T.R.
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF SOIL SCIENCE AND PLANT NUTRITION

BIOREMEDIATION OF SOILS BY USING THE METAL-TOLERANT AND PAHS DEGRADING BACTERIA ISOLATED FROM THE LONG-TERM CHEMICALLY CONTAMINATED SOIL: METAGENOMICS ASSAY AND EXPERIMENTAL MODELING

Master's Thesis

Jawaria JOHAR

Supervisor
Assoc. Prof. Dr. Svetlana SUSHKOVA
II. Supervisor
Prof. Dr. Rıdvan KIZILKAYA

SAMSUN
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This thesis was supported by the European Union project of Erasmus Mundus Joint Master Degree in Soil Science (emiSS) with project number 610528-EPP-1-2019-1-TR-EPPKA1-JMD-MOB.

SAMSUN
2022

ACCEPTANCE AND APPROVAL OF THE THESIS

The study entitled **"BIOREMEDIATION OF SOILS BY USING THE METAL-TOLERANT AND PAHS DEGRADING BACTERIA ISOLATED FROM THE LONG-TERM CHEMICALLY CONTAMINATED SOIL: METAGENOMICS ASSAY AND EXPERIMENTAL MODELING"** prepared by **Jawaria JOHAR**, and supervised by **Assoc. Prof. Dr. Svetlana SUSHKOVA** and **Prof.Dr.Rıdvan KIZILKAYA**, was found successful and unanimously accepted by committee members as Master thesis, following the examination on the date 20.6.2022.

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Member	Prof. Dr. Orhan DENGIZ Ondokuz Mayıs University Department of Soil Science and Plant Nutrition		☒ Accept ☐ Reject

This thesis has been approved by the committee members that already stated above and determined by the Institute Executive Board.

APPROVAL
… / … / …
Prof. Dr. Ali BOLAT
Head of Institute of Graduate Studies

DECLARATION OF COMPLIANCE WITH SCIENTIFIC ETHIC

I hereby declare and undertake that I complied with scientific ethics and academic rules in all stages of my Master's Thesis , that I have referred to each quotation that I use directly or indirectly in the study and that the works I have used consist of those shown in the sources, that it was written in accordance with the institute writing guide and that the situations stated in the article 3, section 9 of the Regulation for TÜBİTAK Research and Publication Ethics Board were not violated.

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Yes ☐ (If it necessary, please add appendices.)

No ☒

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Jawaria JOHAR

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ÖZET

TOPRAKLARIN UZUN VADEDE KİMYASAL OLARAK BULAŞMIŞ TOPRAKTAN İZOL EDİLMİŞ METAL TOLERANSLI VE PAHS YAYICILI BAKTERİLERİ KULLANARAK BİYOREDİMASYONU: METAGENOMİKLERİN TEST VE DENEME MODELLEMESİ

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Toprak, ekosistemin temel ve gerekli bir bileşenidir. Organik ve inorganik kirleticilerle kirlenme, toprak mikrobiyal topluluk yapısını önemli ölçüde değiştirmektedir. Bu değişimler antropojenik baskıya işaret etmekte toprak ıslahı için yeni olasılıkların keşfedilmesine yardımcı olmaktadır. Bu araştırma, Atamanskoye Gölü'nden toplanan Spolic Technosol toprağından izole edilen mikrobiyal izolatların biyoremediasyon potansiyeli üzerinedir. *Pseudomonas putida* ve *Rhodococcus erythopolis* olarak adlandırılan spesifik iki bakteri izolatı, PAH indirgeyici ve metal toleranslı olarak seçilmiştir. Deneme modeli, kontrol uygulaması (Chernozem toprağı), kirli toprak uygulaması (Spolic technosols), Bakteriyel izolat uygulaması, Biochar uygulaması (ayçiçeği kabuğu) ve kombine bakteriyel izolat ve biochar uygualamasıbı içerecek şekilde 5 konuyu kapsamaktadır. Deneme, Arpa bitkisi (Hordeum vulgare) yetiştirilerek laboratuvar koşullarında bir ay süreyle yürütülmüştür. Uygulamaların toprak mikrobiyomu üzerindeki etkisini değerlendirmek için çeşitli parametreler kullanılmıştır. Bakteri ve biyokömürün birlikte uygulanmasında kökler ve sürgün uzunluğu önemli ölçüde artması gibi daha belirgin sonuçlar bulunmuştur. Toplam PAH'lar ve BaP, toprakta 5-6 kat azalırken, bitki kısımlarında da aynı eğilim gözlenmiştir. Aynı şekilde kombine uygulamalarda toplam ağır metal içeriği ve özellikle Cu miktarı azaltılmıştır. Bakteriyel uygulamada heterotrofik mikrobiyal sayı, kombine uygulamalarla karşılaştırıldığında iki kat daha fazla iken, kontrole göre kombine uygulamalarda toprak enzimatik aktivitesi %50 daha az iken, bakteriyel ve biochar uygulamalarında %25 daha az olduğu belirlenmiştir. 16s rRNA metagenomik çalışmaları, mantar topluluklarının başta Ascomycota, Chytridiomycota ve Basidiomycota olmak üzere kombine uygulamalarında daha yüksek olduğu, sadece Ascomycota , Mortierellomycota ve Monoblepharomycota filumları arasında taksonlara göre örneklerdeki OTU ile toprak özellikleri arasında yüksek bir korelasyon belirlenmiştir. Bakteri ve biyokömürün birlikte uygulanmasının PAH'ları ve ağır metalleri azaltmada etkili olduğu sonucuna varılabilir. Daha detaylı sonuçları elde etmek için toprak mikrobiyomunun daha fazla metagenomik çalışma yapılması önerilmektedir.

Anahtar Sözcükler: Poliaromatik hidrokarbonlar, Ağır metaller, Biyoremediasyon, Metagenimik, 16s rRNA

ABSTRACT

BIOREMEDIATION OF SOILS BY USING THE METAL-TOLERANT AND PAHS DEGRADING BACTERIA ISOLATED FROM THE LONG-TERM CHEMICALLY CONTAMINATED SOIL: METAGENOMICS ASSAY AND EXPERIMENTAL MODELING

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Soil is a basic yet essential component of the ecosystem. The contamination with organic and inorganic pollutants changes significantly soil microbial community structure. These shifts indicate anthropogenic pressure and help to discover new possibilities for soil remediation. Current research addresses the study of the bioremediation potential of microbial strains isolated from Spolic Technosols soil collected from lake Atamanskoye. Two specific bacterial strains i.e., *Pseudomonas putida* and *Rhodococcus erythopolis* were selected as PAH degrader and metal tolerant. Experiment model was designed with 5 treatments consisting of control variant (Chernozem soil), polluted soil variant (Spolic technosols), Bacterial strains variant, Biochar variant (sunflower husk) and combined bacterial and biochar variant. The experiment was based *Hordeum vulgare* and conducted for one month under laboratory conditions. Various parameters were measured in order to assess treatments ‘effect on soil microbiome. Prominent results were found in combined application of bacteria and biochar such as roots and shoot length were significantly increased. Total PAHs and BaP was reduced 5-6 times in soil while same trend was observed in plant parts. In the same way, total heavy metal content and especially Cu was reduced in combined variant. Heterotrophic microbial number were twice more in bacterial variant as compared to combined variant. Soil enzymatic activity was 50% less in combined variant as compared to control while 25% less in bacterial and biochar variant. 16s rRNA metagenomic studies revealed the fungal communities mainly *Ascomycota* , *Chytridiomycota* and *Basidiomycota* showed high percentage in combined variants. A high correlation was found between OTU in the samples by taxa and soil properties only among the phyla *Ascomycota* , *Mortierellomycota ,* and *Monoblepharomycota.* It can be concluded combined application of bacteria and biochar do has effect in mitigating PAHs and heavy metal. Further metagenomics study of soil microbiome is recommended to see proper results.

Keywords: Polyaromatic hydrocarbons, Heavy metals, Bioremediation, Metagenimics, 16s rRNA

ACKNOWLEDGEMENT

"All praises to Allah Almighty who is Lord of the Worlds, The Beneficent and the most Merciful."

Task can only be a task until it has been executed in a beautiful way and become an "art". Words are not enough to acknowledge the emotional support from my parents and family during the whole time of my degree. Without their prayers, I would be still struggling to accomplish my thesis is a good way.

I would like to express my deepest gratitude to the head of soil science research group, Prof. Dr. Tatiana Minkina, head of Soil science department and coordinator of EmisS program, Prof. Dr. Coşkun Gülser. I am truly grateful to my supervisors Dr. Svetlana Sushkova and Prof. Dr. Rıdvan Kizilkaya for their support throughout my research work and provide me with all necessary materials to complete my thesis. Besides this, I am very thankful to Dr. Yanina Delegan and Yulia Kocharovskaya who helped me in Bioinformatics while huge thanks to Dr. Andrey Gorovtsov and Konstantin Demin for helping me with microbiology laboratory work.

Big thanks to Dr. Mahmoud Mazarji for his professional guidance especially his instant responses to all my queries. Indeed it was a great pleasure to work with a visionary and progressive man.

I am deeply indebted to the European Union program, Erasmus Mundus Joint Master Degree (EMJMD) for providing funding to pursue my Masters' Degree in Erasmus Mundus Master in Soil Science.

I would be remiss in not mentioning Dr. Anatoly Barakhov for assisting me during my stay in Rostov-on-Don. Besides this, I am also very thankful to Tamara Dudnikova who was the person standing by my side and helping me with all necessary procedures throughout my research work.

Last but not least, I would like to say thanks to close friends and fellows for their time and support to be present in time of need.

Jawaria JOHAR

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SYMBOLS AND ABBREVIATIONS

BaP	: Benzo(a)pyrene
Bp	: Base pair
CFU	: Colony forming unit
CO_2	: Carbon dioxide
$CuSO_4$	: Copper sulphate
DHA	: Dehydrogenase activity
DNA	: Deoxyribose nucleotide acid
EPA	: Environmental Protection Agency
FAO	: Food and Agriculture Organization of the United Nations
Hm	: Heavy metals
HMW PAHs	: High molecular weight PAHs
ISO	: International Organization for standardization
LMW PAHs	: Low molecular weight PAHs
MBC	: Microbial biomass carbon
OTU	: Observed taxonomical units
qCO_2	: Metabolic quotient
PAHs	: Polycyclic aromatic hydrocarbons
PCR	: Polymerase chain reaction
RNA	: Ribose nucleotide acid
16s rRNA	: 16s ribosomal unit of ribose nucleotide acid
UR	: Urease activity

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1. INTRODUCTION

1.1. Soil degradation by toxic pollutants

Environmental contamination is one of the world's most significant problems, and soil is the most important component of our ecosystem. Soil is a nonrenewable resource as it cannot recover quickly from degradation and loss over the course of a human lifetime. Soil pollution is a huge concern to humanity, and it is becoming increasingly contaminated as a result of several anthropogenic causes. Poly aromatic hydrocarbons (PAHs) and heavy metals are two of the most persistent pollutants. (S. Sushkova et al., 2021; P. Zhang & Chen, 2017).

1.2. Occurrence of Polyaromatic hydrocarbons and heavy metals

Because of their persistent nature, toxicity, and carcinogenic qualities, PAHs are regarded a pervasive and hazardous contaminant. These pollutants are categorized according to their benzene ring structure, with low molecular weight (LMW) pollutants having 2 and 3 benzene rings and high molecular weight (HMW) pollutants having 4,5 and 6 benzene rings. (S. N. Sushkova et al., 2021) The US Environmental Protection Agency's (EPA) gave list of 16 priority PAHs, is often used to analyze these PAHs in contaminated soil locations. Only two PAHs, anthracene and phenanthrene, are the simplest, as both are made up of three fused benzene rings. Because of their prevalence in soil samples, the majority of research investigations have focused on simplest PAHs. (Abdel-Shafy & Mansour, 2016).

1.2.1. Effect of PAHs and HMs on living organisms

Heavy metal is second major pollutant in soil contamination. Both heavy metals and PAHs are notorious for their persistent nature and toxicity in soil as well as for plants. They are not only disturbing soil microbiome but also drinking water, hence a great hazardous for living organisms (Kadi, 2009; Rajput et al., 2021). Heavy metals including zinc (Zn), copper (Cu), cadmium (Cd), chromium (Cr), mercury (Hg), lead (Pb), and arsenic (As) are abundant in our agriculture and urban sectors. (Cristaldi et al., 2017; Kaur et al., 2019). Heavy metals don't itself a problem in soil because a specific required amount of them is need in soil for plant growth for example Zn, Cu but if it exceeds from threshold this enhance their bioavailibity soil for example Pb, Cd, Ni can cause diseases both for humans, animals and toxicity problems for sensible plants (Cristaldi et al., 2017). PAH and Heavy metal co-contamination is a big concern and present on soil sites where there is more in urban environment, tanneries factories and oil delivery work (Biswas et al., 2015).

1.4. Study of various polluted soil sites by studying soil microbes

The soil microbial community has recently caught the interest of the research community. (Jansson & Hofmockel, 2018). They are altering the soil microbiome in order to restore the disrupted ecosystem that has resulted from PAH and HM pollution. (Calderón et al., 2017). It has been seen that microorganism play an extremely important role in proper function of ecosystem. Soil bacteria provide a variety of functions, including nitrogen and carbon fixation, hydrocarbon degradation, and heavy metal bioavailability reduction. (Jansson & Hofmockel, 2018).

As a result, metagenomics is assisting researchers in investigating the soil microbial community by giving analytical and bioinformatic tools to understand the soil microbiome. Metagenomics allows for the functional evaluation and taxonomic identification of soil microbiota in a variety of polluted soils.

Many soil microbes have been investigated, and strains from polluted locations have been identified that are involved in degrading PAH and tolerating metal. The majority of bacterial genera are important participants, performing effectively in co-contaminated environments and degrading hydrocarbons in the presence of heavy metals (Czarny et al., 2020). Metagenomics was a substantial contribution, with analyses revealing microbial community performance in degrading PAHs and a shift in alpha and beta diversity profiles (Sazykina et al., 2021). In the same way functional metagenomics and bioinformatics reveal how bacterial strains are involved distantly and these bacterial phyla are interacted for hydrocarbons degrading pathways and favoring PAH mineralization(Zafra et al., 2016).

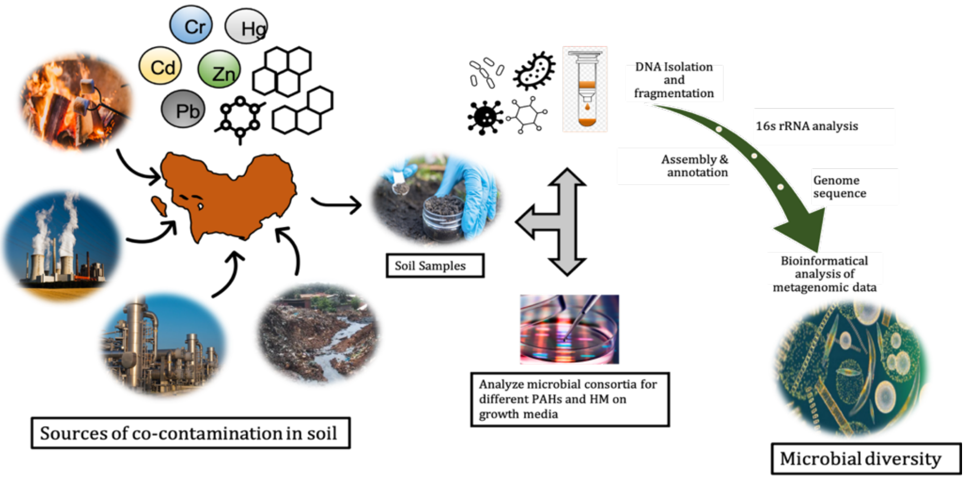

Figure 1. 1. Comprehensive cycle to show sources of contamination sources in soil, extraction of soil microbial DNA and study metagenomics data to see effect on soil microbial diversity.

The objective of this study is to analyze the soil properties during model experiment with combine application of the biochar, isolated metal-resistant and PAHs degrading microbial strains for the approbation of the developing bioremediation method. This work addresses the study of the bioremediation potential of microbial strains isolated from Spolic Technosols formed at the territory of a former industrial sludge reservoir near the Kamensk-Shakhtinsky (Southern Russia). Metagenomic studies were carried out which revealed the microbial community of the soil samples from contaminated sites.

2.LITERATURE REVIEW

2.1. Bioremediation approaches for restoration of contaminated soils

Because of concerns about food safety and soil ecological prosperity, remediation of soils polluted with heavy metals and PAHs has become an expanding livelihood need that must be fulfilled quickly (Huang et al., 2021). Traditional commercial physical (e.g., leaching, soil replacement and fixation), chemical (e.g., oxidation-reduction, electrokinetic and stabilization), and agricultural correction have all been used to restore the ecological vitality and potential production of contaminated soils (e.g., crop rotation, low accumulation planting, and intercropping) . For polluted sites, numerous bioremediation approaches are utilized, such as phytoremediation, microbial remediation, biochar aided remediation, and so on. For the cleanup of hazardous contaminants (Dai et al., 2020), phytoremediation has been acknowledged as a cost-effective, environmentally benign, and long-term solution. Many researchers explored the effect of phytoremediation of contaminated soils with multiple heavy metals and PAHs (Ashraf et al., 2019; Mesa-Marín et al., 2019).

