Université de Montréal

Phytoremediation of soil contaminated with petroleum hydrocarbons and trace elements

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Résumé

L'urbanisation rapide et les activités industrielles ont abouti à la contamination de l'environnement par les hydrocarbures pétroliers (HP) et les éléments traces (ET). Ces composés sont particulièrement toxiques en raison de leur persistance dans l'environnement, et de leurs effets toxicologiques sur les êtres vivants quand les concentrations de ceux-ci dépassent des seuils critiques.

Les préoccupations de plus en plus croissantes sur la contamination de l'environnement ont favorisé le développement de plusieurs technologies de remédiation des sites contaminés par les approches biologiques, physico-chimiques et par l'excavation et l'entreposage. Dans cette thèse, des options douces d'assainissement des sols (ODA) ont été utilisées à différentes échelles pour la r*emédiation* des sols contaminés par des mélanges des HP et des ET.

Dans la première partie de ma thèse, des expériences en laboratoire ont été effectuées dans le but de caractériser les sols contaminés et les micro-organismes autochtones (bactéries et champignons) qu'ils contiennent. Malgré la contamination ancienne du sol, les résultats obtenus montrent des effets négatifs des contaminants sur le développement des lombrics et la biomasse de *Lepidium sativum*. En outre, une respiration élevée de microorganismes, attribuée à la transformation / minéralisation de la matière organique et / ou des polluants organiques a été observée. Cette présence de micro-organismes viables dans les sols contaminés suggère leur adaptation aux contaminants. Toutefois, d'autres résultats ont montré que l'exposition à long terme des microorganismes du sol à de fortes concentrations en HP et le type de milieu de culture utilisé pour l'isolation n'influencent pas la capacité des isolats microbiens à dégrader efficacement les HP. Cette capacité de biodégradation des HP est liée à la phylogénie des microorganismes.

Dans la deuxième partie de cette thèse, les études préliminaires en serre ont été réalisées dans le but d'évaluer l'efficacité de phytoremédiation en utilisant *Medicago sativa* assistée par l'ajout du compost. Les résultats ont montré dans cette expérience que l'ajout du compost dans le sol favorise la dégradation des HP, la croissance et la survie de *M. sativa*, ainsi que la phytoextraction des ET. L'évaluation des risques résiduels après la phytoremédiation a

également montré un effet positif de l'amendement du sol en compost sur la croissance des plantes et le développement des lombrics.

L'expérience pilote réalisée sur le terrain dans la troisième partie de ma thèse a permis une réduction de 80% des HP et de 20% des ET après 17 mois.

Ma thèse a démontré que la luzerne (*M. sativa*) et le tournesol (*Helianthus annus*) sont des choix judicieux de plantes pour la phytodégradation des HP et pour la phytoextraction des ET. Les résultats qui en résultent sont utiles pour d'autres études de phytoremédiation à grande échelle.

Mots-clés : Hydrocarbures Pétroliers, Eléments Traces, Options d'Assainissement Douces, Hydrocarbures Aromatiques Polycycliques, Respirométrie, Ecopile, Bactéries, Champignons.

Abstract

The rapid urbanization and industrialization has led to an increase of disposal petroleum hydrocarbons (PHC) and trace elements (TE) into the environment. These pollutants are considered as the most toxic contaminants in the world due to their persistence in the environment, and the long range of toxicological effects for living beings when their concentrations exceed critical thresholds.

Recent concerns regarding the environmental contamination have initiated the development of several remediation technologies, including physico-chemical, biological and *Dig and Dump* approaches. In my thesis, gentle soil remediation options (GRO) were investigated at different scales for the reclamation of PHC and TE co-contaminated soil.

In the first part of my thesis, laboratory experiments were performed to characterize PHC and TE contaminated soil as well as the indigenous microorganisms (bacteria and fungi) present in these contaminated soils. It was found that the studied aged contaminated soil had a negative effect on earthworm's development and *Lepidium sativum* biomass. Moreover, a high respiration of microorganisms attributed to the transformation/ mineralization of organic matter or/and organic pollutants was observed. This presence of viable microorganisms suggested an adaptation of microorganisms to the contaminant. Further results showed that the long-term exposure of soil microorganisms to high PHC concentration and the type of isolation culture media did not influence the ability of isolates to effectively degrade PHC. However, phylogenic affiliation had a strong effect on PHC biodegradation.

In the second part of my thesis, preliminary studies in greenhouse trials were performed to investigate the ability of *Medicago sativa* assisted by compost in the greenhouse aided-phytoremediation of PHC and TE. The results clearly showed that compost amendment into the soil promoted PHC degradation**,** *M. sativa* growth and survival, and phytoextraction of TE. Residual risk assessment after the phytoremediation trial also showed a positive effect of compost amendment on plant growth and earthworm development.

Pilot-scale ecopile experiment carried out in the third part of this thesis allow a reduction of up to 80% of PHC and 20% of metals after 17 months.

My thesis showed that alfalfa (*M. sativa*) and sunflower (*Helianthus annus*) plants were suitable for phytodegradation of PHC and phytoextraction of TE. The outcomes of my thesis

can be extend to other plants and they bring a new level of understanding that can be helpful for further full-scale phytoremediation studies.

Keywords: Petroleum Hydrocarbon, Trace Elements, Gentle Soil Remediation Options, Polycyclic Aromatic Hydrocarbon, Respirometry, Ecopile, Bacteria, Fungi.

Svensk sammanfattning

Den snabba urbaniseringen och industrialiseringen har lett till en ökning av petroleumkolväten (PHC) och olika spårämnen (TE) i miljön. Dessa föroreningar anses vara de mest giftiga föroreningarna i världen på grund av att de stannar kvar i miljön samt att de har toxikologisk påverkan på levande varelser.

På senare tid har oron för dessa miljöföroreningar lett till utvecklingen av flera saneringstekniker, såsom fysiska, kemiska och biologiska metoder. I denna avhandling undersöktes enkla marksaneringsalternativ (GRO) på olika nivåer, för återvinning av PHC och TE från förorenad jord.

I den första delen av denna avhandling, utfördes laboratorieförsök för att karakterisera PHC- och TE-förorenad jord samt av de inhemska mikroorganismerna (bakterier och svampar) som förekommer i dessa förorenade jordar. Det konstaterades att den studerade förorenade jorden hade en negativ inverkan på daggmaskars utveckling och biomassan av *L. sativum*. Dessutom kunde den höga respirationen bland mikroorganismerna tillskrivas omvandlingen och mineraliseringen av organiskt material och/eller de organiska föroreningar som observerades. Denna närvaro av livsdugliga mikroorganismer antydde att mikroorganismerna anpassat sig till föroreningssituationen på platsen. Ytterligare resultat visade dock att den långvariga exponeringen av höga PHC-koncentrationer i isolerade odlingsmedier, för mikroorganismer i jorden, inte påverkade förmågan för dessa att effektivt bryta ned PHC. Dock hade den fylogenetiska tillhörigheten en stark påverkan på bionedbrytning av PHC.

I den andra delen av denna avhandling genomfördes preliminära studier i växthus där förmågan hos *M. sativa* undersöktes, med hjälp av kompost, gällande den växthusstödda fytosaneringen av PHC och TE. Resultaten visade att inblanding av kompost i jorden främjade nedbrytningen av PHC, tillväxten och överlevnadsgraden av *M. sativa* och fytoextraktion av Pb. Återstående riskbedömning efter fytosaneringen visade också en positiv effekt, när komposten användes, på växternas tillväxt och daggmaskarnas utveckling.

Ett experiment med eco-bädd utfördes för den tredje delen av avhandlingen. Denna studie visade på en minskning på upp till 80% av PHC och 20% av metallerna.

Denna avhandling visar att *M. sativa* och *H. annus* var lämpliga för nedbrytning av PHC och fytoextraktion av Pb och Cu.

Resultaten från denna avhandling förväntas vara användbara för ytterligare studier av fytoremediering i fullskala.

Nyckelord: petroleumkolväten, metaller, GRO, polycykliska aromatiska kolväten, respiration, eco-bädd, bakterier, svampar.

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List of acronyms and abbreviations

As	arsenic
C	carbon
Ca	calcium
CCME	canadian council of ministers of the environment
Cd	cadmium
CEAA	canadian environmental assessment agency
CEPA	canadian evironmental assessment act
CERCLA	compensation, and liability act
CERCLIS	comprehensive environmental response, compensation and liability information system
Co	cobalt
COD	chemical oxygen demand
Cu	cupper
CW	change in weight
DO	dissolved oxygen
DW	dry weight
EC	electrical conductivity
ECR	enrichment coefficient for root
EDTA	ethylenediaminetetraacetic acid
EEA	european environment agency
EU	european union
ESEG	environmental science and engineering group
ET	éléments traces (trace elements)
Fi	fine
GC-MS	gas chromatography coupled to mass spectrometry
GRO	gentle soil remediation options
Fe	iron
Fig.	figure
FW	fresh weight

g	gramme
h	hour
H^+	hydrogen ions
ha	hectare
Hg	mercury
HSD	honestly significant difference
HP	hydrocarbures pétroliers (petroleum hydrocarbons)
IPC-MS	coupled plasma mass spectrometry
K	potassium
Kg	kilogramme
L	liter
Lip	lignin peroxidase
mg	milligramme
Mg	magnesium
mg kg^{-1}	milligramme per kilogramme
ml	milliliter
mm	millimeter
mM	millimolar
Mn	manganese
MNP	manganese-dependent peroxidase
N	nitrogen
Na	sodium
Ni	nickel
NPL	national priorities list
ODA	options douces d'assainissement (gentle soil remediation options)
OH^-	hydroxyl ion
OD	optical density
OM	organic matter
OUR	oxygen uptake rate
P	phosphorus
PAH	polycyclic aromatic hydrocarbons

Pb	plomb, lead
PCR	polymerase chain reaction
PHC	petroleum hydrocarbons
PCB	poly chlorinated biphenyl
SL	swedish limits in sensitive lands
SR	survival rate
TE	trace elements
TF	translocation factor
TOC	total organic carbon
SARA	superfund amendments and reauthorization act
SEPA	swedish environmental protection agency
sp.	species
μl	micro liter
USEPA	us environment protection agency
v	volume
VP	versatile peroxidase
w	weight
XRF	x-ray fluorescence
Zn	zinc

Dedicated to my mothers Seke Suzanne and Bediang Marguerite

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"If we stand tall it is because we stand on the shoulders of many ancestors" African Proverb

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Chapter 1: General introduction

Soil contamination through industrial production, human activities, improper waste disposal or environmental accidents has become a serious concern that threatens human health and ecosystems. Today, many contaminated sites are often tainted with a complex mixture of organic and inorganic compounds, including petroleum hydrocarbons (PHC), pesticides, trace elements (TE) such as lead (Pb), copper (Cu), Zinc (Zn), mercury (Hg) and cadmium (Cd). When they exceed certain concentrations, many of these compounds become a major concern for plants, animals and human beings due to their detrimental biological effects, toxicity, carcinogenicity, mutagenicity and teratogenicy. PHC and TE-contaminated soil may pose risks and hazards to humans and ecosystem through; direct ingestion, or contact with soil, the food chain (soil-plant-human or soil-plant-animal-human), drinking of contaminated ground water and reduction of land usability. The adequate protection and restoration of contaminated soils require their characterization and remediation. In the last decade, efforts have been made towards the reduction of pollutants directly at the source and the establishment of new environmental guidelines for contaminated site remediation. Several technologies such physical, chemical and biological techniques have been developed to remediate these sites. These technologies include the source control (*in situ* and *ex situ* treatment) and the containment remedies. Generally, these approaches have limitations i.e. the high costs, the applicability to high contaminant concentrations, the applicability to mixed wastes (organics and inorganics) and the irreversible changes in soil physicochemical properties. Excavation and landfill known '*Dig and Dump'* approach is largely utilized, although this method doesn't remediate the soil but only transfers pollutants from one place to another. My thesis focuses on the remediation of contaminated soil using non-destructive, less disruptive to the soil and low-cost technologies.

1.1 Delimitation of this thesis

Among the different approaches to the restoration of PHC and TE-contaminated soils, physico-chemical methods are costly, may create further waste, and in many cases, simply transfer pollutants from one phase to another. In the view of these considerations, special attention is drawn in this thesis to the gentle soil remediation options (GRO) for the reclamation

of PHC and TE-contaminated soil (Fig.1.1). GRO includes *in situ* contaminant stabilization ("inactivation" using biological or chemical processes) and plant-based options (i.e. phytoremediation). In the current investigation, plants and their associated microorganisms such as fungi, bacteria have been used to degrade organic pollutants and either sequestrate or extract TE from soils. The focus was also given to toxicity effects posed by PHC and TE contaminated soils before and after treatment.

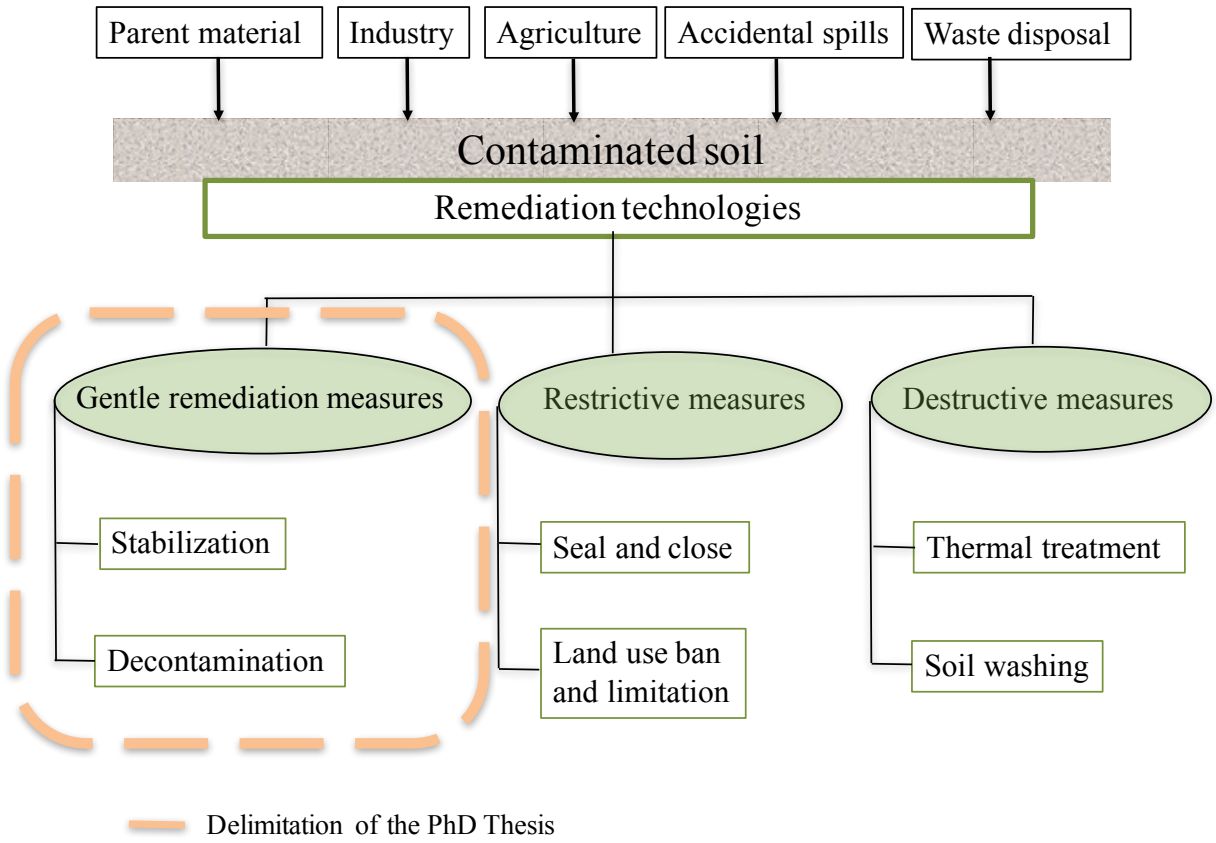


Figure 1.1. The context of the thesis within the contaminated soil remediation strategies.

1.2 Overview of the thesis

The present thesis is the result of the collaboration/partnership between Université de Montréal, Canada and Linnaeus University, Sweden. My thesis started on January 2013 in the Biodiversity Center of the Université de Montréal, Canada as a part of the GenoRem project.

GenoRem project brings together researchers from several institutions and fields with the objective of improving and developing new green technologies for the remediation of polluted soils. From January 2014, the author also worked as a member of the Environmental Science and Engineering Group (ESEG) at Linnaeus University, Sweden. This thesis investigated the remediation of contaminated site in a multi-scale perspective: laboratory, greenhouse and field scale. The general scheme is showed in the figure 1.2. Laboratory investigations at the laboratory run by the Biodiversity Center have been performed to the isolation and characterization of microorganisms (paper II, annex 1) and to the screening of the most efficient microorganism for the degradation of PHC (paper III). Laboratory, greenhouse and field experiments that resulted in the papers I, IV and V respectively was performed at Linnaeus University.

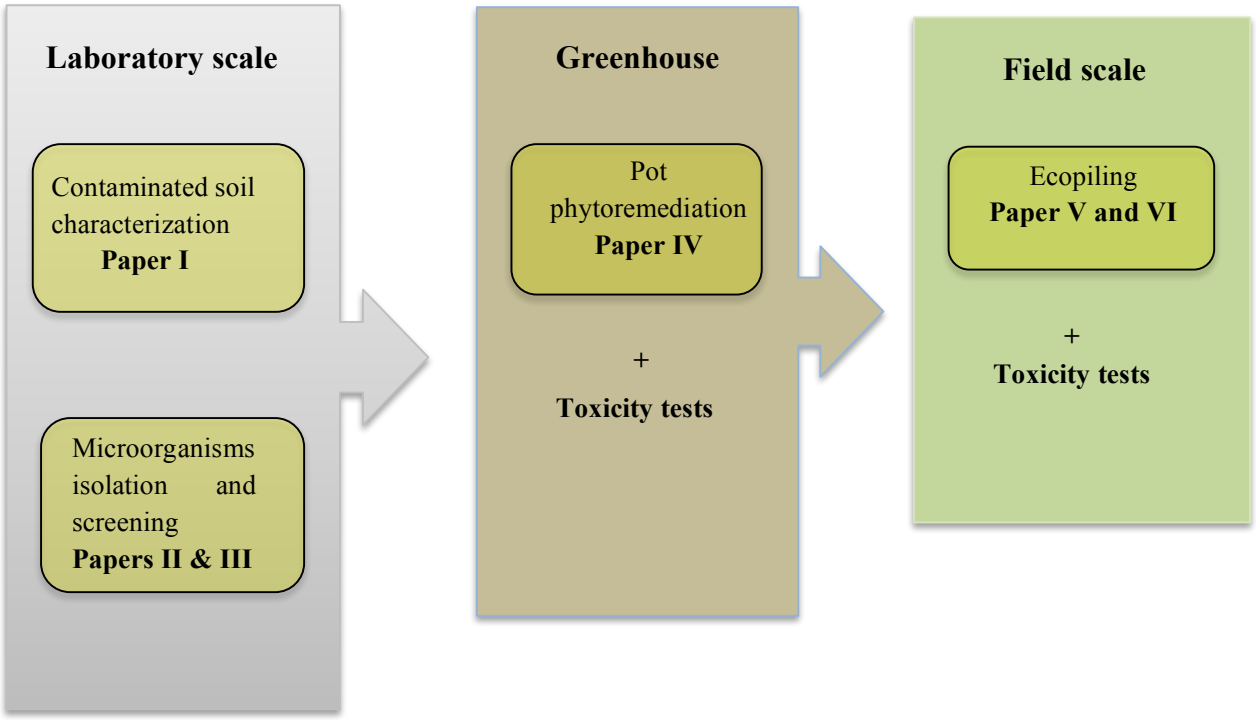


Figure 1.2. General scheme of the work described in the thesis.

1.3 Challenges and originality of the research

Although phytoremediation is becoming increasingly popular for cleaning-up of contaminated soils, many challenges related to the site-specific condition, nature of pollutants, timeline and efficiency have not been overcome yet. Many of the polluted sites contain a heterogeneous and complex mixture of thousands compounds with broad physico-chemical characteristics, toxicity and availability levels. Site with mixed contamination pose technical challenges associated with the nature of pollutants plus the new problems that arise due to the present of two (or many) classes of contaminants with different properties (Chirakkara *et al.* 2016b). The presence of both organic and inorganic compounds increases interaction effects that could lead to an increase or decrease the efficiency of the remediation technology. The presence of organic contaminants may positively or negatively affect the transport and removal of TE in soils while the inherent toxicity of TE can inhibit the biodegradation of organic contaminants by the microorganisms in soil (Chirakkara *et al.* 2016b). Degradation of pollutant mixture has been considered in many investigations where researchers used phytoremediation technique to treat pollutant-spiked soils (Wei and Pan 2010; Peng *et al.* 2009). However, by contrast to freshly contaminated soil, the remediation of PHC that have been present in the soil for aged is still difficult due to the fact that they are less available. Thus, very little is known about the plant metabolism pathways involved in the degradation and toxicity of these compounds (Chigbo and Batty 2013). In addition to the challenges related to the use of aged co-contaminated soils in this study and the complexity of these pollutants, soil characteristics bring another level of complexity that has to be solved. In PHC contaminated soils, the carbon (C): nitrogen (N) and phosphorus (P) ratios become imbalanced due to the high input of C into the system, leading to N and P immobilization or depletion through microbial activity (Adam and Duncan 2002). Current research in phytoremediation is often requiring a high input of N into the soil to significantly reduce pollutant concentration. Investigation of the degradation of PHC by using plants adapted for nutrient acquisition (nitrogen fixation for instance) is beneficial to sustain phytoremediation. Choosing plant species and varieties for phytoremediation is also challenging because the introduction of non-native or genetically modified plants into the environment could be an issue (Hakeem *et al.* 2015). Furthermore, phytoremediation is a site-specific process and feasibility studies are required before full-scale remediation can be applied

successfully. Preliminary studies in laboratory scale can be used to predict degradation rates but it does not reflect field conditions, therefore we should use phytoremediation as a multi-scale perspective. Finally, it is important to complement and integrate phytoremediation studies with ecotoxicological analysis taking into account the effect of bioavailable contaminants and their interaction. Despite to large number of articles dealing with phytoremediation, only a few papers have been published assessing the efficiency of phytoremediation using both chemicals and ecotoxicological analysis.

1.4 Goal and objectives

The overall aim of my thesis is to increase the scientific knowledge and develop sustainable solution for PHC and TE polluted soils in order to minimize the impact of these compound on the environment. The main focus was given on the development of *in situ* GRO as a tool to treat mixed contaminated soils at three different scales (lab, greenhouse and field trials). A particular attention was given to the evaluation of the toxic effects posed by these contaminants before and after the remediation.

Specific objectives are to:

1. Characterize PHC and TE contaminated soil.

   Hypothesis:

   Aged contamination with PHC and TE compounds have a toxic effect on soil microorganisms, earthworms and plants.

2. Isolate, screen and select microorganisms with high potential in degradation of PHC.

   Hypotheses:

   (i) The use of selective culture media allows to isolate the most effective microorganisms to degrade petroleum hydrocarbons.

   (ii) The microorganisms isolated from high contaminated soils are most efficient to degrade PHC compared to those isolated from low contaminated soils.

3. Study the ability of *M. sativa* assisted by compost in the greenhouse aided-phytoremediation of PHC and TE.

   Hypothesis:

   M. sativa cultivation combined with compost amendment increase PHC degradation and represent an advantage for reduce toxicity of contaminants in soil.

4. Investigate the efficiency of *M. sativa* co-planted with *H. annuus* and assisted by compost in field site experiment.

Hypothesis:

M. sativa co-planted with *H. annuus* increase PHC degradation and TE removal as compared to *M. sativa* monoculture.

This thesis is divided in seven chapters. After this first introduction chapter, the second chapter covers literature review of the actual knowledge in contaminated soil remediation technologies and legislation. Then each of these specific objectives will be studies in the chapter 3 to 6 respectively. The last chapter is the general discussion and conclusion.

Chapter 2: Literature review

2.1 Soil environment

Soil is the soft material that covers the surface of the earth. All soils begin with solid rock, which is eroded into smaller pieces by physical and chemical weathering (Parker 2009). Soil has a direct and practical importance for living organisms. It supports plant growth, human and animal life. It provides many other ecological services such as (i) food, fibre, fuel essential for homes and industries, (ii) biodiversity, (iii) water filtration, (iii) nutrient cycling, (iv) organic matter decomposition, (v) chemical buffering capacity (equilibrium between compounds adsorbed to soil surfaces and released into the soil solution) (Whalen and Sampedro 2010). Soil is a major environmental agent that interacts with chemical content in or where there are added according to its properties (Duffy 2011). This interaction depends on these physical, chemical and biological properties.

2.1.1 Soil physical properties

The main physical properties of soil are composition, texture, structure and permeability.

Soil composition

Soil is composed of three phases: (i) solid (50% mineral particles and 5% organic matter); (ii) liquid (25% water) and gas (20% air) (Paria 2008). Soil organic matter is defined as the plant, animal and microbial residues found in soil, both decomposed and undecomposed (Whalen and Sampedro 2010). Although small in amount soil organic matter plays a major role in soil structure as it acts as glue that binds together soil particles.

Soil texture

Soil contents particles of different sizes and characteristics. These particles are divided into three fractions: sand, silt and clay (Fig.2.1). Soil texture refers to the amount of these fractions. The spaces between soils particles are directly related to its texture and determine how easily substance moves through soil (Parker 2009). Sand, mainly composed of quartz and feldspar is relatively inert, low-nutrient, low water retention (but good drainage) and is easy to aerate (Duffy 2011). Particles less than 0.01mm (clays and silts) small size are called colloids and high chemical reactivity due to their high exchange surface (McCauley *et al.* 2005). Textural

name of soil is determined by the soil texture triangle (fig.2.2). Soils located in the middle of triangle are ideal for the growth of plants but have a chemical reactivity due to clay (Duffy 2011).

