 2008.

<table>
<thead>
<tr>
<th>Location</th>
<th>% Sand (2.0-0.05 mm)</th>
<th>% Silt (0.05-0.002 mm)</th>
<th>% Clay (&lt;0.002 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leavittsburg*</td>
<td>25 (8)</td>
<td>70 (10)</td>
<td>5 (3)</td>
</tr>
<tr>
<td>Leavittsburg**</td>
<td>30 (13)</td>
<td>66 (16)</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Lowellville*</td>
<td>27 (3)</td>
<td>70 (4)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>Lowellville**</td>
<td>63 (9)</td>
<td>32 (8)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>Lowellville (composite sample)</td>
<td>39 (0)</td>
<td>56 (2)</td>
<td>5 (1)</td>
</tr>
<tr>
<td>Warren</td>
<td>18 (5)</td>
<td>75 (8)</td>
<td>7 (3)</td>
</tr>
<tr>
<td>Youngstown</td>
<td>18 (8)</td>
<td>76 (13)</td>
<td>6 (6)</td>
</tr>
</tbody>
</table>
Fig. 1 Chemical structure of some PAHs commonly found in contaminated sediments.
CHAPTER II: SOURCES CHARACTERIZATION AND ECOLOGICAL RISK ASSESSMENT OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) IN LONG-TERM CONTAMINATED RIVERBANK SEDIMENTS

Abstract

Polycyclic aromatic hydrocarbons are recalcitrant pollutants common in aquatic ecosystems. Although there is a vast literature on PAH contamination, there is a scarcity of information from long-term contaminated ecosystems. To our knowledge this study is the first detailed characterization of PAHs and their sources from riverbank sediments with a historic legacy of pollution. We quantified 12 PAHs and identified sources using distribution patterns, PAH ratios, and principal component analysis (PCA). High PAH concentrations (94,000 to 560,000 µg/kg) were found in riverbank sediments. Prevalence of pyrolytic sources of PAHs was indicated by the large relative contribution of four ring compounds while high levels of low molecular weight PAHs also suggest input from petrogenic sources. High concentrations of PAHs ranked this sediment as one of the most polluted aquatic ecosystems in the world. Sediment guidelines, carcinogenic PAHs and risk quotients were used to assess the ecological risk of PAHs. This assessment overwhelmingly indicated that the riverbank sediments of the Mahoning posed a very high ecological risk to aquatic organisms, even at what it was previously considered an unpolluted site. These results indicate that there is a great need for implementation of remediation strategy of the riverbanks.

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2 This chapter has been submitted to the Environmental Pollution Journal and is under peer review. The list of authors is as follows: Gloria P Johnston, David Lineman, Carl G Johnston and Laura Leff. Dr. Johnston (Youngstown State University-Biology Dept.) contributed laboratory space and equipment. Dr. Lineman (Hickory High School, Hermitage, PA) provided a GC-MS for analyses and quantification of PAHs. Dr. Leff (Kent State University) provided guidance throughout the analysis and editorial suggestions. I collected sediment samples, extracted PAHs, processed and analyzed data as well as wrote the chapter.
Introduction

Polycyclic aromatic hydrocarbons (PAHs) are of continued interest because of their prevailing occurrence in various aquatic ecosystems, and their toxic, mutagenic and carcinogenic effects (Grimalt et al. 2004; Guo et al. 2007; Cao et al. 2010). The United States Environmental Protection Agency (U.S. EPA 2005), under the National Waste Minimization Program, includes 20 PAHs in its Toxic Release Inventory, and 7 PAHs under its Priority Chemical List.

PAHs enter aquatic ecosystems via atmospheric deposition, and can be transported as gases or aerosols over great distances (Wang et al. 2012). PAH contamination is widespread in industrialized settings, such as harbors, steel mills and shipping ports (DeBruyn et al. 2009). Numerous studies have measured composition of PAHs in river sediments around the world (e.g., Xu et al. 2007; Luo et al. 2008; Badawy and Emababy 2010). However, in most studies, the rivers were either sources of drinking water (Ma et al. 2008 and reference therein) or were currently being contaminated (e.g. Charriau et al. 2009; Witt and Gründler 2005). To gain insight into the nature and source of contamination, some of these studies also examined the sources of PAHs (based on composition of the PAH pool) to predict whether PAHs were of petrogenic or pyrogenic origin (Guo et al. 2009). Pyrolytic PAHs including compounds with four, five and six rings are the result of combustion processes. Petrogenic PAHs (two and three aromatic rings) are derived from petroleum and its products (Chen et al. 2012).

The chemical nature of PAHs make them permanent in aquatic ecosystems; due to their hydrophobicity, PAHs can be bound to organic matter and soil particles (Chen et al. 2013) and be deposited in the benthos. PAHs, usually present as complex mixtures, exhibit a variety of physico-chemical properties that posses toxic effects on aquatic organisms (Neff et al. 2005). Bacterial degradation in anaerobic sediments is limited (Husain 2008) and when sorbed PAHs are released into solution the effect on biota is increased due to food network mechanisms (e.g. bioaccumulation). The toxicity of PAHs in sediments is related directly to their composition and concentrations. The higher number of aromatic rings, the more toxicity they possess. Because PAHs mostly affect aquatic biological communities, several tools have been implemented by government agencies (i.e. U.S.EPA) to estimate ecological risk assessment including
hazard quotients, sediment guidelines for toxicity and risk quotients (Neff et al. 2005; Chen and Chen 2011).

For almost two decades the USACE, environmental agencies, and government councils have proposed different strategies for sediment remediation of PAH, including capping riverbank sediments, which would alter natural stream dynamics. Instead, bioremediation could be a promising alternative for treating PAH-contaminated sediments. This option would cause minimal disruption to the riverbanks and river sediments and perhaps reduce high costs associated with traditional remediation techniques. However, to implement any type of bioremediation strategy it is crucial to document the distribution and types of PAHs. It is also important to determine what sites are in dire need of remediation because of their ecological risk.

In this study, we focused on historically contaminated riverbank sediments of the Mahoning River (Northeast Ohio, USA) that have not received major sources of PAHs since the late 1970s when the regional steel industry collapsed (U.S. Army Corps of Engineers [USACE] 2001). The Ohio Department of Health has issued a fish consumption advisory (1988-present; J. Kwolek, personal communication) because of high concentrations of polychlorinated biphenyls (PCBs) and mercury in fish tissues. Likewise, the Ohio Environmental Protection Agency has posted a fish consumption and a wade/swim ban because of high levels of PCBs and PAHs in the sediments (Ohio EPA 2013). The aims of this research were to characterize the concentrations of individual PAHs, identify possible sources of PAHs (based on composition of the PAH pool), and evaluate the ecosystem risk and toxicological and biological impacts in the highly contaminated riverbank sediments of the Mahoning using sediment guidelines, risk quotients based on carcinogenicity of certain PAHs.

Materials and Methods

Study site

The Mahoning River (located in Northeastern Ohio, USA) is 174 km long with a watershed of 2,965 km² (Ohio EPA 2011). The Mahoning forms the Shenango River, which forms 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 (USACE 2001).

Between 1900 and 1975, the lower Mahoning River intensively supported one of the highest productions of steel worldwide (reviewed by the USACE 1999). Industries used the river as an industrial sewer and source of coolant. As a result, high concentrations of heavy metals, petroleum hydrocarbons, cyanide, PAHs, PCBs and phenols remain in the sediments (reviewed by Amin and Jacobs 2013). The USACE (1999) reported PAH concentrations in river sediments from the river channel and described as as “oil-soaked banks” and it was inferred that the riverbank sediments contained higher concentrations of PAHs than the river channel, however, this report did not measure PAH data from the bank sediments.

Sampling

Sediment cores were collected during July and August 2012 from 3 sites along the Mahoning River (Fig. 1). A selection of characteristics for the sampling sites is listed in Table 1. Two sites (Lowellville and Girard) were chosen based on previous reports of contamination levels with PAHs (USACE 2001), and accessibility. A third site (upstream of the contaminated stretch of the Mahoning River at Newton Falls), was selected as a control site, where theoretically there was no PAH contamination. Sediment cores were collected using a manual auger device with stainless steel liners (diameter = 5 cm, length = 15 cm) from the surface of the riverbank. At each site, three sediment cores (30 to 50 m apart from each other) were collected at three different depths depending on accessibility for sampling and depth of the bedrock (Table 1). Immediately after sampling, cores were capped with stainless steel liners and plastic caps and were placed in Ziploc bags to minimize contact with oxygen. In the laboratory, cores were placed in a glove box under nitrogen and were thoroughly homogenized for analyses. From each depth duplicate measurements of PAHs and sediment parameters were determined.

Chemical standards

Surrogate standards, 2-fluorobiphenyl, nitrobenzene-d5, and terephenyl-d14, were purchased as a certified high concentration mix (1 mg/mL) from Restek (Bellefonte, PA). Internal standards were
deuterated PAHs, also from Restek, Corp. as a certified high concentration mixture (2mg/mL). All solvents used for sample extraction and analyses (dichloromethane, acetone, methanol) were HPLC optima grade (Sigma, USA).

PAH extraction and analyses

PAHs were extracted by a modification of a sonication-assisted extraction method described in USEPA Method 3550C. This was chosen because is fast, relatively inexpensive, and uses low solvent and sediment volumes (Crespo et al. 2006). Approximately 4g of thawed wet and precisely weighed sediment samples were placed in VOA EPA type vials (Sigma-Aldrich, Saint Louis, MO). To remove water, anhydrous granular sodium sulfate was added to the vials and stirred with a spatula until sediment reached a “sand-like” consistency. 10 µl of surrogate standard mix was added directly to the sample, followed by 20 ml of dichloromethane. Samples were extracted in an ultrasonic bath (Aquasonic 750 HT, WVR) for 15 min at 18 °C. Extracts were filtered through a Whatman filter No 1 (Sigma-Aldrich, Saint Louis, MO), collected and measured in clean VOA vials. This step was repeated three times. Samples were analyzed according to the guidelines set forth in USEPA Method 8270D, using internal standard quantitation. We used a Gas Chromatograph Hewlett-Packard 5890, a Mass Spectrometry Hewlett-Packard 5970B, and a Restek XTI-5, 30m x 0.25mm ID x 0.25 µm df column. Sample extracts were analyzed in positive EI full scan mode, using helium as the carrier gas. 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, and then ramped at 10 °C/min to a final temperature of 310 °C, and held for 10 min. A 1µl injection of each sample extract was made in splitless mode, using a CTC-103 autosampler (Zwingen, Switzerland). The precision and accuracy of the analytical procedure were determined by calculating the percent recovery of surrogates (80 - 90%) as well as the relative percent standard deviations (%RSD) which ranged from 5 to 20%. PAHs were quantified based on internal standards, were not corrected for surrogate standard recoveries and are expressed on a dry-weight basis.
Other analysis

Organic matter, volatile solids, fixed solids and moisture content were determined by standard methods for soil analysis (Carter 2000).

Data analyses

Previous studies have indicated that organic carbon plays a role in sorption in sediment particles (Johnston and Johnston 2012); therefore, PAH concentrations were normalized based on organic matter content before performing statistical analyses. Pearson correlations were determined between individual PAHs, sediment characteristics and total PAHs. Diagnostic ratios and principal component analysis (PCA) were used to predict the source contribution to the PAH pool. One factor MANOVA was used to determine if there were significant differences among sites and depths in organic matter, fixed solids, and individual PAH concentrations. PAH concentrations in sediments were compared against effects-based guidelines (effect range low [ERL] and effect range median [ERM]) values set by the US National Oceanic and Atmospheric Administration (NOAA 1999) to estimate potential biological effects. In addition, we used risk quotients (established by Kalf et al. 1997), ecosystem risk based on carcinogenic PAH (U.S. EPA, 2013), and EPA PAH-benchmarks for sediments (U.S. EPA 2010) in order to assess potential ecosystem risk of PAHs to aquatic organisms. Statistical analyses were performed using SPSS 12.0 software for Windows.

Results and discussion

Sediment characteristics

Sediment characteristics are summarized in Table 1. Lowellville (LW) and Girard (GR) sediments were oily, black, viscous, and with a strong odor that persisted in the laboratory, while Newton Falls (NF) sediments did not have an oil coating or odor. Organic matter content (OM) was high for LW (6.8 to 13.2%) and GR sediments (7.2 to 10.8%) and much lower for NF sediments (1.1 to 3.2%). The high organic content in sediments from LW and GR sites, in addition to the apparent grease content (not measured) indicated that other organic compounds (besides PAHs) accumulated and altered sediment
properties. In contrast, NF sediments had much lower organic matter content and visually appeared to have very different sediment particle size distribution (not quantified). OM in LW and GR sediments were consistent with values reported for similar organic-compounds-contaminated environments. Baniulyte et al. 2009 reported OM values ranging from 5.6 to 7.7% from dredged river sediments at a former US steel site in Illinois, US. Interestingly, OM in NF sediments (upstream of the industrial contaminated section of the Mahoning) was comparable to reported values from PAH-contaminated sites. For instance, Guo et al. (2007) found that organic content in PAH-contaminated sediments in the Daliao River, China, ranged from 0.1 to 2.6%; Guo et al. (2009) also reported comparable values (0.19 – 5.01%) for a different stretch of the same river. In the current study, the influence of organic matter in PAH composition was apparent from the significant Pearson correlation between total PAHs and OM (r=0.503, p=0.007), suggesting that OM played an important role in the distribution of PAHs in these sediments.

Volatile solids are usually reported after combustion of organic matter typically from biological sources. In this study, volatile solids are derived from substantial amounts of hydrocarbons and oils present in the sediment; in turn fixed solids are composed of inorganic materials such as sand, gravel, and salt. Fixed solids in LW, GR (86.8 to 93.2%) were statistically significantly lower (p<0.05) than for NF sediments (97.3 to 98.9%) indicating differences in sediment quality and properties.

Concentrations and distribution of PAH

Several specific PAHs were detected and quantified, including naphthalene (Naph), acenaphthylene (Aceph), acenaphthene (Ace), fluorene (Fl), phenanthrene (Phen), anthracene (Ant), fluoranthene (Flu), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chr), benzo[b,k]fluoranthene (BkF), and benzo[a]pyrene (BaP). Average PAH concentrations and RSD% are given in Table 2. Mean concentration of total PAHs in LW sediments ranged from 94,300 to 560,000 µg/kg dry weight, 46,000 to 120,000 µg/kg dry weight in GR sediments and 18,000 to 74,000 µg/kg dry weight in NF sediments. PAH concentrations were heterogeneous among cores and depths, indicating spatial variation from continuous to discontinuous layers of contamination (Amin and Jacobs 2013). However, higher concentrations were found in the deepest sediments at all sites and in all cores (Fig. 2). Results of the Pearson correlation
analyses showed a significant correlation between $\sum$PAHs and depth ($r=0.821$, $p=0.001$), indicating that deeper contamination was due to PAH-contaminated sediment deposition with greater contamination in the past.

PAH concentrations found in LW and GR sediments greatly exceeded the highest category for pollution levels (> 5000 µg/kg) according to the classification suggested by Baumard et al. (1998). However, when individual concentrations of PAHs were considered, some GR and NF cores were classified only as moderate and high level pollution (100 - 1000 µg/kg and 1000-5000 µg/kg respectively). To our surprise, PAH concentrations in NF sediments also surpassed the highest category for pollution levels (> 5000 µg/kg). NF is located upstream from the contaminated stretch of the river, and was designated as an uncontaminated baseline site (USACE 1999). Previous studies also indicated that only 51 km of the Mahoning River exhibited organic pollutant contamination (USACE 2001), and it was inferred that there was no direct input of PAHs (or other organic pollutants) upstream. Other pathways of contamination (e.g. deposition) might have contributed to high levels of specific PAHs in NF sediments. More studies need to be implemented to evaluate if/how biota have responded to pollution at additional sites along the river.

Although it is potentially problematic to directly compare PAH concentrations among sites around the world, our findings ranked Mahoning Riverbank sediments as the second most contaminated freshwater ecosystem in the world (Table 3). Our ranking was performed only with 12 PAH identified in this study, while most studies have reported concentrations for 16 PAHs, and are based on reported literature from the last nine years. Nevertheless, PAHs in LW and GR sediments were four time higher than those found in riverbank sediments in Germany (Pies et al. 2008) and five times higher than in river sediments in France-Belgium (Charriau et al. 2009), but much higher (200X) than many river sediments in China, where PAHs ranged from 133 to 10,757 µg/kg (Li et al. 2010; Sun et al. 2012; Zeng et al. 2013). Concentrations of total PAHs in NF sediments also exceeded reported values in similar environments (Sanctorum et al. 2011). Overall, in our study total PAH concentrations were much higher (2 to 700X) compared to PAH concentrations reported in several studies including harbors (Chen et al. 2013), estuaries (Luo et al. 2006) and seas (Mohammadi et al. 2010; Bihari et al. 2006) but approximately 3X lower than the highest total
PAHs reported by Shi et al. (2005) in the North Pollution Canal in China. This might be due to the relatively intensive industry localized in certain sites (such as Lowellville and Girard) that lasted a long period of time (~100 years). In addition to hydrological features of the Mahoning River including its meanders and laminar flow, the presence of dams did have an effect in generating pockets of contamination.

Composition of PAHs

To study PAH composition, 12 PAHs were classified according to the number of aromatic rings (AR) as follows: naphthalene (2-AR), acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene (3-AR), fluoranthene, pyrene, benzo[a]anthracene, and chrysene (4-AR), benzo[b,k]fluoranthene and benzo[a]pyrene (5-AR). In general, 4-AR PAHs were the most abundant across sampling sites and depths (Fig. 2) at each site. Unexpectedly, NF sediments had similar high percentage of 4-AR PAHs across sites (64%), while GR sediments had the highest of all (69%) and LW sediments the lowest (58%). One factor MANOVA of PAHs, OM, FXS, and depth (using site as a fixed factor) revealed that these parameters were all significant statistically different across sites (Wilks’ Lambda = 0.003, $F$ = 12.93, $p$ = 0.000).

Individually, the order of abundance of the most common PAHs in LW were fluoranthene > pyrene > phenanthrene > benzo[a]anthracene > and chrysene, while in GR and NF sediments were pyrene > fluoranthene > phenanthrene > benzo[b,k]fluoranthene > and chrysene (Fig. 3A). However, naphthalene and fluorene concentrations in LW sediments were also high. On average (Fig. 3B), high molecular weight (HMW) PAHs (4 and 5-AR) accounted for 70, 80 and 80% of the total PAHs in LW, GR and NF sediments respectively, while low molecular weight (LMW) PAHs (2 and 3-AR) contributed 30, 20 and 20% in LW, GR and NF respectively. These results are similar to those from other contaminated aquatic ecosystems. Cao et al. (2010) found that HMW PAHs were the most abundant in river sediments and bank soils in the Luan River basin, China, where 4-6 ring PAHs accounted for 57% of the total PAHs. Similarly, Chen et al. (2004) reported that 4-ring PAHs were dominant in freshwater sediments in China. This pattern is widespread because LMW PAHs are found in lower amounts in sediments and soils, have lower octanol-
water partition coefficient, have higher water solubility, and tend to volatilize more easily than HMW PAHs (Li et al. 2010). In contrast, HMW PAHs more easily undergo sorption in sediment, tend to bind to organic matter and soil minerals (Peng et al. 2008; Haritash and Kaushik 2009; Bathi et al. 2012) and are resistant to degradation (Johnston and Johnston 2012).

Estimates of sources PAHs

Several factors influence distribution of PAHs in an ecosystem. However, there are two main processes that define their origins: PAHs are mainly formed by long-term low-temperature petroleum conditions (petrogenic) and by short term high temperature combustion processes (pyrolytic) (Witt and Gründler 2005). Extremely high temperatures (~2000°C), as found in the burning of coal, leads to 4-6 ring PAH formation (HMW) (Ribeiro et al. 2012). In contrast, lower temperatures (< 800°C) yield alkylated and LMW PAHs (Muri and Wakeham 2009). Thus, low molecular weight PAHs are commonly associated with petrogenic origins, whereas HMW PAHs are associated with combustion processes (Sanctorum et al. 2011). Estimators to identify sources of PAHS have been widely used (Wang et al. 2010; Zhang et al. 2005) and are shown in Table 4. Ratios of some isomers such as Ant/(Ant+Phen) to Flu/(Flu+Pyr) and Phen/Ant to Fl/Pyr have been commonly applied to predict sources of PAHs (e.g. Yunker et al. 2002). Flu/(Flu+Pyr) is also used to discriminate between types of combustion and petrogenic origins of PAHs (Luo et al. 2008 and references therein). A Flu/(Flu+Pyr) ratio of 0.4 is considered the transition point between petroleum combustion and biomass/coal combustion. A ratio less than 0.4 corresponds to petroleum pollutant and higher than 0.5 corresponds to biomass (e.g. grass, wood) or coal combustion, while a ratio between 0.4 and 0.5 is attributed to combustion of liquid fossil fuels such as crude oil, gasoline, kerosene (Shen et al. 2007 and references therein).

