BENZENE AND TOLUENE BIODEGRADATION

LIMITATIONS WITH HEAVY METAL

CO-CONTAMINATION

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To my family,

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Abstract: Bioremediation depends on microorganisms. Low bioavailability of pollutants, high heavy metals' concentration, and insufficient nutrients for microorganisms lead to biodegradation limitations of co-contaminants. Bioremediation is underutilized in co-contaminated sites because of the suspicion and disagreement over its efficiency. Understanding the effect of high concentrations of lead, cadmium, and zinc on the biodegradation of benzene and toluene is needed to improve and enhance the utilization of existing bioremediation practices for co-contaminants. This study identifies native soil aerobic degraders of individual benzene (876.4 mg/kg) and toluene (869.8 mg/kg) in the presence of various concentrations (5.1 mg/kg; 51 mg/kg; 510 mg/kg; and 5,100 mg/kg) of Cd and Pb. The study analyzes the microbial community structure and diversity in aerobic microcosms using several molecular biology techniques. The study also demonstrates and assesses bioremediation potential for benzene (876.4 mg/kg) and toluene (869.8 mg/kg) in Cd (between 7.9 and 341.8 mg/kg), Pb (between 187.5 and 8,772 mg/kg), and Zn (between 860 and 79,341 mg/kg) long-term polluted soils collected from Tar Creek Superfund site in Oklahoma. The bioremediation potential is studied on a laboratory scale in aerobic microcosms utilizing molecular biology and analytical methods. Individual benzene or toluene without heavy metal co-contaminants displayed similar bacterial community distribution. The bacterial community distribution was significantly different statistically when Cd was present, depending on the Cd concentration. *Lysobacter* sp., *Rhodococcus* group, *Caulobacter daechungensi, Pseudomonas* sp., *Pseudomonas putida, Mesorhizobium* sp., *Adhaeribacter* sp., *Flavobacterium limicola,* and *Flavobacterium granulensis* presented high ($<10^4$) 16S rRNA genes/g, indicating a viable bacterial population for an efficient bioremediation process in co-contaminated sites. *Rhodospeudomonas* sp., *Zoogloea* sp.*, Cupriavidus* sp.*, Nitrosospira* sp.*, Dechloromonas* sp.*, Nitrosovibrio* sp.*,* and *Nitrospira* sp. were presented in high abundance in the experimental soils selected for the bioremediation study. They demonstrated the ability to degrade a mixture of benzene (876.4 mg/kg) and toluene (869.8 mg/kg) without limitation in the presence of long-term heavy metal-contaminated soils. Benzene degradation was linear for natural attenuation, while toluene degradation was linear for biostimulation and natural attenuation. The degradation rates between biostimulation and natural attenuation were significantly different ($P<0.05$) in soils polluted with the lowest and highest heavy metal concentrations.

TABLE OF CONTENTS

Chapter Page

LIST OF ABBREVIATIONS AND ACRONYMS

Acetyl-CoA – Acetyl Coenzyme A: important molecule in biochemical reactions and in metabolism
ARRA – American Recovery and Reinvestment Act
ATSDR – Agency for Toxic Substances and Disease Registry
Bph Dox – Biphenyl Dioxygenase
BTEX – Benzene, Toluene, Ethylbenzene, Xylene
CDC – Centers for Disease Control and Prevention
CERCLA – Comprehensive Environmental Response, Compensation, and Liability Act (also known as Superfund)
CY – Cubic Yard
DGGE – Denaturing Gradient Gel Electrophoresis
DI – Deionized Water
EPA – the United States Environmental Protection Agency
GC-FID – Gas-Chromatograph Flame Ionization Detector
HM – Heavy Metals
ICP-OES – Inductively Coupled Plasma Optical Emission Spectrometry
IRIS – Integrated Risk Information System
NADH – Nicotinamide Adenine Dinucleotide (NAD) plus Hydrogen (H): a reducing agent to donate electrons during metabolic reaction
NCBI – National Center for Biotechnology Information
NMDS – Non-Metric Multidimensional Scaling
NPL – National Priority List
ODEQ – Oklahoma Department of Environmental Quality
OTU – Operational Taxonomic Unit
PCR – Polymerase Chain Reaction
qPCR – quantitative Polymerase Chain Reaction
VOC – Volatile Organic Compound (such as benzene and toluene)
VPH – Volatile Petroleum Hydrocarbons (such as benzene and toluene)

LIST OF TABLES

Table Page

Table Page

Table Page

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

Petroleum contains aromatic hydrocarbons, asphaltenes, and non-hydrocarbon compounds (such as sulfur, nitrogen, and oxygen). Benzene and toluene are aromatic hydrocarbons and natural components of crude oil (Speight, 2014; Brezonik, & Arnold, 2011). It is expected that crude oil is present together with heavy metals (Speight, 2014). There was a time when lead (Pb) was added to refined petroleum as a standard fuel additive. It resulted in a high-efficiency combustion material in car engines and increased vehicle performance (Khudur et al., 2018). Petroleum has a natural metal content of 10% w/w as well, which includes iron (Fe), copper (Cu), nickel (Ni), and vanadium (V) (Speight, 2014).

Co-contamination refers to multiple contaminants in the same environment, when soils are polluted with organic pollutants and heavy metals (Roane, Josephson, & Pepper, 2001). Single contamination rarely occurs in polluted areas (Madrid, Rubio-Bellido, Villaverde, Tejada, & Morillo, 2016). Contaminants can be released accidentally or intentionally into the environment (Ahmed et al., 2019).

Petroleum and its combination with heavy metals contaminate soil through human activities such as drilling, mining, transportation, and crude oil processing. It is not an unusual event when petroleum contaminates soil together with heavy metals (Speight, 2014). Co-contamination causes global problems for humans and the environment. It pollutes soils, ground- and surface waters. The biological effects of pollutants can accumulate over time in living organisms (Ma, Li, Mao, Wang, & Wang, 2018). The impacts of pollutants on the individuals depend on the duration of exposure received until the end of life (Carson, 1962).

Remediation of co-contaminants is complicated because heavy metals and organics need different chemical treatment and remediation technologies (Ma, Li, Mao, Wang, & Wang, 2018). Bioremediation is a promising remediation technique for co-contaminants since it is more cost-effective than chemical treatments and harmless to the environment (National Research Council, 1993; Leal et al., 2017; Environmental Protection Agency [EPA], 2001). Bioremediation is a common technique for remediating petroleum and its products' pollution (Leal et al., 2017). There are limitations of co-contaminants biodegradation due to the low bioavailability of pollutants, high concentration of heavy metals, insufficient nutrients, and microbial electron donors or acceptors (Ma et al., 2018). Bioremediation techniques are underutilized (Elekwachi, Andersen, & Hodgman, 2014) due to technical complications of applying the technology and uncertainties of completion (National Research Council [NRC], 1993). Understanding the long-term effect of lead (Pb), cadmium (Cd), and zinc (Zn) on the biodegradation of benzene and toluene is necessary to improve and utilize existing bioremediation practices for co-contaminants.

Aromatic hydrocarbons and mixed isomers are priority contaminants in many Superfund sites in the United States (Shim et al., 2005; EPA, 2020). Superfund sites are contaminated areas caused by improper hazardous waste management. The sites include manufacturing facilities,

processing plants, landfills, and mines. Thousands of Superfund sites exist in the United States (EPA, 2020). There are approximately 1,410 sites contaminated with aromatic hydrocarbons that are active and archived. Some of the contaminated sites are not on the National Priority List (NPL), some of them were deleted from the NPL, a few are on the NPL, and some only partially belong to an NPL site. Approximately 658 of the 1,410 Superfund sites have not been remediated yet. There are 16 Superfund sites located in Oklahoma, and the site remediation has not been started yet on three of 16 (EPA, 2018).

Most polluted soils are co-contaminated with more than one type of pollutant. There is still a lot left to discover and understand regarding the effects of co-contamination and their biodegradation processes. The number of published studies about co-contaminants increases each year (Madrid et al., 2016; Carvajal et al., 2018; Czarny et al., 2020; Zukausaite et al., 2008; Bamforth & Singleton, 2005; Ekperusi & Aigbodion, 2015) to contribute to our understanding of co-contaminants' degradation. It is not uncommon to examine the effect co-contaminants have on biodegradation and the relationship between heavy metals and degradation of oil products (Zukausaite et al., 2008; Bamforth & Singleton, 2005; Khudur et al., 2018; Madrid et al., 2016, Czarny et al., 2020; Ekperusi & Aigbodion, 2015). Co-contaminants were added to most of the soils in these studies (Madrid et al., 2016; Zukausaite et al., 2008; Czarny et al., 2020; Ekperusi & Aigbodion, 2015) in laboratories that created short-term pollution to investigate the developed research questions.

Another reason for added known concentrations to the samples was that heavy metals' concentration was naturally low in the studied soils (Zukausaite et al., 2008). Co-contaminants were added to the samples, which caused a short-term and controlled co-contamination produced in laboratories. These studies could not demonstrate a good result for the bioremediation potential

of long-term and highly co-contaminated sites. Further research is required to test the limitations of bioremediation techniques for long-term co-contaminations and utilize their bioremediation potential (Bamforth & Singleton, 2005). The edaphic and microbial characteristics of the co-contaminated soils also need further study to test the bioremediation potential of co-contaminants.

Public perception

Atari, Luginaah, Gorey, Xu, and Fung (2013) studied the relationship between odor annoyance and BTEX concentrations in the "Chemical Valley" of Sarnia, Ontario, Canada. The study respondents were self-assessed based on their degree of odor annoyance. People who lived close to the chemical plants responded with high odor annoyance. The results demonstrated that odor annoyance was a function of the actual BTEX concentrations. Females were more likely to report odor annoyance than their male counterparts. The authors mentioned some limitations their study had. They did not study that the odor annoyance might have been related to other pollutants correlated to BTEX. The other limitation of the study was the use of postal codes instead of personal monitoring to develop air pollution estimates since the use of postal codes might have led to exposure misclassification (Atari et al., 2013).

The public view on environmental cleanup technologies is dependent on the following factors (Kocher, Levi, & Aboud, 2002):

- new technology should be better than the existing one,
- the technology should be compatible with the people's needs,
- the technology must be explained well to be relatively easy to understand by everyone,
- the technology must be suitable for limited testing,

- and the effects of the technology should be visible (Kocher, Levi, and Aboud, 2002).

One crucial factor that can affect residents' opinion about cleanup technologies is trust. Residents are often skeptical about cleanup plans due to inadequate information, experience, and general institutional distrust (Kocher, Levi, & Aboud, 2002; Pretty et al., 2007; Ronneau & Bitchaeva, 1997).

Elekwachi, Andersen, and Hodgman (2014) conducted a global survey on the preferred remediation methods for different pollutants (aromatic hydrocarbons, heavy metals, and fertilizers). The survey targeted individuals, multinational companies, government agencies, universities, Not-for-Profit Organizations and Non-Governmental Organizations, agriculturists and research groups involved in bioremediation technologies. Half of the survey respondents (46 out of 92 from six continents) answered they would prefer to use bioremediation to other treatments. Practically other treatments are being used more than bioremediation. Chemical treatments are used because of various factors, including the risk of the contaminated land's nature and difficulties in the project design. The project design includes identifying effective microorganisms, optimizing environmental conditions, confirm the magnitude of eventual clean-ups, insufficient understanding of all the mechanisms and processes bioremediation has. The lack of knowledge is why there are difficulties in bioremediation processes to model, simulate or control for a better outcome and implementation (Elekwachi, Andersen, & Hodgman, 2014).

Problem statement

Abiotic and biotic conditions influence the bioremediation efficiency of soils co-contaminated by petroleum products and other pollutants (Lebkowska et al., 2011). Native, aerobic (surface) microbial population in soils can enhance the biodegradation process of aromatic

hydrocarbons (Fountoulakis, Terzakis, Kalogerakis, & Manios, 2009). Combining native plants and microbes that are metal-resistant and better adapted to local pedoclimatic conditions should be used for bioremediation strategies (Mohamad et al., 2017).

Scientists must have a better understanding of the diversity, structures, and metal stress level of native soil microbial communities in co-contaminated soil to be able to design a successful and sustainable bioremediation strategy. The concentration and types of co-contaminants need to be studied to have a better understanding on microbial changes (Schwarz et al., 2019). Knowledge of the microbial structure, characteristics, and behavior on co-contaminants is essential. Understanding pollutant transformation and inhibitory effects improves bioremediation technologies to reduce total energy and water use, reduce waste, and decrease remediation time and cost (EPA, 2020). Few studies compare different bioremediation techniques for the inhibitory effects of long-term co-contaminants in high concentrations from field samples. New studies must seek to understand benzene and toluene biodegradation limitations with Cd, Pb, and Zn co-contamination to fill this gap. It is essential to improve existing bioremediation techniques by discovering heavy metals' limitations on benzene and toluene biodegradation in heavily co-contaminated soils. Demonstrating bioremediation potential for soils exposed to long-term co-contamination in high concentrations is crucial because it will allow scientists to understand the microbial changes and design a better remediation strategy.

Research objectives and hypotheses

This research project focuses on identifying native aerobic benzene- and toluene-degrading microbial communities in the presence of different concentrations of Cd, Pb, and Zn. The study compares the differences between benzene and toluene degrading microorganisms under stress due to various heavy metal concentrations (Cd, Pb, and Zn), focusing on communities specific for the

co-contaminants. It demonstrates and assesses bioremediation potential for heavily and long-term co-contaminated soils on a laboratory scale. The overall purpose of this dissertation is to address the limitations of native aerobic degraders of benzene and toluene in soils' long-term exposed to Cd, Pb, and Zn in high concentrations. The specific objectives of this dissertation are:

1.) Identify native soil aerobic degraders of benzene and toluene in the presence of various concentrations of Cd and Pb. DNA extracts were obtained from aerobic microcosms inoculated with benzene or toluene as a sole carbon source but differed in heavy metals' concentration. The general approach was to analyze the extracted DNA through a combination of molecular biology methods (denaturing gradient gel electrophoresis [DGGE], high throughput 16S rRNA gene amplicon sequencing, and quantitative PCR [qPCR]). This objective is presented in Chapter III of this dissertation.

2.) Determine the bioremediation potential of benzene and toluene in soils long-term impacted with heavy metal contamination at the Tar Creek Superfund site, Oklahoma. The general approach is to collect soils from the Superfund site containing a wide range of heavy metal contamination. Selected soils were then incubated in aerobic microcosms under biostimulated conditions (amended with necessary trace elements and yeast extract for nutrients) and natural attenuation conditions (amended with deionized water) on a laboratory scale. Biodegradation of a mixture of benzene and toluene was then measured until completion. Chapter IV presents this study and analysis.

3.) Determine the in situ microbial community composition of the Tar Creek Superfund site soils and the community enriched on the mixture of benzene and toluene from the selected soils. The microbial communities will be determined and compared in the collected soils

and the aerobic microcosms after benzene and toluene degradation through a 16S rRNA gene amplicon sequencing approach. Chapter IV presents this study and analysis.

Successful completion of the three objectives mentioned above will facilitate a better understanding of how native aerobic microbial communities are altered in response to long-term co-contaminations exposure in different concentrations. It will provide a comprehension of the diversity of the native microbes, their community structures, and stress levels caused by different concentrations and types of co-contaminants. The research findings will help enhance bioremediation practices for long-term exposed, co-contaminated areas or Superfund sites. This data will ultimately assist scientists and remediation specialists to design bioremediation techniques and enhance their application for co-contaminated sites.

Objective 1

Identify native soil aerobic degraders of benzene and toluene in the presence of various concentrations of Cd and Pb.

Hypothesis

Unique bacteria will be enriched from a single soil under different concentrations of Cd and Pb co-contaminated with benzene or toluene as a sole carbon source.

Objective 2

Determine the bioremediation potential of benzene and toluene in soils long-term impacted with heavy metal contamination at the Tar Creek Superfund site, Oklahoma.

Hypothesis

Biostimulation would induce higher degradation rates of benzene and toluene than natural attenuation. Soils with high amounts of heavy metals would also have a negative effect on the degradation rates.

Objective 3

Determine the *in-situ* microbial community composition of the Tar Creek Superfund site soils, and the community enriched on benzene and toluene from soils selected from the site.

Hypothesis

Heavy metals would have a high impact on the microbial communities in-situ and affect the microbial community members enriched on benzene and toluene. I expect a more negligible difference between soils under biostimulated conditions compared to natural attenuation conditions, where in situ levels of N and P may limit specific bacterial growth in addition to metal stressors.

CHAPTER II

REVIEW OF LITERATURE

Examination of co-contamination problems of benzene, toluene, and heavy metals

Soil pollution is a global problem. The most common types of pollutants found in soils are oil, pesticides, heavy metals, high levels of salt, and fertilizers (Orgiazzi et al., 2016). Among soil contaminants, petroleum remains a grave environmental issue (Varjani & Upasani, 2017). Petroleum is the equivalent of crude oil, consisting of complex mixtures of hydrocarbons. Benzene and toluene are aromatic hydrocarbons and natural constituents of crude oil (Speight, 2014; Brezonik & Arnold, 2011). They are also used as a solvent in petroleum refining and petrochemical industries (Speight, 2014).

Elekwachi, Andersen, and Hodgman (2014) conducted a global survey in 2014 about the utilization of bioremediation technologies for addressing different environmental pollutions. They received feedback from all continents except Antarctica. Heavy metals and aromatic hydrocarbons proved to be the most common pollutants and greatest concerns worldwide (*Figure 1*) according to the results of the survey (Elekwachi, Andersen, & Hodgman, 2014).

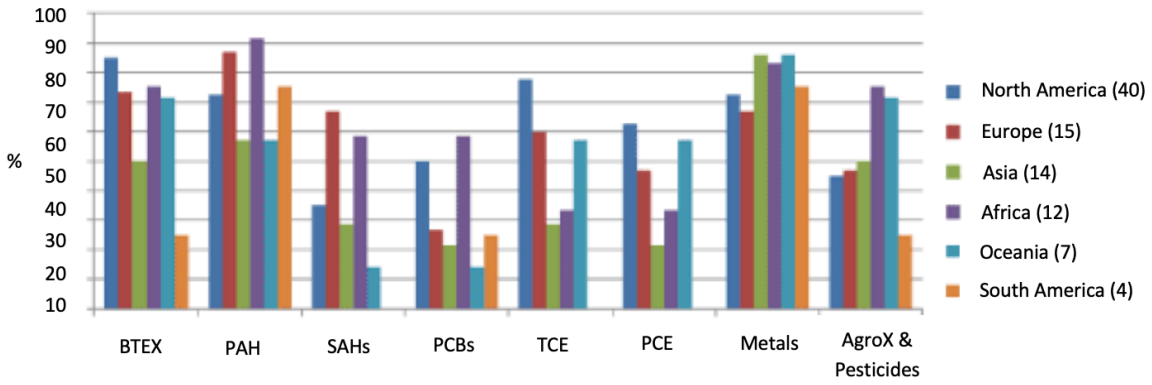

Figure 1 Major contaminants encountered and distributed according to continents. All results presented as percentages of total respondents in each category (Elekwachi, Andersen, & Hodgman, 2014).

Approximately 40% of hazardous waste sites are co-contaminated with organic pollutants and heavy metals in the United States (Sandrin & Maier, 2003). Congress approved the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), also known as Superfund, in 1980. Superfund sites are contaminated areas caused by improper hazardous waste management. The sites include manufacturing facilities, processing plants, landfills, and mines (EPA, 2020). The estimated total number of reported, documented, proposed, and deleted Superfund sites in the United States is approximately 1,871 (*Table 1*), and among them, 1,810 are on the National Priority List (NPL). Around 1,410 sites that are active and archived are co-contaminated with benzene and toluene or their mixed isomers. Approximately 658 of them have not been remediated yet (EPA, 2020). The most common heavy metals in the co-contaminated Superfund sites are: As, Ba, Cd, Cr, Hg, Pb, Ni, and Zn (Sandrin & Maier, 2003).

Table 1 Proposed, current, and deleted NPL Sites in the USA (EPA, 2020)

Status	Non-Federal (General)	Federal	Total
Proposed NPL Sites	48	3	51
NPL Sites	1178	157	1335
Deleted NPL Sites	407	17	424

Adverse health effects of benzene, toluene, Cd, Pb and Zn

Environmental problems also cause negative impacts on human health. Numerous environmental health problems are caused by hazards that are associated with human activity since the industrial age. Chemicals have both direct and indirect impacts on humans, individually and collectively. It is hard to predict the effects of lifetime exposure to chemicals. Exposure to chemicals can cause acute poisoning if the amount and concentration are large enough. The biological effects of pollutants can accumulate over time in living organisms. Their impacts on the individuals depend on the duration of exposure received until the end of life (Carson, 1962). The United States Environmental Protection Agency (EPA) looks for solutions to environmental problems, which overall protect human health and the environment. Those solutions include long-term effectiveness and performance, toxicity, mobility, volume reduction of pollutants, implementation ability, cost, and compliance with the standards (EPA, 2003).

Aromatic hydrocarbon compounds, such as benzene and toluene, are toxic to living organisms and have adverse effects on the environment (Ahmed et al., 2019). The potential of benzene exposure is a larger risk for humans since benzene is a component of gasoline. The primary route of exposure is through inhalation; however, it is also possible for benzene to enter via oral or dermal routes (EPA, 2002). The EPA (2003) evaluates benzene as a potential human carcinogen based on the Integrated Risk Information System (IRIS). Several studies (Chambers et al., 2018; Mochalski et al., 2018; Sauer et al., 2018; Zarth et al., 2014; Ress, Witt, Xu, Haseman, & Bucher, 2002; Lovreglio et al., 2020) proved the carcinogenic effect of benzene. Long-term exposure to benzene in high concentrations causes leukemia in humans through inhalation or drinking water (*Table 2*, EPA, 2003).

Table 2 Health risk estimates of benzene for humans (EPA, 2003)

Drinking water unit risk	4.4×10^{-7} to 1.6×10^{-6} per (μg/L)
Oral slope factor	1.5×10^{-2} to 5.5×10^{-2} per (mg/kg)/day

Sauer et al. (2018) revealed benzene has immune-carcinogenic effects, as they found specific proteins in the blood of individuals exposed to benzene. B7.1 and B7.2 proteins serve as a biomarker for benzene toxicity. Reduced gene expression of tumor suppressor genes was found in workers exposed to low benzene concentration through inhalation. Zarth et al. (2014) analyzed the 7-phenylguanine (7-PhG) produced when benzene oxide reacts with DNA. This product is generated after the transient hydroxyl cyclohexadienyl intermediate is dehydrated. DNA isolated from mice and treated with benzene, DNA from cells exposed to benzene oxide, and DNA from human leukocytes was assessed. The 7-PhG in DNA reacted with benzene oxide, but it did not react with DNA obtained from bone marrow, liver, or lung of mice after being treated with benzene for four weeks daily. It developed cancer after being exposed for 103 weeks. A higher dose, such as 10 mM of benzene, reacted with 7-PhG in the DNA, contrary to when DNA cells were exposed to 100 μM to 1 mM benzene (Zarth et al., 2014).

Benzene has other adverse effects on human health besides a risk of cancer. It causes blood toxicity, neurotoxic effects, genotoxicity neurotoxicity, reproductive toxicity, developmental toxicity, and chronic inhalation (*Table 3*, EPA, 2002). The EPA defined the benchmark concentration for benzene exposure, stating that the chronic oral reference dose is 4×10^{-3} mg/kg per day for an adult (EPA, 2002). Debarba et al. (2020) demonstrated on mice samples that chronic benzene exposure influences severe metabolic imbalance associated with central hypothalamic inflammation and endoplasmic reticulum stress, depending on gender. D'Andrea and Reddy (2013) examined the human exposure to benzene in blood samples after an incident occurred in the British Petroleum Refinery in Texas. Children who were exposed to benzene experienced significantly different blood profiles, liver enzymes, and somatic symptoms. Benzene accounted for higher risk of developing hepatic or blood-related disorders (D'Andrea & Reddy, 2013).

The EPA did not categorize toluene as a carcinogen or genotoxic in the IRIS. Toluene has some concerning health effects on human exposure (EPA, 2005). The general toxicity of toluene is relatively low (Low, Meeks, and Mackerer, 1988; EPA, 2005). Gericke et al. (2001) recruited

volunteers randomly selected from the German Federal Professional Association of the Printing and Paper Processing Industry to evaluate toluene health effects on human health. The employees were exposed to toluene occupationally for over a decade. Gericke et al. (2001) studied employees who worked next to the printers and their helpers. The age differed for the selected subjects. Every volunteer was a man. The exposure assessment included psycho-physiological and blood tests. There was no convincing evidence for defined chronic disease in heavily toluene-exposed workers compared to workers with low exposure. The apparent adverse health effects of toluene in workers exposed over 20 years were not observed (Gericke et al., 2001).

Table 3 Developmental toxicities caused by benzene in humans (EPA, 2002)

Population	Number	Exposure	Effects	Reference
Pregnant worker	1	Exposure to benzene during entire pregnancy	Maternal effects included severe pancytopenia and increased chromosomal aberrations; no fetal effects	Forni et al., 1971
Employees in university laboratory (~745 subjects, 1160 pregnancies), divided into those with and without exposure to organic solvents	745	Responders to questionnaire reported exposure to at least 14 solvents; 41 workers remembered using benzene during the first trimester of pregnancy and 5 workers used phenol	All women exposed to solvents had slight but not statistically significant difference in miscarriage rate over those not exposed (RR = 1.31, 95% CI = 0.89–1.91); 35 of 41 workers exposed to benzene delivered, 1 had induced abortion, and 5 miscarried (miscarriage rate for benzene-exposed subjects, 12.2%; miscarriage rate for all responders to questionnaire, 11.1%; miscarriage rate for unexposed responders, 10.1%); all 5 workers exposed to phenol delivered; exposure to solvents did not affect perinatal death rates or the incidence of malformations	Axelsson et al., 1984
Female gluing operators	360	Exposure to gasoline (a major source of benzene) and chlorinated hydrocarbons via skin and inhalation; benzene levels, < 5 mg/m3; 40% of chlorinated hydrocarbon measurements exceeded permissible limits by 1.2- to 2.4-fold; controls had no chemical exposure	Spontaneous abortions and premature births (17.2% vs. 4.9% in controls), incidence of late membrane rupture, and intrauterine asphyxia of the fetus increased with exposure duration	Mukhameto va & Vozovaya, 1972
Adult female workers and 14 of their children	29	Adults exposed to benzene and other organic solvents during pregnancy (compared with 42 control adults and 7 of their children)	Lymphocytes from adults exhibited approximately twofold increase over controls in incidence of chromosomal aberrations and breaks; their children exhibited increased frequency of chromatid breaks, isochromatic breaks ($p < 0.01$, 14 children), and sister chromatid exchanges ($p<0.001$, 4 children) in lymphocytes	Funes-Cravioto et al., 1977
32-year-old pregnant worker	1	Personal interview revealed exposure to benzene and other solvents (dichloromethane, methanol, and ether) in laboratory during first trimester of pregnancy; compared with matched control	Stillborn anencephalic fetus	Holmberg, 1979
23-year-old female	1	21 intramuscular injections of benzene in an unsuccessful attempt to induce abortion during first trimester of pregnancy	Following normal delivery, infant exhibited slight dysmorphic (hypotelorism and deep nasal bridge), moderate axial hypotonia and abnormal ocular movements; at 1.5 months of age, child was microcephalic, had severe axial hypotonia, severe peripheral hypertonia, and bilateral optic atrophy, and CT scanning revealed bilateral porencephalic cavities that created communication between lateral ventricles and subarachnoid space; interventricular septum lacking; child died from aspiration pneumonia at 2 months of age	Bordarier et al., 1991

Filley, Halliday, and Kleinschmidt-Demasters (2004) found that long-term and higher exposure to toluene vapors via spray paint had a severe impact on the central nervous system in the human brain. They recognized toluene as a neurotoxin to the cerebral white matter in humans. Patients got affected through inhalation of spray paint. Long-term and higher exposure to toluene causes dementia, leukoencephalopathy, and more (Filley, Halliday, & Kleinschmidt-Demasters, 2004). Flowers (2005) stated that the most acute effects of toluene on humans through inhalation were neurologic effects such as altered color vision, dizziness, fatigue, headache, and declined performance in neurobehavioral tests (Flowers, 2005).

Cadmium is classified as toxic to the cell. It causes cellular necrosis, as well as a probable human carcinogen, according to the IRIS (EPA, 1989). Zhang, Du, Zhai, and Shang (2014) evaluated Cd exposure levels and their health effects in a group of residents who lived in a Cd-contaminated area since the 1960s. The subjects of the study were exposed to Cd for over 45 years. One of the major exposures to Cd is through food sources. The average concentration of Cd in rice in the exposed area was 0.59 ± 0.41 mg/kg above the critical limit (*Table 4*) in 2006. The subjects developed renal dysfunctions from long-term Cd exposure. The glucose levels increased in urinary and blood samples that suggested glucose metabolism disorders as a biomarker of chronic Cd toxicity (Zhang, Du, Zhai, & Shang, 2014).

Cabral et al. (2015) studied Cd and Pb concentrations to evaluate the health effects of co-exposure of these two heavy metals. Blood and urinary samples were studied to measure oxidative stress and nephrotoxicity. The study subjects were exposed to heavy metals due to massive solid waste disposal in the outskirts of Dakar city, Senegal, Africa, which included industrial and hospital wastes. The waste was not appropriately managed, and residents became exposed to pollution. The subjects of the study were both men and women between the age of 17 and 70. The subjects had a higher Cd and Pb level than the controls in the blood and urine samples. The studied heavy metals caused oxidative stress conditions because of the overproduction of reactive oxygen species. The

overproduction disrupted the antioxidant defense system and lipid peroxidation in the patients. The results showed signs of impaired renal functions in the subjects (Cabral et al., 2015).

The IRIS (EPA, 2004) evaluated the daily exposure of Pb to the human population. Lead has specific toxic effects that cause cellular necrosis. The Centers for Disease Control and Prevention (CDC) determined 10 μg/dL as a benchmark for Pb concentration in children's blood. The EPA identified Pb as a possible human carcinogen and supported their suggestion with human and animal carcinogenicity data in their report (EPA, 2004). The major routes of Pb for human exposure are food consumption, drinking water (*Table 4*), and inhalation from the air. Lead toxicity level in human blood is associated above 100 μg/L (Vries, Romkens, & Schutze, 2007).

Table 4 An overview of food and drinking water quality criteria for Cd and Pb for human health effects (Vries, Romkens, & Schutze, 2007)

Receptor	Unit	Critical limit Cd	Pb	Source
Wheat	mg/kg	0.2	0.2	Food quality criteria, EU 2001
Vegetables (e.g., endive, spinach, lettuce, etc.)	mg/kg	0.2	0.3	Food quality criteria, EU 2001
Drinking water	μg/L	3	10	WHO 2004

Khan, Quayyum, Saleem, Ansari, and Khan (2010) studied blood lead (Pb) levels in children and workers exposed to Pb to find adverse health effects. The studied subjects lived and worked in the nearby industrial area, frequently exposed to Pb. The control subjects lived 30 km away from the studied area. A significant Pb increase was found in children's blood compared to the controls. These children got exposed to Pb indirectly because they live close to the industrial area, or their fathers occupationally were exposed to Pb. The children exposed to Pb developed hematopoietic, renal, and hepatic malfunctions (Khan, Quayyum, Saleem, Ansari, & Khan, 2010).

Lead (Pb) also harms wildlife. Finkelstein et al. (2012) studied one of the rarest birds on the Earth, the California condor (*Gymnogyps californianus*). The condor became endangered partially due to significant environmental hazards, which are not sufficiently mitigated. Finkelstein

et al. (2012) demonstrated data on the frequency, magnitude, sources of Pb exposure, and related health effects on the California condor (*Gymnogyps californianus*). The California condors (*Gymnogyps californianus*) are chronically exposed to Pb due to lead-based ammunition. The birds developed significant subclinical health effects. The health effects included inhibition of the heme biosynthetic enzyme δ-Aminolevulinic acid dehydratase. Lead (Pb) poisoning increased morbidity and mortality rates in this endangered species (Finkelstein et al., 2012).

Zinc is a vital element in a healthy human diet. It has a recommended dietary allowance of 11 mg/day for adult men and 8 mg/day for adult women. It is a daily nutritional requirement and an essential trace element to survival and health maintenance in all animal species. Severe Zn deficiency causes adverse health effects. The negative impacts are bullous pustular dermatitis, diarrhea, alopecia, mental disturbances, and impaired cell-mediated immunity (EPA, 2005). The principal anthropogenic sources of Zn pollution come from metal smelters and mines. High levels of Zn consumption cause clinical symptoms of gastrointestinal distress. Low levels of Zn consumption affect the status of other vital nutrients in the human body, such as iron and copper. Both Zn deficiency and high concentrations can increase receptiveness to carcinogenesis. The EPA calculated the reference dose for Zn as 0.3 mg/kg/day (Choudhury et al., 2005).

Heim et al. (2015) analyzed the DNA-damaging properties of zinc oxide nanoparticles. Zinc oxide nanoparticles dissolve rapidly to form hydrated Zn^{2+}. Zinc in this form accumulates in the nucleus and mediates and damages DNA, depending on the cellular intake of dissolve Zn^{2+} ions (Heim et al., 2015). Van der Merwe, Carpenter, Nietfeld, and Miesner (2011) studied Pb and Zn poisoning in Canada geese (*Branta canadensis*). The study area was the Tri-State Mining District in Kansas, Oklahoma, and Missouri. The mining district includes Tar Creek Superfund site. Van der Merwe, Carpenter, Nietfeld, and Miesner (2011) looked for observable Pb and Zn poisoning signs in the Canada goose (*Branta canadensis*). Higher lead levels were found in the tissues and caused inhibited blood enzyme activities in birds that resided in the contaminated area.

Histopathologic signs of Zn poisoning, such as fibrosis and vacuolization, were observed in the waterfowls (van der Merwe, Carpenter, Nietfeld, & Miesner, 2011).

Global economic costs of benzene, toluene, and heavy metals bioremediation

Environmental problems have socioeconomic and ecological consequences as well (Carolan, 2004). Many economists (Faber, 2008; Seneca & Taussig, 1974; Costanza, 1992) view environmental problems as economic issues. Economists believe market failure leads to environmental and economic problems. Economists want to correct the market failure by making goods' prices represent economic and social costs (Faber, 2008; Seneca & Taussig, 1974; Costanza, 1992; Sagoff, 1981). These costs also contain pollution and remediation costs. It is a long-time approach to choose the best available strategy, which maximizes the satisfaction of the market's preference (Sagoff, 1981). Contractors are looking for cost-effective remediation strategies. The cost and performance of biological treatment technologies are a long-term interest (EPA, 2003).

Bioremediation is a biological treatment that can enhance the eco-efficiency of polluted land management (Simpanen et al., 2016). Pollutants can be removed with certain microbes during bioremediation. Bioremediation is a natural element of respiration or adaptation, carbon, or metal redox cycling. There are different types of bioremediation techniques; natural attenuation, bioaugmentation, and biostimulation. Bioaugmentation and biostimulation are significant parts of bioremediation to remove contaminants (Krzmarzick, Taylor, Fu, & McCutchan, 2018). Bioremediation techniques also work for oil and oil product pollution. It is a promising remediation technique, among others, because it is not harmful to the environment (Leal et al., 2017) and is an economical and effective solution for soil pollution (Ma et al., 2018).

The remediation market, including bioremediation, was in an early stage in Europe in 2001. The costs were based on very few implementations of technologies at that time. The costs modified throughout the years when the remediation techniques started to develop in Europe. The remediation costs vary between countries and depending on several factors. These factors are regulation, market trends, and landfill prices. Attempts had been made to standardize the costs of

remediation technologies throughout the European Union. The attempts failed due to confusion in the calculation of the price of the remediation techniques. The first effort was to calculate the price by the volume of the contaminated soil, but several cases calculated the total volume of the area, including the "clean" soil. Another attempt was to calculate the median price of the technologies. One of the most cost-effective solutions was in-situ bioremediation, among other remediation technologies, after several calculation attempts in the early 2000s (*Figure 2*). *Table 5* summarizes the average costs of bioremediation technologies, where ex-situ bioremediation was the most expensive and in situ natural attenuation was the most cost-effective technique. In situ bioremediation was the most cost-effective in Germany, and Finland was the most expensive (Summersgill, 2005).

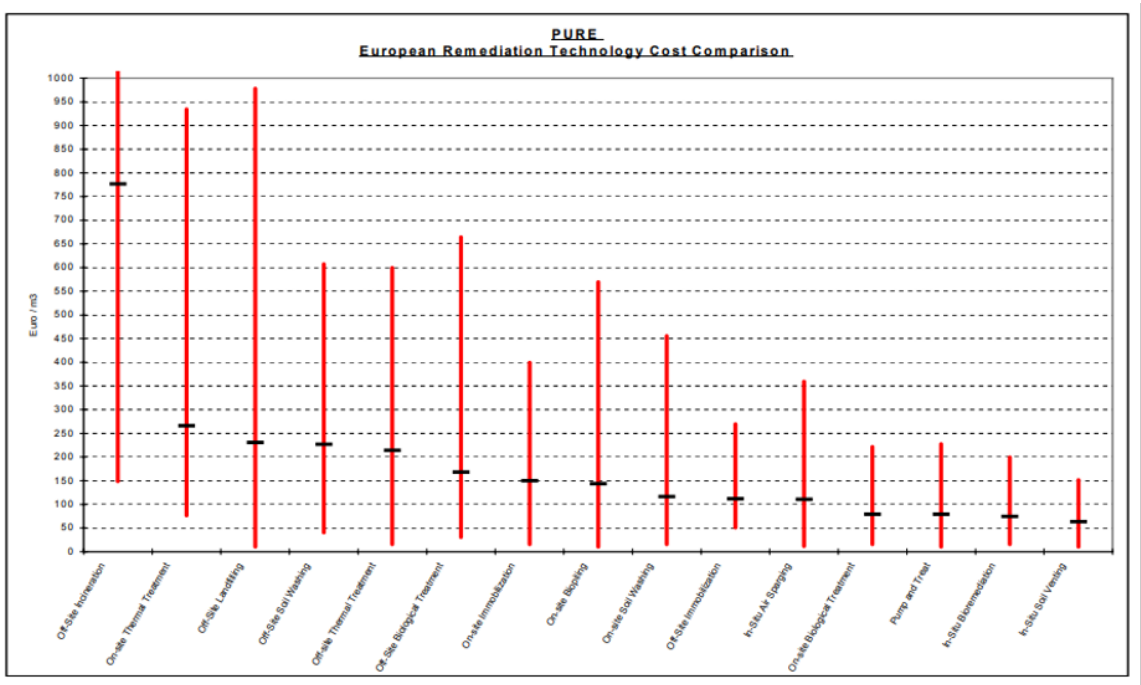

Figure 2 Cost comparison of remediation technologies within European countries in the early 2000s (Summersgill, 2005). [1]

[1] X axis shows Euro/m^3, y axis demonstrates the remediation technologies from left to right: Off-site Incineration, On-site Thermal Treatment, Off-site Landfilling, Off-site Soil Washing, Off-site Thermal Treatment, Off-site Biological Treatment, On-site Immobilization, On-site Biopiling, On-site Soil Washing, Off-site Immobilization, In-situ Air Sparging, On-site Biological Treatment, Pump and Treat, In-situ Bioremediation, and In-situ Soil Venting.