2.1.1. Use of biochar in bioremediation of PAHs and HM

Biochar is a solid, made up of carbon molecules (carbonaceous) at different pyrolysis temperature from waste (Pandey et al., 2020) of any kind (crops, vegetable of fruits). Biochar consists of very unique characteristics, generated soil immobilizes and removes metal ions through a variety of processes, including ion exchange, complexation, electrostatic attractions, and precipitation. Pore filling, hydrophobic effect, electrostatic attraction, hydrogen bond, and partitioning are some of the PAHs remediation methods. Although traditional solutions exist to deal with contaminated soils, many scientific experts believe that using biochar as an effective renewable adsorbent for improved bioremediation is a viable technique for dealing with heavy metals/PAHs co-contaminated soils (Anae et al., 2021).

2.2. PAHs and heavy metal co-contamination in soil

These are often discovered and evaluated during contaminated soil site study. Phenanthrene, 7,12-dimethylbenzo anthracene and benzo[a]pyrene have all been the subject of much investigation. PAHs should not have a total level of more than 200 mg kg^{-1}, yet most research on PAHs have found that soils affected by industry have a total content of up to 6000 mg kg^{-1} (S. N. Sushkova et al., 2021). Sources of PAHs in environment can be natural or anthropogenic activities (Aina et al., 2006; Khpalwak et al., 2018).

2.2.1. Main sources of PAHs

Three following types are the main sources of PAHs contamination in our soils; petrogenic, pyrogenic and biological. During oil maturation and processing, some PAHs are formed they are known as petrogenic. They spread in environment by the usage, transportation and storage of oil. Because of oil spilling, they can be found in oceans, coastal areas and in freshwater. At lower temperatures and incomplete combustion of crude oil also a factor that led to production of pyrogenic PAHs. Urban areas are of great concern because they are major concentration found in industrial areas. While biological PAHs are not described much in literature but can be produced. They are synthesized by some bacteria or plants or they can be by-product of degradation of organic matter (Abdel-shafy & Mansour, 2016).

2.2.2. Contaminated sites having high PAHs amount

In research studies, it has been found that PAHs are mostly present in aged-contaminated soil sites (Haleyur et al., 2019). Diverse environments such as beaches on coastal areas, mangrove sediments (H. F. Dos santos et al., 2011; Tam et al., 2001; Verâne et al., 2020), and forest soils are experiencing these PAHs. Oil spilling (Alonso-Gutiérrez et al., 2009) on coastal areas is main reason of contamination while forest fires are big source of PAHs release in soils as they are found commonly in ashes (Andreolli et al., 2015; Campos et al., 2019; Chaber & Gworek, 2020). These soils are becoming part of these biome. Most of them are in urban areas resulted from industrialization because of high usage of crude oil, fresh fuel, diesel oil, coal tar and petroleum (Alonso-Gutiérrez et al., 2009; Antizar-Ladislao et al., 2008; Bento et al., 2005; Debruyn et al., 2007; Lee et al., 2018; Wong et al., 2002). PAHs are releasing into the soil through several gas manufacturing plants, petrochemical sites, oil refineries, coking and coal fired thermal power plants (Blyth et al., 2015; Cecotti et al., 2018; Cheung & Kinkle, 2001; Douglas et al., 2019; Gune et al., 2019; F. Li et al., 2020; Thavamani, Megharaj, et al., 2012).

2.2.3. Sources responsible for heavy metals

Heavy metals also cause pollution and they are located near large industries (Barsova et al., 2019). Heavy metals are not only present in urban soils but also in rural soils. Waste disposal, metal mining, sewage sludge, smelting, industrial effluents, transportation, and vehicle exhaust are the main sources in urban soils, whereas pesticides, agrochemical impurities, fertilizer application, ore extraction animal/livestock manure, and distribution by surface runoff are sources in rural soils (Hou et al., 2017; Sun et al., 2019). Mixed contamination is not only limited to agriculture or urban soils but it is also becoming part of marine environment, river sediments, mangrove sediment and forest soils (Dell'anno et al., 2020; Khan et al., 2020; Lasota et al., 2020; Marzuki et al., 2020; Rahman, 2020; Rahman & Singh, 2019).

2.2.4. Anthropogenic sources of co-contamination

Anthropogenic sources of the co-contamination are usually occurred when the different types of industries are present on the same location. But mostly it is found that some sectors, such as manufactured gas plants, are sources of both PAHs and heavy metal pollution, where Pb and Cd and contamination seen in long-term polluted soils. Essential metals like Zn are present which are beneficial for microbial life but at the same time there is elevated concertation of Zn led to contamination (X. Li et al., 2017). Besides this, oil pollution is a great problem in spreading this co-contamination in soil for example crude oil (diesel and petroleum industry) is seen to be participating in contaminating soils with heavy metals (Cristaldi et al., 2017; Gran-scheuch et al., 2020; Nwaichi et al., 2016).

Investigations revealed that open burning sites as well as electronic waste (e-waste recycling) are also producing by-products of PAHs and heavy metals (C. Wang et al., 2018). These are prevailing in various extents and patterns in environment and becoming cause of soil contamination specifically for Cd, Cu, Hg, Pb and Zn as studied in several land-use studies (Liu et al., 2017; Thavamani, Malik, et al., 2012a; C. Wang et al., 2018; Y. Wu et al., 2021). For example, in cement industry, co-processed waste was being used in china as an alternative fuel but the unintendedly it was releasing some organic compound and heavy metals. Later, Hg emission was found in cement kiln area (C. Wang et al., 2018). In this whole scenario, due to the anthropogenic load of PAHs and heavy metals, urban soils are the more vulnerable to this mixed contamination (S. Wu et al., 2019).

2.3. Co-contamination effect on soil microbial community diversity

Soil is a wide sink which accumulates all the contaminants from the environment. Because of this property, soil able to respond against environmental stress as it contains millions and trillions of microbes. Thus, these contaminants produce alterations in the metabolic system of microbes and change in metabolic activities which are considered as signals for prevailing contamination in surrounding ecosystem (Stefanowicz et al., 2020). Such as presence of the heavy metal like Pb, Cr, Cd and hydrocarbons create increase CO_2 production and it is indication of high metabolic activities of soil microbes and a reaction to stress. This is a clear evidence that soil pollutants have a significant impact on its soil microbiota (Gorovtsov et al., 2021). In response, soil microorganisms have ability to degrade these environmental contaminants. Hence soil microorganisms are involved in biodegradation of most organic compounds such as PAHs and heavy metals which are spreading day by day in soil through crude oil and its by-products. For example, soil microbes play a role in reducing the charge of metal as conversion of Cr (iv) to its non-toxic form Cr (iii) (E. Da c. Dos santos et al., 2012).

Co-existence of polyaromatic hydrocarbon (PAHs) with heavy metals are caused to decease the efficacy of microflora to biodegrade the contaminants. These chemical mixtures are influencing the total soil microbial community(Thavamani, malik, et al., 2012b). Studies showed that co-existence of the heavy metals with PAHs not only affect the overall biomass of the soil microbial community bust also decrease their intensity. This also implement a selective pressure as it is revealed by the dominance of few bacterial genotypes in the soil and these population start increasing biomass and activity(E. Da c. Dos santos et al., 2012; Thavamani, malik, et al., 2012b).

2.3.1. Soil enzymatic activities under co-contamination

Scientists commonly estimate soil enzymatic activities as one of the markers to assess microbial activity. The enzymes, which are found in soil, perform important metabolic functions. They contribute to the reduction of organic molecules such as PAHs and the conversion of hazardous heavy metals to less toxic or harmless forms (Ciarkowska et al., 2014).

There are very few studies are documented in literature which are focusing on reporting soil enzymatic activity in long term co-contaminated soils. Contrary to this, researchers reported results separately from heavy metal polluted soils and PAHs (diesel or crude oil) contaminated soils. Among heavy metals, cadmium (Cd) is most studies metal in combination with Pb, Zn and Cr while in PAHs, fluoranthene, phenanthrene and benzo[a]pyrene are studies in literature (Cao et al., 2008; Krujatz et al., 2012; Lu et al., 2013; Shen et al., 2005). It has been seen that combined effect are more toxic and dangerous to soil microbes than PAHs alone.

2.3.2. Soil biological properties

Soil biological properties are observed by multiple enzyme tests used in the laboratory on the soil samples to visualize the effect of mixed contamination on the soil microbial community. These include ureases, dehydrogenase activity, microbial biomass carbon, nitrification, microbial respiration, lipases, metabolic quotient (qCO_2), arylsulfatase activity and acid phosphatase and

According to studies (Gran-scheuch et al., 2020; Lu et al., 2013; Thavamani, malik, et al., 2012b), highly contaminated soils showed increased DHA has been seen while some studies(E. Da c. Dos santos et al., 2012) observed low DHA in laboratory experiment in presence of crude oil and chromium and copper. Low microbial biomass carbon (MBC) and UR were also observed. Despite the fact that nitrification was minimal in all cases (no significant changes). High baseline respiration (CO2) and metabolic quotient (qCO2) were found in the research (Lu et al., 2013). Cadmium poisoning resulted in decreased biological activity and microbial mass production (E. Da c. Dos santos et al., 2012; Lu et al., 2013).

Interaction of PAHs and heavy metal has an effect on soil enzymes activities in form of high microbial activates. (Pratt et al., 2012). Besides DHA, UR, MBC, aryl sulfatase, lipase, acid and alkaline phosphatase also expressed microbial activity in contaminated soil (E. Da c. Dos santos et al., 2012; Markowitz et al., 2016). They found lower activity of aryl sulfatase, lipase activity and also lower acid phosphatase activity in contrast to other study (Markowitz et al., 2016). Overall, respiration increased in mixed contamination which showed that there is a declined in population but some selected members increased in population and activity. It is believed that soil microbial community adapt accordingly in long-term contamination and it is because of change in their structure and composition rather microbial diversity (X. Li et al., 2017).

2.3.3. Biodiversity indices

Soil microbial diversity is as important as their activity during contamination. Biodiversity indices are used to assess the quality of a microbiological community. The most frequent indices are evenness index €, richness, Shannon-weaver (H'), and Simpson's index (D). Various microbial diversity analysis methods have been designed and developed, including fluorescence in-situ hybridization (FISH), denaturing gradient gel electrophoresis (DDGE), stable isotope probing (SIP), phospholipid fatty acid analysis (PFLA), terminal restriction fragment length polymorphism (T-RFLP), quantitative PCR (qPCR), and automated version of ribosomal intergenic spacer analysis (ARISA). (Dubey et al., 2020a).

Thavamani (2012b) observed evenness and H’ index low while there as high D index which explained that some genotypes become dominant in ecosystem of specific area while this is also a death cause of other microbes perhaps high contamination and indicates survival of some members. On contrary, Li (2017) observed no significant differences in control and mixed contaminated samples which explained that microbial community adapted the change in long-term contamination. High richness and evenness, H and D index were found (Gorovtsov et al., 2021) in both control and contaminated soil samples which were negatively correlated with PAHs, indicated the soil microbial community effected by heavy contamination.

2.4. Role of bioinformatics

Recent advances and methodologies have enabled researchers to learn about microbes behavior, composition, and structure. New methodologies are emerging, and one of the strong tools being used by researchers to examine the soil microbiome is metagenomics (Hemmat-jou et al., 2018). Because it's hard to isolate and characterize bacteria using medium culture, there was a desire to adopt a technique that didn't have these limitations. As a result, metagenomics meets all of these requirements and provides metagenomics profile of the microbial population. It allows researchers to see the entire genetic variety as well as metabolic activities, pathways, and genes involved. (Abbasian et al., 2016).

2.4.1. Metagenomics to study microbes of co-contaminated sites

Metagenomics is a hub of omics approaches to understand clearly and closely about evolutionary and ecological changes going in microorganisms. This is a less explored biological zone in microbial world which needs to be understand as the composition of the microbes id made up of diverse millions of reads and their functional effect on other community members. Metagenomics offers a full package to identify microbiome profile completely, shown their evolutionary history in form of phylogenetic tree, species abundance, composition and their diversity (table 2,1). Besides this, it also infers functional characterization of microbial community in particular environmental situations (Dubey et al., 2020b).

Table 2.1. General bioinformatic tools to analyze and perform different tasks on metagenomic data

Tasks	Tools	Web address	References
Assembly & Annotation	Genovo	-	(Laserson et al., 2011)
	khmer	-	(Pell et al., 2012)
	MetaGeneAnnotator	http://metagene.nig.ac.jp/	(Noguchi et al., 2008)
	SILVA	https://www.arb-silva.de/	(Pruesse et al., 2007)
	Greengenes	http://greengenes.lbl.gov	(DeSantis et al., 2006)
	SOAPdenovo		(R. Li et al., 2010)
	Meta-IDBA	http://www.cs.hku.hk/~alse/metaidba	(Peng et al., 2011)
	M5nr	ftp://ftp.metagenomics.anl.gov/data/M5nr/current/M5nr.gz.	(Wilke et al., 2012)
	The SEED	-	(Overbeek et al., 2005)
	KEGG	https://www.genome.jp/kegg/	(Aoki & Kanehisa, 2005)
	UniProt	https://www.uniprot.org/	(Magrane & Consortium, 2011)
	RDP database	http://rdp.cme.msu.edu/	(Cole et al., 2009)
Taxonomic profiling	Amphora2	http://wolbachia.biology.virginia.edu/WuLab/Software.html	(M. Wu & Scott, 2012)
	CARMA3	http:// webcarma.cebitec.uni-bielefeld.de	(Gerlach & Stoye, 2011)
	MetaCluster	-	(Y. Wang et al., 2012)
	INDUS	http://metagenomics.atc.tcs.com/INDUS/	(Mohammed et al., 2011)
	MetaPhlAn	http://huttenhower.sph.harvard.edu/metaphlan/	(Segata et al., 2012)
	Treephyler	http://www.gobics.de/fabian/treephyler.php	(Schreiber et al., 2010)
	NBC	http://nbc.ece.drexel.edu	(Rosen et al., 2011)

Functional profiling	HUMAnN	-	(Abubucker et al., 2012)
	metaSHARK	http://bioinformatics.leeds.ac.uk/shark/	(Hyland et al., 2006)
	PRMT	-	(Larsen et al., 2011)
	RAMMCAP	http://tools.camera.calit2.net/camera/rammcap/	ramm(W. Li, 2009)
Statistical test	Metastats		(Paulson et al., 2011)
	Shotgun FunctionalizeR	http://shotgun.zool.guhttp://shotgun.zool.gu.se	
	SourceTracker	-	(Knights et al., 2011)
Database	CAMERA	http://camera.calit2.net	(Seshadri et al., 2007)
	MG-RAST	http://metagenomics.anl.gov	(Meyer et al., 2008)
	EBI-Metagenomics	http:// www.ebi.ac.uk/metagenomics/	(Hunter et al., 2014)
	IMG/M	http:// img.jgi.doe.gov/m	(Markowitz et al., 2012)
	GenBank	https://www.ncbi.nlm.nih.gov/genbank/	

Various methods are used to analyze different levels of microbial community to get information under specific situation such as PAH and leavy metal contaminated soils. Most popular, cheap and common method is 16s rRNA used by researchers. Other methods such as whole-genome shotgun analysis (WGS), whole-transcriptome (metatranscriptome) shotgun and metaproteomic analysis are also used to understand gene sequences, taxonomic profiling and phylogeny of soil microbes (Xu et al., 2014). Different bioinformatic analysis tools are present to specially analyze only 16s rRNA metagenomic data and furthermore tools are available for specie and strain-level metagenomic data analysis (Table 2.2). Metagenomic studies revealed phylogenetic relation between different bacterial species and their interaction with other microbial communities as well as provide us with the information of percentage of communities present in specific soil sample (Fig 2).

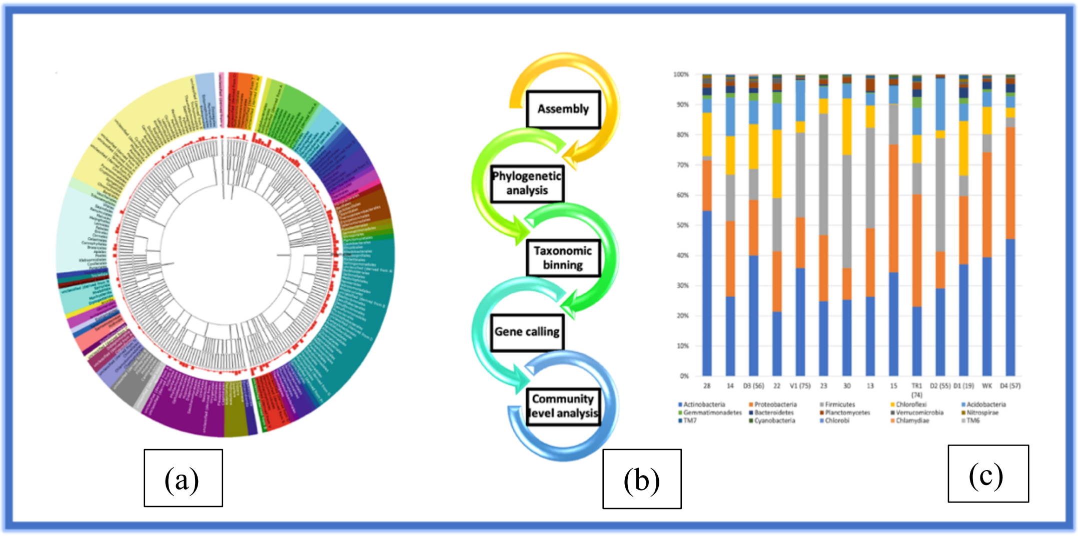

Figure 2.1. (a) Illustration of phylogenetic tree (b) Process to visualize metagenomic data (c) Relative abundance of soil microbes (biodiversity)

Table 2.2. Bioinformatic tools to visualize 16s rRNA data on different levels (Niu et al., 2018).