The Flu/Pyr ratio in LW sediments indicated that our samples came from pyrolytic sources (ranging from 1.15 to 1.66) while the same diagnostic ratios in GR and NF sediments (ranging from 0.75 to 0.89) indicated petrogenic sources (Fig. 4A). This finding is further explained by the high amounts of phenanthrene in the sediments, which is indicative of petrogenic origins (Ribeiro et al. 2012). Fig. 4B shows the distribution of Ant/(Ant+Phen) and Flu/(Flu+Pyr) ratios in sediments from the three study sites.
Ant/(Ant+Phen) ratios at LW were higher than 0.1 (ranging from 0.15 to 0.30) indicating pyrolytic origins while most GR samples had ratios lower than 0.1 (ranging from 0.00 to 0.11) associated with petrogenic sources. Mixed ratios (ranging from 0.06 to 0.16) suggested both pyrolytic and petrogenic sources in NF sediments. Elevated concentrations of fluoranthene, pyrene, benzo[a]anthracene, chrysene and benzo[k]fluoranthene, associated with combustion PAHs (Hwang and Foster 2006) provided further evidence of higher inputs of combustion derived PAHs in LW and GR sites. More specifically, ratios of Flu/(Flu+Pyr) in LW sediments (located near to what it was a coke plant) ranged from from 0.54 to 0.62 indicated that most likely coal combustion processes contributed to PAHs while ratios in GR and NF sediments (ranging from 0.41 to 0.47) suggested that petroleum combustion was a possible source of PAHs. Most sediments samples from LW had a Chr/BaA ratio similar to ratios described for pyrolytic sources, while GR and NF sediment samples had ratios higher than 1 (data not shown) indicating PAH derived from petroleum activities, such as fuel spills and emission of unburned fuels, contributed to the PAH contamination in the river.

Studies of distribution and sources of PAHs have used relationships between principal components and PAHs to indirectly relate PCA factor loadings to source composition (Larsen and Baker 2003; Boonyatumanond et al. 2006). High loadings of Pyr, Flu, Fl, Chr, and BaP are usually associated with pyrogenic sources of PAHs (Fang et al. 2007) while heavy loadings of Naph, Phen, Ant and Ace indicate petrogenic sources (De Luca et al. 2005). In the present study, the majority of the variance (90%) was explained by two principal components with the first principal component (PC1) explaining 65% and the second (PC2) 35% (data not shown). Flu, Pyr, BaA, Chr, BkF and BaP (all associated with pyrolytic origins) were strongly associated positively with PC1. These same PAHs (Flu, Pyr, BaA, Chr, BkF and BaP) had a strong coefficient correlation with total PAHs ($r > 0.85$, $p<0.00$), indicating that PC1 can be taken as a quantitative correlation component of the PAHs (Xu et al. 2007). In contrast PC2 was influenced by Naph, Ace, Aceph and Fl (all indicators of petrogenic sources).

Both diagnostic ratios and PCA confirmed that the PAH contamination in bank sediments along the Mahoning River resulted from a mixture of pyrolytic and petrogenic sources. These results are in agreement with other studies from contaminated sediments. Xu et al. (2007) reported a mixed source of
PAHs in a heavy industrial setting. They attributed the sources to discharge from factories along the river, atmospheric deposition, and leakage of crude oil into the river sediments. Pies et al. (2008) analyzed PAHs in riverbank soils near a former coal plant indicating a mixed of petrogenic and pyrolytic sources, derived from runoff and shipping activities. This situation is very similar to LW, which had coke plants operating at the end of the 1800s (2013, L. Shroeder, personal communication). In the case of the Mahoning River, the mixed source of PAHs and their distribution are in agreement with the history of contamination in this system as well as in other riverine systems. This long-term pollution caused by discharge of oils, greases and other industrial wastewaters over decades is reflected in high concentrations of PAHs with mixed origins left in the sediments. Interestingly, NF sediments (upstream and far from major sources of pollution) showed similar distribution and sources of PAHs, suggesting that atmospheric deposition and other forms of PAH transport might have contributed to PAH accumulation in these sediments. More recently, there has been observation of PAH deposition in regions remote from direct sources of emission (Scott et al. 2012). Regional chemical transport models were used to investigate PAH distribution in two remote sites in Oregon and Washington State (Genualdi et al. 2009). During six months, both remote sites were influenced by PAH and pesticide emissions from forest fires in Siberia and regional fires in Oregon and Washington State suggesting a trans-Pacific and regional atmospheric transport of emissions. Another possibility is that PAHs might travel and reach more distant regions (from the source of pollution) by not well-understood mechanisms such as gas-particle partitioning and oxidation (Friedman and Selin 2012).

Ecosystem risk assessment and sediment biological effects based on PAHs

Sediment guidelines have been used to evaluate effects of contamination on aquatic biota (Zeng et al. 2008). The US National Oceanic and Atmospheric Administration sediment guidelines uses two values to assess biological effects: the ERL is a concentration at which toxicity is found about 10% of the time; and ERM a concentration at which toxicity is found 50% of the time (Long et al. 1995). In this study, total PAHs in LW and GR sediments exceeded by far the standards ERL value (4000 µg/kg) and ERM value (44,792 µg/kg) indicating that negative effects on aquatic organisms will most likely occur. Some samples from NF sediments did not exceed the ERM value but did exceed by far the ERL value, suggesting that
biological effects would occur occasionally and frequently. Individual concentrations of most PAHs except Acenaphthylene from LW sediments exceeded both ERL and ERM. Seven out of 12 PAHs measured in GR sediments were below the ERL value while 5 PAH concentrations were higher than both ERL and ERM (Table 6). Concentrations of 5 PAHs in NF sediments were below ERL and concentrations of 7 PAH were above the ERL but lower than ERM values indicating that toxic effects might be rare and occasionally.

To verify the ecosystem assessment using sediment guidelines, we also used reference values established by Kalf et al. (1997), which compared bank sediments concentrations against their quality values. The ecosystem risk assessment is characterized by risk quotient (RQ) of each PAH and is defined by RQ = [PAH]/[QV], where [PAH] is the concentration of PAH and [QV] is the correspondent quality values of PAHs. Two quality values have been established previously (Kalf et al. 1997) to estimate risk potential: negligible concentration values (NCs) and maximum permissible concentrations (MPCs). NCs indicates the concentration in the environment below which risk of adverse effects are considered negligible; MPCs refer to concentrations of pollutants above which risk of adverse effects are unacceptable to ecosystems health (Crommentuijn 2000). For our study, we used an improved ecosystem risk calculation (Cao et al. 2010), which takes into account risk contribution of individual PAHs defined in the following equations:

\[
RQ_{\sum PAH(NCs)} = \sum RQ_i(NCs) \ldots (i= 1 \ldots 12)
\]

\[
RQ_{\sum PAH(MPCs)} = \sum RQ_i(MPCs) \ldots (i= 1 \ldots 12)
\]

This risk assessment can be used to classify individual PAHs as well as total PAHs in a system, facilitating comparison among different systems (Table 5). The RQ(NCs) mean values calculated for individual sediments in LW, GR and NF are presented in Table 7. In general, individual RQ(NCs) and RQ(MPCs) in LW sediments surmounted greatly the quality reference (> 1) indicating that severe toxicity to aquatic organisms might be attributed to PAHs. Similarly in GR sediments individual RQ(NCs) and RQ(MPCs) were all greater than 1 except for chrysene which might cause only moderate risk to aquatic biota. Individual RQ(NCs) and RQ(MPCs) in NF sediments were also greater than 1, however naphthalene, acenaphthylene, acenaphthene and chrysene were much lower and closer to 1 than other PAHs. As shown in Table 6, despite the fact that chrysene is a HMW PAH, and is considered mutagenic and carcinogenic,
LMW possessed higher ecosystem risk values because they are more volatile and with higher solubility than HMW PAHs. HMW PAHs tend to sorb into soil and sediment particles making biodegradation more difficult but with bioavailability less of a concern.

RQ∑PAH(NCs) and RQ∑PAH(MPCs) in all sites were greater than 800 and greater than 1 respectively indicating high ecosystem risk. However, the mean ecosystem risk in LW was approximately 4 times the risk in NF and 3 times the risk in GR. These findings indicate clearly that bank sediments in the Mahoning River although very different in PAH composition profiles all carry an increased ecological risk to aquatic organisms. RQ∑PAH(MPCs) were much higher in sites nearby former manufacturing zones (LW and GR); in contrast RQ∑PAH(MPCs) than found for the less industrially-impacted upstream site (NF).

Sediment potential toxicity based on carcinogenic PAHs

There are very few studies that have described sediment toxicity in PAH-polluted ecosystems (i.e. Chen and Chen 2011). However, toxicity equivalent factors (TEFs) in combination with measured concentrations of potentially carcinogenic PAHs (BaA, Chr, BkF, and BaP) have been used in other types of studies to assess toxicity. For instance, TEFs and air concentrations of carcinogenic PAHs were used to determine indoor and outdoor PAH exposure in human populations (Jung et al. 2010). In this study, potential toxicity of the sediments was estimated using the total benzo[a]pyrene equivalent (TEQc) according to the following formula: TEQc-CPAHs = Σ(Ci x TEFci), where Ci is the CPAH concentration and TEFci is the toxicity equivalent factor of CPAHs relative to benzo[a]pyrene. Benzo[a]pyrene is the only potential carcinogen that has toxicological data used to derive other carcinogenic toxicity factors (Peters et al. 1999).

Carcinogenic PAHs (CPAHs) in LW sediments (ranging from 80,139 to 340,000 µg/kg) represented 20 to 36% of the total concentrations of PAHs measured. CPAHs in GR sediments (ranging from 12,370 to 34,198 µg/kg) accounted for 22 to 31% of total concentrations of PAHs while in CPAHs in NF (ranging from 3382 to 54,659 µg/kg) represented 18 to 49% of the total concentrations of PAH. In this study, the highest averaged TEQc was found in LW sediments (varied from 3142 to 45,214 µg/kg dry
weight), followed by GR sediments with a TEQc ranging from 3184 to 12,457 µg/kg dry weight, and NF sediments with a TEQc from 442 to 26,149 µg/kg dry weight. These values are much higher than others reported for similar contaminated ecosystems. For instance, Chen et al. (2012) indicated that the TEQc for a contaminated harbor in Taiwan ranged from 3.9 to 1970 TEQc µg/kg dry weight. Wang et al. (2010) also reported TEQc ranging from 3 to 485 µg/kg dry weight from sediment samples in the Luqiao River in China.

Sediment toxicological assessment using benchmarks

The EPA provided benchmark values (chemical concentrations above which possibility of risk to humans or wildlife exist) to assess potential risk to aquatic organisms in contact with PAHs and other contaminants (U.S. EPA 2010). Benchmark values are exceeded when the sum of toxicity PAH values is greater than 1. We used normalized PAH concentrations to organic matter content and applied acute and chronic toxicity divisors to estimate ecological risk in Mahoning sediments (data not shown). PAH-benchmarks values were greatly exceeded in LW, GR and NF (57.5, 7.5 and 31.6 respectively) indicating that PAH mixtures in these sediments are indeed of high concern to wildlife and humans. Individually, naphthalene, fluorene, phenanthrene, fluoranthene, pyrene and chrysene were greater than 1 in LW and NF sediments, while naphthalene and phenanthrene were greater than 1 in GR.

Conclusions

Extremely high concentrations of PAHs were found even at what it was considered an unpolluted site. These concentrations generally exceeded what has been reported in the scientific literature for other contaminated riverbank sediments around the world in the last ten years.

Source identification of PAHs in long-term polluted environments is complex, even more so in riverbank sediments where deposition over time influences PAH accumulation. Use of PAH fingerprinting ratios and PCA allowed us to determine that riverbank sediments in three sites along the Mahoning River originated mainly from coal and petroleum combustion. This finding corroborates historical anecdotes and other unpublished sources that indicated heavy pollution from coke and steel plants in this area during last
century. We used several approaches including sediment guidelines, risk quotients and carcinogenic equivalent factors to provide a rapid identification of low sediment quality and high ecological risk to aquatic biota, nonetheless, more comprehensive ecological risk assessment and remediation are needed in order to guarantee ecological health in the banks of the Mahoning River.

Acknowledgments

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References


United States Army Corps of Engineers (USACE). 1999. Mahoning River Environmental Dredging Reconnaissance Study.


Table 1 Sediment characteristics from the three study sites in the Mahoning River. Cores (15 cm long) were taken at different depths from the surface riverbank. Sites were selected based on PAH concentrations measured from the river channel as reported by the USACE (2001)

<table>
<thead>
<tr>
<th>Site</th>
<th>Depth (m)</th>
<th>Latitude/Longitude</th>
<th>Organic Matter (%)</th>
<th>Fixed Solids (%)</th>
<th>Sediment Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowellville</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>LW1</td>
<td>2.1</td>
<td>41° 2'18.42&quot;N/80° 32'28.7&quot;W</td>
<td>9.1</td>
<td>90.9</td>
<td>Very oily, black viscous, strong odor</td>
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<tr>
<td></td>
<td>2.4</td>
<td></td>
<td>8.1</td>
<td>91.9</td>
<td></td>
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<td>2.7</td>
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<td>8.3</td>
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<tr>
<td>LW2</td>
<td>1.5</td>
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<td>12.0</td>
<td>88.0</td>
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</tr>
<tr>
<td></td>
<td>1.8</td>
<td></td>
<td>10.8</td>
<td>89.2</td>
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<td></td>
<td>2.4</td>
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<td>11.7</td>
<td>88.0</td>
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<tr>
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<td>86.8</td>
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<td>93.2</td>
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<td>2.4</td>
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<td>7.4</td>
<td>92.3</td>
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<td>Girard</td>
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<td></td>
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<td>GR1</td>
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<td>41° 9'18.06&quot;N/80° 42'23.5&quot;W</td>
<td>7.8</td>
<td>92.2</td>
<td>Very oily, black viscous, strong odor</td>
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<td>7.2</td>
<td>92.8</td>
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<tr>
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<td>1.2</td>
<td>41° 9'16.97&quot;N/80° 42'23.22&quot;W</td>
<td>10.8</td>
<td>89.2</td>
<td>Very oily, black viscous, strong odor</td>
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<td>1.5</td>
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<td>10.2</td>
<td>89.8</td>
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<td>7.4</td>
<td>92.6</td>
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<tr>
<td>GR3</td>
<td>1.2</td>
<td>41° 9'16.39&quot;N/80° 42'23&quot;W</td>
<td>9.4</td>
<td>90.6</td>
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<td>10.2</td>
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<td>41° 8'6.59&quot;N/ 80°58'1.96&quot;W</td>
<td>1.8</td>
<td>98.2</td>
<td>Gray, clay, no odor</td>
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<td>98.5</td>
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### Table 2

Range, mean and relative standard deviation of PAH concentrations (µg/kg dry weight) in riverbanks from three sites along the Mahoning River

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<th>No aromatic rings</th>
<th>Lowellville Range</th>
<th>Mean</th>
<th>RSD (%)</th>
<th>Girard Range</th>
<th>Mean</th>
<th>RSD (%)</th>
<th>Newton Falls Range</th>
<th>Mean</th>
<th>RSD (%)</th>
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<td>304 - 1687</td>
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<td>34 - 425</td>
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<td>Aceph</td>
<td>391 - 4243</td>
<td>1790.7</td>
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<td>392 - 843</td>
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<td>7373.2</td>
<td>62.1</td>
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<td>59.1</td>
<td>34 - 425</td>
<td>167.9</td>
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</tr>
<tr>
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<td>169 - 1236</td>
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Table 4 Values of selected ratios used to determine petrogenic and pyrolytic origins in sediments. Values estimated in riverbank sediments from three sites along the Mahoning River

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<th>Reference</th>
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<td>Girard</td>
<td>Newton Falls</td>
<td></td>
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<tr>
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<td>Flu/Pyr</td>
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<td>&gt; 1</td>
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<td>&gt; 0.1</td>
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Table 5 Risk classification of individual and total PAHs used in this study

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<td>Moderate risk</td>
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<tr>
<td>High risk</td>
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Table 6 Comparison of sediment guidelines (ER-L, effects range low and ER-M, effects range median) and average PAH concentrations (µg/kg dw) measured in riverbank sediments from three sites along the Mahoning River

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<th>Sediment guidelines (µg/kg)</th>
<th>Average concentration (µg/kg) in riverbanks</th>
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Table 7 Comparison of average PAH guideline values of risk quotients RQ(NCs) and RQ(MPCs)) and PAH concentrations (µg/kg dry weight) measured in riverbank sediments from three sites along the Mahoning River

<table>
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<tr>
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<th>Newton Falls</th>
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Fig. 1 Map of the study area and sampling locations along the Mahoning River
Fig. 2 Spatial distributions (core data) of total PAHs grouped by number of aromatic rings (2-AR=clear diamonds, 3-AR= clear squares, 4-AR= filled triangles, 5-AR= clear circles) showing in the X-axis concentrations of PAHs in μg/g dry weight (thousands) and in the Y-axis depth in meters. Top panel Lowellville (LW), middle panel Girard (GR) and bottom panel Newton Falls (NF)
Fig. 3 Average PAH composition at each site (Lowellville-LW, Girard –GR, and Newton Falls –NF) in µg/kg dry weight (Panel A) and expressed in percentage (Panel B) and classified according to the number of their aromatic rings (AR): naphthalene (2-AR), acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene (3-AR), fluoranthene, pyrene, benzo[a]anthracene, and chrysene (4-AR), benzo[b,k]fluoranthene and benzo[a]pyrene (5-AR).
Fig. 4 PAHs cross plots for the ratios of Flu/Pyr vs. Phen/Ant (panel A) and Flu/(Flu+Pyr) vs. Ant/(Ant+Phen) (panel B) found in riverbank sediments in Lowellville (LW) filled diamonds, Girard (GR) clear squares, and Newton Falls (NF) filled triangles.
CHAPTER III: BACTERIAL COMMUNITY COMPOSITION AND BIOGEOCHEMICAL HETEROGENEITY IN PAH-CONTAMINATED RIVERBANK SEDIMENTS

Abstract

Predicting response of microbial communities to pollution requires an underlying understanding of the linkage between microbial community structure and geochemical conditions. In this study, we used 16S rDNA-based terminal restriction fragment length polymorphism analysis and sequencing to characterize bacterial communities in highly PAH-contaminated subsurface riverbank sediments. We measured environmental parameters to establish possible correlations between bacterial communities and environmental geochemistry. Non-metric dimensional scaling analyses revealed that bacterial community composition was strongly influenced by PAH concentration. Sulfate, organic matter, pH and moisture also were related to community composition. Groups of bacteria identified by phylogenetic analysis included δ-proteobacteria, firmicutes, bacteriodetes and caldiserica. We also observed high number of phylotypes associated with sulfate reducing bacteria, some of which have been previously described as important in PAH degradation. Our study suggests that despite intense PAH pollution, bacterial community composition did exhibit temporal and spatial variations and was influenced by sediment geochemistry including sulfate concentration, moisture, and pH. Significant positive relationships between bacterial community composition and PAH concentrations suggests that, potentially, extant microbial communities may contribute to natural attenuation and/or bioremediation of PAHs.

3 This chapter has been submitted to the Journal of Soils and Sediments and is under peer review; the author list is as follows Gloria P Johnston and Laura G Leff. Dr. Leff provided guidance in choosing terminal restriction fragment length polymorphism (T-RLFP) as the fingerprinting technique selected and access to lab expertise on this method. I collected samples, conducted geochemical and molecular analyses, processed and analyzed data, wrote chapter.
Introduction

Sediments contaminated with polycyclic aromatic hydrocarbons (PAHs) are a common problem in freshwater and marine environments worldwide (Eggleton et al. 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 (Christen and Dalgaard 2012; Buckley et al. 2012). 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). Bacteria are central to biogeochemical cycles, constitute a major portion of biomass in sediments, and under extreme conditions of contamination can be very active. For example, Mosher et al. (2006) found higher levels of microbial activity (respiration measured as 2-(p-iodophenyl)-3(p-nitrophienyl)-5-phenyl tetrazolium chloride reduction) in PAH-contaminated sediments than in less contaminated sediments. They also suggest that hydrocarbon and metal pollution can increase microbial biomass if pollutants are used as carbon sources or decrease microbial biomass if pollutants are toxic. Over long periods 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 (Bernhanrd et al. 2005) and function (Machado et al. 2012; Lors et al. 2010). Because many bacteria are capable of transforming and mineralizing PAHs (Bamford and Singleton 2005; Dell’Anno et al. 2009) bioremediation via bacterial degradation is a common approach (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 *Nocardiopsis otidiscaviarum* 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 produce biosurfactants therefore increasing PAH bioavailability (Ho et al. 2000; Bastiaens et al. 2000) and by forming biofilms (Johnsen and Karlson 2005). Collectively, these direct and indirect effects suggest that PAHs have an important impact on bacterial community composition.

Microbial biodegradation of PAHs is of immense potential, thus information of microbial community composition is critical to get knowledge before any bioremediation strategy can be implemented. 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 contaminated riverbank sediments. These properties were related to concentrations and composition of PAHs and other environmental variables of the Mahoning River (NE Ohio, USA), which was long exposed to contamination (USACE 2001). We hypothesized that because bacterial communities are embedded in oily deep anaerobic highly contaminated with PAHs, they would not be affected by environmental factors in their surroundings.

**Materials and Methods**

**Study Site**

The Mahoning River (Northeastern Ohio, USA) 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 (USACE 2001). Between 1920 and 1970, the lower Mahoning River supported one of the highest productions of steel worldwide (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 (USACE 1999).

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) at 1.2 m
below the surface (Fig. 1). On each date, nine cores (20 cm apart from each other) were collected. 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 sterile technique. Once
homogenized, sediment was partitioned for geochemical analysis and DNA extraction.