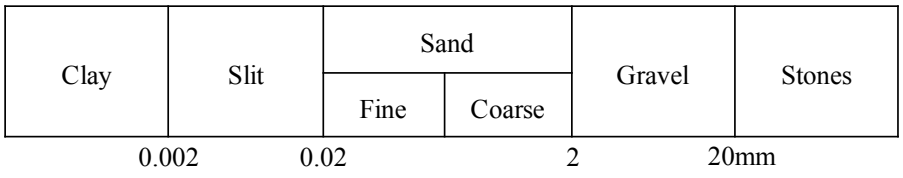


Figure 2.1. Soil particles classification according to the *International Society of Soil Science*. Soil consists of particles having a maximum of 2mm

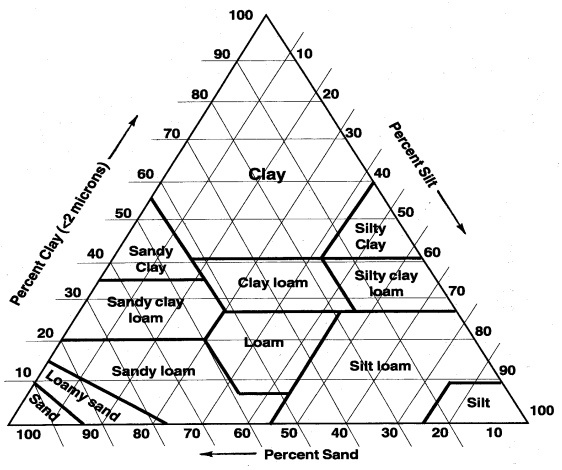


Figure 2.2. The U.S. Department of Agriculture (USDA) soil texture triangle. The percentage of sand, silt and clay present in the soil are determined then, these amounts are plotted on the soil triangle

Soil structure

Structure refers to the arrangement of soil particles. It influences water absorption and air circulation. These aggregates increase stability against soil erosion, improve soil fertility and carbon sequestration, maintain the movement of water and porosity (McCauley *et al.* 2005). The porosity of soil is a quantity of void or pore. A desirable structure should have a high proportion of medium-sized aggregated and a significant number of large pores (Parker 2009).

Permeability

The permeability or hydraulic conductivity is the ability of soil to conduct water. A soil with a good structure is more permeable than soil without structure. Texture and structure are the main factors that influence the transport of water and contaminants in soil (Glatstein and Francisca 2014).

2.1.2 Soil chemical properties

Most chemical interactions occur on colloids soil surfaces due to their large surface exchange. Soil-contaminant interactions are generally made by (i) sorption, (ii) complexion and (iii) precipitation (Tan 2000). Complexion and precipitation are applied to inorganic contaminants. When TE are added to soils, some of them may chemically or physically interact with the natural compounds of soils, being immobilized or forming compounds that have low solubility (fig.2.3). Organic contaminants, such as PHC interact with the soil by sorption. Sorption is the process by which contaminants interact with the interface of the solid particles of the soil. This interaction gives rise to different physical forms which are shown in figure 2.4 (Volkering *et al.* 1997). Unlike chemical adsorption occurs by chemical bonding, physical adsorption occurs when contaminants are attracted to the surface of soil particles. PHC are adsorbed physically due to forces on the hydrophobic surface. This interaction has a direct effect on the main soil chemical properties such the cation exchange capacity, the pH, the content of total and bioavailable elements, and the salinity.

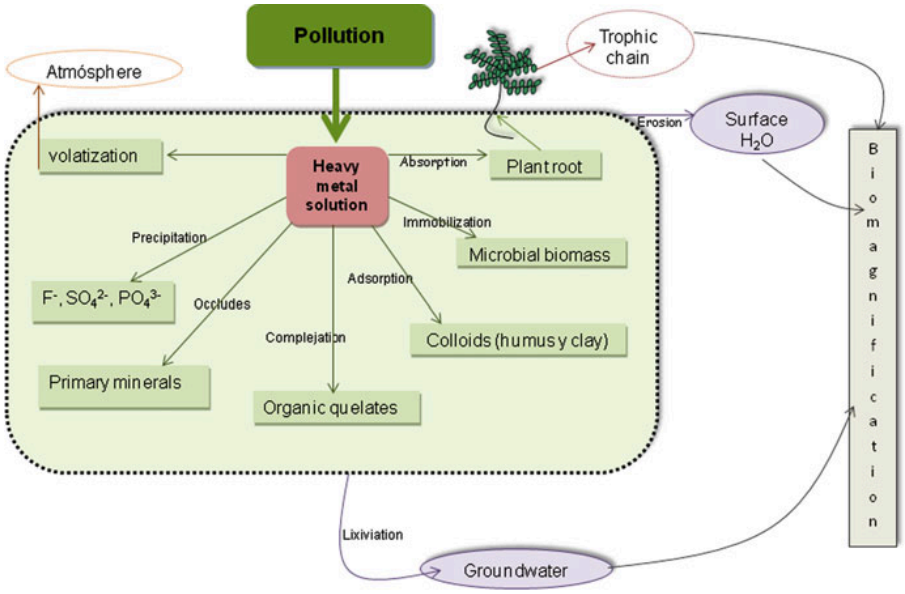


Figure 2.3. Dynamics of TE in soil (Gupta 2013).

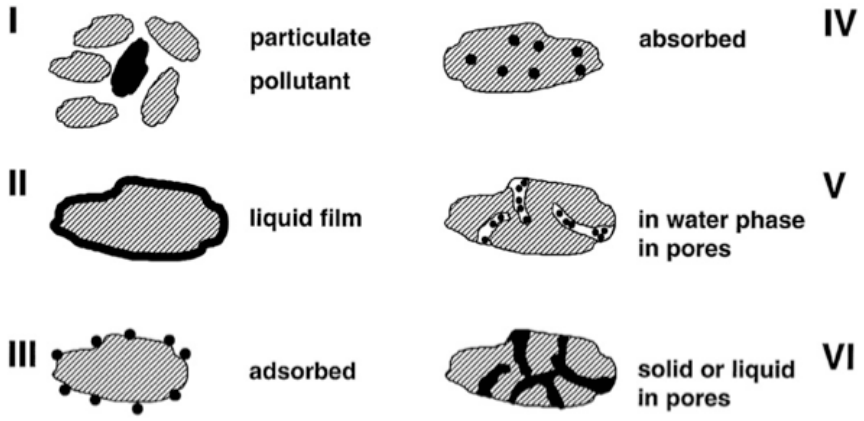


Figure 2.4. Different physical forms of organic pollutants in soil:(I) solid particles; (II) liquid film; (III) adsorbed onto soil; (IV) absorbed into soil; (V) in soil macropores; (V) in soil micropores (Volkering *et al.* 1997).

Cation exchange capacity

The cation exchange capacity (CEC) is the electrostatic capacity of the soil to fix and exchange positive ions on the surface (Duffy 2011). This function is provided by the colloids that have a predominantly negative charge, and enable them to attract cation present in the soil (Fig. 2.5) (McCauley *et al.* 2005). The CEC depends on the amounts and kinds of clay and organic matter present (Parker 2009). When organic matter increases, CEC increase (Parker 2009).

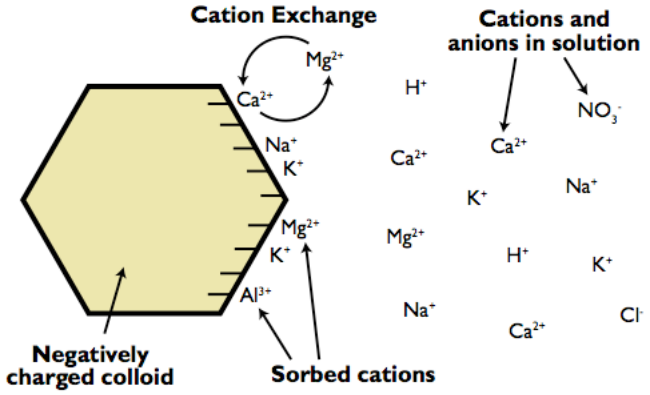


Figure 2.5. Representation of the cation exchange capacity (McCauley *et al.* 2005).

Soil pH

Soil pH is a measure of hydrogen ions (H^+) present in the soil (McCauley *et al.* 2005). The pH varies between 0 and 14. A large quantity H^+ corresponds to a low pH and vice versa. Soils containing more H^+ than hydroxyl ion (OH^-) are acidic (pH less than 7). Neutral soils have pH 7 and those at above 7 are alkaline. Soil pH can affect the CEC by altering the surface charge of the colloids. For example, a high concentration of H^+ neutralizes the negative charges of colloid (McCauley *et al.* 2005). In addition, the pH is a very important chemical property because it influences (i) the growth of soil organisms; (ii) the degradation of organic contaminants; (iii) the availability of nutrients and contaminants (Cao *et al.* 2009).

Total and bioavailable elements

In general, the study of a soil begins by determining the total elements. The composition of the soil depends on the rock from which it comes, but also process it has undergone over time (Whalen and Sampedro 2009). The constituents of the soil mineral phase can be major (e.g. aluminum, iron) or minor (like zinc, copper). Soil elements as contaminants are not all available. The available portion is a part of total soil elements involved in physico-chemical and biological reactions (Duffy 2011). PHC are a mix of thousands compounds that each has a different availability. Physico-chemical properties of the soil such as its nature or the period during which it is contaminated influences remained availability of elements (Chigbo and Batty 2013).

Soil salinity

The three main types of salts that can affect soil are saline, sodic and saline-sodic. Saline soils contain high amounts of soluble salts such as calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^+), while sodic soils are dominated by the sodium ions (Na^+). The saline-sodic soils have both a high concentration of salts and sodium. These salts affect the structure, the porosity and the water content of the plant which results in the decrease of productivity (McCauley *et al.* 2005).

All these physicochemical properties determine the activity of soil organisms.

2.1.1 Soil biological properties

Soil environment is the home of many forms of life (Fig.2.6): plants (flora), animals (fauna) and microorganisms (microbiota) that each plays an important role.

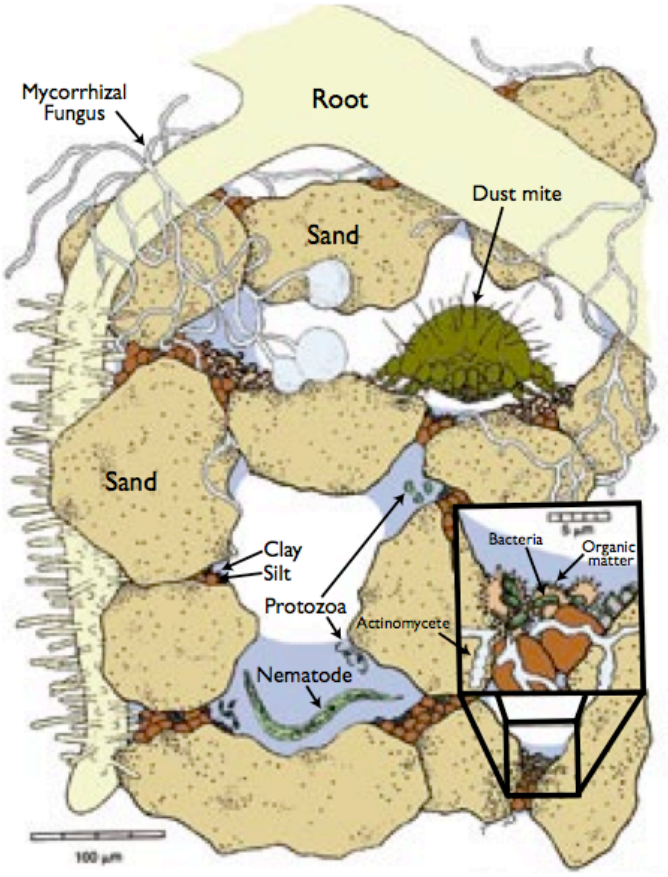


Figure 2.6 Soil organisms and their environment (Sylvia 2005). Soil organisms are divided into two main groups according to their size and where they live in the soil. Microorganisms such as bacteria, fungi are associated with clay and silt particles while soil microfauna (protists, nematodes) live in water films around soil particles. Soil mesofauna such as arthropods moves in soil macropores while soil macrofauna such as earthworms moves through soil cracks.

Soil flora

Plants have a dominant role in soil formation. They are primary producers in terrestrial ecosystems (Oleszczuk 2008). They improve the structure, porosity and provide additional organic matter to the soil through its residue leaves and roots. Plant roots allow the movement

of water and air (Whalen and Sampedro 2009). They stabilize the ground by the formation of aggregates which improve the structure and porosity (Duffy 2011). The root zone is the the most biologically active soil region in direct contact with the roots of plants. Root exudates are a nutrient source for soil organisms (McCauley *et al.* 2005).

Soil fauna

Soil animals are mainly insects, nematodes, arthropods and earthworms. Earthworms are considered the most important because they produce channels that increase soil porosity, they increase the biotic activities by transforming organic matter into small fragment and in secreting substances rich in nutrient (McCauley *et al.* 2005). Animals regulate soil (i) bacterial and fungal population; (ii) initialize the degradation of dead plants and animals; (iii) mix soil layers and increase their aggregation (Whalen and Sampedro 2009).

Soil microbiota

It is the largest and most diverse group in the soil. Soil microbes include bacteria, archea, protozoa, algae, fungi and oomycetes, etc. (Sylvia 2005). Bacteria are the most diverse group of soil microbes. They are very important in the decomposition of organic matter, nutrients and processing the aggregation of small soil particles. Some bacteria such as rhizobia are studied because they are associated with the pulse crops and enable the nitrogen fixation. Fungi are numerically the most dominant soil microorganisms (Whalen and Sampedro 2009). They are very important in the degradation of organic matter and the stability of the aggregates. In general, microorganisms enhance soil structure by secreting organic compounds (mainly sugars) which bind together the soil particles (McCauley *et al.* 2005). Stimulation of soil microorganisms in particular by adding a nutrient source increases the hydraulic conductivity (Glatstein and Francisca 2014).

All these soil physicochemical and biological properties influence the rate and fate of pollutants such TE and PHC.

2.2 Petroleum hydrocarbons in the soil

2.2.1 Sources

Petroleum (also called crude oil) is a fossil fuel made naturally from the slow transformation remains of prehistoric plants and animals. It is a complex mixture of thousands compounds that can be categorized into four groups: asphaltenes (phenols, fatty acids), resins (pyridines, quinolines) saturates (alkanes) and aromatics (Singh 2006c; Wrenn and Venosa 1996). Other minor compounds as TE are also present in petroleum oil. Asphaltenes unlike resins have the highest molecular weight and are heaviest and are the most polar constituents in petroleum. Hydrocarbons (compounds consisting of only carbon and hydrogen) are the most abundant components of petroleum (between 65 and 95% of its composition) (Fig.2.7). The benzene ring is the basic structure of aromatic hydrocarbons and two or more fused cycles form polycyclic aromatic hydrocarbons (PAH) (Haritash and Kaushik 2009). PAH are formed during the incomplete combustion at high temperatures (500-800°C) of the organic substances for long period (Haritash and Kaushik 2009; Megharaj *et al.* 2011). It can occur with anthropogenic activities such as the burning of fossil fuel or municipal solid waste incineration and also be produced naturally in the environment by volcanic eruptions, forest fires, or exudates from trees (Haritash and Kaushik 2009). Organic pollutants in the environment are mostly anthropogenic. Sources of these pollutants include accidental release (e.g. diesel, solvents), industrial activities (e.g. petrochemical, pharmaceutical), agriculture (e.g. pesticides, herbicides) and military activity (e.g. explosives) (Pilon-Smits and Freeman 2006).

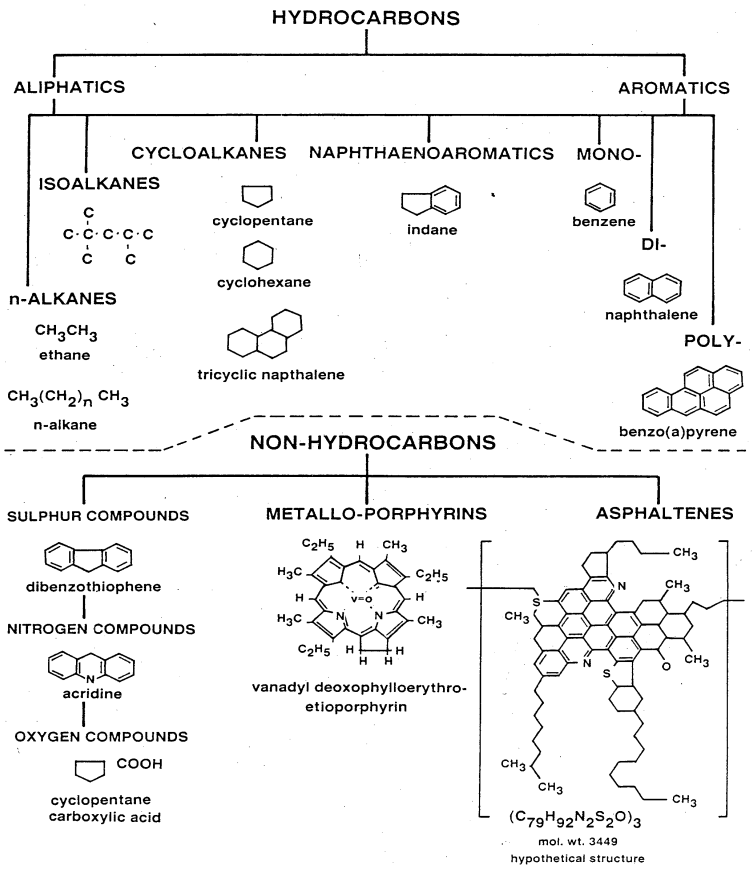


Figure 2.7. Main compounds of petroleum: non-hydrocarbons and hydrocarbons. Hydrocarbons are the most abundant components of the crude oil: they represent between 65 and 95% of its composition. From Geraci and Aubin, 1988.

2.2.2 Social economic interests

The exploitation and usage of petroleum have grown exponentially since the beginning of the last century. Today, it is the economic driver of many petroleum rich countries. Petroleum is primary used as a source of energy. It is considered the mother of all the commodities because it is an important raw material for wide varieties of chemical compounds like benzene (Adebayo and Tawabini 2013). Benzene is used in the plastics industry but also to manufacture medicines, rubber, lubricants, detergents, dyes and explosives. Benzene is also a source of other compounds such as toluene, phenol (used to make resins and adhesives), aniline, naphthalene and styrene (used to make polymers) (Adebayo and Tawabini 2013). Petroleum is also used as an herbicide.

Thus a mixture of oil fraction with low aromatic compounds are used for controlling weeds while the heavier fractions like diesel oil are used as total weeds killers (Adebayo and Tawabini 2013). In addition to insecticides and pesticides, petroleum is also used in the production of fertilizers and ammonia in agriculture (Singh 2006c). Petroleum mulches are also used in agriculture to promote the favourable soil moisture and temperature and to increase the yields. PAH are major constituents of creosote, widely used in the world for the treatment of wood product (Gevao and Jones 1998). Petroleum products include diesel fuel, kerosene, that is daily used in the transport sector. Bitumen is largely used to prevent soil erosion and used as binder in road construction.

Despite these major economic issues, oil and these derivatives, however, have negative effects on wildlife and human health.

2.2.3 Toxicity

Petroleum is the mixture of different chemical compound such as poly chlorinated biphenyl (PCB), TE, PAH which are known to pose harmful effects on the quality of life. Aromatics hydrocarbons are major concern because of their toxicity and tendency to bioaccumulation (Wrenn and Venosa 1996). This is due to the fact that they are poorly soluble in water but very soluble in oil and fat (Singh 2006a). Indeed, the solubility of the aromatic compounds in water decreases with increasing of molecular weight (Wild and Jones 1995). Therefore, due to their hydrophobicity, PAH remain in the aquatic environment to the surface of water or adsorbed to the surface of the sediment and form a reservoir (Borja *et al.* 2005).

Based on the structure and mechanism of activation, many PAH exhibit mutagenic, tumorigenic and carcinogenic properties (Singh 2006c). In soil, they can also adhere to organic matter and in this case, they are not available for microorganisms, plants or leaching (Wild and Jones 1995). US Environment Protection Agency (EPA; www.epa.gov) provides complete removal of 16 specific PAH: acenaphthene, acenaphthylene, anthracene, benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(ghi)perylene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, and pyrene (Table 2.1) listed among the 126 priority pollutants and 5 of them are

listed among the 25 hazardous substances thought to pose the most significant potential threat to human health (Pazos *et al.* 2010).

Table2.1. Properties and chemical structures of the 16 USEPA PAH (Pazos *et al.* 2010).

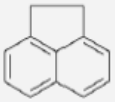
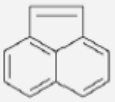
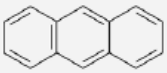
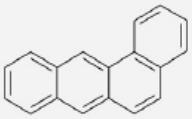
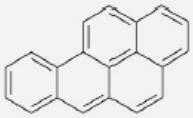
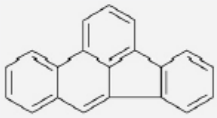
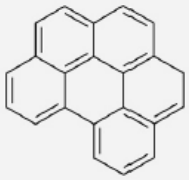
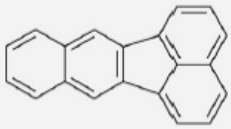
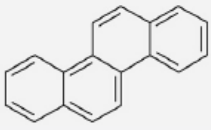
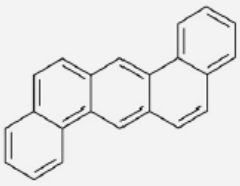
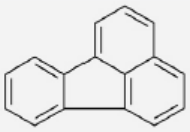
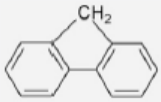
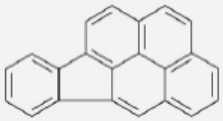
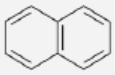
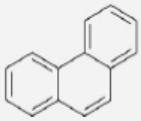
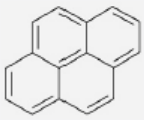
Name	CAS	Chemical structure	Molecular weight (g/mol)	Boiling point (°C)	Melting point (°C)	Water solubility (mg/L)	Vapor pressure (mm Hg)	Partition coefficient, logK_{OW}	Henry's law constant, H (atm-m^3/mol)
Acenaphthene	82-32-9		154.21	277	95	1.93	4.47×10^{-3}	3.98	7.91×10^{-5}
Acenaphthylene	208-96-8		152.2	265–275	92–93	3.93	0.029	4.07	1.45×10^{-3}
Anthracene	120-12-7		178.2	342-340	218	0.0076	1.7×10^{-5}	4.45	1.77×10^{-5}
Benz(a)anthracene	56-55-3		228.29	435	162	0.010	2.2×10^{-8}	5.61	1×10^{-6}
Benzo(a)pyrene	50-32-8		252.3	495	179–179.3	0.0023	5.6×10^{-9}	6.06	4.9×10^{-7}
Benzo(b)fluoranthene	205-99-2		252.3	481	168.3	0.00125×10^{-7}		6.04	1.22×10^{-5}
Benzo(ghi)perylene	191-24-2		276.34	550	273	0.00026	1.3×10^{-10}	6.50	1.44×10^{-7}
Benzo(k)fluoranthene[a]	207-08-9		252.31	481	215–217	–	–	–	–
Chrysene	218-01-9		228.3	448	255–256	0.0028	6.3×10^{-7}	5.16	1.05×10^{-6}
Dibenz(a,h)anthracene	53-70-3		278.35	524	262	0.0005×10^{-10}		6.84	7.3×10^{-8}

Table 1 (continued).

Name	CAS	Chemical structure	Molecular weight (g/mol)	Boiling point (°C)	Melting point (°C)	Water solubility (mg/L)	Vapor pressure (mm Hg)	Partition coefficient, log K_{OW}	Henry's law constant, H (atm-m^3/mol)
Fluoranthene	206-44-0		202.26	375	111	0.20–0.26	5.0×10^{-6}	4.90	6.5×10^{-6}
Fluorene	86-73-7		166.2	295	116–117	1.68–1.98	5.0×10^{-6}	4.90	6.5×10^{-6}
Indeno(1,2,3-cd) pyrene	193-39-5		276.3	530	163.6	0.062	10^{-11}–10^{-6}	6.58	6.95×10^{-8}
Naphthalene[a]	91-20-3		128.17	218	80.5	–	–	3.37	–
Phenanthrene	85-01-8		178.2	340	100	1.20	6.8×10^{-4}	4.45	2.56×10^{-5}
Pyrene	129-00-0		202.3	393	156	0.077	2.5×10^{-6}	4.88	1.14×10^{-5}

2.3 Trace elements in the soil

2.3.1 Sources

Metals are used chemically to define elements, which conduct electricity, have a metallic luster, are malleable and ductile, form cationic and have basic oxides (Duffus 2002). Metals are characterized and distinguished from nonmetals and metalloids (elements with properties intermediate between metals and nonmetals) by these properties, especially the temperature-dependent conductivity (Appenroth 2010). TE refers to cationic metals and oxyanions that naturally occur in soils and plants in concentration less than 1g kg^{-1} (Appenroth 2010). In this definition, there is no relationship between the specific density of the elements and various concepts of physical chemistry commonly used for the definition of heavy metals (Duffus 2002; Cabral *et al.* 2015). The TE are natural components in soils and rock formations. Some of these TE like Zn, Cu, cobalt (Co), manganese (Mn) and iron (Fe) are required in small concentrations

by living beings (Table 2.2; Palmer and Guerinot 2009). However, other TE such as Pb, Hg, Cd and metalloids arsenic (As), have unknown biological role (Duffus 2002; Clemens and Ma 2016). Soil pollution with TE results to natural processes such as volcanism or weathering of parent materials as well as anthropogenic activities (Mather and Pyle 2004). The latter include metal mining and use of industrial waste, smelting activities, combustion of coal accidental chemical spill, sewage sludge, pesticides, herbicides and fertilizer mostly in agriculture sector (Rizwan *et al.* 2016). The most common TE founds at contaminated sites in order of abundance are: Pb, chromium (Cr), As, Zn, Cd, Cu and Hg

Table 2.2. Essential TE for plants (Palmer and Guerinot 2009).