Date Analysis	Software	Availability	Package	Web address
16S rRNA amplicon analysis	QIIME	Open sources, tutorial and database	Yes	http://qiime.org/
	UPARSE	Tutorial	-	http://drive5.com/uparse/
	MOTHUR	Open source and tutorial	Source code	http://www.mothur.org/wiki/Main_Page
	DADA2	Basic and tutorial	-	http://benjjneb.github.io/dada2/index.html
	MED	Basic and tutorial	-	http://merenlab.org/software/med/
Species-level metagenomic data analysis	MetaPhlAn2	Open source, basic and tutorial	Yes	http://segatalab.cibio.unitn.it/tools/metaphlan2/
	kraken	Open source, basic and tutorial	Source code	https://ccb.jhu.edu/software/kraken/

	CLARK	Open source, basic and tutorial	Source code	http://clark.cs.ucr.edu/
	SUPER-FOCUS	Open source, basic and tutorial	Source code	http://edwards.sdsu.edu/superfocus/
	MG-RAST	Open source	-	http://metagenomics.anl.gov/
Strain-level metagenomic data analysis	StrainPhlAn	Open source and tutorial	Yes	http://segatalab.cibio.unitn.it/tools/strainphlan/
	PanPhlan	Open source and tutorial	Yes	http://segatalab.cibio.unitn.it/tools/panphlan/
	Constrains	Open source and tutorial	Yes	https://bitbucket.org/luo-chengwei/constrains
	Sigma	Open source and tutorial	-	http://sigma.omicsbio.org/
	LSA	Open source and tutorial	Yes	http://latentstrainanalysis.readthedocs.io/en/latest/

2.4.2. Microbial composition study from 16s rRNA metagenomic data

Exploration of metagenomic data with the use of these tools revealed that different microbial species are dominated on these contaminated soils with polyaromatic hydrocarbons (PAHs) and heavy metals. These dominated bacteria/soil microbes are representing the degrading bacteria community and those having heavy metal resistant genes. Many of them are reported in PAHs contaminated sites such as *Actinobacteria spp*., *Pseudonocardia spp. Halomonasspp spp., Mycobacterium spp.* and *Streptomyces spp. Desulfotomaculum spp., Nocardia spp., Nocardioides spp., Dietzia spp., Rhodococcus spp. Aeromicrobium spp., Pseu-domonas spp., Pseudoxanthomonas sp*p. and *Bacillus spp*. Khudhur (2018) reported that bacterial genera *Bacillus spp., Balneimonas spp.* and *Geodermatophilus spp.* are correlated to copper (Cu), *Sphingomonas spp.* to lead (Pb) and *Rubrobacter spp.* correlated to zinc (Zn).

After 16s rRNA analysis, composition of bacterial community was found under diesel contaminated soils with heavy metals as Proteobacteria, Bacteroidetes, Actinobacteria, Chloroflexi, Acidobacteria and they isolated *Sphingobium spp*. having high capacity to degrade PAHs under cadmium and chromium (Gran-Scheuch et al., 2020). Recent metagenomic studies showed that *Burkholderia fungorum* FM-2 strain is capable to degrade phenanthrene in acidic contaminated soils with Zn (Rahman, 2020). It has been seen that Proteobacteria is the most dominated phylum present in contaminated soils with the abundance rate of more than 70% (Tipayno et al., 2018). Phylogenetic studies of topsoil microbial community metagenomic data showed the same relative abundance of Actinobacteria and Proteobacteria in contaminated lake soils under Zn metal pollution but it also showed negative correlation of some phyla such as Gemmatimonadetes, Verrucomicrobia, and Nitrospira with total polyaromatic hydrocarbon level (Gorovtsov et al., 2021). Indication of these bacteria phyla showed that these bacteria could be helpful in degrading PAHs in presence of specific heavy metal as they contain degrading and metal resistant genes. These studies give benefit to locate different bacterial consortia which can be used for soil reclamation which they become polluted with different potential toxic elements and heavy metals.

3. MATERIAL AND METHODS

3.1. Introduction

The contamination with organic and inorganic pollutants changes significantly soil microbial community structure. These shifts indicate anthropogenic pressure and help to discover new possibilities for soil remediation. So, the objective is to study soil properties during model experiment and combine application of the biochar and isolated metal-resistant and PAHs degrading microbial strains for the approbation of the developing bioremediation method.

3.2. Site study

Application of bioremediation technology was tested on Technosols which are chemically polluted soils with PAHs at extreme high levels, which was next to a chemical industry operating on the area of Atamanskoye Lake (Fig. 3.1) in Rostov Region since 1964. The bioremediation activity of microbial strains was investigated for the first time in this co-contaminated area with PAHs and Heavy metals.

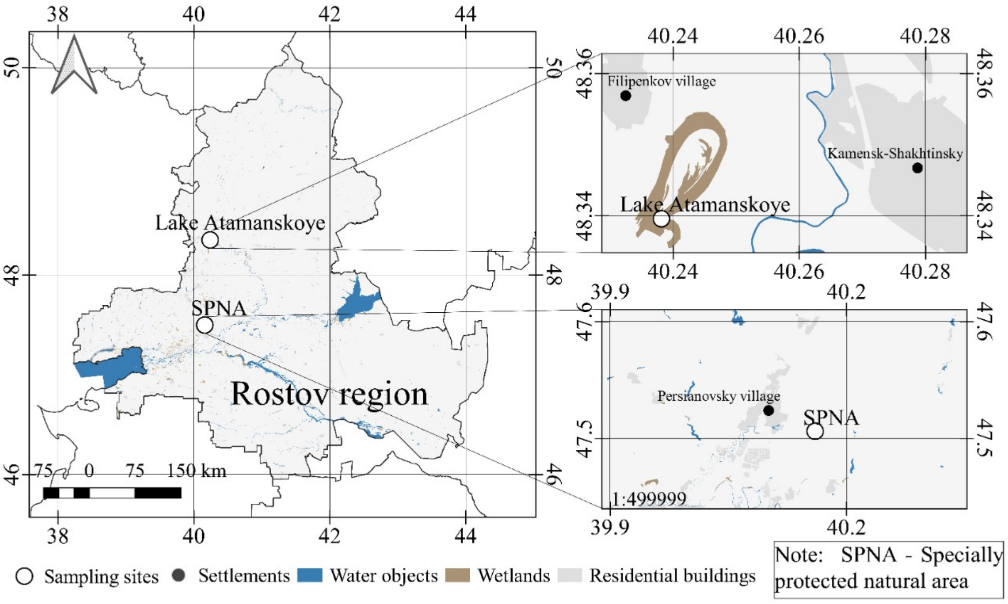

Figure 3.1. Map of soil sampling site- 57, Atamanskoye lake.

3.2.1. Type of contaminated soil - Spolic technosols

Bacterial strains were obtained from Spolic Technosols developed on the site of a former industrial sewage reservoir near the Kamensk-Shakhtinsky (Southern Russia). The impact of HM and PAH concentration variables on microbial community composition was evaluated in relation to soil parameters (organic carbon content) and phylum-phylum interactions for this aim. Control soil was collected from a background area classified as Chernozem which has low amount of PAHs and HM (Table 3.1).

Table 3.1. Concentration of heavy metals in Chernozem vs Spolic Technosols (Minkina et al., 2019).

Heavy metal	Concentration (mgkg^{-1}) Control soil (Chernozem)	Concentration (mgkg^{-1}) Polluted soil (Technosols)
Mn	1426.42 ± 51.7	709.10 ± 66.9
Zn	620321 ± 403.6	118.40 ± 8.1
Cr	154.08 ± 13.8	106.81 ± 7.3
Cu	146.90 ± 10.2	52.36 ± 3.6
Pb	1591.17 ± 129.3	29.66 ± 2.1
Ni	80.54 ± 10.4	65.53 ± 3.4
Cd	10.56 ± 1.2	0.32 ± 0.01

Metagenomic analysis was performed on soils with a high anthropogenic load, in this study. As a result, the majority of the pollutants collected in the flood plain lakes' bottom sediments, which have been a serious source of groundwater secondary contamination, which is utilized for the water supply of Kamentsk-Shakhtinskii city for many years.

From the early 1960s till the mid-1990s, Atamanskoye lake served as a sink for the chemical plant's industrial waste. The quantity of technogenic waste in the lake is estimated to be between 400.000 and 420.000 m^3, with up to 98.000 mg kg^{-1} zinc, 1050 mg kg^{-1} lead, 13.6 mg kg^{-1} mercury, 52 mg kg^{-1} arsenic, and 303 mg kg^{-1} copper (Minkina et al., 2019). Furthermore, as compared to typical lithospheric values, soils on the lacustrine mud of dried-up Atamanskoye lake had hundreds of times more Zn concentration, ten times more Cd and Pb content, and several times higher Cu, Ni, and Cr concentration (Bauer 2018).

During the period 2015-2017, high pollution levels for PAHs were observed in 10 monitoring sites (S. Sushkova et al. 2020 the concentration level of the 16 PAHs varied from 499 to 7178 g kg^{-1}, with exceptionally high values exceed the recommended concentration for benzo[a]pyrene over 150 times (Sushkova et al. 2020a). PAHs content in soil within the diameter of 20cm topsoil layer, at Atamanskoye lake in year 2018 demonstrated an extensive deposition of 16 major

PAHs ranging from 2001 to 4229 g kg^{-1} which were evaluated in the five monitoring plots(S. Sushkova et al. 2020). While at the dried lake, Technosols were developing specific microbial community.

3.2.2. Sample collection and site description

The sampling depth ranged from 0 to 20 cm and was chosen according to ISO 10381-1. (2002). Samples were transported to the laboratory in plastic bags. The soil samples were homogenized, air-dried, sieved through a 2 mm sieve, and mixed thoroughly. Chemical and physical properties of the soil were assessed using ISO guide 34 (2009) protocol at a qualified analytical laboratory (Certificate No. C RU 0001.511127). The pipette technique (with pyrophosphate preparation) was used to determine the particle size distribution (Shein, 2009). Carbonates, Fe, and Al oxides ad organic debris were not removed prior to the pipette method. The pH in an H2O suspension was evaluated using a glass electrode in a 1:5 (volume fraction) suspension of sample in water, by following ISO 10390 (2005). Organic carbon content was measured by sulfur-chromic oxidation with guide of ISO 14235 (1998), the carbonates by using a Scheibler method, ISO 10693 (1995), and ISO 23470 (2011) guide for exchangeable cations were measured by using a hexamine cobalt trichloride solution.

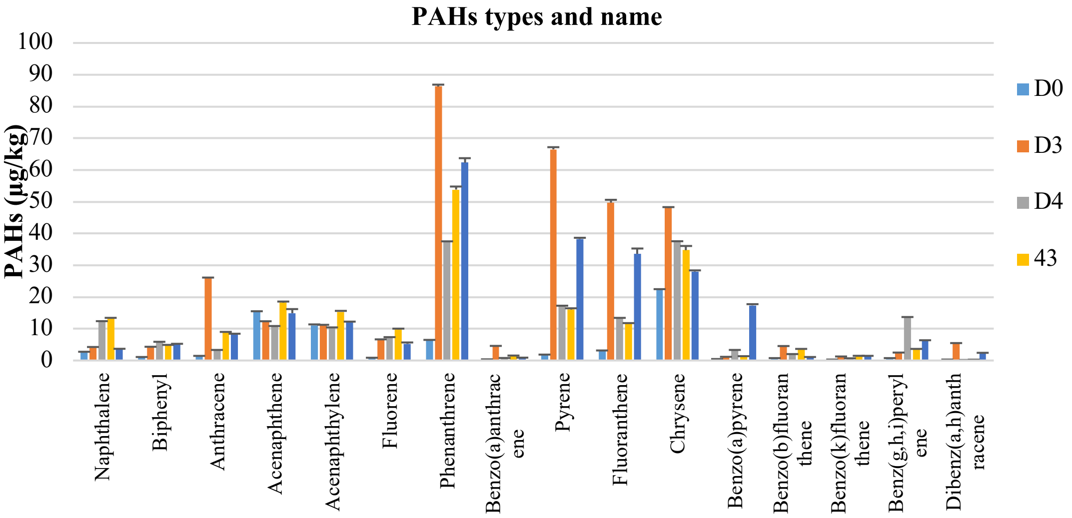

Figure 3.2. Dynamics of 16 PAHs concertation above ground part and hanging bars present the average mean standard error value from three replications

3.3. Model experiment setup

A model experiment was done in the laboratory conditions. Growth chamber was maintained with homogenized temperature, light and humidity. Spolic Technosols soil samples were already collected from the polluted site of Lake Atamanskoye plot number 57. While the control soil (unpolluted soil) Haplic Chernozem was selected from plot number 3. Sunflower husk biochar with concentration of 2.5% was also used in experiment. Inoculate of two bacterial strains metal resistant and PAH degrading i.e., *Pseudomonas putida* and *Rhodococcus erythopolis* was also prepared to introduce them in soil pots as Naphthalene and Phenanthrene degrading strains.

Total five treatments were made in combination with three factors with the following scheme (Fig. 3.3):

Treatment 1: Control (unpolluted soil)

Treatment 2: Polluted soil with PAHs and HMs

Treatment 3: Polluted soil + Bacterial strains inoculate

Treatment 4: Polluted soil + Biochar 2.5%

Treatment 5: Polluted + Bacterial strains + Biochar 2.5%

Plastic vessels (pots) were selected for this experiment. Drainage system was installed in all pots. Each pot was filled with 1 kg of well-mixed soil and seeds of barley were selected to germinate till seedling stage.

At a concentration of roughly 10^10 CFU kg^{-1} soil, two HMs tolerant bacterial strains were applied to the soil. Bacterial strains were injected into the soil in the following order: the metal-resistant bacteria were cultured in separate flasks on nutrient broth for three days, then the media was centrifuged and the biomass was obtained. The number of cells were calculated using a densitometer to measure the optical density of the biomass sample after serial dilution. The cell count was calculated using calibration curves generated before the experiment for each strain using densitometry and plate count.

After getting the desired concentration of the bacterial strains (R.E $4.65 \text{x} 10^{10}$ and P.P 3.81 10^{10} CFU/ml), 0.5 ml from each strain dilution was diluted with 49ml of water to get the 50 ml of bacterial solution. This was the final inoculum prepared with the biomass to achieve the even proportion of both strains. The amount of water was pre-calculated according to the soil moisture, given to the pots, to 60% maximum water holding capacity. Before the pouring of soil, sorbent was added in the bottom layer of pots. Drainage pipe was also installed in the pot, over that soil was added with the already prepared solution of bacterial strains in layered pattern such that after every layer of soil, bacterial suspension was spayed equally over the soil. This was the way to achieve maximum uniformed distribution of both components within the pot. For the rest of treatments with biochar and bacterial inoculum, first the dry soil was mixed thoroughly with biochar. After mixing, bacterial inoculum was incorporated in soils in the same way as mentioned above. While in the control pots, simple water was added instead of bacterial inoculum.

Hordeum vulgare L. seeds were sown in the pots. First, they were germinated in dark for 3 days later transferred into pots. No pre-treatment was given to the seeds. Approximately 20 germinated seedlings were sown. Then the pots were transferred to growth chamber for incubation for almost one month (30 days).

3.3.1. Plants condition in growth chamber

Experiment was performed in uniformed and homogenized growth chamber where temperature was maintained ranging from 25 to 20 ± 3°C. Normal lighting was provided with proper aerobic conditions and relative humidity. 60% moisture according to maximum water field capacity was maintained with distilled water during whole incubation period. There was no use of additional fertilizers and pesticides in this experiment. Experiment was based on completely randomized design (CRD) where each treatment was in triplicate mode. Experiment design and allocation of treatments in each pot was totally random and according to standard procedure.