PAH extraction and analyses

PAHs were extracted by Soxhlet extraction Method 3540C (USEPA 1996). We chose this method
because Soxhlet has been considered the "industry standard" in extraction techniques in the environmental
field (U.S. EPA 2012), is relatively simple and inexpensive. When performed correctly, it is assumed to be
an exhaustive technique (Shen and Shao 2005). Approximately 8g of thawed sediment were placed in
Whatman cellulose extraction thimbles (Sigma, USA). Anhydrous granular sodium sulfate was added to the
thimbles until sediments reached a “sand-like” consistency to remove water. 10 µl of surrogate standard
mix (Restek, Bellefonte, PA) was added directly to the sample, followed by 200 ml of dichloromethane.
The solvent was then heated and refluxed for 18 hours. Sample extracts were concentrated to a final
volume of 30 mL and were analyzed according to the guidelines set forth in USEPA Method 8270D
(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, PA). We used a Gas Chromatograph Hewlett-Packard 5890, a Mass Spectrometry Hewlett-Packard 5970B, and a Restek XTI-5, 30m x 0.25mm ID x 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, and then ramped at 10 °C/min to a final temperature of 310 °C, and held for 10 min. 1 µl 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 ranged from 5 to 20%). High molecular PAHs (5 rings) as well as naphthalene showed high RSD values compared to most 3 and 4-ring PAHs.

Geochemical analysis

Total nitrogen (TN) and total carbon (TC) were determined, from sediments dried at 60 °C, by combustion a 900 °C and measured on an ECS-4010 elemental analyzer (Costech Analytical, Valencia, CA USA). Sulfate was measured by extracting 2 g of sediment with 0.016 M Ca(H₂PO₄)₂·H₂O 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). 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). Moisture content determination followed standard methods for soil analysis (Carter 2000).

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 minutes at low frequency (VWR Ultrasonics Cleaner, PA), and centrifuged for 30 sec at 1000 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, CA), stained with 4,6-diamidino-2-phenylindole (DAPI; 1 mg/ml final concentration) for 5 min, and rinsed with filtered autoclaved water (Lemke and Leff 2006). An epifluorescence microscope (Olympus DP71, PA, USA) was used to count bacteria in 10 randomly selected fields.

Molecular Analyses

Total DNA was extracted from sediment using the PowerSoil DNA Isolation Kit (Mo-Bio Laboratories, Carlsbad, CA, USA), according to the manufacturer’s instructions with minor modifications. Duplicate DNA extractions per core were performed (a total of 18 extractions per sampling date). DNA extracts were stored at -20 °C until analysis.

Overall differences in bacterial community composition were examined using T-RFLP (terminal restriction fragment length polymorphisms (Liu et al. 1997). 16S rRNA genes were amplified using the primers 8F (5’ AGAGTTTGTACATGGCTCAG-3’ and 1492R (5’-GGCTACCTTGCCACGACTTC-3’)(Zhang et al. 2008). The 8F primer was 5’labeled with 6-carboxy- fluorescein phosphoramidite (FAM).

Each PCR reaction (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, WI, USA) 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-RFLP 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, WI, USA). Digested PCR products were sent to the Ohio State University Plant Microbe Genomics Facility (Columbus, OH) for fragment analysis on a 3730 DNA Analyzer (Applied Biosystems, Inc.) using a LIZ1200 size standard and minimum peak height of 50 fluorescence units. Peaks between 50 and 600 bp were included in the analyses if they represented >1% of the cumulative peak height for the sample as established by Dr. Blackwood’
Laboratory protocol. Data was processed, sorted by peak intensity and formatted using Gel ComparII (Applied Maths, Austin, TX) before statistical analysis.

Sediment from three selected cores from each sampling date (January, August, and October) was used for bacterial 16S rDNA gene amplification using the same primers, 8F and 1492R (Zhang et al. 2008), both unlabeled, followed by PCR as indicated above. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Three replicate PCR products were performed for each sample (3 cores from January, 3 from August and 3 from October), and these were pooled before cloning ligation reaction (total of three pooled samples for each sampling date).

Clone library

Products were cloned into competent Escherichia coli cells using the StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA, USA) 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 4µl of 2% of X-gal 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, 0.1mg/ml of ampicillin and 10% glycerol, shaken for 24 h at 37 °C. The insert sizes of selected colonies were determined by PCR as above. We isolated 471 clones (188 from August, 140 from January and October) however only 160 clones from August were sequenced at the University of Kentucky Advanced Genetic Technologies Center, Lexington, KY. Taxonomic affiliations were assigned to bacterial OTUs using the Basic Local Alignment Search Tool (BLAST)(http://www.ncbi.nlm.nih.gov). Nucleotide sequences were checked using Vector NTI Software (Life Technologies Corp., NY, USA) and manually curated. Sequences were aligned using MUSCLE. The phylogenetic tree was constructed with the MEGA software version 5.0 (Kumar et al. 2004) using 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.
Statistical Analysis

One factor 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 SPSS 12.0 for Windows.

Nonmetric multidimensional scaling (NMDS) was used to compare T-RFLP fragment composition among seasons. T-RFs community composition NMDS dimension scores were assessed as variables and correlated (using Spearman correlation coefficients) with total bacteria, sulfate, total 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) × 100 (Jiang et al. 2009), where n is the number of clones present only once and N is the total number of clones recovered.

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.

Results

Sediment characteristics

Contaminated sediments samples were black, viscous, and had a strong petroleum odor that persisted in the laboratory. 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 [TC] (ranging from 12% in January to 15% in October), which corresponded to the high amount of organic matter also (14% to 16%); pH of sediments did not vary substantially (7.3 to 7.9) while moisture content did exhibit greater variability over time (39 to 66%). Sulfate concentrations were highest in
October sampling, ranging from 57 to 1008 µg g\(^{-1}\); in contrast, lowest sulfate concentrations were found in August (2 to 13 µg g\(^{-1}\)). Total nitrogen (TN) was low on all dates. Total bacterial abundance ranged from 4.2 \(\times\) 10\(^6\) to 1.2 \(\times\) 10\(^8\) cells (dry weight g\(^{-1}\)).

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 (2-ring PAHs); phenanthrene and anthracene, fluoranthene (3-ring PAHs); pyrene, benzo[a]anthracene, chrysene, benzo[b,k]fluoranthene, (4-ring PAHs); benzo[a]pyrene and dibenzo[a,h]anthracene (5-ring PAH) and benzo[ghi]perylene (6-ring PAHs). Dibenzo[a,h]anthracene and benzo[ghi]perylene were only detected on Jan 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, 4-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 (HMW) PAHs (4, 5 and 6-ring) accounted for 67% of the total PAHs in January, 41% in August and October, while low molecular weight (LMW) PAHs (2 and 3-ring) contributed 33% and 59% in January and August respectively.

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 (Wilk's Lambda = 0.003, F= 20.241, p=0.000). In addition, significant Pearson correlations coefficients between TN, TC, sulfate, moisture, 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 concentration (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 2-ring PAHs, 3-ring and 4-ring PAHs.

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. 3). 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 NMS 2 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, sulfate, TN, TC, total PAHs, moisture, organic matter and total bacteria) were used to create ordination distances (Fig. 4) and represent the association of these variables with bacterial community composition. As shown in Figure 4, total PAHs, 2-ring PAHs, 3-ring PAHs, and sulfate significantly correlated (p<0.01) with NMS2 while 4-ring PAHs, pH, moisture and total bacteria also correlated (p<0.05) with NMS2. In the ordination plot PAHs and pH appeared to be associated with bacterial communities sampled in August while moisture and sulfate were more associated with bacterial communities in January. Nitrogen, total carbon and organic matter were not significantly correlated with neither NMS1 nor NMS2, indicating that perhaps these environmental conditions were not as associated with the bacterial communities in Lowellville.
Bacterial taxa

Sequences from 160 bacterial clones (August sampling) were manually revised for quality and only 151 were used for analyses. Bacterial sequences were binned into operational taxonomic units (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 5). The CD-Hit analyses resulted in 62 OTUs represented in 8 clusters, which at the same time represented 61% of the sequences (Table 5). The homologous coverage from our study was 71% and the rarefaction curve did not reach a plateau indicating that the bacterial diversity in these sediments is greater than revealed by our analyses (Fig. 5). 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 7 clusters (Table 5), with highest sequence identity to that of bacteriodetes-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).

Recoverd 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. 6), the clone library included organisms related to the proteobacteria (50%), firmicutes (27%), bacteriodetes (11%), acidobacteria (3%), actinobacteria (3%) and caldiserica (3%). Other groups of bacteria included few clones and were phylogenetically related to chloroflexi (3 clones), and nitrospirae (1 clone) and elusimicrobia (1 clone). 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 few sequences. 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, chromatales, 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.

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 (Pratt et al. 2012; Pies et al. 2008). 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 examined 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.

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, and organic matter did not appear to play an important role in shaping the bacterial community as reported also for other contaminated sediments with PAH, where organic matter had very little effect in microbial communities (Wang and Tam 2012). Total carbon in our sediments was much higher compared to 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 in August appeared to be associated to environmental conditions including pH and mostly PAHs (2 and 3 ring) while bacterial communities in January were more associated with moisture and sulfate. The pattern observed in temporality respect to PAHs was unusual.
since PAHs tend to sorb to sediments and do not change much in 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 also indicate that among the environmental conditions measured sulfate had a strong correlation with differences in community composition. In particular, sulfate concentrations were the lowest in August, when we also detected higher number of T-RFs. Perhaps August bacterial communities were using sulfate for respiration reflected in low concentrations measured. 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 temporally (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), and in both studies had an effect on microbial communities.

We found that, although bacteria were not abundant (ranged from $10^6$ to $10^8$ dry weight g$^{-1}$), numbers were highly correlated with PAH concentration, indicating that perhaps bacterial abundance was not limited by PAH contamination. Similar results from other studies have shown that biomass and bacterial cell counts in harbor sediments contaminated with petroleum and in sites heavily contaminated with petroleum hydrocarbons appeared to be unaffected by metal contamination (Shi et al. 2005; Feris et al. 2004).

T-RFLP analysis is a reproducible and sensitive method for comparing temporal changes in bacterial community composition (Liu et al. 1997). NMDS analysis of T-RFLPs suggests that microbial communities were subject to temporal effects despite the extent of contamination. The aggregation or clustering found in bacterial communities in August correlated with the number of T-RFs recovered at the same time, indicating that some groups of bacteria might have been favored (higher relative abundance) during August. Interestingly, our results are in agreement with others that have described clustering of bacterial communities temporally (marked in summer months) (Hullar et al. 2006) and transitional patterns marked by temporal variations (Smoot and Findlay 2001).
In similar contaminated systems, hydrocarbon pollution has been shown as the main driving force for changes in microbial community structure (Wentzel et al. 2007). More specifically, in "hot spots" of contaminated sediments (where high PAH levels are found), 3 and 5-ring PAHs appeared to be most important factor determining microbial community composition (Wang and Tam 2012). In our study, bacterial community composition appeared to be influenced strongly by concentrations of 2, 3 and 4-ring PAHs.

A limited clone library analysis revealed that some bacterial groups including proteobacteria, firmicutes, acidobacteria and bacteriodetes also reported in higher abundances in similar contaminated environments. As described for other contaminated sites (Porat et al. 2010; Edlund and Jansson 2006), 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 addition bacteriodetes, another phylogenetic group associated with PAH contamination (Haller et al. 2011), was also found in Mahoning riverbank sediments, although in less relative abundance.

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 nitrate or sulfate (Gieg et al. 2014).

We also observed phylotypes (20 sequences) affiliated with desulfobacteriales, desulforomonadales and desulfurellales. Some members of these groups have been described as sulfate-reducing bacteria (SRB) involved in PAH degradation (Acosta-Gonzalez et al. 2013; Gomes et al. 2013). For example, SRB that are members of the desulfobacteriales are responsible for most anaerobic mineralization of hydrocarbons in marine sediments (Zhang et al. 2008). In agreement with these results, we found culturable sulfate reducing bacteria (SRB) ranging from 2 to 9x 10^4 cells/g (data not shown) from contaminated sediments in Lowellville. In fact, it has been observed that hydrocarbon contamination (Suarez-Suarez 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.

**Conclusions**

This is the first study that measured environmental conditions and identified bacterial communities (via molecular tools) of highly contaminated PAH riverbank sediments. We showed that specific environmental variables (PAHs, sulfate, pH and moisture) were strongly correlated to bacterial community composition and despite high pollution bacterial communities did respond to environmental changes in their surroundings, indicating that temporality is a stronger factor than pollution in shaping bacterial community composition. Community composition was elucidated by the number of T-RFs recovered from the sediment and followed temporal changes. Within the limited number of 16S rDNA sequences recovered (151), the majority was related to δ-proteobacteria, firmicutes and bacteriodetes. These three groups of bacteria have been also reported in other types of contaminated sediments. In addition, among those three groups of bacteria, we identified SRB sequences that had been previously described as having a role 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.

**References**


Shen J, Shao X. 2005. A comparison of accelerated solvent extraction, Soxhlet extraction, and ultrasonic-assisted extraction for analysis of terpenoids and sterols in tobacco. Anal Bioanal Chem 6, 1003-1008.


United States Army Corps of Engineers (USACE), 1999. Mahoning River Environmental Dredging Reconnaissance Study, USACE Publications.


Table 1 Average values and standard deviations (in brackets, n=9) of environmental variables measured in riverbank sediments at different dates

<table>
<thead>
<tr>
<th>Sampling Date</th>
<th>pH</th>
<th>Organic matter %</th>
<th>TN%</th>
<th>TC%</th>
<th>Moisture %</th>
<th>SO$_2^-$ (μg g$^{-1}$)</th>
<th>Total PAHs (μg dry weight g$^{-1}$)</th>
<th>Total Bacteria (cells dry weight g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>7.4 (0.2)</td>
<td>16.3 (3.8)</td>
<td>0.2 (0.0)</td>
<td>12.4 (1.8)</td>
<td>56.1 (12.1)</td>
<td>83.6 (11.8)</td>
<td>35251.1 (11234.8)</td>
<td>7.5X10$^9$ (3.5x10$^9$)</td>
</tr>
<tr>
<td>August</td>
<td>7.8 (0.2)</td>
<td>14.6 (4.4)</td>
<td>0.2 (0.0)</td>
<td>12.6 (1.2)</td>
<td>44.9 (5.6)</td>
<td>9.1 (3.4)</td>
<td>65314.8 (18879.1)</td>
<td>3.2x10$^7$ (1.8x10$^7$)</td>
</tr>
<tr>
<td>October</td>
<td>7.6 (0.2)</td>
<td>14.3(1.7)</td>
<td>0.3 (0.0)</td>
<td>14.5 (1.5)</td>
<td>46.8 (6.9)</td>
<td>327.5 (390.1)</td>
<td>62207.4 (29107.8)</td>
<td>5.6x10$^7$ (5x10$^7$)</td>
</tr>
</tbody>
</table>

Table 2 Range concentrations of PAHs (expressed in μg/kg dry weight) per sampling date in riverbank sediments from Lowellville, Mahoning River

<table>
<thead>
<tr>
<th>Compound</th>
<th>No benzene rings</th>
<th>January</th>
<th>August</th>
<th>October</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>2</td>
<td>976 - 1928</td>
<td>1708 - 4886</td>
<td>1855 – 8650</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>2</td>
<td>239 - 355</td>
<td>146 - 438</td>
<td>221 - 512</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>2</td>
<td>174 - 648</td>
<td>1141- 3598</td>
<td>371 - 7745</td>
</tr>
<tr>
<td>Fluorene</td>
<td>2</td>
<td>392 -1261</td>
<td>1695 - 4648</td>
<td>724 - 8939</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>3</td>
<td>916 - 5806</td>
<td>6228 - 14759</td>
<td>2793 - 18399</td>
</tr>
<tr>
<td>Anthracene</td>
<td>3</td>
<td>642-2095</td>
<td>2498 - 7086</td>
<td>1160 - 6798</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>3</td>
<td>2723 - 8079</td>
<td>5417 - 17291</td>
<td>4891 - 16987</td>
</tr>
<tr>
<td>Pyrene</td>
<td>4</td>
<td>5076 - 10711</td>
<td>4322 - 13016</td>
<td>5443 - 14238</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>4</td>
<td>3469 - 5287</td>
<td>2655 - 9089</td>
<td>3658 - 8862</td>
</tr>
<tr>
<td>Chrysene</td>
<td>4</td>
<td>3341 - 6000</td>
<td>2761 - 9829</td>
<td>3869 - 9543</td>
</tr>
<tr>
<td>Benz(b,k)fluoranthene</td>
<td>4</td>
<td>1433 - 5312</td>
<td>1128 - 4280</td>
<td>1938 - 3824</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>5</td>
<td>1427 - 4388</td>
<td>417 - 3786</td>
<td>1277 - 2938</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>5</td>
<td>1033 -2534</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Benzo(ghi)perylene</td>
<td>6</td>
<td>937 - 2123</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>∑ PAHs</strong></td>
<td><strong>19712 - 55277</strong></td>
<td><strong>30046 - 86344</strong></td>
<td><strong>30057 – 102, 346</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table 3 Significant correlation coefficients (Pearson) between environmental variables: pH, moisture, organic matter, sulfate, total nitrogen (TN), total carbon (TC), PAHs grouped by benzene rings and total PAHs. Five and six ring PAHs were not included in the analysis since they were not detected in all cores. Significant correlations at the 0.01(*) and 0.05 level (**) respectively

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Organic matter</th>
<th>TN</th>
<th>TC</th>
<th>Sulfate</th>
<th>Bacteria</th>
<th>Total PAHs</th>
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</thead>
<tbody>
<tr>
<td>Moisture</td>
<td></td>
<td></td>
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<tr>
<td>TC</td>
<td></td>
<td></td>
<td>0.202</td>
<td>0.706*</td>
<td></td>
<td></td>
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<tr>
<td>Bacteria</td>
<td></td>
<td></td>
<td></td>
<td>0.806**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PAH</td>
<td></td>
<td></td>
<td>0.686*</td>
<td>0.834**</td>
<td>0.879**</td>
<td></td>
<td></td>
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<tr>
<td>2-ringPAH</td>
<td>0.42*</td>
<td></td>
<td>0.698*</td>
<td>0.965**</td>
<td></td>
<td></td>
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<tr>
<td>3-ringPAH</td>
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<td></td>
<td></td>
<td>0.515*</td>
<td>0.928**</td>
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<td>4-ringPAH</td>
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Table 4 Significant correlation coefficients (Pearson) of selected terminal restriction fragments (T-RFs) and environmental variables

<table>
<thead>
<tr>
<th>T-RF (bp)</th>
<th>Moisture</th>
<th>Bacteria</th>
<th>Sulfate</th>
<th>Total PAHs</th>
<th>2-ring PAH</th>
<th>3-ring PAH</th>
<th>4-ring PAH</th>
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</thead>
<tbody>
<tr>
<td>198</td>
<td></td>
<td></td>
<td></td>
<td>0.552</td>
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<td></td>
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<td>0.534</td>
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<td>205</td>
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<td>0.619</td>
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<tr>
<td>214</td>
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<td>0.720</td>
<td>0.873</td>
<td>0.600</td>
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<tr>
<td>216</td>
<td>0.599</td>
<td></td>
<td></td>
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<tr>
<td>221</td>
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<td>0.557</td>
<td>0.515</td>
<td>0.507</td>
<td>0.527</td>
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<tr>
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<tr>
<td>231</td>
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<tr>
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<td>0.575</td>
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<tr>
<td>304</td>
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<td>0.593</td>
<td></td>
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<tr>
<td>334</td>
<td>0.653</td>
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<tr>
<td>360</td>
<td>0.628</td>
<td>0.686</td>
<td>0.501</td>
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</table>
Table 5. Phylogenetic affiliation of clones amplified from one riverbank sediment site (Lowellville)
Clustered sequences represents 61% of the total number of retrieved sequences

<table>
<thead>
<tr>
<th>Clusters</th>
<th>No of clones in library</th>
<th>Phylogenetic affiliation</th>
<th>Representative clone</th>
<th>Closest environmental sequences</th>
<th>Closest culture relative</th>
<th>GenBank ID</th>
<th>Habitat</th>
<th>GenBank ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1</td>
<td>33</td>
<td>δ-proteobacteria</td>
<td>MRS80 (542bp)</td>
<td></td>
<td>Syntrophus aciditrophicus (94%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster 2</td>
<td>12</td>
<td>Bacteriodetes</td>
<td>MRS92 (1485bp)</td>
<td></td>
<td>Alistipes shahii WAL 8301 (85%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster 3</td>
<td>11</td>
<td>Firmicutes</td>
<td>MRS154 (1495bp)</td>
<td>Thermincola potens JR (84%)</td>
<td>Sludge reactor Hot springs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster 4</td>
<td>10</td>
<td>Caldiserica</td>
<td>MRS33 (1509bp)</td>
<td>Caldisericum exile (82%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster 5</td>
<td>7</td>
<td>δ-proteobacteria</td>
<td>MRS133 (567bp)</td>
<td>Syntrophobacterium aromaticivorans UI (93%)</td>
<td>Methanogenic degrading enrichment culture n.a.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster 6</td>
<td>6</td>
<td>δ-proteobacteria</td>
<td>MRS124 (1525bp)</td>
<td>Desulfobacterium anilini (94%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cluster 7</td>
<td>5</td>
<td>δ-proteobacteria</td>
<td>MRS156 (1528bp)</td>
<td>Smithella propionica LYP (90%)</td>
<td>Anaerobic digestor</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cluster 8</td>
<td>4</td>
<td>δ-proteobacteria</td>
<td>MRS90 (1526bp)</td>
<td>Desulfobacca acetoxidans (90%)</td>
<td>Freshwater</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1 Sampling location and scheme of how sediment samples were collected from riverbanks in Lowellville
Fig. 2 Composition pattern of PAHs by ring size in Lowellville riverbank sediment samples (2-ring: naphthalene, acephthylene, acenaphthene, fluorene; 3-ring: phenanthrene, anthracene, fluoranthene; 4-ring: pyrene, benzo[a]anthracene, chrysene, benzo[b,k]fluoranthene; 5-ring: benzo[a]pyrene, dibenzo[a,h]anthracene; 6-ring: benzo[ghi]perylene). Data presented in does not include 6-ring PAHs for all sampling dates. Data averaged per sampling date is presented.
Fig. 3 Bacterial community composition from riverbank sediments in Lowellville. 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).
Fig. 4 NMDS ordination showing the similarity and distribution of microbial communities (T-RFs of the 16S rRNA gene) in Lowellville 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.
Fig. 5 Rarefaction curve of the 16S rDNA gene libraries constructed from summer clones. OTUs were defined at 97% sequence identity.
Fig. 6 Phylogenetic tree of selected bacterial 16S rRNA gene sequences retrieved from PAH-contaminated riverbank sediments in Lowellville. 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)
CHAPTER IV: ANALYSIS OF DISSIMILATORY SULFATE REDUCTASE GENES (DSRAB) IN RIVERBANK SEDIMENTS IMPACTED BY LONG-TERM PAH AND METAL CONTAMINATION

Abstract

We investigated diversity of sulfate reducing bacteria (SRB) in cores taken from three polyaromatic hydrocarbon (PAH) and heavy-metal contaminated riverbank sediments along the Mahoning River: two sites were heavily contaminated sites and one less contaminated. The abundance of SRB was quantified by enumeration of culturable bacteria and by phylogenetic analyses of the dissimilatory sulfite reductase genes (dsrAB). The effect of PAHs and metal concentrations on bacterial community structure (based on analyses of the 16S rRNA genes) was also evaluated. We found that bacterial community structure appeared to be influenced by metal and PAH contamination; yet diversity based on analyses of the 16S rRNA genes by terminal restriction fragment length polymorphisms appeared not to be affected by metal contamination. In addition, dsrAB diversity also appeared to be related to high PAH and metal concentrations in the sediments. Gene libraries for dsrAB revealed that most sequences recovered were related to unknown, uncultured SRB found in similar contaminated environments. SRB diversity indices were lower for contaminated sites than in the uncontaminated site. These results suggest that although microbial communities seemed to be adapted to high contamination levels, SRB communities were perhaps affected by pollution. Furthermore, it may be possible to use dsrAB genes as more sensitive indicators of pollution than other conserved genes in impacted aquatic ecosystems.