Element	Biologically relevant oxidation states	Lithosphere[a] (mg kg^{-1})	Typical plant[b] (mg kg^{-1})	Transporter family	Examples
Cu	Cu^{+}, Cu^{2+}	50	6	COPT, HMA	Plastocyanin, cytochrome oxidase, SOD
Fe	Fe^{2+}, Fe^{3+}	45,000	100	FRD3, NRAMP, OPT, VIT, YSL, ZIP	Cytochromes, Fe-S proteins, SOD
Mn	Mn^{2+}, Mn^{3+}, Mn^{4+}	950	50	CAX, NRAMP, ZIP	Water-splitting enzyme in PSII, SOD
Mo	Mo^{4+}, Mo^{6+}	1.5	0.1	MOT	Nitrate reductase, sulfite oxidase, xanthine dehydrogenase, aldehyde oxidase
Ni	Ni^{2+}	80	0.1		Urease
Zn	Zn^{2+}	75	20	ZIP, HMA, MTP	RNA polymerase, alcohol dehydrogenase, carbonic anhydrase, SOD

[a]Figures taken from Table 1.3 in *The Handbook of Trace Elements*[100]. [b]Figures taken from Table 1.3 in *Mineral Nutrition of Higher Plants*[1].

2.3.2 Social economic interests

TE plays a vital role in living organisms. Essential TE known as micronutrients are required in trace amounts for normal plant and animal growth development (Rizwan *et al.* 2016). They mediate vital biochemical reactions by acting as enzyme cofactors or in the maintenance of functional metabolism (Palmer and Guerinot 2009; Cabral *et al.* 2015). For example, Fe is important for oxygen transport, DNA synthesis and the formation of blood; Cu is also used for transportation of oxygen by Fe, and the defence against free radicals while Zn play a key role in a host of biological processes such as the regulation of carbohydrate conversion in the body or the induction of the synthesis of metallothione in which is an excellent scavenger of hydroxyl radical (Saghiri *et al.* 2015a, 2015b). The five most industrial produced TE are Fe, Cu, Al, Zn and Pb (Wuana and Okieimen 2011). TE is a raw material in pharmaceutical preparations, skincare products and cosmetics, manufacture of plastic, fertilizers and pesticides. TE can

usually be recycled and are included in many of the everyday articles. This is due to their common properties such as the high electrical and thermal conductivity, the ability to be malleable and ductile. They are used in the transport sector to build railways or bridges and to produce batteries. Electrical and electronic equipment such as electrical cables, computers, mobile phones, is one of the largest uses of TE. However, insufficient or inappropriate disposal of TE could lead to potential harmful impacts on both the environment and human health

2.3.3 Toxicity

Trace element (TE) pollution has harmful effect on biological systems and does not undergo biodegradation. Toxic TE can be differentiated from other pollutants, since they cannot be biodegraded but can be accumulated in living organisms, thus causing various diseases and disorders even in relatively lower concentrations. They are also known to have effect on plant growth, ground cover and have a negative impact on soil microflora (Khan *et al.* 2015a). TE toxicity depend on the bioavailable concentrations, the speciation or chemical form of the element, the pH, the oxidation–reduction conditions and others factors such as the soil type (Swaine 2000). As, Cd, Pb, and Hg are all highly toxic to both plants and humans in their ionic forms. As and Hg are also toxic in their methylated forms (Kopittke *et al.* 2010). Whereas methylated As, at least in the pentavalent state, is generally considered less toxic than As(III) and As(V), methylated Hg is more toxic than Hg(II) for most organisms (Clemens and Ma 2016; Wang *et al.* 2012a). Plants acquire essential beneficial elements from soil but because their selectivity is imperfect, they also absorb elements witch have no known biological function and are known to be toxic at low concentrations (Peralta-Videa *et al.* 2009). The deficiency of essential elements or the accumulation of non-essential TE may cause diseases. Most of the reactions produced in plants stressed by TE are due to the replacement of protein cationic centers or the increase of reactive oxygen species (Fig. 2.8) (Peralta-Videa *et al.* 2009). Exposure to TE is mainly due to the consumption of foods or in some case water with high levels of the contaminant (Vázquez *et al.* 2015; Feng *et al.* 2008). The phytotoxicity of the trace metals followed the trend (from most to least toxic): Pb≈Hg >Cu >Cd≈As >Co≈Ni≈Zn >Mn, with median toxic concentrations of (μM/kg): 0.30 Pb, 0.47 Hg, 2.0 Cu, 5.0 Cd, 9.0 As, 17 Co, 19 Ni, 25 Zn, and 46 Mn (Kopittke *et al.* 2010). Over the years, as a result of the high

industrialization elevated amounts toxic pollutant are released into the environment. Thus, few regulations arise for the remediation of contaminants in soils, wastes and sediments.

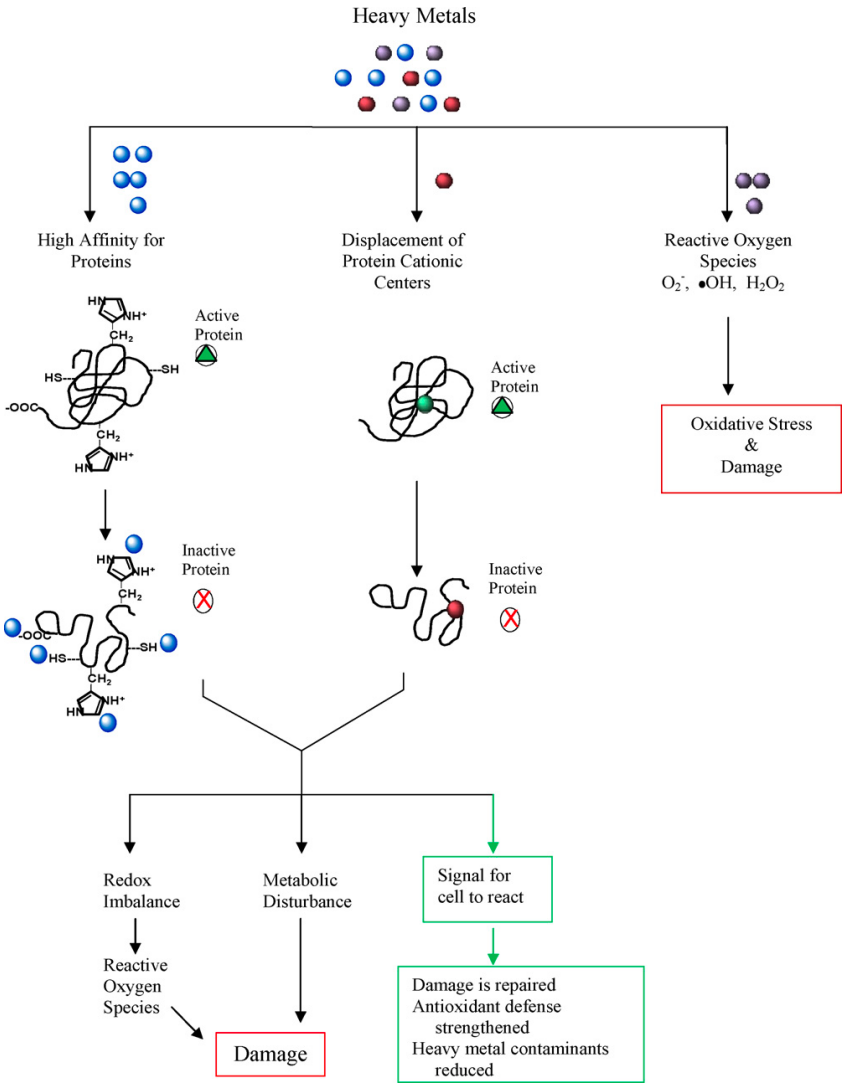


Figure 2.8. TE toxicity in plants. Purple spheres indicate redox active metals and red and blue are redox inactive metals. The green sphere is a metal centre that is displaced by a TE (red). The affinity for TE will alter the activity of the protein and create imbalances and disruption that will lead to macromolecular damage. However the cell may adjust to the toxic metals and signal for reaction to prevent damage(Peralta-Videa *et al.* 2009).

2.4 Legislation

2.4.1 Canada

The Canadian Environmental Assessment Agency (CEAA), created in 1994 prior to the adoption of the Canadian Environmental Assessment Act (CEPA) in 2000, provides high-quality environmental assessments that contribute to informed decision making, in support of sustainable development. The purpose of the CEPA is to prevent, reduce or control environmental and human health impacts of new and existing chemical substances, marine pollution, emissions from vehicles, engines and equipment, fuels and hazardous wastes. Canadian Council of Ministers of the Environment (CCME) provides guidance at the federal level but each province set most specific regulatory regimes for the assessment and remediation of contaminated land. In Québec, the new *Soil Protection and Contaminated Sites Rehabilitation Policy* is continuing the work that began in 1988 with the introduction of the *Contaminated Sites Rehabilitation Policy*. This policy contributes to the sustainable development of Québec society by underlying the following principles: prevention, rehabilitation-reclamation, polluter-pays and fairness principles.

2.4.2 United States of America

The United States Environmental Protection Agency (USEPA) manage the remediation of contaminated sites, conducts environmental assessment, research program, write and enforce regulations based on laws passed by congress. US federal government created in 1980 the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), also known as Superfund for the purpose of protecting human health and the environment. This program oversees the clean-up of land contaminated by hazardous waste and created a tax on the chemical and petroleum industries. Superfund also maintained the Comprehensive Environmental Response, Compensation and Liability Information System (CERCLIS). CERCLIC is a database containing the new contaminated sites, the current status of cleanup efforts or the National Priorities List (NPL) of the land eligible for federal funding to pay for remediation. CERCLA was amended by the Superfund Amendments and Reauthorization Act

(SARA) in 1986. SARA made several important changes to the program like underlined the importance of permanent remedies and innovative treatment technologies; increased the focus on human-health problems posed by hazardous waste sites; and encouraged greater citizen participation in how sites are cleaned up. In 1990, one year after the Exxon Valdez ecological catastrophe, which spills 42 000 m^3 of crude oil into Alaska, the congress passes the Pollution Prevention Act. In 1994 Superfund reform applied the principle of "polluter pays" wherever possible and collected most of fund for land reclamation from the tax on the petroleum chemical industries. Orphan or abandoned contaminated site are remediated by the USEPA. Today, all these federal laws have been complemented subsequently by State-based site contamination laws in most State in USA (Hasegawa *et al.* 2016).

Government, industry, and the public now recognize the potential hazards that PHC and TE pose to the environment. In response to a growing need to address environmental contamination, many efforts have been undertaken to develop remediation technologies to reduce or to manage these contaminants in soil.

2.4.3 European Union

The European Environment Agency (EEA) oversees the site remediation EU countries. The EEA estimates that 250,000 sites are contaminated and more than 80,000 sites have been cleaned up during the last 30 years in EU countries. EU Directive 2004/35/EC establish a comprehensive liability regime for damage to the environment. This directive applies a "polluter pays" principle, according to which the polluter is responsible when environmental damage occurs and leaves significant discretion for implementation to the Member States. However, a considerable share of remediation expenditure, about 35% on average, comes from public budgets in EU countries when occurred and when legally responsible polluters no longer exist, cannot be identified, or are insolvent. In addition to this directive, several other EU directives, government and non-government resources exist in Europe to support the prevention and the remediation of contaminated lands. In Sweden, the Swedish Environmental Protection Agency (SEPA or Naturvårdsverket in Swedish) is a government agency responsible for environmental issues. This agency implemented in 1999 the environmental code and the environmental quality objectives for a sustainable society. This code applies the polluter pays principle and put

pressure on the polluters. Among the 16 environmental objectives for 2020, the most pertinent for contaminated soils is a non-toxic environment which aims to have an environment free from man-made compounds.

2.5 Remediation technologies for hydrocarbons and trace elements contaminated soil

Efforts have been undertaken to develop both *in situ* and *ex situ* remediation technologies for contaminated soil (Pazos *et al.* 2010). *Ex situ* technologies involve excavation and removal of contaminated soils and this is treated or buried off-site, while *in situ* treatments remediate contaminants in place and/or on site (He *et al.* 2015). Although less extensively, in situ technologies are often the preferred treatment options because they are practical, more cost-effective and less intrusive to the environment (Cabrejo *et al.* 2010). The main remediation techniques for organic compounds in the soil are volatilization, photo-oxidation, chemical oxidation, adsorption on soil particles, leaching and microbial treatment (Singh 2006c; Gan *et al.* 2009). Unlike PHC, TE cannot be degraded in the environment, and therefore, remediation must involve either physical removal or transformation into nontoxic compounds (Wang *et al.* 2012a). Methods of remediation of contaminated soil can be mainly classified in three types: physical, chemical and biological methods.

2.5.1 Physical methods

Physical methods aim to separate contaminant from the contaminated solid by exploiting differences in physical characteristics between the contaminant and the native soil.

Physical sorting

Physical sorting is based on the fact that organic contaminants and few TE such as Pb are preferentially bound to the organic materials which are in the fine particle size fraction of soil (ADEME 2009). The fine fraction of the soil is then removed and treated off site.

Soil washing:

Soil washing a treatment method for separating contaminants (particularly TE) from soil *via* chemical leaching, physical separation or physicochemical procedures (Dermont *et al.* 2008).

The specific application of soil washing depends on the form of TE in the waste being remediated. Chemical extraction is primarily applicable when target TE exist in an ionic form, whereas physical separation is suitable for particulate forms (Dermont *et al.* 2008). Physical separation used soil parameters such as the size, the density, and the magnetism. Solvents are also employed to solubilize specific contaminants (Khan *et al.* 2004b). Surfactants are often used in the case of petroleum residues (ADEME 2009). Chelators such as ethylenediaminetetraacetic acid (EDTA) that can form a strong complex with a variety of TE including alkaline-earth cations such as Al^{3+}, Ca^{2+}, Fe^{2+} and Mg^{2+} and target TE such as Pb,Cd, Ni, Zn and Mn are used in the washing of TE contaminated soil (Wang *et al.* 2012a).

Electrochemical/electro-kinetic

Electro-kinetic remediation consists of the controlled application of low intensity direct current through the soil between appropriately distributed electrodes (Pazos *et al.* 2010). The system consists of three compartments; a soil compartment placed between two electrodes (another and cathode) compartments. During electro-kinetic soil treatment, the electric gradient initiates movement of contaminants by electro-migration (charged chemical movement), electro-osmosis (movement of fluid), electrolysis (chemical reactions due to the electric field), and diffusion (movement of the ionic species in the soil solution caused by concentration gradients formed by the electrically induced mass transport) (Moghadam *et al.* 2016). This technique initially used for remediation of TE contaminated soil, is now also applied in the remediation of organic species, especially water-soluble compounds (Pazos *et al.* 2010). Recent electro-kinetic remediation was combined to others treatments such as phytoremediation, soil flushing for removal of PAH and TE simultaneously (Moghadam *et al.* 2016).

2.5.2 Chemical methods

Chemical methods aim to the immobilization, reduction of the bioavailability of the contaminant upon chemical reactions with (1) sulphur-containing ligands; (2) reducing agents; (3) adsorbing agents.

Chemical oxidation and reduction

Oxidation is a chemical reaction in which an electron-deficient compound (oxidant) receives electrons from a compound, which is oxidized as well. Chemical oxidation is a technique to degrade aromatic compounds using oxidizing agents such as hydrogen peroxide (H_2O_2) and permanganate (MnO_4^{-}) (ADEME 2009). In contrast, chemical reduction consists to make a reducing environment by adding electron sources to the soil. Some compounds are then degraded more easily by reducing medium (ADEME 2009). Like chemical oxidation, photochemical degradation happens often in the environment.

The photochemical degradation

Photochemical degradation is the chemical conversion of a compound under the action of sunlight. This transformation pathway include photodimerisation and photooxidation (de Bruyn *et al.* 2012). PAH, which strongly absorb ultraviolet rays, undergo two types of responses (Clark *et al.* 2007). In the environment, an increase temperature, oxygen concentration or time of exposure to radiation leads to an increase of the photochemical degradation (Clark *et al.* 2007).

The advantages of physico-chemical techniques are that: (1) the processes generally attempts to permanently remove contaminants from soil and can allow the recycling of metal in certain cases; (2) the processed soil can be returned to the site; and (3) the process duration is typically short to medium-term compared to biological methods. However, the disadvantages of these methods include: (1) the high consumption of energy and raw materials; (2) the greatly alteration of soil properties; (3) the generation of large volumetric sludge(4) the high cost, and in many cases these techniques transfer the pollutant from one phase to another (Haritash and Kaushik 2009). The excavation and subsequent disposal of contaminated soil to a landfill site is

linked to the migration of contaminants from landfill into an adjacent environment. An alternative to these techniques is the use of biological methods.

2.5.3 Biological methods

Gentle soil remediation options (GRO) are used to remove or stabilize contaminant into less hazardous/ non-hazardous form with less input of chemicals, energy, and time (Ali *et al.* 2013; Wang *et al.* 2012b; Gerhardt *et al.* 2009; Kidd *et al.* 2015b). GRO encompass a number of technologies which include the use of plant (phyto-), fungal (myco-) or microbiologically-based methods, with or without chemical additives, for reducing contaminant transfer to local receptors by *in situ* stabilisation (using biological or chemical processes) or extraction of contaminants (Cundy *et al*., 2013). Microorganisms are able to degrade organic compounds by mineralization or co-metabolism (Johnsen *et al.* 2006). In mineralization, microorganisms use pollutant as source of carbon and energy. On the other hand, co-metabolism requires a second substance as a source of carbon and energy to degrade the pollutant at the same time (Borja *et al.* 2005). Microorganisms are capable of more easily degrade PAH with two to three aromatic rings. The incomplete degradation of the more recalcitrant compounds by co-metabolism has the disadvantage to allow the formation of toxic metabolites (Haritash and Kaushik 2009). In general, use of microorganisms with different metabolic partways and through the production of enzymes lead to completely degrade organic compounds into H_2O, CO_2 (aerobic) or CH_4 (anaerobic) (Fig.2.9) (Cao *et al.* 2009). Bacteria oxidize aromatic compounds in order to acquire their atoms while fungi for their detoxification. Although, these microorganisms cannot degrade metals they can alter their chemical properties via different mechanisms like biosoption, bioleaching, biomineralization or enzyme-catalysed transformations.

Bacterial metabolism

Bacteria extracted TE from contaminated soil by the production of mobilizing substance such as organic acids. These substances mobilize TE. Highly specialized bacteria like *Thio-bacillus* species can also generate TE-leaching sulfuric acid from the oxidation of elemental sulfur. This process is also called bioleaching has been used for many centuries to leach metals from low-grade ores, and currently supports a lucrative market in mineral extraction.

Biosorption or the metabolism-independent sorption of TE to biomass is another mechanism used by microorganisms. This is due to the negative microorganism's cell surface charge that adsorbs positive charged cationic TE.

Indeed, microorganisms evolved a wide range of biochemical reactions to protect themselves from potentially toxic TE. Many of these detoxification processes involve efflux or exclusion of TE ions from the cell, which in some cases can result in high local concentrations of TE at the cell surface where they may react with biogenic ligands and precipitate. These detoxification processes also involve redox transformations where the solubility of TE is biologically reduced (bioreduction). This enzymatic reduction of TE by bacteria is catalyzed by a class of enzymes such as *c*-type cytochromes. Biomineralization based microbial induced calcite precipitation (MICP) as a consequence of bacterial metabolic activity is another promising method for metal remediation. The microorganisms secrete one or more metabolic products that react with TE resulting in the subsequent deposition of mineral particles. Calcite, a biomineralization product, can strongly adsorb on its surfaces and incorporate this TE ion into its crystal structure.

Many bacterial genera are involved in the degradation of aromatic compounds (Cao *et al.* 2009). Usually bacteria are more efficient than other microorganisms to degrade aromatic compounds. Indeed, they are able to assimilate PAH as sole carbon source and energy (Singh 2006c). A dioxygenase is responsible for the first aerobic reaction of PAHs degradation (Haritash and Kaushik 2009) (Fig. 2.9). But some bacteria like Mycobacterium are able to degrade PAHs under the action of a cytochrome P450 monooxygenase (Cao *et al.* 2009). The genus of *Pseudomonas* is extensively studied because it contains species capable to effectively degrade a wide variety of PAH (Cao *et al.* 2009). The species of the genera *Nocardia*, *Rhodococcus*, and *Alcaligenes*, are also responsible for the degradation of many organic compounds. Most of these organisms were isolated from contaminated soil or sediments. Chronic exposure of microorganisms to aromatic compounds improves their degradation ability (Singh 2006c).

Although the bacterial metabolism degrades many aromatic compounds, it has several limitations. Bacterial degradation by co-metabolism often results in the accumulation of toxic intermediate such as chlorobenzoic acids (ACBs) (Cvancarova *et al.* 2012). Unlike fungi, bacteria need to be in contact with a sufficient amount of pollutants to initiate synthesis of degradation enzyme (Singh 2006c). Moreover, bacterial degrading enzymes are very specific and cannot degrade a large variety of compounds. Bacteria need to metabolize pollutants prior

degradation (because they secrete intracellular enzymes) and the rate of aromatic compounds absorption is slow (Cvancarova *et al.* 2012). Fungi that have different metabolisms to degrade aromatic compounds are often used with bacteria.

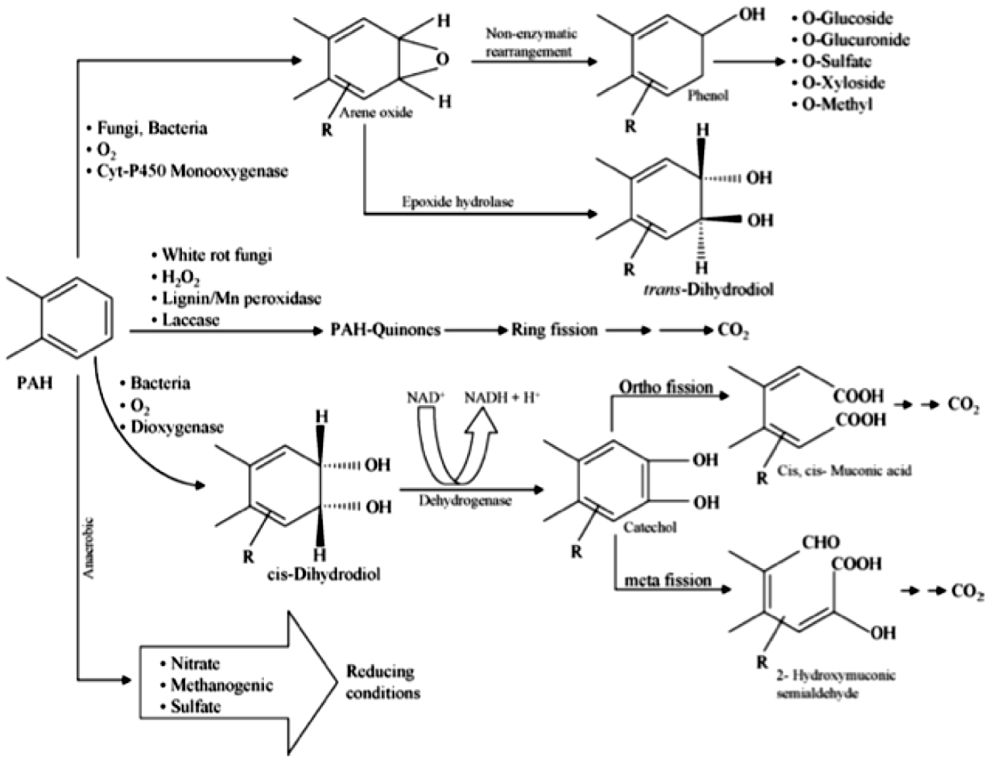


Figure 2.9. Proposed partways for microbial degradation of PAHs (Haritash and Kaushik 2009).

Fungal metabolism

Fungi are eukaryotic organisms able to grow on different substrates and degrade a wide variety of compounds (wood, plastic, textile etc.) via a process called mycodegradation (Singh 2006c). Mycelium is fungal vegetative structure that allows regulating the nutrient and energy flow. It also allows metabolizing pollutants by increasing the exchange surface of the soil. The fungal growth is very slow and often requires a different material for the co-metabolism. Like for bacteria, the remediation of TE by fungi involves the change in TE speciation and mobility.

Few mechanisms such as (i) organic precipitation with oxalates, (ii) inorganic precipitation with carbonates, phosphates or hydroxides, (iii) redox immobilization, (iv) sorption at cell walls and associated polymeric substances, and (v) bioaccumulation allow the immobilization of TE in soil. However, some microorganisms may, in turn, mobilize metals through excretion of H^+ and carboxylic (e.g., citrate) ligands and redox conversion to mobile forms (Gupta 2013).

Numerous fungi are capable of degrading aromatic compounds. Many fungal species able to tolerate environmental stress as *Fusarium*, *Penicillium*, *Aspergillus*, *Trichoderma* are good candidates for mycoremediation. Conversely, fungi as *mucorales* which include *Rhizopus* and *Mucor* genera growing very fast but are inefficient in degradation (Singh 2006c).