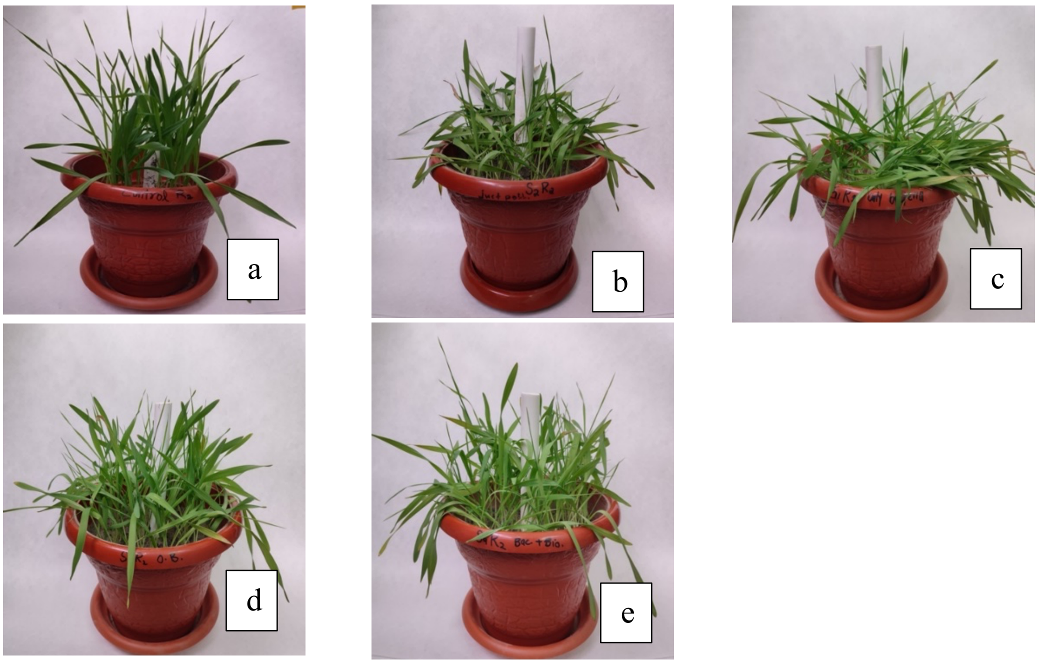

Figure 3.3. Experiment model for soil bioremediation with the help of bacteria and biochar, where (a) Control, (b) Polluted soil, (c) Bacteria, (d) Biochar 2.5% and (e) Bacteria + Biochar 2.5%

Table 3.1. Completely randomized design (CRD) of experiment for soil bioremediation with help of bacteria and biochar

Soil type	No.	Treatment	Bacterial inoculate	Biochar	Plant being cultivated
Haplic Chernozem soil from sample site №3, Atamanskoye lake	1	Control	-	-	*Hordem vulgare*
	2	Control (R2)	-	-	
	3	Control (R3)			
Spolic Technosols (soil from sample site №57, Atamanskoye lake)	4	Untreated soil (R1)	-	-	
	5	Untreated soil (R2)	-	-	
	6	Untreated soil (R3)	-	-	
	7	Soil + Biochar (R1)	-	2.5%	
	8	Soil + Biochar (R2)	-	2.5%	
	9	Soil + Biochar (R3)	-	2.5%	
	10	Soil + bacterial inoculum (R1)	50 ml of $1x10^{10}$ CFU	-	
	11	Soil + bacterial inoculum (R2)	50 ml of $1x10^{10}$ CFU	-	
	12	Soil + bacterial inoculum (R3)	50 ml of $1x10^{10}$ CFU	-	
	13	Soil + bacterial inoculum + biochar (R1)	50 ml of $1x10^{10}$ CFU	2.5%	
	14	Soil + bacterial inoculum + biochar (R2)	50 ml of $1x10^{10}$ CFU	2.5%	
	15	Soil + bacterial inoculum + biochar (R3)	50 ml of $1x10^{10}$ CFU	2.5%	

** Each pot contains 1 kg of sieved soil; 50 ml of H2O were mixed soil alternatively with and without bacteria strains and biochar*

3.3.2. Hordeum vulgare as a selected plant

Hordeum vulgare L. (spring barley) is a 2-row variety crop belongs from family *Poaceae*. This is one of the important crops after wheat in term of grain and cash crops. It has been considered as an efficient heavy metal accumulator because it plays a significant role when it comes to phytoremediation. As a hyperaccumulator, barley has a potential to accumulates Zn and Cd in it’s different parts of plant (Rajput et al., 2018). International standard ISO 11269-1 recommend barley to use for bio-testing. Besides this, Ratnik barley variety is widely grown cop in Rostov region.

As the model experiment was planned to conduct in co-contaminated soil, which was highly polluted with HMs ad PAHs, therefore, barley was selected as test crop. There were more chances to see the clear effects of biochar-microbes interaction and to do better evaluation of HM toxicity, accumulation and PAHs effect on growth of barley. From previous experiments, it has been proved that barley provide data on distribution of different heavy metals such as Zn, Cd, Cr, Pb, Ni, Cu and Mn in plant tissues.

3.3.3. Application of biochar

Biochar application can be helpful in the growth of plants especially it is under highly polluted soil. This could also enhance the bioavailability of the nutrients and microbial activity within soil (Lucchini et al., 2014; B. Wang et al., 2017). Biochar is a dark color, carbon-rich material whose addition in soil can improve the soil physio-chemical properties as well as helps in degradation of pollutants (Zhu et al., 2017). The biochar which is used in this model experiment was made from sunflower husk under the pyrolysis temperature of 700°C. Elemental composition is described in Table 3.3.

Table 3.2. Elemental composition of sunflower husk biochar produced at the pyrolysis temperature 700°C

Element	App Conc.	Intensity	Weight%	Weight% Sigma	Atomic%
C K	36.91	1.1482	66.71	1.43	76.63
Mg K	0.93	0.8075	2.38	0.2	1.35
Si K	0.27	0.9317	0.6	0.11	0.3
K K	1.93	1.0503	3.81	0.23	1.34
Ca K	1.29	0.9584	2.8	0.21	0.96
Mo L	0.56	0.8315	1.41	0.31	0.2
Totals			100		

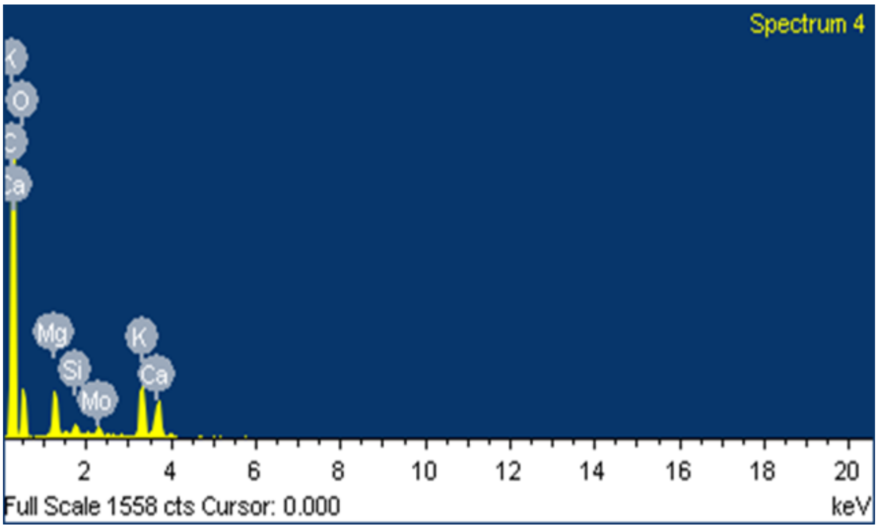

Figure 3.4. Volume or peak response of the elements of the sunflower husk biochar

Structure of the biochar was studies under the scanning electron microscopy (SEM) ad transmission electron microscopy (TEM). Image from biochar surface captured through the TEM showed the various pores that represents the PAHs adsorption in the soil.

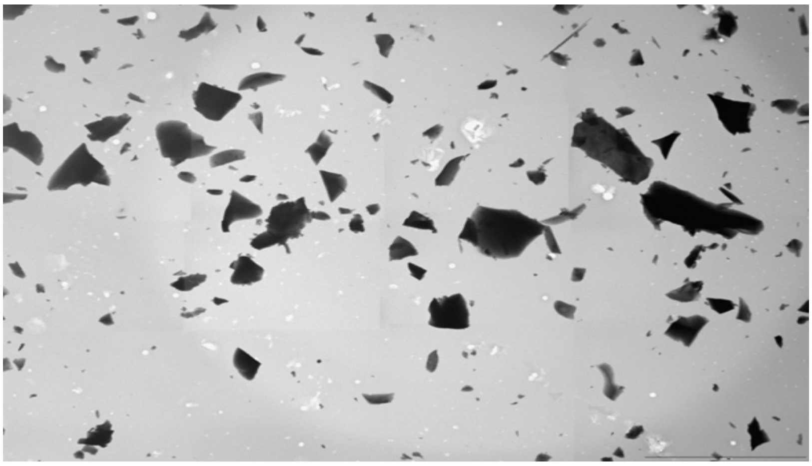

Figure 3.5. TEM imaging of sunflower husk biochar produced at the pyrolysis temperature 700°C

Sunflower husk biochar surface morphology was also studied under scanning electron microscopy (Carl Zeiss EVO-40 XVP) with confocal microscopy having a 3D laser (KEYENCE VK-9700 Generation II, Japan). Topographic (surface) and morphologic (microgeometry) features of bio-sorbent fragments were analyzed. Results recording was done for the low-contrast and non-conductive samples under standard conditions (15kV, low vacuum, increased emission).

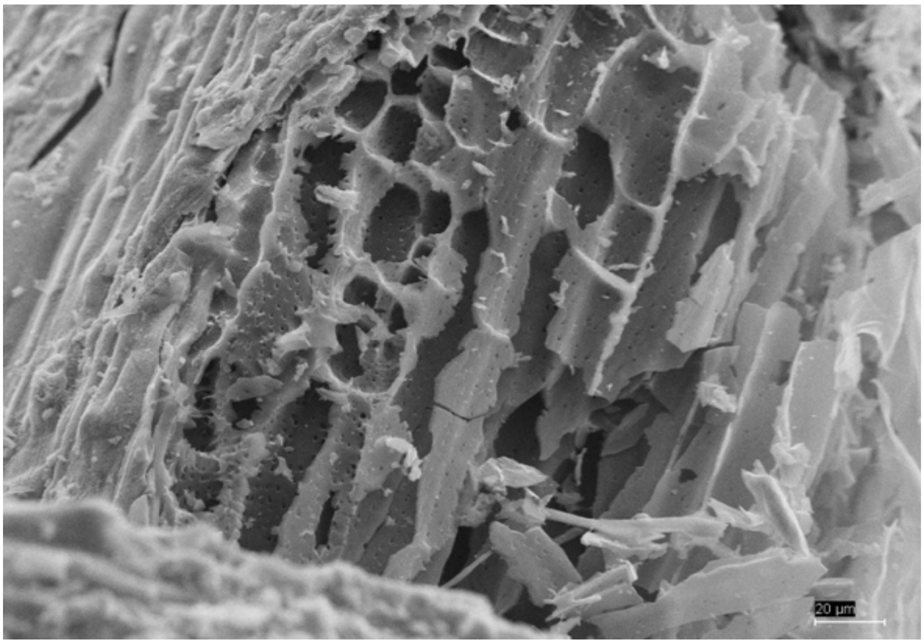

Figure 3.6. SEM imaging of sunflower husks biochar produced at the pyrolysis temperature 700°C

3.4. Measurement of plants morphometric data

Plant growth parameters are necessary to measure as they are used further to compare any difference between control and the treatments as well as among the treatments. These parameters can be a phenotypic indication of influence of biochar or bacterial strains or combined effect on the plant growth. Two morphometric parameters were measured i.e., root and shoot length. After the one month/ 30 days of the growth, plants were removed from the pots and separated from each other gently. Roots and shoots' length were measured with the help of the scale.

3.5. 16s rRNA metagenome assembly and annotation

For isolation of the microbial genomic DNA, DNeasy® PowerSoil® Pro Kit was used. Total DNA was isolated by following manufacturer's instructions. Assays are studied by analyzing the 16S rRNA genes specifically prokaryotic, that is about 1500 bp length and have 9 variables regions. From those regions, mostly V3-V4 is amplified for PCR. These regions are used to study phylogenetic classification such as phyla , family of diverse microbial communities.

This procedure explained how samples were prepared for sequencing variable V3 and V4 regions. This procedure can likewise be used to sequence other regions using primers unique to those regions. This methodology, when paired with a benchtop sequencing device, along with primary and secondary analysis using MiSeq or BaseSpace, created a full 16S rRNA amplicon sequencing process. Library was organized by amplifying the V3-V4 region, limited cycle number of PCR, added Illumina adapters and dual index bar codes.

Gene amplicons for Miseq system, V3-V4 region of 16S rRNA was performed using prokaryotic primers.

16S Amplicon PCR Forward Primer = 5'

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

16S Amplicon PCR Reverse Primer = 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Sequencing was performed using MiSeq platform (Illumina) with v3 reagents (600 cycles) at Federal Budget Institution of Science STATE RESEARCH CENTER FOR APPLIED BIOTECHNOLOGY AND MICROBIOLOGY. Genome was assembled and annotated with the help of QIIME version 2.0.

3.6. Isolation, selection and identification of metal tolerant and PAHs degrading strains

Soil samples were collected from the highly polluted part of the dried lake Atamanskoye which is located in Kamenskii region of Rostov, Southern part of Russia. Bulk portion of the soil was collected from a depth of 0-5cm and transported to the laboratory immediately for isolation of bacterial strains.

For isolation procedure, soil was mixed thoroughly with sterilized water in ratio of 1:10 (10 grams of soil to 100 ml H_2O). Soil-water mixture was ground by rubber pestle in order to separate attached bacterial cells from particles of soil. Resulted soil suspension was poured in conical flask to shake at rotatory shaker for a time of 30 minutes. Further, dilutions were made and spread on nutrient agar.

To assess the metal resistance of the isolated strains, they were grown on a solid nutrient medium with the addition of copper in the form of $CuSO_4$ taken at 10, 25, 50, 75, or 100 MPC (which corresponds to 118, 295, 590, 885, and 1180 mg/kg for $CuSO_4.5H_2O$). The strains capable of growing at these MPCs of copper were assumed to be metal-resistant.

Two strains out of all showed more tolerance (table 3.4) against the CuSO4 (later identified as *Pseudomonas putida* and *Rhodococcus erythropolis*). Plates were incubated at 30°C and colonies of the metal tolerant bacterial strains were observed after five days. Pure cultures were extracted and shifted to the same media.

Table 3.3. Metal resistant bacterial strains and their maximum tolerance

Bacterial strains	Maximum metal tolerance mg/kg
Pseudomonas putida	590
Rhodococcus erythropolis	885

On the other hand, soil dilutions were spread on a solid basal salt medium to isolate PAH degrading strains. Media was containing naphthalene as a sole source of carbon and energy (Table 3.5). PAHs were diluted in sterile DMSO (dimethyl sulfoxide) separately as they couldn't be autoclaved and aseptically added to the autoclaved medium with basal salts. The medium also contained triphenyl tetrazolium chloride (TTC), which was used to stain the most metabolically active colonies on the plates. After 5-7 days, deep red colonies were isolated assuming directly utilizing naphthalene as well as phenanthrene. Pure cultures were transferred and maintained on the same media.

Table 3.4. Media used to grow and identify PAH degrading bacterial strains

Content	Gram/liter of dH_2O
NH_4NO_3	2
KH_2PO_4	2
Na_2HPO_4	3
NaCl	1
$MgSO_4$	0.2
Na_2CO_3	0.1
$CaCl_2$	0.01
$MnSO_4$	0.02
$FeSO_4$	0.01
TTC	0.03
PAH source	0.1% (w/v)

3.7. Identification of the strains for metal tolerance and PAHs degradation

The identification of the metal-tolerant and PAHs degradation bacterial strains was performed on the base of genotypic and phenotypic characterization. All the isolates were decided to be used further for investigation.

3.7.1. Phenotypic characterization

Strains from the selection stage were maintained on the media with naphthalene. Two of all showed the best performance as they continued their growth on naphthalene as well as phenanthrene containing media. These actively growing strains were

Rhodococcus erythropolis and *Pseudomonas putida*. Based on that, strains were used in model experiment. Suspension of these two strains (final cell concentration was 10^{10} cells in each variant, with strain ratio 1:1) was added to the soil mix for model experiment.

3.7.2. Mass spectrometry MALDI-TOF

Metal tolerant strains were identified phenotypically with the help of mass spectrometry and colonies formation on the media. To obtain the samples for MALDI-TOF mass spectrometry, sufficient biomass of the bacterial strains was collected by incubating culture with an inoculum of 24-hour culture on nutrient agar for 16 hours at 30 °C. Raw spectral data was analyzed on device Microflex LT (Bruker Daltonics GmbH, Leipzig, Germany) using Biotyper software (version 3.0). Device has a default setting which use a library of 5989 spectra where database was updated in 2016 by manufacturer. For identification, small number of the cells were picked form the bacterial colonies with the help of a pipette tip. Biomass was spread on steel plates of MSP 96. Thin layer was covered with 1µl of matrix which was saturated solution of α-cyano-4-hydroxycinnamic acid (in 2.5% trifluoroacetic acid- 50% acetonitrile). At room temperature, plates were allowed to dry. Spectral data was taken and analyzed by the software.

After identification, strains were grown on media to analyze their colonies formation. Both strains were gram-negative and non-spore forming. *Pseudomonas putida* was forming slimy round colonies while the *Rhodococcus erythropolis* showed two types of colonies morphology: one with sharp pale-pink colonies and the second with slimy pale-pink colonies.

3.7.3. Genotypic characterization

DNA extraction was performed by using AmpliTube RV M-SorbTube kit (Sintol, Russia). DNA was extracted. DNA amount was evaluated by using Qubit (version 3.0) fluorimeter. Further amplification was performed by PCR. Master solution was prepared as: 25 mM solution of nucleotides 10xdNTP- 2 µl; DreamTaq polymerase- 0.2 µl (ThermoFisher), 0.5 µl forward (F) primer and reverse (R) primer (0.5 µm) (Table 3.6), DNA template 1 µl (5 ng µl-1), 10x DreamTaq PCR buffer (20 mM MgCl2) (ThermoFisher) and H2O (DD): 18.3 µl. total volume was 25 µl. Amplification was performed in below mentioned cycle (T100 Thermal Cycler; Bio-Rad, USA).

1. First denaturation at 95°C- 2 min

2. Denaturation at 95°C -30 sec.
3. Primer annealing at 57°C- 30 sec
4. Elongation at 72 ° C- 1.5 min.
5. Final elongation at 72°C- 3 min.
6. 32 cycles

Table 3.5. Primers' sequence to amplify amplicon region

Primer	Primer sequence (5 '→ 3')	Annealing temp. (° C)
27F	AGAGTTTGATCCTGGCTCAG	57
1492R	ACGGYTACCTTGTTACGACTT	

Amplified products through PCR were further purified by using "ExoSAP" enzymatic method.