4 This chapter is in preparation to be submitted to Water Research. The list of authors is as follows: G. Patricia Johnston, C.G. Johnston, J. Simeonsson and L. Leff. Dr. Johnston (Youngstown State University Biology Dept.) contributed with laboratory space, equipment (IC), and supplies. Dr. Simeonsson (Youngstown State University-Chemistry Dept.) provided an ICP-MS and other equipment for metal analyses. Dr. Leff supervised the study providing guidance in the use of T-RFLPs as fingerprinting method of choice and the use of a functional gene (dsrAB) to expand information about the microbial communities. I was responsible for collecting sediments, metal and PAH analysis, molecular analysis, statistical analyses, and writing the chapter.
Introduction

Dissimilatory reduction of sulfate plays an important role in the sulfur cycle, contributes to organic matter mineralization (Miletto et al. 2008; Jiang et al. 2009), and indirectly constrains methane production (Moreau et al. 2010). Sulfate-reducing prokaryotes (SRP) are a very diverse group of anaerobic microorganisms that use sulfate as the terminal electron acceptor for oxidation of various carbon sources (Rabus et al. 2006). SRP thrive under many different environmental conditions including marine and freshwater sediment, (Muyzer and Stams, 2008, Leloup et al. 2006), hydrothermal vents (high temperature) (Dhillon et al. 2003), microbial mats (Foti et al. 2007), and acid mine effluents (Sanchez-Andrea et al. 2012), thus exhibiting a wide phylogenetic diversity. A small number of SRP also have hydrocarbon degradative capabilities including PAHs (Chang et al. 2001; Chang et al. 2002).

Genes encoding the α and β subunits of the enzyme dsrAB (1.9 kb DNA fragment of the chromosome) have been found in all known SRP, including five bacterial and two archaeal phyla (Jiang et al. 2009). The dsrAB genes encode enzymes that catalyze reduction of sulfite to sulfide (Rampinelli et al. 2008), a critical step in anaerobic sulfate reduction. The dsrAB genes are highly conserved across SRP, making them excellent markers for phylogenetic studies (Dar et al. 2007) and allowing the discovery of new sulfate reducing organisms (Nercessian et al. 2005). Previous phylogenetic analyses have shown that dissimilatory sulfite reductase is a key ancient enzyme that supported early origins of sulfate and sulfite respiration (Sitte et al. 2010). In addition, fingerprint methods such as terminal restriction fragment length polymorphisms (T-RFLP) have been used to investigate microbial composition and expand our knowledge of SRP communities (Pérez- Jiménez and Kerkhof 2005; Wagner et al. 2005; Geets et al. 2006).

The majority of SRP community studies have focused mostly on the analysis of sulfate reducing bacteria (SRB) in acid mine impacted environments (Baker and Banfield 2003), engineered systems [mostly bioreactors used in acid mine drainage experiments] (Neculita et al. 2007), marine systems (Leloup et al. 2009) and uranium-contaminated soils (Cardenas et al. 2010). In contrast, SRB composition and diversity in riverbank sediments under long-term heavy metals and PAH contamination has not been described. Such studies might contribute information for bioremediation practices as well as elucidate how geochemical parameters and microbial populations interact.
Previous work in the Mahoning River indicated that sediments contain high concentrations of sulfate and the microbial community composition includes organisms related to SRB (Johnston and Leff. 2014). We investigated diversity of SRB, based on phylogenetic inference from dsrAB gene sequences, in riverbank sediments impacted by heavy metals and PAHs for over a century. T-RFLP analyses were used to assess bacterial community structure from sediments cores taken along the riverbanks. Environmental sediment parameters, PAHs and metal concentrations were related to the distribution of SRB.

Methods

Sampling sites and environmental data

The samples analyzed in this study were collected between July- August 2012 from 3 sites along the Mahoning River (Fig. 1). Two contaminated sites Lowellville (LW) and Girard (GR) and one with minimal contamination, Newton Falls (NF), were chosen based on previous reports of contamination levels with PAHs (Johnston et al. 2014). Riverbanks along the river, showed similar vegetation, but were not fully homogenous with respect to their hydrodynamics (water height, discharge, and flow). Sediment cores were collected using a manual auger device with stainless steel liners (diameter = 5 cm, length = 15 cm) from the surface of the riverbank. At each location, three cores were collected 50 m apart from each other (Fig. 1). To maintain anaerobic conditions of the sediments, immediately after sampling, cores were capped (using stainless steel plates 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 -80 °C until analysis. For analysis, cores were thawed; both ends discarded and inner core was homogenized under nitrogen (to maintain anaerobic conditions) using sterile technique. Once homogenized, sediment was partitioned for environmental data and DNA extraction.

Sediment variables measured in each location included moisture (MC), concentration of total nitrogen (TN), organic matter (OM), total carbon (TC), sulfate (TS), pH, PAHs and metal concentrations. PAHs were extracted by a modification of a sonication-assisted extraction method set forth in USEPA Method 3550C and quantified by Gas Chromatography Mass Spectrometry (as described in Johnston et al. 2014). Total nitrogen and total carbon concentrations were determined from sediments dried at 60 °C, by
combustion a 900 °C and measured on an ECS-4010 elemental analyzer (Costech Analytical, Valencia, CA USA). Sulfate was measured by extracting 2 g of sediment with 0.016 M Ca(H$_2$PO$_4$)$_2$·H$_2$O and quantified by using Ion Chromatography ( Dionex Ion Chromatograph Model ICS-1100, Dionex Corp) as established in Li et al. (2009). 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). Moisture content determination followed standard methods for soil analysis (Carter 2000).

Metal analyses

Metal concentrations were determined by microwave digestion EPA Method 3015A using nitric acid (Fisher Scientific, Pittsburgh, PA) as solvent extraction. Sediment samples were oven-dried and ground using a tissue homogenizer (Precellys®24, Michigan, US). Approximately 0.1 g of homogenized sediment was extracted using 10 ml of trace metal ultrapure nitric acid. After microwave digestion, triplicate sample digested products were adjusted to 50 ml using ultra clean water. Samples were diluted and analyzed for Be, Al, Cr, V, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Sr, Cd, Ag, Ba, Pb, Tl and U by inductively coupled plasma mass spectrometry (iCAP ICP-MS, ThermoScientific, Waltham, MA). Quality controls were analyzed in accordance with method requirements. Recoveries for standard reference materials (NIST Buffalo River No 2704) were between 85 ± 5% for most metal concentrations. All reagents were of analytical grade. Ultrapure water (Milli-Q System, Millipore) was used for the preparation of solutions and dilutions. The element standard solution (SPEX CertiPrep, Metuchen, NJ) used for calibration was prepared by diluting stock solution of 10 mg/l. Glassware were cleaned by soaking in 10% HCl for at least 24 hr and rinsed liberally with ultra pure water.

Bacterial Enumeration

Total bacterial numbers were determined by preserving sediment samples with paraformaldehyde (8% final concentration) and phosphate buffered saline followed by cell counts using an epifluorescence microscope (Olympus DP71, PA, USA) as in Lemke and Leff (2006).
Most probable number of culturable SRB

Most probable numbers of culturable SRB were determined from a slurry solution in 96-well microtiter plates (Johnsen 2010; Almeida et al. 2013). A modified reducing media (Fortin et al. 1996) was used as the growth medium. Ten-fold serial dilution of samples was performed, and the plates were inoculated with 200 µl. A sterile control was included in uninoculated wells. MPN plates were incubated anaerobically using a GazPak EZ Pouch System (BD Technologies, Franklin Lakes, NJ) at 37 °C until growth was observed. Positive wells (by presence of black precipitates) were scored after overnight and 2 days incubation using statistical tables (USDA, 2007).

DNA extraction

For each core, DNA was extracted twice from 0.25 g wet weight of sediment using the Power Soil DNA Isolation Kit (MoBio, Laboratories, Carlsbad, CA, USA) according to the manufacturer’s instructions. DNA from replicate extractions from a core were pooled and used for further analyses.

T-RFLP

Bacterial composition was examined using T-RFLP (Liu et al. 1997). 16S rRNA genes were amplified using the primers 8F (5’ AGAGTTTGATCATGGCTCAG-3’ and 1492R (5’-GGCTACCTTGCCAGACTTC-3’)(Zhang et al. 2008). The 8F primer was 5’labeled with 6-carboxy-fluorescein phosphoramidite (FAM). Each PCR reaction contained: 0.5 µl of each primer (10 mM), GoTaq Green Master Mix, 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, WI, USA) 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. 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, WI, USA). Digested PCR products were sent to the Ohio
State University Plant Microbe Genomics Facility (Columbus, OH) for fragment analysis on a 3730 DNA Analyzer (Applied Biosystems, Inc.) using a LIZ1200 size standard and minimum peak height of 50 fluorescence units. Peaks between 50 and 600 bp were included in the analyses if they represented >1% of the cumulative peak height for the sample as established by Dr. Blackwood’ Laboratory protocol. Data was processed, sorted by peak intensity and formatted using Gel ComparII (Applied Maths, Austin, TX) before statistical analysis.

Phylogenetic analysis using 16S rDNA genes

Sediment from each core (three cores sampling site) was used for bacterial 16S rRNA gene amplification using the same primers, 8F and 1492R (Zhang et al. 2008), both unlabeled, followed by PCR as in Chapter III. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Three replicate PCR products (from two DNA extractions per core) were pooled before cloning ligation reaction. Products were cloned into competent Escherichia coli cells using the StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA, USA) 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 4µl of 2% of X-gal incubated overnight at 37 °C. Control plasmids and competent cell controls were also included. We isolated a total of 752 clones: 282 from LW (from 3 cores), 282 from GR (from 3 cores) and 188 from NF (from 3 cores). White recombinants were transferred into 96-well plates containing LB medium, 0.1mg/ml of ampicillin and 10% glycerol, shaken for 24 h at 37 °C. Two set of subsamples (50 µl each) were stored at -80 °C for further analyses. However, no further analyses were completed due to the budgetary constraints.

dsrAB functional gene clone library

dsrAB gene fragments were PCR amplified from DNA extracted from sediments from each core (three cores from each sampling site) using primer pairs DSR1F/DSR4R (Jiang et al. 2009). All reactions were carried out in 25 µl mixtures containing: 0.5 µl of each primer (10 mM), GoTaq Green Master Mix, 2
µl of DNA, and 9.5 µl of molecular grade water. Negative controls were performed using nuclease free water. Positive controls were performed using SRB from an anaerobic culture using specific SRB reducing media (Fortin et al. 1996). The PCR conditions were as follows 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min, followed by a final extension step at 72 °C for 10 min. Reaction products were checked by agarose gel electrophoresis. PCR products were cloned into the pSC-A-amp/kan vector supplied with the StrataClone PCR Cloning Kit TA cloning kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer’s instructions. Ligation mixtures were used to transform competent cells of *Escherichia coli*. A total of 470 positive clones including 188 from LW, 94 from GR and 188 from NF containing *dsrAB* gene fragments were picked randomly and transferred into 96-well plates containing LB medium, 0.1mg/ml of ampicillin and 10% glycerol, shaken for 24 h at 37 °C. Two set of subsamples (50 µl each) were stored at – 80 °C for further analyses. A small subset of 110 clones (from LW, GR and NF combined) was chosen for sequencing (Advanced Genetic Technologies Center, Lexington, KY).

**Phylogenetic analyses**

*dsrAB* gene sequences were submitted to the Ribosomal Database Project II (http://rdp8.cme.msu.edu/cgis/chimera) to detect the presence of chimeric artifacts. A BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST) of the GenBank database was conducted to identify the phylogenetic relationships of the recovered sequences. Nucleotide sequences were checked using Vector NTI Software (Life Technologies Corp., NY, USA) and manually curated. Sequences were aligned using MUSCLE and evolutionary distance was constructed by the neighbor-joining method (Saitou and Nei 1987). Bootstrap resampling analysis of 100 replicates was performed to estimate the confidence of tree topologies. The phylogenetic tree was constructed with MEGA (molecular evolutionary genetics analysis; http://www.megasoftware.net/index.html) software, version 5.0 (Kumar et al. 2004).

**Statistical Analysis**

Principal component analysis (PCA) was performed because it is a powerful technique for pattern recognition designed to transform original variables into uncorrelated axes (principal components).
(Chabukdhara and Nema 2012) without losing representativeness and not requiring to use a model (Cohen and Moerner 2007). PCA has been widely used to establish links among pollutant concentrations and environmental variables (Singh et al. 2004). Unrotated PCA and untransformed data was used for PCA (based on the Euclidean distance matrix) was used to explain geochemical parameters variance. One way- multiple analyses of variance (MANOVA) was used to determine if there were significant differences among sampling sites in organic matter, MC, TS, TC, TN, pH, total PAHs and total metals. We also calculated Pearson correlation coefficients for the relationships between organic matter, MC, TS, TC, TN, pH, PAHs, metals, bacteria and SRB. Nonmetric multidimensional scaling (NMDS) was used to compare T-RFLP fragment composition among sites and among cores. T-RFs community composition NMDS dimension scores were assessed as variables and correlated (using Spearman correlation coefficients) with total bacteria, sulfate, total PAHs, TN, TC, MC, pH, \( \sum \) metals and \( \sum \)PAHs to determine their influence on microbial community composition. We used the following formula to measure homologous coverage (C) in the clone library \( C = 1 - (n_1/N) \), where, \( n_1 \) is number of phylotypes that occurred only once in the clone library and \( N \) the total number of clones recovered (Jiang et al. 2009). We also estimated Shannon Wiener and Simpson diversity indexes and evenness (Begon et al. 1996) of TRFs fragments and \( dsrAB \) sequences at each site. Statistical analyses were performed using SPSS 12.0 for Windows.

Nucleotide sequence accession numbers

\( dsrAB \) nucleotide sequences determined in this study are currently being annotated to be deposited in GenBank.

Results

Soil geochemistry

The average pH of the deep sediments in LW and GR were similar (ranging from 6.8 to 7.3) while NF sediments exhibited values below neutral (Table 1). The highest moisture content (MC) in sediments was found in GR (~53%), followed by LW (~40%) and NF (~22%). No substantial differences among sites
in nitrogen content among core samples were observed. Average total carbon in LW and GR sediments were relatively similar (9%) and much higher (9X) than NF sediments. Sediment sulfate (ranging from 17 to 310 ug g⁻¹) showed high heterogeneity within and between cores and among sites. There was no particular trend with sediment depth and sulfate concentrations. PAH concentrations are described in more detail Johnston et al. (2014), and they were much higher in LW sediments than GR and NF sediments.

Some metal concentrations were particularly much higher (including Fe, Mn, Al and Zn) than other metals concentrations (Table 2). In general, most metals in LW and GR were 5X to 10X higher than metal concentrations in NF. Surprisingly, GR showed the highest concentrations for heavy metals including uranium. Arsenic was also much higher in each core in comparison to cores from Lowellville and Newton Falls.

One-way MANOVA of organic matter, pH, TS, TN, TC, MC, total PAHs, and individual metal concentrations revealed that there were significant differences among sites (Wilks’ Lambda = 0.000, \( F = 48.55, p=0.020 \)). To obtain information about the relationships among the environmental variables measured, unrotated PCA was performed for MC, TS, TN, TC, \( \sum \)PAHs, organic matter, pH, individual PAH concentrations, and individual metal concentrations of the three sampling sites. PCA yielded five PCs explaining 53%, 30%, 6%, 4% and 3% of the cumulative variance, respectively. In order to have clear visualization of the data trends, only the first two principal components are shown in Fig. 2. Geochemical parameter data segregated by site and by depth. The scores and loadings of the principal components for the three sites clearly discriminated by site corroborating results from the MANOVA, which consistently identified site differences between environmental parameters. Strong positive statistically significant Pearson correlations were found (Table 3) between total moisture and TC (r=0.888, p<0.01), organic matter (r=0.793, p<0.05) and metals (r=0.877, p<0.01) respectively. TN strongly correlated with moisture (r=0.929, p<0.01), TC (r=0.939, p<0.01), organic matter (r=0.927, p<0.01), pH (r=0.690, p<0.05), and total metals (r=0.959, p<0.01). TC strongly correlated with organic matter (r=0.900, p<0.01) and metals (r=0.894, p<0.01). Organic matter strongly correlated with pH (r=0.737, p<0.05) and metals (r=0.928, p<0.01).
Bacterial Abundance

Total bacteria estimated by DAPI enumeration varied from $1.5 \times 10^7$, $2.6 \times 10^7$, and $2.6 \times 10^7$ number of cells (per g dry weight) in LW, GR and NF respectively (Fig. 3). The abundance of bacteria did not increase with depth and differences were not statistically significant among sites. Enumeration of SRB by MPN revealed that the abundances of cultivated SRB in average were the highest in LW sediments and differed from GR and NF sediments ($3.8 \times 10^2$, $1.8 \times 10^2$, $1.5 \times 10^2$ respectively).

T-RFLP analysis for spatial variation of 16S rRNA gene diversity

Analysis of T-RFLP profiles differentiated the sediment samples according to their site (Fig. 4) according to the NMS plot. T-RFs from the three sites were not tightly clustered. The scatter plot mapped LW communities above the NMS1 axis while GR and NF communities were not clearly separated. A correlation matrix was used to examine the relationships between the geochemical and microbiological variables and the NMS axes (which represent the microbial communities) (Table 4). MC, TN, bacteria, and the majority of metals were highly negatively correlated with NMDS1. These results indicated that these variables were the main gradients of NMDS1. SRB, pH, depth (D), total PAHs, TC, Mn and Sr were highly positively correlated with NMDS2. The most abundant terminal restriction fragments (T-RFs) were found at LW (36) followed by NF (33) and GR (31). Accordingly, the Shannon Wiener index (Table 5) showed the highest at LW (H= 2.99), followed by NF (H=2.98) and GR (H= 2.61). Shannon Wiener evenness was similar for Lowellville and Newton Fall communities (as reflected in the number of T-RFs recovered from each site) while Girard had a lower evenness value. Similarly, the Simpson diversity indexes for LW and NF (13.97 and 14.98 respectively) were higher than Girard's Simpson diversity index (7.3). The same trend was observed for the Simpson evenness; higher values calculated for LW and NF (0.37 and 0.44 respectively) and much lower value for GR (0.24).

$dsrAB$ sediment community

Partial $dsrAB$ sequences were analyzed to identify sediment-associated SRB. Three clone libraries were constructed with LW, GR and NF screened sequences (26, 35 and 48 clones respectively), resulting in
58, 85 and 23% coverage (Table 6). Sequences grouped into 14, 7 and 40 operational taxonomic units [OTUs] (LW, GR and NF respectively) were closely related to uncultured SRB from a variety of contaminated environments including uranium-mining sites, a leachate-polluted aquifer, and sediment from polluted harbors. *dsrAB* clones did not match known sequences of SRB or SRB-affiliated sequences. Occurrences of OTUs at each site are presented in Table 7. As shown in Table 6, and based on the limited number of sequences, the Shannon Wiener diversity index calculated using the number of OTUs recovered from each site revealed that NF had the highest *dsrAB* diversity (3.62) followed by LW (2.14) and Girard (0.85). The same trend was observed for evenness values.