Table 5 Average costs comparison of bioremediation costs in Europe in the early 2000s (Summersgill, 2005).

Technology	Euro/m^3		
	Minimum	**Maximum**	**Average**
Ex situ biological treatment	20 (~16.6 $/cy)	665 (~551.95 $/cy)	167 (~138.61 $/cy)
In situ biological treatment	11 (~9.13 $/cy)	222 (~184.26 $/cy)	76 (~63.08 $/cy)
In situ bioremediation	15 (~12.45 $/cy)	200 (~166 $/cy)	73 (~60.59 $/cy)
In situ natural attenuation	15 (~ 12.45 $/cy)	25 (~ 20.75 $/cy)	20 (~16.6 $/cy)

Bioremediation technologies are started to be utilized more frequently in the United States to remediate hazardous waste sites such as Superfund sites. The second most common type of Superfund sites where bioremediation techniques are applied is petroleum-contaminated sites, according to the EPA (2001). Benzene and toluene are two of the most frequent pollutants that pollute Superfund sites in the United States (*Figure 3*). Bioremediation technologies have been utilized in the U.S. since the mid-1980. Implementing different bioremediation techniques was not common in the mid-1980s until their usage started to rise in 1989. Each year 8 to 12 bioremediation techniques are applied on Superfund sites ever since. There is not much information about the cost of bioremediation. A total of 67 Superfund sites made their cost (*Table 6*) implementation available for the EPA (EPA, 2001). *Tables 7* and *8* include a summary of a couple of available cost data on bioremediation projects. These projects were applied to remediate sites polluted with benzene, toluene, and heavy metals.

There is an increase in using bioremediation techniques in recent years. There is a lack of available information about the cost and performance of bioremediation techniques carried out twenty years ago. This lack of knowledge was because of the limited number of research papers on the topic. More bioremediation research and field demonstrations have been conducted during the last two decades (EPA, 2001). Another reason for the increase in using bioremediation is the received project funds. The Obama administration signed the American Recovery and Reinvestment Act (ARRA) in 2009. The act included $7.22 billion for the EPA projects. This covered $100 million for cleanup, revitalization, and sustainable reuse of brownfields; $600 million

for hazardous waste sites remediation; and $200 million for petroleum leaks from underground storage tanks (EPA, 2018).

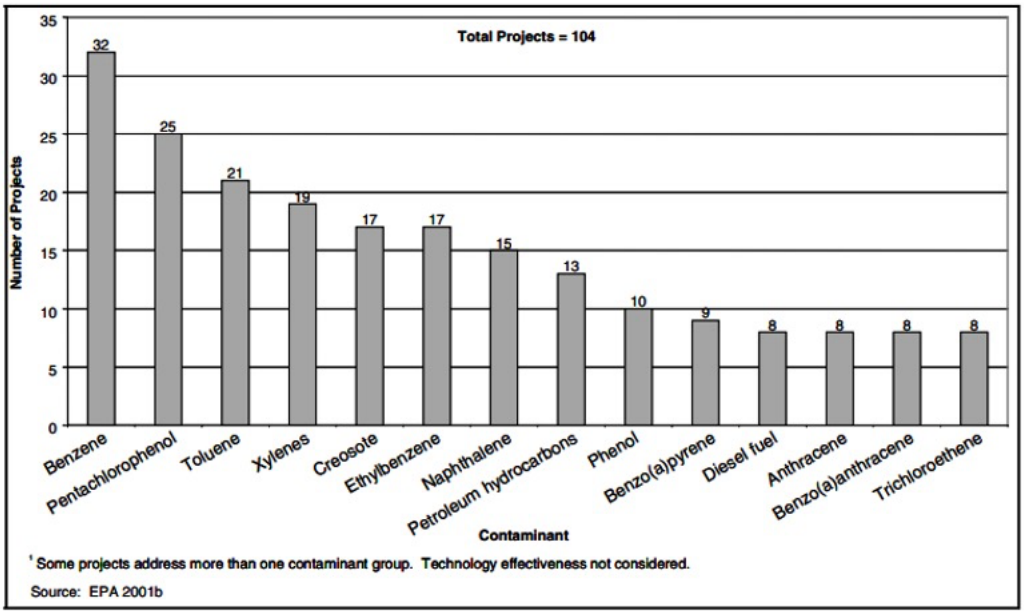

Figure 3 Pollutants most treated by bioremediation techniques at Superfund sites in the United States between 1982 and 1999 (EPA, 2001).

Table 6 Estimation of different remediation techniques for treating polluted soil. Based on case studies from 1998 (EPA, 2001). cy indicates for cubic yard.

	Estimation for unit costs for soil bioremediation	
***In situ* (bioventing) projects**	from $2/cy to $300/cy	most sites cost less than $40/cy
***Ex situ* projects**	from $13/cy to $500/cy	most sites cost less than $300/cy

	Estimation for unit costs for soil remediation	
Thermal Desorption *(in and ex situ)*	from $38/cy to $642/cy	average $256/cy
Soil vapor extraction (*in situ)*	from $37/cy to $1,200/cy	average $300/cy
On-site Incineration	from $184/cy to $1,610/cy	average $628/cy

The EPA included two examples in their 2001 report when the proposed remediation technology was changed to bioremediation due to cost and effectiveness. One of the examples occurred in Louisiana in 1995. The first project defined incineration to remediate soils and sludge. Both soils and sludge were contaminated with benzene, toluene, mercury (Hg), lead (Pb), chromium (Cr), arsenic (As), barium (Ba), and several other organic compounds. The on-site

incineration appeared to be an expensive solution. Incineration was changed to bioremediation to make the project cost-effective. The other example occurred in a petrochemical facility in Texas. The primary pollutant of the site was benzene. The EPA signed the original remediation plan of the site in 1991. The EPA added *in situ* bioremediation techniques for treating the aquifer later in 1998. The original remediation techniques did not prove to be adequate for this project (EPA, 2001).

Table 7 Summary for some examples of *ex situ* soil bioremediation costs (EPA, 2001)

Site name	State	Cleanup program	Status	Contaminants	Start year	Area cost factor	Technology cost ($) (Source)	Volume treated (cy)	Unit cost ($/cy)	Comments
Ex situ soil bioremediation – land treatment										
Dubose Oil Products Co.	FL	Superfund	Complete	Benzene, toluene, ethylbenzene, xylene, chlorinated VOCs, other VOCs	1993	0.87	4,990,000	13,137	380	Composting treatment system, leachate collection, inoculant generation, vacuum extractions, wastewater treatment
Fort Greely Underground Storage Tank Soil Piles	AK	Other	Complete	BTEX	1994	1.60	749,000	9,800	76.4	Operation and maintenance only in summer months; no liner
Fort Wainwright, North Post Site Soil Remediation	AK	Other	Complete	BTEX	1993	1.60	433,000	4,240	102	Activities included liner construction, drainage, tilling, and biostimulation
Havre Air Force Station, Remove Abandoned Underground Storage Tanks	MT	Other	Complete	BTEX	1992	1.14	48,700	1,786	27.3	Application mainly consisted of soil plowing and tilling
Lowry Air Force Base	CO	Other	Ongoing	BTEX, petroleum hydrocarbons	1992	1.03	130,000	5,400	24.1	Conducted on plastic sheeting, biostimulation, aeration
Matagora Island Air Force Base	TX	Other	Complete	BTEX	1992	0.82	77,600	500	155	Cost of entire project including monitoring

Table 8 Summary for some examples of *in situ* soil bioremediation costs (EPA, 2001)

Site name	State	Cleanup program	Status	Contaminants	Start year	Area cost factor	Technology cost ($) (Source)	Volume treated (cy)	Unit cost ($/cy)	Comments
In situ soil bioremediation – bioventing										
Dover Air Force Base Area 6	DE	Superfund	Demonstration complete	Chlorinated VOC, heavy metals	1996	1.02	551,000	1,667	331	Direct injection of air and propane; co-metabolic aerobic, pilot test
Hill Air Force Base, Site 280	UT	Not specified	Ongoing	BTEX, petroleum hydrocarbons	1990	1.03	271,000	Not reported	Not calculated	Interim costs
Hill Air Force Base, Site 914	UT	Other	Complete	BTEX, petroleum hydrocarbons	1989	1.03	863,000	5,000	173	Early bioventing application, combined with soil vapor extraction
Lowry Air Force Base	CO	Other	Complete	BTEX, petroleum hydrocarbons	1992	1.03	75,300	Not reported	Not calculated	Interim costs; high initial contaminant concentrations; used horizontal trenches

Limitations for heavy metals' concentration in US soils

The United States Environmental Protection Agency (EPA) works with facilities to address soil exposure routes that cause negative impacts on the environment. The EPA focuses on cleanup activities to limit exposure to pollutants. Ecological Soil Screening Level (Eco-SSL) is a collaborative work led by the EPA. The EPA issued the Eco-SSL for its Superfund programs. The Eco-SSL was developed for several contaminants frequently found in the Superfund sites' soils. The EPA developed an online database where specific contaminants and their effects can be searched (EPA ECOTOX Knowledgebase, 2020). This dissertation focuses on Cd, Pb, and Zn as the studied heavy metals. ECOTOX database has standard concentration levels for these before-mentioned metals. The ECOTOX user guide (Elonen, 2020) helps researchers and scientists understanding the context of the data retrieved from ECOTOX for data analyses or summary projects purposes (EPA, 2020).

Earthworms are known bioindicators of many metals in soils. These animals accumulate certain essential metals and non-essential metals from the soils. Earthworms have a crucial role in terrestrial ecotoxicological risk assessment. The heavy metal bioavailability can be assessed as relative toxicity or lethality index or more sensitivity or endpoints in earthworms (Suthar, Singh, and Dhawan, 2008). Peres et al. (2011) recorded a range of earthworm responses to environmental changes, like heavy metal contamination at both community and individual levels. The studied heavy metals were Cd, Pb, and Zn. The relevance of earthworm descriptors is much dependent on the range of heavy metals concentrations and their bioavailability (Peres et al., 2011). The regulatory concentration of Cd, Pb, and Zn in soils and ecotoxicological levels in earthworms are discussed in the following sections.

Cadmium in total 3,500-35,000 mg/kg concentration caused mortality for worms and standard test species (bioindicators) found in natural soils as the contaminated medium type, reported by Hartenstein, Neuhaser, and Narahara (1981). The duration of contamination lasted 56 days within laboratory conditions. Another concentration (between 1,800-18,000 mg/kg) was

measured for Cd, which resulted in growth inhibition for worms and test species in the same study (Hartenstein, Neuhaser, & Narahara, 1981). The average Cd concentration found in natural soil was between 0-0.3 mg/kg in other studies reported to the ECOTOX Knowledgebase, which affected accumulation in worms. The most extended observation period for 0-0.3 mg/kg Cd concentration in natural soil was 578.36 days, measured by Beyer, Hensler, and Moore (1987), which resulted in Cd accumulation in earthworms.

Lead concentration regulatory levels in soils are 400 mg/kg for in play areas and 1,200 mg/kg for non-play areas in an urban environment. These regulation levels apply to cleanup projects as well (EPA, 2000). Beyer, Hensler, and Moore (1987) studied worms in natural soils treated with different kinds of heavy metals. The total concentration of Pb was between 8.3 and 32 mg/kg, which was the highest among the reported studies in the ECOTOX database, where earthworms were exposed to lead for 578.36 days. The lead accumulated in the worms and the accumulated concentrations were positively correlated with the soil Pb and organic matter (Beyer, Hensler, & Moore, 1987).

Zinc concentration ranges between <5 and 400 mg/kg with a mean of 36 mg/kg in the U.S. cultivated soils. These levels are between <10 and 2,000 mg/kg with a mean of 51 mg/kg in uncultivated soils, according to the U.S. Agency for Toxic Substances and Disease Registry (ATSDR, 2011). Zinc can complex with inorganic soil components, such as carbonates, sulfates, hydroxides, to assemble precipitates or positively charged complexes, according to the EPA (2021). Hartenstein, Neuhaser, and Narahara (1981) studied the highest Zn concentration levels (between 1,300 to 26,000 mg/kg) in worms within laboratory conditions reported in the ECOTOX database. The Zn concentrations between 1,300 to 13,000 mg/kg caused adverse effects in growth rate, while 26,000 mg/kg concentration level had a fatal effect on earthworms (Hartenstein, Neuhaser, & Narahara, 1981).

Heavy metal toxicity on microorganisms

The presence of heavy metals inhibits the degradation of individual benzene or toluene. Some metals (such as Cr, Co, Cu, Fe, Mn, Mo, Ni, Se, V, and Zn) in a small quantity can serve as micronutrients in metabolic reactions and enzyme stabilization (Voica, Bartha, Banciu, & Oren, 2016). The micronutrients can function as redox centers for metalloproteins and iron-sulfur proteins. These proteins have a crucial role in electron transport (Srivastava & Kowshik, 2012). Other heavy metals (such as As, Cd, Hg, and Pb) become toxic in high concentrations for every organism. Lead and cadmium are toxic for all microorganisms and do not have significant biological roles (Voica et al., 2016; Srivastava & Kowshik, 2012). They can substitute essential metals from their binding site or react with other specific ligands (Voica et al., 2016; Sandrin & Maier, 2003). Toxic metal cations can replace physiologically vital cations in the enzyme, such as Cd^{2+}, in exchange for Zn^{2+}. The replacement causes defects in the enzyme function. Microorganisms will be exposed to oxidative stress because of heavy metals' presence (Sandrin & Maier, 2003).

Heavy metals can be classified based on their toxicity level to the environment and beings. The range of heavy metal toxicity can vary between extremely poisonous (such as Cd) (Ashraf et al., 2019) and relatively harmless (such as Ag). Their toxicity also depends on the period of contamination (Chen et al., 2013) and the organisms (Gadd & Griffiths, 1978). Rathnayake, Megharaj, Krishnamurti, Bolan, and Naidu (2013) studied existing growth medium to observe whether the media are accurate enough to determine heavy metal toxicity. Three bacteriological media that are commonly used in toxicological studies was utilized in this study. A new minimal bacteriological medium was created for the experiment. The genus Bacillus (*Bacillus megaterium*, *B. thuringiensis*, and *B. simplex*) was selected for the experiment. The Cd and Pb were added to the medium with increased concentrations, from 0.05 to 8 mg/L. Most growth medium used for the heavy-metal toxicity tests have undefined organic components or high levels of phosphate. These components can chelate heavy metals. The bonding will lead to an overestimation of the toxicity

level of heavy metals. The newly created minimal medium had high free-metal ion activity. The medium proved to give a more accurate determination of the heavy metals' concentration (Rathnayak et al., 2013).

Heavy metals can be present in different chemical and physical forms in soils. These forms are separate-phase solids, soil-absorbed species, colloidal solutions, soluble complexes species, or ionic solutes. The physical and chemical states of the heavy metals are impacted by the environmental condition of the polluted site. These conditions are pH, ionic strength of the water phase, and soil properties such as ion exchange capacity, soil physical type, and organic matter content. Heavy metal ions can bond tightly to sulfhydryl (-SH) groups of enzymes, which are vital for the metabolism of microorganisms (Sandrin & Maier, 2003). Heavy metals can prevent biodegradation of contaminants due to disrupting enzyme activities directly involved in biodegradation. Heavy metals also disturb enzymes that participate in general metabolisms (Amor, Kennes, & Veiga, 2001). The toxicity of heavy metals originates from the concentration of ionic species and not the total or total soluble concentrations of heavy metals. It involves metal-organic complexes, which cannot bind to enzymes. The other concern is the concentration of heavy metals that can bind to enzymes and intervene in microbial activity (Sandrin & Maier, 2003).

High concentrations of heavy metals disrupt cell membranes, alter enzymatic specificity, and harm DNA (Voica et al., 2016). The presence of heavy metals inhibits a wide range of microbial processes. The microbial processes include methane metabolism, microbial growth, conversion of nitrogen and sulfur, dehalogenation, and reductive processes (Sandrin & Maier, 2003). Baath (1989) presented that low enzymatic activity in the soil can occur because of the low concentration of the enzyme or metal inhibition of the enzyme. Enzyme synthesis also appeared to decrease highly because of heavy metals present. The reduction of enzyme activities mainly impacted a declined enzyme synthesis related to prevented microbial growth than to direct enzyme inhibition due to heavy metals (Baath, 1989).

The concentration of the heavy metals must be bioavailable to be removable from the environment (Sandrin & Maier, 2003). Microbes will not degrade heavy metals but transform metals from one organic complex or oxidation state to another. Heavy metals can become less toxic, volatile, water-soluble, or bioavailable during this transformation; they can be removed when it happens. B. thuringiensis showed an increase in Cd and Zn contaminated soils extracted from Cd-rich soils. The number of *Azotobacter vinelandii* increased in the presence of Zn (II). Sulfate-reducing bacteria, like *Desulfovibrio desulfuricans*, can convert sulfate to hydrogen sulfate that can react with Cd and Zn to form them insoluble in water (Chibuike & Obiora, 2014). *Pseudomonas aeruginosa* can enhance pollutant remediation via a different mechanism, according to Agnello, Bagard, van Hullebusch, Esposito, & Huguenot (2016). It can produce metal-chelating siderophores that improve metal bioavailability (Agnello et al., 2016).

Heavy metals affect the abundance and biomass of fungi and bacteria. Fungi show more tolerance to heavy metals than bacteria. Some heavy metals, such as Cd, Pb, and Zn, especially in high concentrations, can cause changes in the species microfungal composition. The number of species and the diversity of higher fungi reduces in heavy metal contaminated soils. Metal pollution has a biological effect, such as an impact on the production of sporophores. Both fungi and bacteria can adapt to heavy metal pollution. Fungal species can only alter to a certain degree of pollution, but competition is always present with other organisms. A sensitive species can adapt to heavy metals but still be less competitive than those species, which can already resist the contaminant. This competition is incredibly intense in the soil system (Baath, 1989).

Some microorganisms develop resistance (*Figure 4*) to heavy metals. Bacteria and Eukaryote withstand heavy metals through decreased influx or enhanced efflux and enzymatic detoxification. Intracellular chelation also demonstrated resistance to metals in many microorganisms. The chelation occurs by different cysteine-rich metal-binding peptides, such as metallothioneins and phytochelatins. Metallothioneins are small molecular weight polypeptides and are classified based on the number of cysteine-residue. Two cysteine-rich domains are usually

present and bind to the heavy metals through mercaptide bonds. The N-terminal β-domain typically binds to three metal ions, while the C-terminal α-domain binds to four metals. Microbial cells can also produce metal sequestering proteins. These proteins are siderophores and DNA-binding proteins from the nutrient-starved cells. Heavy metals produce the multimerization of metal-chelating proteins in Archaea. The proteins are CutA- and DpsA- like, resulting in the protein-metal complex's precipitation (Srivastava & Kowshik, 2012). Hydrogen sulfide (H_2S) produced by microbes has a significant effect on heavy metal toxicity. It is because heavy metals form insoluble sulfides with H_2S. The H_2S-generating microorganisms demonstrate tolerance to heavy metals (Gadd & Griffiths, 1978).

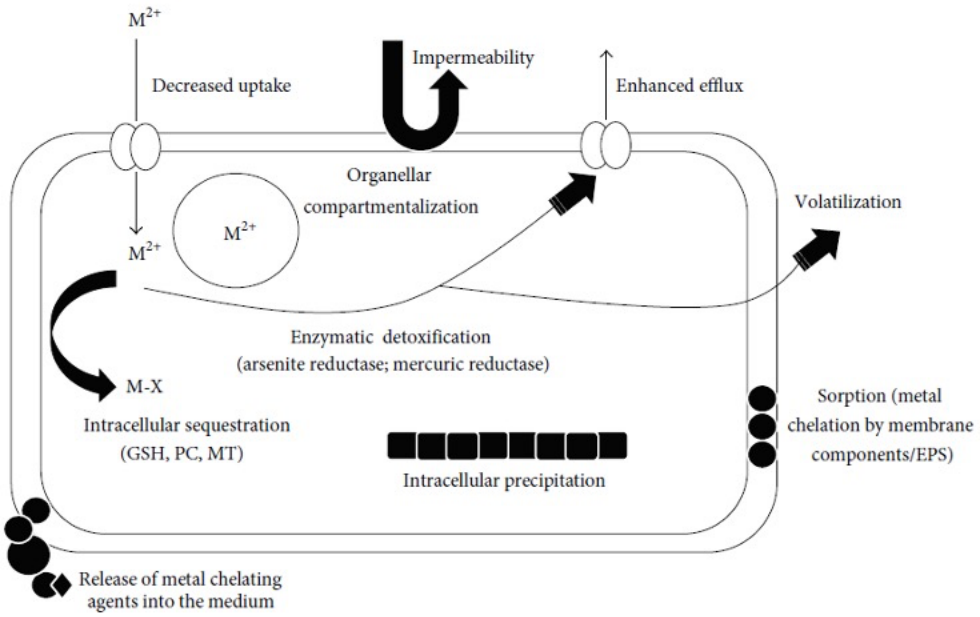

Figure 4 General mechanisms adapted by bacteria, eukaryotes, and archaea for metal resistance (Srivastava & Kowshik, 2012).

Baath, Diaz-Ravina, Frostegard, and Campbell (1998) studied the effect of metal-rich sewage sludge amendments on the soil microbial community. The studied soils were contaminated for over 20 years with Cu, Zn, and Ni as the principal heavy metals. The effects of the metals were examined in two different concentrations (low and high) to observe if the heavy metals generate a variety of responses in the microbial community. Zinc's mean (dry) concentration was 16 g/kg, Cu

was 8 g/kg, and Ni was 4 g/kg in soils treated with sewage sludge for over 20 years. Two different soil types were analyzed: a silty loam and a sandy loam. The bacterial community tolerance increased in all metal-polluted treatments compared to the control due to the experiment. The control sample was uncontaminated sludge. Baath et al. (1998) demonstrated that the tolerance of microbial communities was the highest, where the concentration of metals was the highest. Microbes resistant to high Zn concentrations were also tolerant to both Cu and Cd. Communities that showed resistance to high Cu levels were tolerant to Cd. The threshold levels for the sandy loam soil were 20 mg/kg for Cu tolerance, 60 mg/kg for Ni, and 140 mg/kg for Zn (Baath et al., 1998).

Degradation pathways of benzene and toluene

Biodegradation is a vital natural process in soils that microorganisms carry out. There are genomes of specific microbes that have extra blocks of genetic materials. The extra blocks are part of the chromosome. The blocks, also known as chromosomal islands, include clusters of genes for a specialized function. One of their specific activities is to encode the biodegradation of contaminants, like aromatic hydrocarbons. Petroleum is a rich organic matter source, and microorganisms can utilize hydrocarbons as electron donors (Madigan, Martinko, Bender, Buckley, & Stahl, 2015; Brezonik & Arnold, 2011).

Microbes generate reductants that act extracellularly, like porphyrins, corrinoids, and enzymes. The reductants are capable of decreasing contaminants (Brezonik & Arnold, 2011). Hydrocarbons need to be oxygenated before they can be catabolized. Oxygen (O_2) plays an essential role as a reactant in the catabolism of hydrocarbons. Oxygenase enzymes catalyze O_2 to combine into organic compounds. Two classes of oxygenase are known. The first one is dioxygenase that catalyzes the incorporation of both atoms of O_2 into the molecule. The second one is monooxygenase that catalyzes the addition of only one of the two oxygen atoms. These oxygen atoms are added into an organic compound with the second atom of O_2, reduced to H_2O. The necessary electron donor is NADH or NADPH for most monooxygenase (Madigan et al., 2015).

One of the atoms of the O_2 is incorporated at the last carbon atom as the first oxidation step of the saturated aliphatic hydrocarbon. Monooxygenase catalyzes this reaction. The product of this reaction is a fatty acid of the same length as the original hydrocarbon (*Figure 5*). Beta-oxidation oxidizes fatty acid as the second step. The next step is the formation and oxidation of NADH in the electron transport chain. This step occurs because of energy conservation. The β-oxidation releases acetyl-CoA and a new fatty acid, two carbons shorter than the original one. The process is repeated, and another acetyl-CoA will be released. The produced acetyl-CoA can be used to create new cell material or oxidize via the citric acid cycle (or Krebs cycle). β-oxidation is the primary essential step among all these mentioned reactions for aerobic and anaerobic hydrocarbon catabolism (Madigan et al., 2015).

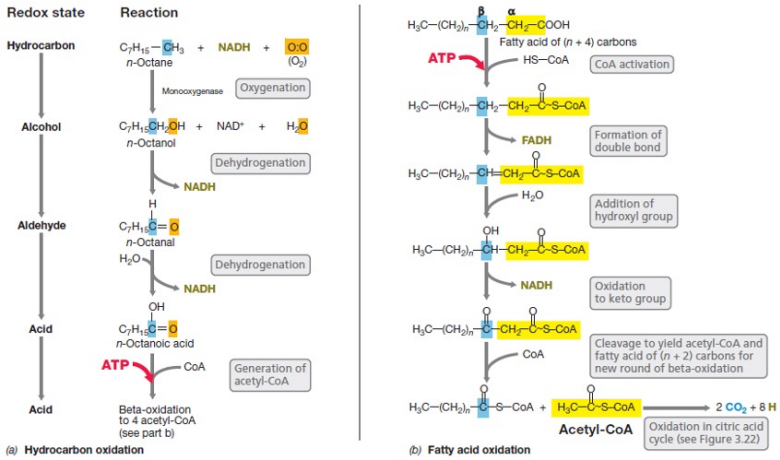

Figure 5 Aerobic hydrocarbon catabolism a) first oxidation step of an aliphatic hydrocarbon, catalyzed by monooxygenase, b) and fatty acid oxidation (Madigan et al., 2015).

Microorganisms use aromatic hydrocarbons as electron donors. The hydroxylation of benzene forms catechol by a monooxygenase (*Figure 6*). NADH is the electron donor in this case. Catechol can break and further degrade after it is generated. The formed compounds, such as acetyl-CoA, can enter the Krebs cycle. Many steps in the aerobic catabolism of aromatic hydrocarbons require oxygenase. The aromatic compounds oxidize ultimately to CO_2, whether single- or multi-ringed (Madigan et al., 2015). Several studies (Oh, Shareefdeen, Baltzis, & Bartha, 1994; Carvajal,

Akmirza, Navia, Perez, Munoz, & Lebrero, 2018; Chen, Abriola, Alvarez, Anid, & Vogel, 1992; Chang, Voice, & Criddle, 1992; Alvarez & Vogel, 1991; Yu, Kim, & Rittmann, 2001) proved that individual benzene and toluene degrade entirely or near completely when no inhibitory effect or other factors are involved in the polluted site. Bacteria and yeast can oxidize aromatic hydrocarbons to fatty acids. The produced fatty acids become acetate. Some of the acetates are then metabolized to CO_2 and water, where energy is produced. The generated energy is utilized to build new cell materials (Rosenberg, 1993).

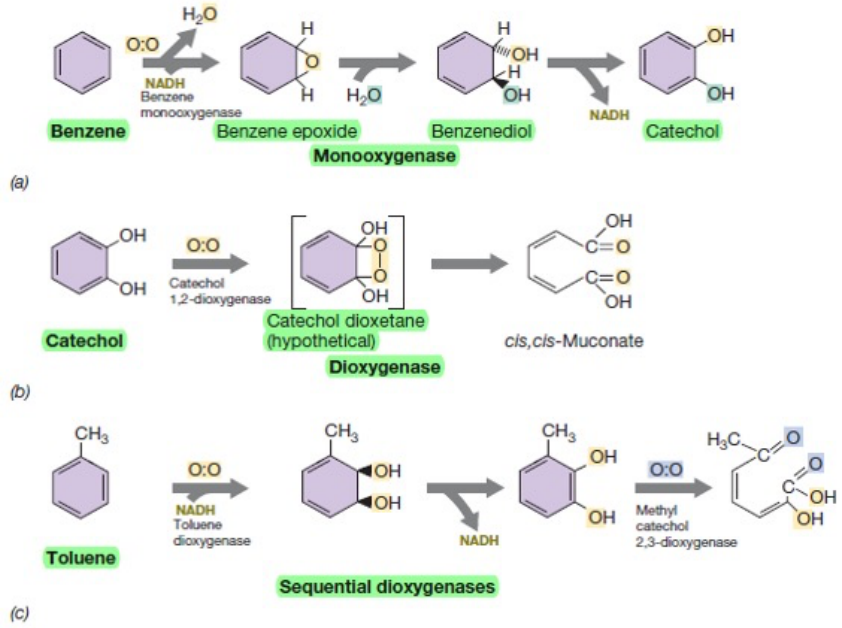

Figure 6 Catabolism of aromatic hydrocarbons a) hydroxylation of benzene to catechol, b) degradation of catechol, and c) toluene (Madigan et al., 2015).

Suenaga, Mitsuoka, Ura, Watanabe, and Furukawa (2001) studied the evolution of Biphenyl Dioxygenase to enhance the degradation of benzene and toluene. The clone of *E. coli* (pSHF1072) demonstrated the potential to degrade benzene and toluene. The modification in one amino acid at position 376 in BphA1 helped to degrade monocyclic aromatic hydrocarbons. The increased degradation rate of benzene and toluene was also observed in the combination of Gln-255, Ile258, Ala-268, Tyr-277, and Thr-376. The toluene dioxygenase from F1 presented high

oxygenation activities toward benzene and toluene, such as pSHF1072 Bph Dox (Suenaga et al., 2001).

Phylogenetic diversity of benzene and toluene degraders

Benzene and toluene can be degraded by microorganisms, depending on the types of the degrading enzyme. *Pseudomonas* species, for instance, can degrade benzene and toluene using toluene-4-monooxygenase, toluene/o-xylene monooxygenase, benzene monooxygenase BmoA, xylene monooxygenase, and naphthalene monooxygenase (Yoshikawa et al., 2017). Bacteroidetes and Actinobacteria are bacterial phyla that significantly contribute to the microbial communities in benzene and toluene-contaminated soils (Borowik et al., 2019). The combinations of *Burkholdera cepacia* LB400 Bph Dox (Biphenyl Dioxygenase) and *Pseudomonas pseudoalcaligenes* KF707 genes in culture indicate ability for complete degradation of benzene and toluene (Suenaga, Watanabe, Sato, Ngadiman, & Furukawa, 2002; Verma & Kuila, 2019). Bacteria are very well studied when it comes to bioremediation research of contaminated sites. Archaea also plays an essential role in bioremediation. Bacteria are better known to degrade hydrocarbons, while Archaea, specifically methanogens, are often part of the degradation activity (Krzmarzick et al., 2018). Fungi also can degrade benzene and toluene. A study showed that Ascomycota was a predominant fungal phylum that degrades toluene (Zhang et al., 2019).

Anaerobic degradation of benzene and toluene differs from aerobic degradation since microorganisms can degrade these aromatic hydrocarbons without using oxygen. Anaerobic microorganisms need other sources, such as nitrogen (N_2) and carbon dioxide (CO_2). Hydrogenotrophic and acetolactic methanogens transform hydrogen and acetate to methane gas within anaerobic conditions (Madigan et al., 2015). A syntrophic relationship often exists between Bacteria and Archaea, where Bacteria degrade aromatic hydrocarbons and methanogenic Archaea eliminates the waste products of the initial degradation. Krzmarzick et al. (2018) reviewed the critical function of Archaea in soils polluted with hydrocarbons. Petroleum contaminated soil was

enriched with *Methanosarcinales* strains when it was undergoing remediation. *Methanomicrobiales, Methanosarcinales, Methanobacteriales, Thermoplasmatales*, and *Methanosaeta* were also discovered in aromatic hydrocarbons polluted soil. Archaea populations increased under biostimulation conditions. Archaea population can decrease when temperature rises or fertilizers are applied (Krzmarzick et al., 2018).

Khudur et al. (2018) did not find any dominant genera in the studied Western Australian soils co-contaminated with heavy metals and total petroleum hydrocarbons. *Azospirillum* spp. was present in 85% and *Conexibacter* spp. was present in 82% of all soils despite the different soil types. *Saccharopolyspora* ssp. and *Solirubrobacter* spp. were present in 78% of the co-contaminated soils. All of them are members of the Bacteria domain. *Conexibacter* spp., *Saccharopolyspora* spp., and *Solirubrobacter* spp. are in the phylum of Actinobacteria, while *Azospirillum* spp. belongs to Proteobacteria. The presence and abundance of these genera are due to the uniqueness of the sites and the weather condition. These genera are common in soils from hot to extreme arid regions (Khudur et al., 2018).

Several hydrocarbon-degrading bacteria were found in co-contaminated soils, including *Acinetobacter* spp., *Pseudonocardia* spp*., Halomonas* spp., *Mycobacterium* spp., *Streptomyces* spp., *Desulfotomaculum* spp., *Nocardia* spp., *Nocardioides* spp., *Dietzia* spp., *Rhodococcus* spp., *Aeromicrobium* spp., *Pseudomonas* spp. and *Pseudoxanthomonas* spp. All of them belong to the Bacteria domain. *Acinetobacter* spp., *Halomonas* spp., *Pseudomonas* spp., and *Pseudoxanthomonas* spp. are members of the Proteobacteria phylum. *Pseudonocardia* spp., *Mycobacterium* spp., *Streptomyces* spp., *Nocardia* spp., *Nocardioides* spp., *Dietzia* spp., *Rhodococcus* spp. and *Aeromicrobium* spp. belong to Actinobacteria. *Desulfotomaculum* spp. is a member of the *Firmicutes* phylum (Khudur et al., 2018).

Actinobacteria is one of the largest taxonomic units, which belongs to the Bacteria domain. Many Actinobacteria (such as the genera *Streptomyces, Micromonospora, Rhodococcus,* and

Salinispora) can be found in soils and aquatic environments. Actinobacteria population density depends on the environment and climate conditions. Other factors such as temperature, pH, and soil moisture also impact the growth of Actinobacteria. Actinobacteria are mesophilic, like many soil bacteria, with an optimal temperature between 25ºC and 30ºC. There are some species in this phylum, which can grow in temperatures ranging between 50ºC and 60ºC. Most of the Actinobacteria species grow in soils with neutral pH. Actinobacteria has great significance in biotechnology and is utilized as a source of antibiotics, insecticides, bioherbicides, and antifungal agents. *Streptomyces* have been isolated from acidic soils (Barka et al., 2016). Actinobacteria and Proteobacteria are the most abundant phyla in BTEX polluted soils (Carvajal et al., 2018; Hendrickx et al., 2006; Cupples, 2011). Firmicutes are also commonly found in soils contaminated with BTEX (Carvajal et al., 2018; Cupples, 2011).

Proteobacteria have many iron-oxidizing bacteria that have different responses to oxygen and optimal pH levels for growth. Proteobacteria has been an attractive focus for research since the discovery of the first species in the 1940s. The phylum has a special significance in biotechnology and its role in environmental pollution. Proteobacteria obtain energy from the oxidation of ferrous iron alone. Another way to get energy is when Proteobacteria coupled to the reduction of molecular oxygen. Proteobacteria can also couple the oxidation of reduced sulfur compounds to the depletion of ferric iron in anoxic environments. Proteobacteria had a significant influence on the geochemical evolution of the Earth as iron-oxidizing bacteria. Proteobacteria still have an important impact on the terrestrial and aquatic environments. The current utilization of Proteobacteria is in biotechnology. Proteobacteria are used to solubilize metals from mineral ores, especially in the case of gold, to pre-prepare metals for chemical extraction (Hedrich, Schlomann, & Johnson, 2011). Proteobacteria is one of the major bacterial phyla in soils. The phylum has a wide range of morphological, physiological, and metabolic diversity. Proteobacteria are essential to global carbon, nitrogen, and sulfur cycling (Spain, Krumholz, & Elshahed, 2009).

Firmicutes are one of the most common bacterial phyla found in soils. Teixeira et al. (2010) observed that Firmicutes could survive within severe conditions for a long time (Teixeira et al., 2010). A study (Desai, Parikh, Vaishnav, Shouche, & Madamwar, 2009) showed Firmicutes is one of the five significant phyla with 52.75% in loamy sand polluted with 10,703.03 mg/kg of Cr, 73.63 mg/kg of Zn, and 0.18 mg/kg of Cd. Firmicutes were one of the nine main phylogroups with 31.25% in loamy sand contaminated with 6,291.30 mg/kg Cr, 12.65 mg/kg Cu, and 72.15 mg/kg Zn. Firmicutes were also retrieved from loamy sand with 358.65 mg/kg Cr, 5.36 mg/kg Cu, 65.36 mg/kg Zn and 0.14 mg/kg Cd contamination, one of the twelve major bacterial lineages with 6.25%. Desai et al. (2009) concluded that the increased number of Firmicutes indicates the loss of other sensitive species because of heavy metal stress in soils (Desai et al., 2009).

Bioremediation techniques for benzene and toluene contamination

Bioremediation is a biological treatment that can enhance the eco-efficiency of polluted land management (Simpanen et al., 2016). There are two main types of bioremediation techniques: *ex-situ* and *in-situ*. Both *ex-situ* and *in-situ* bioremediation are applicable for benzene and toluene remediation. Natural attenuation (passive approach), biostimulation (addition of amendments), bioaugmentation (addition of specific microbes), and biosparging (air injected to the soil subsurface to stimulate microbial activity) are *in-situ* bioremediation techniques. Bioventing (stimulation of airflow) is applicable for toluene pollution. The *ex-situ* bioremediation technique for benzene and toluene degradation is a bioreactor. Bioreactors can be a batch reactor, fed-batch, sequencing batch, continuous flow, and multistage (Azubuike, Chikere, & Okpokwasili, 2016).