3.7.4. 16S rRNA gene sequencing

Primary nucleotide sequences were determined by sequencing method called Sanger sequencing. Gene specific primers (table 1) along with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) were used to perform sequencing. Reaction mix was prepared to perform PCR with below mentioned protocol:

1. Preliminary denaturation at 96ºC- 1 min;
2. Denaturation at 96ºC- 10 sec,
3. Annealing temperature at 50ºC- 5 sec,
4. Elongation at 60ºC- 4 min.
5. 26 cycles

Mixture was further purified to get the desire PCR product by following the protocol: 40 µl of 0.125 M ammonium acetate (88% ethanol) was used for every 10 µl of the PCR mixture. After couple of centrifuges and washing with ethanol, pellet was recovered. With the help of capillary electrophoresis (ABI PRISM 3730, Applied Biosystems, USA), sequencing products were detected. Whole procedure was done in the laboratory of Interdisciplinary Center for Collective Use of Kazan Federal University, Russia.

3.8. Mold and bacterial colonies plate count

3.8.1. Sample and media preparation

Soil samples were collected from the pots at the end of experiment when plants were removed for morpho-biometric measurements. Soil was mixed with the help of spatula thoroughly in the pot to get uniform and homogenized sample. Approximately 150 grams of the soil was taken, added in the plastic bags and shifted to refrigerator (4°C) to store.

Colonies plate count was performed for all samples from each replication. Total 15 samples were evaluated for the mold and bacterial plate count. Each sample was then tested for three different media in 3 replicates. Malt extract, Czapek and Starch ammonium (ISP4) medium were prepared. Hence, total 135 plates were observed for the bacterial and yeast plate count. For every 5 soil samples, recipes were made for 500 ml (Table 3.7).

Table 3.6. Media recipe to count molds and bacteria used in the plate count method

Malt extract media		Czapek Media		Starch Ammonium agar (ISP4)	
Compound	Weight (g)	Compound	Weight (g)	Compound	Weight (g)
Malt extract	0.5	Maltose/sucrose	10	Starch	5.0
Yeast extract	0.2	K_2HPO_4	0.5	$(NH_4)_2SO_4$	1.0
Glucose	0.2	$MgSO_4$	0.25	K_2HPO_4	0.5
Agar-agar	8.0	KCl	0.25	$MgSO_4$	0.5
		$NaNO_3$	1.5	NaCl	0.5
		$FeSO_4$	Trace amount	$CaCO_3$	1.5
		Agar-agar	10	Agar-agar	12.5

Ingredients were added in 500 ml distilled water in the flask, closed it with stopper and covered with aluminum foil. Flasks along with the test tubes having 9 ml of distilled water were also closed with stopper (later used for serial dilutions) and put in autoclave for 30 minutes at 2 atm and 121°C.

Serial dilutions were prepared from soil suspension for each soil sample. Flasks containing soil suspension were put on shaker for 40-50 min. At 150 rpm. Dilutions for three media were 10^{-2} dilution for Czapek media, 10^{-4} dilution for Malt extract media and Starch ammonium agar. 1 ml of soil suspension was taken from the flask with the help of pipette and added into the first test tube with 9 ml of distilled autoclaved water. In the same way, all dilutions were prepared.

Mediums were transferred to sterilized chamber with UV light. Before pouring into the plates, streptomycin as antibiotic was added into Czapek media to prevent the bacterial growth while amphotericin B was added into Malt extract and Starch ammonium mediums. For inoculation, 50μl of 2nd dilution 10^{-2} was used in Czapek media and spread equally on plate with the help of sterilized glass spatula. In the same way, 4th dilution 10^{-4} was used for Malt extract and Starch ammonium agar (ISP4). Later plates were shifted to separate incubator for bacterial and fungal growth for incubation of 4 days for fungal growth and 1 week for bacterial growth. Mold, yeast, bacterial and actinomycetes colonies were counted after end of incubation period.

3.9. Determination of soil enzymatic properties

Four different kinds of the enzymes activity were measured in the soil samples in the laboratories of Academy of Biology and Biotechnology. Studied enzymatic activities were: Catalase activity, Peroxidase activity, Invertase activity and Urease activity. Key method of determination, classification and units are briefly explained below.

3.9.1. Catalase

One (1) gram of soil placed in a glass flask was mixing with 5 ml of a 3% hydrogen peroxide solution. Soil and peroxide mixed with each other at 20°C. The mixture is shaken during the whole experiment. The released oxygen was displacing water from the burette, the level of which was noted after 1 and 2 minutes. The soil sterilized by dry heat (180°C) served as the control.

3.9.2. Peroxidase

One (1) gram of soil was added in 50 ml conical flasks along with freshly prepared 1% hydroquinone 10 ml and 1 ml of 0.05% hydrogen peroxide solution and mixed thoroughly for 30 min at 30°C. Control sample, having no soil but mixture. Later 10 ml ethanol was added. The mixture was then filtered or centrifuged. Then colorimetry of the yellow alcohol extract on a photo-colorimeter with a filter of blue light (wavelength 460 nm) in contrast to control solutions was performed. Amount of parabenzoquinone thus calculated from a standard curve drawn using pure parabenzoquinone solution.

3.9.3. Invertase

One (1) gram of the prepared soil, 5 ml of a 3% freshly prepared sucrose solution and trace amounts of toluene added in 50 ml flasks and placed in a thermostat for 24 hours at 30°C. Controls were soil sterilized by dry heat at 180°C for 3 hours. After incubation, 25 ml of distilled water were added to the flasks, shaken, and filtered. 6 ml of the filtrate and 6 ml of Felling's reagent then added into a test tube, which then for

10 min. heated in a boiling water bath. After the tube cooled, the contents were centrifuged for 1-3 minutes at 1500-3000 rpm. Colorimetry was done at 630 nm in 1 cm wide cuvettes. The amount of glucose (mg/ml) is determined from the calibration curve. The data obtained is multiplied by 30 (total volume of the solution).

3.9.4. Urease

One (1) gram of the prepared soil were placed in 50 ml flasks, 5 ml of 3% urea and trace amounts of toluene were added. Sterilized soil (180°C, 3 hours) served as the control. Samples were placed in a thermostat at 30°C for a time of 24 hours. After incubation, 15 ml of sodium hydroxide (1 M) was added in the flasks. Flasks were centrifuged for 5-10 min at 3000 rpm. 10 ml of centrifugate solution was poured into a 50 ml volumetric flask and diluted to 30 ml with dH2O. Then, 2 ml of a 30% solution of potassium-sodium tartrate along with the 2 ml of Nessler's reagent and solution was mixed thoroughly.

Colorimetry carried out on a photoelectric colorimeter in cuvettes 30 mm wide with a blue light filter (wavelength 400 nm). The amount of NH3 is calculated from a pre-compiled calibration curve. Standard solution: 0.1 mg NH3 in 1 ml water. Expressed as NH3 = (x-y)×d×10/a, where x is mg NH3 from curve, y is mg NH3 in control from curve, d - dilution, a - initial amount of soil.

3.9.5. Dehydrogenase

Six (6) g of soil samples were taken in a sterilized tube. 2.0 ml and 1.5 ml of 3% aqueous triphenyl tetrazolium chloride (TTC) and 2% solution of glucose respectively, was added in the samples. In contrast, control tube was having 3.5 ml of distilled water instead of solution of glucose and TTC. In modification, sealed tubes were used without any space left, filled with reaction mixture. While in original method, open tubes are put in vacuum desiccator. Tubes together with reaction mixture were mixed and left for 24 hours incubation on 30C. Produced triphenyl tetrazolium formazan was extracted and washed with ethanol on a filter paper. Extracted TTF was adjusted by adding equal volume of ethanol. Beckman Coulter DU800 spectrophotometer at wavelength of 490 nm was used to determine absorbance.

3.10. PAHs extraction and determination method

PAHs extraction method was performed on soil samples for 16 priority PAHs. Alkaline saponification method was used which was already described in our previous research works (Sushkova et al., 2020). This method was first performed to check the amount of co-extracted substances such as lipids or gummy soil as well as to improve the quality of PAHs and their recovery.

To extract PAHs, 2% KOH in ethanol, 20 ml solution was prepared and added in 1 gram of dry soil sample. Resulted soil mixture was heated for 3 hours under reflux. Solution was cooled down to room temperature. Resulted supernatant was transferred to an Erlenmeyer flask. Another solution of distilled water (5 ml) and n-hexane (15 ml) was prepared and added in the flask for layers separation. Further separated mixture was put onto the rotary shaker for 10 minutes. N-hexane layer was added into separate flask. In the same way, residue was re-extracted in the flask twice. Extracted n-hexane was collected in separate small jar and washed with distilled water. Obtained solution was left in dark for five hours in a glass vessel. Five grams of anhydrous Na_2SO_4 was added to extract/removed the water from the solution. After rest of eight hours, resulting extract was added in the round-bottomed flask for evaporation until it was fully dried by using the hot rotary bath machine at temperature of 40°C. Residue was dissolved in 1ml of acetonitrile and put on shaker for 30 min.

PAHs contents were determined by external standardized method (liquid chromatography, 2008 is used to measure the benzo(a)pyrene content in all kinds of soils). Below equation is used to measure the PAHs' content:

$$\mathbf{Cs = kS_i \times C_{st} \times V / (S_{st} \times m)} \qquad \text{\_\_\_\_\_\_\_\_\_\_\_\_\_\_} \quad \mathbf{(1)}$$

Where Cst and Cs are standard solution of PAH and PAH conc. determined in samples respectively ($\mu g\ kg^{-1}$). While S_i and S_{st} representing the area of PAH of studied sample and standard solution respectively. V stands for acetonitrile volume; k stands for recovery factor and m represents to sample mass.

Concentrated extract was analyzed for the 16 PAHs included two-ring and four-ring as benzo[a]pyrene, phenanthrene, naphthalene, benzo[k]fluoranthene, benzo[b]fluoranthene, a acenaphthylene, chrysene, anthracene, pyrene, acenaphthylene, fluoranthene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, fluorine, benzo[a]anthracene and biphenyl. Samples were analyzed by high-performance liquid chromatography (HPLC) 1260 Agilent, Infinite system, Germany, equipped with reversed-phase column (150 x 4.6mm, 5 µm) Hypersil BDS C 18 with acetonitrile and ultrapure water mixture for mobile phase. This is coupled with fluorescence detector following ISO 13859, 2014).

According to the Agilent Application solution (ISP 13877, 2005), quality control of every HPLC was performed. Solution was purchased from Sigma-Aldrich (Merch). To calibrate the retention time, obtain certified refence and Calibration curves, PAH standard solutions were used and injected in machine. This produced the quantification limits (LOQs) and detection limits (LODs), proposed previously by (Minkina et al., 2019). A random component for measurement error was also estimated in concentration of 2-200 μg kg^{-1} and range 3.5 – 14%.

3.11. Chlorophyll fluorescence measurement

Chlorophyll fluorescence is main factor to determine the plant physiological state during its interaction with the different kind of contaminations. To assess, maximal quantum yield of PS II (Fv/Fm), an important parameter plays its major role. This tool determines the accurate physiological state of plants. By comparing the values between control and different treatments, we can evaluate plant's behavior under contamination.

Chlorophyll-a-fluorescence was measured in the plants after 14 and 24 days of sowing date. Before measuring, plant samples were dark-adapted in a dark room with no source of artificial light for 20 minutes. Darkness ensured that photosynthetic reactions were on ground state such as complete oxidation of PQ, QA and QB pool behind photosystem II (PS II) reaction center. Room temperature was in accordance the temperature of plant growth chamber, during the measurements. Pulse amplitude-modulated (PAM) fluorometer (model Diving PAM, Waltz, Germany) was used to measure the maximal quantum yield of PS II (photosystem II). Red measuring light was having photosynthetic photon flux density (PPFD) of 0.15 mmol photons m^{-2} s^{-1} while the white saturating flash PPFD was 1800 mmol photons m^{-2} s^{-1}. Thirty reading were taken from the fresh leaves and all the measurements were recorded in same conditions.

3.12. Statistical Analysis

Statistical processing of the obtained results was carried out using STATISTICA 7 and Sigma plot 12.5 software. The normality of the distribution in the samples was calculated using the Shapiro–Wilk test. To analyze the differences between the mean lengths of roots and stems of barley, one-way ANOVA was used, followed by the determination of differences between groups by post-hoc test Tukey HSD. While for data visualization, graphs for plate count, enzymes and PAHs and Cu data were made through Microsoft excel.

For sequence analysis, PIPITS software version 2.3 was used. Further data was assembled and annotated with the help of QIIME version 2.0.

4. RESULTS AND DISCUSSION

4.1. Effect on Overall Plant growth parameters

Using a single-factor ANOVA, significant differences were established between the morpho-biometric parameters of barley growing in the soils of various experimental variants (Table 1). It was found that the length of roots and stems in barley of the control variant is 14.9 cm and 30.4 cm, respectively (Figure 4.1). Using the Tukey criterion, at a p-level < 0.05, it was shown that plants growing on Technosols have significantly lower root and aboveground lengths compared to the control variant, with an average difference of 5.4 cm and 5.8 cm, respectively. The remediation methods used reduce the toxicity of Spolic Technosols. The best result is achieved when biochar is introduced into the Spolic Technosols together with bacteria. At the same time, the observed average values of morphometric parameters are close and there are no statistically significant differences between Technosols and control plants.

Table 4.1. Effect of pollution level on morpho-biometric parameters (root and shoot length).

Plant Parts	F	p
Root length	38,0	>0,00001
Shoot length	190,6	>0,001

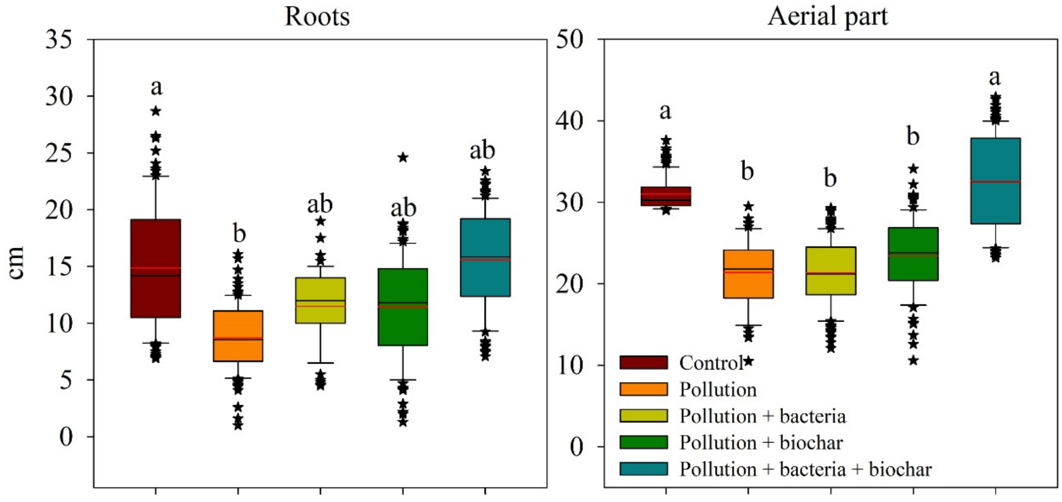

Figure 4.1. Morpho-biometric indicators of barley on various variants of the experiment

Note: Different letters indicate significant differences ($p < 0.05$) resulting from the post hoc Tukey's—honestly significant difference (HSD) test.

4.2. Colonies plate count of culturable microorganisms at different treatments level

Heterotrophic soil microbial counts were determined on the base of plate-count method (Maletić et al., 2021). Total heterotrophic microorganisms were measured in all three mediums which were prepared to analyze the fungal and bacterial colonies. Colony forming units (CFU) measurement unit was 10^{10} CFU/mL as the bacterial strains inoculation (*Pseudomonas putida* and *Rhodococcus erythropolis*) was made with this concentration for the bioremediation treatment.

Soil samples were analyzed at the end of the experiment (30 days). Plate count for fungal (mold and yeast) colonies was done at the 4 days of incubation while for bacteria and actinomycetes after 7 days. Initial concentration for P. putida was 3.81×10^{10} CFU/mL and for R. erythropolis was 4.65×10^{10} CFU/mL. Further data from plate-count was processed to check the amount of bacteria (soil suspension 10^{-4} dilution) and fungi (soil suspension 10^{-2} dilution) in per gram of soil (on the base of each media dilution).

B = V x 20 x 100 ___________ (1)

Where *V* is the value from each sample of each replication, *times 20* because the aliquot was 50 microliters; *times 100* because the dilution of initial soil was 10^2.

B x MC ___________ (2)

Where *MC* is the soil moisture content of each sample (from treatments) to calculate the amount in one gram of dry soil.

4.2.1. Effect on fungal number due to contamination of PAHs and HMs

Means values of molds in showed the highest fungi number in Spolic Technosols (polluted with PAHs and heavy metals) as compared to control. While their number is less in treatment having external bacterial strains (Figure 4.2). Mold number is more or less same in biochar treatment as well as in combined treatment of bacteria + biochar. This explained that adding of biochar does support soil microbes to increase their number but it is still less than alone biochar present in treatment.