**Discussion**

This study employed the T-RFLP technique to assess bacterial community structure and diversity to test the existence of spatial heterogeneity in the overall bacteria and *dsrAB*-bearing communities. T-RFLP was chosen because it is a robust technique, is a reproducible method and allows for a fast analysis and monitoring of community structure (Osborn et al. 2000). We also measured SRB diversity based on the *dsrAB* gene since it has been used to characterize SRB and to investigate the diversity of SRB in a variety of complex sedimentary environments, including microbial mats, estuaries, and everglades (Foti et al. 2007; Dhillon et al. 2003). To our knowledge, this is the first study describing diversity of SRB in heavy-metal and PAH contaminated riverbank sediments.

The three sampling sites (LW, GR and NF) contained a different biogeochemical matrix. Heterogeneity of the sediments was reflected in a wide range of biogeochemical parameters. Some parameters did not show any particular trend among sites (TN and pH). Other variables, including MC, TC, PAHs, metals and sulfate, exhibited statistically significant differences among sites. Higher PAH concentrations were found at deeper sediments consistent with previous studies that indicated that deposition processes affected how sediments accumulated over time in contaminated sites (Amin and Jacobs 2013). In the same manner, high sulfate correlated with PAHs; this trend has been observed with other contaminated sediments, where levels of contamination were associated with high sulfate concentrations (Vishnivetskaya et al. 2011). Interestingly, total metal concentrations were also highly
correlated with total carbon. The Mahoning River supported for many years steel industries that contributed to the pollution in the riverbanks. Accordingly, oils and total hydrocarbons (not measured in this study) were always combined with metal wastes deposited in the river. Collectively, geochemical parameters did show a clear strong environmental gradient that might have contributed to bacterial community composition. Thus, analysis of bacterial communities using T-RFLPs and NMDS showed that high metal concentrations and PAH were highly associated with bacterial community structure. This was also observed in other studies where microbial communities seemed to be affected by the level of contamination (i.e. high concentrations of PAHs). Wang and Tam (2012) identified PAHs as the most important factor influencing bacterial sediment communities. Similarly, Ji et al. (2013) indicated that PAH contamination had the highest impact on bacterial community structure, where MC also had a secondary role. In fact, results from this study indicated that MC and pH also appeared to be responsible for bacterial community composition. Changes in soil moisture can affect differently and can vary between taxonomic groups (DeBruyn et al 2011) therefore resulting in changes in the community (Vinas et al. 2005). This suggests that soil physical–chemical factors do have a strong impact on composition of bacterial communities in these habitats.

The number of T-RFs recovered from each location did not vary significantly (ranging from 31 to 36) which was also reflected in diversity indexes. We found that bacterial diversity based on T-RFs appeared not to be influenced by the degree of contamination at each site since the number of T-RFs was similar. Lowellville and Girard bacterial communities were similar in composition, while the Newton Falls (with low contamination) community showed very similar diversity to Lowellville (high contamination). The detection of SRB via analysis of the functional marker gene dsrAB supported previous studies that indicated SRB as an important bacterial group in contaminated sediments (Zhang et al. 2008; Leloup et al. 2004). Interestingly, we recovered fewer sequences from the LW and GR sites (high metal contamination), while a higher number of OTUs were described for NF sediments (low metal contamination). dsrAB diversity estimated from the three sampling sites revealed that NF sediments had the highest diversity followed by LW and GR (Table 6). In agreement with this finding, Sitte et al. (2010) also concluded that dsrAB diversity was greatly affected by metal concentrations. Similarly Cabrera et al. (2006) reported toxic
effects of dissolved metals on SRB. Nevertheless, our findings should be taken cautiously because diversity indexes were calculated only with a small number of OTUs; in addition clone coverage for each site was also low indicating that the diversity in these sediments is much greater than what we were able to measure. What we can infer certainly is that from the limited data provided, the majority of sequences recovered were related to unknown SRB. This finding is consistent with reports from other contaminated environments including uranium mining tailings (Chang et al. 2001; Carlson et al. 2005) and metal-contaminated aquifers (Geets et al. 2006). Similarly Perez-Jimenez and Kerkhof (2005) reported that 20% of the \( \text{dsrAB} \) genes detected from sediments collected in 16 locations in four continents did not have affiliation in the GenBank database. Concurrently, culturable SRB abundance was within the same range as that in samples from uranium-contaminated subsurface sediments (Sitte et al. 2010). SRB communities under intense anthropogenic impact represented 50% of the bacterial population found in uncontaminated sites (Perez-Jimenez and Kerkhof 2005). Although detection of \( \text{dsrAB} \) sequences indicated the presence of SRB in these sediments, this does not provide evidence of active population; further study is needed to quantify their metabolic activity. In addition, 16S rRNA genes and \( \text{dsrAB} \) indexes do not provide information on the possible function that these communities have in the environment. Nevertheless, by integrating them with their surroundings, valuable information about their diversity and function can be elucidated.

Conclusion

Riparian zones that converge with contaminated riverbank sediments represent a microbial habitat important to the cycling of nutrient and pollutants in streams. To date, the majority of studies describing \( \text{dsrAB} \) communities have focused on estuaries and marine sediments (Jiang et al. 2009; Leloup et al. 2009). Fewer studies have attempted to describe effects of metals on \( \text{dsrAB} \) communities using pure cultures. Molecular characterization of \( \text{dsrAB} \)-bearing communities may have important implications for river restoration approaches, because without understanding of the ecology of heavily contaminated environments, implementing strategies for remediation will not be successful. Here, we used moderate resolution molecular techniques to demonstrate a correlation between bacterial community structure and
diversity in riverbank sediments and sedimentary metal contamination. The information presented provides insights for future experiments and improves our knowledge of long-term effects of metal contamination on river ecosystems on the impact on microbial communities.

References


United States Department of Agriculture (USDA). 2007. Laboratory Guidebook: Most Probable Number Procedure and Tables. Laboratory Quality Assurance Division (LQAD), Athens GA.


United States Environmental Protection Agency (USEPA) Microwave assisted acid digestion of aqueous samples and extracts Method 3015A.


Table 1 Averaged (n=9) geochemical parameters measured in riverbank sediments from three sites along the Mahoning River: Lowellville (LW), Girard (GR) and Newton Falls (NF)

<table>
<thead>
<tr>
<th>Site</th>
<th>Core</th>
<th>Latitude/Longitude</th>
<th>MC %</th>
<th>TS (µg/g)</th>
<th>TN %</th>
<th>TC %</th>
<th>Total PAHs (µg/kg)</th>
<th>Depth (m)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowellville</td>
<td>LW1</td>
<td>41° 2’18.42”N/80° 32’28.7”W</td>
<td>38.2</td>
<td>309.6</td>
<td>0.2</td>
<td>8.9</td>
<td>416290.2</td>
<td>2.7</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>LW2</td>
<td>41° 2’17.71”N/80° 32’24.75”W</td>
<td>42.9</td>
<td>129.2</td>
<td>0.2</td>
<td>10.2</td>
<td>218037.4</td>
<td>2.4</td>
<td>7.2</td>
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<td></td>
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<td>36.0</td>
<td>25.0</td>
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<td>6.9</td>
<td>122275.0</td>
<td>2.1</td>
<td>7.3</td>
</tr>
<tr>
<td>Girard</td>
<td>GR1</td>
<td>41° 9’18.06”N/80°42’23.5”W</td>
<td>50.2</td>
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<td>Newton Falls</td>
<td>NF1</td>
<td>41° 8’6.59”N/ 80°58’1.96”W</td>
<td>20.4</td>
<td>25.1</td>
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<td>1.0</td>
<td>102451.8</td>
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<td>6.6</td>
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</table>
Table 2 Metal concentrations expressed in µg/g dry weight measured in riverbanks from three sites along the Mahoning River: Lowellville (LW), Girard (GR) and Newton Falls (NF)

<table>
<thead>
<tr>
<th>Site</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
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<th>Mean</th>
<th>SD</th>
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<th>SD</th>
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<td>2.1</td>
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Table 2 Metal concentrations expressed in µg/g dry weight measured in riverbanks from three sites along the Mahoning River: Lowellville (LW), Girard (GR) and Newton Falls (NF)
Table 3 Correlations between geochemical parameters measured in riverbank sediments from three sites along the Mahoning River: Lowellville, Girard and Newton Falls. Significant correlation coefficients are underlined (double at the 0.01 level, single at the 0.05 level)

<table>
<thead>
<tr>
<th></th>
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<th>TC</th>
<th>TS</th>
<th>pH</th>
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Table 4 Correlation matrix between environmental conditions and bacterial communities (represented by NMS dimensions 1 and 2). Significant negative correlations in bold and positive correlations underlined.

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Table 5 Diversity indexes based on the number of terminal restriction fragment (T-RFs) recovered from riverbank sediments at Lowellville, Girard and Newton Falls

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<th>Lowellville</th>
<th>Girard</th>
<th>Newton Falls</th>
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<tr>
<td>Depth (m)</td>
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<td>No of T-RFs</td>
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Table 6 Diversity indexes based on dsrAB gene fragments recovered from riverbank sediments at Lowellville, Girard and Newton Falls

<table>
<thead>
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<th>Site</th>
<th>Lowellville</th>
<th>Girard</th>
<th>Newton Falls</th>
</tr>
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<tbody>
<tr>
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<tr>
<td>No of OTUs</td>
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Table 7 Distribution of *dsrAB* sequences recovered from riverbank sediments at three sites along the Mahoning River: Lowellville, Girard and Newton Falls

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<th>Newton Falls</th>
<th>Total number of occurrences</th>
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Fig. 1 Sampling sites along the Mahoning River. Three sediment cores at three different depths were taken at each site: Lowellville (high contamination), Girard (high contamination) and Newton Falls (low contamination)
Fig. 2 Principal components PC1 and PC2 extracted from environmental parameters explaining 53% and 30% of the variation in the data respectively. Data segregated by site Lowellville (blue), Girard (orange) and Newton Falls (green). Vectors represent environmental parameters measured at each site.
Fig. 3 Abundance of bacterial cells and sulfate reducing bacteria (clear and filled symbols respectively) enumerated from riverbank sediments along the Mahoning River: Lowellville (circles), Girard (triangles) and Newton Falls (squares)
Fig. 4 NMDS plot constructed with terminal restriction fragments (T-RFs) recovered from Lowellville (blue), Girard (orange) and Newton Falls (green). T-RFs that significantly correlated with NMS dimensions are shown as vectors.
Fig. 5 T-RFLP based diversity indexes and evenness measured in Lowellville (LW), Girard (GR) and Newton Falls (NF): Shannon Wiener (H) filled triangles, Shannon Wiener evenness (E-H) clear triangles, Simpson (D) filled squares and Simpson evenness (E-D) clear squares
Fig. 6 Phylogenetic tree of bacterial dsrAB gene sequences retrieved from PAH-metal contaminated riverbank sediments in Girard along the Mahoning River. The tree was constructed using the neighbor-joining method. Bootstrap values (in %) are based on 100 replicates each (distance and minimal evolution) and are shown at the nodes with > 50% bootstrap support.
Fig. 7 Phylogenetic tree of bacterial \textit{dsrAB} gene sequences retrieved from PAH-metal contaminated riverbank sediments in Newton Falls along the Mahoning River. The tree was constructed using the neighbor-joining method. Bootstrap values (in %) are based on 100 replicates each (distance and minimal evolution) and are shown at the nodes with > 50% bootstrap support.
CHAPTER V: EFFECT OF ANTHRAQUINONE-2,6-DISULFONATE [AQDS] ON DEGRADATION OF PHENANTHRENE AND FLUORENE BY INDIGENOUS MICROBIAL COMMUNITIES FROM CONTAMINATED SEDIMENTS

Abstract

Humic substances are one of the most abundant organic compounds in any ecosystem on Earth. In addition to serving as a relatively recalcitrant pool of organic matter, humics can be used as terminal electron acceptors during anaerobic microbial degradation of pollutants. Anthraquinone-2,6-disulfoante (AQDS), a humic analogue, has been linked to degradation of toxics in the environment, and humic-respiring microbes are widely distributed in soils and sediments, including contaminated ecosystems. In this study, we report enhanced anaerobic degradation of phenanthrene and fluorene when AQDS was used as an acceptor. Moreover, detection and enumeration of indigenous anaerobic PAH degrading bacteria isolated from riverbank sediments indicated that microbial communities were responsible for PAH degradation, suggesting that there is a potential for using humic compounds in bioremediation of PAH-contaminated ecosystems. This is the first report that indicates anaerobic degradation of PAHs by addition of AQDS. Information gathered in this study may be of potential use for the remediation of similar contaminated sites elsewhere.

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5 This chapter is in preparation and will be submitted to the Biodegradation Journal; the author list is as follows: Gloria P. Johnston, David Lineman, Jessica Drohn, Laura G. Leff and Carl G. Johnston. Dr. Johnston (Youngstown State University-Biology Dept.) provided laboratory space and equipment. Ms. Drohn (Youngstown State University undergraduate) helped with GC-MS analyses and anaerobic culturing at Youngstown State University. Dr. Leff provided guidance in selecting microbiological methods for culturing as well as general advice during the study. I was responsible for sediment collection, sediment analyses, experimental study, as well as all statistical analyses and writing.
**Introduction**

Humic substances are complex, ubiquitous organic compounds of high molecular mass that comprise 80% of organic matter in soils and sediments (Mulholland 2003). By accepting and donating electrons exogenously, humic substances play an important role in oxidation of organic materials when Fe(III) or Mn(IV) are abundant (Liu et al. 2007). Humics can act as reducing agents of Fe(III) to Fe(II), and also can be oxidized and used as terminal electron acceptors for respiration (Cervantes et al. 2000).

The role of humics as electron shuttling compounds has been elucidated by studies of the humic model compound, anthraquinone-2,6-disulfonate [AQDS] (Lovley 2000). Studies with dissimilatory iron reducing bacteria (e.g. *Geobacter* spp) showed that microbial cells can reduce AQDS (oxidized form) to AQDSH$_2$ which then shuttles electrons to Fe(III) oxides. Fe(III) oxides can be reduced by different terminal electron acceptors, such as fumarate (Lovley 1999), regenerating AQDS (Lovley et al. 1996).

Humic respiring bacteria have been identified from a variety of marine and freshwater ecosystems and are mostly iron reducers (Lovley 2000) related to the Geobacteriaceae (Coates et al. 1998); these organisms are also capable of reducing AQDS. For example, iron-reducers, such as the facultative anaerobe *Pantoea agglomerans* SP1, can use AQDS as an alternative electron acceptor for anaerobic oxidation of acetate and hydrogen (Francis et al. 2000) while *Deinococcus radiodurans*, can reduce AQDS with lactate as the substrate (Fredrikson et al. 2000). Fermentative bacteria, including *Propionibacterium freudenreichii*, *Lactococcus lactis*, and *Enterococcus cecorum* (Benz et al. 1998), as well as Gammaproteobacteria, *Shewanella* spp, sulfate reducing bacteria (SRB) and some methanogenic bacteria (Cervantes et al. 2002) can use humic acids as terminal electron acceptors under anaerobic conditions.

Research suggests that electron shuttling by humic compounds is an important process for Fe(III) reduction in sediments (Cervantes et al. 2008). Because of their higher solubility, quinones establish better contact with insoluble Fe(III) oxides making them more available to bacteria (Lovley et al. 1996). In addition, humics can improve degradation of contaminants in the environment by increasing bioavailability of pollutants with low solubility (Luu and Ramsay 2003). For instance, mineralization of vinyl chloride and dichloroethene was achieved, with addition of AQDS, by a humics-respiring consortium from streambed sediments (Bradley et al. 1998). Pentachlorophenol PCP dechlorination rates were enhanced by addition of
AQDS to indigenous microbial communities from soils; response was attributed to dechlorinating and iron reducing bacteria (Chen et al. 2010).

Degradation of polycyclic aromatic hydrocarbons (PAHs) using humic substances has been reported under aerobic conditions by Ortega-Calvo and Saiz-Jimenez (1998) and Haderlein et al. (2001). In contrast, under anoxic conditions, only one study has been reported which demonstrated phenanthrene degradation by the humic-reducing bacterium, *Pseudomonas aeruginosa* strain PAH-1 when AQDS was the sole electron acceptor (Ma et al. 2011). Apart from these studies, there is no other information on anaerobic degradation of PAHs using AQDS.

PAHs are toxic and carcinogenic hence research on their environmental fate and remediation is of interest. Although microbial degradation is more extensive under aerobic conditions, contaminated sites are usually anaerobic (Johnston and Johnston 2012); more importantly in the environment, the interaction of Fe(III) and humics are of higher complexity than in pure cultures (Luu and Ramsay 2003). Thus, approaches that include indigenous microbial communities in the anaerobic degradation of PAH are needed and provide fundamental information for bioremediation.

Because humic compounds are ubiquitous in natural systems their potential application in bioremediation of pollutants is of great interest. In addition, electron shuttling through humic compounds is a key process in sediments or soils with high organic matter content (Luu and Ramsay 2003). To determine the potential for humic substances to aide in remediation of PAHs, we investigated the role of AQDS in anaerobic degradation of two PAHs, fluorene and phenanthrene, by indigenous bacterial communities from riverbank sediments rich in organic matter and with high PAH and iron oxides concentrations. We hypothesized that addition of AQDS would promote electron shuttling therefore increasing fluorene and phenanthrene disappearance. We chose deuterated fluorene and phenanthrene as markers to measure degradation over time. We also enumerated and cultured PAH-degrading bacteria under anaerobic conditions.
Materials and Methods

In August 2012, sediment samples were collected from the Mahoning River at Lowellville, Ohio. We chose this site because of high concentrations of PAHs (Johnston et al. 2014). We collected sediments using a manual auger core device at 2.3 m depth from the riverbank sediments. Sediments were collected anaerobically and kept on ice until laboratory analysis. In the laboratory sediments were homogenized in a glove bag under nitrogen. Biogeochemical parameters including sulfate, total PAHs, moisture content, organic matter content and pH were measured to establish sediment properties. Moisture content and organic matter were determined by standard methods for soils (Carter 2000). Sulfate was measured by extracting 2 g of sediment with 0.016 M Ca(H₂PO₄)₂·H₂O 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). 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).

Glassware for bacterial analyses

All glassware used for the experiments was sterilized at 121 °C for 15 min. Ultrapure filtered (0.2-µm) and autoclaved water was used for dilutions and to prepare solutions.

Bacterial enrichment

Experiments were performed in triplicate sacrificial 100 mL serum bottles containing 5 mL of mineral salt medium (MSM), 1 g of sediment, and 25 mg/ml of a mixture of the two deuterated PAHs (dPAHs), phenanthrene and fluorene (Table 1). dPAHs are heavier isotopes that have a similar fate as their respective undeuterated analogues (Gomez-Eyles et al. 2010) and can be used as tracers instead of using radiolabeled PAHs. Physical properties of fluorene and phenanthrene are shown in Table 2.

To evaluate anaerobic degradation of fluorene and phenanthrene, three experimental conditions were included: i) to evaluate the effect of AQDS sediment slurries were amended only with AQDS (final concentration 20mM); ii) to evaluate the effect of sulfate and AQDS sediment slurries were amended with
AQDS (20mM) and sulfate (30mM) final concentrations; iii) to evaluate natural attenuation sediment slurries did not receive any amendment.

The experimental medium (MSM) added to all experimental conditions consisted of (in g/L):

\[
\begin{align*}
\text{MgCl}_2.4\text{H}_2\text{O}, & \quad 0.25; \\
\text{CaCl}_2.4\text{H}_2\text{O}, & \quad 0.1; \\
\text{FeCl}_2.4\text{H}_2\text{O}, & \quad 0.25; \\
\text{K}_2\text{HPO}_4, & \quad 0.1; \\
\text{KH}_2\text{PO}_4, & \quad 0.1; \\
\text{(NH}_4\text{)}_2\text{Mo}_7\text{O}_{24}.4\text{H}_2\text{O}, & \quad 0.01; \\
\text{NH}_4\text{NO}_3, & \quad 0.2; \\
\text{MgSO}_4.7\text{H}_2\text{O}, & \quad 0.25. \\
\end{align*}
\]

pH was adjusted to 7.0 before autoclaving.

All sediment mixtures were purged with high purity nitrogen several times in an anaerobic glove bag to achieve complete anaerobiosis. Fluorene and phenanthrene were added into the incubation bottles under nitrogen. Immediately after addition of the PAHs, bottles were sealed with PTFE rubber stoppers, crimped with aluminum caps, and homogenized by vortexing for 1 min. Serum bottles were incubated anaerobically using a GazPak EZ Pouch System (BD Technologies, Franklin Lakes, NJ) at 25 °C for 40 days in the dark. The bottle contents were sacrificially sampled after 0, 7, 22, and 40 days. At each time point, as described below the bacterial community was examined and PAH concentrations measured. The oxygen indicator of the GazPak system was continuously monitored to ensure anaerobic conditions were maintained over the length of the experiment.

Bacterial community

Culturable heterotrophic bacteria were enumerated from sediment slurries from the unamended natural attenuation treatment on day 40. Ten-fold serial dilutions were conducted using 1 ml of slurry and 9 ml of 0.2 µm filtered autoclaved water. Nutrient agar plates (Difco) were incubated at 30 °C from 48 to 72 hrs and colony forming units (CFUs) were recorded (Method 9215, APHA 1999).