Fungi are the largest polymer plant decomposers such as lignin, cellulose and hemicellulose. Two main enzymatic systems allow this degradation: cytochrome P450 for non-lignonolytic fungi and lignonolytic system for lignolytic fungi (Haritash and Kaushik 2009). Dehydrogenases and oxygenases are the main degradative enzymes involved in the degradation of aromatic compounds by non-lignonolytic fungi (Singh 2006c). Lignolytic fungi are able to degrade lignin as well as different compounds that have a similar structure as PAH. This is due to the fact that lignonolytic fungi synthetize extracellular enzymes with very low substrate specificity (Haritash and Kaushik 2009; Cajthaml *et al.* 2006). Lignonolytic system consists of three main peroxidases: (i) lignin peroxidase (Lip), (ii) manganese-dependent peroxidase (MNP) and (iii) phenoloxidases also called versatile peroxidase (VP) including lacases and tyrosinases (Haritash and Kaushik 2009; Cvancarova *et al.* 2012). These enzymes were discovered in *Phanerochaete chrysosporium*, which is now used as model in mycoremediation because it is able to degrade toxic compounds more efficiently than other fungi (Singh 2006c). The degradation ability of other lignonolytic fungi such as *Irpex lacteus, Trametes versicolor, Pleurotus ostreatus* was also highlighted.

Mechanism of fungal aromatic compounds degradation has many advantages compared to the bacterial system. Fungi are able to (i) degrade completely these compounds; (ii) secrete a non-specific extracellular enzyme and non-dependent on pollutant concentration and (iii) to adjust the environment pH.

Plant metabolism

Phytoremediation is the use of plants and their associated microorganisms, soil amendments

and agronomic techniques to clean up environmental contaminants (Wang *et al.* 2012b; Gerhardt *et al.* 2009). Plants promote dissipation of contaminants by immobilization, removal and promotion of organic compounds microbial degradation (Megharaj *et al.* 2011) (Fig.2.10). Some compounds are removed by the roots of plants and translocated to aboveground plant tissues, which are subsequently harvested (phytoextraction) (Pilon-Smits and Freeman 2006). Partial or complete degradation of organic contaminants by enzyme can be involves by phytodegradation (plant enzyme) or phytostimulation (microbial activity). In phytovolatilization, contaminants are uptake by plants roots and then release as volatile chemicals. Finally by phytostabilization, plant roots allow accumulation or precipitation of contaminants to prevent their mobilization (Pilon-Smits and Freeman 2006). Also plants can solubilize TE for uptake by decreasing pH within the rhizosphere or by various organic chelators (root exudates), such as carboxylates or phytosiderophores from the mugineic acid family (Gupta 2013). Plant rhizosphere is the soil closest to the root system. This zone plays an important role in organic contaminants degradation because root exudates support growth and metabolic activity of microbes (Johnson *et al.* 2005). The root plants exudates to stabilize, demobilize and bind the contaminants in the soil matrix, thereby reduce their bioavailability (Tangahu *et al.* 2011).

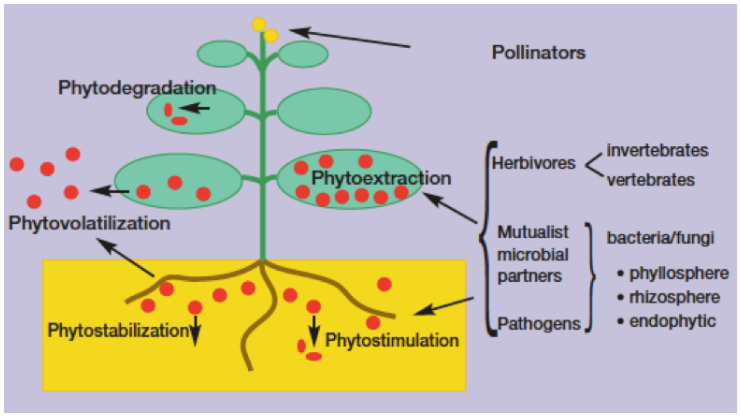


Figure 2. 10. Overview of phytoremediation methods(Pilon-Smits and Freeman 2006).

Plants act both as TE "excluders" (restrict contaminant uptake into their biomass) and "accumulators" (survive despite concentrating contaminants in their aerial tissues and can

biodegrade or biotransform the contaminants into inert forms in their tissues) (Tangahu *et al.* 2011). Plants have evolved highly specific and very efficient mechanisms to uptake, translocate and store TE. Plant roots, aided by plant-produced chelating agents and plant-induced pH changes and redox reactions, are able to solubilize and take up TE from very low levels in the soil, even from nearly insoluble precipitates (Tangahu *et al.* 2011). Following mobilization, the initial contact of the TE ion with root cell involves its biosorption at the cell wall via ion-exchange and chelation at cellulose, hemicellulose, pectin, and some minor polymers (Gupta 2013). The transport of TE into plant cell involved proton pumps, co- and antitransporters, as well as channels (Fig.2.11).

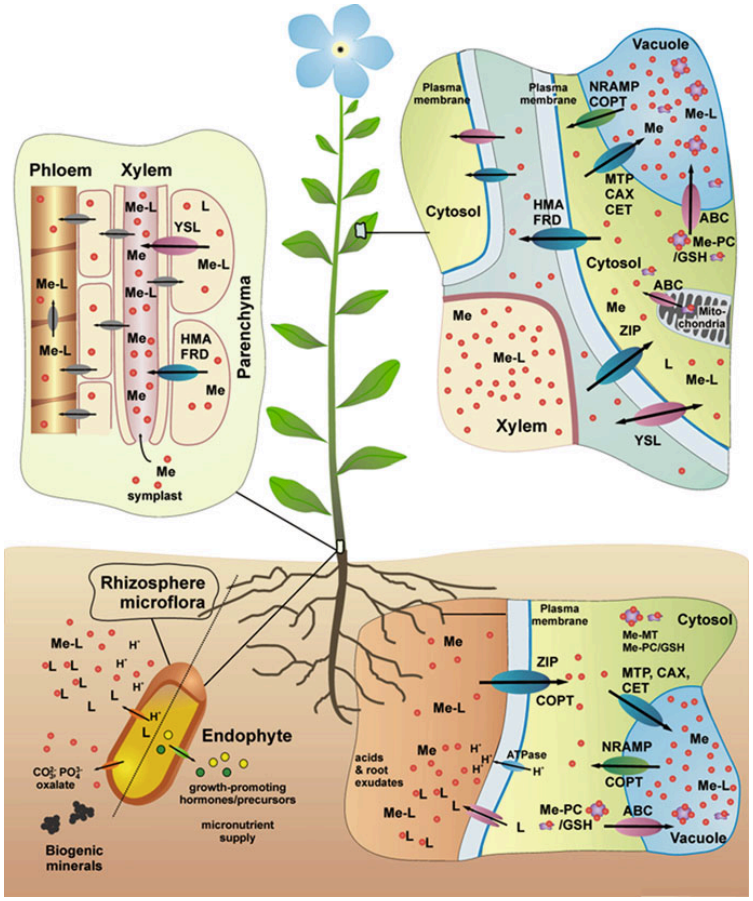


Figure 2.11. Molecular determinants and mechanisms involved in metal–plant interaction. The metal is mobilized in the rhizosphere by secreted acidic or chelating molecules of both plant (root exudates) and microbial origin. The soil microflora may also trigger formation of organic or inorganic secondary minerals (carbonates, phosphates and hydroxides increases, oxalates), rendering the metal less available to plant. Endophytic bacteria inhabiting the plant mainly contribute

mainly by promoting plant growth (support, e.g., the acquisition of micronutrients or the production of plant hormones). Root-to-shoot translocation of metals, either as hydrated ions ormetal–ligand complexes, occurs via the xylem. Metals reaching the aboveground apoplast are than differentially captured in different cell types, moving also cell-to-cell through plasmodesmata(not shown). Transporters and transporter families involved in uptake and redistribution of metals within plant body and cells are indicated: ZIP zinc-regulated, iron-regulated transporter protein family; COPT copper transporter family (syn. CTR); HMA heavy metal ATPase of P1B-type(Gupta 2013).

Physical, chemical and biological remediation techniques have been used for many years in order to mitigate or remove pollutants from soils. Selection of the best method for remediation depends on many factors such as soil characteristics, concentration of pollutants, future use of contaminated land, purpose of remediation, allowable amount of contaminants in the medium, type of pollutants, available methods, economic conditions, and time to remediate (Moghadam *et al.* 2016). Since remediation of co-contaminated soil is complex, mixing few remediation technologies promises to be the most effective method. However, is important to carefully consider the factors that may influence the remediation process.

2.6 Factors influencing hydrocarbon and trace elements remediation

The extent and rate of bioremediation depend on many factors which can be divided into three groups (i) physico-chemical factors (chemical structure, concentration of pollutants, physical and chemical properties of the soil); (ii) environmental conditions (temperature, pH, oxygen and nutrient availability); and (iii) micro-organisms (genetic composition, number and type of microorganisms, interactions between them (Haritash and Kaushik 2009; Singh 2006c). Thus, the heterogeneity of pollutants, their concentration and variety of environmental conditions limit the bioremediation. Few of the most important factors will be developed below.

2.6.1 Biological factors

Plants and microorganisms can degrade a large variety of contaminant but native organisms have the high capacity of degradation. The success of the bioremediation technique depends upon the identification of suitable species that remove, stabilize or accumulate contaminant and produce large amounts of biomass (Tangahu *et al.* 2011). Acclimatization of microorganisms due to the exposition to higher levels of pollutants may result in genetic adaptability (Haritash and Kaushik 2009). Enzymes secreted by microorganisms influence the hydrocarbon biodegradation because most of them are substrate specificity and active at different temperatures (Haritash and Kaushik 2009). Surfactant-like compounds produced by some microorganisms when growing on aromatic hydrocarbons solubilize the PAH and leads to increase in concentration in the medium (Haritash and Kaushik 2009). It could, also at times, cause inhibition of the degradation process (Carmichael and Pfaender 1997).

2.6.2 Physico-chemical factors

Bioavailability of pollutant is an important factor in the bioremediation. Bioavailability is directly related to the balance between the amount of the pollutant adsorbed and dissolved organic matter. The primary soil factors controlling the potential bioavailability of TE are soil organic matter content, soil pH, the accessibility and character of sorption sites on soil surfaces, the contents of Fe and Al oxyhydroxides, clay fraction content, and the cation exchange capacity (Alvarez *et al.* 2009). As a result, the bioavailability of TE ions in soil is limited, because of their presence in mineral form, formation of hydrous oxides at pH >5, and strong binding to soil components like humic and fulvic acids (Gupta 2013). The bioavailability of organic and inorganic pollutants which are in prolonged contact of the soil is reduced because they are bound to the soil particles (Haritash and Kaushik 2009). This phenomenon called sequestration it mainly due to the organic matter but also to the cation exchange capacity (CEC), the micropore volume, the soil texture and the surface area (Chung and Alexander 2002). In the case of PAH, the increase of molecular weight results in the decrease of the solubility and the bioavailability (Haritash and Kaushik 2009). The slow rate of remediation in soil is primarily due to the slow rate of desorption of pollutants from the soil particles and not due to the slow rate of degradation

by microorganisms (Haritash and Kaushik 2009). The concentration of pollutants also modifies the biodegradation rate: low pollutant concentrations are insufficient to induce the synthesis of degradation enzymes by competent bacteria. On the other hand, high concentrations of compounds are toxic for organisms (Borja *et al.* 2005). Studies also showed that the presence of PAH in a mixture produces interactive effects, which can either increase or decrease the rate of degradation of individual PAHs. For example, the presence of phenanthrene inhibited the bacterial degradation of pyrene (McNally *et al.* 1999). Anthracene degradation by *Rhodococcus* sp., was also inhibited by the presence of fluoranthene (Dean-Ross *et al.* 2002). In contrast, pyrene and phenanthrene degradations by *Pseudomonas putida* strain KBM-1 were stimulated by phenanthrene. During they co-metabolism study, Yuan *et al.* (2002) reported that the biodegradation is more efficient when fluorene, phenanthrene, acenaphthene, anthracene and pyrene are present simultaneously compares to the individual rate degradation.

Temperature is an important environmental factor in the bioremediation of organic and inorganic compounds. The temperature affects the growth of plants and microorganisms as well as the catalytic activity of enzymes involved in the degradation of PAH. Under aerobic conditions, low temperatures induce a decrease in the biodegradation of PAH. This is due to the reduction of (i) microbial metabolism and (ii) bioavailability of the less soluble PAH (Eriksson *et al.* 2003). However, in anaerobic culture conditions, bacterial biodegradation of PAHs is similar in low and high temperatures(Eriksson *et al.* 2003). Oxygen is essential for the absorption of nutrient at the root zone and for aerobic oxygenation of hydrocarbons in the environment. The lack of oxygen limited biodegradation of PAHs with more than three rings (Wiegel and Wu 2000). Moreover, availability of nutrients as carbon sources in contaminated soil is known to enhance the rate of hydrocarbons degradation. Agronomical practices such as oxygen and pH adjustment, addition of chelators, fertilizers are developed to enhance remediation. For example, the addition of compost for example helps to enhance hydrocarbons degradation by providing nutrients to the microbial population, oxygen transfer, inhibit formation of extractable polar intermediates and enhance soil texture (Haritash and Kaushik 2009). The pH is important for both organic and inorganic remediation because it induces a change in the adsorption and desorption (Borja *et al.* 2005). Since the bioavailability of TE in soils decreases above pH 5.5–6, to reduce lead uptake by plants, the pH of the soil is adjusted with lime to a level of 6.5 to 7.0 (Tangahu *et al.* 2011).

Preface (Chapter 3)

Soil is an essential component of the environment. It has long been studied because it is the medium in which many biogeochemical cycles occur, the support of many forms of life and the basis of agriculture. However, the losses of contaminants during industrial and commercial activities and inadequate storage lead to soil contamination by toxic elements. The risks posed by these elements in soils generally come from the presence of more than one TE and PHC. Some auto scrap yards have been abandoned without any continuing management and have caused TE and PHC pollution in neighboring agricultural soils and crops. A crucial step in the remediation of these contaminated areas is their characterization. This characterization is essential to obtain information about the physico-chemical composition of the soil as well as the nature of toxic effects of TE and PHC. It is also a crucial step in the planning of any remediation process. This chapter focus on the physicochemical and toxicological characterization of PHC and TE- contaminated soil. Results of this study are expected to contribute or provide some insight to the planning and selection of remediation process.

Chapter 3: Physicochemical and ecotoxicological characterization of petroleum hydrocarbons and trace elements contaminated soil

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Author contributions:

Conceived and designed the experiments: CM, WH. Performed the experiments: CM. Analyzed the data: CM, WH, YJ, FK. Contributed reagents/materials/analysis tools: WH, MH. Wrote the paper: CM, WH, YJ, FK, MH.

Abstract

Underground storage tanks used for auto oil spill waste contain many hazardous materials, including polycyclic aromatic hydrocarbons (PAHs) trace elements. These compounds pose a significant threat to the environment and affect negatively human health. This study was aimed at characterization of two soil samples from a former auto scrap yards in which oil spill tank leakage occurred. The soil samples collected from an area of $5m^2$ around the oil tank (Soil-N5 samples) were highly contaminated with petroleum hydrocarbons (PHC) and TE (Co and Pb) while the soil samplings at about 500 m from the tank (Soil-N500 samples) were contaminated with Co. The characterization of these soil samples included the analysis of the relevant physico-chemical soil properties as well as ecotoxicological tests with plant (seed germination and seedling growth of *Lepidium sativum*), earthworm (*Eisenia fetida* survival and development) and soil microorganisms (oxygen uptake rate in soil and aqueous suspension). Toxicity tests showed that contaminants had strongly negative effects on earthworm's development and *L. sativum* shoots dry biomass in both N5 and N500. These two parameters were the most sensitive in reflecting toxicity of study soils. Oxygen uptake rate (OUR) in aqueous phase was four times higher than that of the solid phase even though a similar trend was observed in both phases (aqueous and solid). Moreover, microorganism's respiration was high in N5 in comparison to N500 due to the mineralization of readily available OM and/or organic pollutants as well as the inhibitory effect of TE on soil respiration. The combination of chemical analyses with different toxicity tests proved to be adequate to assess the quality of these PHC and TE contaminated soils.

KEYWORDS: Cobalt, Lead, Oxygen uptake rate (OUR), Polycyclic aromatic hydrocarbon (PAHs), Toxicity tests.

Introduction

Petroleum hydrocarbons (PHC) and trace elements (TE) are considered as the most hazardous contaminants in the world due to the long range of toxicological effects for plants, animals and human beings (Cobbina *et al.* 2015; Marchand *et al.* 2011; Jakasa *et al.* 2015). Many polycyclic aromatic hydrocarbons (PAHs) such as benz(a)anthracene, benzo(a)pyrene and chrysene are toxic, carcinogenic, teratogenic and mutagenic pollutants (Masiol *et al.* 2012; Adisa *et al.* 2015). Some of the TE are essential for plants and animals like manganese (Mn) and cobalt (Co) while others like arsenic (As) and lead (Pb) are known to have no role in living beings and they are highly toxic (Khan *et al.* 2015b; Pourrut *et al.* 2011; Hanumanth Kumar and Pramoda Kumari 2015). It was found that TE cause major environmental and human health problems because of their trophic transfer in organisms, biomagnification in food chains and toxic effects on living (Song *et al.* 2009; Li *et al.* 2010a). The toxic effects of TE include growth retardation (Di Salvatore *et al.* 2008), cell plasmolysis (Basile *et al.* 2012), alteration of plant photosynthesis machinery and membrane permeability (Hanumanth Kumar and Pramoda Kumari 2015), neuronal degeneration in brain (Cobbina *et al.* 2015) and carcinogenic healthy risks (Bhattacharjee *et al.* 2013). In the view of all these considerations, the remediation of contaminated soils with PHC and TE has become a priority.

Soil characterization is a crucial step in the planning of any remediation process (Mao *et al.* 2009). It is widely known that soil characterization is measured by the determination of the total concentration of pollutant (Qiao *et al.* 2011). However, the actual risk exposures, which depend on the bioavailable fraction of pollutant, are more important than the total concentration. Different researchers highlighted the fact that toxicity is influenced by the available contaminants in soil which depends in turn on the soil characteristics (e.g. organic matter and clay content) and the contaminants residence time in soil which is often known as “ageing” (Marti *et al.* 2013; Stella *et al.* 2015). Therefore, it has been recommended to characterize any contaminated soil by combining the chemical analyses with different levels of toxicity tests (Eom *et al.* 2007; Masakorala *et al.* 2014; Plaza *et al.* 2010). Many guidelines were developed to find the environmental risks of contaminant by using different species at different levels of biological organization (Romero-Freire *et al.* 2015; Fernández *et al.* 2005). For example, soil organisms like the collembolans; the earthworms and the plants were used to test the toxicity of

contaminants in soils due to the high sensitivity of these organisms to any changes in the ecosystem (Masakorala *et al.* 2014; Eom *et al.* 2007). Other soil organisms were also used like algae and bacteria (Kaczala *et al.* 2011; Ding *et al.* 2015). Soil biological properties such as soil respiration have been successfully used as biological indicator of soil quality(Soler-Rovira *et al.* 2013; Plaza *et al.* 2010). The respirometer is a highly sensitive and non-disruptive technique which can generate qualitative and quantitative information on the microbial activities in contaminated soils (Taccari *et al.* 2011). But due to the fact that respirometer is expensive, troublesome, needs constant maintenance and frequent calibration (Barrena Gomez *et al.* 2006), earthworm and plant toxicity tests have been widely used as an alternative (Oleszczuk 2008; Eom *et al.* 2007; Li *et al.* 2009a; Hirano and Tamae 2011). These two methods are simple, cost effective and allow *in situ* monitoring of the soil real contaminated conditions (Hentati *et al.* 2013).

Characterization of contaminated soils with petroleum hydrocarbons and TE has been in focus of many researchers but most of these studies were done by physicochemical analyses (Mao *et al.* 2009; Qiao *et al.* 2011). Available information on both chemical and toxicological analyses of former yards is rare. Therefore, the goal of the present study was to characterize the PHC and TE-contaminated soils through the analysis of both physicochemical and eco-toxicological properties as an essential step for initiating a field scale remediation project. The combined approach should orient decision support for the selection of the most appropriate site-specific remediation approaches.

Materials and Methods

Site description and sampling

The studied area was an auto scrap yard located in the city of Nybro, in the southestern part of Sweden (56°45′0” N; 15°54′0” E). The site has been used as an automobile repairing shop since 1984. In the yard, an underground tank was used to collect waste oils from vehicles. The tank’s overfilling protection system was out of order, which made large amounts of oils to spill down and contaminating the area around the tank. Soil samples were collected by excavating an area of $5m^2$ around the tank (Soil-N5 samples). Ten samples were randomly collected from a depth of 1 m to produce three independent composite samples 50 kg fresh

weight (FW) each. Also another 10 samples were collected from the soil at about 500 m from the tank (Soil-N500 samples) at a depth of 0.5 m to produce three composite samples. All samples were stored in plastic bags, and transported to the laboratory at Linnaeus University (Kalmar, Sweden). Then, the samples were immediately homogenized by manual mixing and sieved through an 8-mm mesh. All the samples were kept at 4 ± 1 °C between sampling and analysis.

Chemical and physical characterization of soil samples

Solid Phase

The studied soils were characterized for texture by using the protocol described by Colorado Master Gardener Notes (Whiting *et al.* 2011). The texture was found by measuring the relative proportion of sand, slit and clay(Whalen and Sampedro 2010). The soil dry weight (DW) was determined by taking the difference in weight for the samples before and after warming in a ventilated oven at 105 ± 5 °C until reaching to constant mass (ISO 11465 1993). Organic matter (OM) was found by determining the difference in the weight of the pre dried samples at 105 °C before and after 16h in a muffle furnace at the temperature of 550°C (ASTM D 2974, n=3)(Cheng *et al.* 2008). PHC concentration was measured by gas chromatography coupled to mass spectrometry (GC-MS) using a commercial service provided by AGAT Laboratories Montreal, QC, Canada. TE concentration was measured by X-Ray fluorescence (XRF) equipment (XRF model Olympus DS-4000, Innov-X Systems, Inc. USA). The XRF method was applied for fluorine and heavier elements with a typical detection limit of 0.01% (w/w)(Kaartinen *et al.* 2013). All analyses were performed in triplicates for each composite soil sample except for PHC concentration, which was done in duplicate.

Aqueous Phase

Aqueous phase from each soil sample was extracted by mixing 100 g of air-dried soil with 500 ml of deionized water for 1 hour in a magnetic stirrer (Cheng *et al.* 2008). The suspensions were centrifuged twice at 16.000 x g for 15 minutes at 13°C (Avanti J-25, Beckman Coulter, Inc. California, USA). Dissolved oxygen (DO), pH and the electrical conductivity (EC) of water extracts were analysed with an HQ11d portable meter (Hach Company, USA). Total organic carbon (TOC) and chemical oxygen demand (COD) were analysed with Dr Lange

cuvette tests (Dr Bruno Lange, GmbH& CO. KG, Dusseldorf, Germany). Cuvettes were measured spectrophotometrically with a HACH XION 500 spectrophotometer. Lange methods were validated according to ISO 8466-1 (1990), DIN 32645 (1996) and DIN 38402 A51 (1986). All analyses were performed in triplicate for each composite soil sample.

Ecotoxicological characterization

Plant toxicity test

Seed germination and seedling growth tests were conducted with the cress (*Lepidium sadivum*) based on the procedures described in the ISO 11269-2 (ISO, 1995). Seeds were obtained from Weibulls Seed Company (Sweden). Soils (500g FW) were placed in 0.5 L plastic pots while the remaining 50% of the pots was left for the water holding capacity. Fifteen cress seeds were sowed on the surface of each wetted soil in a greenhouse that was kept at 25 ± 2 °C, with a photoperiod of light to darkness of 16:8 (h) and photosynthesis active radiation of 270 umol m^{-2} s^{-1} provided by Lu400W/PSL/T40 (Lucalox^{TM}). The standardized field soil LUFA 2.3 (Landwirtschaftliche Untersuchungs- und Forschungs-Anstalt, Speryer, Germany) with its sandy loam was used as a control soil. Deionised water was added every day (50 % water holding capacity). Seed germination was determined by visual seedling emergence and after 3 days the number of germinated seeds was recorded. Then, the cress seedlings were thinned and only five of the most uniform plants per pot were left for further measurements of plant growth at the end of a 21-day period. Roots and shoots of each individual plant were harvested, washed with deionized water, blotted and the fresh biomass was immediately weighed. Dry biomass was determined after oven drying at 70°C for 48h. All experiments were carried out in triplicate for each composite soil sample.

Earthworm test

Acute toxicity test with earthworm *Eisenia fetida* was carried out according to the OECD 207 – "Earthworm, Acute Toxicity Tests". Earthworms were obtained from an earthworm-culturing farm located in Ljungby city in Sweden. First all the earthworms were rinsed with distilled water, and maintained on Whatman No1 filter in the dark at room temperature for 24 h to void of gut contents. Ten earthworms adult (0.3± 0.05g) were washed and weighed before being transferred into 250 ml glass vessel containing 400 g DW of soil sample. The standardized

field soil LUFA 2.3 was used as a control. The experiments were carried out in triplicate for each composite soil sample, with loose lids placed over the test vessels. After 14 days, the mortality registered, the surviving worms were washed and weighed and the change in weight (CW, %) was determined.

Respirometry

Respirometric test was carried out to evaluate the toxicity of the studied soil samples through measuring O_2 uptake rate (OUR), which is an indicative for the microorganisms biological activity (Paletski and Young 1995). Respirometric measurements were performed directly in soils according to (Sanchez Arias, Fernandez et al. 2012) and in aqueous suspension according to (Cantin *et al.* 2004). The respirometric test was done in duplicate for each composite soil sample at 25 °C with a residence time of 21 days using a pulse-flow headspace respirometer with accompanying software (PF-8000, Respirometers System and Applications, Fayetteville, AK, USA). The temperature was kept constant at 25 ± 1°C by using an appropriate water bath. Magnetic stirrers were used with an agitation speed of 200 rpm to avoid soil settling.