An *ex-situ*-based remediation techniques are more common in most European countries than *in situ*-based treatments (Simpanen et al., 2016). Superfund sites in the United States received *in-situ* treatments in half of the documented cases between 2009 and 2011 (EPA, 2001). *Ex-situ* remediation techniques are easier to control and monitor; however, they are usually expensive, have health risks, and contribute to waste production and ecosystem disturbance at the cleanup site

(Simpanen et al., 2016). It is a principle to compare bioremediation cost and success to the physical and chemical remediation techniques. The applicability of bioremediation techniques differs due to unfavorable conditions of the polluted site. A comprehensive understanding of the site conditions will optimize bioremediation and achieve a more effective result (Bamforth & Singleton, 2005).

Ekperusi and Aigbodion (2015) studied the bioremediation of diesel oil and heavy metals with earthworm (*Eudrilus eugeiae*). A mixture of 5 mL of diesel oil with 1 kg of soil was analyzed in the study. Distilled water was added to the mixture until the soil reached the maximum water holding capacity. The experimental soil was left to be exposed to the diesel oil for seven days after the treatment. Cow dung was utilized to fertilize the treated soil after seven days, then earthworms (*Eudrilus eugeiae*) were added to the soil. The experimental soils were monitored for 90 days, twice a day. Atomic absorption spectrophotometer and gas chromatography with a flame ionization detector measured heavy metals and BTEX concentrations. No mortality of the earthworms was observed during the experiment. The concentration of Zn, Mn, Cu, Ni, and Cr was detected at minimal levels. The concentration of Cd, V, Pb, Hg, and As were below the detection level. Benzene remained in the experimental soils until the end of the experiment, which did not degrade completely, unlike toluene, ethylbenzene, and xylene (Ekperusi & Aigbodion, 2015).

Genovese et al. (2008) evaluated the removal efficiency of fuel hydrocarbons from a jet fuel contaminated site in Italy. The bioaugmentation technique was carried out in biopile in a field experiment. Biopile is the addition of amendments to the soil. Samples were analyzed with high resolution of gas chromatograph mass spectrometry (GC-MS). Several molecular biology techniques were applied, such as DNA extraction, PCR, and DNA sequencing. The concentration of the studied BTEX was 980 µg/g in the soil. The study reported that attempts for natural attenuation failed. The explanation of the failure was the scarcity of the available nutrients or adverse environmental conditions that occurred during the experiment. *In-situ* bioaugmentation was presented as a potential bioremediation method for total hydrocarbons. Adding three microbial

populations to the soil: *Pseudomonas* sp., *Rhodococcus* sp., and *Acinetobacter* sp. can enhance the degradation of BTEX. The significant degradation rate of BTEX started to develop on the 15th day. Genovese et al. (2008) concluded that using biopile improved the efficiency of bioremediation and demonstrated suitability for removing hydrocarbons (Genovese et al., 2008).

Wolicka, Suszek, Borkowski, and Bielecka (2009) isolated aerobic consortia of BTEX degraders from a petroleum product contaminated site in Poland. The concentration of benzene was 8 mg/L, ethylbenzene was 1.2 mg/L, and xylene was 5.5 mg/L. The field samples were contaminated with petroleum products for over 30 years. An optimal medium should be selected by isolating autochthonous microorganisms from the contaminated soil to achieve the maximum biodegradation rate of BTEX. The medium should not contain any chemical compounds (such as lactate, ethanol, or acetate) because that could act as a potential carbon source for the microorganisms instead of the BTEX. Other organic chemical compounds would inhibit the biodegradation process of BTEX. The high effectiveness of *in-situ* bioaugmentation for BTEX biodegradation was reported as a bioremediation technique (Wolicka et al., 2009).

Problems with co-contamination for bioremediation

Describing any organic compound's physical, chemical, and biological behavior is complicated because it requires knowledge of multiple property values, coefficients, and constants. Some fundamental constants can help to understand and interpret the observed environmental behavior of most organic compounds. The constants also assist in predicting how organic compounds will behave in the environment. Acid dissociation constant (K_a), Henry's law constant (KH), and the octanol-water partition coefficients (K_{ow}) are important equilibrium constants among the key ones. Many physical-chemical characteristics of organic compounds are used to predict how the compounds will react chemically or biologically. The reaction includes the vulnerability of organics toward chemical or biological degradation and toxicity to organisms. The acid dissociation constant, Henry's law constant, and the octanol-water partition coefficients assist in

predicting the behavior of the organic pollutants in aquatic systems or closed systems, such as a batch reactor. The degree of ionization is another impact that affects the chemical reactivity of organic pollutants to oxidation or reduction. The degree of ionization can adsorb or desorb from different surfaces and its bioavailability to microorganisms (Brezonik & Arnold, 2011).

Environmental factors can influence the biodegradation of aromatic hydrocarbons. The abiotic factors are weathering, lack of water in soils, low or high pH, and low phosphorus and nitrogen both in water and soil. The abiotic factors have an impact on the rates of microbial growth and enzymatic activities. The factors affect the biodegradation rate of aromatic hydrocarbons. The stability of petroleum contaminants in soils depends on the quantity and quality of the hydrocarbon mixture and the characteristics of the affected environment. The features of the polluted soil and the abiotic factors will determine the persistence of petroleum hydrocarbons, whether aromatic hydrocarbons will biodegrade in a couple of hours or days or not at all (Atlas, 1981).

Some studies (Oh et al., 1994; Chang et al. 1992; Yu et al., 2001) demonstrated when benzene and toluene are present together in the same sample, their degradation changed. Oh, et al. (1994) presented that toluene inhibited the microbial use of benzene, much more than benzene inhibited the degradation of toluene. Oh, et al. (1994) found that the saturation constant (K) was over 20 times higher in benzene samples than in the toluene mixture. The result indicated that the biodegradation of benzene was significantly prevented in the presence of toluene (Oh et al., 1994). The study of Chang et al. (1992) also revealed that the degradation rate of benzene or toluene was slower when they were present together in the same substrate (Chang et al., 1992). Another study (Yu et al., 2001) presented catechol, as an intermediate, must be mineralized for successful and complete individual benzene or toluene biodegradation (Yu et al., 2001).

Toluene transformed into intermediates within two days of incubation when it was present individually in the anaerobic experiment of Grbic-Galic and Vogel (1986). These intermediates were *p*- and *o*-cresol, benzoic acid, 2-methyl cyclohexanol, and hexanoic acid. Grbic-Galic and

Vogel (1986) observed that the transformation of individual benzene was slower than toluene, and phenol was produced concomitantly within four days. The complete degradation of benzene occurred after 34 days and the degradation of toluene after 64 days. Grbic-Galic and Vogel (1986) did not find any intermediates in the sterile chemical or biological controls. The study showed eight aromatics, five alicyclic, and ten aliphatic compounds in the toluene cultures. Some aromatic compounds are degraded by the end of the incubation time. Grbic-Galic and Vogel (1986) observed benzene and phenol throughout the incubation period. Two alicyclic compounds (methylcyclohexane and cyclohexene) appeared in the samples after 57 days, representing partial substrate reduction products. All aliphatic acids are reduced or degraded by the end of the incubation. Phenol was the dominant aromatic intermediate in the benzene cultures. Cyclohexanone and propanoic acid were additional compounds in the benzene samples (Grbic-Galic & Vogel, 1986).

Collins and Daugulis (1999) studied substrate inhibition in two-phase bioreactor systems. Immiscible organic phase was used to remove inhibitory end-products from the aqueous phase during the fermentation. The overall concentrations of the inhibitory substrates in the system were very high. The substrates can be maintained well below inhibitory levels in the aqueous phase. Collins and Daugulis (1999) investigated substrate inhibition to induce bioremediation techniques for benzene and toluene polluted sites. Some solvents (like dipentyl ether, ethyl heptanoate, 1-Decyne, Jasmone, Adol 85 NF, 2-Decanol, 2-Decanone, and 2-Undecanon) were not bioavailable. The microbes were inhibited by *p*-xylene the most, then benzene and toluene the least. Collins and Daugulis (1999) suggested *Pseudomonas* sp. ATCC 55595 to induce the biodegradation of benzene. Toluene degraded faster than benzene or *p*-xylene. The presence of toluene was desirable during the fermentation of benzene and *p*-xylene (Collins & Daugulis, 1999).

The availability of contaminants for microbes is the key to bioremediation success. Important concepts associated with contaminants desorption rates are 1) the initial distribution of

the solute concentration within the polluted region and 2) the length of time a site has been exposed to the contaminant source. Contaminated sites exhibit lower rates of desorption flux even in the latest stages of remediation. Haws, Ball, and Bouwer (2007) suggested that "aging" (long-term exposure) of the contaminants should be considered when assessing the long-term effectiveness of remediation strategies (Haws, Ball, & Bouwer, 2007).

Haws, Ball, and Bouwer (2007) demonstrated that diffusion from sequestered regions is often limiting for remediation strategies. It is because diffusion controls the speed at which pollutants become available for uptake and removal. One of the most challenging difficulties with remediation strategies is the awareness of the exact initial compounds' distribution within the sequestered region. The initial concentration of the contaminants is usually unknown. "Aging" (refers to long-term) contaminants make the pollution less available for remediation purposes or biological uptake. Remediation strategies are dependent on case, contamination time and type, and the physical and chemical characteristics of the polluted sites (Haws, Ball, & Bouwer, 2007). Sorption of organic contaminants has significant influences on the fate of chemical pollutants in the environment. These influences include a direct impact on bioavailability, transport by fluids, and rates of transformation (Nguyen, Sabbah, & Ball, 2004).

Heavy metals can be present together with aromatic hydrocarbons as co-contaminants. Heavy metals affect the degradation of benzene and toluene. Zukausaite, Jakubauskaite, Belous, Ambrazaitiene, and Stasiskiene (2008) investigated the effects of micro-elements (Co, Cu, Mn, and Mo) on the biodegradation of two oil products, black oil, and diesel fuel, from coastal soil. The concentration of the black oil was 28 g/kg, and the diesel fuel was 46 g/kg in every sample. Manganese (Mn) had the best condition for the biodegradation of both oil products after 30 days. The most significant difference between the control samples and treated ones occurred after 30 days in each sample. Manganese (Mn) increased the effectiveness of diesel fuel degradation after 120 days, and the degradation was near complete (90%). The effect of Mn on black oil was more

negligible after 120 days, with only a 63% degradation rate. Diesel fuel degradation was 86%, and for black oil, it was 59%, while for the controls, it was 34% in the samples treated with copper (Cu). The control samples had low concentrations of each metal. The microbial growth was intense in the treated co-contaminated soils compared to the controls. A determined concentration of heavy metals could influence the growth of the microorganisms, but high concentrations of heavy metals impact the growth rate negatively. Small amounts of micro-elements can significantly decrease the biodegradation of oil products (Zukausaite et al., 2008).

Khudur et al. (2018) evaluated co-contaminated soils in Western Australia for total petroleum hydrocarbons and concentrations of heavy metals. The study focused on the ecotoxicity these co-contaminants have on soils remediated with natural attenuation. The concentration of heavy metals was as follows: Cr was in the range of 6-338 mg/kg, Cu was between 7-153 mg/kg, Pb was 2.5-151 mg/kg, Ni was 8-162 mg/kg, and Zn was between 19-130 mg/kg. High concentrations of Pb and Zn co-contaminated with total petroleum hydrocarbons had a strong correlation with ecotoxicity. The other metals (Cr, Ni, and Cu), even in high levels, did not show any relation to toxicity. Soil samples with a higher concentration of total petroleum hydrocarbons had an increased number of copies of the alkB gene than soil samples with lower concentrations of total petroleum hydrocarbons. The diversity and richness of the bacterial communities differed regardless of the toxicity and the presence of pollutants (Khudur et al., 2018).

Co-contamination is not a unique problem. Single contamination is rarely found in polluted soils. Understanding the effects of co-contaminants on the degradation processes is essential for soil remediation. Single contaminations created within laboratory conditions do not reflect the complexity of pollutant dissipation in the natural environment. The presence co-contaminants affect the biodegradation rate of other compounds. Inhibitory effects of co-contaminations must be considered because co-contaminants decrease the success of bacterial microflora in mitigating contamination, depending on the mixtures (Madrid et al., 2016).

CHAPTER III

IDENTIFICATION OF NATIVE SOIL AEROBIC DEGRADERS OF BENZENE AND TOLUENE IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF CD AND PB

Introduction

Studying bacterial communities in BTEX contaminated soils is common and is necessary to improve bioremediation practices. Several studies have identified individual genera (Lhotsky et al., 2017; Hendrickx et al., 2006; Sperfeld et al., 2018). These studies found that aerobic bacteria's widespread presence in BTEX polluted soil at a genus level include *Aminobacter, Burkholdera* sp., and some anaerobic bacteria like *Geobacter*. The most critical aerobic aromatic-degrading genera are *Pseudomonas* and *Rhodococcus*, commonly found in soils (Lhotsky et al., 2017; Hendrickx et al., 2006). It is also usual to find species and strains of the phyla of *Actinobacteria, Proteobacteria*, and *Firmicutes* in BTEX contaminated soils (Hendrickx et al., 2006; Sperfeld et al., 2018) as dominant species. A comprehensive characterization of site-specific degraders can help determine if the bioremediation processes are efficient enough or it needs to be enhanced (Lhotsky et al., 2017).

Mohan et al. (2020) showed that microorganisms' growth rate decreases as benzene and toluene concentration increases. No significant microbial growth was observed beyond the 700 mg/L concentration of the BTEX mixture (Mohan et al., 2020). Another study reported that BTEX concentration above 500 mg/L results in substrate inhibition due to bacterial toxicity (Shim, Shin, & Yang, 2002). Lin, Lin, and Lai (2007) presented microbial community changes after degrading MTBE (methyl tertiary butyl ether) contaminated with BTEX and heavy metals in water samples. Different types of substrate concentrations and mixtures resulted in different microbial community structures. The substrate's removal was higher when MTBE was present together with toluene, but the removal efficiency decreased when MTBE was contaminated with benzene. Microbial cultures polluted with benzene generated lower band numbers than other substrates, such as toluene (Lin, Lin, & Lai, 2007).

Aromatic compounds with high degradation rates often produce communities with simple structures by favoring the growth of few dominant species (Lin, Lin, & Lai, 2007). Native species could use the substrate gradually increase in portion and became the dominant population when benzene or toluene was the sole carbon source. The MTBE removal rates were lower when the substrate was present with heavy metals (Al^{+3} and Zn^{+2}). Lin, Lin, and Lai (2007) and Amor, Kennes, and Veiga (2001) both experienced the same inhibitory effect, which was heavy metal concentration-dependent, causing a more significant effect at higher concentrations (between 5 to 10 mg/L). Their samples generated distinctly different community profiles when heavy metals were present together with co-substrates (BTEX, heavy metals and MTBE) than when MTBE was the only substrate with or without heavy metals. The presence of heavy metals decreases bacterial communities' diversity and produces a difference in the community structure (Lin, Lin, & Lai, 2007; Amor, Kennes, & Veiga, 2001; Kozdroj & van Elsas, 2001).

Ferreira et al. (2017) hypothesized that some bacteria species isolated from coal mining areas with low pH (3-9) and high (from 1 to 15 mg/L) heavy metal content (Cr, Cd, Pb, Zn, Cu, and Ni) were tolerant to these conditions in vitro. A total of 18 isolates were found to be efficient

as biological nitrogen fixers. The results of the phytoremediation project showed that soils with the most extended restoration period (20 years re-vegetation) had the highest number of bacterial strains. The shortest re-vegetation time resulted in the lowest number of isolates. A significant number of bacterial strains grew at a higher metal concentration. A total of 18 bacterial strains were phylogenetically related to nitrogen-fixing bacteria. The selected strains belonged to the genera *Rhizobium, Bradyrhizobium*, and *Burkholdera*. Ferreira et al. (2017) suggested that the higher number of bacteria strains may be related to restoring of the studied areas (Ferreira et al., 2017).

Mesorhizobium metallidurans was the dominant species in Zn and Pb mine soils located in France and remediated with common kidney vetch (*Anthyllis vulneraria*); a legume associated with nitrogen-fixing bacteria. *Mesorhizobium* species demonstrated metal-tolerant populations in highly contaminated Zn (between 14 and 40,237 mg/kg), Cd (between 1.3 and 82 mg/kg), and Pb (between 46 and 15,551 mg/kg) mine soils. Increased metal concentrations determined the microbial populations by influencing their growth, abundance, diversity, and activity. Heavy metals pressure microorganisms, resulting in more metal-tolerant populations but with a lower diversity than unpolluted soils. The ratio of metal-resistant and sensitive microbes may be a bioindicator of the degree of pollution. Mohamad et al., 2017, reported a positive correlation between metal tolerant rhizobia and levels of metals in the environment on a microbial community scale. *Mesorhizobia* species demonstrate high variability and capacity to adapt to local and extreme edaphic conditions, site-specific. *Mesorhizobia* demonstrated a related but distinct set of species at respective contaminated sites. Combining native plants and microbes that are metal resistant and better adapted to local pedoclimatic conditions should be used for bioremediation strategies (Mohamad et al., 2017).

The preliminary study (Fiddler, 2016) found that native aerobic microorganisms can degrade benzene and toluene individually in the presence of various concentrations (5.1 mg/kg; 51 mg/kg; 510 mg/kg; and 5,100 mg/kg in wet soil) of Cd and Pb. Cadmium had little to no effect on the biodegradation of individual benzene and toluene. Benzene samples degraded within ten days.

Toluene samples required an average of three days for complete biodegradation. Lag time was not observed during the preliminary experiment. Samples that had high Cd concentration demonstrated a higher toluene degrading population. The microbial population seemed to be inhibited at higher Pb concentrations. The preliminary study demonstrated that heavy metals negatively affect the biodegradation of benzene and toluene if the microbial population is less diverse. Microorganisms are less affected by heavy metal toxicity in a diverse and populated environment (Fiddler, 2016).

The utilization of heavy metal-persistent and good degrading microorganisms is a prerequisite for successful bioremediation. It is essential to identify microorganisms that can effectively degrade organic substances at contaminated sites from samples obtained from the field (Lee, Lee, & Jeon, 2019). This chapter's primary objective is to identify, characterize, and compare the differences in native benzene or toluene degrading microorganisms under various Cd and Pb concentrations. This chapter examines the microbial population and community changes after complete degradation in the aerobic microcosms during the preliminary experiment. The hypothesis is that unique bacterial communities enrich a single soil inoculum under different Cd and Pb concentrations co-contaminated with benzene or toluene as a sole carbon source. Several molecular biology techniques and statistical analysis were used to investigate the hypothesis.

Material and methods

Sample collection

One type of soil was collected from a Walmart parking lot runoff in Stillwater, Oklahoma, during the preliminary (Fiddler, 2016) study. Microcosms were created of 2 g of the collected soil and 100 mL of mineral medium. Each medium was spiked with 876.39 mg/kg (wet soil) individual benzene and 869.80 mg/kg (wet soil) individual toluene. Each microcosm was treated with different Cd and Pb concentrations (5.1 mg/kg, 51 mg/kg, 510 mg/kg, and 5,100 mg/kg wet soil, as shown in *Table 9*). A 1 mL sample of the mixed aerobic medium was collected from each microcosm after

complete degradation was observed and analyzed. The samples were stored in a commercial freezer at -20 °C until DNA extraction (Fiddler, 2016).

Table 9 The 54 samples that were being used for the first objective.[2]

Benzene (876.39 mg/kg wet soil) co-contaminated with		**Toluene (869.80 mg/kg wet soil) co-contaminated with**	
5.1 mg/kg Cd	5.1 mg/kg Pb	5.1 mg/kg Cd	5.1 mg/kg Pb
51 mg/kg Cd	51 mg/kg Pb	51 mg/kg Cd	51 mg/kg Pb
510 mg/kg Cd	510 mg/kg Pb	510 mg/kg Cd	510 mg/kg Pb
5,100 mg/kg Cd	5,100 mg/kg Pb	5,100 mg/kg Cd	5,100 mg/kg Pb
0 mg/kg Cd and Pb		0 mg/kg Cd and Pb	

DNA extraction and quantification

DNA extraction was performed on a total of 54 soil samples collected from the aerobic mineral medium, obtained from the preliminary study, with a DNeasy PowerSoil Kit 100 (by Qiagen N. V.) according to the manufacturer's protocol. All genomic DNA samples were kept in a -20ºC freezer until further analysis after extraction. DNA concentration was measured in triplicates using a Quantus Fluorometer (Promega Corporation, Madison, WI) with dsDNA System reagents according to the manufacturer's instruction. The extracted DNA samples of bacterial 16S rRNA were amplified using the PCR (Polymerase Chain Reaction) technique.

16S rRNA microbial community analysis

Three universal primers were used in Polymerase Chain Reaction (PCR): one forward Bac338 (5'-ACT CCT ACG GGA GGC AG-3', Kim et al., 2010), and two reverses Bac518R without clamp (5'- ATT ACC GCG GCT GCT GG, Bakke et al., 2011) and with GC clamp, to amplify the targeted DNA. The primers are commonly used for generating PCR products for DGGE analysis (Bakke et al., 2011, Kim et al., 2010), which was the next step after the PCR was carried out.

[2] The table shows individual Benzene and Toluene samples were co-contaminated with different concentrations of Cd and Pb. Total 18 samples were analyzed in this study. Abbreviation of samples can be found in the bracket.

The PCR method was carried out with BioRad T100 Thermal Cycler for two and a half hours. The PCR mixture contained 2 μL of $MgCl_2$ (25 mM), 10 μL of reaction buffer (5), 2.50 μL of bovine serum albumin (20 mg/mL), 0.40 μL of deoxynucleotide triphosphates (10 mM), 0.25 μL of GoTaq polymerase (5 units/ μL, Promega, USA), 1 μL of the extracted DNA, 1.25 μL of PCR primers (10 μM) and PCR grade water up to the final volume of 50 µL. The cycling parameters for primers Bac 338 and Bac 518 R with GC clamp were as follows: the first cycle of pre-denaturation ran at 94°C for 5 minutes, and the second cycle of denaturation ran at 92°C for 30 seconds. The third annealing cycle ran at 55°C for 30 seconds, and the fourth elongation cycle ran at 72°C for 2 minutes. The third cycle was followed by repeating the cycles of the second until the fourth cycle 35 times. The final cycle was the extension step, which occurred at 72°C for 5 minutes.

The samples were kept at 4°C indefinitely after each cycle was completed. The PCR amplification of the products were verified with electrophoresis using a 1.5% agarose gel and stained with SYBR Gold nucleic acid gel stain (ThermoFisher, USA). The size and yield of PCR products were verified with a 100 bp DNA Ladder (Promega, USA). The prepared PCR products were used for Denaturing Gradient Gel Electrophoresis (DGGE) analysis with Bio-Rad D Gene System (Bio-Rad Laboratories, USA).

DGGE was performed on the previously prepared PCR products with Bac 518 R with GC clamp. DGGE is a molecular fingerprinting method, which separates PCR-generated DNA products. DGGE separates the DNA sequence. Each band on the gel profile represents many of the dominant bacterial populations. DGGE divides PCR products based on sequence differences, which results in distinctive denaturing characteristics of the DNA. DGGE is also helpful to explore a wide range of phylogenies or specifically targeted organisms (Muyzer & Smalla, 1998).

Ten µL of PCR products were obtained and mixed with 3 µL of 6 blue loading dye (Bio-Rad Laboratories, USA). DGGE was performed in a DCode Universal Mutation Detection System (Bio-Rad Laboratories, USA) utilizing 10% polyacrylamide gels with a urea/formamide denaturing gradient of 30-55% superimposed with a porous gradient of acrylamide/bisacrylamide. A total of

six 16/16 cm gels were electrophoresed separately in a 1 TAE buffer at 60°C and 135 V for 7 hours. The six gels were stained separately with SYBER Safe Gold dye (Thermo Fisher Scientific) in a 0.5 TAE (Tris-acetate EDTA) buffer for 30 minutes, after the 7 hours running time was over. All gels were placed in Bio-Rad Gel Doc XR+ Molecular Imager to capture the image of the profiles of the gels (*Figure 25* shown in the Appendices) with Image Lab Software.

Next generation DNA sequencing data analysis

DNA sequencing was outsourced to Molecular Research DNA (Shallowater, TX) and performed with Thermo Fisher Scientific Ion S5 XL next-generation sequencing system, a semiconductor sequencing technology. The system does not use fluorescent-labeled nucleotides. The sequencing is based on detecting the hydrogen ion, which is released during the sequencing process (Kchouk, Gibrat, & Elloumi, 2017). A total of 10 composite samples were prepared for DNA sequencing containing 21 µL of DNA extract. The samples included a total number of five samples of benzene and five samples of toluene, co-contaminated with low (51 mg/kg) and high (5,100 mg/kg) concentrations of Cd or Pb, and control samples without heavy metals (triplicate samples were mixed). Data results were received in MS Excel file format for further analysis.

Quantitative real time Polymerase Chain Reaction (qPCR)

A total of 46 primers (*Tables 33* and *34* are shown in the Appendices) for quantitative Polymerase Chain Reaction (qPCR) were designed by using the online tool Primer-BLAST by the National Center for Biotechnology Information (NCBI) and MEGA-X freeware by Molecular Evolutionary Genetics Analysis. Primers were developed based on the results of the bacterial community composition analysis. Each qPCR mixture totaled 10 µL, which contained 5 µL of 2X iTaq SYBER Green Supermix with Rox master mix (Bio-Rad Laboratories, Inc, USA), 0.5 µL of bovine serum albumin (20 mg/mL), 0.15 µL of the Forward primer, 0.15 µL of the Reverse primer, 3.2 µL PCR grade water, and 1 µL of 10 times diluted DNA extract or the standards. DNA extracts were diluted in 0.5 TE (Tris EDTA) buffer.

Standards for each qPCR were prepared from known concentrations of plasmids extracts containing the 16S rRNA gene of interest. The standards were prepared for PCR. The PCR products were verified with 1.5% agarose gel. The PCR products were cleaned with Ultra Clean PCR Clean-up Kit (MO Bio Laboratories, Inc., USA), according to the manufacturer's protocol. Each standard was quantified with Quantus Fluorometer (Promega Corporation, Madison, WI). All procedures were described in detail in the above sections. Standards were serially diluted (10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, and 10^{-1}) in 0.5 TE buffer.

A total number of 138 samples were analyzed in 96-well qPCR plates vessels with CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The cycling parameters for the designed primers were as follows: the first cycle of pre-denaturation ran for 3 minutes, the second cycle of denaturation for 30 seconds, both at 95ºC. The third cycle of annealing ran for 30 seconds at 59ºC. The cycle was followed by additional 39 cycles to the first pass through (second and third cycle). The last cycle consisted of extension for 5 seconds at 95 ºC and then once again at 95 ºC for 5 seconds. Analysis of the results was performed with Bio-Rad CFX Manager Software. The melting curves were analyzed after each complete run to ensure primer-dimers were not amplified and the amplification was specific. Each sample was analyzed with qPCR in triplicates, including the standards. The triplicates were then log10 transformed and averaged. All data obtained were exported to MS Excel file format for further statistical analysis.

Diversity and statistical analysis

The difference in bacterial community composition between treatments was analyzed with non-metric multidimensional scaling (NMDS) and performed using the VEGAN package in R version 1.2 in the phylum level (*Figure 9*). The NMDS plot helped to visualize the DNA sequence data using Bray-Curtis similarities. The top three OTUs (Operational Taxonomic Unit) in the DNA sequence were identified and selected for each sample. Computing ecological indices with metabarcoding data involves clustering DNA reads into Operational Taxonomic Units (OTU) (Keck, Vasselon, Rimet, Bouchez, & Kahlert, 2018). The OTUs were selected at least 50 times

higher relative read depths in the heavy metal co-contaminated samples than the control samples. The selection was made using the IF logical function in MS Excel. The analysis distinguished which aerobic microbial communities are abundant and might be specific for different co-contaminants than the controls. The selected OTUs served to design Primers (*Tables 33* and *34* are shown in the Appendices) mentioned above. The selected OTUs were then analyzed with qPCR assays. The qPCR assays were limited to the OTUs closely related to the bacteria strains (>97% identity).

Linear regression was carried out to create standard curves for the qPCR analysis in MS Excel (parameters of the qPCR assays are reported in the Appendices in *Tables 35* and *36*). Triplicate samples were averaged, and standard deviation was calculated in MS Excel. A normality test was carried out to examine normal distribution within the dataset in MS Excel. One-way analysis of variance (ANOVA) was performed in R version 4.0.4 for all qPCR gene targets. Tukey's honestly significant difference (HSD) test was conducted to test the difference in mean gene concentrations between each possible pair of soil samples co-contaminated with different concentrations of heavy metals. Results were considered significant statistically when *P*-value <0.05 (*Tables 10* and *11*).

Dendrogram and heatmap analysis was performed with the HEATMAP.2 function with average clustering using the GPLOTS package in R version 4.0.4 for the qPCR data analysis. The dendrogram served to visualize clustering and display the distances among individuals. The heatmap assists in describing the whole expression matrix, the samples vs. gene targets. The heatmap is a color-coded two-dimensional mosaic (*Figure 10*) with different color intensities according to the copy numbers of the gene targets in the samples. The data set shown in the heatmap is mean-centered (Bergkvist et al., 2010).

Results and discussion

Microbial community composition

A total of 7,165 different OTUs (operational taxonomic units) were identified in all samples. Three archaeal phyla (*Crenarchaeota, Euryarchaeota, Thaumarchaeota*), 15 Eukaryotes, and 26 bacterial phyla were identified (*Figure 7*). *Actinobacteria* and *Proteobacteria* were the dominant phyla in each treatment, as demonstrated in other BTEX biodegradation studies (Carvajal et al., 2018; Czarny et al., 2020; Hendrickx et al., 2006; Weelink, van Eekert, & Stams, 2010). The increased number of *Firmicutes* indicates the loss of other sensitive species because of soil heavy metal stress (Desai et al., 2009). An average of 3,163 and 2,586 OTUs were identified in the benzene and toluene samples, respectively *(Figure 8*).

A total of 44 phyla were found in the samples, but only 16 of them were present when heavy metal contamination was involved. Six OTUs are within the family *Pseudomonadaceae*, which includes aerobic bacteria found in soils that can degrade a variety of low-molecular-weight organic compounds and hydrolytic products (Cousin, 1999). Five OTUs are within the family of *Bacillaceae*, which are resistant to heat, radiation, chemicals, and drought, and commonly found in soils. The family consists of primarily aerobic bacteria and participates in the carbon, nitrogen, sulfur, and phosphorous cycles in soils (Mandic-Mulec, Stefanic, & Drik van Elsas, 2015). Four OTUs were found within the family of *Xanthomonadaceae*, which were reported to degrade chlorinated phenols and other aromatics in soils. Members of the family can accelerate biodegradation and, in combination with metal ions, affect nutrient transport and accessibility to plants (Sharma & Garg, 2018).

Three OTUs were identified within the family of *Burkholderaceae, Chitinophagaceae*, and *Flavobacteriaceae*. Lunsmann et al. (2015) found that members of the *Burkholderaceae* were the main degraders of toluene and their abundance remained stable throughout the three years experiment (Lunsmann et al., 2015). The family of *Chitinophagaceae* might suggest the presence of hydrocarbons degrading potential (Aburto-Medina et al., 2012). Sun et al. (2019) demonstrated

that members of the *Flavobacteriaceae* family were present in a nitrifying consortium to degrade aromatic hydrocarbons. Two OTUs were identified in the *Rhodocyclaceae* and *Caulobacteraceae.* Tancsics et al. (2018) found that the members of the Rhodocyclacea family had a central role in hypoxic toluene degradation in a BTEX contaminated aquifer (Tancsics et al., 2018). Martinez-Pascual et al. (2015) identified members of the *Caulobacteraceae* family as one of the native microbial communities demonstrating resilience to alkylbenzene-polluted soils and water (Martinez-Pascual et al., 2015). One OTU belongs to the family of *Oxalobacteraceae, Hyphomicrobiaceae, Micrococcaceae, Bacteroidaceae, Nocardiaceae, Verrumicomicrobia* subdivision 3, *Cytophagaceae, Cryomorphoceae, Nitrosomonadaceae,* and *Phyllobacteriaceae.*

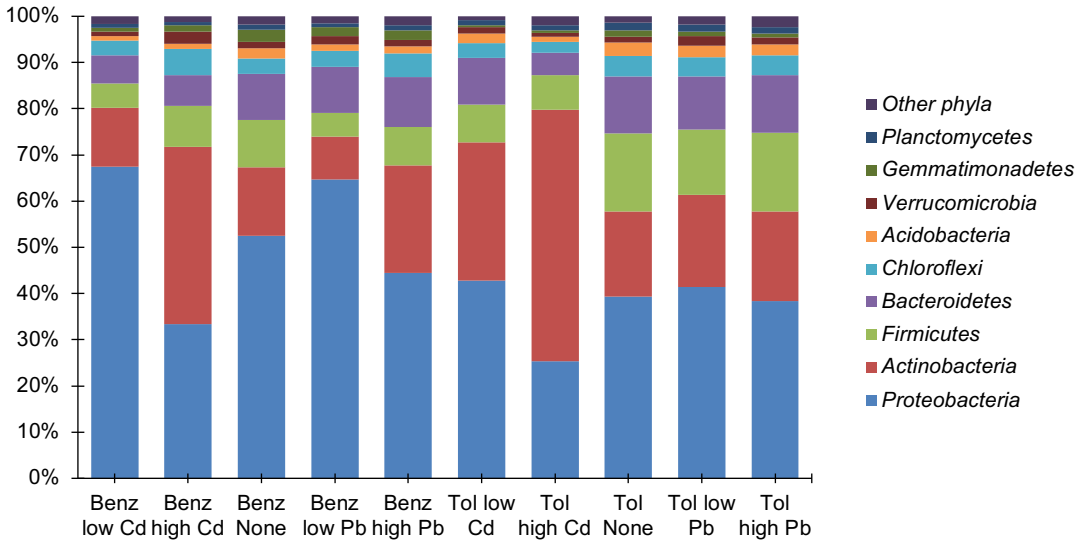

Figure 7 Native phyla found in aerobic microcosm, which were treated with benzene or toluene co-contaminated with various concentrations of Cd and Pb. Results of the DNA sequence.

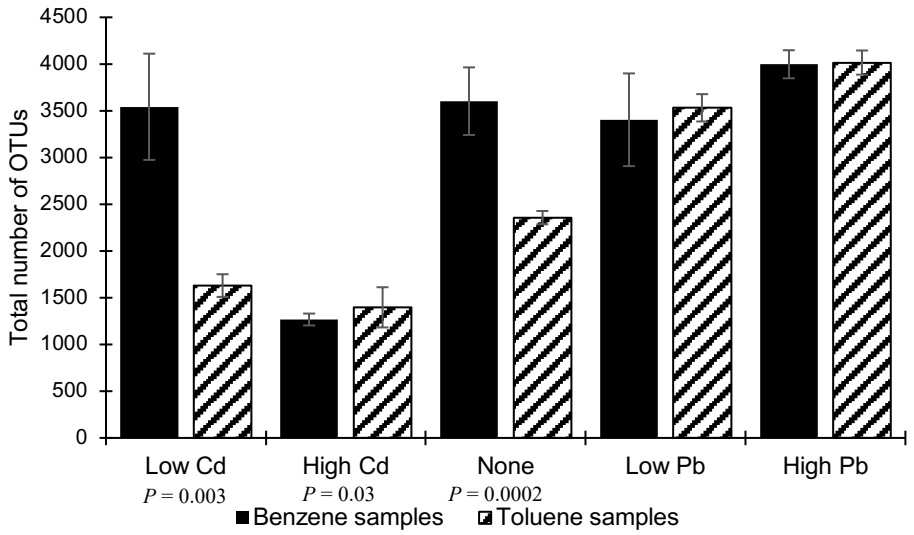

Figure 8 The total number of OTUs found in each sample.[3]

Table 10 Pairwise comparison of OTU numbers between benzene and toluene samples polluted with different concentrations of Cd and without Cd.[4]

	Toluene with low Cd	Benzene with high Cd	Benzene control	Toluene control
Benzene with low Cd	**0.003**	**0.0006**	0.94	
Toluene with high Cd	0.36	**0.03**		0.74
Toluene control	**0.03**		**0.0002**	
Benzene control		**0.0000002**		

Table 11 Pairwise comparison of OTU numbers between benzene and toluene samples polluted with different concentrations of Pb and without Pb.[5]

	Toluene with low Pb	Benzene with high Pb	Benzene control	Toluene control
Benzene with low Pb	0.64	0.28	0.70	
Toluene with high Pb	0.30	0.56		**0.00001**
Toluene control	**0.0000001**		**0.0002**	
Benzene control		**0.04**		

[3] Low concentration equals to 51 mg/kg, high concentration is 5,100 mg/kg, and None indicate the control samples, which do not have any heavy metals (0 mg/kg).

[4] . Individual cells contain *P*-values from one-way ANOVA test comparing OTU numbers from the samples indicated in the column and row for each cell. Statistically significant (P<0.05) are shown in bold.[4]

[5] Individual cells contain *P*-values from one-way ANOVA test comparing OTU numbers from the samples indicated in the column and row for each cell. Statistically significant (P<0.05) are shown in bold

The pairwise differences in the number of OTUs are shown in *Tables 10* and *11*. There is a significant (P=0.003) difference statistically in the total number of OTUs between toluene co-contaminated with low Cd concentration (51 mg/kg) and benzene co-contaminated with low Cd concentration. There is a significant (P=0.0006) difference statistically between benzene co-contaminated with low and high (5,100 mg/kg) Cd concentration. The difference is significant statistically (P=0.0002) between the benzene and toluene control. The difference was also statistically demonstrated (P=0.0000002) between the benzene control and benzene co-contaminated with high Cd concentration. The difference is significant statistically (P=0.04) between benzene control and benzene co-contaminated with high Pb concentration. There is a significant difference statistically (P=0.0000001) between toluene control and toluene co-contaminated with low Pb concentration. The difference is also significant statistically (P=0.00001) between the toluene control and toluene co-contaminated with high Pb concentration.