To determine the total number of bacteria, 1 ml of sediment slurry (from natural attenuation treatment on day 40) was preserved with equal parts of paraformaldehyde (8% final concentration) and phosphate buffered saline. Samples were vortexed, sonicated for 10 minutes at low frequency (VWR Ultrasonics Cleaner, PA), and centrifuged for 30 sec at 1000 rpm. Samples were filtered with 0.2-µm (25 mm) pore black polycarbonate filters (Poretics, Livermore, CA), stained with 4,6- diamidino-2-phenylindole (DAPI; 1 mg /ml final concentration) for 5 min, and rinsed with filtered autoclaved water
An epifluorescence microscope (Olympus DP71, PA, USA) was used to count bacteria in 10 randomly selected fields.

We enumerated sulfate-reducing bacteria (SRB) by the most probable number (MPN) technique from sediment slurries. The SRB medium of Fortin et al. (1998) consisted of (in g/L): Bacto™ Tryptone, 10, 5.9 mL 60% Na-lactate, MgSO$_4$·7H$_2$O, 2.0, FeSO$_4$·7H$_2$O, 0.5, Na$_2$SO$_3$, 0.5, ascorbic acid, 0.5, and thioglycollic acid, 0.5. The pH was adjusted to approximately 7. Medium was autoclaved in MPN tubes, vials were allowed to cool, and degassed under nitrogen before inoculation with sediment slurry. A three-tube MPN dilution was performed for each sample. Negative controls received filtered autoclaved water as inoculum. Growth (indicated by presence of black precipitates) was scored using statistical tables (USDA 2007).

Enumeration of PAH degraders by most probable number

We enumerated PAH degraders (as established by Haines et al. 1996) from a single sediment slurry incubation bottle under natural attenuation conditions on day 40 only. A subsample (1 ml) of the slurry was used in ten-fold serial dilutions using sterile glass culture tubes and mineral salt medium (as described above). Undeuterated PAHs were dissolved in hexane (5 mg/ml) and stored in amber bottles to protect against photo-oxidation. Dissolved phenanthrene, fluorene, and pyrene (20 µl) were added into separate microtiter plates to avoid cross-contamination through the gas-phase (Johnsen et al. 2002). A plate containing 20 µl hexane was used as a negative control. Microtiter plates were placed in a laminar flow hood to allow for evaporation so PAHs could form a crystal layer on each well. Coated plates were inoculated (200 µl) from serial dilutions in microtiter wells followed by anaerobic incubation using a GazPak EZ Pouch System (BD Technologies, Franklin Lakes, NJ) at 25 °C for four weeks. Growth-positive wells were determined by addition of 50 µl of tetrazolium salt (p-iodonitrotetrazolium violet-INT- (3mg/ml) after a 24-hr incubation at 25 °C. Positive wells (by presence of red/orange coloration) were scored using most probable number statistical tables (USDA, 2007).
Cultivation of PAH degraders by spray plate technique

We cultured phenanthrene, naphthalene and pyrene degraders from a single sediment slurry incubation bottle under natural attenuation treatment on day 40 only. A spray plate assay was used to assess the abundance of culturable PAH-degrading bacteria (Srujana and Khan 2012). Phenanthrene (C_{14}H_{10}), naphthalene (C_{10}H_{8}) and pyrene (C_{16}H_{10}) were dissolved in hexane (ultra pure) to a final concentration of 5 mg/ml. Phenanthrene, naphthalene, pyrene and hexane (control plates) were sprayed onto a solid agar medium containing either a low carbon media (regular nutrient agar media with only 1/10 of nutrient ingredients) or R2A medium. The PAHs and hexane were allowed to evaporate under a sterile hood to form a crystal layer. A subsample (1 ml) of the slurry was used in ten-fold serial dilutions using sterile glass culture tubes and mineral salt medium (as described above). After inoculating by streak plating, bacteria were incubated anaerobically using a GazPak EZ Pouch System (BD Technologies, Franklin Lakes, NJ). Petri dishes (per triplicate) in their respective anaerobic bags (one per each PAH) were placed at 25 °C in the dark for 60 days. Growth was monitored weekly. PAH degraders were visualized by formation of a distinctive clearing zone surrounding individual colonies. Some morphological characteristics of individual colonies were examined to determine the shape of cells, colony morphology and color.

PAH extraction and analysis

Chemicals and glassware

All reagents used for the chemical analysis were of HPLC grade including dfluorene (purity >99%), dphenanthrene (purity >96%), fluorene (purity >99%), phenanthrene (purity >99%), pyrene (purity >99%), dichloromethane (DCM) (purity >99.5%) and hexane (purity >97%), and were purchased from Sigma–Aldrich Corporation (St. Louis, MO). The stock solutions of both dfluorene and dphenanthrene were prepared in hexane with a concentration of 25 g/L. Glassware used for PAH extraction was rinsed several times using DCM.

PAHs were extracted by a two-stage modified Bligh Dyer method as in Pratt et al. (2012). In the first stage, dichloromethane (DCM) and methanol (1:2 v/v) were used as extraction solvents including a surrogate standard mix (Restek, Bellefonte, PA) followed by sonication (VWR Ultrasonics Cleaner, PA)
for three 10-min pulses at low frequency. In the second stage, DCM and ultrapure water (18.2 MΩ cm) (1:1 v/v) were used to separate the organic portion of the mixture followed by incubation at 4 °C overnight. The next day, the organic layer was separated using a solid phase extraction (SPE) system (Supelco, Sigma Aldrich, St Louis, MO) with Na₂SO₄ as the drying agent and DCM as the solvent. Extraction efficiencies were estimated by recording DCM volumes before and after extraction procedure. Samples were concentrated under a flow of nitrogen at 37 °C before quantification.

Gas chromatography–mass spectrometry (GC-MS)

To quantify fluorene and phenanthrene disappearance the GC-MS analyses were performed on a Saturn 2000 (Varian-Agilent, Wilmington, DE). The chromatographic column was a 30m, 0.25 mm inner diameter, 0.25 lm film thickness HP-5MS capillary column (#19091s-433, Agilent Technologies, Santa Clara, CA). Helium was used as the carrier gas at a constant flow rate of 1.2 mL per min. The injector port was held at 275 °C, with an initial oven temperature of 50 °C. The oven was held at 50 °C for 5 min, and then ramped at 10 °C/min to a final temperature of 315 °C, and then held for 2.44 min. The MS was operated in the electron impact mode at 70 eV. The ion source temperature was held at 275°C. The mass spectra were recorded at a scan mode covering the range of 60–650 mass units for sediment samples. A 1ml injection of each sample extract was made in splitless mode, using an A200S autosampler (IET, Vernon Hills, Illinois). Quantification was performed using naphthalene d-8 as an internal standard. Retention time and the identity of PAHs were confirmed using PAH standards.

Statistical analyses

MANOVA was performed to determine statistical significance between experimental conditions using day and treatment as fixed factors followed by One-way ANOVA of the day*treatment interaction as one factor. Statistics were performed using SPSS 12.0 for Windows.
**Results**

Sediments were characterized in terms of sulfate concentrations, organic matter content, moisture content, total PAHs, and pH (Table 3). As previously reported (Johnston and Leff 2014) high sulfate and PAH concentrations characterized these sediments. Organic matter content and pH were similar to other contaminated sediments elsewhere (Guo et al. 2007). Moisture content was similar to previous values reported for these sediments (Johnston and Leff 2014). Sediments used in the slurry experiment were consistently oily and mostly characterized by silt and clay as expected (Amin and Jacobs 2013).

Enumeration of total bacteria yielded values ranging from $8.7 \times 10^7$ to $2.6 \times 10^8$ cells/g dry weight. Total heterotrophic bacteria ranged from $2 \times 10^6$ to $3 \times 10^6$ while SRB enumerated by the MPN technique were $1 \times 10^4$ cells/g (Fig. 1). Two approaches were used to determine if bacteria from sediment slurries had PAH-degrading capabilities. By the modified MPN technique, we found that fluorene degrading bacteria had the greatest abundance ($6 \times 10^3$ cells/g) followed by phenanthrene degrading bacteria ($3 \times 10^3$ cells/g) and pyrene degrading bacteria ($1 \times 10^2$ cells/g). Our second approach was based on a spray plate technique to identify colony-forming units of PAH-degrading bacteria using two types of media. Growth and clearing zone formation was observed on LC media for phenanthrene degraders after 30 days and 25 days (Table 4) while pyrene degraders showed some growth and clearing after 21 days on R2A media. There was no clear zone formation (indicative of PAH utilization) for pyrene and naphthalene using LC media and for phenanthrene and naphthalene using R2A media. Morphological characterization of the phenanthrene degrading colonies isolated from sediments (Table 4) suggested that our isolates might be related to similar phenanthrene degrading bacteria as described by Um et al. (2010).

**Anaerobic incubations with of fluorene and phenanthrene**

Fluorene and phenanthrene concentrations decreased under each experimental condition over the 40-days anaerobic incubation (Fig. 2 and 3). Degradation of PAHs was calculated based on their total concentrations (PAHs remaining in the aqueous slurry sediment after incubation for 0, 7, 22 and 40-days). The concentration of fluorene (initial = 6.8 µg/ml) remained almost constant in the natural attenuation experiment and did not show a significant ($p>0.05$) decrease (final =6.3 µg/ml) during the incubation
period. In contrast, phenanthrene under natural attenuation conditions did show a slight decrease from 4.0 µg/ml to 2.9 µg/ml, which corresponded to 25% disappearance (Table 5), but was not significant (p>0.05).

In the incubations with AQDS and sulfate, fluorene dropped significantly from 9 µg/ml to 4.7 µg/ml, which corresponded to 48% disappearance (one-way ANOVA, p<0.000) while phenanthrene concentration decreased from 4.7 µg/ml to 1.9 µg/ml indicating a 59% disappearance (one-way ANOVA, p<0.000) (Fig. 3). The highest decrease in concentrations of fluorene and phenanthrene was obtained by the addition of AQDS only. Fluorene with an initial measured concentration of 9.9 µg/ml decreased over time to 5.4 µg/ml (45% decrease) (one-way ANOVA, p<0.000); in the same manner phenanthrene with an initial concentration of 7.4 µg/ml decreased to 2.5 µg/ml (66% decrease) over the 40-day anaerobic incubation (one-way ANOVA p<0.000). In general, fluorene and phenanthrene rapidly decreased from 0 to 22 d followed by a slower decrease from 22 to 40 d.

Discussion

PAHs are arguably the most common pollutants in a variety of aquatic environments. PAHs are diverse and those with lower molecular weight are more easily degraded than those with high molecular weight are more recalcitrant and require more time to be degraded.

Previous studies of anaerobic biodegradation of PAHs have used pure or mixed cultures under sulfate and nitrate reducing conditions (Rothermich et al. 2002; Coates et al. 2001). However, this does not represent realistic environmental conditions and does not consider the influence of indigenous microorganisms and their interactions (Thavamani et al. 2012). Moreover, only one study has focused on degradation of PAHs with humic substances as electron acceptors (Ramsay et al. 2005). Humics are abundant and can serve as terminal electron acceptors in microbial respiration (Van Trump et al. 2006). Furthermore, through electron shuttling humics may be key compounds in anaerobic biodegradation of organic pollutants including PAHs (Ma et al. 2011). In this study, we measured the effect of AQDS (electron shuttle) on the anaerobic degradation of fluorene and phenanthrene. We detected enhanced PAH degradation in sediments with AQDS added compared to sediments with sulfate and AQDS and sediments
under natural attenuation conditions. Recovery of PAH-degrading bacteria by culturing suggests that indigenous microbial communities might have played an important role in the biodegradation.

Experiments were carried out under closed, anaerobic conditions in the dark to minimize other possible pathways of PAH disappearance (photooxidation, volatilization, chemical oxidation). Results from the anaerobic incubations indicated that biodegradation of fluorene and phenanthrene did occur under each of the three experimental treatments (AQDS only, AQDS and sulfate and natural attenuation). Because extreme care was taken during the experiment, we ruled out that other pathways of PAH disappearance might have occurred. Dark conditions were provided to avoid photooxidation, and anaerobiosis was ensured to minimize volatilization. We did not detect intermediate metabolites of fluorene and phenanthrene degradation (p-cresol and phenol) yet their concentrations decreased over time under three experimental conditions confirmed biotic degradation process. Because only marginal increases in PAH degradation have been observed with addition of organic carbon sources (Thavamani et al. 2012) we did not include any carbon addition as a source of electrons since we expected that the microbial communities could use PAHs as the sole carbon sources. Sediment slurries with spiked deuterated PAHs were chosen as the approach since previous attempts using only sediment were not successful in large part due to heterogeneity in the concentrations of PAHs (data not shown). Our preliminary experiment using sediment only (no liquid added) resulted in high heterogeneity presumably because of limited mixing of sediment with the PAHs added, which did not allow us to quantify disappearance over time. In that study, we did determine that sulfate may be used as terminal electron acceptor because of its disappearance over 131-days experimental anaerobic conditions.

The indigenous microbial community was able to use preferentially AQDS as source of energy and this was reflected in the use of fluorene and phenanthrene as carbon sources (Fig. 2 and 3). The slurry type incubations we performed did not allow us to measure AQDS reduction, which is usually indicated by the color change of the culture liquid from colorless to yellow (Ma et al. 2011). However, since there was no other addition of either organic substrate or carbon source, we inferred that degradation of fluorene and phenanthrene was enhanced by addition of AQDS. Similarly but to a lesser degree, the addition of both AQDS and sulfate together also stimulated the microbial communities and therefore fluorene and
phenanthrene degradation. This suggests that sulfate did not further enhance respiration in support of PAH degradation although present in abundance. In agreement with Cervantes et al. (2008), AQDS respiration was preferred over sulfate during anaerobic oxidation of PAHs. The high concentration of AQDS (20mM) might have inhibited use of other alternative electron acceptors including methane (Cervantes et al. 2000). For this microbial community, AQDS appeared to be more favorable than sulfate under the AQDS-sulfate conditions. In contrast, under natural attenuation conditions no major stimulation occurred. Microbial communities might be initially limited by carbon availability, as the sediment received more bioavailable dPAHs, a slight decrease in both fluorene and phenanthrene was observed, indicating that microbes have the potential to use PAH as carbon sources. Nevertheless, phenanthrene degradation was much higher than fluorene degradation. Fluorene, as well as other PAHs with a pentane ring in the middle (Table 1) tends to resists complete microbial attack (Thavamani et al. 2012) making biodegradation more difficult and reflected in lower degradation rates over time.

Microbial-mediated reduction of humic material is related to anaerobic biodegradation of organic or inorganic compounds (Wang et al. 2009). AQDS (and humic substances) can be utilized by microbes as efficient electron acceptors or shuttles for the degradation of organic pollutants including vinyl chloride, 1,2-dichloroethylene, toluene (Cervantes and Dijksma 2001; Cervantes et al. 2000) and also for degradation of complex molecules such the explosive XRD (Kwon and Finneran 2008). Therefore, in rich with organic matter, anaerobic sediments where humics are abundant (such as the Mahoning River), it can be possible that degradation of both PAHs was also likely performed by humic-reducing microbes. Humic-reducers are phylogenetically diverse and some are also SRB such as Desulforomonas acetexigens (Lovley et al. 1998). In fact, SRB may be involved in the biotransformation of fluorene and phenanthrene under AQDS+sulfate conditions. This was supported by the relative high number of SRB found in the sediments used in this study (Fig. 1) and with other studies indicating that SRB play a major microbial role in the anaerobic degradation of PAHs (Coates et al. 1996; Chang et al. 2008). It is also plausible that, along with humic reducers and SRB, other more specialized groups of bacteria were responsible for phenanthrene and fluorene degradation. For instance, phenanthrene and pyrene degradation rates were stimulated by a strain relatively close to Clostridium pasteur, which used both PAHs as carbon sources (Yuan et al. 2007).
Although in relatively lower abundances, we detected phenanthrene, fluorene and pyrene degraders that used PAHs as sole carbon sources. Collectively, these results suggested that anaerobic degradation of fluorene and phenanthrene was indeed a biotic process.

Conclusions

The addition of AQDS to stimulate the anaerobic biodegradation of PAHs is a promising approach. Humic reducing bacteria are found in a variety of ecosystems and are phylogenetically diverse, even in contaminated environments. Our results suggest that microbial reduction of AQDS was responsible for higher fluorene and phenanthrene degradation compared to natural attenuation conditions. We conclude that indigenous microbial communities showed the potential for anaerobically PAH degradation, which can be enhanced by AQDS addition.

References


United States Department of Agriculture (USDA). 2007. Laboratory Guidebook: Most Probable Number Procedure and Tables. Laboratory Quality Assurance Division (LQAD), Athens GA.


Table 1 Experimental conditions used in the fluorene and phenanthrene degradation study

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>PAHs Compounds (mg/ml)</th>
<th>MSM (ml)</th>
<th>Sulfate (mM)</th>
<th>AQDS (mM)</th>
<th>No. reps</th>
<th>Analyses days</th>
<th>No. of sacrificial incubations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural attenuation</td>
<td>Flu &amp; Phen</td>
<td>25</td>
<td>5</td>
<td>_____</td>
<td>3</td>
<td>0, 7, 22, 40</td>
<td>15</td>
</tr>
<tr>
<td>+ AQDS</td>
<td>Flu &amp; Phen</td>
<td>25</td>
<td>5</td>
<td>_____</td>
<td>20</td>
<td>0, 7, 22, 40</td>
<td>12</td>
</tr>
<tr>
<td>+ AQDS + Sulfate</td>
<td>Flu &amp; Phen</td>
<td>25</td>
<td>5</td>
<td>30</td>
<td>20</td>
<td>0, 7, 22, 40</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 2 Structures and properties of the PAHs used in the degradation study

<table>
<thead>
<tr>
<th>PAH</th>
<th>Structure</th>
<th>Molecular weight</th>
<th>Log Kow</th>
<th>Boiling point</th>
<th>Melting point</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorene</td>
<td><img src="image" alt="Fluorene Structure" /></td>
<td>176.3</td>
<td>4.2</td>
<td>295 °C</td>
<td>116 °C</td>
<td>1.9 mg/L</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td><img src="image" alt="Phenanthrene Structure" /></td>
<td>188.3</td>
<td>4.6</td>
<td>340 °C</td>
<td>100 °C</td>
<td>1.6 mg/L</td>
</tr>
</tbody>
</table>

Table 3 Physicochemical characteristics indicating average values and standard deviations (SD) in brackets of sediments used in the degradation study

<table>
<thead>
<tr>
<th>Sediment characteristics</th>
<th>Average (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.2 (0.4)</td>
</tr>
<tr>
<td>Total PAHs</td>
<td>218,000 µg/g (120,000)</td>
</tr>
<tr>
<td>Moisture content</td>
<td>49 % (7.69)</td>
</tr>
<tr>
<td>Sulfate concentration</td>
<td>45 µg/g (5.1)</td>
</tr>
<tr>
<td>Organic matter</td>
<td>12 % (0.25)</td>
</tr>
</tbody>
</table>
Table 4 Phenanthrene (Phen), pyrene (Pyr) and naphthalene (Naph) degrading bacteria grown from a single sediment slurry from the Mahoning riverbanks on two types of media low carbon (LC) and R2A

<table>
<thead>
<tr>
<th>Media</th>
<th>Growth on PAH</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC</td>
<td>R2A</td>
<td>Phen</td>
<td>Pyr</td>
<td>Naph</td>
<td>Phen</td>
</tr>
<tr>
<td>Colony morphology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow, convex, 0.5 mm circular</td>
<td>25+++</td>
<td>60-</td>
<td>60-</td>
<td>60-</td>
<td>21+</td>
<td>60-</td>
</tr>
<tr>
<td>Pale white transparent, 1mm circular</td>
<td>30+</td>
<td>60-</td>
<td>60-</td>
<td>60-</td>
<td>60-</td>
<td>60-</td>
</tr>
</tbody>
</table>

Time needed for clear zone formation (day) and abundance of the clear surrounding each colony: (-) no clear zone, (+) clear zone with radius smaller than colony itself (++) clear zone with radius larger than colony itself

Table 5 Percentage of fluorene and phenanthrene disappearance under anaerobic conditions over 40-days period

<table>
<thead>
<tr>
<th>PAH</th>
<th>0 days Fluorene (µg/ml)</th>
<th>40 days Fluorene (µg/ml)</th>
<th>0 days Phenanthrene (µg/ml)</th>
<th>40 days Phenanthrene (µg/ml)</th>
<th>% Disappearance Fluorene</th>
<th>% Disappearance Phenanthrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural attenuation</td>
<td>6.8</td>
<td>6.3</td>
<td>4.0</td>
<td>2.9</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td>AQDS + sulfate</td>
<td>9.0</td>
<td>4.7</td>
<td>4.7</td>
<td>1.9</td>
<td>48</td>
<td>59</td>
</tr>
<tr>
<td>AQDS</td>
<td>9.9</td>
<td>5.4</td>
<td>7.4</td>
<td>2.5</td>
<td>45</td>
<td>66</td>
</tr>
</tbody>
</table>
Fig. 1 Bacterial populations enumerated (average and standard deviations (except for SRB)) from sediment under natural attenuation conditions: total bacteria, HPC (heterotrophic bacteria), SRB (sulfate reducing bacteria), FLU-deg (fluorene degrading bacteria), PHEN-deg (phenanthrene degrading bacteria) and PYR-deg (pyrene degrading bacteria)
Fig. 2 Percentage of fluorene remaining in the sediments after 40-day anaerobic incubation under different experimental conditions. Statistically significant differences (One-way ANOVA (p<0.000)) between treatments are shown (*)
Fig. 3 Percentage of phenanthrene remaining in the sediments after 40-day anaerobic incubation under different experimental conditions. Statistically significant differences (One-way ANOVA (p<0.000)) between treatments are shown (*)
CHAPTER VI: SYNTHESIS AND FUTURE WORK

Ongoing global changes indicate that ecosystems will not remain at equilibrium (Tilman et al. 2006). Among these environmental changes, contamination of rivers sediments with toxic organic compounds and metals present a complex long-term environmental challenge that translates into loss of ecosystem health and habitat degradation. On the other hand, microbial communities are diverse, widely distributed, and are responsible for critical functions, including biodegradation of pollutants. In order to predict response of affected ecosystems, and ultimately the underlying processes involved, scientists need to understand and quantify the linkage between microbial community response and change in sediment properties.