Statistical analysis

Statistical analyses were performed with GraphPad Prism (version 6.0 for mac OS, GraphPad Software Inc., San Diego, USA). The differences among studied soils in relation to their physico-chemical and eco-toxicological characteristics were verified through One-Way ANOVA tests. Whenever significant differences were found ($p<0.05$), a post hoc Tukey's HSD test was used to further elucidate differences among means ($p<0.05$). Conditions of normality and homoscedasticity of data were checked in all cases.

Results and Discussion

The Soil physico-chemical properties and the total contaminants content

The investigated soils – (N5 and N500) were characterized in terms of texture, physico-chemical properties, TE and PHC content. As shown in table 3.1, comparing the results of the concentration of PHC and TE with the threshold limits established by the Swedish EPA (2009) and the Canadian Ministry of the Environment (2011) it was observed that the concentrations

of As and Pb in the soil-N5 were higher than that of the N500 and the Swedish EPA. Only the concentration of Co in the soil of N500 was higher than that of the N5 and the concentration in both soils were higher than the allowable value of the Swedish and the Canadian limits. The observed concentrations of TE in this current study were in agreement with previously reported investigations addressed to contaminated soils in auto scrap yard areas (Chicharro Martín *et al.* 1998; Jaradat *et al.* 2005). The elevated concentrations of TE in this area could be attributed to stores of scrap iron and the activity related to them e.g. Pb from electric batteries and Co from car paint and motors (Jaradat *et al.* 2005). In addition to inorganic contaminants, Chicharro Martín *et al.* (1998) revealed the presence of engine oil and break liquids as the main organic contaminants. Here, results showed that the PHC C10-50 (alkanes), the medium (M) and the high (H) molecular weight PAH concentrations in soil-N5 were higher than the Swedish EPA values in residential soils and the Canadian ministry of the environment (Canadian Ministry of the Environment 2011; Swedish Environmental Protection Agency 2009).

The results showed that there was a slight difference in the pH value between the N500 and N5 soil, 7.1 and 6.5 respectively. The pH value of N500 soil was comparable to those obtained for similar areas by Jaradat *et al.* (2005), where the pH ranged from 7.63 to 7.73. However, the lower pH of N5 soil contrast with these values and also with those findings reported by Masakorala *et al.* (2014) who reported a significant pH increase in crude oil-contaminated soil. This difference could be explained by the chemical reactions between PHC, TE and soil elements. As reported by Chandrasekaran and Ravisankar (2015) a reduction of the soil adsorptive capacity towards TE such Co and Mg usually is a result of a decrease in pH values. In a previous study, Chuan *et al.* (1996) found that the pH-dependent TE adsorption reaction was the main mechanism controlling the release of TE from soils. Generally TE solubilities were high under slightly acidic conditions (pH = 5.0) and neutral conditions were not favorable for metal solubilization (Chuan *et al.* 1996). In the present research the pH values of the studied soils were almost neutral. According to Murata *et al.* (2003) at this pH range, the mobility of the most TE is low.

Table 3.1. Physicochemical properties, petroleum hydrocarbons and TE content in soil-N500 and soil-N5. Values represent the arithmetic means of the different soils with standard deviation (N=3 for each category except for PAH: N=4).

		Soil-N500	Soil-N5		
	Unit	Solid phase properties		SL[a]	CL[b]
Texture		Sandy Loam	Silt Loam		
Sand	%	66 ± 2	14.3 ± 0.5		
silt		22.6 ± 2.1	76 ± 1		
Clay		11.3 ± 0.6	9.6 ± 1.1		
pH (1:5,w/v)		7.1 ± 0.4	6.5 ± 0.3		
Moisture	%	8.2 ± 0.1	60.9 ± 0.4		
OM		9.5 ± 0.6	31.4 ± 2		
Arsenic	mg/kg	Nd	12.3 ± 3.0	10	18
Manganese		223.5 ± 9.2	378.3 ± 36.5	-	
Cobalt		149 ± 28.9	92 ± 9.5	15	21
Lead		17.5 ± 0.46	195 ± 80.7	50	120
Iron		11107 ± 691.9	11117.6 ± 8.1		
Chromium		21 ±2.6	16.6 ± 1.5	80	70
Copper		13 ± 1	18.33 ± 2.5	80	92
Zinc		51.3 ± 2.8	139 ± 10.3	250	290
PHC C10-50		Nd	27066.6 ± 3625.3	3-100	10-240
PAH-L[c]		Nd	2.1 ± 1.8	3	0.255
PAH-M[d]		Nd	7.7 ± 3.8	3	2.53
PAH-H[e]		Nd	5.0 ± 2.4	1	5.42
		Aqueous phase[f] properties			
EC	us/cm	65.9 ± 2.4	133.1 ± 14.6		
DO	mg/L	6.6 ± 0.1	4.8 ± 0.2		
COD		234.3 ± 39.3	86100 ± 556.7		
TOC		33.2 ± 4.9	1221 ± 8.7		

[a]Swedish limit in sensitive land (Swedish Environmental Protection Agency (2009)

[b] Canadian limit in residential/industrial land (Canadian Ministry of the Environment 2011)

[c]PAH-L: naphthalene, acenaphthene and acenaphthylene

[d]PAH-M: fluorene, phenanthrene, anthracene, fluoranthene and pyrene

[e]PAH-H: benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene,benzo (a) pyrene, dibenz (ah) anthracene, benzo (ghi) perylene and indeno (123cd) pyrene.

[f] Water-extractable (1: 5; 25 °C); PHC C10-50, petroleum hydrocarbons C10-C50; EC, Electrical conductivity; DO, dissolved oxygen; COD, chemical oxygen demand; TOC, total organic carbon; Nd, not detected.

Another parameter which significantly affect TE bioavailability is the content of OM through the sorption/desorption processes (Alexander 2000). The percentage of organic matter (OM) was higher in N5 (31.4%) compared to N500 (9.5%). As a result, with an increase in the OM content the sorption reactions increased and the rates of chemical release to the aqueous phase decreased (Stella *et al.* 2015). Previous authors have also reported the sorption of TE such as Pb to OM since its surface functional groups have high affinity (Minkina *et al.* 2006; Kim *et al.* 2015). The N500 texture was classified as sandy loam while the N5 was classified as silt loam on the basis of silt, sand and clay contents. Both sand and silt particles are considered to contribute little to chemical reaction due to their low solubility and lack of absorptive surface (Whalen and Sampedro 2010). However, silt particles abundant in N5 exhibited some plasticity, cohesion and adsorption. In comparison, sandy loam N500 with a small surface area and high porosity facilitate the passage of oxygen and water (Whalen and Sampedro 2010). As a consequence, the percentage of moisture was higher in N5 (60.9%) compared to N500 (8.2%). In addition to the soil texture, the high moisture content in N5 is characteristics of the disturbed soils. Sheppard *et al.* (2000) reported a moisture content twofold higher in TE contaminated soil in comparison to the uncontaminated soil and attributed this to soil compaction.

The results regarding water extraction from the contaminated soils showed that contents of dissolved oxygen (DO) were lower in N5 compared to N500 (Table 3.1) that may be attributed to the soil texture e.g. sandy loam N500 facilitates the passage of oxygen and water; but mainly to the amount of organic matter. COD, which is an indication of oxygen demand to oxidize chemically organic and inorganics contents, indicates the mass of oxygen consumed per liter (Jiang *et al.* 2015). This parameter and TOC are widely used as indicator of water quality (Kaczala *et al.* 2011; Svensson *et al.* 2015). The COD values were 86100 and 234.3 mg/L for N5 and N500 respectively, shown in table 1. Furthermore, the TOC results ranged between 1221

mg/L and 33.2 mg/L for N5 and N500 respectively, which confirmed the higher amount of carbon contents in the water extract. In a previous study, Liu *et al.* (2015) reported a high level of COD in the Taizi River due to the presence of high toxic organic pollutants such as PAH. Thus, the higher contents of COD and TOC in N5 in comparison to N500 (Table 3.1) could be attributed to the high amount of PHC C10-50, PAH-M and PAH-H, in N5. The EC of the water extract indicates the relative water-soluble salt content of the soil (Sheppard *et al.* 2000). The EC for N5 and N500 was lower that those previously found by Jaradat *et al.* (2005) and by Chicharro Martín *et al.* (1998) in the scrapyard, which may indicate lower content of soluble salts.

Ecotoxicological analysis of soils

Plant toxicity test

In order to have full characterization of both studied soils (N5 and N500), the physico-chemical analyses were complemented with eco-toxicological analyses for the aqueous and solid phases. Three levels of eco-toxicological analyses were conducted: plant (*Lepidium sativum*), earthworms (*Eisenia fetida*) and soil microorganisms. As shown in Fig.3.1 the toxicity results of the *L. sadivum* seed germination rates were 93.3%, 44.4% and 71.1% for the standard field soil LUFA 2.3, N500 and N5 respectively (Fig.3.1a). The germination rate in N5 had not significant difference in comparison to N500. However, the contaminants in the N5 soil posed negative effects to shoots and roots of the *L. sativum* (Fig.3.1b-c). The present results are in accordance with Oleszczuk (2008) who found that PAHs and TE did not show any significant adverse effect on the seed germination even though growth reduction was observed. This may be explained by the fact that seed coats have selective permeability meaning that pollutants may have inhibitory effects on root growth but the germination is not affected due to impossibilities of passing through the seed coats (Lin and Xing 2007).

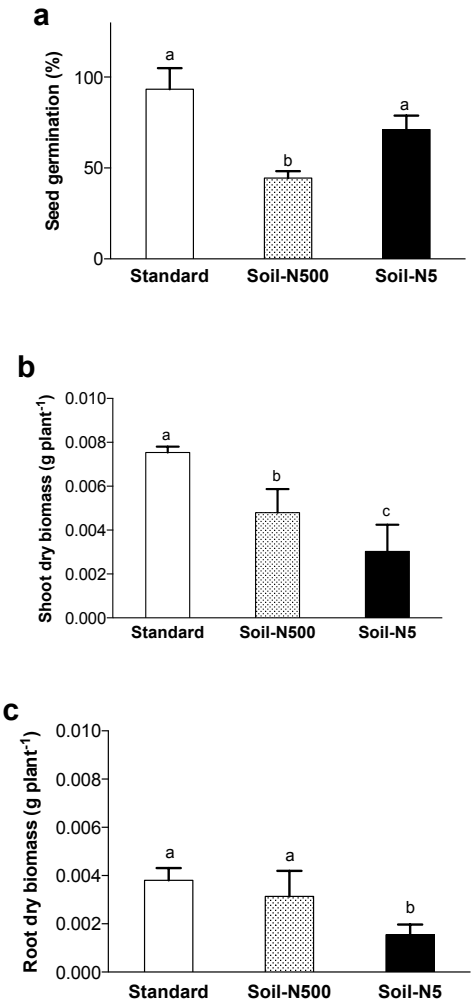

Figure 3.1. Seed germination (%, a), shoot (b) and root (c) biomasses (g plant^{-1} DW) produced by *Lepidium sativum* after a 21-day growth in the two studied soils (N5 and N500). Mean for each treatment are the means of three replicates and error bars represent the standard deviations. The different letters above bars indicate significantly differences means according to Tukey's HSD test ($p<0.05$).

Since roots are the first tissue directly exposed to contaminants, the toxic effects may appear in roots rather than in shoots. This may explain why the roots biomass in N5 highly affected by the contaminants (Fig.3.1b-c). Contrariwise in N500, no significant effects were observed on the roots biomass but the seed germination and the shoots dry biomass were lower compared to the standard soil. As previously observed by Li *et al.* (2009a), the shoots biomass of barley, oilseed rape and tomato was significantly inhibited by Co. The Co^{2+} is absorbed by

plants roots through an active transport across cell membranes (Li *et al.* 2009b). Toxicity symptoms of Co^{2+} in plants can be caused by competition between Co and Fe for the same physiological binding sites (Chatterjee and Chatterjee 2003; Sree *et al.* 2015). Thus, the excess Co in plant induced Fe deficiency symptoms e.g. chlorosis on young leaves, decrease of leaf biomass (Sree *et al.* 2015).

Earthworm test

Additional to plant toxicity test, many studies have considered earthworms as one of the most important soil invertebrates because they produce channels that increase soil porosity, increasing the biotic activities by transforming organic matter into small fragment and secreting substances rich in nutrient (McCauley *et al.* 2005). No mortality was registered when earthworms (*E. fetida*) were exposed to standard soil, N5, and N500. As shown in Fig.3.2, high loss in the weight of the earthworms was observed after 14 days of exposure to both N5 and N500 (Fig.3.2). The loss in weight in the N5 was higher than that of the N500 by 3%. On the other hand, an increase in the weight of the earthworms (9.3) was observed in the standard field soil LUFA 2.3. In a previous study, (Luo *et al.* 2014) also found a significant weight loss of the earthworms after 28 days of exposure to Pb.

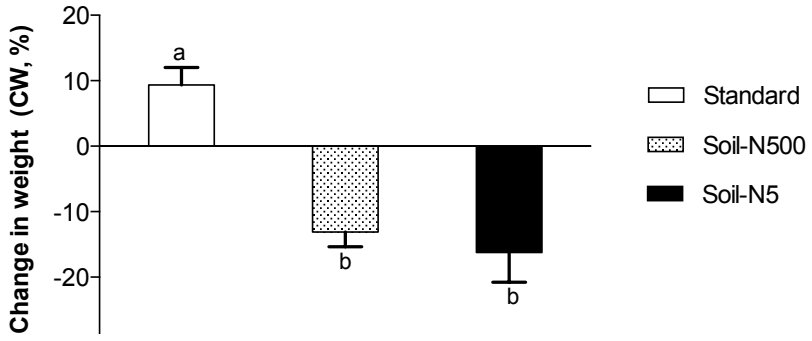

Figure 3.2. Earthworm's change in weight (CW%) after a 14-day growth in a standard uncontaminated soil and in the two studied soils (N5 and N500). Mean for each treatment are the means of three replicates and error bars represent the standard deviations. The different letters above bars indicate significantly differences means according to Tukey's HSD test ($p<0.05$).

Respirometry test

Like the earthworms, soil microorganisms play vital role in different processes such as organic matter decomposition, nutrient cycling and modification of soil physical structure (Plaza *et al.* 2010). Microbial respiration has been previously used to assess the toxicity of PHC and TE in soil (Montserrat *et al.* 2006). The results obtained during the respirometric tests with both soils N5 and N500 are shown in Fig. 3.3. The variation of the oxygen uptake rates OUR in the aqueous phase were four times higher than that of the solid phase even though similar uptake patterns were observed in both phases. In the aqueous phase, OUR values were high in the beginning (6.7 for N500 and 7.7 mgO2/L-h for N5) and then decreased with time (Fig.3.3a). As previously found by (Chica *et al.* 2003) the oxygen uptake rate was at the maximum when the experiment started in aqueous suspension then decreased throughout the process due to respiratory activity. Respirometry data from both soils has shown the same kinetics but OUR at the beginning was higher in N5 (Fig.3.3b). Mukherjee *et al.* (2014) reported similar patterns when they measured microbial activity in an aged creosote-contaminated soil. They found that with high contaminant exposure the diversity of microorganisms decreased with increasing the total microbial activities in soils (Mukherjee *et al.* 2014). Moreover, Plaza *et al.* (2010) found a high viable microorganisms in PHC contaminated soils by two fold than in TE contaminated soils when they performed ecotoxicological and microbiological characterization of soils from TE and PHC contaminated sites. They attributed the presence of these viable microorganisms to the adaptation of contaminant. This high respiration in N5 could be also due to the transformation/mineralization of readily OM and/or organic pollutants. The low viable microorganisms found in TE contaminated soils by Plaza *et al.* (2010) might be explained by the TE inhibition effect on soil respiration found by Kızılkaya *et al.* (2004). When TE content in soil increase, soil respiration decreased (Kızılkaya *et al.* 2004; Doelman and Haanstra 1984). After the first high respiration phase, a decrease of readily biodegradable substrate causes a decrease of OUR in both soils at different rates depending of the pollutants content. Thus, the respiration rate in N5 decreased faster than in N500 (Fig.3.3b).

Previous researchers highlighted the fact that the toxicity is influenced by the availability of contaminant for soil organisms, which depends on the soil properties and composition (Marti *et al.* 2013). It can be clearly visible in the data presented in (Fig.3.1-3) that the results of the

bioassays depend on the type of soil (N5 or N500) and the sensitivity of the parameters tested (seed germination, plant growth, earthworm mortality or biomass production and oxygen uptake rate) a negative effect of organic and inorganic contamination was most clearly visible on the inhibition of earthworm biomass production and on shoot dry biomass.

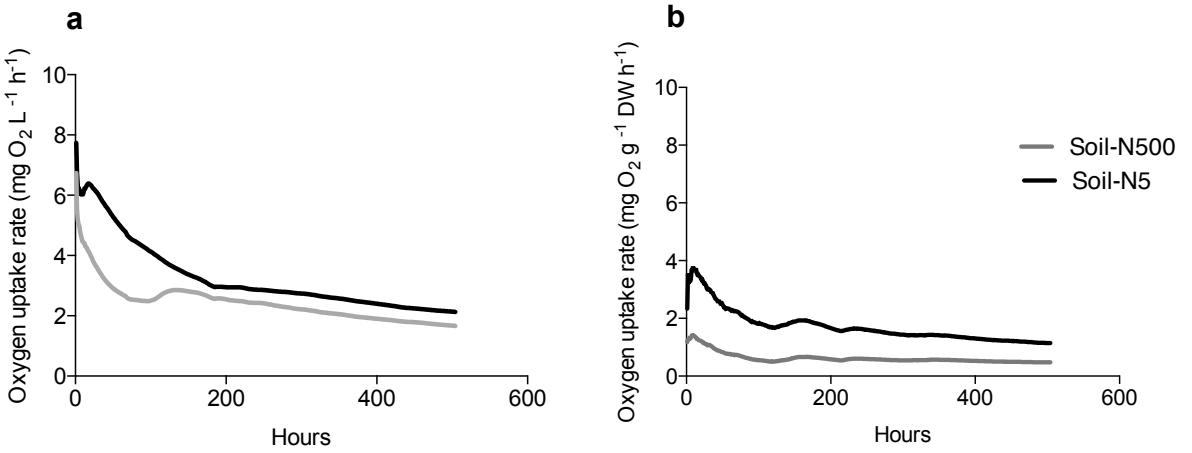

Figure 3.3. Oxygen uptake rate (OUR) related to microbial activity in the two studied soils (N5 and N500) for the aqueous phase (a) and for the solid phase (b). The values are the average of two replicates.

Conclusion

This study has provided physico-chemical and ecotoxicological characterization of contaminated soils in a former auto scrap yard with the main focus on PHC and TE (Co, As, Pb). This combined approach provided interesting information on the negative impact of PHC and TE contaminants. Results showed that microorganisms activity, *E. fetida* growth and shoot dry biomass of *L. sadivum*, were negatively affect by the contaminants in both N5 and N500. These bioassays were adequate to obtain fast answers with low costs, suggesting it should be used as a screening tool to assess soil contamination or its remediation. This study provides a comprehensive assessment of PHC and TE toxicity in a former auto scrap yard and is helpful for environmental management.

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Preface (Chapter 4)

The previous chapter showed that PHC and TE containing in the soil had a negative effect on earthworm's development and *L. sativum* shoot dry biomass. Moreover, a high respiration of microorganisms attributed to the transformation/ mineralization of organic matter or/and organic pollutants was observed. This presence of viable microorganisms suggested an adaptation of microorganisms to the pollutant. Since microbial diversity and abundance can be reducing in contaminated environment, cultured hydrocarbon degraders can be a promising approach to enhancing hydrocarbon biodegradation. However, the culture and isolation of microorganisms indigenous from contaminated site remained unclear. In a paper II (annex1), nutrient-rich (standard culture media) and impoverished media, supplemented with various hydrocarbons form and concentration (selective culture media) were used in other to assess the effectiveness of culture-based methods at recovering indigenous microorganisms from PHC contaminated soil. One of the best approaches to restoring contaminated soil is to make use of microorganisms, which are able to degrade a complex mixture of recalcitrant pollutants. The inherent capabilities of these microorganisms depend namely on (i) the availability of chemicals for the biodegradation; (ii) the quantity of these microorganisms, and (iii) their activity level (Bidoia *et al.* 2010). Currently, one of the limiting factors in the bioremediation is the identification of microorganisms having the metabolic capacity to degrade the recalcitrant organic pollutants. These highly efficient inoculants could be used *in situ* and *ex situ* to accelerate the remediation of zones polluted with high concentrations of hydrocarbons. They could be also used for further functional studies to improve actual knowledge on the molecular mechanisms involved in the degradation of organic pollutants. Although few studies have isolated microorganisms from contaminated soils and screened them for hydrocarbons biodegradation capacity, no study has specifically investigated the importance of using selective instead of standard culture media for the isolation of the most effective candidates. This chapter compared the degradation efficiency of PHC by bacterial and fungal isolated with standard and selective culture media.

Chapter 4: Petroleum biodegradation capacity of bacteria and fungi isolated from petroleum-contaminated soil

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Author contributions:

Conceived and designed the experiments: CM, MH, MSA. Performed the experiments: CM. Analyzed the data: CM. Contributed reagents/materials/analysis tools: MH. Wrote the paper: CM, TB, MH, MSA, WH.

Abstract

We investigated the potential for petroleum hydrocarbon biodegradation by 95 bacterial and 160 fungal strains isolated from a former petrochemical plant. We tested whether soil origin, culture media type, and strain taxonomy influenced the degradation of added petroleum hydrocarbon compounds. Preliminary screening was based on two colorimetric tests using 2,6-dichlorophenolindophenol and p-iodonitrotetrazolium indicators, to assess microbial strain tolerance to crude oil. Top-performing strains in these screening assays were then assessed for their ability to mineralize a mixture of four polycyclic aromatic hydrocarbons (PAH) for 49 days, using GC-MS quantification. The aerobic activity of these candidate strains was also assessed by respirometry over the first 24 days of incubation. On average, PAH degradation by microbial isolates from soil that was lightly, moderately, and highly contaminated with petroleum was equally efficient, and the type of culture medium used did not significantly impact mean biodegradation. Phylogenetic affiliation had a strong and significant effect on PAH biodegradation. Fungal isolates belonging to the group *Sordariomycetes,* and bacterial isolates belonging to the groups *Actinobacteria*, *Betaproteobacteria,* and *Gammaproteobacteria* showed high potential for PAH degradation. Three of the strains tested by GC-MS, *Rhodococcus* sp., *Trichoderma tomentosum*, and *Fusarium oxysporum,* significantly degraded all four PAH compounds in the mixture compared to the control.

Keywords: Bioremediation, respirometry, polycyclic aromatic hydrocarbon (PAH), Crude oil, colorimetric tests.

Introduction

The widespread exploitation, transportation, and consumption of crude oil has attracted public attention to the fate of petroleum hydrocarbon (PHC) compounds in the environment. Crude oil contains a complex mixture of many thousands of chemicals, including aromatics such as polycyclic aromatic hydrocarbon (PAH) compounds. PAHs are of concern to human health and the environment, since they are highly persistent, can negatively impact soil functions, and pose mutagenic risks to microorganisms, plants, and animals (Williams *et al.* 2013; Cao *et al.* 2009). Consequently, avenues for rehabilitating PHC-contaminated soils are of wide general interest.

A number of technologies have been developed for the rehabilitation of PHC-contaminated sites, including physical, chemical, and biological approaches (Gan *et al.* 2009; Rayu *et al.* 2012; Li *et al.* 2010b). Physico-chemical methods are expensive, may create further waste, and in many cases, simply transfer pollutants from one phase to another (Haritash and Kaushik 2009; Khan *et al.* 2004a). The use of living organisms for the rehabilitation of contaminated sites, also known as bioremediation, has attracted considerable research interest over the last decade, as a sustainable and cost-effective alternative to chemical treatment (Chikere *et al.* 2011; Kanaly and Harayama 2010). Biodegradation of PHCs by natural populations of bacteria and fungi is well known (Megharaj *et al.* 2011; Morelli *et al.* 2013; Palanisamy *et al.* 2014), as many microorganisms are able to use hydrocarbons as both energy and carbon sources (Montagnolli *et al.* 2015b). Through different extracellular and intracellular enzymatic activities reviewed by Fritsche and Hofrichter (2008), these microorganisms can mineralize PHCs (i.e. fully degrade them, with CO_2 as an end-product). The rate and extent of mineralization depend of the metabolic abilities of the microorganisms (Dobler *et al.* 2000). Numerous studies have demonstrated the potential for PAH bioremediation by bacteria (Palanisamy *et al.* 2014; Khan *et al.* 2013; Lu *et al.* 2011) and fungi (Sayara *et al.* 2011; Isola *et al.* 2013; Morelli *et al.* 2013; Lee *et al.* 2015). However, determining the biodegradation potential of microorganisms remains challenging, since the most recalcitrant pollutants are degraded much more slowly than the more labile components. In addition to abiotic factors that limit complete degradation, indigenous microorganisms that can effectively tolerate and/or rapidly degrade PHCs may be present at very low abundance, or may only be able to degrade

certain compounds (Tahhan *et al.* 2011; Sayara *et al.* 2009). To overcome slow innate PHC biodegradation in soil, adjustments to nutrient concentrations, oxygen, pH and/or temperature (biostimulation), along with microbial inoculation (bioaugmentation), may be used (Sayara *et al.* 2010; Betancur-Galvis *et al.* 2006; Tahhan *et al.* 2011).