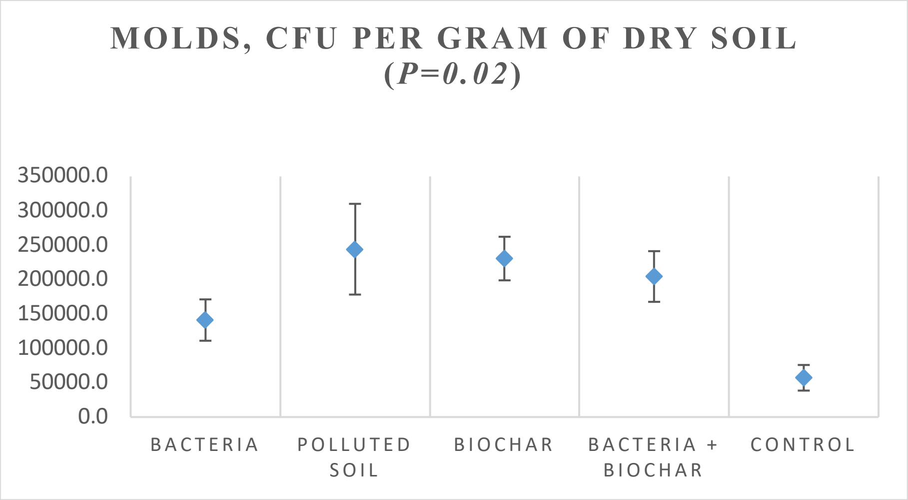

Figure 4.2. Amount of molds in Spolic Technosols (polluted soil) separately with bacterial strains, biochar and combined with biochar.

4.2.2. Effect on bacterial and actinomycetes in the presence of co-contamination

When bacterial and actinomycetes colonies (Figure 4.3 and Figure 4.4) were measured in all the treatments, it was found that number of the bacteria and actinomycetes is more in bacterial strains variant. Which showed that bacteria continue to grow in presence of PAH degraders and heavy metal tolerance. This showed that bacterial strains do have effect as compared the soils. While in both media, biochar didn’t show the great number of bacterial and actinomycetes colonies.

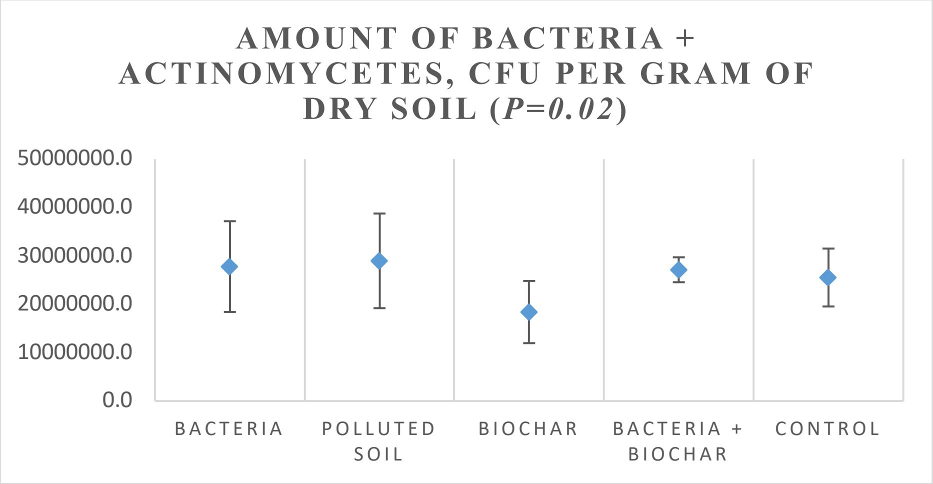

Figure 4.3. Amount of bacteria and actinomycetes in Spolic Technosols (polluted soil) separately with bacterial strains, biochar and combined with biochar.

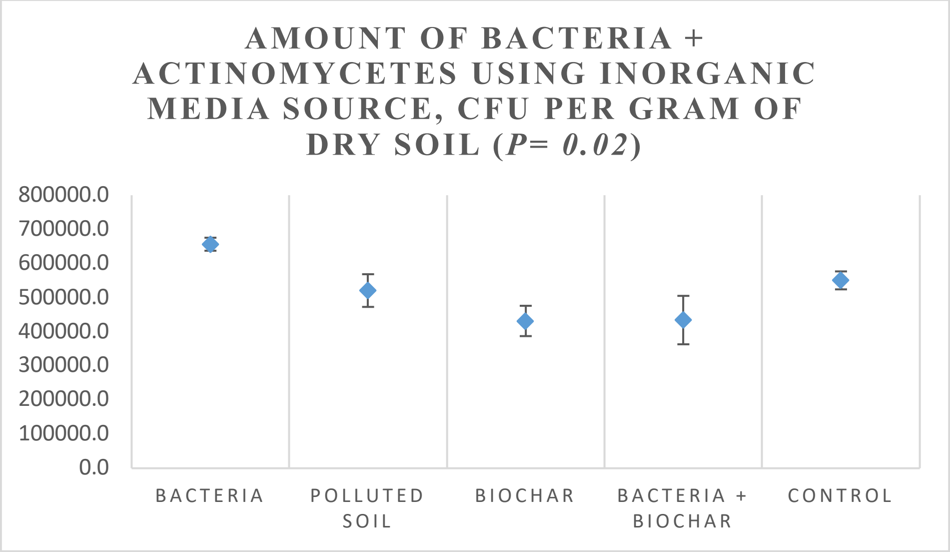

Figure 4.4. Amount of bacteria and actinomycetes in presence of inorganic media source in Spolic Technosols (polluted soil) separately with bacterial strains, biochar and combined with biochar.

With the addition of chemical oxidants, the quantity of indigenous bacteria in the soil fell at first, then grew. On the impact of chemical oxidants on bacteria, there are two points of view. One is that chemical oxidation can result in unfavorable environmental circumstances (pH and oxidation potential) that prevent typical microorganisms from growing and functioning properly.

Bacteria studies are consistent with their studies that found the same bacterial effect during the experiment in not only co-contaminated soils but also in oil, petroleum and diesel contaminated soil (Machado et al., 2020; Varjani & Upasani, 2019; G. Zhang et al., 2018)having high number PAHs. Therefore, these studies showed that bacterial strains do have an impact in soil bioremediation but the effect is less in combined treatment with biochar.

4.3. Polyaromatic hydrocarbons and heavy metal analysis in all the variants of experiment

PAH decomposition constant in the soil (K_p, g^{-1}) was calculated according to the equation: $K_p = - \ln (C_t / C_{ref})/t$, where C_{ref} and C_t are concentrations of BaP in the soil: initial and experiment, t-time. The half-life of PAHs in soil (T_{50}) was calculated by the equation: $T_{50} = 0.693/ K_p * 364$ (days).

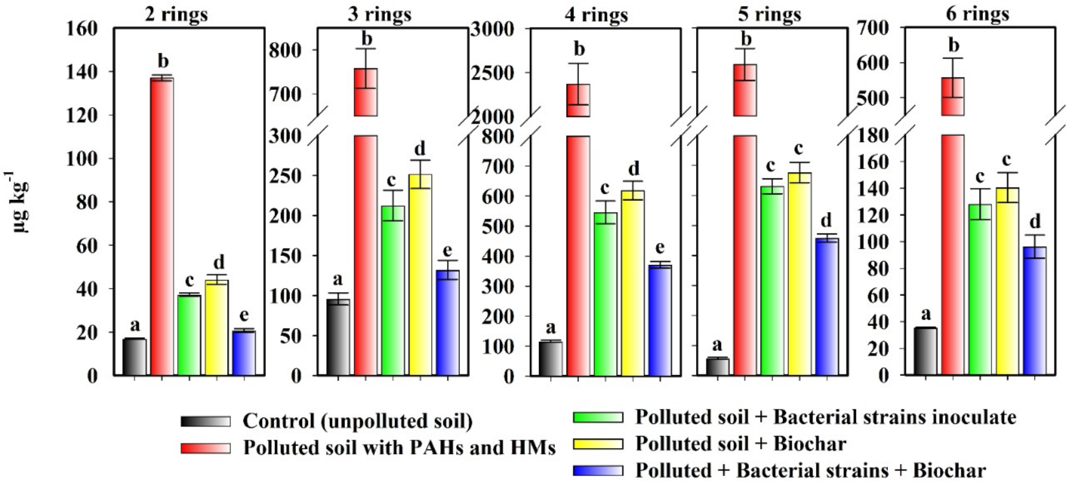

Figure 4.5. The content of PAHs of various annularity in spolic technosols when bacteria are introduced separately and together with biochar

Note: tank - bacteria; letters indicate significant differences ($p < 0.05$) obtained as a result of calculating Student's t-test

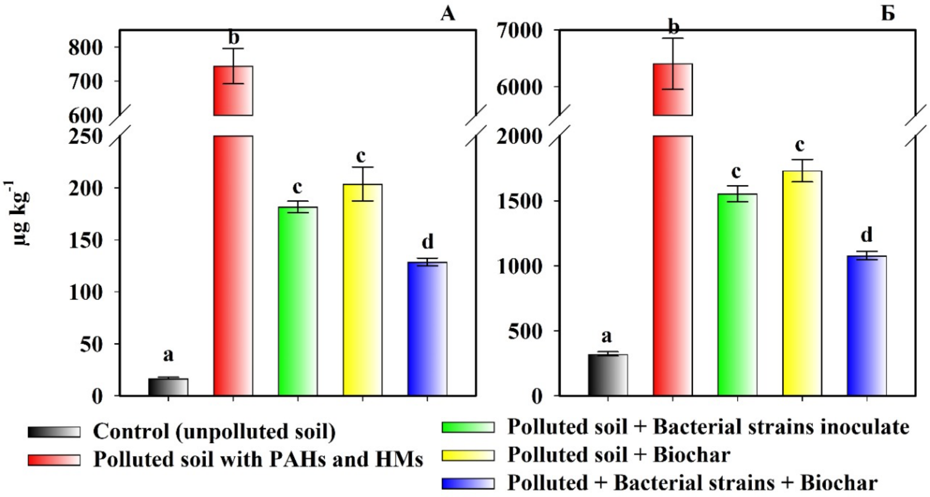

Figure 4.6. The content of benzo(a)pyrene (A) and the total content of 16 priority PAHs in spolic technosols when bacteria are introduced separately and together with biochar

Note: tank - bacteria; letters indicate significant differences ($p < 0.05$) obtained as a result of calculating Student's t-test

Table 4.2. Amount loosely bound compounds present in polluted soil with addition of bacterial strains separately and with the combined biochar.

Treatments	Total content, mg/kg	Loosely bound compounds, mg/kg			
		Exchange	Complex	Specific sorption	Amount of HC, % of the total content
Control	57.6±2.3	0.5±0.03	2±0.1	4.8±0.3	13
Polluted Soil	2256±90.2	163±9	290±16	315±18	34

Bacteria	2304±96.8	147±8	273±15	298±17	31
Biochar	2312±92.5	55±3	137±8	160±9	15
Bacteria + Biochar	2258±90.3	27±2	91±5	165±9	13

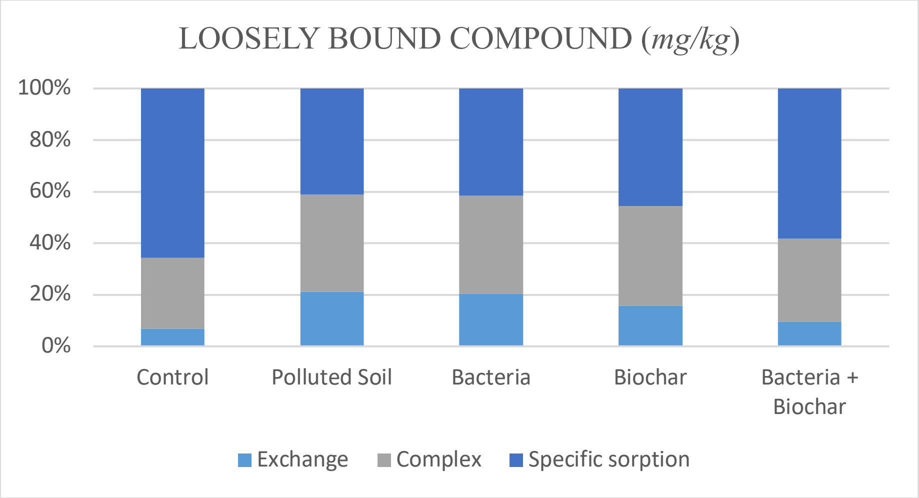

Figure 4.7. Percentage of the loosely bound compounds in all treatments.

Table 4.3. Amount of BaP, total PAH and Cu in above ground (shoot) and below ground (roots) barley.

Treatments	BaP		PAH amount		Cu	
	Root	Shoot	Root	Shoot	Root	Shoot
Control	1.5±0.1	1.1±0.1	125±5.6	74±3.6	11.8±0.8	9.5±0.7
Polluted Soil	85±3.0	41±1.7	954±50.1	568±45.5	90.6±6	62.4±4
Bacteria	30±0.9	26±1.2	278±12.3	194±8.4	52.7±4	29.8±2
Biochar	35±2.3	25±1.2	301±14.7	206±6.3	74.5±5	53.1±4
Bacteria + Biochar	28±1.0	15±0.5	154±6.5	62.3±2.8	35.1±2	21.5±1

Note: Bold indicates excess of MRL Cu (30 mg/kg)

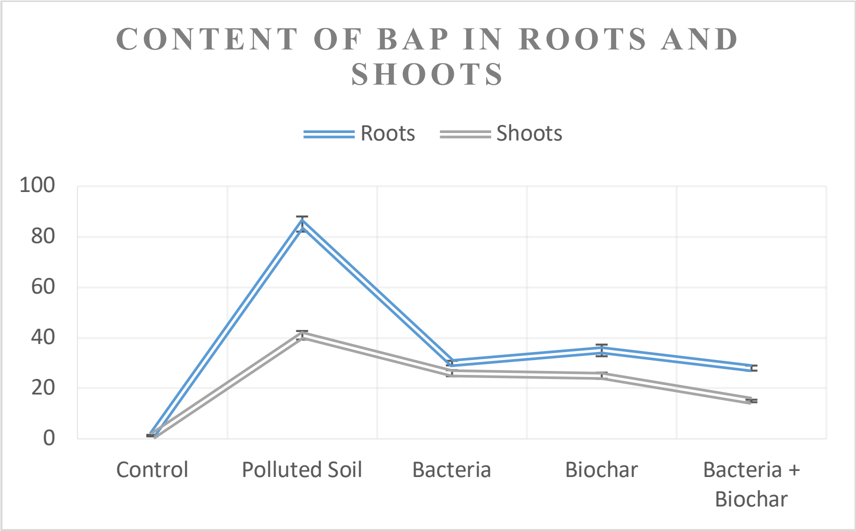

Figure 4.8. BaP content in roots and shoots of barley plant.

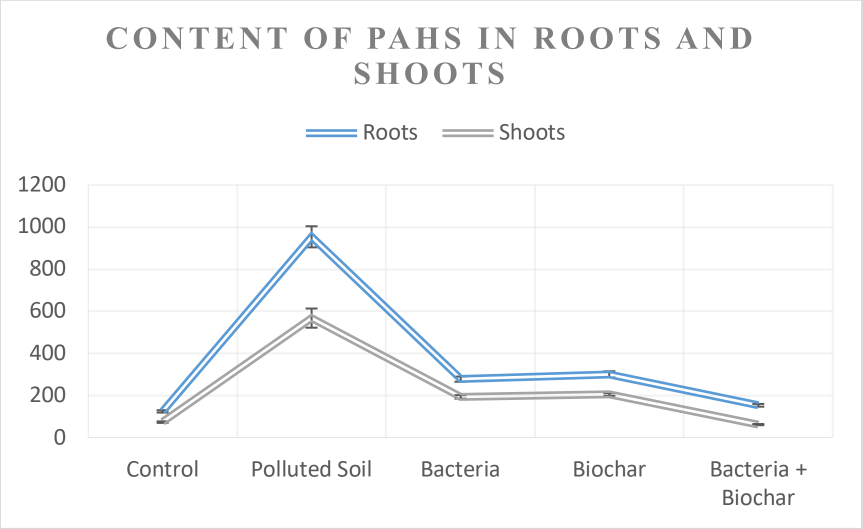

Figure 4.9. Total PAHs content in roots and shoots of the barley plant

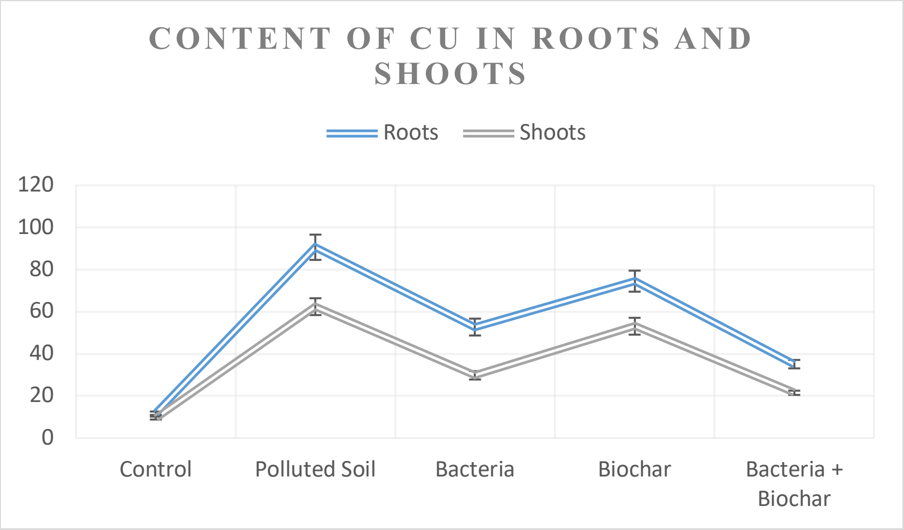

Figure 4.10. Copper content in roots and shoots of the barley plant.