The composition of the bacterial communities in the samples was assayed by Denaturing Gradient Gel Electrophoresis (DGGE). The non-metric multidimensional scaling (NMDS) analysis of bacterial community composition as determined by DGGE is shown in *Figure 9.* The points represent different co-contaminant types and concentrations. The samples like one another are ordinated closer together. The axes are arbitrary, as is the ordination of the plot. The stress value is 0.16, which is considered fair and not random (Ramette, 2007). The microbial communities affected by Cd and Pb diverged prominently and uniquely and differed from the controls. The microbial communities of the controls (benzene and toluene without heavy metals) did not delineate from each other. Benzene and toluene without heavy metals (the controls) displayed similar bacterial community distribution. The samples co-contaminated with Cd presented a significant difference statistically (P=0.02) among bacterial community composition depending on the concentration. The distinction within Pb co-contaminated with individual benzene or toluene was not as straightforward as in the Cd samples.

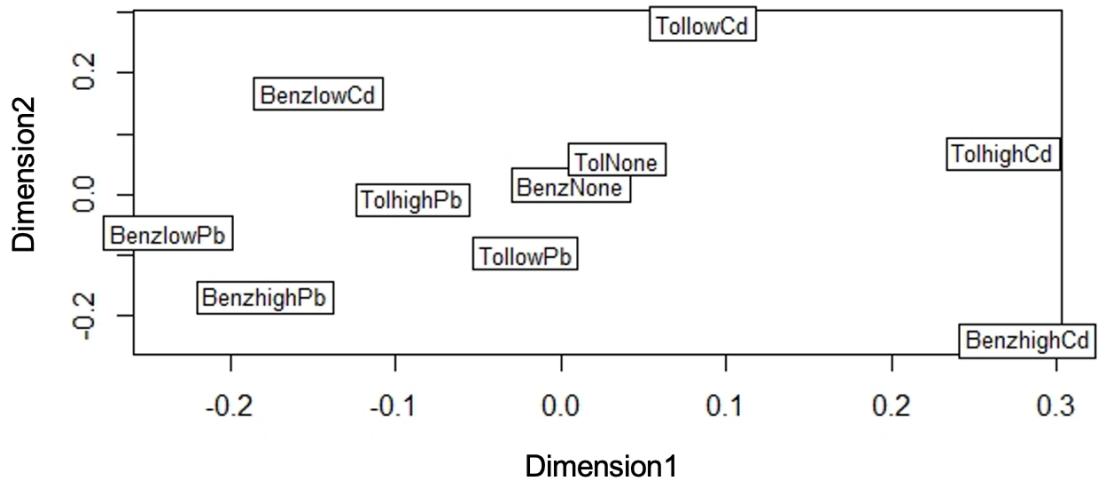

Figure 9 Results of non-metric multidimensional scaling (NMDS) analysis of bacterial community composition as determined by Denaturing Gradient Gel Electrophoresis (DGGE). [6]

The presence of co-contaminants in the soil modifies the structure of microbial communities and leads to a unique and less diverse population of microorganisms. Different substrate concentrations and mixtures resulted in different microbial community structures in Lin, Lin, and Lai's (2007) BTEX study.

Several studies (Carvajal et al., 2018; Czarny et al., 2020; Hendrickx et al., 2006; Weelink, van Eekert, & Stams, 2010) presented the dominant aerobic and anaerobic BTEX degraders. This study focuses on the aerobic benzene and toluene degraders that demonstrated resistance to high heavy metals concentrations. It is essential to find unique communities specific for co-contamination type (benzene and Cd, benzene and Pb, toluene and Cd, toluene and Pb) and heavy metal concentration (5.1 mg/kg, 51 mg/kg, 510 mg/kg, and 5,100 mg/kg wet soil). QPCR analysis was conducted to investigate the hypothesis further.

[6] Samples polluted with benzene (identified as “Benz”), and toluene (identified as “Tol”), co-contaminated with different concentrations of Cd (identified as “lowCd” and “highCd”) and Pb (identified as “lowPb” and “highPb”).

Quantitative PCR

Quantitative real-time Polymerase Chain Reaction (qPCR) was conducted to observe differences in aerobic soil microbial communities regarding the co-contamination type and concentrations. Target microbial communities (OTUs) were selected more than 50 times greater in the composite DNA extract from benzene or toluene samples versus the composite DNA extract from the control. The communities are listed in the *Appendices* and the parameters of the qPCR assays in *Tables 35* and *36*. Many of the qPCR assays measured more than one OTU.

The difference was significant statistically between samples and 16S rRNA genes/g for the following qPCR targets (shown in *Table 12*):

Table 12 Comparison of 16S rRNA genes/g derived from the qPCR analysis between benzene and toluene samples.[7]

qPCR target	Benzene samples (mean±std dev)	Toluene samples (mean±std dev)	Significance (*P*<0.05)
Azoarcus subgroup 1	0.22±0.43	1.78±0.58	0.0003
Thauera group	0.91±0.17	0.40±0.26	0.03
OTU 21 *Lysobacter* sp.	2.36±0.16	1.60±0.51	0.01
OTU 3504 *Lysobacter* sp.	4.40±1.09	3.15±1.63	0.04
Arthrobacter group	2.31±0.02	1.66±0.24	0.001
OTU 1 *Pseudomonas* sp.	1.92±0.37	0.83±0.15	0.0003
OTU 6205 *Pseudomonas putida*	2.52±1.48	1.40±0.23	0.02
OTU 6872 *Pseudomonas putida*	1.65±0.13	0.72±0.37	0.001
Rhodococcus group	4.40±0.01	3.42±0.22	0.00001
OTU 11 *Bacillus* sp.	1.45±0.69	0.44±1.17	0.04
OTU 254 *Pedosphaera* sp.	2.65±0.07	2.09±0.31	0.02
OTU 147 *Cauloacter* sp.	2.41±0.04	1.54±0.31	0.0004
OTU 6151 *Caulobacter daechungensis*	4.17±0.39	3.36±0.32	0.01
OTU 89 *Mesorhizobium* sp.	3.56±0.02	2.95±0.21	0.001
OTU 330 *Adhaeribacter* sp.	1.28±0.17	2.22±0.71	0.01
OTU 36 *Flavobacterium limicola*	3.66±0.05	2.51±1.13	0.006
OTU 117 *Flavobacterium granulensis*	2.83±0.16	2.22±0.14	0.004
OTU 3740 *Ferruginibacter* sp.	1.97±1.14	0.32±0.56	0.002

[7] *P*-values are from one-way ANOVA test. *P* values are considered significant if *P*<0.05. Std dev indicates for standard deviation. The following table shows the results that were significantly different statistically.

The difference was not significant statistically (P<0.05) between samples and 16S rRNA genes/g for the following qPCR targets:

Table 13 Comparison of 16S rRNA genes/g derived from the qPCR analysis between benzene and toluene samples (*Table 12* continued). [8]

qPCR target	Benzene samples (mean±std dev	Toluene samples (mean±std dev)	Significance (P<0.05)
Massilia group	1.96±1.43	1.50±2.18	0.48
Azoarcus subgroup 2	0.12±0.15	0.13±0.22	0.97
Burkholdera group	0.00003±0.05	-0.08±0.03	0.41
Lysobacter group	2.38±0.30	1.79±0.55	0.07
OTU 121 *Xanthomonas* sp.	1.63±0.91	1.50±0.35	0.72
OTU 110 *Pseudomonas* sp.	2.49±0.20	2.23±0.83	0.46
OTU 6940 *Pseudomonas* sp.	3.09±0.43	2.58±0.12	0.06
Bacillus Sporo group	2.36±0.05	2.03±0.19	0.06
OTU 16 *Bacillus* sp.	0.93±0.45	0.50±0.38	0.18
OTU 4705 *Bacillus* sp.	1.98±0.39	1.41±1.30	0.21
OTU 5793 *Bacillus* sp.	1.81±0.85	1.50±0.92	0.50
OTU 141 *Nitrosovibrio* sp.	1.34±0.25	1.09±0.27	0.31
OTU 3353 *Cytophaga* sp.	1.81±0.11	2.49±1.35	0.11
OTU 123 *Flavobacterium* sp.	2.71±0.18	3.01±0.25	0.18
OTU 7 *Chitinophaga* sp.	2.38±0.34	2.03±0.49	0.27
OTU 90 *Fluviicola* sp.	3.70±0.15	3.29±0.34	0.10
OTU 61 *Pedobacter* sp.	3.85±0.29	3.33±0.59	0.12
Pedobactero group	1.15±0.22	1.39±0.42	0.37
OTU 391 *Hyphomicrobium* sp.	1.06±0.05	0.75±0.34	0.15
Noviherbaspirillum group	1.33±0.27	0.81±0.33	0.07

OTU 3504 *Lysobacter* sp., OTU 1 *Pseudomonas* sp., OTU 6205 *Pseudomonas putida*, *Rhodococcus* group, OTU 6151 *Caulobacter daechungensis*, and OTU 89 *Mesorhizobium* sp. had the highest 16S rRNA genes/g (as shown in *Table 35 and 36* in the Appendices) in the benzene samples. Only OTU 330 *Adhaeribacter* sp. 16S rRNA genes/g was high (>10^4 16S rRNA genes/g in soil) in the toluene samples. The copy numbers of OTU 36 *Flavobacterium limicola* and OTU 117 *Flavobacterium granulensis* were high in the benzene samples and the toluene samples when high concentrations of Pb (510 mg/kg and 5,100 mg/kg) was present. High 16S rRNA genes/g is a

[8] P-values are from one-way ANOVA test. P values are considered significant if P<0.05. Std dev indicates for standard deviation. The following table shows the results that were not significantly different statistically.

good indicator for a viable bacterial population for a sustainable and more efficient bioremediation process (Koshlaf et al., 2019).

The microbial community changes in oil-polluted soils (Militon et al., 2010). Volatile petroleum hydrocarbons decrease and shape microbial diversity. Bacterial species respond uniquely to different volatile petroleum hydrocarbons. Mangse, Werner, Meynet, and Ogbaga (2020) demonstrated high 16S rRNA genes/g of *Rhodococcus, Mesorhizobium, Lysobacter,* and *Pseudomonas* in petroleum hydrocarbon affected soil at a genus level (Mangse, Werner, Meynet, & Ogbaga, 2020). OTU 3504 *Lysobacter* sp., OTU 1 *Pseudomonas* sp., OTU 6205 *Pseudomonas putida, Rhodococcus* group, OTU 6151 *Caulobacter daechungensis*, and OTU 89 *Mesorhizobium* sp. demonstrated the 16S rRNA genes/g (>10^4 16S rRNA genes/g in soil) in my benzene samples and identified as the viable communities. OTU 330 *Adhaeribacter* sp. demonstrated the 16S rRNA log unit copies/g in my toluene samples. OTU 36 *Flavobacterium limicola* and OTU 117 *Flavobacterium granulensis* displayed a high copy number in benzene and toluene samples co-contaminated with high Pb concentration (5,100 mg/kg wet soil).

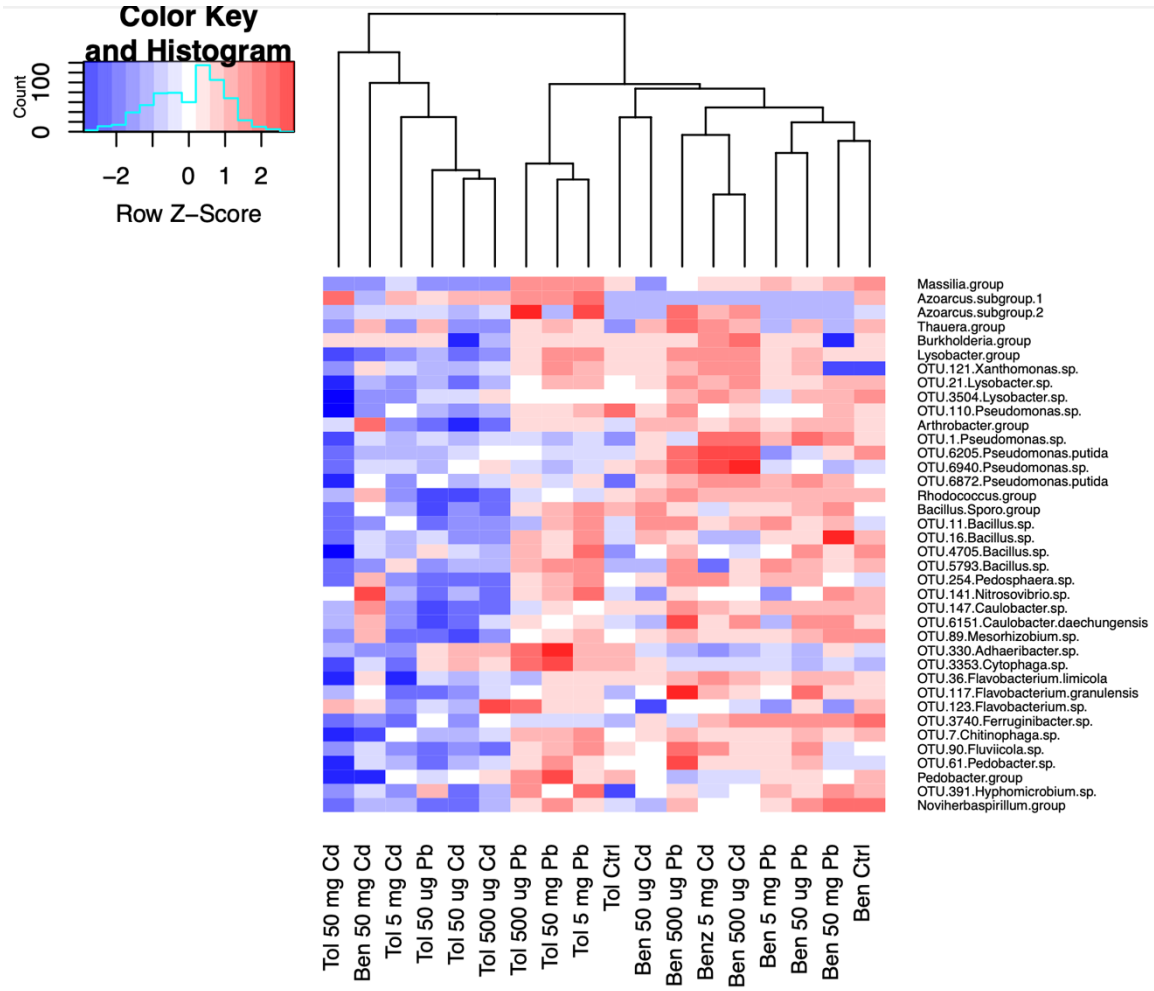

Figure 10 Dendrogram and heatmap (correlation distance) analysis of the 38 Bacteria in an OTU level most abundant and unique for the co-contaminants' type and concentration. [9]

A dendrogram and heatmap analysis on the qPCR targets at the OTU level is shown in *Figure 10*. The dendrogram indicates that the qPCR target microbial community amended with benzene with 5,100 mg/kg Cd differed the most from other benzene-amended microcosms, which was also indicated from the NMDS analysis in *Figure 9*. The microbial community of the benzene

[9] The scale bar and heatmap indicates the log-unit increase or decrease for each OTU from 16S rRNA in the sample, derived from qPCR analysis, compared with control soil (Ctrl). The rows show the targeted genes, the columns are the DNA sequence samples. The data is mean centered in the samples dimension. Z score is a measure of distance in standard deviations. Dark blue corresponds to low expression, dark red corresponds to high expression. There are several genes that are high in benzene or toluene samples for all species and low in other regions.

sample co-contaminated with 5.1 mg/kg Cd showed similarity to the toluene control, where no heavy metal was present. The microbial community of the benzene sample co-contaminated with 5,100 mg/kg Pb was like the benzene control, where no heavy metal was present. The microcosms of benzene co-contaminated with 5,1 mg/kg Pb showed similarity to benzene co-contaminated with 510 mg/kg Pb. The benzene co-contaminated with 51 mg/kg Cd microcosms were like benzene co-contaminated with 510 mg/kg Cd. Toluene samples co-contaminated with Cd clustered distinctly from toluene samples co-contaminated with a high concentration of Pb. Toluene sample co-contaminated with 5.1 mg/kg Pb demonstrated similarity to toluene samples co-contaminated with Cd. The same results can be indicated from the NMDS analysis in *Figure 9* as well.

Conclusions

This study distinguishes and determines the bacterial community composition and diversity in benzene and toluene samples co-contaminated with Pb and Cd using molecular biology techniques. Samples were collected from aerobic microcosms set up during the preliminary biodegradation study conducted in 2016 (Fiddler, 2016). The preliminary study used one genetic type of soil collected in Stillwater, OK. Each aerobic medium was spiked with 876.39 mg/kg (wet soil) individual benzene and 869.80 mg/kg (wet soil) individual toluene from stock solutions. The aerobic microcosms were inoculated individually with different Cd and Pb concentrations (5.1 mg/kg, 51 mg/kg, 510 mg/kg, and 5,100 mg/kg wet soil) that resulted in a short-term co-contamination.

Individual benzene or toluene without heavy metal co-contaminants (controls) displayed similar bacterial community distribution. The bacterial community distribution was significantly (P= 0.003 for 51 mg/kg and P=0.03 for 5,100 mg/kg Cd concentration) different statistically from the controls when Cd was present, depending on the Cd concentration. The presence of co-contaminants in the soil modifies the structure of microbial communities and leads to a unique and

less diverse microbial population (Lin, Lin, & Lai, 2007; Amor, Kennes, & Veiga, 2001; Kozdroj & van Elsas, 2001).

A total of 18 OTUs were identified as specific for co-contaminants type and concentration. A total of 9 (from the 18 OTUs) unique bacteria were identified and presented high ($>10^4$) 16S rRNA genes/g in wet soil derived from qPCR analysis. The high 16S rRNA genes/g is a good indicator for a viable bacterial population for an efficient bioremediation process in co-contaminated sites (Koshlaf et al., 2019). This study demonstrated unique bacterial communities, which enrich from a single soil inoculum under different Cd and Pb concentrations co-contaminated with benzene or toluene as a sole carbon source. Benzene samples had six OTUs (OTU 3504 *Lysobacter* sp., OTU 1 *Pseudomonas* sp., OTU 6205 *Pseudomonas putida*, *Rhodococcus* group, OTU 6151 *Caulobacter daechungensis*, OTU 89 *Mesorhizobium* sp.). The six OTUs demonstrated high ($>10^4$) 16S rRNA genes/g in wet soil and were significantly different statistically (P=0.01) from the toluene samples. Toluene demonstrated one OTU (OTU 330 *Adhaeribacter* sp.) high ($>10^4$) 16S rRNA genes/g in wet soil and was significantly different statistically (P=0.01) from the benzene samples. OTU 36 *Flavobacterium limicola* and OTU 117 *Flavobacterium granulensis* were present in high ($>10^4$) 16S rRNA genes/g in wet soil in benzene and toluene samples co-contaminated with 5,100 mg/kg concentration of Pb.

Understanding the structures of native microbial communities and their diversity based on the concentration and types of co-contaminants will help design a successful and sustainable bioremediation strategy (Schwarz et al., 2019). Knowledge of the microbial structure, characteristics, and diversity in co-contaminated soil is critical (EPA, 2001). A comprehensive characterization of site-specific degraders can help determine if the bioremediation processes are efficient enough or it needs to be enhanced (Lhotsky et al., 2017). Further research is required to demonstrate bioremediation potential for long-term co-contaminants in soils collected from a Superfund site and define the critical microbial community members for heavy metal co-contaminants. Future research will look for the nine unique OTUs identified in this study to

investigate whether they are specific for benzene and toluene when high concentrations of heavy metals are present.

CHAPTER IV

DEMONSTRATION OF BIOREMEDIATION POTENTIAL FOR CO-CONTAMINANTS IN SOIL SAMPLES COLLECTED FROM THE FIELD AND DETERMINING THE *IN-SITU* MICROBIAL COMMUNITY COMPOSITION

Introduction

In situ bioremediation is an economical, faster, and safer remediation technology than the conventional cleanup methods. Bioremediation is neither universally understood nor trusted by those who must approve its use. Bioremediation is clouded by disagreement over what it does and how well it works due to its dependence on microorganisms. The full potential of bioremediation technologies cannot be realized because of the previously mentioned disagreement. Microorganisms can be utilized to degrade hazardous pollutants or transform them into less dangerous forms. Microbes can destroy contaminants when they have access to a variety of materials. These materials can be compounds that help produce energy or nutrients to build more cells. Natural environmental conditions at the polluted site sometimes can have all the vital elements in enough quantities for microorganisms that the site does not require human intervention. This process is called *intrinsic* bioremediation. Polluted sites need *engineered* bioremediation most of the time when microbe-simulating materials must be supplied to encourage microbial growth and optimize the environmental conditions (National Research Council, 1993).

A contaminated site's bioremediation depends on the pollutants' biodegradability, the site's geological and chemical characteristics. It is crucial to understand that not a single set of site characteristics will favor the bioremediation of every contaminant (National Research Council, 1993). The applicability of bioremediation techniques differs due to unfavorable conditions of the polluted site. A comprehensive understanding of the site conditions will optimize bioremediation and achieve a more effective result (Bamforth & Singleton, 2005).

It is essential to consider how the chosen bioremediation technique will perform under different and not perfectly known conditions. The fundamental criterion of a successful bioremediation project is when microorganisms are mainly responsible for the cleanup. No strategy can reduce the uncertainties around bioremediation techniques, even for the best-designed system. Microorganisms may selectively degrade the compound from multiple contaminants that are the easiest to digest or provide the most energy. Benzene and toluene are easy to biodegrade because they are relatively soluble, serve as the primary electron donor, and degrade fast if oxygen is available. Microbes cannot degrade heavy metals, but they can modify their reactivity and mobility (National Research Council, 1993).

Long-term heavy metal contamination influences microbial diversity and composition. The heavy metals' impact on the microorganisms depends on the metals' bioavailability. The bioavailability is influenced by the climate, soil type and structure, organic matter, pH, and plant roots (Jansen, Michels, van Til, & Doelman, 1994; Mohamad et al., 2017). Mohamad et al. (2017) studied a legume (*Anthyllis vulneraria*) associated with nitrogen-fixing rhizobia to find metal tolerant rhizobia belonging to *Mesorhizobium metallidurans* or other sister metal tolerant species in long-term Zn and Pb contaminated mine soils. Legumes associated with nitrogen-fixing rhizobia together can offer an adapted biological material for mine-soil phytostabilization through limiting metal pollution. The studied mine-border soils were less contaminated than the mine soils. Mohamad et al. (2017) suggested that the mine-border soils with higher organic matter might decrease metal mobility and metal availability in the soil. Soils with higher organic matter result in

a more heterogeneous soil environment with hotspots of metal-available niches interspersed among niches with less metal availability at the microscale (Mohamad et al., 2017).

The bacterial community tolerance increased in metal-polluted samples compared to the control (which was un-contaminated) in Baath et al. (1998) study. The tolerance of microbial communities was the highest, where the concentration of metals was the highest. Microbes resistant to high Zn concentrations were also tolerant to both Cu and Cd. Communities that showed resistance to high Cu levels were tolerant to Cd. The threshold levels for sandy loam soil were 20 mg/kg for Cu tolerance, 60 mg/kg for Ni, and 140 mg/kg for Zn (Baath et al., 1998). The number of *Bacillus thuringiensis* increased in Cd and Zn contaminated soils extracted from Cd-rich soils. *Azotobacter vinelandii* increased in the presence of Zn (II) (Chibuike and Obiora, 2014). Heavy metals affect the abundance and biomass of fungi and bacteria. Fungi show more tolerance to heavy metals than bacteria. Some heavy metals, such as Cd, Pb, and Zn, especially in high concentrations, can cause changes in the species microfungal composition. Both fungi and bacteria can adapt to heavy metal pollution. A sensitive species can adapt to heavy metals but still be less competitive than those species, which can already resist the contaminant (Baath, 1989).

Khudur et al. (2018) did not find any dominant genera in the ten studied Western Australian soil co-contaminated with heavy metals and total petroleum hydrocarbons. *Azospirillum* spp. was present in 85%, and *Conexibacter* spp. was 82% of all soils despite the different soil types. *Saccharopolyspora* ssp. and *Solirubrobacter* spp. were present in 78% of the co-contaminated soils. Several hydrocarbon-degrading bacteria were found in co-contaminated soils studied by Khudur et al. (2018). These bacteria included *Acinetobacter* spp., *Pseudonocardia* spp., *Halomonas* spp., *Mycobacterium* spp., *Streptomyces* spp., *Desulfotomaculum* spp., *Nocardia* spp., *Nocardioides* spp., *Dietzia* spp., *Rhodococcus* spp., *Aeromicrobium* spp., *Pseudomonas* spp. and *Pseudoxanthomonas* spp. (Khudur et al., 2018).

Genovese et al. (2008) reported a failed attempt for natural attenuation of BTEX contaminated soils. The explanation of the failure was the scarcity of the available nutrients or

adverse environmental conditions during the experiment. Genovese et al. (2008) suggested in situ bioaugmentation as a potential bioremediation method for total hydrocarbons. Adding three microbial populations to the soil: *Pseudomonas* sp., *Rhodococcus* sp., and *Acinetobacter* sp. can enhance the degradation of BTEX. The significant degradation rate of BTEX started to develop on the 15th day (Genovese et al., 2008).

Daghio et al. (2018) could not link the complete removal of *o*-xylene to microbial activity since it also degraded from the abiotic controls. The removal of benzene and ethylbenzene from sediments was slower than the other BTEX compounds. Daghio et al. (2018) could not achieve a complete benzene and ethylbenzene degradation during

the duration (150 days) of the experiment with a bioelectrochemical system. A short lag phase (about 4 days) was observed. The highest removal rate constants were achieved at 0.8 V. The degradation rate for toluene was 0.4 ± 0.1/days, 0.34 ± 0.09/days for *m*-xylene, and 0.16 ± 0.02/days for *p*-xylene. It is hard to untangle if the lower degradation rate of benzene and ethylbenzene is because of inhibition or lack of a particular degradation pathway (Daghio et al., 2018). Other studies observed the following degradation rates for benzene and toluene within different environmental conditions (demonstrated in *Table 14*):

Table 14 First-order coefficient values for benzene and toluene found in other degradation studies (Salinatro et al. 1997; Morgan, Lewis, and Watkins 1993; Edwards and Gribic-Galic 1992, Essaid et al. 2003; Patterson et al. 1993).

Reference	Condition	Compound	k_1 (first order kinetic constant, day^{-1})
Salinatro et al. (1997)	In aquifer soil microcosms: methanogenic	Benzene	0.16-0.20
		Toluene	0.037-0.17
Morgan, Lewis, and Watkins (1993)	In groundwater microcosms: nitrate-reducing	Benzene	0.022
		Toluene	0.046
Edwards and Grbic-Galic (1992)	In enrichment cultures microcosms: sulfate-reducing	Benzene	0.20
		Toluene	0.04
Essaid et al. (2003)	In natural attenuation: methanogenic	Benzene	0.00065
		Toluene	0.19
Patterson et al. (1993)	In a column study: nitrate-reducing	Toluene	3.4
deNardi, Zaiat, and Foresti (2007)	In a bioreactor study: methanogenic	Benzene	8.4-10.0
		Toluene	9.7-11.3

Daghio et al. (2018) observed a notable change in the composition of the microbial communities after biodegradation. The anode and cathode used in the bioelectrochemical experiment potentially influenced the evolution of the microbial communities. Hydrogen can be produced at the cathode and consumed by sulfate-reducing bacteria when an anode is utilized as an electron acceptor. Microorganisms linked to the sulfur cycle (*Desulfobulbaceae* and *Desulfuromonadaceae)* were principally observed in the bioelectrochemical study. Putative cable bacteria were involved in the electron transfer from the bulk to the anode (Daghio et al., 2018). The microbial community structure was significantly (P<0.05) affected by different volatile petroleum hydrocarbons in the study of Mangse et al. (2020). At least 60% similarity between microbial communities was observed for each volatile petroleum hydrocarbon treatment. The volatile petroleum hydrocarbons demonstrated that they are statistically (P<0.01) significant factors forming the microbial communities in soils (Mangse et al., 2020).

This chapter's primary objective is to evaluate and demonstrate the bioremediation potential of benzene and toluene in heavy metal long-term impacted soils collected from Tar Creek Superfund site, Oklahoma. The chapter examines the microbial community composition in the chosen Superfund site. It compares the bacterial community changes before and after simulating two bioremediation techniques (natural attenuation and biostimulation) in a batch microcosm experiment. The hypothesis is that sites with high benzene and toluene biodegradation rates will have key microbial community members for heavy metal co-contaminants compared to sites with low biodegradation rates. The chapter also examines whether the biodegradation of benzene and toluene is limited in aerobic microcosms due to the high concentrations and long-term contamination of Cd, Pb, and Zn found in the Tar Creek Superfund site’s soil.

Material and methods

Description of the sampling area

Tar Creek Superfund Site, located in Ottawa County, Oklahoma, requires immediate action since the 1970s. Originally Pb and Zn mining operation was on the site that produced bullets and weapons for both World Wars. The mine was ceased after 1970, and mining waste, also known as "chat," was left behind (EPA, 2019). The chat contains Cd, Pb, and Zn, which contaminates surface and groundwater, and the soil (Oklahoma Department of Environmental Quality [ODEQ], 2019). The EPA placed the Tar Creek Superfund site on the Administrator's Emphasis List as it requires immediate action. Tar Creek Superfund site is one of the most polluted and long-term exposed sites in the United States. The site still releases heavy metals to the environment and has a high risk for the environmental ecology and health (EPA, 2019). The heavy metal contamination in Tar Creek Superfund site is estimated to be in the range of 0.1 mg/kg to 10 mg/kg Cd, 1 mg/kg to 1,000 mg/kg Pb, and 10 mg/kg to 1,000 mg/kg Zn (Beattie et al., 2017).

Soil sampling

Random sample points were created using the Fishnet tool in ArcGIS Desktop 10.8 before the soil sampling. Composite soil samples were collected on September 11th, 2020, from Tar Creek Superfund site, Picher, Oklahoma, following the EPA's guidelines (EPA-230-R-95-005 Guidelines). It was raining for 2-3 days before sample collection. It did not rain on the day of collection. The position of 26 randomly selected sites was detected using Gaia GPS v2021.3 on a personal smartphone. Each sampling location was recorded and shown in *Figure 34* in the Appendices. A total of 26 composite soil samples were collected from the topsoil to a depth of 6 inches (from 0 to 0.10 m) with an ethanol-sterilized metal scoop close to the mine tailings (chat piles). Soil samples were collected in labeled sterile plastic bottles and transferred to the laboratory (located in Advanced Technology Research Center, Oklahoma State University, Stillwater, Oklahoma) immediately after collection. Each sample was labeled as S1, S2, etc., where S identifies

the site, and the numeral (1-26) are in ascending order according to the heavy metals' concentration. Wet samples were stored in a commercial refrigerator at 4 °C for future analysis.

DNA extraction and quantification

DNA extraction was performed on a total of 26 soil samples collected from Tar Creek Superfund site immediately after sample collection, with a DNeasy PowerSoil Kit 100 (by Qiagen N. V.) according to the manufacturer's protocol. The genomic DNA was also extracted from 18 chosen samples (the selected 6 soil samples for the bioremediation study are highlighted in the Appendices in *Table 39*) after completing the bioremediation study. A total of 1.5 mL samples from each aerobic microcosm were collected for microbial community analysis. The samples were centrifuged at 10,000 g for 10 minutes. The liquid upper layer was discarded, and the slurry left in the tubes was vortexed for a couple of seconds before the DNA extraction. All genomic DNA samples were kept in a -20ºC commercial freezer after extraction for the microbial communities' characterizations. DNA concentration was measured in triplicates using a Quantus Fluorometer (Promega Corporation, Madison, WI) with dsDNA System reagents according to the manufacturer's instruction.

Next generation DNA sequencing data analysis

DNA sequencing was outsourced and performed with Thermo Fisher Scientific Ion S5 XL next-generation sequencing system, a semiconductor sequencing technology. The system does not use fluorescent-labeled nucleotides. The sequencing is based on detecting the hydrogen ion, which is released during the sequencing process (Kchouk, Gibrat, & Elloumi, 2017). A total of 28 composite samples were prepared for DNA sequencing containing 20 µL of pure DNA extract. The samples included two randomly selected samples from S1 and S4 and were sent in duplicates. The 28 DNA extracts were collected before the bioremediation study. A total of eighteen 20 µL of pure DNA extracts were also collected from the aerobic microcosms after the four-week bioremediation study was completed. Data results were received in MS Excel file format for further analysis.

Analyzing soil pH and moisture content

Soil pH value was measured in a portion (between 10 g and 15 g wet soil) of the collected soil samples using a pH meter after diluting the soil samples to a 1:2 soil-solution (deionized water) ratio. SevenCompact pH/Ion meter S220 (from Mettler Toledo) was calibrated before measuring the samples. Results can be found in *Table 39* in the Appendices. Soil texture and color were determined and observed on the field during the sample collection on September 11th, 2020. Approximately 15 g of wet soil samples were measured using an aluminum weighing boat and dried overnight in a Thermo scientific oven at 105°C. Soil moisture was calculated based on the Gravimetric Soil Moisture equation:

$$\%\, Moisture = \frac{weight\ of\ wet\ soil\ (g) - weight\ of\ oven\ dried\ soil\ (g)}{weight\ of\ oven\ dried\ soil\ (g)} \times 100$$

Acid soil digestion for the heavy metal analysis

All the before-mentioned oven-dried soil samples were crushed with a mortar and pestle. Approximately 2 g dried soil was measured separately in a weigh-boat using a digital scale. Soil samples were digested using EPA's guidelines for the aqua regia ratio (1:3) and following the acid digestion procedure. All soil samples were placed in a labeled 50 mL digestion tube. All digestion tubes were transferred under the fume hood, and 2.5 mL nitric acid (HNO_3) and 7.5 mL hydrochloric acid (HCl) was added to the soil samples. Digestion tubes were closed tightly. Soil and acid solutions were swirled gently to mix the soil with the acid. Digestion tubes were left to rest for 24 hours under the fume hood at room temperature. Digestion tubes were opened the next day to release pressure under the fume hood, then closed again tightly and transferred to a heating plate at 50°C for 1.5 hours. Digested samples were removed from the heating plate and filtered through a 0.45 μm cellulose filter with a syringe. 5 mL of the digested soil solution was diluted with distilled water into a clean and labeled plastic tube until volumed up to 50 mL. The acid digested soil samples were prepared to measure Cd, Pb, and Zn concentration with an Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) instrument (Perkin Elmer Optima

7000DV). The digested soil samples were transferred and analyzed in Accurate Labs and Training Center in Stillwater, Oklahoma, to determine the total heavy metal concentrations.

Experimental setup for the bioremediation study

Six different genetic types of soil samples collected from Tar Creek Superfund site located in Picher, Oklahoma, were chosen for the bioremediation study. Genetic soil type refers to the soil body with a certain sequence of diagnostic horizons (Gerasimova, 2013). The soil characteristics are determined in the Appendices in *Table 39*. The design of the bioremediation study was as follows (*Table 15*):

Table 15 The setup of the bioremediation study.[10]

Biostimulation in triplicates (25 mL of aerobic mineral medium + B&T)	**Natural attenuation in triplicates (25 mL of DI water + B&T)**	**Sterile controls in duplicates (25 mL of aerobic mineral medium + B&T)**	**Microbial controls in duplicates (25 mL of aerobic mineral medium)**
S2 low HM and high OM	S2 low HM and high OM	S2 low HM and high OM	S2 low HM and high OM
S4 low HM and low OM	S4 low HM and low OM	S26 high HM and low OM	S4low HM and low OM
S12 medium HM and high OM	S12 medium HM and high OM	-	S12 medium HM and high OM
S13 medium HM and low OM	S13 medium HM and low OM	-	S13 medium HM and low OM
S24 high HM and high OM	S24 high HM and high OM	-	S24 high HM and high OM
S26 high HM and low OM	S26 high HM and low OM	-	S26 high HM and low OM

Aerobic mineral medium (*Table 16*) was made for the microbes to provide proper nutrients. The aerobic mineral medium contained trace elements (*Tables 17* and *18*) required for microbial growth.

[10] S indicates site, HM indicates heavy metals, and OM indicates organic matter; DI indicates deionized water. 25 mL of aerobic mineral media was added to the bottles for the biostimulation, sterile and microbial controls. 25 mL of deionized water (DI) was added to the bottles for simulating natural attenuation. Each aerobic media, except for the microbial controls, was spiked with a mixture of 0.5 mL benzene (876.39 mg/kg wet soil) and 1 mL toluene (869.80 mg/kg wet soil). Sterile controls served to ensure that only biological activity was the cause of the degradation in the microcosms. B&T indicates for benzene and toluene.

Table 16 The composition of the aerobic mineral medium.

Aerobic Mineral Medium (volumed up to 1 L with DI water)		
Compound Name	**Chemical Symbol**	**Concentration**
Sodium Chloride	NaCl	1,000 mg/L
Magnesium Chloride Hexahydrate	$MgCl_2*6H_2O$	500 mg/L
Potassium Bicarbonate	$KHCO_3$	147 mg/L
Dipotassium Phosphate	K_2HPO_4	10 mg/L
Ammonium Chloride	NH_4Cl	300 mg/L
Potassium Chloride	KCl	300 mg/L
Calcium Chloride	$CaCl_2$	15 mg/L
Trace Elements A	-	1 mL/L
Trace Elements B	-	1 mL/L
Yeast Extract	-	100 mg/L

Table 17 The composition of trace elements solution A.

Trace Elements Solution A	
Compound	**Concentration**
$FeCl_2*4H_2O$	1.5 g/L
$CoCl_2*6H_2O$	0.19 g/L
$MnCl_2*4H_2O$	0.1 g/L
$ZnCl_2$	70 mg/L
H_3BO_3	6 mg/L
Na_2MoO_4	36 mg/L
$NiCl_2*6H_2O$	24 mg/L
$CuCl_2*2H_2O$	2 mg/L
25% HCl	10 ml/L

Table 18 The composition of trace elements solution B.