For bioaugmentation to be efficient in practice, inoculated microorganisms must tolerate contaminants, efficiently degrade compounds of interest, and thrive in the target environment (Yao *et al.* 2015; Bisht *et al.* 2015). These microbial traits have been evaluated using culture-based assays, molecular methods, and analytical chemistry techniques like gas chromatography mass spectrometry (GC-MS) (Sayara *et al.* 2011). Since some of these techniques are costly, colorimetric methods have also been used to rapidly estimate the biodegradation capacity of microorganisms (Montagnolli *et al.* 2015b; Puškárová *et al.* 2013). Respirometry has also been used as a sensitive and effective method for quantifying PHC biodegradation (Coello Oviedo *et al.* 2009; Oyelami *et al.* 2013), by assessing CO_2 and O_2 production by organisms exposed to PHCs (Montagnolli *et al.* 2015a).

Although only a small portion of the microorganisms found in soil environments can be characterized through cultivation, a large number of microorganisms have been isolated from contaminated sites using different nutrient-rich (standard) and impoverished media, supplemented with various types and concentrations of PHCs (selective) (Stefani *et al.* 2015; Jacques *et al.* 2009). Polluted soils are of particular interest as sources for cultivation, since microbes in these soils are more likely to have developed multiple tolerance mechanisms, allowing them to survive and function effectively in the presence of PHCs (Caliz *et al.* 2012; Oriomah *et al.* 2014). Although bioremediation has become a crucial technology for *in situ* PHC removal, difficulties in identifying organisms that degrade high molecular weight compounds could limit our ability to enhance biodegradation. Current research in this area is limited by the identification of organisms that degrade complex aromatic structures.

This investigation aimed to evaluate the petroleum biodegradation efficiency of 95 bacterial and 160 fungal strains isolated from a former petrochemical plant that is highly petroleum-contaminated. We used screening tests based on colorimetric and quantitative analyses to determine the utility of these isolates in PHC biodegradation. Our specific objectives were to: (i) compare the potential of standard and selective media to isolate effective PHC-degrading microorganisms, (ii) evaluate the relationship between soil contaminant

concentration and strain phylogeny with petroleum biodegradation efficiency, and (iii) identify a consortium of two to four microbial strains that could be useful for further *in vitro* assessments of hydrocarbon degradation pathways and potentially for *in situ* bioaugmentation.

Materials and Methods

Experimental site and isolation of microorganisms

Bacterial and fungal strains used in this study were isolated from soil samples obtained at the site of a former petrochemical plant in Varennes, Quebec, Canada (45°41'56"N, 73°25'43" W). This site has been contaminated for the last forty years by a variety of petroleum waste products related to the petro-chemical industry. Experimental design and sampling, DNA isolation, amplification, and sequencing of microbial isolates was previously described by Stefani *et al.* (2015). Briefly, the study area of approximately 2500 m^2 was divided in five plots of 300 m^2 each. The 24 soil samples from each plot were pooled to obtain representative composite soil samples and analysed for F1-F4 hydrocarbons (sum of all aromatic and aliphatic hydrocarbon compounds with chain lengths of C10-C50) by Maxxam Analytics (Montreal, Quebec, Canada). Results from hydrocarbon analyses (Table 4.1) revealed an increasing contamination gradient from plots 1 through 5, which led us to classify the blocks into three discrete contaminant levels: slightly contaminated (plots SC1, SC2), contaminated (plot C3), and highly contaminated (plots HC4, HC5). Bacterial strains were isolated from these three sections on standard media (tryptic soy agar, TSA, containing 30 g L^{-1} of tryptic soy broth (TSB)) and selective media (plates containing 2- to 30-fold diluted TSB (1 to 15 g L^{-1}) along with various concentrations of diesel engine oil or crude oil, or that were coated with crude oil). Fungal strains were isolated on standard media (potato dextrose agar, PDA, containing 24 g L^{-1} of potato dextrose broth (PDB)) and selective media (plates containing 3- to 39-fold diluted PDA (1 to 12 g L^{-1} of PDB) along with various concentrations of diesel engine oil or crude oil, or that were coated with crude oil). Sanger sequencing data have been deposited in GenBank under the accession numbers KP177318-KP177405 and KP177406-KP177454 for bacteria and fungi, respectively (Stefani *et al.* 2015).

Selection of microorganisms and inoculum preparation

A total of 781 bacterial strains and 279 fungal strains were isolated and sequenced from SC, C, and HC soils using standard and selective media. Among these isolates, 95 bacterial and 160 fungal strains were selected for this screening test. The criteria for selecting these microbial isolates were: (i) they were not classified as known pathogens of humans, animals, or plants; and (ii) they covered an array of major groups, allowing phylogenetic comparison. Selected fungal isolates belonged to three major taxonomic groups: 45 *Dothideomycetes*, 73 *Sordariomycetes*, and 42 *Mucoromycotina*. Selected bacterial isolates belonged to five major taxonomic groups: 30 *Actinobacteria*, 23 *Bacilli*, 9 *Alphaproteobacteria*, 10 *Betaproteobacteria,* and 23 *Gammaproteobacteria*.

Before performing screening tests, bacterial and fungal cultures were grown for two weeks in Tryptic Soy Broth (TSB) and Potato Dextrose Broth (PDB), respectively (Table 4.1). Bacterial isolates were then transferred to 96-well plates containing 200 µL of TSB in each and incubated at 25°C under aerobic conditions in a rotary shaker adjusted to 100 rpm. At the exponential growth phase (optical density at 600 nm of 0.5-0.9), plates were centrifuged at 3500 rpm for 10 min and the supernatant was removed. Bacterial pellets were rinsed with 200 µL of Phosphate-buffered saline (PBS) (Sigma-Aldrich, Montreal, Canada), and re-centrifuged at 3500 rpm for 10 min. The supernatant was removed and 200 µL of minimum culture medium Bushnell-Haas (BH) (Table 4.1) was added to each well. The density of bacteria in each well was estimated by counting cells using a hemocytometer and light microscopy at 100x magnification.

Fungal isolates were cultivated in 250 ml flasks filled with 100 ml of PDB and inoculated with a mycelium disk (5 mm diameter). Cultures were incubated at 22°C for two weeks under agitation at 100 rpm. Fresh mycelia were harvested using 1 µL sterile inoculation loops (Sarstedt, Montreal, Canada), washed with sterile deionized water, centrifuged at 3500 rpm for 15 min, and used as described below in each test.

Colorimetric screening test for fungal and bacterial isolates

Crude oil degradation was evaluated using a technique based on the redox indicator 2,6-dichlorophenolindophenol (DCPIP) described by Wrenn and Venosa (1996). The origin of the crude oil was the Gulf of Mexico (provided by Portland-Montreal Pipeline). DCPIP is an

enzyme-catalyzed redox electron acceptor that can be used as an indicator of microbial metabolism (Montagnolli *et al.* 2015b). Oxidation of the carbon source by microorganisms involves redox reactions, in which electrons are transferred to terminal electron acceptors such as oxygen, nitrates, and sulfates (Montagnolli *et al.* 2015b). Initially blue in its oxidized form, DCPIP is reduced by electrons abstract from the respiratory chain to a colorless liquid during the biodegradation process, due to a change in the molecular structure of the compound, from a double to a single carbon-nitrogen bond (Isola *et al.* 2013). Adding DCPIP to the culture medium allowed us to determine the ability of fungi to use PHCs as a carbon and energy source for growth by observing the color reduction. A colorimetric test with DCPIP was performed on solid oil-coated medium 0.1% PDA- 5% COM (crude oil was mixed with the medium before solidification, Table 4.1) containing 1g L^{-1} of DCPIP at pH 7. A fresh mycelium disk (5 mm diameter) was placed in the center of each Petri dish. Three replicates were performed for each isolate. Negative controls consisted of culture medium with DCPIP added, but with no fungal inoculation. All cultures were incubated for 15 days at 25°C. The radius of the area of discoloration of DCPIP was measured for each Petri dish.

A mixture of four PAH compounds (anthracene, phenanthrene, fluorene, and pyrene) at 0.2 g L^{-1} (0.05 g L^{-1} of each PAH) was used to evaluate the degradation efficiency of bacteria as described by Wrenn and Venosa (1996). This colorimetric method uses p-iodonitrotetrazolium chloride (INT), which is oxidised by NADH in bacteria. When bacteria degrade hydrocarbons, INT is reduced to an insoluble red to purple compound called formazan (Haines *et al.* 1996). Thus, the INT reduction reflects the ability of bacteria to use PAHs as the carbon source for their growth (Haines *et al.* 1996).

The INT screening test was performed in 96-well plates. Each well contained 200 µL of BH medium containing 10^6-10^7 bacterial cells in the exponential growth phase, and 10 µL of the PAH mixture. This mixture is composed (per litre of culture medium) of 1g of each PAH compound (anthracene, phenanthrene, fluorene, and pyrene) dissolved in pentane (1:1 w/v). Pentane quickly evaporates, leaving only the PAH compounds in each well. After 21 days of incubation at room temperature, 50 µL of INT was added to each well. Three replicates were carried out for each bacterial isolate. Negative controls consisted of a minimal BH culture medium with the PAH mixture added, but without bacteria. Plates were photographed, and each well was scanned using Image J 1.45 software to measure light intensity. The colour intensity

of negative controls was subtracted from the values of inoculated wells to normalize between plates.

Quantitative screening test for fungal and bacterial isolates

In order to identify a consortium of a few microbial strains that could be useful for further *in vitro* assessments of hydrocarbon degradation pathways and potentially for bioaugmentation *in situ*, top-performing strains belonging to each bacterial group and to the *Sordariomycetes* (*Fungi*) were selected for advanced screening. *Sordariomycetes* were specifically selected, since fungi belonging to this group (i) metabolized significantly more PAHs than the *Mucoromycotina* and *Dothideomycetes* and (ii) have often been identified as important PAH-degraders. Six bacterial (*Achromobacter piechaudii* KP177321 isolated from HC soil; *Bacillus mycoides* KP177359 isolated from C soil; *Pseudomonas* sp. KP177395 isolated from HC soils; *Rhodococcus* sp. KP177337 isolated from C soils; *Rhodococcus qingshengii* KP177336 isolated from SC soil; *Sphingomonas* sp. KP177347 isolated from SC soil) and two fungal (*Fusarium oxysporum* KP177421 isolated from HC soil; *Trichoderma tomentosum* KP177420 isolated from HC soil) strains were used for thorough quantification of PAH biodegradation using GC-MS to quantify remaining PAH concentrations and for analysis of aerobic heterotrophic microbial activity through a respirometry assay.

Respirometry assay

A respirometric test was carried out to determine the oxygen produced during the microbial degradation of the PAH mixture. The test was conducted using a pulse-flow headspace respirometer and the accompanying software (PF-8000, Respirometers System and Applications, Fayetteville, AK, USA). Respirometry measurements were performed in closed 500 ml glass bottles filled with 250 ml of BH medium containing 0.05 g L^{-1} of the PAH mixture and the appropriate microbial inoculum (10^6-10^7 bacteria in exponential growth phase or a 0.5 cm^2 mat of fresh mycelium harvested from two-week old subcultures in PDB). The temperature was kept constant at 25 ± 1°C using a water bath. To avoid the settling of liquid samples within the test bottles, magnetic stirrers were used with an agitation speed of 100 rpm. The oxygen uptake rate (OUR) was registered every 10 minutes for 24 days. Three replicates were carried

out for each isolate as well as a control consisting of the culture medium with the PAH mixture but without microbial inoculation.

GC/MS analysis

GC-MS analysis was performed to quantify the PAH compounds before and after microbial degradation. The test was performed in 250 ml Erlenmeyer flasks filled with 30 ml of BH medium containing 0.2 g L^{-1} of the mixture of PAHs (0.05 g L^{-1} of each PAH) and microbial inoculum (10^6-10^7 bacteria in exponential growth phase or a 0.5 cm^2 mat of fresh mycelium). Erlenmeyer flasks were hermetically sealed with precision seal rubber septa (Sigma-Aldrich, Montreal, Canada) in order to avoid evaporation. Three replicates were carried out for each isolate as well as a no-inoculation control. All cultures were incubated at 25°C for 7 weeks under 100 rpm agitation. The number of bacteria in the medium at the end of the experiment was determined by direct counting using a hemocytometer while the dry biomass of each fungal isolate was determined after drying the mycelia at 60°C for 2 days. The PAH concentrations were quantified by GC-MS at the end of the test using a commercial service provided by AGAT Laboratories (Montreal, Quebec, Canada).

Chemical properties of the culture media were also determined after centrifuging at 16,000 x g for 15 minutes (Avanti J-25, Beckman Coulter, Inc. California, USA) at 13°C. Dissolved oxygen (DO), pH, and conductivity were analyzed with an HQ11d portable meter (Hach Company, USA). Chemical oxygen demand (COD) was analyzed using the LCK114 Dr. Lange's cuvette tests (Dr. Bruno Lange, GmbH& CO. KG, Dusseldorf, Germany). Cuvettes were measured spectrophotometrically with a HACH XION 500 spectrophotometer. All analyses were performed in triplicate.

Statistical analysis

One-way ANOVAs followed by Tukey HSD post-hoc tests were performed to compare the means of DCPIP discoloration radius between fungal isolates or INT coloration values between bacterial isolates. To determine how PHC degradation was related to the isolation environment, we removed the effect of phylogeny by separately comparing bacterial and fungal groups present inside SC-C; C-HC and SC-HC soils. To assess how PHC degradation was

related to the phylogenetic origin of the strains, we separately compared bacterial and fungal groups present inside SC, C and HC soils. To determine the potential usefulness of culture media, we first compared bacterial and fungal groups isolated on standard and on all selective culture media using Student's T-tests. Secondarily, one-way ANOVAs, followed by the Dunnett post-hoc tests, were used to compare differences in bacterial and fungal groups isolated on standard culture media (used as controls) with those isolated on selective media. One-way ANOVAs followed by the Dunnett post-hoc test were used to compare differences in cumulative microbial respiration, oxygen uptake rate, pH, DO, EC, COD, and the percentage of PAH compounds degraded. Fungal dry biomass and the number of bacteria in the culture medium at the end of the experiment were compared using Student's T-tests and one-way ANOVAs followed by Tukey HSD post-hoc tests, respectively. Conditions of normality and homoscedasticity of the residuals were checked in all cases. Differences were considered statistically significant at $p<0.05$. Statistical analyses were performed with JMP v11.0 software (SAS Institute, Cary, NC) and GraphPad Prism (version 6.0 for mac OS, San Diego, USA).

Results and Discussion

Colorimetric screening of the fungal and bacterial isolates

Effect of isolation culture media

Significant efforts have been made to isolate organisms that degrade highly recalcitrant pollutants, such as PAHs (Stefani *et al.* 2015; Lang *et al.* 2016; Beškoski *et al.* 2012). In this experiment the potential usefulness of standard and selective culture media to isolate effective PHC-degrading microorganisms was investigated. During this colorimetric screening test, we observed that the color of the control without microbial inoculation remained constant, indicating that the dyes were not reduced abiotically or by microbiota that eventually existed in the oil. This shows that DCPIP discoloration and INT coloration change was reflective of microbial growth and activity. Figure 4.1 represents the results of fungal DCPIP decolorization (Fig. 4.1a-b) and bacterial INT colorization assays (Fig. 4.1c-d) according to the standard and selective isolation media. PHC use by fungal and bacterial isolates was equally efficient in standard and selective culture media, as seen in Figure 4.1a-c. By separately comparing all of the selective media used to isolate microorganisms, only 0.1% PDA-5% OD had a DCPIP

decolorization that was significantly higher than the standard PDA media (Fig. 4.1b). This result suggests that the use of 0.1% PDA containing 5% of diesel engine oil allowed the isolation of fungi that were able to metabolize some compounds of crude oil more efficiently than those isolated on PDA media. However, it is not possible to definitively identify the compound(s) used, due to the complexity of crude oil. Palanisamy *et al.* (2014) evaluated the effects of various culture parameters, such as initial hydrocarbon concentration, on the biodegradation of diesel oil, and reported optimal growth by the bacterial strain *Acinetobacter baumanii* at an initial hydrocarbon concentration of 4% which is close to the concentration available in the 0.1% PDA-5% OD medium.

Table 4.1. Polycyclic aromatic hydrocarbons (PAHs) and C10-C50 hydrocarbons recorded in slightly contaminated (SC; plots 1, 2), contaminated (C, plot 3), and highly contaminated (HC; plots 4, 5) soils. From (Stefani et al., 2015; Annex 1).

PAHs and C10–C15 hydrocarbons	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5
	Slightly contaminated		Contaminated	Highly contaminated	
Acenaphthene (mg/kg)	0	0	16.9	110	110
Acenaphthylene (mg/kg)	0.1	0.1	1.3	6.5	5.6
Anthracene (mg/kg)	0.1	0.4	9.9	53.6	45.5
Benzo[a]anthracene (mg/kg)	0	0	0.8	3.3	2.4
Benzo[a]pyrene (mg/kg)	0	0	0.2	0.7	0.4
Benzo[b,j,k]fluoranthene (mg/kg)	0	0	0.2	0.9	0.6
Benzo[c]phenanthrene (mg/kg)	0	0	0.3	1.4	1
Benzo(g,h,i)perylene (mg/kg)	0	0	0	0	0
Chrysene (mg/kg)	0	0	1	5.2	3.2
Dibenzo[a,h]anthracene (mg/kg)	0	0	0	0	0
Dibenzo[a,i]pyrene (mg/kg)	0	0	0	0	0
Dibenzo[a,h]pyrene (mg/kg)	0	0	0	0	0
Dibenzo[a,l]pyrene (mg/kg)	0	0	0	0	0
7,12-Dimethylbenz[a]anthracene (mg/kg)	0	0	0	0	0
Fluoranthene (mg/kg)	0	0	3.5	18.3	14
Fluorene (mg/kg)	0	0	11.3	85.5	90.2
Indeno[1,2,3-cd]pyrene (mg/kg)	0	0	0	0	0
3-Methylcholanthrene (mg/kg)	0	0	0	0	0
Naphthalene (mg/kg)	0	0	6.3	59.6	93
Phenanthrene (mg/kg)	0.1	0.2	60.6	318	314
Pyrene (mg/kg)	0	0.1	5.2	27.4	20.7
1-Methylnaphthalene (mg/kg)	0	0	14.5	81.9	85.9
2-Methylnaphthalene (mg/kg)	0	0	14.6	71.2	91.3
1-3-Dimethylnaphthalene (mg/kg)	0	0	23.4	92.2	69,0
2,3,5-trimethylnaphthalene (mg/kg)	0	0	6.6	28.2	21.6
Acenaphthene-D10 (%)	104	117	134	94	107
Fluoranthene-D10 (%)	102	121	104	126	110
Perylene-D12 (%)	112	115	93	103	93
Hydrocarbons C10 – C50 (mg/kg)	<100	<100	2870	6400	5900

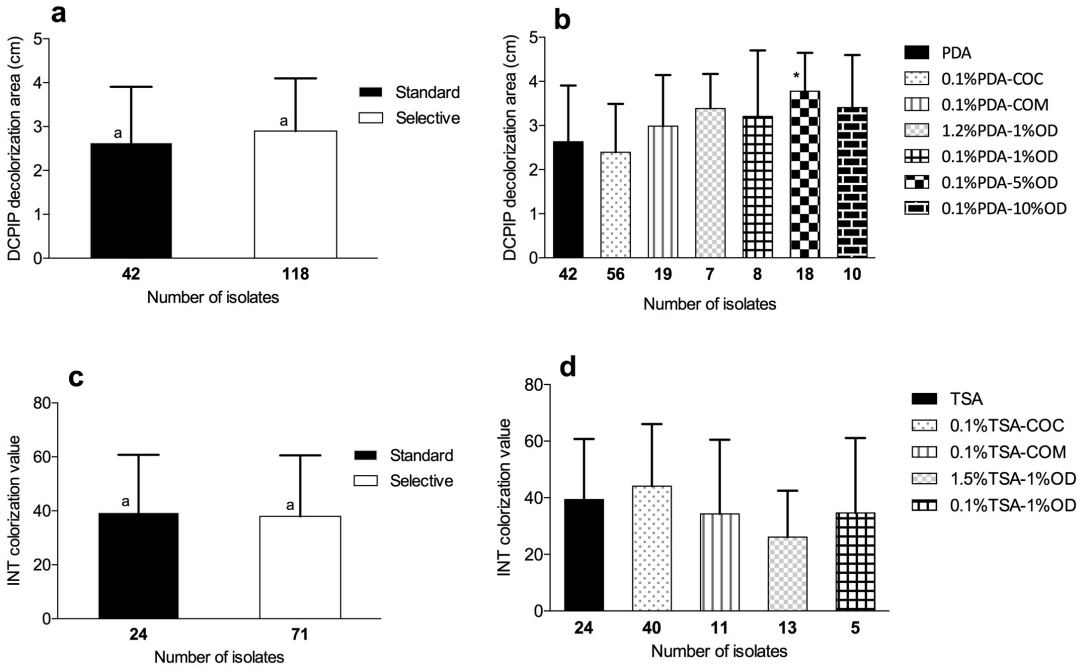

Figure 4.1. DCPIP decolorization area (fungal; a and b) and INT colorization values (bacteria; c and d) of cultures isolated on standard (tryptic soy agar, TSA for bacteria and potato dextrose agar, PDA for fungal) or selective culture media (TSA and PDA along with various concentrations of diesel engine oil (OD) or crude oil (COM), or that were coated with crude oil (COC)). Details on the media are available in Table 1, Table S2 and in Stefani *et al.* (2015). Values between standard and selective media were compared using a Student's T-test (a and c); no significant difference was found between media at the α=0.05 level. Standard culture media were also compared to each selective media (b and d) using a Dunnett post-hoc test; an asterisk (*) denotes a significant difference between an isolation medium and the standard. The number of isolates obtained from each media type is indicated along the x-axis. Each strain was analyzed in triplicate and the mean was determined. Mean values represented are the averaging of number of isolates obtained from each medium type. Bars are standard deviations of this mean.

Effect of environment

Although microorganisms have been isolated from different environments, it is unknown whether isolation from soils with different concentrations of long-term PHC contaminants would influence the ability of isolates to effectively degrade PAHs. Figure 4.2 shows the fungal DCPIP decolorization area (Fig. 4.2a) and bacterial INT colorization values (Fig. 4.2b) of isolates taken from the slightly contaminated (SC), contaminated (C), and highly contaminated (HC) soils. To limit the effect of phylogeny, we separately compared bacterial

and fungal groups present inside SC-C; C-HC and SC-HC soils. Figure 4.2 represents the result obtained by comparing bacterial groups present inside SC-C soils (*Actinobacteria*, *Bacilli*, *Alphaproteobacteria*, and *Gammaproteobacteria*) and fungal groups present inside SC-C and C-HC soils (*Dothideomycetes*, *Sordariomycetes*, and *Mucoromycotina*). Results obtained by comparing bacterial groups present inside SC-HC (*Bacilli*, *Alphaproteobacteria*, *Betaproteobacteria,* and *Gammaproteobacteria*) and C-HC soils (*Bacilli*, *Alphaproteobacteria*, *Gammaproteobacteria*) and fungal groups present inside SC-HC soils (*Sordariomycetes*) are shown in Figure S4.1 (Supporting information).

Fungal DCPIP decolorization and bacterial INT colorization did not differ based on the origin of isolation, except for the fungal group *Sordariomycetes*, which was present in both the SC and HC soils (Fig. 4.2 and Fig. S4.1). This suggests that except for the fungal group *Sordariomycetes*, long-term exposure of soil microorganisms to high PHC concentrations did not significantly influence their biodegradation potential, at least in those microorganisms that we were able to isolate. As previously observed by Cruz *et al.* (2014), exposure of *Bacillus subtilis* to diesel, biodiesel, and petroleum over 60 days allowed selection of isolates of this species able to metabolize these contaminants. Patel *et al.* (2015) also reported that organisms isolated from pristine ecosystems could not use high molecular weight hydrocarbon compounds as carbon sources, compared to organisms isolated from polluted samples on the same media. They suggest that microorganisms in the polluted sediment adapted to high PAH concentrations, and became able to use them as carbon sources. Previous exposure to aromatic hydrocarbons was shown to strongly influence the type and number of hydrocarbon-degrading organisms in soils, which in turn, largely determined the ability of these organisms to metabolize PAH compounds (Zafra *et al.* 2015; Hong *et al.* 2010). Consequently, microorganisms that are naturally selected from PAH-contaminated soils would be more likely to survive and metabolize PAHs than organisms isolated from elsewhere. In contrast to this, our results suggest that exposure to high PAH concentrations did not generally affect the PAH degradation capacity of microorganisms relative to those isolated from soils with low PAH concentrations.

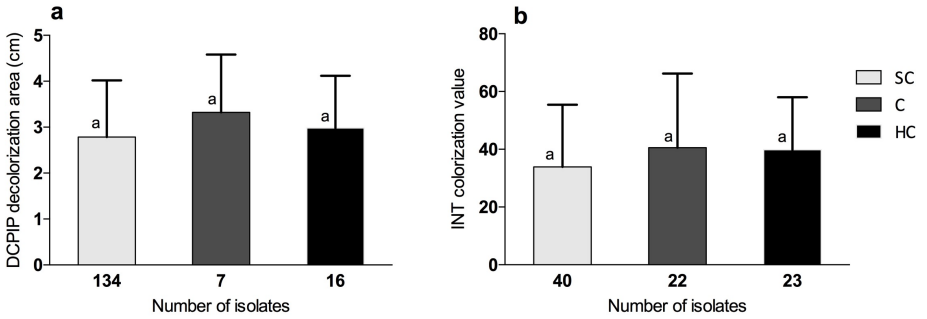

Figure 4.2. DCPIP decolorization area (fungal; a) and INT colorization values (bacteria; b) of cultures isolated from each of the three soil types: slightly contaminated (SC), contaminated (C), and highly contaminated (HC). Mean values are presented with standard deviations for bacterial groups present inside SC-C soils (*Actinobacteria*, *Bacilli*, *Alphaproteobacteria*, *Betaproteobacteria,* and *Gammaproteobacteria*) and fungal groups present inside SC-C and C-HC soils (*Dothideomycetes*, *Sordariomycetes*, and *Mucoromycotina*). Different letters above bars indicate significantly different means according to Tukey's HSD test ($p<0.05$). The number of isolates obtained from each soil type is indicated along the x-axis. Each strain was analyzed in triplicate and the mean was determined. Mean values represented are the averaging of number of isolates obtained from each soil type. Bars are standard deviations of this mean.