The central theme throughout my dissertation research has been integration of microbial community analyses and geochemistry in order to relate function (in my case, degradation of a xenobiotic) to biotic and abiotic conditions so predictions of responses to environmental changes can be elucidated. The purpose of the research described in this dissertation was to characterize bacterial communities in riverbank sediments affected by long-term contamination with PAHs. I used a combination of techniques for: i) molecular determination of bacterial community composition, ii) measurement of environmental parameters that might influence bacterial community structure, and iii) examination of whether or not bacterial communities had the potential to biodegrade PAHs under anaerobic conditions. Novel aspects of the research presented within this dissertation include the first detailed characterization of PAH contamination in riverbank sediments, first molecular approach to identify bacterial communities and patterns in community composition in riverbank sediments, examination of correlations between environmental conditions and bacterial community structure in riverbanks long-term impacted by pollution, and evaluation of the potential for indigenous bacterial communities to anaerobically degrade PAHs.

Community composition was associated with some environmental conditions more than others. Despite intense PAH contamination, bacterial community composition differed among dates and was highly associated with PAHs. However, metal and PAH contamination appeared to have a much stronger
relationship with community composition reflected by spatial segregation of the communities and marked by a strong environmental gradient. Finally, the potential for anaerobic degradation of two PAHs (fluorene and phenanthrene) was documented and indigenous microbes including PAH-degraders and SRB appeared to play a role in the degradation. Details regarding how my findings relate to previous literature and future directions that may enhance these findings are presented below.

**Polycyclic aromatic hydrocarbons (PAHs) and extent of sediment contamination in riverbanks**

An extensive literature review of PAHs (Chapter I) was necessary to understand different aspects of these pollutants. Among pollutants found in sediments, the most common types are PAHs. PAHs are still of great concern because some of them are carcinogenic and mutagenic, even small 2 and 3- ring PAHs have shown toxic effects on humans and animals (Sims and Overcash, 1983), and chronic effects on plants (Henner et al. 1999). PAHs are derived from natural sources (e.g. volcanic eruptions) but mostly from anthropogenic activities related to industrial processes and increased manufactory revolution. PAHs consist of two or more fused benzene rings and their recalcitrance in the environment increases with their molecular weight. Yet, the most important feature of PAHs is their tendency to bind to sediment particles. The affinity for particulate matter by PAHs is described by its octanol-water partitioning coefficient (Kow), which is the basis for predicting the environmental fate of PAHs (Johnston and Johnston 2012). Simpler PAHs have low values of Kow are readily soluble, whereas heavier PAHs have high values of Kow and considered hydrophobic. Sediments are sinks for PAHs so high concentrations are commonly found in a variety of aquatic ecosystems. Remediation of PAHs involves several physical and chemical strategies, nevertheless bioremediation (using microbes) is considered the most important and successful approach in PAH disappearance.

The majority of research that is dedicated to measuring PAHs in sediments has been conducted in marine systems (e.g. DeBruyn et al. 2009; Stark et al. 2003). In fact, most marine settings and estuaries are directly influenced by activities that carry either oil spills and/or petroleum releases. PAHs are associated with both. Another important body of research has focused on the characterization of PAHs in riverine systems, particularly in countries like China where pollution is rampant and regulations are not well
enforced (Luo et al. 2008; Charriau et al. 2009; Xu et al. 2007). Despite this massive amount of information on PAHs, very few studies have evaluated the extent of PAH contamination in riverbank sediments.

Riverbanks are key components in river ecosystems. Given the widespread contamination of aquatic systems (Blocksom et al. 2010), riparian zones not only act like buffers against contamination (Rasmussen et al. 2011) but are also considered key geographic features where aquatic biota interacts (Walters et al. 2008). In fact there is a growing interested in food-web studies and the land-water ecotone (riparian zone). Moreover riparian zone are considered i) critical in regulating aquatic-to-terrestrial contaminant transfers ii) integrators of linked aquatic-terrestrial systems within the context of contaminant exposure risk, iii) fundamental for remediation and long-term conservation of river ecosystems (Alberts et al. 2013).

To address this knowledge gap, a very detailed characterization of PAHs, their distribution and origins was conducted in Chapter II. In addition an ecological risk assessment using several guidelines to establish if riverbanks sediments were potentially harmful to aquatic biota was also performed. Because of limited funds, accessibility and feasibility only two sites, previously considered contaminated, and one uncontaminated site (upstream of the contaminated stretch of the river) were chosen as sampling sites. Our results indicated that a great percentage of PAHs corresponded to high molecular weight PAHs while a relatively small percentage of PAHs were low molecular weight PAHs. These findings were in agreement with other contaminated environments; four out of twelve identified PAHs were classified as carcinogenic, and found in high concentrations even at what it was considered the uncontaminated site. It is important to note here that a previous assessment of the river bottom sediments conducted in 1999 by the USACE (1999) indicated that this upstream site was not contaminated. In fact, the contamination in this site is relatively shallow (~ 90cm); moreover the river goes through a recreational park where picnicking, wading, and fishing are allowed. I plan to share this finding with environmental agencies so appropriate control measures can be taken.

In addition to being pollutant sinks, contaminated sediments can also become sources of contamination when changes in chemical composition in the aqueous phase or environmental disturbances remobilize pollutants. When/if this happens benthic biota might become exposed to PAHs and transfer toxic concentrations through food webs and higher organisms (e.g. fish) (Yang and Baumann, 2005; Bell et
The risk assessment conducted in the riverbanks revealed a high ecological risk to biota; several guidelines including the one introduced by the EPA after the BP oil spill were overwhelmingly exceeded indicating a severe environmental problem in the Mahoning River. In fact, the use of such guidelines is valuable in order to control pollutant sources and prevent future risks either to biota or human populations. It is important to point out that these riverbank sediments are deep, anaerobic, and not uniformly distributed. Yet because of their constant interaction with the main water channel (hydraulic connectivity between shallow aquifers in riverbanks and sediments) any indication of ecological risk should be considered.

In order to improve my findings, future research should include a greater number of sites along the Mahoning River so a complete picture of the extent of contamination can be described. Since PAHs are not deposited uniformly along the 51 km of contamination, it would be useful to sample sediments at different depths including also the surface sediments/soils in order to elucidate rates of deposition. Desorption kinetic analyses should be also performed to understand how sediment organic matter and sediment physical structure affect transport and fate of PAHs.

**Bacterial community composition in response to PAHs and metal contamination**

*16S rDNA bacterial community*

Contaminated soils and sediments are among the most complex and poorly studied habitats of ecosystems. Yet, bacterial communities inhabiting polluted systems are very diverse and involved in a large number of ecological processes (Lavelle et al. 2006). Cultivation-independent community analyses have been used extensively to gather information about which organisms are present and to reveal the metabolic potential of the microbial communities from various environments (e.g., Jiang et al. 2009; Hugenholtz 2002). A number of community profiling techniques have been described to examine PCR products from environmental DNA templates. One of those methods, terminal restriction fragment length polymorphisms (T-RFLP), identifies different sizes of PCR products based on restriction site variability and is one of the most frequently used high-throughput fingerprinting methods, in spite of the limitations described above (Schütte et al. 2008). On the other hand, because the majority of bacteria and archaea remain unculturable,
organisms of key importance in the community might be overlooked if they are unculturable (Vartoukian et al. 2010); but is only through culturing that a comprehensive characterization of physiological properties can be elucidated. Thus, we also included culturing of specific bacterial communities to gain more information about these sediments.

In aquatic systems, temporal changes have a major impact on microbial community structure (e.g. Hullar et al. 2006; Eggleton et al. 2004). However, environmental factors, including moisture, nutrients, and pollutants are also important drivers of variability in community composition (Wang and Tam 2012; Bernhard et al. 2005; Pratt et al. 2012). These correlations have been extensively documented for uncontaminated sites. Although sampling of the clone library was incomplete, this is the first study to report temporal shifts of bacterial communities in heavily impacted sediments with PAHs (presented in Chapter III). A higher number of T-RFs was recovered from this sediment compared to similar studies in uncontaminated sediments (Peng et al. 2012); these T-RFs were used to generate bacterial community profiles.

As stated by Kent and colleagues (2007) and Crump and Hobbie (2005) some changes in community composition are predictable while others are not. Interestingly, we found that despite intense pollution bacterial communities followed a shift over time. At each sampling date, although sediment cores were taken from the same site (25 - 50 cm apart), the number of T-RFs recovered from each core varied. In fact, the sediment physicochemical conditions that were highly variable across the sampling period were also the ones highly associated with bacterial community composition. The magnitude and direction of the community changes with time were not similar and appeared to relate strongly to PAHs, pH, sulfate and moisture. These results indicate that the temporal shifts in community structure was likely the result of environmental conditions as reported by Feris et al. (2003) in a study of microbial communities along a heavy-metal contaminated sediments, and by Wang and Tam (2012) who also reported seasonal shifts in microbial communities in PAH-contaminated sediments.

T-RFLP analysis when coupled with 16S rRNA clone library construction and sequencing do provide additional information on the composition of microbial communities. We included only a small phylogenetic analysis with 150 sequences recovered from highly contaminated sediment to infer taxonomic
information. Different groups of bacteria were present in the sediments including a large proportion of δ-proteobacteria, firmicutes, and bacteriodetes. These three groups have been also found in sites contaminated with heavy metals (Vishnivetskaya et al. 2011), acid mine drainage sites (Druschel et al. 2004), and petroleum contaminated soils and sediments (Barragan et al. 2008). There is no clear evidence as to why these groups tend to be abundant under contamination (Porat et al. 2010; Edlund and Jansson 2006). Although this analysis was based on a small number of sequences, a large proportion of them were closely associated with sulfate reducing bacteria (SRB). Members of the desulfo bacterales, desulforonanadales and desulfurellales have been described as sulfate-reducing bacteria (SRB) involved in PAH degradation (Acosta-Gonzalez et al. 2013; Gomes et al. 2013). Hydrocarbon contamination (Suarez-Suarez et al. 2011) as well as anthropogenic activities such as pollution (Cleary et al. 2012), increases abundance of SRB, which we also corroborated by culturing SRB in relatively high numbers. This was the first cohesive piece of evidence that combining abundant culturable SRB, high concentrations of sulfate and a history of extensive PAH contamination might facilitate anoxic degradation of hydrocarbons. However, because of our limited information about SRB, a closer look at the microbial communities needed to be performed first.

**dsrAB bacterial community**

Studies of functional genes from altered and unaltered aquatic environments have provided new insights into the diversity of sulfate reducing bacteria (SRB) (Leloup et al. 2006, Leloup et al. 2004; Pérez-Jiménez and Kerkhof 2005). Based on PAH distribution in the sediments and phylogenetic information of the bacterial communities provided by findings in Chapter II and III, a study using the functional gene *dsrAB* that encodes enzymes for sulfate reduction was included to gain more information about SRB communities in PAH-contaminated sediments (Chapter IV).

*dsrAB* genes are highly conserved across SRB, encoding enzymes that catalyze reduction of sulfite to sulfide (Rampinelli et al., 2008), a critical step in anaerobic sulfate reduction. *dsrAB* genes are found consistently in sulfate reducing bacteria, making them excellent markers for phylogenetic studies (Dar et al. 2007). The use of genetic data is more powerful when it is integrated with efforts to culture
microorganisms, which can provide clues about the physiology of the uncultured microorganisms (reviewed by Vartoukian et al. 2010). Thus, culturing of SRB was also necessary to obtain information about the SRB communities.

A literature review of similar contaminated sediments indicated that the second most common pollutant in sediments were metals. Furthermore, metals (heavy or transitional) appear to have an effect on bacterial communities (e.g. Sitte et al. 2010; Mosher et al. 2012). For that reason, an analysis of 20 metals was included in the suite of environmental conditions measured.

Analysis of the geochemical parameters, including PAHs and metals, revealed a clear segregation by site; in other words, the two highly contaminated sites and the one low contamination site had very different geochemical signatures. Statistical analyses suggested a relationship between these parameters and bacterial community composition, however, as this was a field survey these relationships were not shown empirically. We were not able to explore specific effects of metal contamination on bacterial communities due to the limited information provided by phylogenetic analysis. However, we recovered fewer sequences from the most highly metal contaminated sites, while a higher number of OTUs were detected at the site with least metal contamination. \textit{drsAB} diversity (Shannon-Wiener based on the number of OTUs) in the least metal contaminated site was higher compared to the two highly contaminated sites. Taking this information into account we could infer that because of the strong environmental gradient found at each site, and the low diversity indices (Shannon-Wiener based on T-RFs and OTUs), metals and PAHs may have impacted these communities.

As also observed in other studies (e.g. Kang et al. 2013), metals appear to influence bacterial community structure and function by increasing the abundance of SRB and the number of OTUs associated with SRB (Minz et al. 1999; Chang et al. 2001). It has also been proposed that indigenous microbial communities that inhabit metal-enriched sediments are capable of biogeochemical reactions that “detoxify” their local environment (Rastogi et al. 2011) perhaps by using some metals for respiration. As a result, bacterial communities become crucial components in determining fate and transport of heavy metals (Rastogi et al. 2010; Sevinc et al. 2007).
The sulfate-reducing community based on analysis of the functional marker gene $dsrAB$ supported previous studies suggesting that SRB are an important bacterial group in sediments like the ones studied in my dissertation (Leloup et al. 2006; Gillan et al. 2005; Nevin et al. 2003; Wang et al. 2010). The majority of sequences recovered here were related to uncultured SRB also described for other contaminated and uncontaminated environment, including uranium mining tailings (Chang et al. 2001; Carlsson and Buchel 2005) and metal-contaminated aquifers (Geets et al. 2006). Concurrently, culturable SRB abundance was within the same range as that in samples from uranium-contaminated subsurface sediments (Sitte et al. 2010). Our finding suggests that indigenous SRB communities were tolerant of the level of PAHs and metal contamination and might play a role in metal biotransformations. Heavy metals are of great concern and efforts such my work are needed to better understand the diverse SRB capable of biogeochemical transformations and the influence that other geochemical parameters have on their fate (Vishnivetskaya et al. 2011).

Given the body of literature showing that SRB are capable of transforming complex molecules (Lovley et al. 1993; Cebron et al. 2009; Révész et al. 2014), it is critical to conduct more research to be able to identify SRB with PAH-degrading capabilities and SRB that can potentially methylate heavy metals (e.g. U and Hg). Future research should include more powerful analyses of the bacterial community composition, using approaches such as pyrosequencing. In addition, identifying archaeal communities would complement information about diversity in contaminated sediments. In fact, there are only few scientific works that focused on archaeal communities in sediments (Porat et al. 2010; Haller et al. 2011). More research is necessary to detect the presence of functional genes associated with anaerobic degradation of PAHs in environmental samples. Implementation of qPCR using target genes would provide information about the genetic potential of microbes under extreme conditions of pollution. To clearly define links between microbial diversity and structure and ecosystem properties (and to generate/test testable hypothesis) stable isotope analyses and mass spectrometry techniques can identify important microbial functions and organisms involved in such functions (reviewed by Chen and Murrell 2010).
Microbial communities involved in ecosystem function measured as degradation of PAHs

One of the main challenges when studying microbial communities is that community structure cannot be separated from function (Boschker et al. 1998). It is almost impossible to determine relative contributions of individual taxa to ecosystem processes (there are many definitions for “bacterial species”). Most processes that involve microbes are usually the result of microbial consortia. Some even argue that “solid evidence” that links ecosystem function and diversity is “practically non-existent” (Schwartz et al., 2000). Nevertheless, relating community structure to function is necessary since bacteria are key elements in almost all biogeochemical transformations in sediments (Torsvik et al. 2002; Curtis et al. 2004). Microbes have the capacity to remove pollutants from the environment by transforming, binding and mostly respiring contaminants (Lovley 2003).

Among microorganisms, bacteria are responsible for most of the PAH degradation processes. Numerous species have been identified as PAH-degraders. For example, Aitken et al. (1998) identified several species (isolated from PAH-contaminated sites) of *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Burkholderia* and *Sphingomonas* as microbes with PAH-degrading capabilities. Because much PAH degradation takes place in the presence of oxygen, aerobic bacterial degradation of PAHs has been extensively studied and demonstrated in a variety of environments, including marshes, marine sediments, brackish sediments, and contaminated soils (Lei et al. 2005; Lovley 2000; Meckenstock et al. 2004). Yet, there is strong evidence of anaerobic degradation of PAHs in sediments (Coates et al. 1997; Ma et al. 2011). When oxygen is not available, electron acceptors are depleted in the following sequence: nitrate, manganese, iron, sulfate, and labile organic compounds (Stumm & Morgan 1981). In fact, laboratory studies have shown anaerobic biodegradation of PAHs under denitrifying, sulfate-reducing, and methanogenic conditions (Coates et al. 1997; Rothermich et al. 2002). Hayes & Lovley (2002) reported biodegradation of naphthalene in marine sediments under sulfate-reducing conditions, indicating that microbes related to the phylotype NaphS2 (from the δ- Proteobacteria), a naphthalene-degrading sulfate reducer, were involved in the degradation. Coates et al. (1997) showed mineralization of fluorine, phenanthrene and fluoranthene of contaminated marine sediments under sulfate reducing conditions. Lei et
al. (2005) reported phenanthrene degradation under sulfate reducing conditions by unidentified indigenous bacteria from river sediments.

Information gained from the studies cited above were needed to finally address the most intriguing question in my dissertation: Do indigenous communities have the potential to anaerobically degrade PAHs? The reason I use the term “intriguing” is because in the last 20 years there have been many attempts to prove that bioremediation of Mahoning River sediments could be feasible. The USACE (2001) has chosen dredging, a remediation alternative that would terribly damage the riverbanks and alter the river ecosystem. Most local communities along the Mahoning River are opposed to these plans and have been looking for alternative solutions. The USACE has not considered bioremediation because there is no study showing if it is feasible, let alone that could be implemented at large scale. Thus, information about the microbial ecology of these sediments (geochemistry and microbiological) was necessary before consideration of an experimental study of degradation. Previous experiments (unpublished data) showed no PAH disappearance, but revealed that microbial communities were using sulfate as terminal electron acceptor. Given the high concentrations of sulfate in the sediments, a study that lasted 131 days was performed to determine if microbes could use sulfate for respiration. In addition, the presence of culturable and unculturable SRB in these sediments suggested that PAH degradation under anaerobic conditions could be feasible.

Literature on anaerobic degradation of PAHs is indeed very limited so finding useful information of how to proceed experimentally was challenging. However, a few scientists have been using a humic model compound (anthraquinone -2,6-disulfonate [AQDS]) to facilitate anaerobic degradation of other high molecular and complex compounds (Bradley et al. 1998; Chen et al. 2012; Kwon and Finneran 2008). So, if degradation of other complex pollutants can be achieved when AQDS is added, would the addition of AQDS also result in enhanced anaerobic degradation of PAHs? This is the first study that used AQDS to facilitate anaerobic degradation of PAHs. To test my hypothesis, AQDS was used as an amendment to evaluate if bacterial communities could degrade fluorene and phenanthrene. These two PAHs were used as models based on other similar degradation studies published (Tsai et al. 2009; Tian et al. 2008).

Enhanced degradation of fluorene and phenanthrene was achieved by the addition of AQDS over
the 40-day anaerobic incubation. Compared to natural attenuation (8 and 20% respectively), when AQDS was added to sediments fluorene concentrations decreased 55% while phenanthrene decreased 66%. For this microbial community, AQDS appeared to be more favorable thermodynamically than other potential electron acceptors present in the sediments. PAHs can dissipate by other mechanisms, including photot oxidation, leaching, and volatilization (Husain 2008). So in order to ensure that these processes did not take place, strict anaerobic conditions were met during the experiment. Culturable PAH-degrading bacteria including fluorene and phenanthrene degraders were enumerated from sediments as well as relatively high numbers of SRB. Collectively, these findings showed that anaerobic degradation of PAHs was likely a biotic process, in which either PAH-degraders, SRB or both were responsible for degrading fluorene and phenanthrene. Although these data do not provide strong evidence that links community structure and function (measured as loss of PAHs), this finding fills in a gap of knowledge about anaerobic degradation of PAHs. Moreover, it contributes to a better understanding of how certain microbial communities (e.g. SRB) might have acquired degradative capabilities due to the extent and length of exposure to PAHs. More importantly, this work improved and expanded our understanding of AQDS as humic model compound in degradation studies. This is of particular interest since the process of humic reduction has been considered an important mechanism for oxidation of organic matter and pollutants in sediments, especially if Fe is present (Coates et al. 1998).