Trace Elements Solution B	
Compound	**Concentration**
Na_2Wo_4*2H2O	8 mg/L
NaOH	0.5 g/L
$Na_2SeO_3*5H_2O$	6 mg/L

Resazurin stock solution was prepared to confirm aerobic degradation in the bottles. 1X phosphate-buffered saline (PBS) solution was made to create a 1:10 dilution with deionized (DI) water. Resazurin stock solution (100X) was produced by dissolving 0.5 g Resazurin sodium salt into 100 mL of 1X phosphate-buffered saline (PBS). The salts and Resazurin stock solution were added to a 2 L flask with a magnetic stirring bar. The 2 L flask was volumed up to 1 L with deionized (DI) water. The aerobic mineral medium turned purple after adding the Resazurin stock

solution to it. The medium was autoclaved once without adding the potassium bicarbonate ($KHCO_3$), trace elements, and yeast extract in a Primus Sterilizer Co. Inc. autoclave. The aerobic mineral medium was then aerated by adding a stirring bar to the 2 L flask and stirring vigorously for an hour. The potassium bicarbonate ($KHCO_3$), trace elements (solutions A and B), and yeast extract were added to the autoclaved and aerated mineral medium. The pH of the medium was adjusted to 7.5 after autoclaving using a 1 M solution of the acid hydrochloric acid (HCl) and was stirred well until everything was dissolved.

The mineral medium was divided into 160 mL serum bottles at 25 mL per bottle. The bottles were rinsed with hexane, methanol, and deionized (DI) water previously. Eighteen bottles (containing the aerobic mineral medium and approximately 2 g of soil) were used for the biostimulation test, 4 bottles for the sterilized controls, and 6 bottles for the microbial controls (as shown in *Table 15*). Another 18 bottles contained 25 mL deionized (DI) water instead of the aerobic mineral medium and 2 g of soil for the natural attenuation test. The chosen 6 soils were prepared in triplicates for each treatment: biostimulation, natural attenuation, and microbial controls. Two chosen soils were prepared in duplicates for the sterile controls.

Stock solutions of benzene and toluene were created separately by filling and sealing a 160 mL serum bottle with deionized (DI) water from Labconco Water Pro PS. A 1.8 mM benzene concentration was injected into a serum bottle to create an 11.22 mM stock solution. 0.755 mM of toluene was inoculated to another serum bottle to make a 4.72 mM stock solution. A Teflon-coated rubber stopper was placed on the serum bottles to seal them. Concentrations of the benzene and toluene stock solutions were determined by following the preliminary study (Fiddler, 2016) to compare microbial communities with the third chapter's community. The prepared stock solutions were placed in an incubator (at 30°C with 19% humidity) on a shaker table until complete mixing for a day. A mixture of 0.5 mL of benzene stock and 1 mL of toluene stock was inoculated to the 25 mL microcosm with a 1 mL glass syringe, bringing the concentrations to 0.2244 mM (876.39 mg/kg wet soil) of benzene and 0.1888 mM (869.80 mg/kg wet soil) of toluene in the microcosms.

Aerobic microcosms were kept in the incubator until complete degradation at 30°C and 19% humidity.

Twelve serum bottles were prepared for benzene and toluene standard curves before the biodegradation analysis with Gas Chromatography (GC-FID). A 100 mL deionized (DI) water was added to six 160 mL serum bottles and adding benzene (with a glass syringe) in decreasing concentrations, creating standard stocks between 0.5121 mM (2,000 mg/kg wet soil) to 0.064 mM (250 mg/kg wet soil). A 100 mL deionized (DI) water was added to six 160 mL serum bottles and adding toluene (with a glass syringe) in decreasing concentrations creating standard stocks between 0.4341 mM (2,000 mg/kg wet soil) to 0.0543 mM (250 mg/kg wet soil) as demonstrated in *Table 19* below:

Table 19 Prepared standard benzene and toluene stock solutions for biodegradation study.

Standard in duplicate	Benzene concentration		Toluene concentration	
	mM	mg/kg wet soil	mM	mg/kg wet soil
A	0.5121	2,000	0.4341	2,000
B	0.3841	1,500	0.3256	1,500
C	0.2560	1,000	0.2171	1,000
D	0.1280	500	0.1085	500
E	0.0640	250	0.0543	250
F (blank)	0	0	0	0

Standard curves were obtained for benzene and toluene using Gas Chromatography (GC-FID). Standard solutions were made in triplicates and injected manually into the GC-FID. Triplicate samples were averaged. A five-point calibration standard curve was generated before the bioremediation experiment. Linear regression was carried out to create standard curves for benzene and toluene stocks in MS Excel. The calibration curves were linear with correlation coefficient (R^2) values 0.9986 for benzene and 0.9989 for toluene and are presented in *Figures 11* and *12.*

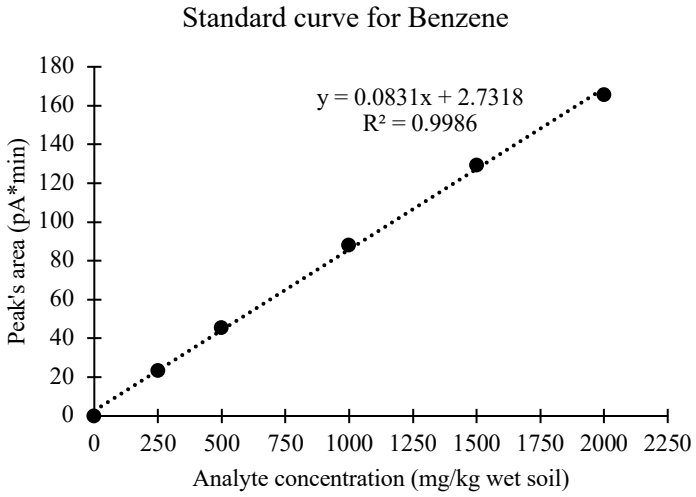

Figure 11 Standard curve prepared for various concentrations of benzene.[11]

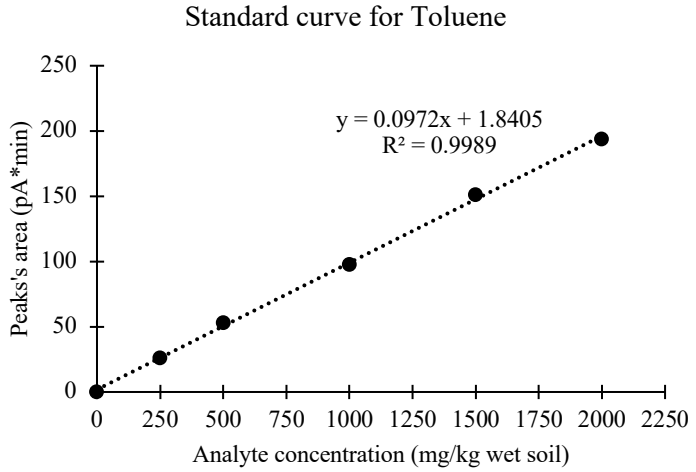

Figure 12 Standard curve prepared for various concentrations of toluene.[12]

Biodegradation analysis with Gas Chromatography (GC-FID)

The concentration of the benzene and toluene was monitored over time. The biodegradation of benzene and toluene in the aerobic microcosms was assayed using an Agilent Technologies 7890B GC System equipped with flame ionization detector (GC-FID) and an Equity™ -5 Capillary

[11] Samples were prepared and measured with gas chromatography (GC-FID) in triplicates and results averaged for the linear regression.

Column (30 m × 0.25 mm × 0.25 μm film thickness by Supelco, Inc.). The operating conditions for the GC-FID are given in *Table 20.* A 100 µL headspace sample was taken manually from the serum bottles three times a day for three days (from day 0 to day 2) using a Hamilton gas-tight syringe, then once a day until complete degradation occurred. A total of 40 serum bottles that had benzene and toluene in them were sampled each time. The other 12 serum bottles did not have either benzene or toluene since they served as microbial controls. A total of 4 extra serum bottles from the standards (two benzene and two toluene from the highest and lowest concentrations) were added to the headspace measurement each time to ensure a better representation of the data. New standards were created if the concentrations deviated more than 10% of the original value. The headspace measurements were injected manually. The experiment lasted for nine days.

Benzene and toluene biodegradation rate was estimated by the zero-order kinetic constant (k) calculated for natural attenuation and biostimulation. The zero-order kinetic constant was calculated by linear regression of time vs. compound concentration in MS Excel. The degradation rates and time are summarized in *Table 25.*

Table 20 The operating conditions for the GC-FID during the bioremediation experiment.

Manual GC-FID specification			
Spitless inlet		**Columns**	
Specification	**Values**	**Specification**	**Values**
Heater	250°C	Constant pressure	
Pressure	10 psi	Flow	0.6194 mL/min
Total flow	18.619 mL/min	Average velocity	19.13 cm/sec
Septum purge flow	3 mL/min	Holdup time	2.59 min
Split vent purge flow	15 mL/min	Helium	100°C
FID detector		**Oven**	
Specification	**Values**	**Specification**	**Values**
Heater	300°C	Temperature	105°C
Air flow	400 mL/min	Equilibration time	0.5 min
H_2 fuel flow	30 mL/min	Maximum oven temperature	300°C
Helium makeup flow	25 mL/min	**FID back signal**	
Helium column flow	0.6194 mL/min	Specification	Values
		Data rate	50 Hz/0.004 min

Enrichment culture maintenance

The microcosms were aerated and re-spiked six times with 0.2244 mM of benzene (876.39 mg/kg wet soil) and 0.1888 mM of toluene (869.80 mg/kg wet soil) after complete biodegradation occurred in the serum bottles. The microcosms were re-spiked to build a robust microbial population before DNA extraction. The microcosms were diluted twice after the sixth re-spike into 40 new microcosms. Each new microcosm was injected by 1 mL of the bacterial community. The diluted microcosms were then re-spiked again twice with 0.2244 mM of benzene (876.39 mg/kg wet soil) and 0.1888 mM of toluene (869.80 mg/kg wet soil). This step ensured identifying the specific microbes responsible for the biodegradation of the mixture of benzene and toluene.

Elemental analysis with micro XRF

The selected 6 soils for the bioremediation study were also studied for elemental analysis. The samples were dried overnight in a Thermo scientific oven at 105°C. The oven-dried soil samples were crushed with a mortar and pestle. Roots and debris were discarded. Approximately 30 g dried and crushed soil was measured separately from every 6 samples in a weigh-boat using a digital scale. The dry soils were pressed and compacted into plastic disposable XRF X-cell sample cups (Thermo Fisher Scientific) using a scoopula. A thin film of plastic was pulled tight over the sample and secured into place with a ring that fits around the rim of the dish.

Micro-XRF analysis of the major, minor, and other elements in the soils was performed with Orbis PC XRF (AMETEK, Inc.). The storage ring current during data acquisition was 1 mA operating at 50kV. The X-ray fluorescence data was processed using Orbis Vision software (AMETEK, Inc.). Microsoft Excel was used to organize the processed data. Results are reported in *Tables 23 and 24.* The device did not have to be calibrated. The instrument can detect spot sizes from 2 mm down to 50 microns. It was necessary to adjust the beam to bring the sample into focus using the optical camera in the device. No reference or standard materials were used. The user identifies the elements by observing the peaks in the spectrum and comparing them to the program's reference spectra. The elemental analysis with micro XRF (Orbis PC XRF from AMETEK, Inc.)

was carried out in Imaging Suite in Advanced Technology Research Center, Oklahoma State University, Stillwater, Payne County, Oklahoma.

Statistical analysis

The collected data were averaged of three replicates ± standard errors in MS Excel. A normality test was carried out in MS Excel to examine normal distribution within the dataset. The characteristics of the reference soils (S3 and S6) were compared to the six soil samples used for the bioremediation experiment. A one-way analysis of variance (ANOVA) test was performed to compare the differences in soil characteristics of the studied and reference soils, followed by a post-hoc Dunnett's T3 test. Post-hoc Dunnett's T3 test was chosen since the experimental and reference soils' sample size was unequal. Non-linear regression was carried out in MS Excel for the first-order degradation kinetics. A one-way analysis of variance (ANOVA) test was performed on the biodegradation rates in R version 4.0.4, followed by Tukey-HSD test (honestly significant difference). The DNA sequence results were tested with two-tailed *t*-test. The statistical significance was based on a P-value < 0.05 in all cases.

Results and discussion

Soil characteristics of the samples collected from Tar Creek Superfund site

The concentration of heavy metals in the collected soils (are shown in detail in *Table 39* in the Appendices) was as follows: Cd was in the range of 7.90-341.82 mg/kg, Pb was 136-8,771.93 mg/kg, and Zn was between 860-79,341.32 mg/kg. The reference (control) soils (S3 and S6) have the following concentration of heavy metals: for S3, Cd was below the detection limit, Pb was 461.72 mg/kg, which is under the limitation of non-play areas in the urban environment defined by the EPA (2000), and Zn was 6,650.72 mg/kg, which is above the limitations defined by the EPA (2000) for cultivated and non-cultivated soils. The heavy metals' concentration for S6 reference soil was as follows: Cd was 24.80 mg/kg, above the EPA limit in natural soils, Pb was 308.14 mg/kg, which is under the limitation of non-play areas in the urban environment, and Zn was

3,996.15 mg/kg, which is above the limitations defined by the EPA (2000) for cultivated and non-cultivated soils.

Soil is a complex system, which has a heterogeneous structure and different compositions in depth. Often the distribution of trace essential and hazardous elements and their deposition are in-depth down to 50 cm. Analysis of soils is necessary due to the relation between soils' chemical and mineralogical composition (International Atomic Energy Agency [IAEA], 1997). I will focus on the six soil samples (S2, S4, S12, S13, S24, and S26) selected for the bioremediation experiment in the following paragraphs. The edaphic and microbial characteristics of the selected six soil samples used for the bioremediation experiment are summarized in *Table 21.* The moisture content ranged between 18.97% to 44.79%, which is between the driest (wilting point) and wettest drained state (field capacity) in clay soils (Brandt, Johnson, Elphinston, & Ratnayaka, 2017). The pH level was between 6.9 and 8.6, considered slightly acidic and slightly alkaline.

Table 21 Edaphic and microbial characteristics of the six soil samples selected and utilized for the bioremediation experiment. [12]

Site	Moisture (%)	pH	Cd (mg/kg)	Pb (mg/kg)	Zn (mg/kg)	DNA before bioremediation (μg/g)	DNA after biostim. (μg/g)	DNA after natural att. (μg/g)
S2	23.65	7.6	7.9	187.5	1,177	11,789	3012	3563
S4	28.23	8.6	15.7	317.2	2,639	18,392	5016	1816
S12	22.80	6.9	80.9	1,646.8	12,798	13,990	3368	2762
S13	25.45	8.0	60.6	1,051.3	10,776	16,634	6787	3327
S24	44.79	7.7	335.8	8,431.4	2,630	19,563	5072	3191
S26	18.97	7.0	341.8	4,316.4	79,341	7,158	1730	787

[12] Biostim indicates for biostimulation, natural att indicates for natural attenuation.

Table 22 Pairwise comparison of DNA concentrations before inoculating the samples with benzene and toluene. [13]

	DNA after biostimulation (μg/g)	DNA after natural attenuation (μg/g)
DNA before bioremediation (μg/g)	**0.0004**	**0.0001**
DNA after biostimulation (μg/g)		0.09

The selected six soil samples 'edaphic characteristics were compared to the reference (S3 and S6) soils' edaphic characteristics. There was not any significant difference statistically in moisture content, pH level, Cd, Pb, and Zn concentration between the reference and selected soil samples. The Pb concentration was below the EPA limitations for the reference soils. There was no significant difference statistically in the DNA concentration between reference and experimental soils before biodegradation. Only the six selected soil samples were utilized for the bioremediation experiment. The DNA concentration in the soil samples was significantly different (P=0.0004) before the bioremediation and after biostimulation. The DNA concentration was also significantly different (P=0.0001) before bioremediation and after natural attenuation. The DNA concentration was not significantly different statistically (P=0.09) between biostimulation and natural attenuation (*Table 22)*. Gong (2012) also encountered a significant decrease in the numbers of soil bacteria in hydrocarbon-contaminated soil compared to the initial increase in the number of degraders at the beginning of biodegradation. Gong (2012) explained the decrease with the disruption of the cellular membrane as a reasonable indicator for cell death.

The spatial distribution of the overall major elements (needed for good soil fertility and plant growth) in the six experimental soils was studied before the bioremediation experiment using micro XRF analysis. *Tables 23 and 24* show the found major, minor, and trace elements (Al, Si, P,

[13] Individual cells contain P-values from a one-way ANOVA test comparing DNA concentrations from the samples indicated in the column and row for each cell. Statistically significant (P<0.05) are shown in bold. Soils were not inoculated with benzene and toluene before bioremediation. The DNA was quantified immediately after sample collection from the Tar Creek Superfund site. DNA was quantified once again after each sample was inoculated with benzene and toluene, and complete biodegradation occurred.

S, Rh, K, Ca, Ti, Fe, Zn, and Pb). The concentration of the major and minor elements found in the six experimental soils was significantly higher statistically (*P*-value <0.05) than the typical concentrations sufficient for healthy plant growth. The results in *Figure 13* do not indicate that all these elements are available for plant uptake. The concentration of mineral elements in soil and their available form for plants are as follows: 0.002 g/g for phosphorus (P), 0.01 g/g for K, 0.005 g/g for Ca, 0.0001 g/g for Fe, 1.69×10^{6} g/g for S and 0.00002 g/g for Zn (Epstein, 1965; Epstein, 1972). The spatial distribution of the major elements in the S26 soil sample, which had the highest heavy metal concentrations, differed from the other soils samples. It had lower concentrations (% by mass (g/g)) of the major elements than the other soils (S2, S4, S12, S14, and S24), but the Si was present in S26 in the highest concentration. Silicon reduces the P sorption in soils and affects the binding of nutrient elements to soil particles (Koski-Vahala, Hartikainen & Tallberg, 2001).

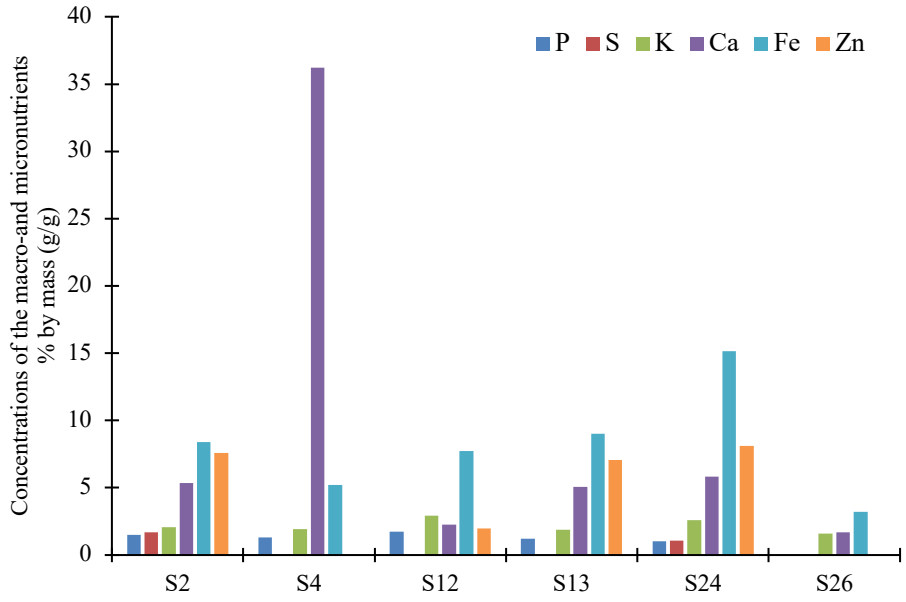

Figure 13 Total concentration % by mass (g/g) of the found major and minor elements in the six experimental soils before the bioremediation experiment was carried out. S numbers indicate for the sample name.

Table 23 The total concentrations of major, minor, and other elements found in the experimental soils before the bioremediation experiment was carried out. [14]

Element	S2			S4			S12		
	% by mass (g/g)	% by abundance (mol/mol)	*P* value <0.05	% by mass (g/g)	% by abundance (mol/mol)	*P* value <0.05	% by mass (g/g)	% by abundance (mol/mol)	*P* value <0.05
Al	4.57±0.86	5.45±0.04		3.66±0.72	4.90±0.03		5.57±1.07	6.29±0.06	
Si	65.54±0.17	75.18±0.13		39.62±0.16	50.99±0.07		75.11±0.23	81.49±0.18	
P	1.51±1.60	1.57±0.02		1.31±1.04	1.53±0.01		1.73±2.25	1.70±0.04	
Rh	n.d.	n.d.		10.71±0.49	3.76±0.05		0.96±0.58	0.28±0.01	
S	1.69±1.05	1.7±0.02		n.d.	n.d.		n.d.	n.d.	
K	2.06±0.81	1.69±0.02	**0.006**	1.94±0.60	1.80±0.01	0.23	2.91±1.02	2.27±0.03	**0.02**
Ca	5.36±0.41	4.31±0.02		36.19±0.12	32.64±0.06		2.24±0.96	1.70±0.02	
Ti	1.02±0.65	0.69±0.01		1.34±0.54	1.01±0.01		1.77±0.72	1.12±0.01	
Fe	8.40±0.13	4.85±0.01		5.22±0.15	3.38±0.01		7.74±0.20	4.23±0.02	
Zn	7.58±0.11	3.74±0.01		n.d.	n.d.		1.98±0.30	0.92±0.01	
Pb	n.d.	n.d.		n.d.	n.d.		n.d.	n.d.	

Table 24 The total concentrations of major, minor, and other elements found in the experimental soils before the bioremediation experiment was carried out (*Table 23* is continued). [15]

Element	S13			S24			S26		
	% by mass (g/g)	% by abundance (mol/mol)	*P* value <0.05	% by mass (g/g)	% by abundance (mol/mol)	*P* value <0.05	% by mass (g/g)	% by abundance (mol/mol)	*P* value <0.05
Al	3.98±1.00	5.35±0.04		5.43±0.91	7.83±0.05		6.79±0.06	4.25±0.86	
Si	55.16±0.20	71.27±0.12		42.27±0.23	58.59±0.11		84.82±0.18	90.06±0.19	
P	1.20±1.77	1.41±0.02		1.02±1.85	1.28±0.02		n.d.	n.d.	
Rh	16.66±0.61	5.87±0.10		16.04±0.60	6.07±0.10		n.d.	n.d.	
S	n.d.	n.d.		1.08±1.25	1.31±0.01		n.d.	n.d.	
K	1.89±0.95	1.76±0.02	**0.01**	2.57±0.80	2.55±0.02	**0.03**	1.59±0.01	1.07±0.77	0.10
Ca	5.06±0.48	4.58±0.02		5.85±0.44	5.68±0.03		1.70±0.01	1.93±0.65	
Ti	n.d.	n.d.		1.35±0.64	1.10±0.01		1.27±0.01	1.01±0.55	
Fe	9.00±0.14	5.85±0.02		15.13±0.11	10.55±0.02		3.19±0.01	1.27±0.18	
Zn	7.05±0.12	3.91±0.01		8.11±0.12	4.83±0.01		n.d.	n.d.	
Pb	n.d.	n.d.		1.17±0.47	0.22±0.01		n.d.	n.d.	

[14] The concentrations are reported with ± standard deviations. Statistically significant difference (*P*-value < 0.05) between the found concentrations and sufficient concentrations for plant growth is shown in bold. *P*-values are from a one-way ANOVA test. "n.d." indicates not detected.

[15] The concentrations are reported with ± standard deviations. Statistically significant difference (*P*-value < 0.05) between the found concentrations and sufficient concentrations for plant growth is shown in bold. *P*-values are from a one-way ANOVA test. "n.d." indicates not detected.

The difference between the concentration of the major, minor, and other elements found in the six experimental soils was not significantly different (*P*-value <0.05) when they were compared with each other.

Benzene and toluene biodegradation through the bioremediation experiment

The initial concentration of benzene ranged between 504 and 570 mg/kg in the aerobic microcosms for the biostimulation experiment. Benzene ranged between 440 and 510 mg/kg in the bottles simulated for natural attenuation. The initial concentration of toluene ranged between 287 and 349 mg/kg in the aerobic microcosms for the biostimulation experiment. Toluene ranged between 238 and 323 mg/kg in the bottles simulated for natural attenuation.

Lag phase was not observed during the bioremediation experiment. Degradation of benzene and toluene was observed after 3 hours of inoculating a mixture of 0.5 mL of benzene stock and 1 mL of toluene stock to the aerobic microcosms (from *Figures 14* to *25)*. The minimum hour required for complete benzene degradation occurred after 73 hours in the S12 soil sample (with "medium"-heavy metal concentrations) simulated for biostimulation. The maximum hour required for complete benzene degradation occurred after 130 hours in the S26 soil sample (with the highest-heavy metal concentrations) simulated for biostimulation. The minimum time required for complete benzene degradation occurred after 89 hours in S13 soil sample (with the second "medium"-heavy metal concentrations) simulated for natural attenuation. The maximum time required for complete benzene degradation occurred after 216 hours in S26 soil sample simulated for natural attenuation (*Table 25*).

The minimum hour required for complete toluene degradation occurred after 73 hours in the S12 soil sample simulated for biostimulation. The maximum hour required for complete toluene degradation occurred after 154 hours in the S4 soil sample (with the second-lowest heavy metal concentrations) simulated for biostimulation. The minimum time required for complete toluene degradation occurred after 73 hours in S13 soil sample simulated for natural attenuation. The

maximum time required for complete toluene degradation occurred after 178 hours in S26 soil sample simulated for natural attenuation (*Table 25*).

Toluene degraded faster than benzene in both scenarios (under biostimulation and natural attenuation conditions). The biodegradation was faster using biostimulation than using natural attenuation. Neither benzene nor toluene biodegradation seemed to be inhibited or limited because of each other's presence or the high levels of heavy metals (Cd, Pb, and Zn). S13 soil sample required less time (73 and 89 hours) to remove benzene and toluene through natural attenuation altogether. S12 soil sample required less time (only 73 hours) to remove benzene and toluene through biostimulation. S26 soil sample produced the slowest complete degradation (130 hours, 178 hours, and 216 hours) for the complete removal of benzene through biostimulation and natural attenuation and the natural attenuation of toluene (*Table 25*).

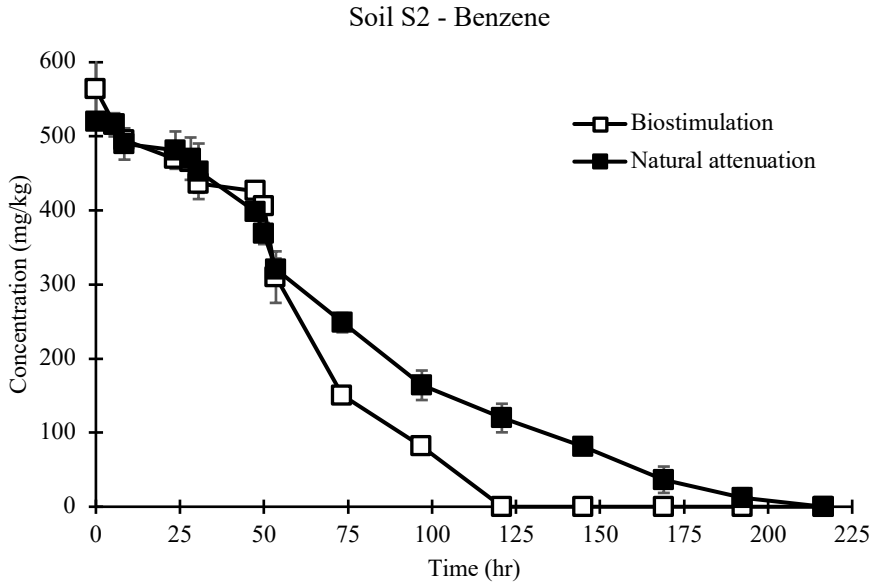

Figure 14 Benzene concentration in soil S2 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [16]

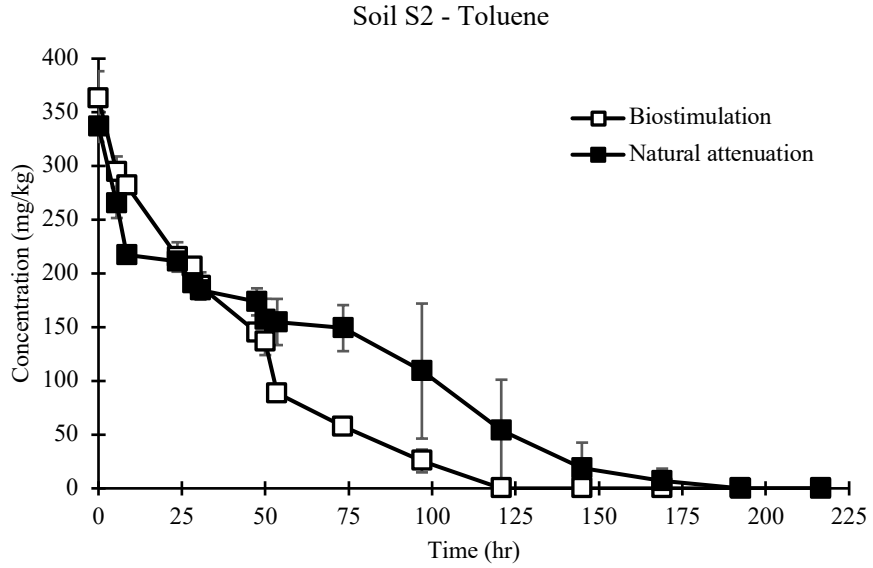

Figure 15 Toluene concentration in soil S2 samples was measured with gas chromatography (GC-FID) in the aerobic microcosms created for natural attenuation and natural attenuation. [17]

[16] The concentration of benzene dropped drastically after 50-hour of incubation at 30°C with 19% humidity. Benzene degraded entirely after 121 hours in the aerobic microcosms created for biostimulation, and 216 hours for natural attenuation. Natural attenuation resulted a linear benzene degradation. The heavy metal concentrations were the lowest in this sample.

[17] The concentration of toluene dropped drastically after 50-hour of incubation at 30°C with 19% humidity. Toluene degraded entirely after 121 hours in the aerobic microcosms created for biostimulation, and 193 hours for natural attenuation. Natural attenuation resulted a linear toluene degradation. The heavy metal concentrations were the lowest in this sample.

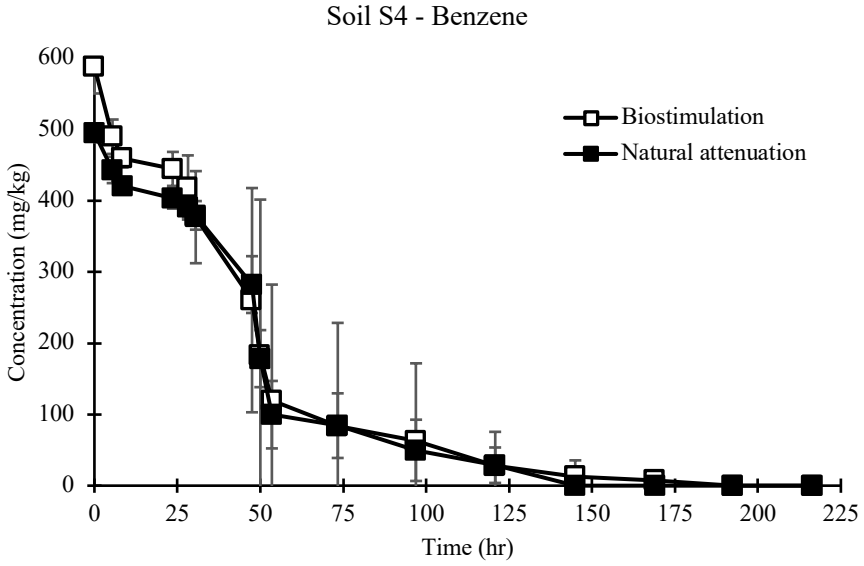

Figure 16 Benzene concentration in soil S4 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [18]

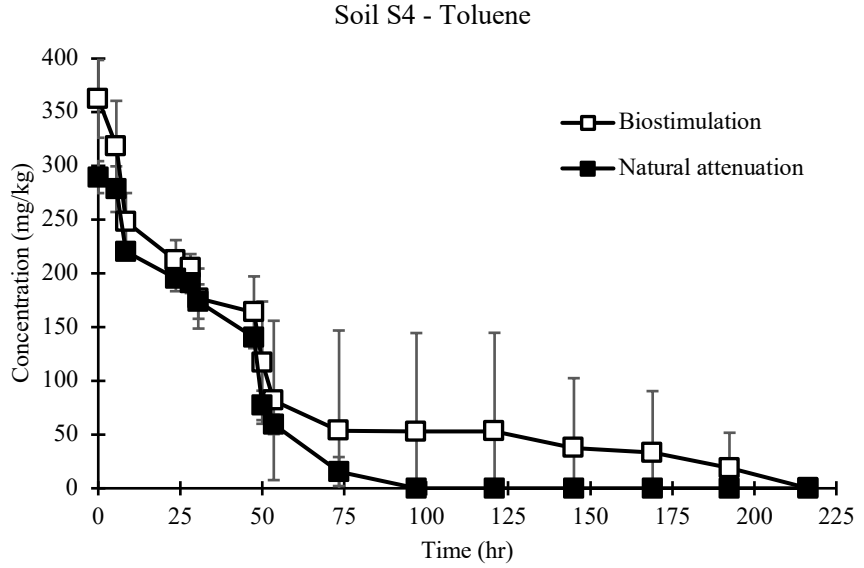

Figure 17 Toluene concentration in soil S4 was measured with gas chromatography (GC-FID) in the aerobic microcosms created for natural attenuation. [19]

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[18] The concentration of benzene dropped drastically after 50-hour of incubation at 30°C with 19% humidity. Benzene degraded entirely after 192 hours in the aerobic microcosms created for biostimulation, and 145 hours for natural attenuation. There was no difference between natural attenuation and biostimulation. The heavy metal concentrations were the second lowest in this sample.

[19] The concentration of toluene dropped drastically after 50-hour of incubation at 30°C with 19% humidity. Toluene degraded entirely after 216 hours in the aerobic microcosms created for biostimulation, and 97 hours for natural attenuation. The heavy metal concentrations were the second lowest in this sample.

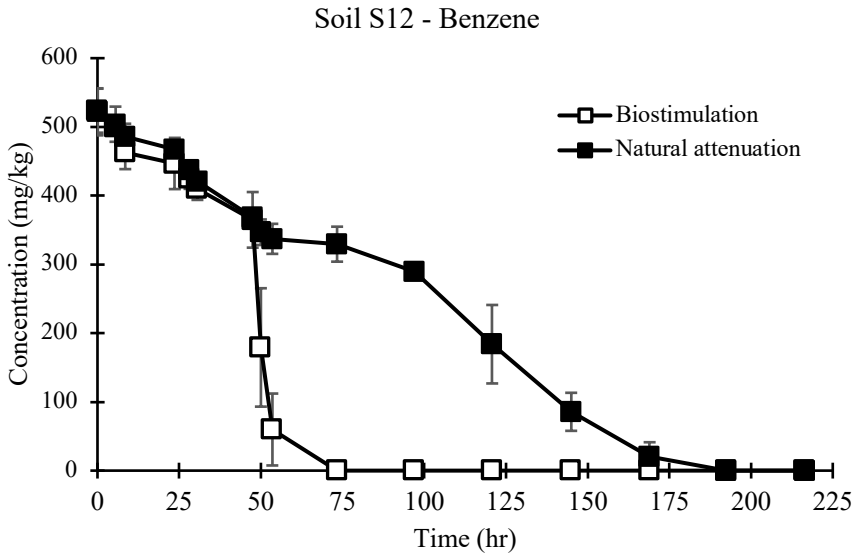

Figure 18 Benzene concentration in soil S12 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [20]

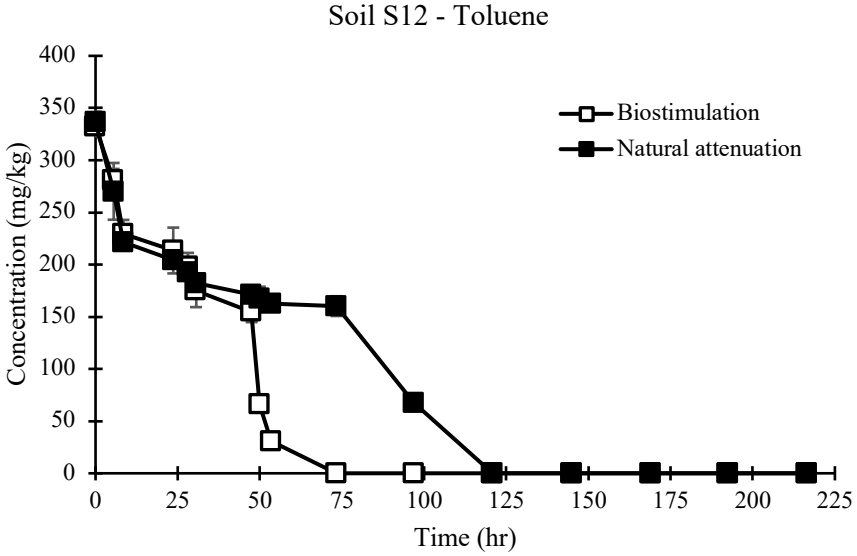

Figure 19 Toluene concentration in soil S12 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [21]

[20] The concentration of benzene dropped drastically after 50-hour of incubation at 30°C with 19% humidity. Benzene degraded entirely after 73 hours in the aerobic microcosms created for biostimulation, and 192 hours for natural attenuation. Natural attenuation resulted a linear benzene degradation.

[21] The concentration of toluene dropped drastically after 50-hour of incubation at 30°C with 19% humidity. Toluene degraded entirely after 73 hours in the aerobic microcosms created for biostimulation, and 121 hours for natural attenuation. Natural attenuation resulted a linear toluene degradation.