Effect of phylogenetic affiliation

Fungal DCPIP decolorization and bacterial INT colorization, as sorted by taxonomic groups, are shown in Figure 4.3. This figure represents a comparison of bacterial and fungal groups present in the SC soil. Similar results were obtained by comparing bacterial and fungal groups present in the C and HC soils (data not shown). Bacterial isolates belonging to the groups *Actinobacteria*, *Betaproteobacteria,* and *Gammaproteobacteria* metabolized significantly more PAHs than the *Bacilli* and *Alphaproteobacteria* (Fig. 4.3b). It has been extensively reported that hydrocarbon contamination often increases the relative abundance of *Proteobacteria* (Jurelevicius *et al.* 2013; Hou *et al.* 2015; Stefani *et al.* 2015). Within the phylum *Proteobacteria*, Patel *et al.* (2015) outlined the prevalence of *Betaproteobacteria* in PAH-contaminated sediments from a shipbreaking yard, as well as a prevalence of *Gammaproteobacteria* in pristine sediments and *Alphaproteobacteria* in both sediments. Interestingly, abundance in the environment may not always correlate with function. Hesselsoe *et al.* (2008) showed with microautoradiography that although *Alphaproteobacteria* were more abundant in a cultured consortium for diesel degradation, *Betaproteobacteria* represented over

half of radioactively labeled cells. Also, bacteria belonging to the *Gammaproteobacteria* such as *Pseudomonas* have often been identified as important PAH-degraders (Niepceron *et al.* 2013; Kang 2014).

Similarly, hydrocarbon degradation by fungi depended on phylogenetic origin (Fig. 4.3a). Fungi belonging to the group *Sordariomycetes* were more efficient than *Mucoromycotina* and *Dothideomycetes* in using crude oil as a carbon source (Fig. 4.3a). *Sordariomycetes*, one of the largest groups of *Ascomycota,* includes species such as *Trichoderma*, *Hypocrea* and *Fusarium,* which have been shown to be highly efficient in degrading hydrocarbon contaminants (Wu *et al.* 2010; Argumedo-Delira *et al.* 2012; Hong *et al.* 2010).

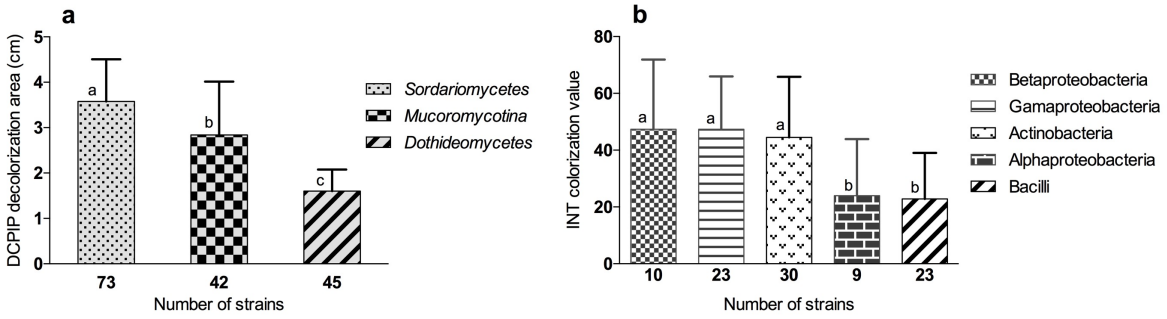

Figure 4.3. DCPIP decolorization area (fungal; a) and INT colorization values (bacteria; b) of cultures isolated according the group-level taxonomic affiliation. Mean values are presented with standard deviations for bacterial and fungal groups obtained from the SC soil. Each strain was analyzed in triplicate and the mean was determined. Mean values represented are the averaging of the number of strains obtained from the SC soil. Bars are standard deviations of this mean. Different letters below bars indicate significantly different means according to Tukey's HSD test ($p<0.05$). The numbers of strains belonging to each group are indicated along the x-axis.

Quantitative screening test for fungal and bacterial isolates

Respirometry

All the eight microorganisms selected for this respirometry test showed a similar metabolic profile, with the highest O_2 uptake occurring at 220h after the start of the incubation, with the exception of *Sphingomonas* sp., for which maximum uptake was observed 75h

following the beginning of the assay (Fig. 4.4). For all isolates, respirometry readings steadily declined after reaching maximum oxygen consumption (Fig. 4.4). There was no significant difference between O_2 uptake over 24 days in culture media containing microorganisms or without microorganisms (Fig.4.4). However, 24 days after the start of the incubation, *Sphingomonas* sp. showed the lowest oxygen uptake, confirming its low growth rate, in line with the fact that its PAH removal efficiency was the lowest among all tested bacterial strains (Fig. 4.4). In contrast, *Achromobacter piechaudii*, *Rhodococcus qingshengii,* and *Rhodococcus* sp. strains had the highest oxygen uptake, which is correlated with the growth rate at the end of the experiment (Table 4.3).

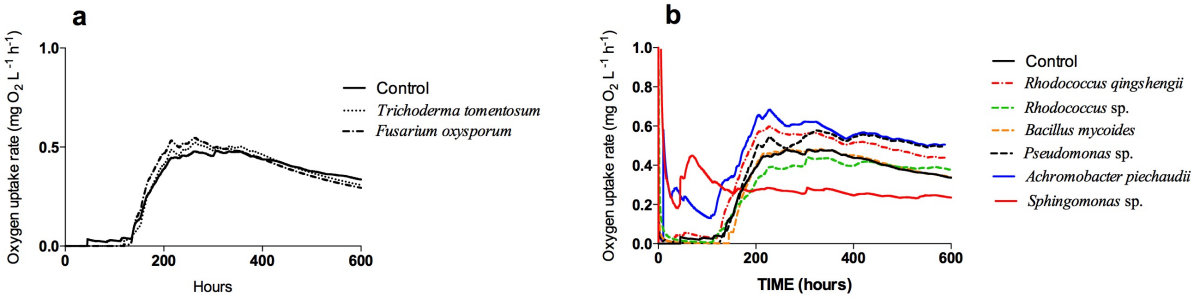

Figure 4.4. Oxygen uptake rate (OUR, mg O_2 L^{-1} h^{-1}) related to fungal (a) and bacterial (b) activity over 24 days of growth (n=3 for each strain, mean values are presented with standard deviations). Control consists of the mixture of PAHs without microorganisms.

GC/MS analysis

Biodegradation of anthracene, phenanthrene, fluorene and pyrene by bacterial and fungal strains after 7 weeks of culture was confirmed by GC-MS analysis (Fig. 4.5). In the control treatment, we observed declines of 33.55%, 43.25%, 49.85%, 77.45% of added pyrene, anthracene, phenanthrene, fluorene, and pyrene, respectively (Fig. 4.5). Since the Erlenmeyer flasks were hermetically sealed during the incubation, this could be due to release of evaporates at the end of the experiment or sorption of the PAH compounds on the glass vessel, as proposed by Qian *et al.* (2011). They found a linear relationship between the sorption coefficients (K_d) of pyrene (9.51mL g^{-1}), anthracene (2.88mL g^{-1}), phenanthrene (2.13mL g^{-1}) and fluorene (0.67mL g^{-1}) on glass surfaces and the corresponding water solubility (log S_w) of -2.44 mol m^{-3}, -1.87

mol m^{-3}, -1.57 mol m^{-3}, -1.17 mol m^{-3} respectively. It is clear from these data presented by Qian *et al.* (2011) that the sorption of PAH on glass surfaces decrease with increase of water solubility. This contrast with the results observed in the control treatment where the decline of PAH increase with the increase of water solubility. As reported by Howard *et al.* (2005) volatilization is the most important process affecting the fate of lower molecular weight PAH in the environment. Therefore, the decline of PAH observed in the control treatment in this study could be mainly due to release of evaporates at the beginning and at the end of the experiment. All of the microbial isolates had a significant positive effect on the degradation of at least one PAH compound. Three strains performed particularly well, significantly degrading all four PAH compounds in the mixture compared to the control. More than 10%, 13%, 8%, and 17% of the loss of anthracene, phenanthrene, fluorene, and pyrene respectively was due to *Rhodococcus* sp., *Trichoderma tomentosum,* and *Fusarium oxysporum* degradation after 49 days (Fig. 4.5). The percentage of PAHs removed by these three strains was significantly higher than what was observed in the negative control (Fig. 4.5). PAH biodegradation by *Bacillus mycoides* was similar, but this strain did not significantly degrade anthracene (Fig. 4.5). A number of bacteria commonly found in contaminated soils, such as *Pseudomonas*, *Bacillus,* and *Rhodococcus* are known to use a diverse array of aromatic compounds (Haritash and Kaushik 2009; Lu *et al.* 2011; Guermouche M'rassi *et al.* 2015). Results of this GC/MS analysis carried out under laboratory conditions showed the bioremediation ability of *Rhodococcus* sp. in PHC degradation. It has been extensively reported that *Rhodococcus* is one of the most promising groups of organisms suitable for the biodegradation of aromatic compounds due to their capacity to acquire a remarkable range of diverse catabolic genes and their robust cellular physiology(Larkin *et al.* 2005; de Carvalho *et al.* 2014). The efficient degradation of aromatic compounds proceeds via multiple pathways and a wide range of dioxygenases (Martinkova *et al.* 2009). *Trichoderma* and *Fusarium* have also demonstrated their potential in the enzymatic degradation of organic pollutants by using cytochrome P450 (Haritash and Kaushik 2009; Marco-Urrea *et al.* 2015).

To confirm PAH degradation by bacteria and fungi, we measured biomass production at the end of the experiment. The number of bacterial cells and the dry biomass of fungi in the culture media after 7 weeks are shown in Table 4.2. The dry biomass of both fungal strains was similar ($p > 0.05$, Table 4.2). However, the number of *Achromobacter piechaudii*, *Rhodococcus*

qingshengi, Rhodococcus sp., and *Pseudomonas* sp., cells in the culture media at the end of the experiment was significantly higher compared to *Bacillus mycoides,* and *Sphingomonas* sp. (Table 4.2). Unlike others strains, a lower number of cells/ml of *Bacillus mycoides* was observed with the PAH degradation (Table 4.2). This could be due to the inhibitory effect of PAH compounds or the production of toxic metabolites during the hydrocarbons degradation. As previously observed by Pumphrey and Madsen (2007) despite being able to use naphthalene as carbon and energy source, *Polaromonas naphthalenivorans* strain CJ2 balance naphthalene utilization against both direct naphthalene inhibition and formation of toxic intermediate metabolites. Although the microbial PAH degradation result in the most case in the utilization of the contaminant for growth, Hanzel *et al.* (2012) pointed out the fact that microbial contaminant degradation act as a protective mechanism against its toxicity.

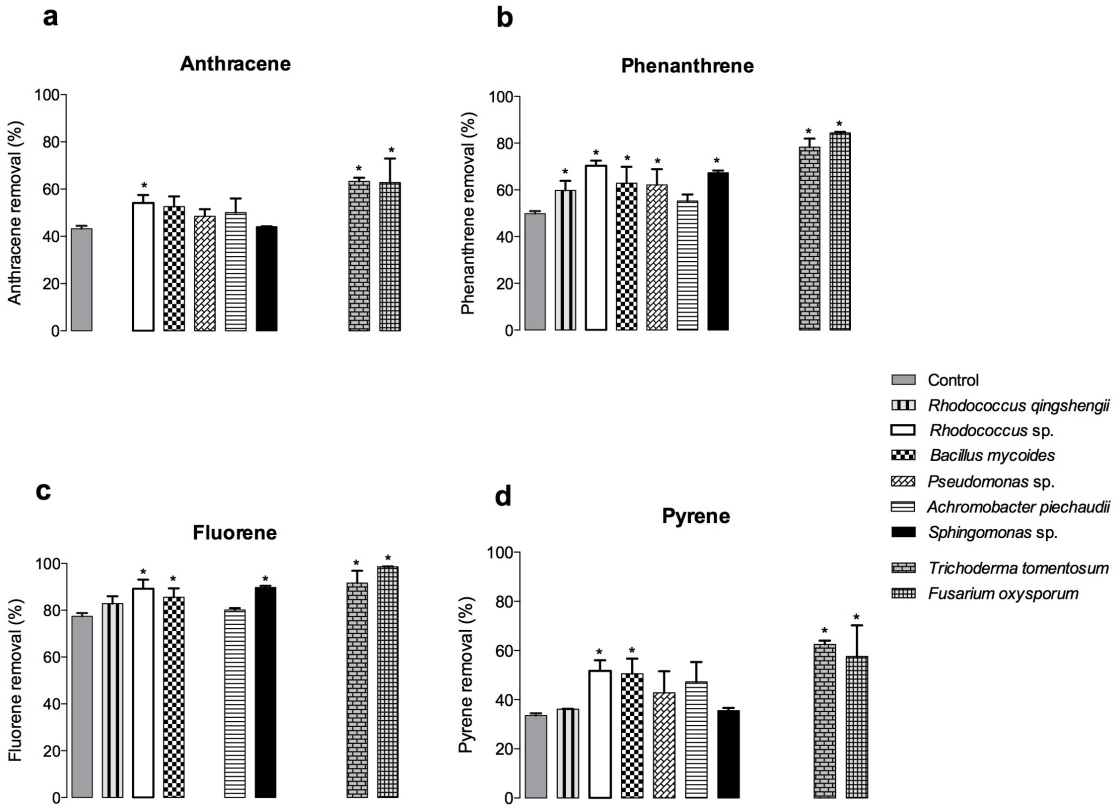

Figure 4.5. Percentage of anthracene (a), phenanthrene (b), fluorene (c) and pyrene (d) removed by bacterial and fungal strains after 7 weeks of culture. The control assay consisted of a mixture of PAHs without microorganisms. Percentage of PAHs removed by each strain is the mean of three replicates and

error bars represent standard deviations. Asterisks (*) denote significant differences between the control and each microorganism at the 0.05 level with a Dunnett's test.

Table 4.2: Biomass of fungal isolates and number of bacterial cells after 7 weeks of culture in the BH medium containing a mixture of PAHs. Mean values are presented with standard deviations, n=3 for each strain. Letters indicate significant differences between each bacteria cell at the 0.05 level with a Tukey's test and the dry biomass of the two fungal strains at the 0.05 level using a Student-T-test.

Fungal		Dry biomass ($g\ L^{-1}$)
	T=0	T=7 weeks
Trichoderma tomentosum	0.006 ± 0.0	0.02 ± 0.01*a*
Fusarium oxysporum	0.006 ± 0.0	0.02 ± 0.0 *a*
Bacteria		Number of cells per ml
	T=0	T=7 weeks
Rhodococcus sp.	$3.44 \pm 0.88 \times 10^5$	$85.33 \pm 16.42 \times 10^6$ ab
Pseudomonas sp.	$3.33 \pm 0.51 \times 10^5$	$145.06 \pm 26.06 \times 10^6$ ab
Achromobacter piechaudii	$4.04 \pm 0.50 \times 10^5$	$220.80 \pm 31.51 \times 10^6$ a
Rhodococcus qingshengii	$3.37 \pm 0.23 \times 10^5$	$67.13 \pm 10.20 \times 10^6$ bc
Sphingomonas sp.	$3.85 \pm 0.55 \times 10^5$	$0.09 \pm 0.02 \times 10^6$ d
Bacillus mycoides	$4.00 \pm 0.53 \times 10^5$	$6.18 \pm 0.77 \times 10^6$ c

Many reports have also pointed out that white rot fungi have evolved to degrade lignocellulose using a myriad of enzymes and complex multi-enzyme systems, including ''feedback'' type enzymes, allowing for simultaneous degradation of both lignin and cellulose (Leonowicz *et al.* 1999; Cajthaml *et al.* 2008; Novotny *et al.* 2004). This versatile, yet complex, machinery of enzymes can be used to attack and metabolize crude oil compounds. However, the fungal isolates used in our study, *Trichoderma tomentosum* and *Fusarium oxysporum,* are filamentous non-lignolytic fungi, whose PAH-degrading efficiency has been shown (Singh 2006b; Jacques *et al.* 2008). These fungal species belong to the order *Hypocreales,* which is

generally characterized by high reproductive rates and low nutritional requirements, allowing them to survive under adverse conditions (Argumedo-Delira *et al.* 2012; Zafra *et al.* 2015). Other studies have demonstrated that non-lignolytic fungi can efficiently remediate PAHs due to their ability to synthesize non-specific enzymes that can degrade a wide range of organic substrates (Hong *et al.* 2010; Marco-Urrea *et al.* 2015; Reyes-Cesar *et al.* 2014). The enzymatic versatility of *Trichoderma* species could explain their advantage in PAH biodegradation (Zafra *et al.* 2015).

Effect of isolation media on biodegradation efficiency of the microbial isolates

The effect of culture media on the biodegradation of PAH compounds after 7 weeks is shown in Table 4.3. Comparing the pH, EC, DO, and percentage of COD reduction of the culture media containing microorganisms with the non-inoculated control, we found that for all eight of the tested microorganisms, DO was significantly higher (Table 4.3). An increase of DO due to enhanced aerobic biodegradation of BTEX compounds (benzene, toluene, ethylbenzene and xylenes) by indigenous microorganisms in petroleum-hydrocarbon contaminated groundwater has also been observed by Chen *et al.* (2010). In addition to DO, COD is widely used as an indicator of water quality (Laohaprapanon *et al.* 2013; Svensson *et al.* 2015). There was a slight reduction in the COD value of culture media containing *Achromobacter piechaudii*, *Rhodococcus qingshengi, Bacillus mycoides,* and *Sphingomonas* sp. However, in the culture media containing *Rhodococcus* sp., *Pseudomonas* sp., *Trichoderma tomentosum,* and *Fusarium oxysporum,* COD reductions of 29.38%, 65.48%, 43.13%, 38.31 were observed, respectively, relative to the COD value of the control (mixture of PAHs without microorganism). Shokrollahzadeh *et al.* (2008) observed a COD reduction of 89% by petrochemical-degrading microorganisms, which mainly belonged to the genus *Pseudomonas*. COD, which is a proxy indicator of the amount of organic compounds in water, indicates the mass of oxygen consumed per litre (Jiang *et al.* 2015). The reduction of COD in the culture media containing *Rhodococcus* sp., *Pseudomonas* sp., *Trichoderma tomentosum,* and *Fusarium oxysporum,* confirmed the lower carbon content in the culture media after 7 weeks. We did not observe any significant difference between EC for the treatment with and without microorganisms ($p > 0.05$, Table 4.3), which indicates no change in the content of soluble salts of the culture media. Interestingly, after 7 weeks in culture with the PAH mixture, the three strains *Rhodococcus* sp., *Trichoderma*

tomentosum, and *Fusarium oxysporum* that showed higher PAH degradation significantly increased the culture pH compared to controls (Table 4.3).

Table 4.3: Effect of selected fungal and bacterial isolates on the chemical parameters of the BH medium containing a mixture of four PAHs compounds (anthracene, phenanthrene, fluorene, and pyrene) after 24 days of growth.

Microbial isolate[a]	pH	DO ($mg\ L^{-1}$)	EC ($ms\ cm^{-1}$)	COD reduction (%)
Rhodococcus sp.	8.27 ± 0.54*	8.4 ± 0.17*	3.35 ± 0.02	29.38 ± 1.07
Pseudomonas sp.	7.78 ± 0.02	8.5 ± 0.26*	3.34 ± 0.02	65.48 ± 1.79
Achromobacter piechaudii	7.68 ± 0.14	7.16± 1*	3.31 ± 0.03	5.89 ± 0.42
Rhodococcus qingshengii	7.38 ± 0.05	7.26 ± 0.23*	3.07 ± 0.37	6.83 ± 0.52
Sphingomonas sp.	7.51 ± 0.16	6.5 ± 1.25*	3.34 ± 0.17	19.84 ± 0.52
Bacillus mycoides	7.84 ± 0.12	8.16 ± 0.15*	3.35 ± 0.03	6.79 ± 0.39
Trichoderma tomentosum	8.28 ± 0.06*	8.26 ± 0.11*	3.37 ± 0.03	43.13 ± 0.94
Fusarium oxysporum	7.90 ± 0.17*	8.53 ± 0.15*	3.36 ± 0.02	38.31 ± 0.69
Control	7.27 ± 0.14	1.33 ± 0.11	3.27 ± 0.02	

[a] Microbial strains selected for the second screening test.

DO, dissolved oxygen; EC, Electrical conductivity; COD, chemical oxygen demand.

Mean values are presented with standard deviations, n=3 for each parameter. Asterisks (*) denote significant differences between the control (mixture of PAHs without microorganism) and microbial strains at the 0.05 level based on Dunnett's test.

Conclusion

This investigation confirmed the biodegradation efficiency of some bacterial and fungal strains in PHC degradation, at least in laboratory conditions. Results of this study suggest that *Rhodococcus* sp., *Trichoderma tomentosum,* and *Fusarium oxysporum* may effectively enhance the biodegradation of PAH compounds. It was also found that the concentration of contaminants in the initial soil from which the microbes were isolated, and the type of culture medium, did not significantly impact the ability of microbial isolates to degrade PAH, while phylogenetic

affiliation had a significant effect on biodegradation of PAH. Further studies examining the potential of the best-performing isolates for PAH bioremediation *in situ* are still needed.

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Preface (Chapter 5)

In the previous chapter, the biodegradation potential of bacteria and fungi was investigated. Results showed that the long term exposure of soil microorganisms to high PHC concentration and the type of isolation culture media did not influence the ability of isolates to effectively degrade PHC. However, phylogenic affiliation had a strong on PHC biodegradation. Thus in the present chapter, compost has been used as a source of microbial inoculants, nutrients and organic matter. The potential of phytodegradation of PHC and phytostabilization or phytoextraction of TE using *M. sativa* singly and combined with compost was examined under a five months' greenhouse trial. For overcoming the low nutrient content in this aged contaminated soil, this leguminous plant was choosen based on its capacity to fix atmospheric N and to form mycorrhizae. The biomasses and ionome of *M. sativa* as well as the residual soil ecotoxicity was determined.

Chapter 5: Effect of *Medicago sativa* L. and compost on organic and inorganic pollutant removal from a mixed contaminated soil and risk assessment using ecotoxicological tests

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Marchand C., Kaczala F., Jani Y., Augustsson a., Hijri M., Hogland W. Phytoremediation of petroleum hydrocarbons contaminated soils with Medicago sativa. Linnaeus ECO-TECH' 14. Kalmar (Sweden)

Author contributions:

Conceived and designed the experiments: CM, WH. Performed the experiments: CM, AA, FK, YY. Analyzed the data: CM, WH, YJ, FK. Contributed reagents/materials/analysis tools: WH, MH. Wrote the paper: CM, WH, YJ, FK, LM, AA, MH.

Abstract

Several Gentle Remediation Options (GRO), e.g. plant-based options (phytoremediation), singly and combined with soil amendments, can be simultaneously efficient for degrading organic pollutants and either stabilizing or extracting trace elements (TE). Here, a 5-month greenhouse trial was performed to test the efficiency of *Medicago sativa* L., singly and combined with a compost addition (30% w/w), to treat soils contaminated by petroleum hydrocarbons (PHC), Co and Pb collected at an auto scrap yard. After five months, total soil Pb significantly decreased in the compost-amended soil planted with *M. sativa*, but not total soil Co. Compost incorporation into the soil promoted PHC degradation, *M. sativa* growth and survival, and shoot Pb concentrations (3.8 mg kg^{-1} DW). Residual risk assessment after the phytoremediation trial showed a positive effect of compost amendment on plant growth and earthworm development. The O_2 uptake by soil microorganisms was lower in the compost-amended soil, suggesting a decrease in microbial activity. This study underlined the benefits of the phytoremediation option based on *M. sativa* cultivation and compost amendment for remediating PHC and Pb contaminated soils.

Keywords: Cobalt; Petroleum Hydrocarbon; Phytoremediation; Polycyclic Aromatic Hydrocarbon; Lead.

Introduction

Auto scrap yards are used to recycle end-of-life motor vehicles worldwide. However, these yards are often subjected to soil contamination with hydrocarbon compounds such as motor oil spillage, brake pads, as well as trace elements (TE), e.g. Pb from electric batteries and Co from car paint and motors (Gottesfeld and Pokhrel 2011; Harper *et al.* 2012). Such soil contamination by TE and petroleum hydrocarbons (PHC) is of concern due to potential adverse effects on: a) soil functions and services; b) functional and structural diversity of soil microorganisms, animals and plant communities and; c) human health (Kidd *et al.* 2007; Yenn *et al.* 2014; Cao *et al.* 2009; Khan *et al.* 2015b). Many PHCs such as the 16 polycyclic aromatic hydrocarbons (PAHs) prioritized by the United States Environmental Protection Agency (USEPA), are toxic and carcinogenic pollutants (Wilson and Jones 1993; Winquist *et al.* 2014). Thus, the remediation of these pollutants is urgently needed.