The process of microbial humic substance reduction is of great interest because it may serve as an important mechanism for organic matter oxidation in contaminated environments where Fe is also present (Lovley et al. 1996) and usually abundant (Zhu et al. 2013; Chabukdhara and Nema 2012; Varol 2011). Humic substances can reduce the toxicity of metals, hydrocarbons and other chemicals (Lipczynska-Kochany and Kochany, 2008; Feificova et al. 2005). Humics directly catalyze the oxidation of toxic compounds (Bradley et al. 1998, Wang et al. 2009; Cervantes et al. 2001) and complex molecules such the explosive XRD (Kwon and Finneran 2008) by a mechanism called “electron shuttling” (Luu et al., 2003, Aulenta et al. 2010). Reduced humic substances can abiotically transfer the electrons gained from microbial reduction to Fe(III) and reoxidize the humic substances while accepting electrons from humic-reducing bacteria (Luu and Ramsay 2003). Humics can also increase solubility of organic compounds and Fe making
them more bioavailable for bacteria (Cho et al. 2002; Lovley et al. 1996). On the other hand, humic reducers are found in most anaerobic sediments, are phylogenetically diverse, and are mostly iron reducers (Lovley 2000). Some have been described as SRB (Lovley et al. 1998) and more recently some (i.e. *Shewanella*) have been used as model organisms for uranium bioremediation studies (Sheng and Fein 2014) and for microbial fuel-cell research (reviewed by Lovley 2008). We attempted to culture humic reducers without success. Humic reducing bacteria, although common in anaerobic environments (Cervantes et al. 2001) are difficult to grow (Coates et al. 1998). Collectively, the information we gathered in this experiment lays the foundation for more detail studies that could more precisely identify the microbial communities responsible for PAH degradation. The use of AQDS and the role of humics and Fe in organic matter cycling in sediments is still a relatively unexplored area of research that calls for more innovation. In that regard, contaminated sediments with metals are great for conducting this type of study. Future research should include identification of humic-reducing or Fe-reducing bacteria, determination of Fe mineralogy, identification of electron shuttling mechanisms, and isolation of Fe or humic reducers. This information would contribute to a deeper understanding of the Fe cycle. This would only be possible if more sensitive instrumentation is used, such as high performance liquid chromatography and high resolution GC-MS.

Culturing methods under anaerobic conditions must be included in order to isolate important microbes or consortia associated with PAH degradation. Enrichment cultures are usually time consuming yet they should be included in future experiments (given the time and budget) so questions that relate identity to function can be answered. Most PAH research has been conducted under aerobic conditions (Lei et al. 2005; Lovley 2000; Meckenstock et al. 2004) and pathways of degradation have been fully described for some PAHs (Lopez et al. 2006; Lee et al. 2008), yet relatively little is known about enzymes, genes, and catabolic reactions during anaerobic degradation of PAHs (Meckenstock et al. 2004; Mouttaki et al. 2012). Future research should aim to provide new information about anaerobic degradation by using: anaerobic experiments using different terminal electron acceptors (under denitrifying, methanogenic, iron conditions), radiolabeled PAHs to measure complete mineralization, using 2-, 3-, 4- and 5–ring PAHs combined with AQDS, and changes in the microbial community throughout the degradation process which could reveal
which microbes are directly associated with degradation. These experiments coupled with more powerful molecular techniques (e.g. pyrosequencing or stable isotope probing) could reveal how they are doing it.

Overall, this dissertation filled in many scientific gaps related to geochemistry and microbial ecology. This study improved our understanding of the ecology of long-term contaminated sediments with PAHs and metals. This work provided important clues into how bacterial communities respond to intensive and extensive pollution and are driven by environmental conditions to different extents. I hypothesize that bacterial communities (specifically SRB and/or PAH-degraders) were likely using sulfate and humics for energy and PAHs as carbon sources, contributing to biogeochemical cycling of sulfur and carbon, and providing an important ecosystem function translated as degradation of PAHs (Fig. 1). This was the first study reporting bacterial composition and diversity as well as dsrAB diversity from important components of river ecosystems: riparian zones heavily impacted by metal and PAH-contamination. More importantly, this is the first report in the literature of anaerobic degradation of PAHs amended with AQDS by indigenous bacterial communities from riverbank sediments.

Limitations of study methods and challenges of interdisciplinary research

Throughout my dissertation work I encountered some limitations that affect and/or restricted the methods I used and the analysis of the data, and in some ways confined the conclusions drawn by this study. I summarized these limitations in the sections below.

**Bacterial communities.** Bacterial communities are not uniformly distributed in sediment particles nor are all types of bacteria uniform in the ease with which their DNA can be extracted. In fact, even when multiple DNA extractions from a sample are performed different microbial communities will be obtained (Feinstein et al. 2009). To overcome this issue, in my dissertation work duplicate or triplicate DNA extractions were performed on each sample. In addition, multiple PCR products were pooled before analyses via T-RFLP or for cloning.

The examination of bacterial communities in this study and in others using the same widely accepted approaches have limitations. Because of their complexity, microbial communities cannot be
completely identified (Ritz et al. 2007). In my dissertation, I used terminal restriction fragment length polymorphisms (T-RFLPs) to determine community composition. T-RFLPs was chosen because: i) it is one of the most frequently used high-throughput fingerprinting methods (Schütte et al. 2008), ii) it has been used widely for analysis of fungal ribosomal genes (Johnson et al. 2004; Kennedy et al. 2005), bacterial 16S rRNA genes (Hullar et al. 2006; Katsivela et al. 2005; Thies 2007), and archaeal 16S rRNA genes (Wu et al. 2006), iii) it is considered suitable for assessing temporal or spatial changes of microbial community structure in highly diverse soil (Lukow et al. 2000; Mummey and Stahl 2003), and iv) it is an excellent method for rapidly comparing the relationships between bacterial communities in environmental samples (Dumbar et al. 2000; Tom-Petersen et al. 2003; Morales et al. 2006). However since T-RFLP is a PCR based technique it has the common flaws of any based PCR-based tool, including bias and underestimation of microbial diversity (Gao and Tao 2012). T-RLFP is considered a partial community analysis method since it tends to underestimates community diversity because only a limited number of bands per gel/capillary (generally <100) can be resolved, and different bacterial species can share the same T-RF length -OTU overlap or OTU homoplasy (Rastogi and Sani 2011). This was a limiting factor in my study since the community composition was partially revealed, so any temporal or spatial patterns observed should be interpreted cautiously. In fact, it is impossible to know if the number of T-RFs recovered from core sediments representing bacterial communities was enough to truly represent microbial community composition. Although the method does provide a robust index of community diversity, T-RFLP results are generally very well correlated when coupled to extensive clone libraries (Fierer and Jackson 2006).

For my phylogenetic analyses, I followed established protocols using cloning kits. This method uses universal bacterial primers that could have also underestimated certain groups of bacteria, especially when diversity is unknown or high (Loisel et al. 2006). Although I isolated a large number of clones (~1400), the cost of sequencing was a limiting factor in fully characterizing bacterial communities. The coverage calculation in Chapter III and IV revealed that more clones were needed in order to have representative information regarding bacterial diversity. In bacterial communities, the number of types of organisms observed tends to increase with more samples (Hughes et al. 2001). With more information from a microbial community, the variance of microbial richness can be calculated and compared. In addition,
because of the low clone coverage the depiction of the composition of the community may be inaccurate so that less common taxa were not detected. Low coverage also means that diversity and evenness indices were also inaccurate (either underestimated and overestimated). Increased sampling is needed to quantify the actual diversity of the community (Hill et al. 2003). Indices combining proportional abundances with phylogenetic diversity such as dissimilarity index (Dmean) (Watve and Gangal 1996), as well as repeated sampling using estimators that are both precise and unbiased such as Chao1 (Hill et al. 2003) should be included in future research.

By elucidating diversity in sediments (or soils), we can infer general functions of microbes. For instance, organic matter is decomposed by a wide range of microbes while specific functions (e.g. denitrification, sulfate reduction) are achieved by specific groups of bacteria (Griffiths et al. 2001). Furthermore, diversity is linked to variation in environmental conditions (Ives and Carpenter 2007). Specifically, variation in chemical and physical properties, disturbance type and strength, and key species for resistance and resilience affect the stability of bacterial communities (Deng 2012). With the limited information of bacterial diversity gathered in my work, we were not able to answer important questions about: i) diversity-stability relationships under extensive contamination, ii) links between geochemistry and diversity, iii) PAHs as a ‘true’ stressor on the bacterial communities, iv) or the influence of particular geochemical parameters versus pollution on bacterial diversity. These are all questions that remain unresolved, but should be included in future work.

Sequencing is a readily available tool for characterizing microbial communities, but it is still costly. Nevertheless, even with these limitations, I was able to identify for the first time bacteria inhabiting riverbank sediments highly contaminated with PAHs. Unfortunately, there are no studies that have examined bacterial diversity in contaminated sediments with PAHs, and only a few studies that have analyzed bacterial diversity in long-term impacted sediments (Pratt et al. 2012; Sutton et al. 2013; Zhu et al. 2013). This was perhaps a major limitation in my study, since there were no other systems for comparison of either community composition or diversity data. Access to other similar sites would have revealed data about the distribution of bacterial communities and how diversity and environmental factors are related. In fact, by comparing these results with those from other contaminated sites, information could
be gathered about the functional diversity of PAH-degrading bacteria and of other functional groups such as SRB or methanogens in this type of system. In addition, information about bacterial communities, functional traits, and responses to disturbance and diversity-stability could have been elucidated. Indeed, it would have been great to be able to access other sediments, but I had very limited funds to collect sediments, and thus was limited to three sites in one river.

More importantly, although there is still research interest in contaminated sediments, the majority of studies focus on rivers that are still being actively contaminated. There is little research on sites with only a legacy of pollution. There are many such sites in the U.S. and worldwide, but there have not been investigations of the microbes inhabiting these environments. That alone is also a limiting factor, since most research grants are geared to “what is happening” not to “what happened and is buried”. Research in sites that have been affected for long time can tell us how microbes have adapted to these extreme conditions (Sutton et al. 2013). In fact, even regulatory agencies do not focus enough on basic research and/or applied research in contaminated sites, unless it is related to homeland security issues such as radioactive waste and sites with potential to find microbes able to reduce uranium and other heavy metals. Learning about the complexity of factors that influence bacterial communities can give us insightful information about key populations for in situ remediation practices (Powell et al. 2006). In addition cultivation-independent molecular approaches in long-term impacted sites can be useful to i) assess the impact of pollution on microbial communities, ii) evaluate natural attenuation (degradation over time without human intervention), and iii) measure degradation potential and activity of indigenous microbes that can be used to test bioremediation technologies.

**PAH extractions.** Throughout my dissertation, I used three different approaches to measure concentrations of PAHs. One technique (Soxhlet) involved 18 hours per sample and high volumes of solvents. Another technique (sonication) was not as exhaustive, but solvent volumes were easier to handle. The last technique (modified Blyer method, Pratt et al. 2012) needed more finesse, and was very elaborate but required very little amount of solvents and was less time consuming. These three methods have advantages and disadvantages and the decision to use of one over another was, in most cases, related to the time and
amount of solvent required. It is important to note that at the beginning of this work, these methods were compared using standard sediments, surrogate and internal standards to verify their extraction efficiencies and repeatability. However, when environmental samples were tested, we were not able to extract 100% of PAHs. This was taken into consideration, and in the case of the Soxhlet method, more hours of extraction were added. In the case of the sonication method, an additional pulse of 10 min was also included. Nevertheless, our recoveries varied from 5 to 20% consistently across sediments. At that point, I selected a method and included the surrogate recoveries and internal standards in the calculations. It would not have been fruitful to spend more time and resources trying to maximize the extraction efficiencies since the intrinsic nature of the sediment (e.g. age, recalcitrance, hydrophobicity) played a key role in the extraction method.

**Soil and sediment standard methods.**- Before collecting data for my dissertation work, all methods were tested to verify repeatability and efficiency. Months were dedicated to adjusting or modifying standard methods for collecting soils and sediments. The sediments from the Mahoning River are very oily and contain numerous pollutants including total petroleum hydrocarbons, dioxins, pesticides, etc., thus they were not easy to work with. For most geochemical parameters, multiple replicates were included in order to reduce heterogeneity and sampling error. This aspect is of particular importance because deep anaerobic contaminated sediments are not distributed uniformly within the riverbank. Although a thorough homogenization of sediments was conducted (via physical measures including vortexing and grinding), it was almost impossible to overcome the presence of tar balls or microscopic pockets of oily sediments. In fact, aged sediments deposited over long periods of time tend to aggregate and are very difficult to homogenize. This issue in particular may have influenced the extraction efficiencies of PAH extraction. For instance, the efficiencies of the Soxhlet method tends to increase with molecular weight, so 2 and 3- ring PAHs might have been underrepresented. Depending on the extraction method some PAHs are extracted preferentially over others (Lau et al. 2010). In addition, strong adsorption of high-molecular-weight PAHs to clay particles results in less detectable PAHs (Spack et al. 1998). This was particularly true for our sediments since we were not able to detect 5 and 6-ring PAH in the majority of samples. Nevertheless,
measurement of total contaminant concentrations should not be taken as measure of bioavailability (Khan et al. 2012). Age-sediments can indeed reduce chemical extractability but does not imply reduce bioavailability. In fact, bacteria have developed different mechanisms to increase bioavailability of PAHs (Johnsen and Karlson 2004). Therefore, although PAH concentrations measured in this study cannot be taken as absolute or actual concentrations, their measurement provided relevant information of the extent of contamination. More importantly, measurement of PAHs combined with information of the indigenous microbial communities and findings from the degradation study (see below) gave useful insights about biotic interactions in these contaminated sediments. Another limitation was the presence of metals in the sediments which made it impossible to measure redox by Fe(II) and Fe(III) ratios, a common approach when the use of electrodes is not feasible (Lovley and Phillips 1987). I was not able to use commercial redox electrodes because the sediments would clog them and make them useless afterwards.

**Degradation study.** Most degradation studies have used pure cultures and have been performed in spiked clean sediments (Johnson and Ghosh 1998; Tian et al. 2008; Tsai et al. 2009), however I decided to use environmental samples (natural sediments), acknowledging upfront that it would be a difficult task. I was able to show loss of PAHs using deuterated PAHs instead of radiolabeled PAHs. Deuterated PAHs are heavier isotopes of regular PAHs and can be used to track loss of PAHs over time (Gomez-Eyles et al. 2011). However, with this technique, it is not possible to detect breakdown products or intermediate metabolites during fluorene or phenanthrene degradation. As a result, we could not infer any ‘possible’ degradation pathway that may have undergone during PAH degradation. In the same manner, using deuterated compounds it is not possible to measure mineralization of PAHs (final conversion to CO₂). I chose only two low-molecular-weight PAHs: fluorene and phenanthrene. I used these PAHs because they have been used in similar anaerobic degradation experiments (Tsai et al. 2009). I did not use a high-molecular-weight PAHs because there is not enough information about their degradation, and the few experiments in the literature were conducted with pure bacterial cultures (Chang et al. 2008; Ambrosoli et al. 2005) over very long periods of time (~160 -250 days).
Other limitations. - There are many reasons why combining geochemistry and microbial ecology is challenging. In general, most geochemical approaches focus on detailed collections of physical and chemical parameters that can be measured continuously (in some cases, via sensory technology) and intensively. On the other hand, microbiological studies that attempt to identify taxa within communities usually do not involve intensive sampling, and rarely involve geochemical measurements that match the number of environmental DNA extractions performed. For instance, organic matter can be measured from sediments at virtually no cost, while a clone library with good coverage from a single sample costs thousands of dollars. In my work, largely because of budget constraints, the number of samples for DNA examination did not match the number of samples for geochemical parameters and clone library coverage was low. This is still an issue that many researchers face when embarking on multidisciplinary microbial ecology projects.

My study evaluated temporal patterns in microbial community structure only in the most contaminated site along the river (Lowellville) in Chapter III. In the analyses of the bacterial communities in Chapter III we did not include a reference site because we wanted to evaluate if temporal patterns were still observable even under extreme sediment contamination. We hypothesized that since these are bacterial communities embedded in deep oily anaerobic sediments with a high degree of contamination, any interaction with environmental factors in the surroundings would be negligible or non-existent. We did not believe a reference site would be necessary since there are plenty of studies (in uncontaminated sites) that show temporal effects on bacterial community structure that we used for comparison. However, if the budget would have allowed it, we could have sampled other contaminated sites perhaps in other rivers across the U.S. Future research should be undertaken including similar PAH-contaminated river sites for comparison in order to draw stronger conclusions about temporal and spatial shifts of bacterial communities in highly contaminated riverbank sediments.

In Chapter III the bacterial community structure was determined by T-RLFP and phylogenetic analyses from one site (Lowellville) and on one sampling date, which limited the outcomes regarding diversity and community structure. In fact, although temporal patterns were observed in T-RFLP fingerprints, because of the low number of clones other important relationships could not be drawn. For
instance, abundance of certain groups of microbes and dominant or redundant species, that might contribute to shifts over time were not shown. In addition, we were not able to identify specific groups of bacteria perhaps associated with PAH degradation (e.g. *Mycobacterium*). Many factors influence the ability of microbes to use pollutants as substrates or cometabolize them (Seo et al. 2009), therefore identifying specific groups of microbes with degradative capabilities is a key factor in determining if/how efficient cleanup efforts of pollutants can be achieved. More clones were recovered from the other two sampling dates (in Chapter III) but I was not able to sequence them due to budget constraints.

In Chapter IV, we wanted to evaluate if metals and PAH pollution had an effect on bacterial community structure and diversity. We chose three sites along the river (two highly contaminated sites and one with low contamination) to establish links between degree of contamination and bacterial communities. We used 16S rDNA genes for T-RFLP analyses and clone libraries from the three sites; we also used the functional *dsrAB* gene to exemplify SRB communities. However, we were only able to illustrate the community structure using T-RFLP of 16S rDNA and identify SRB community by phylogenetic analyses from the three sites. We did not get sequences from clone libraries using the 16S rDNA genes for budgetary reasons. We believed that the sites chosen would provide sufficient information to answer our questions so including other sites (either in the same river or other rivers) was outside of the scope of the study. However, resources permitting, combining contamination gradients in other systems should be pursued in order to draw stronger conclusions about microbial diversity in polluted ecosystems. More phylogenetic information and other more powerful tools (e.g. metagenomics) are needed to characterize the vast microbial diversity, understand their interactions with biotic and abiotic environmental factors, and answer important questions regarding what process are involved in their genetic and metabolic diversity.

Much of my dissertation work represents a field survey, in which relationships between microbial community structure and geochemical data were statistically evaluated. Because most environments are complex, even with complete information of the community structure, causality or direct effects can only be predicted on the basis of a single environmental variable (Prosser et al. 2007). I chose multivariate analyses to show geochemical-community structure relationships as correlation coefficients, and interpreted statistical outcomes as representative of the interactions between geochemistry and bacterial communities.
These statistical analyses only suggested (as in many other published studies Haller et al. 2011; Halter et al. 2011; Besaury et al. 2014; Berthe et al. 2013) that pollution might be a factor affecting microbial community structure.

Correlations coefficients are commonly used to reveal associations between two variables, to measure their covariance, and to measure the strength of any relationship between variables (Zuur et al. 2007). Correlation coefficients are powerful, but only capture linear relationships between variables that are normally distributed and have homogeneity of variances (Robinson and Hamann 2008). Low correlation values do not mean that there is no correlation. In fact, in ecological systems where most environmental factors follow a non-linear distribution (Gurevitch and Hedges 1999), a high degree of dependence between variables exists, resulting in non-significant statistical analysis and low correlation coefficients (Ruan et al. 2006). In my work, there are several reasons (some inherent to the complexity of the sample) that resulted in low correlation coefficients including: randomness caused by the uneven distribution of physical properties of the environment, confounding variables (a third non-measured variable that affects the relationship between two measured variables), difficulty in obtaining accurate measurements of many geochemical properties (e.g. PAHs), presence of ‘outliers’, non linear relationship among variables, co-dependant variables, and effects of other variables not measured (e.g. redox). In addition, since significance is largely influenced by the number of samples (Zuur et al. 2009), some non-significant high correlation coefficients reported in this dissertation were most likely the result of low sampling size since large comparative differences in the relationships between variables were not observed.

Research findings need to be validated by statistical analyses so outcomes can provide meaningful interpretative results. Thus, future research should included improved designs of observational and experimental studies, as well as sufficient sample sizes to obtain a reasonable power so stronger conclusions can be depicted. Experiments manipulating sediments, bacterial communities and mimicking environmental conditions (one or two parameters) are also necessary to measure cause and effect (Peter et al. 2011).
Conclusion

Information gathered in this study is important because, as humans, we are concerned about rapid global changes in our ecosystems. Understanding the complex activity of interacting microbial communities and their pivotal role in biogeochemical transformation on Earth is urgent in order to ensure that our ecosystem functions do not fail. Most ecological studies do not include microbial composition or consider effects of environmental factors on microbial communities, and even less is dedicated to correlating microbial processes to biogeochemical functions that ensure a diverse biosphere. My dissertation work was a small contribution to the demand for research that seeks a deeper understanding of microbial communities, their responses to environmental gradients and their dynamics, elements that are going to be critical to predict how our ecosystems modulates and responds to future environmental conditions. On the applied side, my dissertation followed an interdisciplinary approach that can lead to better and more scientific strategies so bioremediation can be successful; in particular bioremediation efforts need to be based on: i) the knowledge of the microbes that are present, ii) their metabolic capabilities, and iii) their responses to environmental conditions. Finally, I would like to emphasize that microbial ecologists in particular, need to reach to other disciplines and integrate their knowledge to generate more comprehensive research.

References


United States Army Corps of Engineers (USACE). 1999. Mahoning River Environmental Dredging Reconnaissance Study


Fig. 1 Integrated framework of microbial communities and environmental conditions related to ecosystem function found in Mahoning riverbank sediments impacted by PAH and metal contamination.