Table 4.4. Decomposition constant (Kp) and half - life (T50) of BaP and sums of 16 PAHs in Technosols when bacteria are introduced separately and together with biochar

Treatments	Benz(a)pyrene		16 PAHs	
	K_p, g^{-1}	T_{50}, days	K_p, g^{-1}	T_{50}, days
Control	0.02	14922	0.04	6465
Polluted Soil	0.06	2567	0.07	1511
Bacteria	1.46	172	1.49	170
Biochar	1.35	187	1.38	183
Bacteria + Biochar	1.80	139	1.85	136

Table 4.5. Decomposition constant (K r) and half-life (T 50) of PAHs in polluted soil when bacteria are added separately and together with biochar

Treatments Constant, g^{-1}	Number of benzene rings PAH molecule				
	2	3	4	5	6
Control	0.07	0.04	0.05	0.03	0.02
Polluted Soil	0.07	0.07	0.08	0.07	0.03
Bacteria	1.38	1.34	1.55	1.48	1.49
Biochar	1.21	1.17	1.43	1.41	1.40
Bacteria + Biochar	1.96	1.85	1.90	1.80	1.78
T_{50}, days					
Control	3613	6795	5170	9565	14836
Polluted Soil	3398	3568	3000	3574	10031
Bacteria	183	188	163	170	169
Biochar	209	215	177	179	180
Bacteria + Biochar	43	46	43	47	47

Table 4.6. KN coefficient and acropetal coefficient (AK) of PAH, BaP and Cu in barley plants growing on Technosols when bacteria are introduced separately and together with biochar

Treatments	KN	AK	KN	AK	KN	AK
	BaP		PAH amount		Cu	
Control	0.09	0.73	0.39	0.59	1.6	0.8
Polluted Soil	0.11	0.48	0.15	0.60	0.1	0.7
Bacteria	0.17	0.87	0.18	0.70	0.1	0.7
Biochar	0.17	0.71	0.17	0.68	0.1	0.6
Bacteria + Biochar	0.22	0.54	0.14	0.40	0.1	0.6

The effectiveness of the complex method of remediation using biochar and PAH-degrading bacteria has been proven describe regularities of PAHs reduction in soil. It has been established that, in general, the introduction of strains of degrading microorganisms has a more effective effect on the reduction of PAHs in the soil than biochar. For Cu , no significant changes were found upon the introduction of bacteria, while the introduction of a carbonaceous sorbent led to a decrease in the proportion of HC metal compounds by 15%. The best effect was achieved with the combined use of bacteria and biochar on Technosols, where the total content of 16 PAHs and BaP is reduced by 5.8-6.0 times, respectively. At the same time, the mobility of Cu becomes comparable with its mobility in uncontaminated soil (Figure 4.5 – 4.7 , Table 4.2). The decomposition constant of BaP reaches 1.8 g $^{-1}$, which corresponds to the half- life of the pollutant (T $_{50}$) 137 days. C decrease in the content of the most dangerous representative of polyarenes-BaP in Technosols depends on the method of soil bioremediation and forms the following sequence according to T $_{50}$: “Technosols + bacteria + biochar” (139 days) > “Technosols + bacteria” (172 days) > “Technosols + biochar "(187 days) > "Technosols" (2567 days).

Due to the most effective removal of pollutants in Spolic Technosols with the combined introduction of bacteria and biochar, plants growing under these conditions accumulate less pollutants than when only one of the components is remediated in the soil. Thus, the content of Cu in the roots of barley decreased by 61%, and in the aerial part - by 66% and does not exceed the MRL. At the same time, the total content of PAHs is close to the control values in the root (154 ng/g) and above-ground (62 ng/g) parts, and the accumulation of BaP is significantly reduced, but does not reach the control variant (Tables 4.3 - 4.6).

4.4. Relation between the chlorophyll fluorescence and co-contamination of PAHs and heavy metal

It was found that in the first period of shooting (14 days) of photosynthesis parameters in barley of the control variant of the experiment, the maximum quantum yield (Fv / Fm) was 0.81, which is the norm for higher plants (Maxwell1 & Johnson2, 2000). With soil contamination, a significant decrease in Fv / Fm was recorded in barley relative to control plants. This indicates damage to photosystem II reaction centers (Wan et al., 2011). The use of biochar and bacteria separately or in combination in Technosols contributes to an increase in the maximum quantum yield to the level of control values (Figure 4.11).

In the second survey period, a decrease in Fv / Fm is observed , especially for the variant without the use of biochar or bacteria (up to 0.60). The use of remediates contributes to a less intensive decrease in this indicator. At the same time, the best result, close to the control, was achieved when using biochar in combination with bacteria.

4.4.1 Maximum quantum yield efficiency

The maximum quantum yield of a photosystem, defined as the ratio of the number of quanta used in charge separation to the total number of quanta absorbed by the light-collecting complexes. The effective quantum yield of a photosystem shows the efficiency of the functioning of the photosynthetic apparatus - part of the excitation energy of the chlorophyll molecules that make up the photosystems used in the process of electron transport (Matorin et al., 2019). Thus, the observed decrease in the maximum quantum yield with an increase in the effective quantum yield of barley may indicate the adaptation of plants to soil pollution in time.

In general, trends in the responses of plant photosynthetic activity to stress caused by soil pollution obtained for Fv / Fm and photosystem II quantum yield efficiency (Y II) are comparable with each other. The negative effect of PAHs on the Y(II) value may be due to the degradation of photosynthetic pigments or a decrease in the rate of biosynthesis of these pigments, for example, due to the inhibition of enzymes of the chlorophyll biosynthesis pathway, such as NADPH-protochlorophyllide – oxidoreductase (Oguntimehin et al., 2008). However, Y II is a less sensitive parameter for assessing barley stress when soil is contaminated . There is an increase in the value of Y II when applying biochar to polluted soils

By the second measurement period (28 days), there is an increase in the parameter Y II compared with the first measurement period (14 days). At the same time, in variants with the introduction of biochar and bacteria into Technosols Y II in plants is comparable to the control variant.

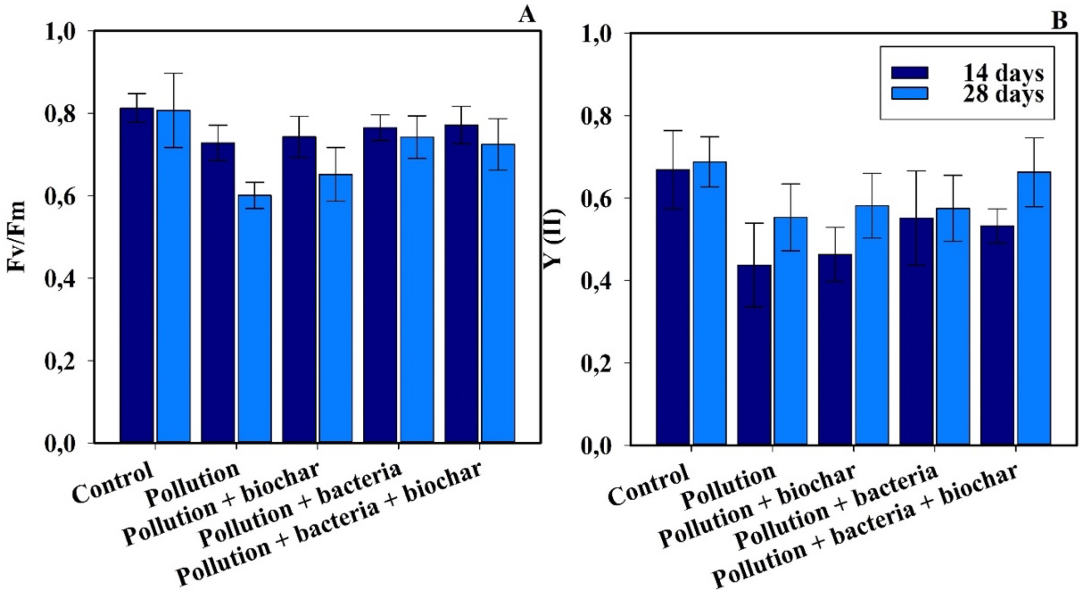

Figure 4.11. Maximum quantum yield of photosystem II (A) and effective quantum yield of photosystem II (Y II) in barley in various variants of the experiment for two periods of measurements

4.5. Effect of different treatments on Enzyme activity

Invertase, catalase, peroxidase, urease and dehydrogenase were measured to characterize the dynamic changes of microbial activity induced by soil additives. Soil biological activities are heavily effected with the addition of biochar as well as the microbes. Biochar helps in upregulating pH, oxygen content and microbial number as providing the space to grow and interact with other microbes, here specifically PAH degrader and ones' helping in mitigating heavy metal content.

4.5.1. Urease activity

Among all the treatments, polluted soil with addition of 2 bacterial strains showed the highest activity while remediated combination (bacteria + biochar) showed the lowest activity (Figure 4.12). This trend has also seen in another study(Guo et al., 2012).

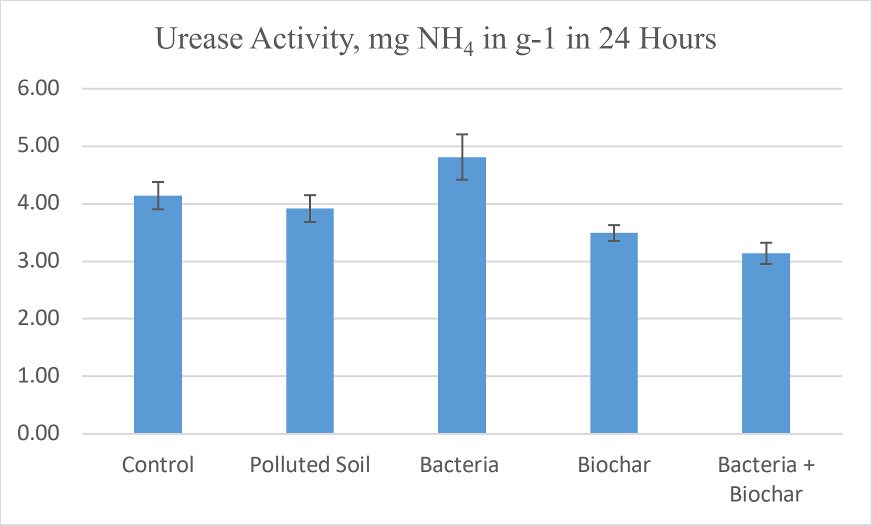

Figure 4.12. Urease activity in Spolic Technosols soil when bacterial strains added separately and combined with biochar

4.5.2. Catalase activity

For this enzyme, variant with addition of bacterial strains showed the high enzymatic activity as compare to control and others while polluted soil showed the least (Figure 4.13).

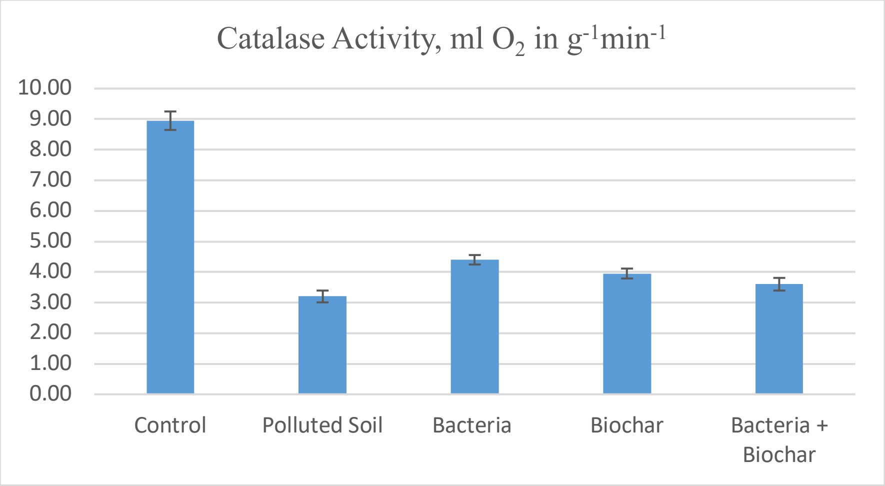

Figure 4.13. Catalase activity in Spolic Technosols soil when bacterial strains added separately and combined with biochar

4.5.3. Invertase activity

Treatment with combined application of biochar and bacteria showed the highest activity in soil as compared to control. Both biochar and bacterial strains increased the enzyme activity and boost up microbial population in this soil (Figure 4.14).

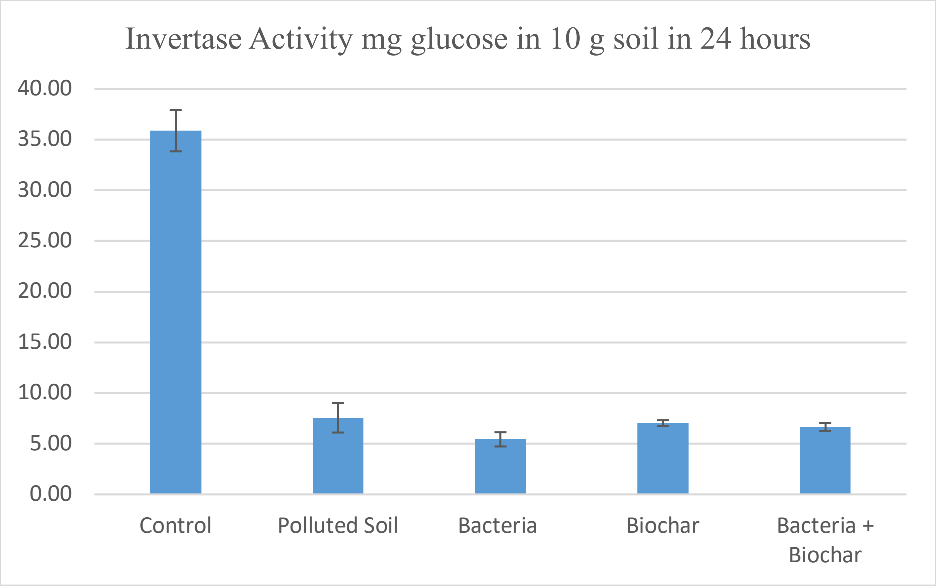

Figure 4.14. Invertase activity in Spolic Technosols soil when bacterial strains added separately and combined with biochar

4.5.4. Peroxidase activity

This enzyme showed the different behavior as of urease and catalase. Here instead of bacteria, only biochar treatment showed the significant effect in soil biologically properties. While bacterial strains variant showed the least activity (Figure 4.15).

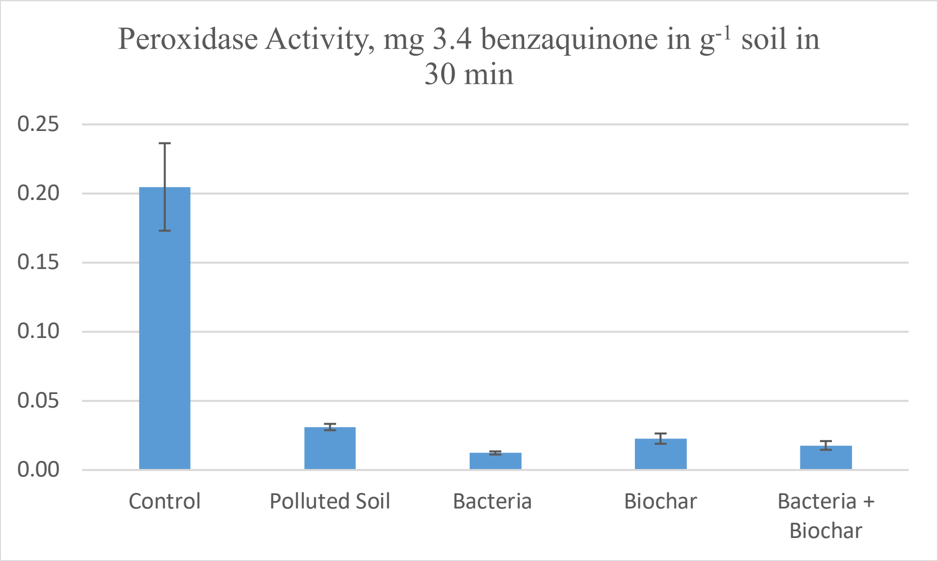

Figure 4.15. Peroxidase activity in Spolic Technosols soil when bacterial strains added separately and combined with biochar

4.5.5. Dehydrogenase activity

For this analysis, one results was lost in combined application of biochar and bacteria. While in the rest of the treatments, activity was very less that it cannot be compared properly. However, if we compare the rest of the treatments (Figure 4.16), it was found that variant with bacterial strains showed the high dehydrogenase activity and biochar treatment showed the least activity, which is consistent with other studies (Dai et al., 2020; Gran-Scheuch et al., 2020; Paneque et al., 2020) .

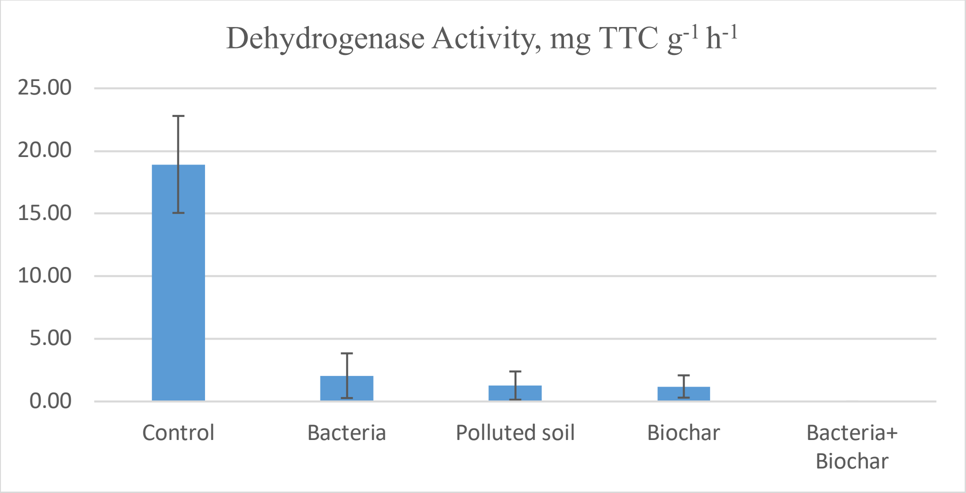

Figure 4.16. Dehydrogenase activity in Spolic Technosols soil when bacterial strains added separately and combined with biochar

The reduction of soil enzymes activity caused by biochar may be attributed to various reasons like, biochar addition directly harms microorganisms, which affect enzymes production (C. Wu et al., 2021).