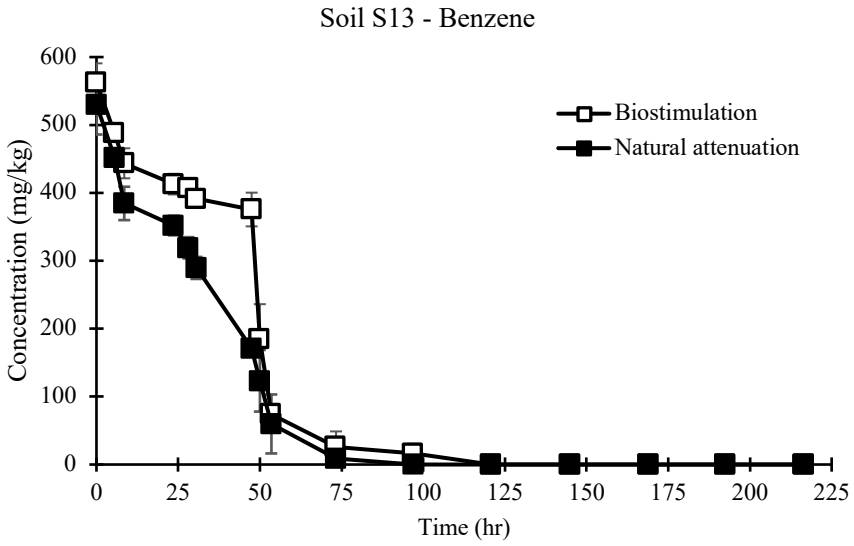

Figure 20 Benzene concentration in soil S13 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [22]

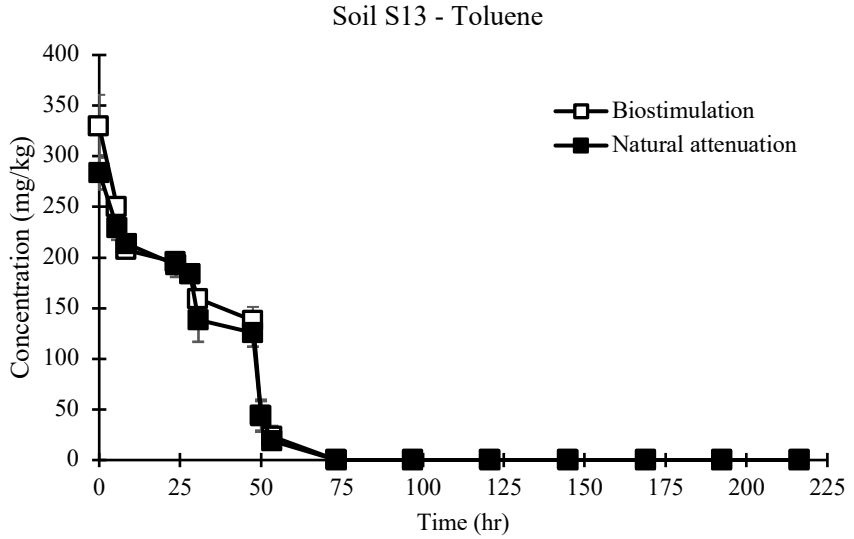

Figure 21 Toluene concentration in soil S13 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [23]

[22] The concentration of benzene dropped drastically after 30-hour of incubation at 30°C with 19% humidity in the aerobic microcosms created for biostimulation, and after 50-hour incubation for natural attenuation. Benzene degraded entirely after 73 hours in the aerobic microcosms created for biostimulation, and 192 hours for natural attenuation. Natural attenuation resulted a linear benzene degradation.

[23] The concentration of toluene dropped drastically after 50-hour of incubation at 30°C with 19% humidity. Toluene degraded entirely after 73 hours in the aerobic microcosms created for biostimulation and for natural attenuation as well.

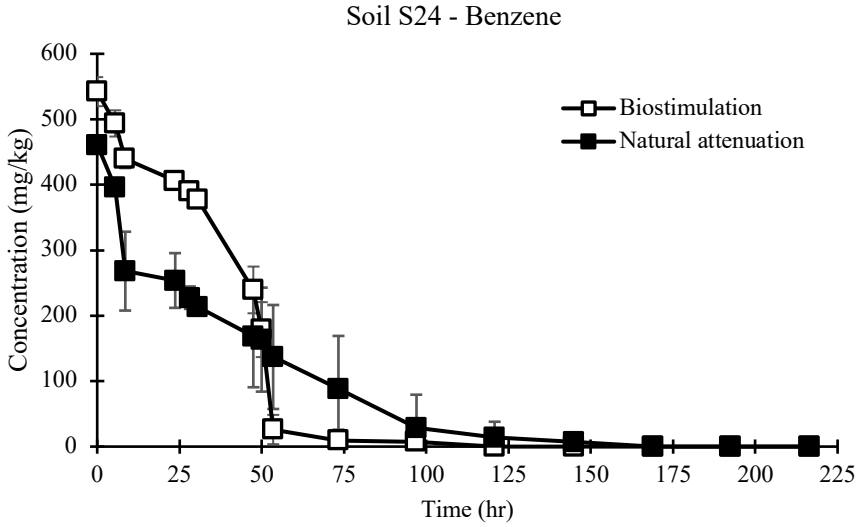

Figure 22 Benzene concentration in soil S24 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [24]

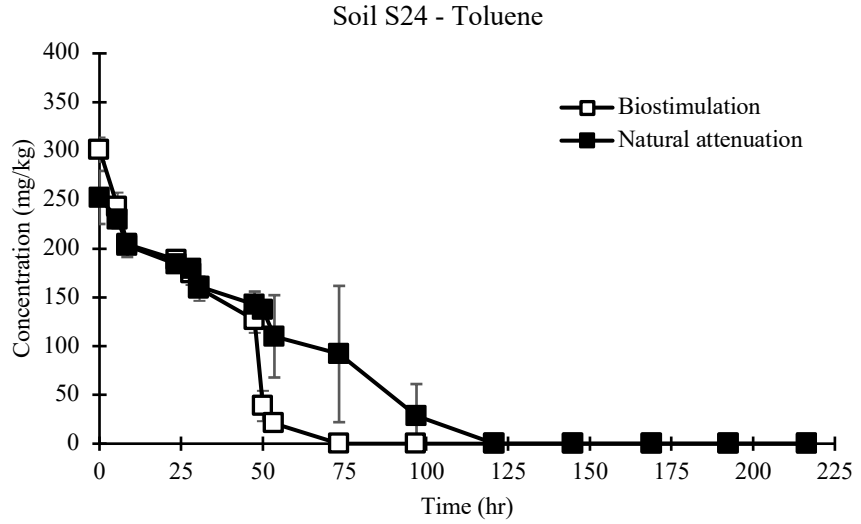

Figure 23 Toluene concentration in soil S24 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [25]

[24] The concentration of benzene dropped drastically after 50-hour of incubation at 30°C with 19% humidity in the aerobic microcosms created for biostimulation. Benzene degraded entirely after 121 hours in the aerobic microcosms created for biostimulation, and 169 hours for natural attenuation. Natural attenuation resulted a linear benzene degradation. The heavy metal concentrations were the second highest in this sample.
[25] The concentration of toluene dropped drastically after 50-hour of incubation at 30°C with 19% humidity in the aerobic microcosms created for biostimulation. Toluene degraded entirely after 73 hours in the aerobic microcosms created for biostimulation, and 121 hours for natural attenuation. Natural attenuation resulted a linear toluene degradation. The heavy metal concentrations were the second highest in this sample.

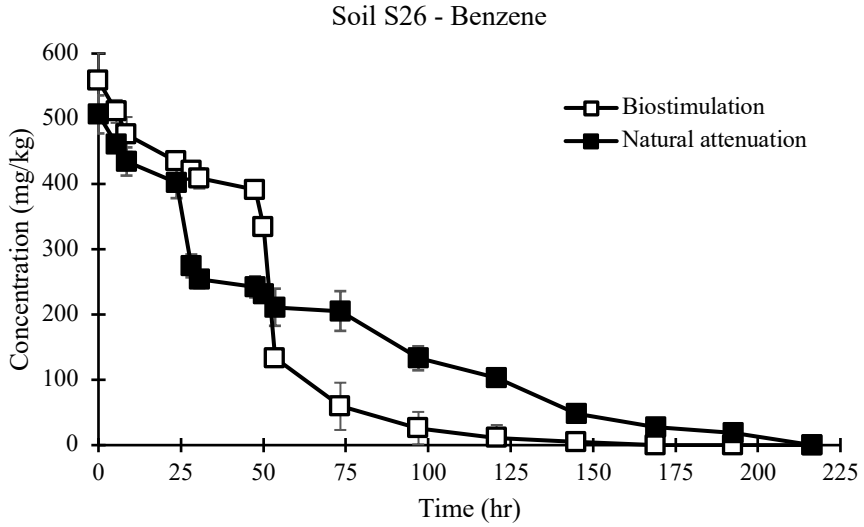

Figure 24 Benzene concentration in soil S26 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [26]

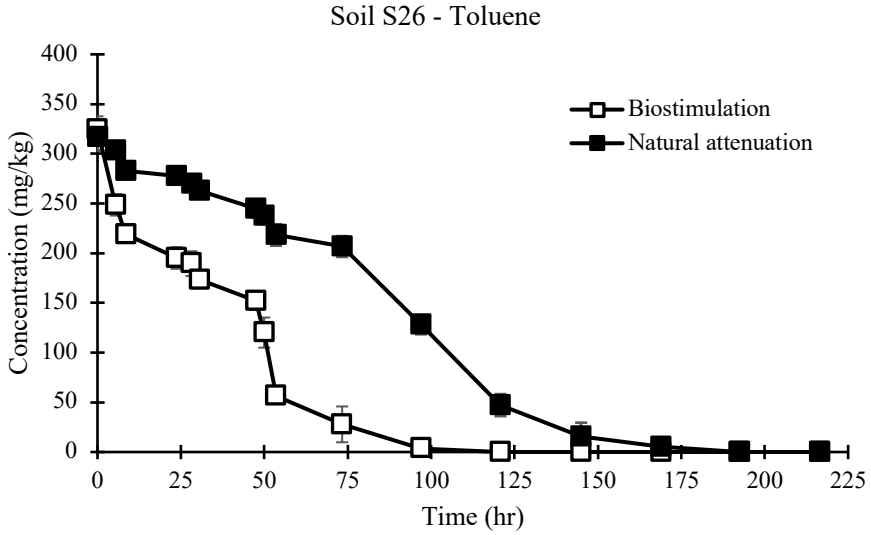

Figure 25 Toluene concentration in soil S24 sample was measured with gas chromatography (GC-FID) in the aerobic microcosms created for biostimulation and natural attenuation. [27]

[26] The concentration of benzene dropped drastically after 50-hour of incubation at 30°C with 19% humidity in the aerobic microcosms created for biostimulation. Benzene degraded entirely after 167 hours in the aerobic microcosms created for biostimulation, and 216 hours for natural attenuation. Natural attenuation resulted a linear benzene degradation. The heavy metal concentrations were the highest in this sample.

[27] The concentration of toluene dropped drastically after 50-hour of incubation at 30°C with 19% humidity in the aerobic microcosms created for biostimulation. Toluene degraded entirely after 121 hours in the aerobic microcosms created for biostimulation, and 192 hours for natural attenuation. Natural attenuation resulted a linear toluene degradation. The heavy metal concentrations were the second highest in this sample.

Table 25 Zero order kinetic constants calculated (and the triplicates were averaged) for benzene and toluene in the aerobic microcosms during the bioremediation experiment.

Samples with heavy metals	Bioremediation technique	Compound	Zero order kinetic constant (hour^{-1})	Complete degradation (hour)
S2	Biostimulation	Benzene	4.755±0.07	121±0
Cd 7.9 mg/kg		Toluene	2.645±0.05	121±0
Pb 187.5 mg/kg	Natural attenuation	Benzene	2.794±0.18	208±13.86
Zn 1,177.2 mg/kg		Toluene	1.617±0.49	161±36.54
S4	Biostimulation	Benzene	6.598±3.07	113±68.78
Cd 15.7 mg/kg		Toluene	3.126±1.69	153±55.24
Pb 317.2 mg/kg	Natural attenuation	Benzene	4.199±1.29	129±27.71
Zn 2,639.2 mg/kg		Toluene	3.311±0.29	89±13.64
S12	Biostimulation	Benzene	4.500±2.75	73±0
Cd 80.9 mg/kg		Toluene	4.112±0.13	73±0
Pb 1,646.8 mg/kg	Natural attenuation	Benzene	2.918±0.22	185±13.57
Zn 12,797.6 mg/kg		Toluene	1.951±0.07	121±0
S13	Biostimulation	Benzene	2.719±0.92	105±27.40
Cd 60.6 mg/kg		Toluene	3.792±0.26	73±0
Pb 1,051.3 mg/kg	Natural attenuation	Benzene	6.026±1.18	89±13.41
Zn 10,775.9 mg/kg		Toluene	3.699±0.164	73±0
S24	Biostimulation	Benzene	3.452±3.08	89±27.40
Cd 335.8 mg/kg		Toluene	3.741±0.07	73±0
Pb 8,431.4 mg/kg	Natural attenuation	Benzene	3.209±0.93	121±41.57
Zn 2,630 mg/kg		Toluene	2.113±0.54	113±13.76
S26	Biostimulation	Benzene	2.597±0.93	129±36.68
Cd 341.8 mg/kg		Toluene	2.683±0.33	105±13.76
Pb 4,316.4 mg/kg	Natural attenuation	Benzene	2.021±0.06	216±0
Zn 79,341.3 mg/kg		Toluene	1.952±0.19	177±27.42

The biodegradation of benzene and toluene did not seem to be inhibited or limited due to the high concentration levels of the heavy metals (as shown in *Table 25* above). Baath et al. (1998) demonstrated that the tolerance of microbial communities was the highest, where the concentration of metals was the highest. Microbes resistant to high Zn concentration were also tolerant to both Cu and Cd. Communities that showed resistance to high Cu levels were tolerant to Cd. The threshold levels for the sandy loam soil were 20 mg/kg for Cu tolerance, 60 mg/kg for Ni, and 140 mg/kg for Zn (Baath et al., 1998). Threshold levels of the microbes could not be defined for the studied heavy metals in this dissertation since the mixture of benzene and toluene degraded entirely at the end of the bioremediation experiment. The degradation rates for both benzene and toluene differed from those found in other studies (Salinatro et al., 1997; Morgan, Lewis, & Watkins, 1993;

Edwards & Grbic-Galic, 1992; Essaid et al., 2003; Patterson et al., 1993; de Nardi, Zaiat, & Foresti, 2007) mentioned in *Table 25* above.

Table 26 Pairwise comparison of degradation rates for benzene biostimulation between the initial rate and final rate for samples S12, S13, S24, and S26. [28]

Samples with heavy metals	Period	Degradation rate ($hour^{-1}$)	*P*-value (rate)	Time (hour)	P-value (time)
S12 Cd 80.9 mg/kg Pb 1,646.8 mg/kg Zn 12,797.6 mg/kg	Initial	2.911±0.48	0.18	48±0	-
	Final	6.090±3.33		73±0	
S13 Cd 60.6 mg/kg Pb 1,051.3 mg/kg Zn 10,775.9 mg/kg	Initial	2.430±0.38	0.51	48±0	**0.02**
	Final	3.007±1.32		105±27.40	
S24 Cd 335.8 mg/kg Pb 8,431.4 mg/kg Zn 2,630 mg/kg	Initial	6.223±0.82	**0.0003**	50±0	0.07
	Final	0.682±0.04		89±27.40	
S26 Cd 341.8 mg/kg Pb 4,316.4 mg/kg Zn 79,341.3 mg/kg	Initial	3.202±0.51	0.11	50±0	**0.02**
	Final	1.992±0.89		129±36.68	

The biostimulation of benzene was not linear for S12, S13, S24, and S26 soil samples. The biostimulation of benzene became linear in S12 and S13 soil samples after 48 hours, and after 50 hours in S24 and S26 soil samples. The degradation rates and time differed statistically in these samples before and after 48 and 50 hours. The degradation time significantly changed (P=0.02) after 48 hours in S13 soil sample, and after 50 hours in S26 soil sample. The degradation rate significantly changed (P=0.0003) after 50 hours in S24 soil sample (*Table 26*). The biostimulation of toluene was linear, as well as the natural attenuation of benzene and toluene. The biodegradation rate and time was significantly different statistically between biostimulation and natural attenuation in S2 (P=0.00006 for rate and P=0.0004 for time), S24 (P=0.01 for rate and time), and S26 (P=0.03 for rate and P=0.02 for time) soil samples. The biodegradation rate was significantly different statistically (P=0.0001) for benzene between biostimulation and natural attenuation in S12 soil

[28] *P*-values were calculated from one-way ANOVA test. Statistically significant if $P<0.05$. "-" means for data that could not be calculated for significance analysis. The statistically significant differences are shown in bold.

sample. The biodegradation time was significantly different statistically for toluene between biostimulation and natural attenuation in S2 (P=0.02) and S12 (P=0.00001) soil samples. The biodegradation time was significantly different statistically (P=0.002) for benzene between biostimulation and natural attenuation in S13 soil sample (*Table 27*).

Table 27 Pairwise comparison of degradation rates and time between biostimulation and natural attenuation for each sample. [29]

Samples with heavy metals	Comp.	Bio-remediation	Complete degradation (hour)	*P*-value (time)	Zero order kinetic constant ($hour^{-1}$)	*P*-value (rate)
S2	Benzene	Bio.	121±0	**0.0004**	4.755±0.07	**0.00006**
Cd 7.9 mg/kg		Nat.	208±13.86		2.794±0.18	
Pb 187.5 mg/kg	Toluene	Bio.	121±0	0.13	2.645±0.05	**0.02**
Zn 1,177.2 mg/kg		Nat.	161±36.54		1.617±0.49	
S4	Benzene	Bio.	113±68.78	0.73	6.598±3.07	0.28
Cd 15.7 mg/kg		Nat.	129±27.71		4.199±1.29	
Pb 317.2 mg/kg	Toluene	Bio.	153±55.24	0.12	3.126±1.69	0.86
Zn 2,639.2 mg/kg		Nat.	89±13.64		3.311±0.29	
S12	Benzene	Bio.	73±0	**0.0001**	4.500±2.75	0.37
Cd 80.9 mg/kg		Nat.	185±13.57		2.918±0.22	
Pb 1,646.8 mg/kg	Toluene	Bio.	73±0	-	4.112±0.13	**0.00001**
Zn 12,797.6 mg/kg		Nat.	121±0		1.951±0.07	
S13	Benzene	Bio.	105±27.40	0.42	2.719±0.92	**0.002**
Cd 60.6 mg/kg		Nat.	89±13.41		6.026±1.18	
Pb 1,051.3 mg/kg	Toluene	Bio.	73±0	-	3.792±0.26	0.62
Zn 10,775.9 mg/kg		Nat.	73±0		3.699±0.164	
S24	Benzene	Bio.	89±27.40	0.33	3.452±3.08	0.90
Cd 335.8 mg/kg		Nat.	121±41.57		3.209±0.93	
Pb 8,431.4 mg/kg	Toluene	Bio.	73±0	**0.01**	3.741±0.07	**0.01**
Zn 2,630 mg/kg		Nat.	113±13.76		2.113±0.54	
S26	Benzene	Bio.	129±36.68	0.01	2.597±0.93	0.33
Cd 341.8 mg/kg		Nat.	216±0		2.021±0.06	
Pb 4,316.4 mg/kg	Toluene	Bio.	105±13.76	**0.02**	2.683±0.33	**0.03**
Zn 79,341.3 mg/kg		Nat.	177±27.42		1.952±0.19	

The abiotic factors have an impact on the rates of microbial growth and enzymatic activities. The factors affect the biodegradation rate of aromatic hydrocarbons. The stability of petroleum contaminants in soils depends on the quantity and quality of the hydrocarbon mixture and the characteristics of the environment. The features of the polluted soil and the abiotic factors

[29] *P*-values were calculated from one-way ANOVA test. Statistically significant if P<0.05. Comp. indicates compound, Bio. indicates biostimulation, and Nat. indicates natural attenuation. "-" means for data that could not be calculated for significance analysis. The statistically significant differences are shown in bold.

will determine the persistence of petroleum hydrocarbons, whether aromatic hydrocarbons will biodegrade in a couple of hours or days or not (Atlas, 1981). The biodegradation was fast for both benzene and toluene. It occurred only within 216 hours in this dissertation's bioremediation experiment, independently from the presence of each other or high concentrations of heavy metals. The temperature in the incubator was 30°C with 19% humidity, which might have caused the fast biodegradation.

Oh et al. (1994) found that the biodegradation of benzene was significantly prevented in the presence of toluene (Oh et al., 1994). The study of Chang et al. (1992) also revealed that the degradation rate of benzene or toluene was slower when they were present together in the same substrate (Chang et al., 1992). Benzene degraded entirely in this dissertation's bioremediation experiment, unlike in the study of Ekperusi and Aigbodion (2015), where benzene did not degrade entirely in the experimental soils. Benzene biodegradation was not prevented or slower due to the presence of toluene in this dissertation's bioremediation experiment.

Microbial community analysis (qualitative assessment)

The DNA sequence results of the original soil samples collected from Tar Creek Superfund site, Ottawa County, northeastern Oklahoma, demonstrated 6,701 different OTUs (operational taxonomic units). These identified OTUs belong to 31 different phyla (*Figure 26*). Twenty-three bacterial phyla, three archaeal phyla (*Euryarchaeota, Thaumarchaeota* and *Crenarchaeota*), and five Eukaryotes were identified in the soil samples before the bioremediation experiment on the mixture of benzene and toluene was carried out.

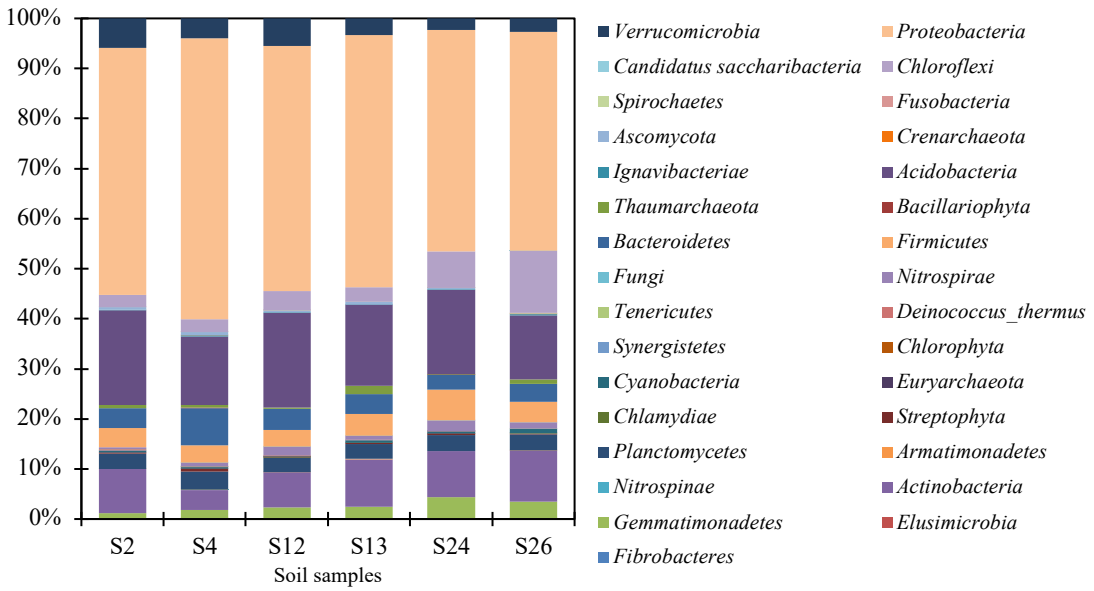

Figure 26 All native phyla were found in the soil samples before the bioremediation experiment was carried out. Results of the DNA sequence. [30]

A total of 2,951 different OTUs were identified in all samples after complete biodegradation. These identified OTUs belong to 26 different phyla. Eighteen bacterial phyla, one archaeal phylum (*Thaumarchaeota*), and seven Eukaryotes were identified in the soil samples after benzene and toluene biodegradation. These DNA sequence results are in accordance with the DNA results obtained in Chapter III, where an average of 3,163 and 2,586 OTUs was identified in the individual benzene and toluene samples, respectively. Chapter III's DNA sequence results demonstrated 16 different phyla, ten less than this chapter's (Chapter IV) DNA sequence results. *Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria*, and *Nitrospirae* were the top five dominant phyla in each soil sample (microbial control, biostimulation, and natural attenuation). *Proteobacteria, Actinobacteria*, and *Firmicutes* are usually the top three dominant phyla in BTEX biodegradation (Carvajal et al., 2018; Czarny et al., 2020; Hendrickx et al., 2006; Weelink, van Eekert, & Stams, 2010) and soil heavy metal stress studies (Desai et al., 2009).

[30] S indicates the sample name, and the numbers indicate the heavy metals' concentration in ascending order (2 indicates the lowest concentration, while 26 indicates the highest).

All phyla found after the complete biodegradation of benzene and toluene were compared to the original soil samples as well (shown in *Figures 27* to *32*). The original soil samples indicate the soil samples collected from the field (Tar Creek Superfund site, Ottawa County, Oklahoma), which had various heavy metal concentrations and were stored in a commercial refrigerator at 4°C. The microbial controls indicate the soil samples collected from the field, stored in glass bottles in an incubator at 30°C with 19% humidity, and mixed with aerobic mineral medium for the duration of the bioremediation experiment.

Five phyla (*Fibrobacteres, Armatimonadetes, Euryarchaeota, Tenericutes*, and *Fusobacteria*) disappeared (*Figure 27*) from the original S2 soil sample (which had the lowest heavy metals' concentration), when a mixture of benzene (876.39 mg/kg wet soil) and toluene (869.80 mg/kg wet soil) stock solution was introduced and degraded using two different bioremediation techniques (biostimulation and natural attenuation). Seven new phyla (*Chytridiomycota, Discosea, Eukaryota, Spirochaetes, Chlamydiae, Elusimicrobia*, and *Oomycota*) appeared, when benzene and toluene were introduced and degraded, which were not present in the original S2 soil sample. Their counts were between 1 and 49.

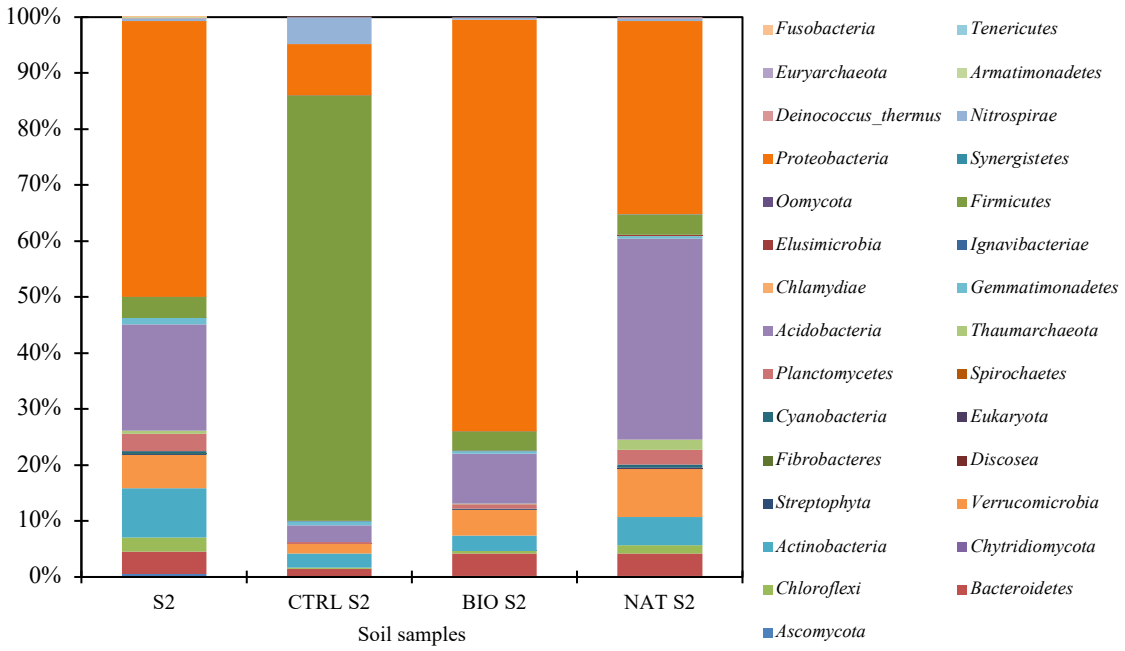

Figure 27 All phyla found in soil sample S2, which indicates the lowest heavy metals' concentration. [31]

Table 28 The top five OTUs identified in the S2 (lowest heavy metals' concentration) soil samples, which had the highest percent reads based on the DNA sequence result. [32]

Soil sample S2	O.T.U. number	Taxonomy identification	Percent reads
Original (S2)	A325	*Acidobacterium* sp.	1.8
	A5	*Dongia* sp.	1.6
	A9	*Acidobacterium* sp.	1.4
	A20	*Pelobacter* sp.	1.2
	A1	*Rhodopseudomonas* sp.	0.9
Microbial control (CTRL S2)	B9	*Symbiobacterium* sp.	17.1
	B10	*Symbiobacterium* sp. ka13	12.7
	B17	*Symbiobacterium* sp.	10.1
	B12	*Nitrospira* sp.	4.7
	B42	*Symbiobacterium* sp. ka13	4.1
Biostimulation (BIO S2)	B2	*Zoogloea* sp.	16.1
	B1	*Pseudomonas taiwanensis* str. bf_s2 eu857417.1	11.9
	B43	*Acidovorax konjaci* str. icmp 7733 af137507.1	2.8
	B40	*Xylophilus ampelinus*	2.8
	B25	*Azoarcus* sp. bh72	2.4
Natural attenuation (NAT S2)	B7	*Acidobacterium* sp.	23.6
	B13	*Nitrosovibrio* sp.	2.2
	B140	*Anaeromyxobacter* sp.	1.8
	B3	*Rhodopseudomonas* sp.	1.2
	B135	*Acidobacterium* sp.	1.1

[31] S2 is the original soil sample collected from Tar Creek Superfund site, Ottawa County, Oklahoma. CTRL indicates the microbial controls for the bioremediation experiment, which only had the heavy metals and mixed with the aerobic mineral media. BIO indicates biostimulation, and NAT indicates natural attenuation. S2 results are from the DNA sequence before the bioremediation experiment was carried out. CTRL S2, BIO S2, and NAT S2 demonstrate the results from the DNA sequence after the complete degradation of benzene and toluene occurred in the soil samples.

[32] The original sample indicates the soil sample collected from the field and only had heavy metals' concentration. A indicates the first DNA sequence before the bioremediation experiment. B indicates the second sequence after the bioremediation experiment was completed.

Table 28 demonstrates the top five OTUs identified in the S2 soil samples, which had the lowest heavy metals concentration. The microbial community changed even when only mineral aerobic medium (microbial control) was added to the S2 original sample. *Symbiobacterium* sp. became the dominant species in the microbial controls with 17.1%, 12.7%, 10.1%, and 4.1% reads, while the original S2 soil sample had *Acidobacterium* sp. as the dominant species with 1.8% and 1.4% reads. *Symbiobacterium* was found in high abundance in alkaline, hot spring soil, which soil was rich in metallic (Mn, Fe, Co, Ni, Cu, Zn, Ba, and Pb) and non-metallic elements. (Rawat & Joshi, 2019). The abundance of *Symbiobacterium* also increased after applying composted tannery sludge for 7 years to the soil. The sludge contained organic and inorganic (such as chromium, Cr) compounds (Miranda et al., 2018).

Acidobacteria were reported in high relative abundance in volatile petroleum hydrocarbons (VPH) contaminated sandy soil and have a significant potential to degrade VPH (Mangse, Werner, Meynet, & Ogbaga, 2020). *Acidobacteria* were also presented in high percent reads in aliphatic hydrocarbon contaminated soils two years after biostimulation in an industrial site in France (Militon et al., 2010). *Acidobacteria* were also among the most abundant bacteria in Pb (from 123 mg/kg to 254 mg/kg), and Zn (from 72 mg/kg to 207 mg/kg) polluted soils collected from the third largest Pb and Zn mine in Iran. The Bama Mine (Isfahan province, Iran) has been exposed to heavy metals for over 50 years (Hemmat-Jou, Safari-Sinegani, Mirzaei-Asl, & Tahmourespour, 2018).

The S2 soil samples inoculated with benzene and toluene and degraded with biostimulation showed a great bacterial variety. *Zoogloea* sp., *Pseudomonas* sp., *Acidovarax* sp., *Xylophilus* sp., and *Azoarcus* sp. became the dominant species with 16.1%, 11.9%, 2.8%, 2.8%, and 2.4% reads, respectively. *Zoogloea* sp. demonstrated as a potential hydrocarbon-degrading bacterium in a Hungarian contaminated site (Farkas et al., 2014). The species also represented as a nitrogen-fixing bacterium (Xie & Yokota, 2006). *Pseudomonas* sp. and *Azoarcus* sp. were also highly abundant in Chapter III's soil samples. *Pseudomonas* species already demonstrated the ability to degrade benzene and toluene in other studies (Yoshikawa et al., 2017; Suenaga, Watanabe, Sato, Ngadiman,

& Furukawa, 2002; Verma & Kuila, 2019). The species also represents nitrogen-fixing strains in soils (Poly, Monrozier, & Bally, 2000).

Acidobacterium sp.*, Nitrosovibrio* sp., *Anaeromyxobacter* sp., *Rhodopseudomonas* sp., and *Acidobacterium* sp. were the top five dominant OTUs, with 23.6%, 2.2%, 1.8%, 1.2%, and 1.1% percent reads, respectively, in S2 soil samples, inoculated with benzene and toluene and degraded with natural attenuation. *Nitrosovibrio* species were also present in Chapter III's samples with high abundance. These species were found in a Pb and Zn contaminated mining site (Hemmat-Jou, Safari-Sinegani, Mirzaie-Asl, & Tahmourespour, 2018), indicating tolerance toward heavy metals.

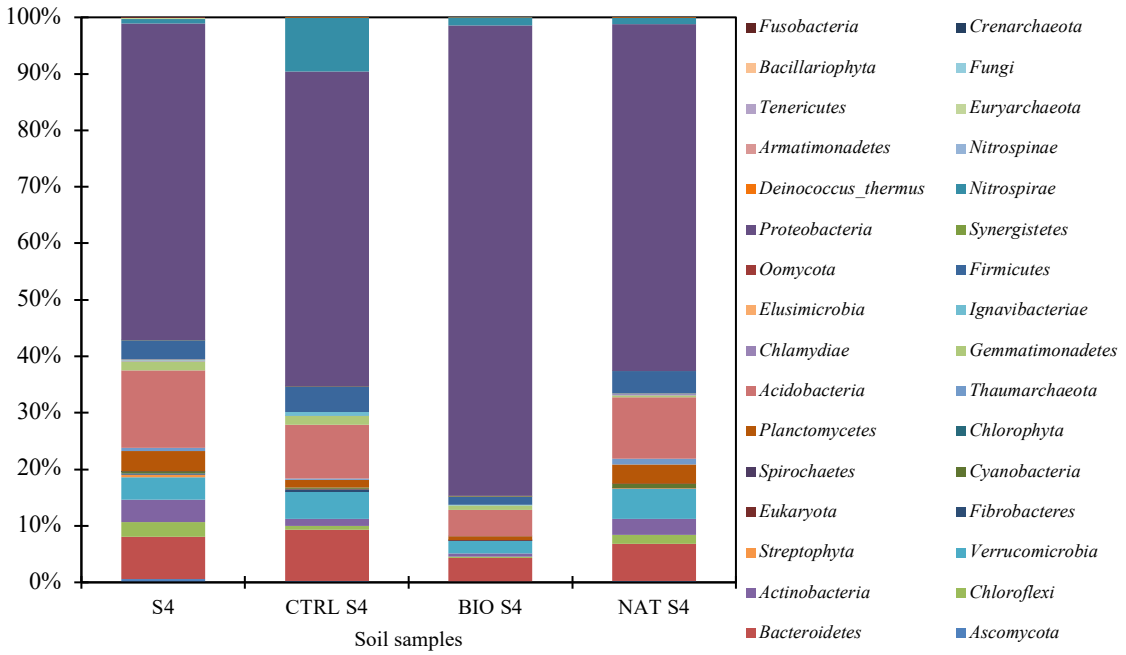

Figure 28 All phyla found in soil sample S4, which indicates the second lowest heavy metals' concentration. S4 is the original soil sample collected from Tar Creek Superfund site, Ottawa County, Oklahoma. [33]

Table 29 The top five OTUs identified in the S4 (the second lowest heavy metals' concentration) soil samples, which had the highest percent reads based on the DNA sequence result. [34]

Soil sample S4	O.T.U. number	Taxonomy identification	Percent reads
Original (S4)	A11	*Nitrosovibrio* sp.	1.2
	A12	*Thiorhodospira* sp.	1.1
	A5	*Dongia* sp.	1.0
	A181	*Pseudomonas putida* str. pc36 dq178233.1	0.7
	A30	*Chitinophaga* sp.	0.6
Microbial control (CTRL S4)	B24	*Nitrospira* sp. rc99 y14643.1	5.8
	B16	*Pseudomonas resinovorans* str. c87 fj950593.1	3.6
	B58	*Nitrosomonas* sp. vkmm063	3.1
	B88	*Spinghomonas* spp. kis08_048 gq385296.1	2.0
	B3	*Rhodopseudomonas* sp.	1.2
Biostimulation (BIO S4)	B1	*Pseudomonas taiwanensis* str. bf_s2 eu857417.1	25.4
	B16	*Pseudomonas resinovorans* str. c87 fj950593.1	6.5
	B69	*Pseudomonas otitidis*	2.6
	B2	*Zoogloea* sp.	2.6
	B25	*Azoarcus* sp. bh72	1.9
Natural attenuation (NAT S4)	B6	*Pseudomonas mendocina* str. b6_1 ef208965.1	18.8
	B25	*Azoarcus* sp. bh72	4.7
	B13	*Nitrosovibrio* sp.	1.7
	B1	*Pseudomonas taiwanensis* str. bf_s2 eu857417.1	1.5
	B21	*Dechloromonas* spp. cu466895.1	1.2

[33] CTRL indicates the microbial controls for the bioremediation experiment, which only had the heavy metals and mixed with the aerobic mineral media. BIO indicates biostimulation, and NAT indicates natural attenuation. S4 results are from the DNA sequence before the bioremediation experiment was carried out. CTRL S4, BIO S4, and NAT S4 demonstrate the results from the DNA sequence after the complete degradation of benzene and toluene occurred in the soil samples.
[34] The original sample indicates the soil sample collected from the field and only had heavy metals concentration. A indicates the first DNA sequence before the bioremediation experiment. B indicates the second sequence after the bioremediation experiment was completed.

Table 29 demonstrates the top five OTUs identified in the S4 soil samples, with the second-lowest heavy metals concentration. The microbial community changed even when only mineral aerobic medium (microbial control) was added to the S4 original sample. *Nitrospira* sp.*, Pseudomonas* sp.*, Nitrosomonas* sp.*, Spinghomonas* sp., and *Rhodopseudomonas* sp. became the dominant species in the microbial controls with 5.8%, 3.6%, 3.1%, 2.0%, and 1.2% reads. The original S4 soil sample had *Nitrosovibrio* sp.*, Thiorhodospira* sp., *Dongia* sp.*, Pseudomonas* sp., and *Chitinophaga* sp. as the dominant species with 1.2%, 1.1%, 1.0%, 0.7%, and 0.6% reads at the same time.