Conversely, in treatment with combined application of biochar and bacterial strains increase in enzyme activities induced by biochar were likely associated with increased substrate availability and microbial population, while the highly variability in soil enzyme activities in combinations treatment may be linked to the interaction as per study (Tang et al., 2020). However, there are also some studies showed that biochar activated soil enzyme activities such as urease and invertase The addition of biochar and bacterial strains significantly improved soil properties, which might in turn affect soil enzyme activities.

4.6. Metagenomics analysis and contamination effect on fungal communities

A universal primer pair was used on the ITS 2 region, which is the internal transcribed spacer (ITS) region:

Forward : GCATCGATGAAGAACGCAGC

Reverse : TCCTCCGCTTATTGATATGC

Sequence analysis was carried out using PIPITS software . v.2.8 (Gweon et al., 2015) and QIIME2 v.2022.2 (Bolyen et al., 2019.). Paired readings were combined by examining overlapping sequence regions. Next, the selected subregion (ITS 2 region) is extracted, if necessary, which is reoriented in the direction 5' - 3', these operations are carried out using the HMMER 3 plugin (Mistry et al., 2013), which not only extracts the subregion of interest, but also compares sequences with a set of patterns built from a number of different subregions of ITS sequences . Filtering by quality is accompanied by the removal of short reads (<100bp) and singletons . Grouped sequences with a 97% similarity threshold, representative sequences for each cluster are subjected to detection and removal of chimeras. The taxonomy is assigned using the RDP classifier v . 05/10/2021, based on the UNITE database .

To assess biological diversity, alpha and beta diversity indices were calculated.

Alpha Diversity:

1) Information-statistical - Shannon's index (Shannon, 1948);
2) Dominance index - Pielou index
3) Qualitative index - Observed OTUs .

Beta Diversity:

1) UniFrac method (Lozupone et al., 2010) in two versions – " unweighted " and " weighted " (Catherine & Rob, 2005).

The results in this study are presented using PCA multivariate statistics – principal components analysis (principal component analysis). Statistical analysis of the significance of differences between groups was carried out using the nonparametric ANOVA / MANOVA (PERMANOVA) method.

Statistical analysis of the data was performed using the non-parametric Kruskal -Wallace test (H -test), which is a generalization of the Mann-Whitney U -test (Kruskal & Wallis, 1952).

Spearman 's analysis was applied to determine the correlation between the abundance of microbial taxa and soil characteristics. Spearman's rank correlation coefficient is applied to samples that do not follow the normal distribution law (Daniel, 1990).

The average number of unprocessed fungal sequences per sample exceeded 290,000, ranging from 34,021 to 448,978. After quality filtering, the average number of sequences per sample was 212,000. The average sequence length after processing was 200 nucleotide bases. The number of sequences with subregion ITS 2 was 1,068,805, from which 546 operational taxonomic units were formed, which belong to 208 phylotypes .

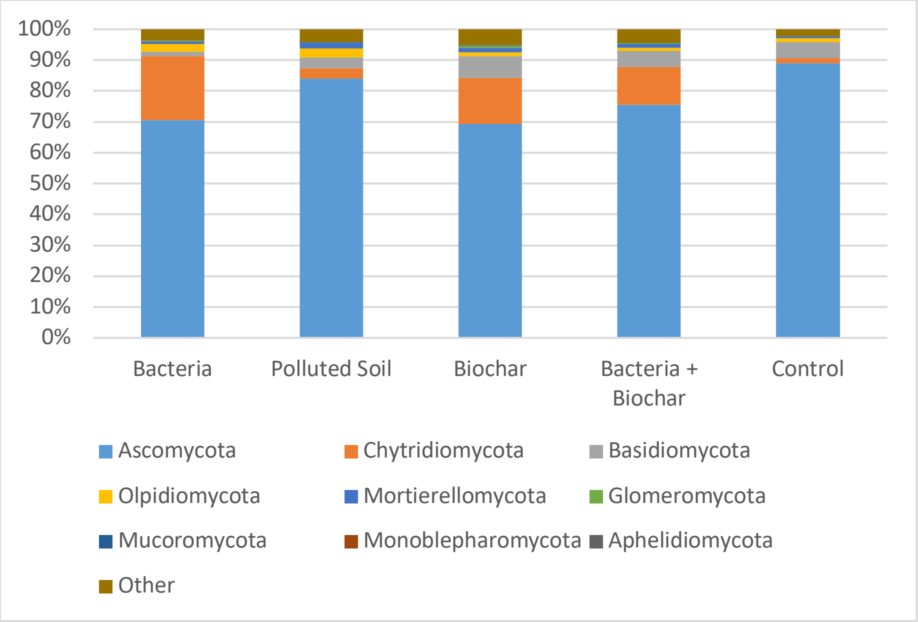

Figure 4.17. Taxonomic structure (at the phylum level) of fungal communities

Fungal communities are formed mainly by the phyla *Ascomycota* , *Chytridiomycota* , *Basidiomycota* and *Olpidiomycota* (Figure 4.17). The *Ascomycota* group has the largest shares in microbiomes , which occupies from 69 to 89% of the total fungal diversity in the samples. They reach their maximum in the chernozem sample . Representatives of the phylum *Chytridiomycota* also have differences in samples - in bacterial variant, their number is 20%, as compared to control (Chernozem soil) which has only 1.6%. *Basidiomycota* present in treatment biochar and in Bacteria + Biochar 6.9% and 5.2%, respectively. The proportion of other groups of organisms are relatively similar in all soil samples. It is interesting to note that the phyla *Monoblepharomycota* and *Aphelidiomycota* found only Spolic technosols soil and Bacteria + Biochar variant, respectively.

When conducting a correlation analysis ($p < 0.05$), high correlations were found between the distribution of the share of OTU in the samples by taxa and soil properties only among the phyla *Ascomycota* , *Mortierellomycota* , and *Monoblepharomycota* . The distribution of *Ascomycota* in the samples has a negative dependence on the content of Ni in soils ($r = -0.72$, $p < 0.05$), while the distribution of *Monoblepharomycota* has a positive dependence on the Cu content ($r = 0.73$, $p < 0.05$). In the *Mortierellomycota group* , a more complex dependence of the abundance of these organisms in soil samples was found, so a high positive correlation was found with the Cu content ($r = 0.84$, $p < 0.05$) and a high negative correlation with the Cd content ($r = -0.73$, $p < 0.05$).

4.6.1. Fungal taxonomic – Alpha and Beta diversity assessment

The assessment of fungal diversity at the family level showed that all 5 samples are characterized by high taxonomic diversity. Figure 2 shows the taxonomic structure of the communities, which shows the 17 most dominant families in the communities, all other families occupy less than 1% of the communities and are grouped into the “Other” group. The most dominant family is *Aspergillaceae* , whose representatives in the studied communities occupy from 14 to 23% of the total fungal diversity. Representatives of the *Rhizophlyctidaceae family vary* significantly in samples, their number is maximum in bacterial variant - 16.2%, and minimum in Chernozem (control) - 0.5%. The *Chaetomiaceae group* has an interesting distribution in the studied samples, so in Technosols soil and Chernozem they occupy 11.2% and 9.3%, respectively, of the total microbial diversity, but in variants bacteria, biochar and combined application of bacteria + biochar, their number is approximately the same and varies from 0 .5% to 1.1%. The proportion of other groups is relatively the same in all five samples.

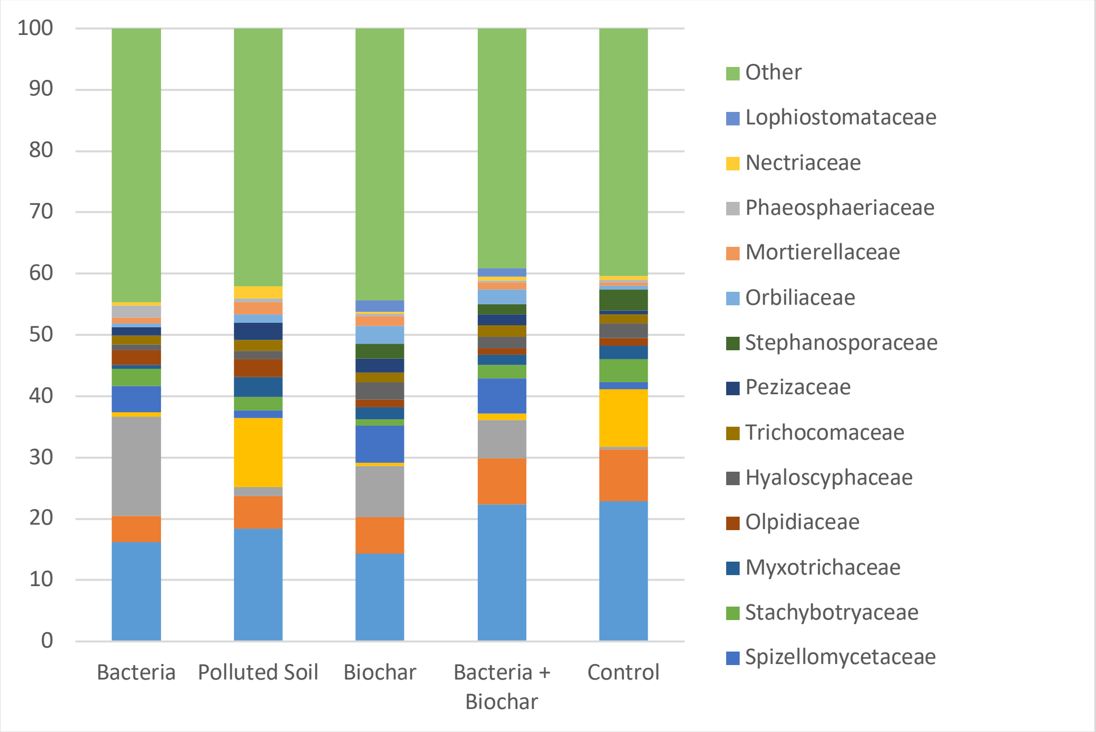

Figure 4.18. Taxonomic structure (at the family level) of fungal communities

In the samples, there is a non-uniformity of the presented taxa in the samples, therefore, the assessment of alpha diversity (Table 4.7) was carried out using the

Shannon index. It was revealed that all samples are characterized by a high abundance of species and a smaller difference in the number of representatives of various taxa. These results are also confirmed by the Observed index. OTUs , as well as the Pielu index.

Table 4.7. Alpha diversity of the studied soil communities

Sample / index	Pielu index	Shannon index	Observed OTUs
Bacteria	0.62	4.9	229
Polluted soil	0.68	5.4	249
Biochar	0.70	5.4	215
Bacteria + Biochar	0.70	5.4	222
Control	0.66	5.1	210

Table 4.8. Results of the Kruskal-Wallis analysis in soil samples as well as for heavy metals

	Shannon index	Pielu index	Observed OTUs
Soil Type	H= 0, 5; p= 0.48	H= 0, 5; p= 0.48	H=2; p= 0.15
Total PAHs	H= 3, 2; p=0, 20	H= 3, 2; p=0, 20	H= 2, 13; p= 0.34
Mn	H= 0.5; p= 0.48	H= 0.5; p= 0.48	H= 2; p=0.1 6
Ni	H= 0.5; p= 0.48	H= 0.5; p= 0.48	H= 2; p=0.1 6
Pb	H= 0.5; p= 0.48	H= 0.5; p= 0.48	H= 2; p=0.1 6
Zn	H=0.33; p=0.56	H=0; p= 1	H=1.33; p= 0.25
Cu	H= 0.5; p= 0.48	H= 0.5; p= 0.48	H= 2; p=0.1 6
Cd	H= 0.5; p= 0.48	H= 0.5; p= 0.48	H= 2; p=0.1 6

The search for the statistical dependence of metagenomic diversity on the main soil characteristics showed (Table 4.8) that, according to the value of test statistics (H) and the achieved level of statistical significance (p), none of the indicators has the required level of reliability ($\alpha < 0.05$). This indicates the absence of dependence of microbial diversity on soil characteristics; therefore, we cannot speak of a clear dependence of alpha diversity on soil environmental conditions.

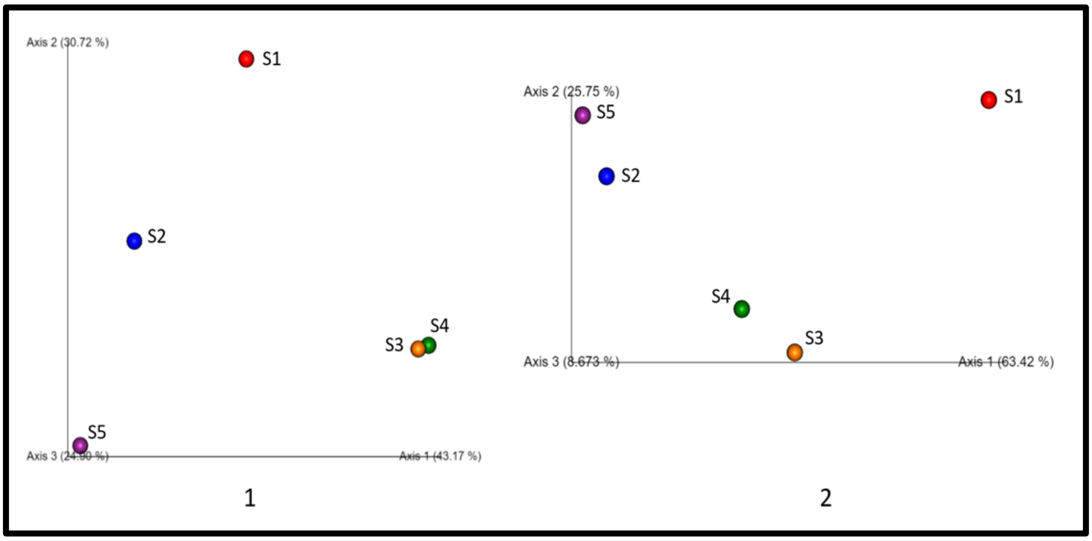

Figure 4.19. Principal Cordinate Analysis (PCoA) " unweighted " (1) and " weighted " (2) for fungal communities. S1 - Bacterial variant, S2 – Polluted soil, S3 – Biochar variant, S4 – Bacteria + Biochar and S5 – control.

Beta diversity assessment was performed using UniFrac 's " unweighted " and " weighted " method , the results of which were visualized on Principal Coordinate Analysis (PCoA) plots. Figure 4.19 presents the data of " unweighted " and " weighted " analyzes of the composition and structure of soil microbial diversity depending on the main soil characteristics. The total diversity recorded by the top two principal coordinates was 73.89% and 89.17%, respectively. Reliable formation of clusters is not observed on any of the graphs, which is confirmed by the PERMANOVA test, according to which the average test values for " unweighted " are F =1.39, R^2 =1.56, p =0.32, and for " weighted " F = 1.07, R^2 = 0.46, p = 0.44, therefore, we do not observe significant differences or similarities in the groups. However, these results should be interpreted with caution, given the limited sample size in this dataset, hence more research is required.

5. CONCLUSION

The current research was designed to find out the potential method for soil bioremediation of co-contaminated soils present in southern region of Russia. Experiment was conducted using bacterial strains and biochar to see the effect of these components on enzymatic activities, PAHs, HMs content in both soil and plant and change in soil microbial communities. It is concluded from the above results and discussion derived from plant morphometric data, PAHs total concentration, BaP and Cu amount in roots and shoots of barley plant as well as in soil showed the promising results in the variant bacterial strains and biochar. This variant showed the least accumulation amount of PAHs, BaP and Cu in roots and shoots. On contrary, Heterotrophic microbial number (molds and bacteria) was more in bacterial variant i.e., 40% more bacterial and actinomycetes in bacterial variant. PS II activity was less affected in bacterial and biochar variant.

As far as, results from microbiome study conducted through 16s rRNA are concerned, results were only discussed form fungal community not bacterial community. We found that fungal diversity with high percentage of phyla *Ascomycota* , *Chytridiomycota* and *Basidiomycota* in bacterial variant as compared to the other treatments. It also supports the results of enzyme analysis where the same variant showed the high enzymatic activity. Alpha and beta diversity showed that addition of external bacteria and biochar do have some effect on overall fungal community in the soil. But the results were not significant that revealed the minimal dependence of communities on soil characteristics. Correlation studies showed high correlations between the distribution of the share of OTU in the samples by taxa and soil properties only among the phyla *Ascomycota* , *Mortierellomycota ,* and *Monoblepharomycota*.

Application of biochar amendment have an effect on the contaminated soils in remediating the effect of heavy metals and PAHs. From these results we can say that biochar and bacteria have effect in remediating the effect of pollution/contamination in the Spolic Technosols soils when compared with the control soil which is chernozem. But we recommend further studies to explore the bacterial communities to understand this phenomenon properly.

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