The S4 soil samples inoculated with benzene and toluene and degraded with biostimulation demonstrated *Pseudomonas* sp. as the dominant OUT, followed by *Zoogloea* sp. and *Azoarcus* sp. with 25.4%, 6.5%, 2.6%, 2.6%, and 1.9% reads, respectively. *Pseudomonas sp*. were also the dominant species followed by *Azoarcus* sp.*, Nitrosovibrio* sp., and *Dechloromonas* sp. with 18.8%, 4.7%, 1.7%, 1.5%, and 1.2% percent reads, respectively, in S4 soil samples, inoculated with benzene and toluene and degraded with natural attenuation.

Nine phyla (*Chlorophyta, Nitrospinae, Armatimonadetes, Euryarchaeota, Tenericutes, Fungi, Bacillariophyta, Crenarchaeota,* and *Fusobacteria*) disappeared (*Figure 28*) from the original S4 soil sample when a mixture of benzene (876.39 mg/kg wet soil) and toluene (869.80 mg/kg wet soil) stock solution was introduced and degraded using two different bioremediation techniques (biostimulation and natural attenuation). Two new phyla (*Eukaryota* and *Oomycota*) appeared when benzene and toluene were introduced and degraded and not present in the original S4 soil sample. Their counts were between 2 and 25.

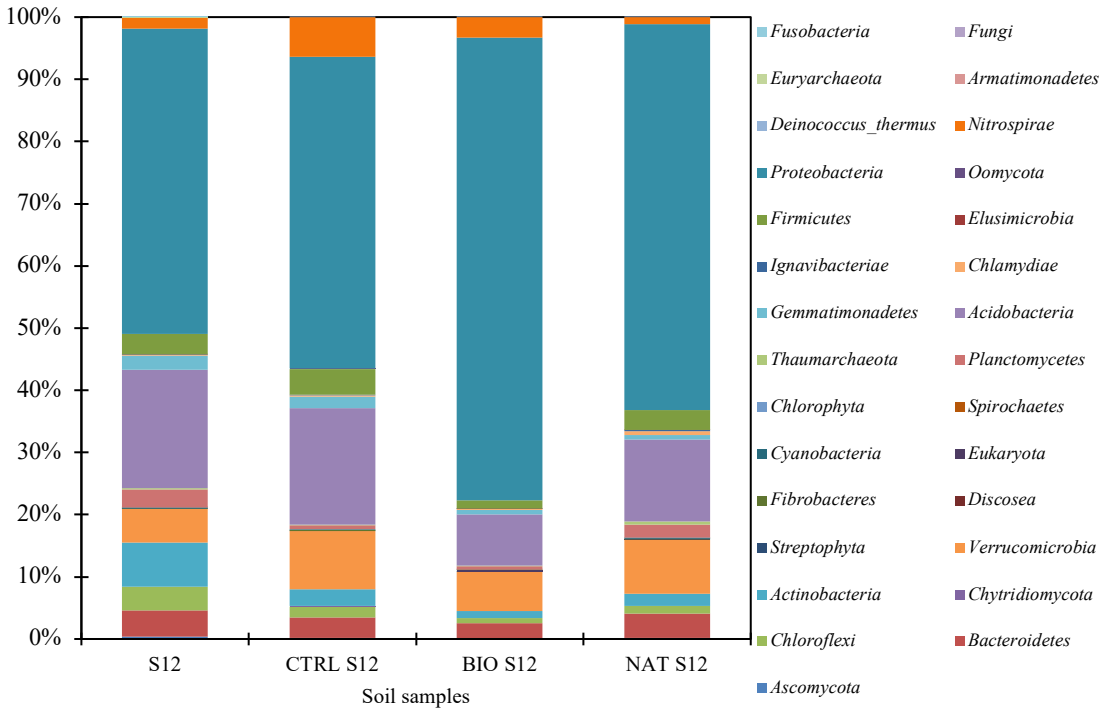

Figure 29 All phyla found in soil sample S12, which indicates medium heavy metals' concentration. S12 is the original soil sample collected from Tar Creek Superfund site, Ottawa County, Oklahoma. [35]

Table 30 The top five OTUs identified in the S12 (the first medium heavy metals' concentration) soil samples, which had the highest percent reads based on the DNA sequence result. [36]

Soil sample S12	O.T.U. number	Taxonomy identification	Percent reads
Original (S12)	A285	*Acidobacterium* sp.	1.8
	A2	*Bradyrhizobium lupini* str. km50 90 (usda 3514) u69637.1	1.7
	A39	*Rhodoplanes* spp. ef019976.1	1.0
	A22	*Nitrospira* sp.	0.9
	A583	*Gemmatimonas* sp.	0.8
Microbial control (CTRL S12)	B29	*Nitrosovibrio tenuis* str. nv1 ay123803.1	5.7
	B12	*Nitrospira* sp.	2.5
	B37	*Acidobacterium* sp.	2.5
	B48	*Anaeromyxobacter* sp.	2.3
	B18	*Cupriavidus respiraculi* str. Au3775 ay860237.1	2.0
Biostimulation (BIO S12)	B2	*Zoogloea* sp.	21.5
	B1	*Pseudomonas taiwanensis* str. bf_s2 eu857417.1	14.6
	B20	*Pseudomonas* sp.	1.9
	B12	*Nitrospira* sp.	1.8
	B71	*Nitrosospira* sp. apg3	1.7
Natural attenuation (NAT S12)	B1	*Pseudomonas taiwanensis* str. bf_s2 eu857417.1	12.6
	B2	*Zoogloea* sp.	8.6
	B13	*Nitrosovibrio* sp.	2.9
	B37	*Acidobacterium* sp.	1.9
	B38	*Prosthecobacter* sp.	1.9

[35] CTRL indicates the microbial controls for the bioremediation experiment, which only had the heavy metals and mixed with the aerobic mineral media. BIO indicates biostimulation, and NAT indicates natural attenuation. S12 results are from the DNA sequence before the bioremediation experiment was carried out. CTRL S12, BIO S12, and NAT S12 demonstrate the results from the DNA sequence after the complete degradation of benzene and toluene occurred in the soil samples.

[36] The original sample indicates the soil sample collected from the field and only had heavy metals concentration. A indicates the first DNA sequence before the bioremediation experiment. B indicates the second sequence after the bioremediation experiment was completed.

Table 30 demonstrates the top five OTUs identified in the S12 soil samples, with medium heavy metals concentration. The microbial community changed even when only mineral aerobic medium (microbial control) was added to the S12 original sample. *Nitrosovibrio* sp.*, Nitrospira* sp., *Acidobacterium* sp.*, Anaeromyxobacter* sp.*,* and *Cupriavidus* sp. became the dominant species in the microbial controls with 5.7%, 2.5%, 2.5%, 2.3%, and 2.0% reads. The original S12 soil sample had *Acidobacterium* sp.*, Bradyrhizobium* sp., *Rhodoplanes* sp., *Nitrospira* sp., and *Gemmatimonas* sp. as the dominant species with 1.8%, 1.7%, 1.0%, 0.9%, and 0.8% reads at the same time.

The S12 soil samples inoculated with benzene and toluene and degraded with biostimulation demonstrated *Pseudomonas* sp. as the dominant OTU, followed by *Zoogloea* sp. and *Nitrospira* sp. with 21.5%, 14.6%, 1.9%, 1.8%, and 1.7% reads. *Pseudomonas* sp. and *Zoogloea* sp. were also the dominant species followed by*, Nitrosovibrio* sp., *Acidobacterium* sp. and *Prosthecobacter* sp. with 12.6%, 8.6%, 2.9%, 1.9%, and 1.9% percent reads, in S12 soil samples, inoculated with benzene and toluene and degraded with natural attenuation.

Four phyla (*Armatimonadetes, Euryarchaeota, Fungi* and *Fusobacteria*) disappeared (*Figure 29*) from the original S12 soil sample when a mixture of benzene (876.39 mg/kg wet soil) and toluene (869.80 mg/kg wet soil) stock solution was introduced and degraded using two different bioremediation techniques (biostimulation and natural attenuation). Four new phyla (*Chytridiomycota*, *Discosea*, *Eukaryota* and *Oomycota*) appeared when benzene and toluene were introduced and degraded and not present in the original S12 soil sample. Their counts were between 1 and 51.

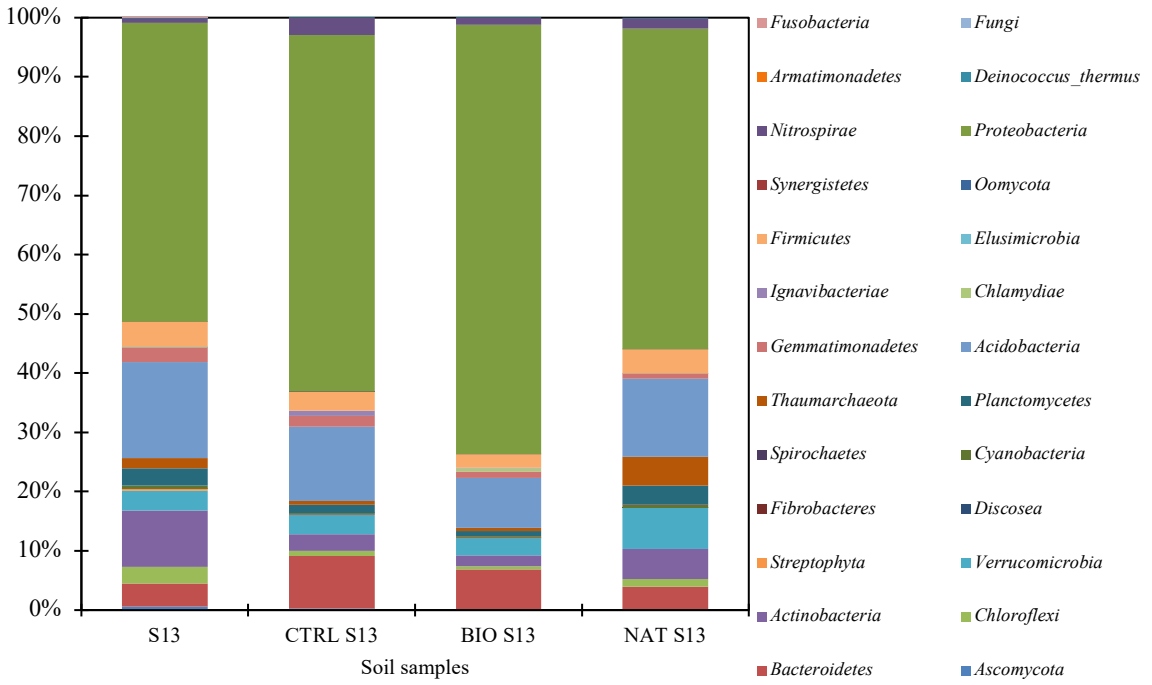

Figure 30 All phyla found in soil sample S13, which indicates the second medium heavy metals' concentration. S13 is the original soil sample collected from Tar Creek Superfund site, Ottawa County, Oklahoma. [37]

Table 31 The top five OTUs identified in the S13 (the second medium heavy metals' concentration) soil samples, which had the highest percent reads based on the DNA sequence result. [38]

Soil sample S13	O.T.U. number	Taxonomy identification	Percent reads
Original (S13)	A1	*Rhodopseudomonas* sp.	4.5
	A4	*Rhodopseudomonas* sp.	1.7
	A3	*Rhodopseudomonas* sp.	1.1
	A314	*Pelobacter* sp.	0.9
	A7	*Steroidobacter* sp.	0.9
Microbial control (CTRL S13)	B3	*Rhodopseudomonas* sp.	9.8
	B11	*Rhodopseudomonas* sp.	5.2
	B4	*Rhodopseudomonas* sp.	3.3
	B26	*Rhodopseudomonas* sp.	2.8
	B19	*Nitrosospira multiformis* str. atcc25196 ab070984.1	2.5
Biostimulation (BIO S13)	B1	*Pseudomonas taiwanensis* str. bf_s2 eu857417.1	14.4
	B3	*Rhodopseudomonas* sp.	6.3
	B11	*Rhodopseudomonas* sp.	3.8
	B4	*Rhodopseudomonas* sp.	2.3
	B26	*Rhodopseudomonas* sp.	2.1
Natural attenuation (NAT S13)	B2	*Zoogloea* sp.	9.4
	B3	*Rhodopseudomonas* sp.	4.3
	B44	*Bdellovibrio bacteriovorus* dq328_109 eu050708.1	3.1
	B11	*Rhodopseudomonas* sp.	2.1
	B45	*Candidatus nitrososphaera* sca1175 u62819.1	1.8

[37] CTRL indicates the microbial controls for the bioremediation experiment, which only had the heavy metals and mixed with the aerobic mineral media. BIO indicates biostimulation, and NAT indicates natural attenuation. S13 results are from the DNA sequence before the bioremediation experiment was carried out. CTRL S13, BIO S13, and NAT S13 demonstrate the results from the DNA sequence after the complete degradation of benzene and toluene occurred in the soil samples.

[38] The original sample indicates the soil sample collected from the field and only had heavy metals concentration. A indicates the first DNA sequence before the bioremediation experiment. B indicates the second sequence after the bioremediation experiment was completed.

Table 31 demonstrates the top five OTUs identified in the S13 soil samples, with the second medium-heavy metals concentration. The microbial community did not change significantly in S13 soil samples when the original samples were compared to the bioremediation experiment's samples. *Rhodospeudomonas* sp. demonstrated as the dominant species in the original, microbial control, and biostimulation samples. It was present as an abundant species in the samples obtained from the natural attenuation experiment but was not as abundant as in the other samples. *Zoogloea* sp., *Bdellovibrio* sp., and *Candidatus* sp. were also the dominant species next to *Rhodospeudomonas* sp., with 9.4%, 4.3%, 3.1%, 2.1%, and 1.8% percent reads, in S13 soil samples, inoculated with benzene and toluene and degraded with natural attenuation. It appears that *Rhodospeudomonas* sp. is becoming the dominant species when the heavy metals concentration starts to increase in the benzene and toluene co-contaminated soils.

Four phyla (*Elusimicrobia, Armatimonadetes, Fungi* and *Fusobacteria*) disappeared (*Figure 30*) from the original S13 soil sample when a mixture of benzene (876.39 mg/kg wet soil) and toluene (869.80 mg/kg wet soil) stock solution was introduced and degraded using two different bioremediation techniques (biostimulation and natural attenuation). Three new phyla (*Discosea*, *Spirochaetes* and *Oomycota*) appeared when benzene and toluene were introduced and degraded and not present in the original S13 soil sample. Their counts, however, were not significant (between 1 and 3).

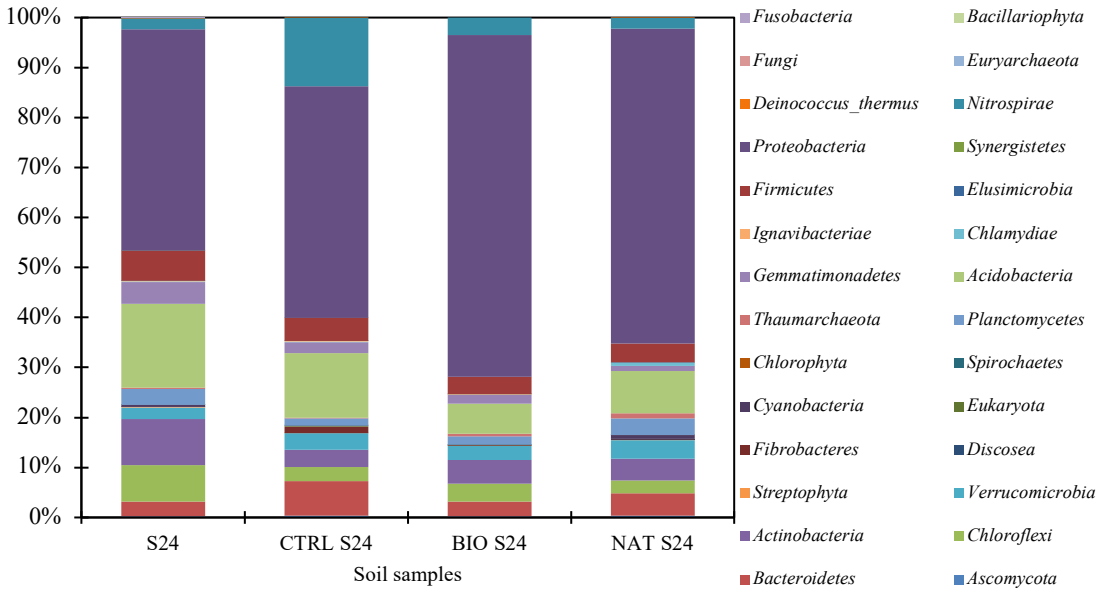

Figure 31 All phyla found in soil sample S24, which indicates the second high heavy metals' concentration before the highest. [39]

Table 32 The top five OTUs identified in the S24 (the second highest heavy metals' concentration) soil samples, which had the highest percent reads based on the DNA sequence result. [40]

Soil sample S24	O.T.U. number	Taxonomy identification	Percent reads
Original (S24)	A52	*Acidobacterium* sp.	3.1
	A6	*Nitrosospira* sp.	2.5
	A8	*Dongia* sp.	2.2
	A14	*Thermaerobacter* sp.	1.7
	A82	*Clostridium* sp.	1.4
Microbial control (CTRL S24)	B14	*Nitrospira* sp.	3.4
	B15	*Nitrosospira* sp.	3.1
	B3	*Rhodopseudomonas* sp.	3.1
	B4	*Rhodopseudomonas* sp.	2.7
	B12	*Nitrospira* sp.	2.7
Biostimulation (BIO S24)	B1	*Pseudomonas taiwanensis* str. bf_s2 eu857417.1	7.7
	B21	*Dechloromonas* spp. cu466895.1	6.8
	B5	*Cupriavidus* sp.	5.4
	B8	*Cupriavidus taiwanensis* str. ngr193a dq665823.1	4.5
	B2	*Zoogloea* sp.	4.4
Natural attenuation (NAT S24)	B5	*Cupriavidus* sp.	6.5
	B4	*Rhodopseudomonas* sp.	5.8
	B3	*Rhodopseudomonas* sp.	4.6
	B34	*Dechloromonas aromatica* str. rcb ay032610.1	3.9
	B15	*Nitrosospira* sp.	3.7

[39] S24 is the original soil sample collected from Tar Creek Superfund site, Ottawa County, Oklahoma. CTRL indicates the microbial controls for the bioremediation experiment, which only had the heavy metals and mixed with the aerobic mineral media. BIO indicates biostimulation, and NAT indicates natural attenuation. S24 results are from the DNA sequence before the bioremediation experiment was carried out. CTRL S24, BIO S24, and NAT S24 demonstrate the results from the DNA sequence after the complete degradation of benzene and toluene occurred in the soil samples.

[40] The original sample indicates the soil sample collected from the field and only had heavy metals concentration. A indicates the first DNA sequence before the bioremediation experiment. B indicates the second sequence after the bioremediation experiment was completed.

Table 32 demonstrates the top five OTUs identified in the S24 soil samples, with second highest heavy metals concentration. The microbial community changed even when only mineral aerobic medium (microbial control) was added to the S24 original sample. *Nitrospira* sp., *Nitrosospira* sp. and *Rhodospeudomonas* sp. became the dominant species in the microbial controls with 3.4%, 3.1%, and 2.7% reads. The original S24 soil sample had *Acidobacterium* sp., *Nitrosospira* sp., *Dongia* sp., *Thermaerobacter* sp. and *Clostridium* sp. as the dominant species with 3.1%, 2.2%, 2.1%, 1.7%, and 1.4% reads, respectively.

The S24 soil samples inoculated with benzene and toluene and degraded with biostimulation demonstrated high abundance in *Pseudomonas* sp., *Dechloromonas* sp., *Cupriavidus* sp. and *Zoogloea* sp. with 7.7%, 6.8%, 5.4%, 4.5%, and 4.4% reads, respectively. *Rhodopseudomonas* sp. was also the dominant species followed by, *Cupriavidus* sp., *Dechloromonas* sp., and *Nitrosospira* sp. with 6.5%, 5.8%, 4.6%, 3.9%, and 3.7% percent reads, in S24 soil samples, inoculated with benzene and toluene and degraded with natural attenuation.

Four phyla (*Euryarchaeota, Fungi, Bacillariophyta* and *Fusobacteria*) disappeared (*Figure 31*) from the original S24 soil sample when a mixture of benzene (876.39 mg/kg wet soil) and toluene (869.80 mg/kg wet soil) stock solution was introduced and degraded using two different bioremediation techniques (biostimulation and natural attenuation). Two new phyla (*Discosea* and *Eukaryota*) appeared when benzene and toluene were introduced and degraded and not present in the original S24 soil sample. Their counts were between 6 and 16.

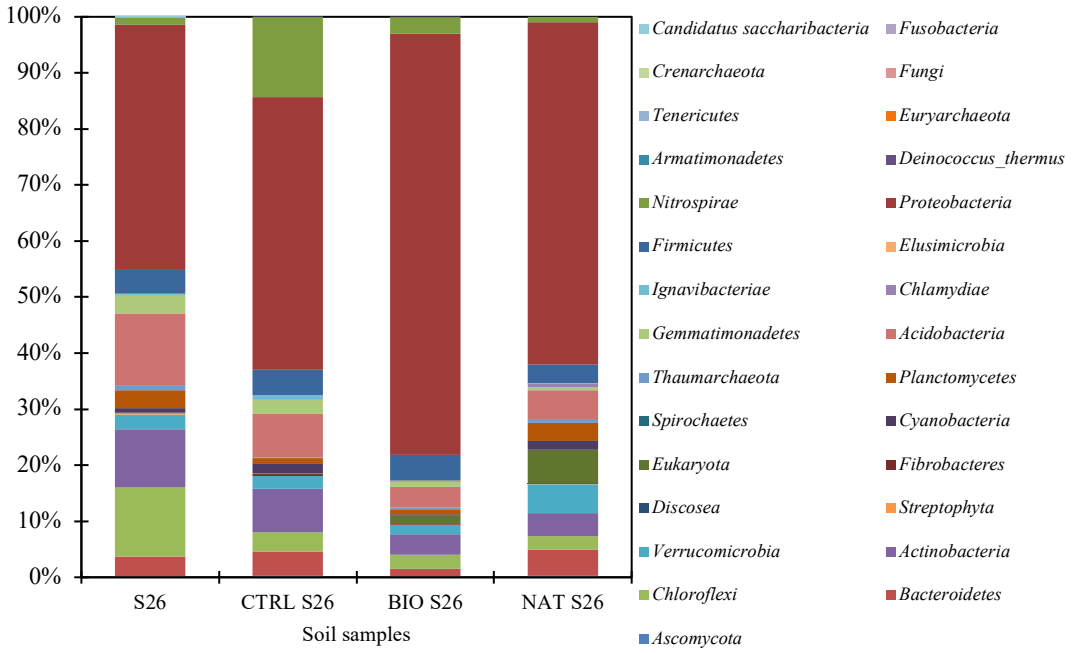

Figure 32 All phyla found in soil sample S26, which indicates the highest heavy metals' concentration. S26 is the original soil sample collected from Tar Creek Superfund site, Ottawa County, Oklahoma. [41]

Table 33 The top five OTUs identified in the S26 (the highest heavy metals' concentration) soil samples, which had the highest percent reads based on the DNA sequence result. [42]

Soil sample S26	O.T.U. number	Taxonomy identification	Percent reads
Original (S26)	A16	*Defluviicoccus* sp.	2.4
	A33	*Chloroflexus* sp.	1.9
	A1	*Rhodopseudomonas* sp.	1.5
	A10	*Rhodopseudomonas* sp.	1.5
	A8	*Dongia* sp.	1.3
Microbial control (CTRL S26)	B14	*Nitrospira* sp.	4.6
	B19	*Nitrosospira multiformis* str. atcc25196 ab070984.1	4.4
	B27	*Nitrospira* sp.	4.2
	B75	*Sphaerotilus* sp.	2.4
	B46	*Dechloromonas* spp. clone 12_orf27 dq376553.1	2.3
Biostimulation (BIO S26)	B1	*Pseudomonas taiwanensis* str. bf_s2 eu857417.1	22.7
	B2	*Zoogloea* sp.	10.0
	B8	*Cupriavidus taiwanensis* str. ngr193a dq665823.1	6.2
	B67	*Azonexus* sp. rv3	2.6
	B20	*Pseudomonas* sp.	2.5
Natural attenuation (NAT S26)	B23	*Sechloromonas* sp.	10.0
	B28	*Thoracosphaera heimii*	6.1
	B3	*Rhodopseudomonas* sp.	3.5
	B4	*Rhodopseudomonas* sp.	3.3
	B21	*Dechloromonas* spp. cu466895.1	2.9

[41] CTRL indicates the microbial controls for the bioremediation experiment, which only had the heavy metals and mixed with the aerobic mineral media. BIO indicates biostimulation, and NAT indicates natural attenuation. S26 results are from the DNA sequence before the bioremediation experiment was carried out. CTRL S26, BIO S26, and NAT S26 demonstrate the results from the DNA sequence after the complete degradation of benzene and toluene occurred in the soil samples.

[42] The original sample indicates the soil sample collected from the field and only had heavy metals concentration. A indicates the first DNA sequence before the bioremediation experiment. B indicates the second sequence after the bioremediation experiment was completed.

Table 33 demonstrates the top five OTUs identified in the S26 soil samples, with the highest heavy metals' concentration. The microbial community changed even when only mineral aerobic medium (microbial control) was added to the S26 original sample. *Nitrospira* sp. and *Nitrosospira* sp. became the dominant species in the microbial controls with 4.6%, 4.4%, and 4.2% reads. The original S26 soil sample had *Defluviicoccus* sp., *Chloroflexus* sp., *Rhodopseudomonas* sp., and *Dongia* sp., as the dominant species with 2.4%, 1.9%, 1.5%, and 1.3% reads, respectively.

The S26 soil samples inoculated with benzene and toluene and degraded with biostimulation demonstrated high abundance in *Pseudomonas* sp., *Zoogloea* sp., *Cupriavidus* sp., *Azonexus* sp., and *Pseudomonas* sp. with 22.7%, 10.0%, 6.2%, 2.6%, and 2.5% reads, respectively. *Rhodopseudomonas* sp. was also the dominant species followed by, *Sechloromonas* sp., *Thoracosphaer* sp., and *Dechloromonas* sp. with 10.0%, 6.1%, 3.5%, 3.3%, and 2.9% percent reads, in S26 soil samples, inoculated with benzene and toluene and degraded with natural attenuation.

Seven phyla (*Armatimonadetes*, *Euryarchaeota, Tenericutes, Fungi, Crenarchaeota, Fusobacteria* and *Candidatus saccharibacteria*) disappeared (*Figure 32*) from the original S26 soil sample when a mixture of benzene (876.39 mg/kg wet soil) and toluene (869.80 mg/kg wet soil) stock solution was introduced and degraded using two different bioremediation techniques (biostimulation and natural attenuation). Three new phyla (*Discosea*, *Eukaryota* and *Deinococcus thermus*) appeared when benzene and toluene were introduced and degraded and not present in the original S26 soil sample. Their counts were between 1 and 1433.

Taxonomy identification for the highest and lowest biodegradation rates

S26 soil sample with the highest heavy metals' concentration had the lowest biodegradation rate for benzene when biostimulation (2.60 $hour^{-1}$) and natural attenuation (2.02 $hour^{-1}$) was carried out. OTU1 and OTU20 *Pseudomonas* sp. demonstrated significant difference statistically (*P* values = 0.003 and 0.0002) between biostimulation and natural attenuation, followed

by OTU1013 *Zoogloea* sp. (*P*=0.03). OTU8, OTU342 and OTU5 *Cupriavidus* sp. were significantly different statistically (*P* values = 0.001 and 0.03), when the low and medium (soil samples S2, S4, S12, and S13) heavy metals' concentrations were compared to the high (soil samples S24 and S26) heavy metals' concentration.

OTU13 *Nitrosovibrio* sp. demonstrated significant difference statistically (*P*=0.01) between biostimulation and natural attenuation. OTU5, OTU8, and OTU342 *Cupriavidus* sp. were significantly different statistically (*P*-values = 0.03, 0.05 and 0.0004), when the low and medium (soil samples S2, S4, S12, and S13) heavy metals' concentrations were compared to the high (soil samples S24 and S26) heavy metals' concentration. OTU34 and OTU30 *Dechloromonas* sp. and OTU 61 *Sphingobacterium* (*P*=0.02) were also significantly different statistically (*P*-values = 0.006 and 0.04) when low and medium heavy metals' concentrations were compared to the high ones.

S13 soil sample with the second medium heavy metals' concentration had the highest biodegradation rate (6.03 $hour^{-1}$) for benzene when natural attenuation was used. S13 soil sample also demonstrated the highest degradation rate (3.70 $hour^{-1}$) for toluene when natural attenuation was carried out. OTU4 *Rhodopseudomonas* sp. demonstrated significant difference statistically (*P* values =0.02), when the low and medium (soil samples S2, S4, S12, and S13) heavy metals' concentrations were compared to the high (soil samples S24 and S26) heavy metals' concentration.

S12 soil sample with the medium heavy metals' concentration had the highest biodegradation rate (4.11 $hour^{-1}$) for toluene when biostimulation was carried out. OTU1 and OTU20 *Pseudomonas* sp. (*P* values = 0.003 and 0.0002), OTU13 *Nitrosovibrio* sp. (*P*= 0.01) demonstrated significant difference statistically between biostimulation and natural attenuation in S12 soil samples.

S4 soil sample with the second lowest heavy metals' concentration had the highest biodegradation rate (6.60 $hour^{-1}$) for benzene when biostimulation was carried out. OTU1 and OTU20 *Pseudomonas* sp. demonstrated significant difference statistically (*P* values = 0.003 and 0.0002) between biostimulation and natural attenuation in S4 soil samples. S2 soil sample with the

lowest heavy metals' concentration had the lowest degradation rate for the biostimulation (2.64 $hour^{-1}$) and natural attenuation (1.62 $hour^{-1}$) of toluene.

Conclusions

Heavy metals can prevent biodegradation of contaminants (Amor, Kennes, & Veiga, 2001; Sandrin & Maier, 2003), especially when they are present in high concentrations. This chapter focused on the bioremediation potential, on a laboratory scale, for the mixture of benzene (876.39 mg/kg) and toluene (869.80 mg/kg) in Cd, Pb, and Zn long-term impacted soils collected from Tar Creek Superfund site in Ottawa county, northeastern Oklahoma. The concentration of Cd ranged between 7.9 and 341.8 mg/kg, Pb between 187.5 and 8,771.9 mg/kg, and Zn between 860 and 79,341.3 mg/kg in the collected Superfund's soils. The presence of long-term and high levels of heavy metals did not prevent the biodegradation of the mixture of benzene (876.39 mg/kg) and toluene (869.80 mg/kg) when biostimulation or natural attenuation techniques were utilized in this study.

Six composite soil samples (S2, S4, S12, S13, S24, and S26) were selected for the bioremediation experiment from different locations in Picher, Ottawa County, Oklahoma (*Figure 34* found the in Appendices). The six selected soil samples edaphic characteristics varied as follows: moisture content was between 18.97% and 44.79%, pH was between 6.9 and 8.6, Cd concentration was between 7.9 mg/kg and 341.8 mg/kg, Pb concentration was between 187.5 mg/kg and 4,316.4 mg/kg, and Zn concentration was between 1,177 mg/kg and 79,341 mg/kg. There was no significant difference statistically (P-value<0.05) between the spatial distribution of the overall major elements between the six experimental soils.

The lag phase was not observed during the bioremediation experiment. Benzene degraded entirely within 130 hours using biostimulation and within 216 hours using the natural attenuation technique. Toluene degraded entirely within 154 hours utilizing biostimulation and within 178 hours utilizing the natural attenuation technique. The presence of toluene did not prevent or limit

the biodegradation of benzene in this dissertation like it was concluded in other studies (Oh et al., 1994; Chang et al., 1992; Ekperusi & Aigbodion, 2015).

The biostimulation of benzene was not linear for S12, S13, S24, and S26 soil samples. The biostimulation of benzene became linear in S12 and S13 soil samples after 48 hours, and after 50 hours in S24 and S26 soil samples. The degradation rates and time differed statistically in these samples before and after 48 and 50 hours. The degradation time significantly changed (P=0.02) after 48 hours in S13 soil sample, and after 50 hours in S26 soil sample. The degradation rate significantly changed (P=0.0003) after 50 hours in S24 soil sample. The biostimulation of toluene was linear, as well as the natural attenuation of benzene and toluene. The biodegradation rate and time was significantly different statistically between biostimulation and natural attenuation in S2 (P=0.00006 for rate and P=0.0004 for time), S24 (P=0.01 for rate and time), and S26 (P=0.03 for rate and P=0.02 for time) soil samples. The biodegradation rate was significantly different statistically (P=0.0001) for benzene between biostimulation and natural attenuation in S12 soil sample. The biodegradation time was significantly different statistically for toluene between biostimulation and natural attenuation in S2 (P=0.02) and S12 (P=0.00001) soil samples. The biodegradation time was significantly different statistically (P=0.002) for benzene between biostimulation and natural attenuation in S13 soil sample.

S4 soil sample with the second lowest heavy metals concentration had the highest biodegradation rate (6.60 $hour^{-1}$) for benzene when biostimulation was carried out. S26 soil sample with the highest heavy metals concentration had the lowest biodegradation rate (2.60 $hour^{-1}$) for benzene when biostimulation was carried out. S13 soil sample with the second medium-heavy metals concentration had the highest biodegradation rate (6.03 $hour^{-1}$) for benzene when natural attenuation was carried out. S26 soil sample with the highest heavy metals concentration had the lowest biodegradation rate (2.02 $hour^{-1}$) for benzene when natural attenuation was carried out.

S12 soil sample with the medium-heavy metals concentration had the highest biodegradation rate (4.11 $hour^{-1}$) for toluene when biostimulation was carried out. S2 soil sample

with the lowest-heavy metals' concentration had the lowest biodegradation rate (2.64 $hour^{-1}$) for toluene when biostimulation was carried out. S13 soil sample with the second medium-heavy metals' concentration had the highest biodegradation rate (3.70 $hour^{-1}$) for toleune when natural attenuation was carried out. S2 soil sample with the lowest-heavy metals' concentration had the lowest biodegradation rate (1.62 $hour^{-1}$) for toluene when natural attenuation was carried out.

The microbial community significantly changed when the original six soil samples from Tar Creek Superfund site, Ottawa County, Oklahoma, was mixed with aerobic mineral medium (microbial control and biostimulation) and inoculated with the mixture of benzene (876.39 mg/kg) and toluene (869.80 mg/kg) (biostimulation and natural attenuation). The top five OTUs in the highest abundance were identified in each six soil samples (S2, S4, S12, S14, S24, S26) and demonstrated in *Tables 28 and 32*. OTU8 and OTU5 *Cupriavidus* sp. (*P* values = 0.001 and 0.03) and OTU4 *Rhodopseudomonas* sp. (*P* values =0.02) were significantly different statistically when the low and medium (S2, S4, S12, and S13) heavy metals' concentrations were compared to the high (S24 and S26) heavy metals' concentration. OTU34 and OTU30 *Dechloromonas* sp. were also significantly different statistically (*P*-values = 0.006 and 0.04) when low and medium-heavy metals' concentrations were compared to the high ones. OTU1 and OTU20 *Pseudomonas* sp. (*P* values = 0.003 and 0.0002), OTU13 *Nitrosovibrio* sp. (*P*= 0.0108), and OTU1013 *Zoogloea* sp. (*P*=0.03) demonstrated a significant difference statistically between biostimulation and natural attenuation.

Several hydrocarbon-degrading bacteria were identified in other studies (Khudur et al., 2018; Farkas et al., 2014; Yoshikawa et al., 2017; Suenaga, Watanabe, Sato, Ngadiman, & Furukawa, 2002; Verma & Kuila, 2019; Hemmat-Jou, Safari-Sinegani, Mirzaei-Asl, & Tahmourespour, 2018) co-contaminated soils, including *Rhodococcus* spp., *Pseudomonas* spp., *Zoogloea* sp., *Acidobacterium* sp., and *Nitrosovibrio* sp. The diversity of the bacterial communities differed regardless of the presence of the co-contaminants, just like in the study of Khudur et al. (2018). *Rhodospeudomonas* sp., *Zoogloea* sp., *Cupriavidus* sp., *Nitrosospira* sp., *Dechloromonas*

sp.*, Nitrosovibrio* sp., and *Nitrospira* sp. might be good indicators for the presence of hydrocarbon and heavy metals co-contaminants in soils. They can degrade benzene and toluene even when heavy metals are present in high concentrations, above the EPA limitations for soils (Beyer, Hensler, and Moore, 1987; EPA, 2000; ATSDR, 2011).

CHAPTER V

SUMMARY AND GENERAL CONCLUSIONS

Petroleum is built up of aromatic hydrocarbons, asphaltenes, and non-hydrocarbon compounds. Benzene and toluene are aromatic hydrocarbons (volatile organic carbons) and natural components of crude oil (Speight, 2014; Brezonik & Arnold, 2011). Petroleum has a natural metal content of 10% w/w. It is not an unusual event when petroleum contaminates soil together with heavy metals (Speight, 2014). Co-contamination refers to multiple contaminants in the same environment when soils are polluted with organic pollutants and heavy metals (Roane, Josephson, & Pepper, 2001). Petroleum, heavy metals, and their combination contaminate soil through human activates such as drilling, mining, transportation, and crude oil processing. Co-contamination causes global problems for humans and the environment (Ma, Li, Mao, Wang, & Wang, 2018).

Approximately 40% of hazardous waste sites are co-contaminated with organic pollutants and heavy metals (Sandrin & Maier, 2003). Aromatic hydrocarbons and mixed isomers are priority contaminants removed from many Superfund sites in the United States (Shim et al., 2005). The Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), also known as Superfund, was approved by Congress in 1980 in the United States. Superfund sites are contaminated areas caused by improper hazardous waste management. These sites include manufacturing facilities, processing plants, landfills, and mines. Thousands of Superfund sites exist in the United States (EPA, 2020).

Bioremediation is a commonly used technique for remediating petroleum and its products' pollution (Leal et al., 2017). The biodegradation of co-contaminants is limited because of the low bioavailability of pollutants, high concentration of heavy metals, insufficient nutrients, and microbial electron donors or acceptors (Ma et al., 2018). Biodegradation, microorganisms carry out, is a naturally occurring breakdown of organic materials and vital soil processes (Madigan, Martinko, Bender, Buckley, & Stahl, 2015; Brezonik & Arnold, 2011). Bioremediation techniques are underutilized by managers and environmental scientists (Elekwachi, Andersen, & Hodgman, 2014) due to the lack of information on the characteristics of the co-contaminated sites. Bioremediation is neither universally understood nor trusted by those who must approve its use. There is a disagreement around bioremediation's efficacy about what it does and how well it works due to its dependence on microorganisms. The full potential of bioremediation technologies cannot be realized because of the disagreement over their efficiency (National Research Council [NRC], 1993).

Understanding the long-term effect of high concentrations of Cd, Pb, and Zn on the biodegradation of benzene and toluene is needed to improve and utilize existing bioremediation practices for co-contaminants. Testing the limitations of bioremediation techniques for long-term co-contamination and utilizing co-contaminants' bioremediation potential is required to improve bioremediation strategy. Understanding the microbial structure, characteristics, diversity, and

behavior of co-contamination is important to help improve bioremediation technologies and design a successful and sustainable bioremediation strategy. The present dissertation identifies native soil aerobic degraders of benzene and toluene in the presence of various concentrations of heavy metals. It analyzes the microbial community structure and diversity of co-contaminants and demonstrates bioremediation potential for them.

The second chapter (Chapter II) introduces other studies done on the biodegradation of benzene and toluene. The chapter also discusses the socioeconomic perspective of the studied co-contaminants (benzene, toluene, Cd, Pb, and Zn). The findings indicated that there is short- and long-term adverse health effects of benzene, toluene, Cd, Pb, and Zn to the human and animal body. Long-term high exposure to high concentrations of benzene, Cd and Pb has carcinogenic effects toluene causes altered color vision, and Zn causes mental disturbances (Chambers et al., 2018; Sauer et al., 2018; Zarth et al., 2014; Lovreglio et al., 2020; Debarba et al., 2020; D'Andrea & Reddy, 2013; Filley et al., 2004; Flowers, 2005; Choudhury et al., 2005). Bioremediation technologies have been utilized in the U.S. since the mid-1980. Implementing different bioremediation techniques was not typical in the mid-1980s until their usage started to rise in 1989. Eight to 12 bioremediation techniques have been applied on Superfund sites since then. *In-situ* bioremediation projects cost less than $40/cubic yard based on case studies from 1998. Most sites with applied *ex-situ* bioremediation projects cost less than $300/cubic yard in 1998. The unit costs for soil remediation cost more than soil bioremediation in 1998. Thermal desorption (*in-* and *ex-situ*) had an average cost of $256/cubic yard. Soil vapor extraction (*in-situ*) costs an average of $300/cubic yard. On-site incineration cost $628/cubic yard before 1998 (EPA, 2001).

The first objective of this dissertation (Chapter III) was to identify native soil aerobic degraders of individual benzene (876.39 mg/kg) and toluene (869.80 mg/kg) in the presence of various concentrations (5.1 mg/kg; 51 mg/kg; 510 mg/kg; and 5,100 mg/kg) of Cd and Pb. The chapter analyzes the microbial community structure and diversity in aerobic microcosms using several molecular biology techniques. The presence of co-contaminants in the soil modifies the

structure of microbial communities and leads to unique bacterial communities. Seven thousand one hundred sixty-five different operational taxonomic units (OTUs) were identified in the microcosms, with an average of 3,163 OTUs in the benzene samples and 2,586 OTUs in the toluene samples. Individual benzene or toluene without heavy metal co-contaminants (control samples) displayed similar bacterial community distribution. The bacterial community distribution was significantly different statistically (P= 0.003 for 51 mg/kg and P=0.03 for 5,100 mg/kg concentration of Cd) when Cd was present, depending on the Cd concentration.

The difference in Pb co-contaminated samples (with benzene or toluene) was not statistically different, unlike in the Cd co-contaminated samples. A total of 18 OTUs were distinguished that are unique for co-contaminants' type and concentration. Nine of the 18 unique bacteria presented high 16S rRNA genes/g ($<10^4$ 16S rRNA genes/g), which is an indicator for a viable bacterial population (Koshlaf et al., 2019) for an efficient bioremediation process in co-contaminated sites. Benzene samples had six OTUs (OTU 3504 *Lysobacter* sp.*,* OTU 1 *Pseudomonas* sp.*,* OTU 6205 *Pseudomonas putida*, *Rhodococcus* group, OTU 6151 *Caulobacter daechungensis*, OTU 89 *Mesorhizobium* sp.). The six OTUs demonstrated high ($>10^4$) 16S rRNA genes/g in wet soil and were significantly different statistically (P=0.01) from the toluene samples. Toluene demonstrated one OTU (OTU 330 *Adhaeribacter* sp.) high ($>10^4$) 16S rRNA genes/g in wet soil and was significantly different statistically (P=0.01) from the benzene samples. OTU 36 *Flavobacterium limicola* and OTU 117 *Flavobacterium granulensis* were present in high ($>10^4$) 16S rRNA genes/g in wet soil in benzene and toluene samples co-contaminated with 5,100 mg/kg concentration of Pb.

The second objective of this dissertation (Chapter IV) was to determine the bioremediation potential for the mixture of benzene (876.39 mg/kg) and toluene (869.80 mg/kg) in soils long-term impacted with heavy metal contamination at the Tar Creek Superfund site in Ottawa county, Oklahoma. The third objective was to determine the *in-situ* microbial community composition of the Tar Creek Superfund soils and the community enriched on benzene and toluene from selected

soils. The bioremediation potential was studied on a laboratory scale in aerobic microcosms utilizing molecular biology and analytical methods. A total of 26 composite soil samples were collected from the topsoil to a depth of 6 inches. The concentration of Cd ranged between 7.9 and 341.8 mg/kg, Pb between 187.5 and 8,771.9 mg/kg, and Zn between 860 and 79,341.3 mg/kg in the collected Superfund's soils. Six different genetic types of soils (from the collected 26) with different heavy metals concentrations were chosen for the bioremediation experiment. The six soils (S2, S4, S12, S13, S24, and S26) were prepared in triplicates for each treatment: biostimulation, natural attenuation, and microbial controls. Two chosen soils were prepared in duplicate for the sterile controls (S2 and S26). The biostimulation bottles had the soil mixed with aerobic mineral medium and inoculated with a mixture of benzene (876.39 mg/kg) and toluene (869.80 mg/kg). The natural attenuation bottles had the soil mixed with deionized water and the mixture or benzene and toluene stock solution. The microbial controls were mixed with mineral medium only, without the inoculation of the benzene and toluene mixtures. The sterile controls were autoclaved three times and mixed with the aerobic mineral medium and the mixture of benzene and toluene.

The lag phase was not observed during the bioremediation experiment. Benzene degraded entirely within 130 hours using biostimulation and within 216 hours using the natural attenuation technique. Toluene degraded entirely within 154 hours utilizing biostimulation and within 178 hours utilizing the natural attenuation technique. The presence of toluene did not prevent or limit the biodegradation of benzene in this dissertation like it was concluded in other studies (Oh et al., 1994; Chang et al., 1992; Ekperusi & Aigbodion, 2015).

The biostimulation of benzene was not linear for S12, S13, S24, and S26 soil samples. The biostimulation of benzene became linear in S12 and S13 soil samples after 48 hours, and after 50 hours in S24 and S26 soil samples. The degradation rates and time differed statistically in these samples before and after 48 and 50 hours. The biostimulation of toluene was linear, as well as the natural attenuation of benzene and toluene. The biodegradation rate and time was significantly different statistically between biostimulation and natural attenuation in S2 (P=0.00006 for rate and

P=0.0004 for time), S24 (*P*=0.01 for rate and time), and S26 (*P*=0.03 for rate and *P*=0.02 for time) soil samples. The biodegradation rate was significantly different statistically (*P*=0.0001) for benzene between biostimulation and natural attenuation in S12 soil sample. The biodegradation time was significantly different statistically for toluene between biostimulation and natural attenuation in S2 (*P*=0.02) and S12 (*P*=0.00001) soil samples. The biodegradation time was significantly different statistically (*P*=0.002) for benzene between biostimulation and natural attenuation in S13 soil sample.

S4 soil sample with the second lowest heavy metals concentration had the highest biodegradation rate (6.60 $hour^{-1}$) for benzene when biostimulation was carried out. S26 soil sample with the highest heavy metals concentration had the lowest biodegradation rate (2.60 $hour^{-1}$) for benzene when biostimulation was carried out. S13 soil sample with the second medium-heavy metals concentration had the highest biodegradation rate (6.03 $hour^{-1}$) for benzene when natural attenuation was carried out. S26 soil sample with the highest heavy metals concentration had the lowest biodegradation rate (2.02 $hour^{-1}$) for benzene when natural attenuation was carried out.

S12 soil sample with the medium-heavy metals concentration had the highest biodegradation rate (4.11 $hour^{-1}$) for toluene when biostimulation was carried out. S2 soil sample with the lowest-heavy metals' concentration had the lowest biodegradation rate (2.64 $hour^{-1}$) for toluene when biostimulation was carried out. S13 soil sample with the second medium-heavy metals' concentration had the highest biodegradation rate (3.70 $hour^{-1}$) for toluene when natural attenuation was carried out. S2 soil sample with the lowest-heavy metals' concentration had the lowest biodegradation rate (1.62 $hour^{-1}$) for toluene when natural attenuation was carried out.

The microbial community significantly changed when the original six soil samples from Tar Creek Superfund site, Ottawa County, Oklahoma, was mixed with aerobic mineral medium (microbial control and biostimulation) and inoculated with the mixture of benzene (876.39 mg/kg) and toluene (869.80 mg/kg) (biostimulation and natural attenuation). OTU8 and OTU5 *Cupriavidus* sp. (*P* values = 0.001 and 0.03) and OTU4 *Rhodopseudomonas* sp. (*P* values =0.02)

were significantly different statistically when the low and medium (S2, S4, S12, and S13) heavy metals' concentrations were compared to the high (S24 and S26) heavy metals' concentration. OTU34 and OTU30 *Dechloromonas* sp. were also significantly different statistically (*P*-values = 0.006 and 0.04) when low and medium-heavy metals' concentrations were compared to the high ones. OTU1 and OTU20 *Pseudomonas* sp. (*P* values = 0.003 and 0.0002), OTU13 *Nitrosovibrio* sp. (*P*= 0.01), and OTU1013 *Zoogloea* sp. (*P*=0.03) demonstrated a significant difference statistically between biostimulation and natural attenuation. *Rhodospeudomonas* sp.*, Zoogloea* sp.*, Cupriavidus* sp.*, Nitrosospira* sp.*, Dechloromonas* sp., *Nitrosovibrio* sp., and *Nitrospira* sp. presented in high abundancy in the soils and demonstrated ability to degrade a mixture of benzene (876.39 mg/kg) and toluene (869.80 mg/kg) in the presence of long-term and highly contaminated soils with heavy metals.

The availability of contaminants for microbes is the key to bioremediation success. Important concepts associated with contaminants desorption rates are 1) the initial distribution of the solute concentration within the polluted region and 2) the length of time a site has been exposed to the contaminant source. Recently contaminated sites exhibit lower rates of desorption flux even in the latest stages of remediation. The contaminants aging (long-term exposure) should be considered when assessing the long-term effectiveness of remediation strategies. Diffusion from sequestered regions is often a limiting process for remediation strategies. One of the most challenging difficulties with remediation strategies is the awareness of the initial compounds' exact distribution within the sequestered region. The initial concentration of the contaminants is usually unknown as well. The aging of contaminants makes the pollution less available for remediation purposes or biological uptake. Remediation strategies are dependent on case, contamination time and type, and the physical and chemical characteristics of the polluted sites (Haws, Ball, & Bouwer, 2007).

The dissertation focused on identifying native aerobic soil degraders of benzene and toluene in the presence of various concentrations of Cd and Pb. It analyzed the microbial

community structure and diversity when benzene (876.39 mg/kg) and toluene (869.80 mg/kg) are co-contaminated with different concentrations of heavy metals. The dissertation conducted a bioremediation study on a laboratory scale to analyze and demonstrate bioremediation potential for soils exposed long-term to high heavy metal concentrations (7.9 to 341.8 mg/kg of Cd, 187.5 to 8,771.9 mg/kg of Pb, and 860 to 79,341.3 mg/kg of Zn). It evaluated the bioremediation potential for co-contaminated soils. It analyzed whether the biodegradation of benzene and toluene is limited due to the high concentrations and long-term contamination of heavy metals (Cd, Pb, and Zn). The results of this study were obtained within laboratory conditions, where the incubator's temperature (30°C), humidity (19%), and brightness were controlled with stable environmental conditions. The concentrations and length of exposure to the heavy metals found in the experimental soils were uncontrolled variables.

It might be possible to predict the unknown initial concentration of benzene and toluene at Superfund/polluted sites with the knowledge of key, unique bacterial communities that enrich the soil when co-contaminants are present in different concentrations. Research has rarely been subjected to inverse modeling (Cozarelli et al., 2002; Essaid et al., 2003; Yang, Samper, & Molinero, 2008) with microbial communities to predict the initial BTEX (benzene, toluene, ethylbenzene, and xylene) compounds concentration and distribution. Some studies (Cozarelli et al., 2002; Essaid et al., 2003) have been done on inverse modeling on BTEX dissolution and biodegradation. The studies predicting the unknown initial concentration of BTEX compounds through the degradation rates of BTEX compounds. Essaid et al. (2003) found that inverse modeling was successful only when a single dissolution coefficient rate was used for all BTEX compounds, and the oil spill was assumed as stationary. The historical dissolved BTEX concentration data were also insufficient. Yang, Samper, and Molinero (2008) tried inverse modeling that relies on the microbial reactive transport model, allowing simultaneous estimation of geochemical and microbial parameters. Yang, Samper, and Molinero's (2008) model results

indicated that both chemical and microbial parameters could be estimated accurately for error-free data.

Future studies could also focus on the correlation between soil characteristics (including genetic soil type, chemical, and physical properties) and microbial community diversity and the distribution of co-contaminated soil to better understand how co-contamination affects soil biodiversity. The data could support and improve inverse modeling of BTEX biodegradation, leading to improved bioremediation performance. Carrying out a field bioremediation experiment could also improve the accuracy of the bioremediation studies. Quantitative PCR analyses are suggested to be carried out on the following microbes: *Rhodospeudomonas* sp., *Zoogloea* sp., *Cupriavidus* sp., *Nitrosospira* sp., *Dechloromonas* sp., *Nitrosovibrio* sp., and *Nitrospira* sp. to study their relevance and uniqueness to S2, S4, S12, S13, S24 and S26 soils used in this study. Carrying out a phytoremediation study with legumes and non-legume plants on a laboratory and field scale is also recommended to study the correlation between native, metal-resistance hydrocarbon-degraders and nitrogen fixation and nitrification. Combining native plants and microbes that are metal-resistant and better adapted to local pedoclimatic conditions should be used for successful bioremediation strategies (Mohamad et al., 2017).

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APPENDICES

Appendix A: Bacterial community analysis for chapter three

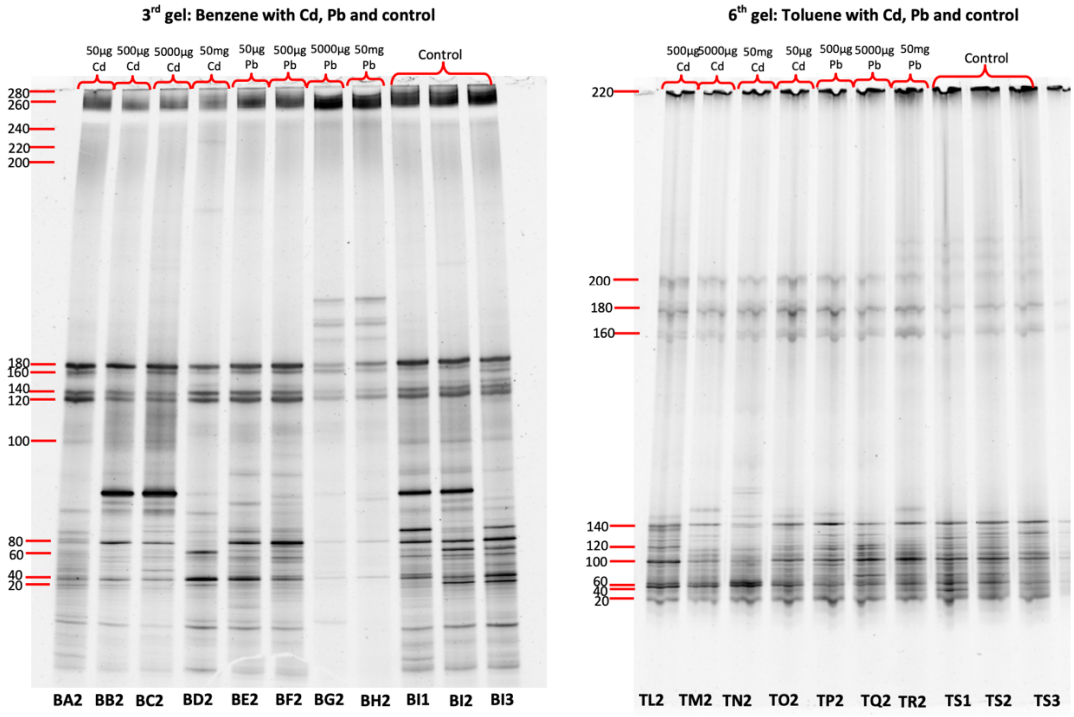

Figure 33 Bacterial DGGE profiles of 16S rRNA amplicons from aerobic mineral medium. [43]

[43] Benzene samples are shown on the left side, while toluene samples are shown on the right side. Upper labels indicate the studied heavy metals concentrations and the control. Lower labels indicate the abbreviations used for identifying the samples.

Table 34 The primers designed and used to target the selected phylogenetic groups.

qPCR Target*	Forward Primer	Reverse Primer	Amplicon Size (bp)
OTU 745	5'- TTAACCTGGGAACTGCGCTT -3'	5'- CGCATTTCACTGCCTACACGT-3'	265
OTU 176	5'- TTAACCTGGGAACTGCGCTT-3'	5'- CGCATTTCACTGCTACACCA-3'	242
OTU 2815	5'- ACCTGGGAATTGCGATGGAG-3'	5'- CGCATTTCACTGCTACCACG-3'	289
OTU 22, 147	5'- TCAACCTGGGAACTGCGTTT-3'	5'- CCATCGGTGTTCCTCCTGAT-3'	149
OTU 327	5'- AACCTGGGAACTGCATTGGT-3'	5'- GACTAGCGCCGGGTATCTAA-3'	253
OTU 1817, 6399	5'- GTAGCGGAATTCCCGGTGTA-3'	5'- GTCAGTGCTGGTCCAGGTAG-3'	289
OTU 121	5'- ATGGCATTGGATACTGGCGG-3'	5'- CGGATGTTCCTCCCGATCTC-3'	132
OTU 21	5'- AGTCTGATGTGAAAGCCCCG- 3'	5'- GAATTCCGCCACCCTCTACC-3'	429
OTU 3504	5'- TAGGCGGTTTTGTTAAGTCTGTC-3'	5'- CTCTAGCTCGCCAGTATTCCATT-3'	208
OTU 110	5'- GGGAACTGCATCCAAAACTACT-3'	5'- CCTCAGTGTCAGTATTAGTCCAGG-3'	128
OTU 1, 4554, 7128	5'- CGCGTAGGTGGTTGGTTAAG-3'	5'- TTCGCCACTGGTGTTCCTTC-3'	242
OTU 6872	5'- CGCGTTAGGTGGTTCGTTAAG-3'	5'- TTCGCCACTGGTGTTCCTTC-3'	82
OTU 6205	5'- GTTAAGTTGAATGTGAAAGCCCCG-3'	5'- TACACAGGAATTCCACCACCTCTAC-3'	135
OTU 5935	5'- ATCGGAATTACTGGGCGTAAG-3'	5'- GTTTTGAATGCAGTTCCCACGG-3'	498
OTU 6940	5'- CAACCTGGGAACTGCATTCAA-3'	5'- TTCGCACTGTGTTCCTTCCT-3'	427
OTU 409	5'- GTCGACTGGAGAGCAGTAGG-3'	5'- GCGTCTGAGCGTCAGATACAG-3'	253
OTU 6850	5'- GAAAGTCCGGGGCTCAACTCC-3'	5'- CTCAGCGTCAGTTAATGCCCAG-3'	289
OTU 2686	5'- AGAGTGCGGTAGGGGAGATT-3'	5'- GCCATTGGTGTTCCTCCTGA-3'	407

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Table 35 The primers designed and used to target the selected phylogenetic groups (Table 33 continued).

qPCR Target*	Forward Primer	Reverse Primer	Amplicon Size (bp)
OTU 9	5'- GTCGCGTCGTCTGTGAAAAC-3'	5'- GGAATTCCAGTCTCCCCTGC-3'	253
OTU 11	5'- GGCGGTTTACCAAGTCTGGA -3'	5'- TTTCGCCACTGGTGTTCCTC -3'	250
OTU 16, 5712	5'- GGCGGTTTTCTAAGTCTGGG -3'	5'- TTTCGCCACTGGTGTTCCTC -3'	106
OTU 4705	5'- GGCGGTTCCTTAAGTTTGGA -3'	5'- TTTCGCCACTGGTGTTCCTC -3'	85
OTU 5793	5'- GGCGGTTCCTTAAGTTTGGG -3'	5'- TTTCGCCACTGGTGTTCCTC -3'	226
OTU 6481, 6853	5'- GTCTGATGTGAAAGCCCACG -3'	5'- TCTCTACGCATTTCACCGCT -3'	260
OTU 254	5'- AGGTGGTGAGGTAAGTCGGA -3'	5'- TGCCTCAGTGTCAGGTGTTG -3'	181
OTU 141	5'- CAACTCAGAGTCTGCAACGG -3'	5'- AGTTATGGTCCAGTGAGCCG -3'	245
OTU 74	5'- GAAACTGCGTCTGAAACTACAGGT -3'	5'- GGATTTTACCCCTACATGCGAA -3'	253
OTU 147	5'- AGGGCTCAACCCTGGAATTG -3'	5'- CACTCGGAGTTCCACTCACC -3'	289
OTU 6151	5'- TTAGTCAGAAGGCTGAAAGCCC -3'	5'- TACACTCGGAGTTCCACACA -3'	1,350
OTU 89	5'- CGCACGTAGGCGGATACTTA -3'	5'- TCCGGACTCGAGATACCCAG -3'	253
OTU 330	5'- TGCCATTGATACTGACGGGC -3'	5'- CTCAGCGTCAGTTACAGCCT -3'	113
OTU 3353	5'- TGCCATTGATACTGACGGCT -3'	5'- CTCAGCGTCAGTTACAGCCT -3'	128
OTU 36	5'- GGTCCGTAGGCGGTCAGATA -3'	5'- GTATCAATGGCCGTTCCACC -3'	60
OTU 117	5'- GGTCCGTAGGCGGTTTAGTA -3'	5'- GTATCAATGGCCGTTCCACC -3'	175
OTU 123	5'- GGTCGCTCAACGATCAAACG -3'	5'- CGTCCATCAGCGTCAATCCA -3'	96
OTU 3740	5'- CGGAAACTGCCATTGATACTATAGA -3'	5'- TGCCTCAGTGTCAATCGATCC -3'	245
OTU 37	5'- GGTGCGTAGGCGGATAAGTAA -3'	5'- CCTCAGTGTCAATCGATCC -3'	94
OTU 7	5'- AGGTTTGCGGAATGGGTCAT -3'	5'- TTTCGAGCCTCAGCGTCAAT -3'	273
OTU 90	5'- AAGTGGGCGGAATGTGTCAT -3'	5'- TAGTGAGCTGCCTACGCAAT -3'	253
OTU 61	5'- AGTCAGAGGTGAAAGCCGGT -3'	5'- GTAAGCTGCCTTCGCAATCG -3'	250
OTU 5367	5'- GGTGCGTAGGCGGCTTATTA -3'	5'- CAGTATCAAGGGCACTGCGA -3'	253
OTU 391	5'- CTCAACCTCGGAACTGCCTT-3'	5'- GCCACCGGTGTTCTTCCTAA-3'	109
OTU 3067, 325, 72, 5847	5'- GCATACGACCTGAGGGTGAAA -3'	5'- TGTGGCTGATCGTCCTCTCA -3'	132
OTU 161	5'- CCCTGGAGTGGGGGATAACT -3'	5'- AGCATGAGGTCTTGCGATCC -3'	87
OTU 1695	5'- TGAGGGGGAAAGTAGGGGAT -3'	5'- TTACGGCGGCGGCTGG -3'	345
OTU 31, 356	5'- GAAACTGCCGGTGACAAACC-3'	5'- CTTCTGGTGGAACCCACTCC-3'	282

*The full list of the qPCR's gene targets in details were as follows:

OTU 745 – *Azoarcus tolulyticus* str. 2fb2 (*Azoarcus* subgroup 1)

OTU 176 – *Sphingomonas* sp. zh0 (*Azoarcus* subgroup 2)

OTU 2815 – *Massilia* group

OTU 22 and 147 – *Thauera aromatica* str. pn_1 and Uncultured *Caulobacter* sp. (*Thauera* like group)

OTU 327 – *Burkholdera cepacia* (*Burkholdera* group)

OTU 1817 and 6399 – *Lysobacter spongicola* str. kmm 329 and *Lysobacter spongicola* str. kmm 329 (*Lysobact*er like group)

OTU 121 – *Xanthomonas* sp. ly3

OTU 21 - *Lysobacter* sp. c1802

OTU 3504 – Uncultured *Lysobacter* sp.

OTU 110 – *Pseudomonas* sp. chol7

OTU 1, 4554, and 7128 – *Pseudomonas* sp. tgr13

OTU 6872 – *Pseudomonas putida* str. atcc 17472

OTU 6205 – *Pseudomonas putida* str. 7

OTU 5935 - *Pseudomonas* sp. ii_43

OTU 6940 - Uncultured *Pseudomonas* sp.

OTU 409 – Uncultured *Longilinea* sp.

OTU 6850 – *Arthrobacter* sp. kfc_75 (*Arthrobacter* group)

OTU 2686 – *Cryobacterium* group

OTU 9 – *Rhodococcus* group

OTU 11 – Uncultured *Bacillus* sp.

OTU 16, and 5712 - Uncultured *Bacillus* sp.

OTU 4705 – Uncultured *Bacillus* sp.

OTU 5793 - Uncultured *Bacillus* sp.

OTU 6481 and 6853– *Bacillus Sporo* group

OTU 254 – Uncultured *Pedosphaera sp*.

OTU 141 – Uncultured *Nitrosovibrio* sp.

OTU 74 – Uncultured *Bdellovibrio* sp.

OTU 147 – Uncultured *Caulobacter* sp.

OTU 6151 – *Caulobacter daechungensis*

OTU 89 – Uncultured *Mesorhizobium* sp.

OTU 330 – Uncultured *Adhaeribacter* sp.

OTU 3353 – Uncultured *Cytophaga* sp.

OTU 36 – *Flavobacterium limicola* str. nbrc 103156

OTU 117 – *Flavobacterium granulensis* str. kw05

OTU 123 – *Flavobacterium* sp. hme7816

OTU 3740 – *Ferruginibacter* sp. ds48_6_4

OTU 37 – *Ferruginibacter* sp. wf24

OTU 7 – Uncultured *Chitinophaga* sp.

OTU 90 – Uncultured *Fluviicola* sp.

OTU 61 – Uncultured *Pedobacter* sp.

OTU 5367 – *Pedobacter* group

OTU 391 – Uncultured *Hyphomicrobium* sp.

OTU 3067, 325, 72, and 5847 – *Cupriavidus* group

OTU 161 – *Noviherbaspirillum* group

OTU 1695 – *Methylophilus* group

OTU 31 and 356 – *Nitrosospira* group

Table 36 Parameters of the qPCR assays for each target group. [44]

qPCR target	Percent Efficiency	R^2	Linear Range Log (copies per µL)
OTU 745	96.98	0.98	1.28-2.83
OTU 176	107.42	0.99	0.01-2.14
OTU 2815	55.54	0.96	0.35-3.60
OTU 22, 147	99.13	0.99	0.001-3.05
OTU 327	99.26	0.99	0.07-0.65
OTU 1817, 6399	102.99	0.99	0.36-3.48
OTU 121	97.35	0.99	0.59-2.61
OTU 21	102.49	0.99	0.13-3.25
OTU 3504	88.95	0.99	1.12-5.85
OTU 110	97.45	0.99	0.48-4.06
OTU 1, 4554, 7128	101.57	0.99	0.03-3.50
OTU 6872	96.91	0.99	0.06-2.31
OTU 6205	96.05	0.99	0.23-5.35
OTU 5935	b.d.	b.d.	b.d.
OTU 6940	80.25	0.98	1.00-4.91
OTU 409	b.d.	b.d.	b.d.
OTU 6850	98.91	0.99	0.67-2.96
OTU 2686	b.d.	b.d.	b.d.
OTU 9	72.55	0.99	2.23-5.25
OTU 11	79.93	0.99	0.01-3.69
OTU 16, 5712	90.01	0.99	0.04-3.35
OTU 4705	88.72	0.99	0.25-3.73
OTU 5793	87.66	0.99	0.29-3.38
OTU 6481, 6853	81.33	0.99	0.53-3.19
OTU 254	72.81	0.99	0.68-3.31
OTU 141	99.56	0.99	0.08-2.53
OTU 74	b.d.	b.d.	b.d.
OTU 147	88.86	0.99	0.18-2.93
OTU 6151	89.41	0.99	1.32-5.99
OTU 89	70.69	0.98	1.67-3.98
OTU 330	93.50	0.98	0.09-4.09
OTU 3353	79.75	0.97	0.45-4.59
OTU 36	44.16	0.98	0.03-4.97
OTU 117	82.48	0.97	0.60-4.21
OTU 123	85.07	0.98	1.09-5.76

[44] Percent efficiency of qPCR reaction based on the standard curve, R^2 value of linear regression of standard curve, and linear range are shown in the table. The lowest level of the linear range is the quantification limit for that assay. b.d. indicates for below detection.

Table 37 Parameters of the qPCR assays for each target group (*Table 35* continued). [45]

qPCR target	Percent Efficiency	R^2	Linear Range Log (copies per µL)
OTU 3740	84.67	0.99	0.03-3.27
OTU 37	b.d.	b.d.	b.d.
OTU 7	81.64	0.97	0.40-3.52
OTU 90	84.77	0.97	1.76-4.69
OTU 61	109.56	0.96	0.92-5.91
OTU 5367	93.30	0.99	0.08-2.56
OTU 391	104.60	0.99	0.03-2.00
OTU 3067, 325, 72, 5847	b.d.	b.d.	b.d.
OTU 161	104.40	0.99	0.03-2.71
OTU 1695	b.d.	b.d.	b.d.
OTU 31, 356	b.d.	b.d.	b.d.

[45] Percent efficiency of qPCR reaction based on the standard curve, R^2 value of linear regression of standard curve, and linear range are shown in the table. The lowest level of the linear range is the quantification limit for that assay. b.d. indicates for below detection.

Appendix B: Soil chemical and physical properties for chapter four

Figure 34 Map of the 26 collection sites in Tar Creek Superfund site, Picher, Ottawa county, northeastern Oklahoma. [46]

[46] A total of 26 composite samples (following EPA-230-R-95-005 Guidelines, 1998) were collected on 11th September 2020. The highlighted sample locations (S2, S4, S12, S13, S24 and S26) indicate for the six samples, utilized in the bioremediation experiment.

Table 38 Description of the sampling sites and weather condition on the sampling date. [47]

Site code	Sampling date	City in OK	Weather condition	Coordinates	Altitude (m)	Site description
S11	09/11/2020	Cardin/Picher	Cloudy/wet	N36°57'15.08" W94°49'51.34"	243	Active gravel mine nearby
S26	09/11/2020	Cardin/Picher	Cloudy/wet	N36°57'29.05" W94°49'52.49"	241	Active gravel mine nearby
S19	09/11/2020	Cardin/Picher	Cloudy/wet	N36°57'39.56" W94°49'50.52"	242	Active gravel mine nearby
S8	09/11/2020	Cardin/Picher	Cloudy/wet	N36°57'55.12" W94°49'51.35"	239	Active gravel mine nearby
S7	09/11/2020	Cardin/Picher	Cloudy/wet	N36°58'0.371" W94°49'57.94"	240	Active gravel mine nearby
S5	09/11/2020	Cardin/Picher	Cloudy/wet	N36°58'11.09" W94°49'51.60"	244	Active gravel mine nearby
S17	09/11/2020	Cardin/Picher	Cloudy/wet	N36°58'21.65" W94°49'50.16"	253	Active gravel mine nearby
S21	09/11/2020	Cardin/Picher	Cloudy/wet	N36°58'21.11" W94°49'53.18"	244	Active gravel mine nearby
S2	09/11/2020	Cardin/Picher	Cloudy/wet	N36°58'38.82" W94°49'47.86"	246	Next to the chat pile
S20	09/11/2020	Cardin/Picher	Cloudy/wet	N36°58'46.96" W94°49'49.55"	247	Former residential area
S13	09/11/2020	Cardin/Picher	Cloudy/wet	N36°58'54.69" W94°49'49.76"	254	Former residential area
S1	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'4.631" W94°49'49.37"	251	Former residential area
S24	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.70" W94°50'1.391"	249	Former downtown
S22	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.74" W94°50'7.295"	251	Former downtown
S18	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'3.380" W94°50'22.74"	256	Former commercial area
S16	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.78" W94°50'45.56"	241	Next to a chat pile
S23	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.96" W94°50'56.51"	244	Former commercial area
S10	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.74" W94°51'6.408"	248	Next to a chat pile
S12	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.85" W94°51'22.57"	249	Former commercial area
S14	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.92" W94°51'44.42"	248	Former commercial area
S15	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.67" W94°52'6.996"	242	Former school nearby
S25	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.31" W94°52'28.09"	243	Former school nearby
S9	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.24" W94°52'41.12"	244	Next to a farm
S4	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.06" W94°53'3.732"	244	Next to a farm
S6	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'13.16" W94°53'48.61"	242	Next to a farm
S3	09/11/2020	Cardin/Picher	Cloudy/wet	N36°59'10.82" W94°56'4.127"	237	Next to a farm

[47] S indicates for site; the numbers indicate for the heavy metals' concentration in ascending order. Sampling sites in the Table are shown in collection order (from the first to the last).

Table 39 Characteristics of collected soils from Tar Creek Superfund site. [48]

Site code	Soil moisture content (%)	pH (at 20.5°C)	Heavy metal concentration (mg/kg)			DNA quantity (μg/g wet soil)	Soil description (texture and color)
			Cd	Pb	Zn		
S11	15.41	7	274.81	1,366.73	56,906.61	10,402.75	Black, with pea gravel
S26	**18.97**	**7**	**341.82**	**4,316.37**	**79,341.32**	**7,158.15**	**Black, gravel**
S19	22.23	7.3	279.67	2,845.33	52,772.37	6,429.02	Black, clayey
S8	33.38	7.3	52.27	793.89	6,508.88	25,817.13	Black, clayey
S7	31.71	7.3	79.60	555.29	6,887.51	13,471.43	Yellowish, clayey
S5	36.04	7.6	57.68	392.53	7,228.34	32,522.53	Rich black topsoil, yellowish under
S17	23.61	8.2	120.45	2,452.65	22,948.03	11,781.13	Brown, gravel
S21	26.06	7.4	238.53	3,452.98	40,663.33	13,794.46	Black, gravel
S2	**23.65**	**7.6**	**7.896**	**187.53**	**1,177.18**	**11,788.53**	**Brown, loam**
S20	19.67	8.2	90.17	3,415.23	20,098.28	9,461.00	Brown, gravel
S13	**25.45**	**8**	**60.58**	**1,051.25**	**10,775.86**	**16,633.54**	**Brown, gravel**
S1	22.77	7.9	109.65	136.00	860.00	11,653.41	Brown, clayey
S24	**44.79**	**7.7**	**335.78**	**8,431.37**	**2,630.00**	**19,563.241**	**Brown, clayey**
S22	25.11	7.6	219.98	5,712.20	40,106.95	24,567.81	Brown, clayey
S18	41.98	7.6	169.46	2,491.37	29,847.06	36,885.36	Black, gravel
S16	12.79	7.6	158.75	2,422.83	32,092.11	6,421.17	Brown, gravel
S23	26.67	7.5	317.11	6,071.78	47,935.10	18,274.13	Brown, gravel
S10	26.31	6.2	58.27	1,878.63	7,978.72	12,756.84	Brownish red, clayey
S12	**22.80**	**6.9**	**80.85**	**1,646.83**	**12,797.62**	**13,989.50**	**Brownish red, clayey**
S14	20.55	7.4	**18,257.46**	1,404.80	10,988.74	10,069.30	Brownish, clayey
S15	27.40	7.6	63.12	2,287.97	10,256.41	14,325.76	Brownish yellow, clayey
S25	32.68	7.6	86.99	8,771.93	15,034.11	8,021.55	Brown, sandy
S9	25.90	7.7	61.56	1,359.67	10,130.88	23,750.85	Brown, gravel
S4	**28.23**	**8.6**	**15.71**	**317.19**	**2,639.23**	**18,391.78**	**Brownish yellow, topsoil clayey, sandy gravel under**
S6	20.12	8.2	B.d.l.	461.72	6,650.72	10,765.96	Brownish red, clayey and gravel
S3	21.96	7.6	24.80	308.14	3,996.15	23,577.69	Brown farm soil

Table 40 Limitations for heavy metals' concentration in US soils (Beyer, Hensler, and Moore, 1987; EPA, 2000; ATSDR, 2011).

Cd (mg/kg)		Pb (mg/kg) (Urban environment)		Zn (mg/kg)	
Average in natural soils	0-0.3	play areas	400	US cultivated soils	<5-400 (average: 36)
		non-play	1,200	US non-cultivated soils	<10-2,000 (average:51)

[48] B.d.l. indicates for below detection limit. Highlighted rows were the selected soil samples for the bioremediation experiment. The highlighted red number is an outlier. S indicates for site; the numbers indicate for the heavy metals' concentration in ascending order. Sampling sites in the Table are shown in collection order (from the first to the last).

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