Many *in situ* remediation techniques have been used for the rehabilitation of contaminated soils, including physical, chemical and bioremediation options (Mulligan *et al.* 2001; Gan *et al.* 2009; Mench *et al.* 2010b; Vangronsveld *et al.* 2009). Physico-chemical remediation options are costly, may create further waste, and in many cases, simply transfer pollutants from one phase to another (Susarla *et al.* 2002; Pilon-Smits and Freeman 2006). GRO including in situ contaminant stabilization ("inactivation" using biological or chemical processes) and plant-based options (i.e. phytoremediation) have been alternatively used for remediating polluted soils (Ali *et al.* 2013; Wang *et al.* 2012b; Gerhardt *et al.* 2009; Kidd *et al.* 2015b). Low-cost, non-destructive GRO using plants to degrade organic pollutants and either sequestrate or extract TE from soils, have been successfully used for different types of soils, climate conditions and pollutants (Doni *et al.* 2012; Kumar *et al.* 2013; Bramley-Alves *et al.* 2014; Hechmi *et al.* 2014). However, GRO have some limitations particularly due to the long period required to achieve effective performance on soil remediation (many years or even decades are required to cleanup TE) (Rayu *et al.* 2012; Ali *et al.* 2013; Marchand *et al.* 2015) as well as pollutant localization in the soil profile vs. the root zone depth. The GRO efficiency depends on many complex parameters: pollutant speciation and concentration, soil properties, nutrient content, climate conditions, plants species and their associated microbes (Bell *et al.* 2013; Gerhardt *et al.* 2009).

Generally, industrial contaminated soils are poor in nutrients, especially nitrogen, and poorly drained (Benyahia and Embaby 2016b; Newman and Reynolds 2004). In this context, leguminous plants belonging to the *Fabaceae* are often appropriate for soil phytoremediation, because they have the potential capacity of fixing atmospheric N using their bacterial symbionts and are able to form mycorrhizae (Hutchinson *et al.* 2001; Smith and Read 2008; Marchand *et al.* 2015). Rhizobial and mycorrhizal symbioses can improve the growth of leguminous plants and stimulate microbial activity, enhancing therefore hydrocarbon degradation (D'orazio *et al.* 2013). Of leguminous species, *Medicago sativa* L. is widely used for phytoremediation of organic (Wei and Pan 2010; Hechmi *et al.* 2014) and inorganic pollutants (Zaefarian *et al.* 2013; Vamerali *et al.* 2011; Bonfranceschi *et al.* 2009). This plant has a fibrous root system suitable for the PHC rhizodegradation (Wang *et al.* 2012b) and can contribute to TE phytostabilisation (Zribi *et al.* 2015). *M. sativa* is widely cultivated as feedstock and cover crop for its high shoot yield, high-quality forage and longevity (D'orazio *et al.* 2013; Campanelli *et al.* 2013). For overcoming the low soil fertility and improving soil phytoremediation, soil amendments (biostimulation) and microbial inoculants (bioaugmentation) can be used (Sayara *et al.* 2010). Compost material with different properties have been widely used in agricultural ecosystems as a source of nutrients and organic matter (Hamdi *et al.* 2012). Increasing the soil organic matter generally promotes soil structural stability, water holding capacity, soil porosity, and consequently oxygen diffusion (Hernández *et al.* 2015). Compost amendment can enhance the biodegradation of organic compounds (Park *et al.* 2011) and reduces the mobility of some metals (Ruttens *et al.* 2006).

Despite numerous investigations on the phytoremediation of PHC and TE-contaminated soils (Mench *et al.* 2010b; Gerhardt *et al.* 2009; Susarla *et al.* 2002; Kidd *et al.* 2015b), only few of them have assessed the process efficiency based on both concentration and toxicity reduction (Hamdi *et al.* 2012; Kumpiene *et al.* 2014). Most of these studies focus on total and available pollutant concentrations; however, the relation of such concentrations and their biological effects is poorly addressed (Megharaj *et al.* 2011). Alone, chemical analyses are insufficient for risk assessment of contaminated soils (Fernández *et al.* 2005; Marti *et al.* 2013). They must be complemented with biological options integrating the effects of bioavailable contaminants and their interactions (Eom *et al.* 2007). Different international (ISO11269-2, OECD 207) and national (ASTM E1367-03, USEPA 2002a) standard guidelines have been

developed for ecotoxicological testing. These guidelines recommend assessing various living organisms at different levels in the trophic chain (Eom *et al.* 2007; Fernández *et al.* 2005). Among the terrestrial tests for assessing soil ecotoxicity, higher plants and invertebrates such as earthworms are frequently used (Eom *et al.* 2007; Luo *et al.* 2014; Mao *et al.* 2009). Respirometric tests were also successfully employed for quantifying the ecotoxicity of PHC- and TE-contaminated soils (Soler-Rovira *et al.* 2013; Marti *et al.* 2013; Taccari *et al.* 2011) and water (Coello Oviedo *et al.* 2009).

This pot study aimed to investigate the potential of *M. sativa* to phytoremediate a PHC and TE-contaminated soil, singly and in combination with a single compost amendment (30% w/w). *M. sativa* was cultivated under a greenhouse for five months on the untreated and compost-amended soils. The biomasses and ionome of *M. sativa* roots and shoots were determined.

Residual soil ecotoxicity was assessed using (1) two plant species, i.e. *Lepidium sativum* L. and *Zea mays* L., (2) earthworm (*Eisenia fetida* Savigny, 1826), and (3) soil respirometry. We hypothesized that both *M. sativa* cultivation and soil amendment with compost would promote PHC degradation and TE removal, thus decreasing soil toxicity.

Materials and Methods

Site and sampling procedures

The studied area was an auto scrap yard located in the city of Nybro, in southern Sweden (56°45′0” N; 15°54′0” E). This auto repair workshop goes back to 1984. An underground tank was used to collect waste oils and during oil drains spilled (the overfilling protection system for the tank has often been out of order). Contaminated soils around the tank were excavated for this study. Three independent composite samples (50 kg FW each, made of ten sub-samples) were randomly collected from sampling depths of 0.5-1 m spatially distributed around the tank. These samples were stored in plastic bags, and transported to the laboratory at Linnaeus University (Kalmar, Sweden). Thereafter, samples were immediately homogenized by manual mixing and sieved through an 8-mm mesh. Compost was purchased from Södra Århults Torv AB Sweden. It contained 55% dark sphagnum, 40% lys sphagnum and 5% zeolite. Soils and compost were kept at 4 ± 1 °C between sampling and analysis.

Chemical and physical characterization of soil samples

Solid phase

The experimental set-up was a fully randomized design of four different treatments: untreated soil (Unt), soil amended with compost at the 30% (w/w, air-dried soil DW) rate (30%C), soil unamended but planted with *M. sativa* (MS) and soil amended with 30% C and planted with *M. sativa* (MS-30%C). The Unt treatment consisted of 5 kg of untreated soils from each composite while the 30% C treatment was made of 3.5 kg of untreated soils from each composite mixed with 1.5 kg of compost (DW/DW). Soils were placed in 12 L plastic pots (25 cm diameter x 25 cm height) (n=3 for each treatment).

Prior to the experiment set up, total PHC and TE concentrations were measured using respectively a commercial service provided by AGAT Laboratories Montreal, QC, Canada and X-Ray fluorescence (XRF) equipment (XRF model Olympus DS-4000, Innov-X Systems, Inc. USA), in both soil treatments and in the compost (PHC n=2 and TE n=3 replicates). Total PHC concentrations (aliphatic hydrocarbon compounds with chain lengths of C10-C50 and 16 PAHs prioritized by the USEPA) were quantified by gas chromatography coupled to mass spectrometry (GC-MS). The 16 PAHs were classified according to the Swedish EPA (2009) in (i) low molecular weight PAHs (PAH-L): naphthalene, acenaphthene and acenaphthylene; in (ii) medium molecular weight PAHs (PAH-M): fluorene, phenanthrene, anthracene, fluoranthene and pyrene; and in (iii) high molecular weight PAHs (PAH-H): benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene,benzo (a) pyrene, dibenz (ah) anthracene, benzo (ghi) perylene and indeno (123cd) pyrene. The XRF equipment is a suitable portable device for detection and quantification of heavier elements with a typical detection limit of 0.01% (w/w) (Kaartinen *et al.* 2013). However, the current study focused only on the TEs that were found in concentrations above those established by Swedish quality standards: As, Co, Pb (Swedish EPA, 2009). The element Mn was also included since it has been extensively used as antiknock agent in petrol by the transportation sector (Padmavathiamma and Li 2010; Gerber *et al.* 2002). Certified reference material NIST 2709-San Joaquin Soil was used to validate the results. The soil texture was determined using the protocol described by the Colorado Master Gardener Notes (Whiting *et al.* 2011). To measure soil pH 50 mL of milli-Q water was mixed to 10 g of air-dried soil and the mixture was allowed to react for 1h before measurements. Soil pH was measured with an HQ11d portable pH meter

(Hach Company, USA). The DW of soil samples was determined in a ventilated oven at 105 ± 5 °C until constant mass (ISO 11465 1993). Organic matter (OM) was determined as sample weight loss (previously oven-dried at 105°C) upon ashing at 550°C for 16h in a muffle furnace (ASTM D 2974, n=3) (Cheng *et al.* 2008).

Aqueous phase

Aqueous phase from fresh soil samples and compost was extracted using 500 mL of deionized water mixed to 100 g of air-dried soil and the mixture was allowed to react for 1h before measurements (Cheng *et al.* 2008). The suspensions were centrifuged twice at 16.000 x g for 15 minutes (Avanti J-25, Beckman Coulter, Inc. California, USA) at 13°C. Dissolved oxygen (DO) and electrical conductivity (EC) in water extracts were analyzed with an HQ11d portable meter (Hach Company, USA). Total organic carbon (TOC), soluble nitrogen (N), soluble phosphorus (P) and chemical oxygen demand (COD) were analyzed using Dr. Lange's cuvette tests (Dr. Bruno Lange, GmbH& CO. KG, Dusseldorf, Germany). The following Dr. Lange kits were used: TOC LCK 387 for TOC; LCK138 for soluble N; with LCK 348, LCK350 for soluble P and LCK114 for COD. Cuvettes were measured spectrophotometrically with a HACH XION 500 spectrophotometer. Lange methods were validated according to ISO 8466-1 (1990), DIN 32645 (1996) and DIN 38402 A51 (1986). All analyses were performed in triplicate for each composite and compost sample. Physicochemical properties of the soils, the compost and water extract are listed in Table 1.

Phytoremediation assay set up

Seeds of *M. sativa* L. were obtained from Weibulls Seed Company, Sweden. Seeds were surface-sterilized in 10% (v/v) hydrogen peroxide for 10 min and then washed with distilled water 7 times (Wei and Pan 2010). Seeds were pre-germinated for four days (week 8) in parafilm covered petri dishes with Whatman No1 filter papers moisturized with distilled water (just enough to prevent desiccation) (Benabderrahim *et al.* 2011). After four days, twenty seedlings of uniform size were then selected and transplanted into each designated pot. To provide the plants with an adequate amount of water, pots were manually watered three times a week with deionized water (50 % water holding capacity). Growth was allowed for 5 months (week 9 to week 29) under greenhouse-controlled conditions: the temperature was set to 25 ± 2 °C, the relative air humidity to 65 ± 5%, and a photoperiod of light:darkness of 16:8 (h) was chosen. A

photosynthesis active radiation of 270 µmol m^{-2} s^{-1} was provided during light hours using Lu400W/PSL/T40 (Lucalox^{TM}).

Measurement of plant growth

After 5 months (week 29), the survival rate of *M. sativa* in each pot (%SC) was recorded as the number of surviving plants relative to the total number of plantlets transplanted (Campanelli et al. 2013). *M. sativa* shoots were harvested, washed with deionized water and blotted with filter paper. Roots were harvested, cleaned with deionized water to remove soil particles adhering to the surface and blotted. Fresh weight (FW) of shoots and roots was determined. Shoots and roots were then oven dried for 48h at 80°C and their DW biomass were determined. Water content of plant parts (%WC) was determined by the weight loss (Novo *et al.* (2013).

Measurement of trace element concentrations in plant tissues

The TE that were found in concentrations above the Swedish limits (Swedish EPA, 2009) (As, Co, Pb) and Mn found in high concentration in soil treatments were analyzed in plant tissues using the commercial service provided by ALS laboratory Scandinavia AB, Luleå, Sweden. The total TE concentrations in shoot and root samples were analyzed by inductively coupled plasma-sector field mass spectrometry (ICP-SFMS) after microwave oven-assisted digestion of air-dried samples with HNO_3/H_2O_2, following US EPA method 200.8. TE translocation factor (TF) from roots-to-shoot was expressed as the ratio between the shoot and root TE concentrations (on a dry weight basis, (Novo *et al.* 2013). Enrichment coefficient for root (ECR), an indication of the root uptake of TE was computed as the ratio between the TE concentration (on a dry weight basis) of plant roots and in the soil (Meeinkuirt *et al.* 2012). TE concentrations in roots and shoots of *M. sativa* grown in untreated soils were not determined due to their low biomass production.

Ecotoxicological tests

Plants

Phytotoxicity tests were conducted on the four treatments after the 5-month phytoremediation trial (week 30) with two plant species which have been widely used in ecotoxicological tests due to their relatively high sensitivity to TE and PHC (Masakorala *et al.* 2013; Visioli *et al.*

2016; Gouider *et al.* 2010). *L. sativum* and *Z. mays*. Seed germination and seedling growth were performed based on the procedures described in the ISO 11269-2 (ISO, 1995). Seeds were obtained from Weibulls Seed Company (Sweden). Soils (500g FW) were placed in 0.5 L plastic pots and 15 *L. sativum* seeds and five *Z. mays* seeds were separately sowed on the surface of each wetted soils in a greenhouse using the same conditions as for the *M. sativa* growth (25 ± 2 °C, with a photoperiod of light/darkness (h) of 16:8 and photosynthesis active radiation of 270 µmol m^{-2} s^{-1}). Deionized water was added every day (50 % water holding capacity). After 3 days of seed germination, *L. sativum* seedlings were thinned and only five of the most uniform plants per pot were left for further measurement of plant growth at the end of a 21-day period. Roots and shoots of each individual plant were harvested, washed with deionized water, blotted and the fresh biomass was immediately weighed. Dry biomass was determined after oven drying at 70°C for 48h. All experiments were carried out in triplicate for each soil treatment.

Earthworms

Acute toxicity test with the earthworm *Eisenia fetida* was carried out in parallel to plant testing according to the OECD 207 method, "Earthworm, Acute Toxicity Tests". Earthworms were obtained from an earthworm-culturing farm located in Ljungby (Sweden). Before the experiment, all earthworms were rinsed with distilled water, and maintained on Whatman No1 filter in the dark at room temperature for 24 h to allow for the voiding of gut contents. Ten adult earthworms (0.3 ± 0.05g) were washed and weighed before being transferred into 250 mL glass vessel containing the soil (400g DW) to be tested, for the four soil treatments after the 5-month phytoremediation trial. The standardized field soil LUFA 2.3 (Landwirtschaftliche Untersuchungs- und Forschungs-Anstalt, Speryer, Germany), which is a sandy loam, was used as an uncontaminated control soil. The experiments were carried out in triplicate for each soil treatment, with loose lids placed over the test vessels. After 14 days, the mortality was registered, the surviving worms were washed and weighed and the change in weight (CW, %) was determined.

Respirometry

A respirometric test was carried out for all soil treatments to measure the O_2 uptake rate (OUR), which is a proxy of soil microbial activity (Paletski and Young 1995). It was conducted with a pulse-flow headspace respirometer and an accompanying software (PF-8000, Respirometers

System and Applications, Fayetteville, AK, USA). Respirometry measurements were performed according to (Sanchez Arias *et al.* 2012) at 25°C and a residence time of 21 days, n=2 for each soil sample.

Statistical analysis

Two-way ANOVAs were performed to compare differences in soil pH and EC, soil moisture and OM content, total soil As, Mn, Co and Pb and also DO, COD, TOC N and P in the aqueous soil phase among the four soil treatments. One-way ANOVAs were used to compare TE translocation factors and enrichment coefficients for roots in *M. sativa*. Additionally, differences in the shoot and root DW biomasses of *L. sativum* and *Z. mays* and changes in earthworm weight developing in soil sampled after the phytoremediation trial were compared across treatments using two-way ANOVAs. ANOVAs were completed by post-hoc Tukey HSD tests to assess multi-comparison of means between treatments. The survival rate and water content in roots and shoots of *M. sativa*, cultivated on untreated and compost-amended soils, were compared using a Student-T-test. Differences in TE concentrations between roots and shoots were evaluated using a Student-T-test for each TE. Conditions of normality and homoscedasticity of data were checked in all cases. Differences were considered statistically significant at $p<0.05$. Statistical analyses were performed with GraphPad Prism (version 6.0 for mac OS, San Diego, USA).

Results and Discussion

Phytoremediation of a mixed contaminated soil-using *M. sativa*

Main soil contaminants were PHC (alkanes and PAHs) and TE (Co and Pb) (Table 5.1). The total concentrations of Co and Pb were higher when compared to the common range of 1-10 and 10-30 mg kg^{-1} DW respectively (Blum *et al.* 2012). Furthermore, Co and Pb exceeded also the Swedish limits established for sensitive lands (SL), which are 15 mg and 50 mg Pb kg^{-1} soil DW of Co and Pb respectively (Swedish EPA, 2009). Surprisingly, Co concentrations in the utilized compost also exceeded the Swedish limits. The studied soils displayed PHC concentrations up to >8000 fold higher than concentrations in residential soils that is considered in Sweden as SL [3 to 100 mg kg^{-1} DW] (Swedish Environmental Protection Agency (2009) Table 5.1). Medium (M) and high (H) molecular weight PAH concentrations were also respectively 2 and 4 fold

higher than their corresponding SL values. Conversely, low (L) molecular weight PAH concentrations remained below their SL value (3 mg kg^{-1} DW, Table 5.1). PHC concentration has been reported to increase soil pH (Masakorala *et al.* 2014; Bauder *et al.* 2005). However in this study, soil pH was slightly acid probably due to chemical reactions between PHC, TE and soil elements as highlighted by Sun and Zhou (2007). As reported by Chandrasekaran and Ravisankar (2015) the soil adsorptive capacity towards TE such as Co usually decreases when pH is also reduced. Both soils and compost showed slightly similar acidic pH values. According to Kim *et al.* (2015) at this pH range, the mobility and the bioavailability of most TE is low. Another parameter that significantly affects TE bioavailability is the OM content through sorption/desorption processes (Alexander 2000; Minkina *et al.* 2006). The percentage of OM in the studied soils and compost varied between 30.9% and 41% (shown in Table 5.1) suggesting to be high enough for increased sorption processes and high rates of chemical release to the aqueous phase (Stella *et al.* 2015). Along with other physicochemical characteristics, soil texture can significantly affect the contaminants behaviour in contaminated soils (Khan *et al.* 2015b; Romero-Freire *et al.* 2015). The studied soils were classified as silt loam on the basis of silt, sand and clay contents (Table 5.1). Silt particles are considered to exhibit some plasticity, cohesion, adsorption and low porosity, which inhibited the passage of oxygen and water (Whalen and Sampedro 2010). As a consequence, the soils moisture content was high. In aqueous phase, EC, DO, TOC and COD varied widely between soils and compost (Table 5.1). COD, which is a proxy indicator of the amount of organic compounds in water, indicates the mass of oxygen consumed per liter (Jiang *et al.* 2015). This parameter and TOC are widely used as indicators of water quality (Kaczala *et al.* 2011; Svensson *et al.* 2015). The COD and TOC results ranged between 53200-86100 mg L^{-1} and 832.6-1221 mg L^{-1} for untreated soil and the compost-amended soil respectively, which confirmed the higher amount of carbon contents in the aqueous phase. The higher contents of COD and TOC could be also attributed to the high amount of PHC in studied soils.

Table 5.1. Physicochemical parameters and contaminant concentrations (mg kg^{-1}) of the compost (C), the untreated soil (Unt) and the compost-amended soil (30%C) before the phytoremediation trial (n=3 for each parameters except for PHC: n=2, mean values are presented with standard deviations).

		C	Unt	30%C	SL[a]
		Solid phase			
Texture			Silt Loam	Silt Loam	
Sand	%		14.3 ± 0.5	21 ± 1.5	
Slit			76 ± 1	64 ± 0.5	
Clay			9.6 ± 1.1	14 ± 2	
pH (1:5,w/v)		6.7 ± 0.2	6.5 ± 0.3	6 ± 0.5	
Moisture	%	62.8 ± 0.6	60.9 ± 0.4	56.3 ± 0.6	
OM		41± 0.6	31.4 ± 2	30.9 ± 3.6	
Arsenic	mg kg^{-1}	Nd	12.3 ± 3.0	10.5± 3.5	10
Manganese		375 ± 145.9	378.3 ± 36.5	293 ± 10.1	—
Cobalt		67.5 ± 3.5	92 ± 9.5	106.3 ± 22	15
Lead		17.6 ± 1.8	195 ± 80.7	114.3 ± 8.5	50
PHC C10-50		Nd	26200 ± 4666.9	25250 ± 3464.8	3-100
PAH-L[c]		Nd	2.5 ± 3.1	0.7 ± 0.1	3
PAH-M[d]		Nd	6.3 ± 5.3	3.2 ± 0.0	3
PAH-H[e]		Nd	4.4 ± 3.6	1.8 ± 0.3	1
		Aqueous phase[b]			
EC	us cm^{-1}	674.6 ± 70.3	133.1 ± 14.6	156.7 ± 0.01	
DO	mg L^{-1}	4.9 ± 0.5	4.8 ± 0.2	6.1 ± 0.03	
COD		5956.6 ± 324	86100 ± 556.7	53200 ± 800	
TOC		1726.3 ± 28.2	1221 ± 8.7	832.6 ± 7	
N		9.7 ± 0.2	4.1 ± 0.8	7.8 ± 0.1	
P		10.5 ± 0.6	2.2 ± 0.02	2.3 ± 0.02	

[a]Swedish limit in sensitive land (Swedish Environmental Protection Agency 2009).

[b] Water-extractable (1: 5; 25 °C). PHC C10-50, petroleum hydrocarbons C10-C50; EC, Electrical conductivity; DO, dissolved oxygen; COD, chemical oxygen demand; TOC, total organic carbon; N, soluble nitrogen and P, soluble phosphorus in (mg L^{-1}).

[c]PAH-L, low molecular weight PAHs: naphthalene, acenaphthene and acenaphthylene.

[d]PAH-M, medium molecular weight PAHs: fluorene, phenanthrene, anthracene, fluoranthene and pyrene.

[e]PAH-H, high molecular weight PAHs: benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene,benzo (a) pyrene, dibenz (ah) anthracene, benzo (ghi) perylene and indeno (123cd) pyrene.

After the 5-month aided-phytoremediation trial, PHC concentrations remained steady in the soil planted with *M. sativa* as compared to the untreated one (Table 5.2). Similarly, while PAH (H) concentrations did not differ between both treatments, PAH (L and M) concentrations tended to numerically decrease in the planted soil. Rhizodegradation of high molecular weight PAH may be hindered due to their sorption to soil particles (D'orazio *et al.* 2013). This confirmed previous findings of Hamdi *et al.* (2012) and Chigbo and Batty (2013) who reported no degradation of PAHs (H) in their phytoremediation trials. Gartler *et al.* (2014) also did not report significant reduction of the ∑ 16 EPA PAH concentrations after a 6-month remediation trial using grass species and leguminous plants, including *M. sativa*. The lack of PHC degradation in our *M. sativa* -cultivated soils contrasts with Wei and Pan (2010) and Moubasher *et al.* (2015) reporting PHC degradation in their spiked soils. However, PHC rhizodegradation efficiency in aged contaminated soils is more complex and difficult to achieve compared to either freshly contaminated or spiked soils (Gerhardt et al. 2009). Similarly, Chigbo and Batty (2013) have compared the phytoremediation potential of *Brassica juncea* using both freshly spiked and aged contaminated soils: the pyrene removal was greater in freshly spiked soils than in aged contaminated soils. This was likely due to a lesser PAH bioavailability in aged contaminated soils as they were bound to the OM and sequestrated into the rhizosphere, which made them less assessable to microbial degradation (Alexander 2000).

Table 5.2: Physicochemical properties and contaminant concentrations (mg kg^{-1}) in the untreated soil (Unt), the unamended soil planted with *Medicago sativa* (MS), the compost-amended soil (30%C) and the compost-amended soil planted with *M. sativa* (MS-30%C) after a five-month phytoremediation trial (n=3 for each category except for PHC: n=2, mean values are presented with standard deviations). The different letters stand for statistical significance between the modalities at the 0.05 level with a Tukey's test.

		Unt	MS	30%C	MS-30%C	*p* value[f]	SL[a]
			Solid phase				
pH (1:5,w/v)		7 ± 0.3	7 ± 0.2	7.2 ± 0.3	6.9 ± 0.4		
Moisture	%	53.3 ± 3.5	48 ± 4.8	60.8 ± 5.6	56.1 ± 4.8		
OM		26 ± 1.8	27.7 ± 1.6	24.8 ± 1.7	25.4 ± 1.9		
Arsenic	mg kg^{-1}	13 ± 5.7[a]	10.2 ± 2.2[a]	8 ± 0.0[a]	9.6 ± 0.5[a]	*	10
Manganese		412.6 ± 157.7	409.5 ± 111.7	313.4 ± 87.2	259.8 ± 38.1		–
Cobalt		104.4 ± 40.2	89.7 ± 19.4	122.8 ± 68.6	94.3 ± 29.0		15
Lead		173 ± 68.2	131.3 ± 24.2	117.1 ± 20.1	106.6 ± 25.2		50
PHC C10-50		23800 ± 7071	20250 ± 1767.8	15600 ± 565.7	12300 ± 424.3		3-100
PAH-L[c]		2.3 ± 1.2	0.7 ± 0.1	0.3 ± 0.2	0.3 ± 0.07		3
PAH-M[d]		7.3 ± 0.3	5.6 ± 0.5	4.2 ± 0.7	3.4 ± 0.1		3
PAH-H[e]		3.5 ± 1.1	4.1 ± 0.6	3.8 ± 0.2	2.8 ± 0.2		1
			Aqueous phase[b]				
EC	us cm^{-1}	118.4 ± 4.6	127.4 ± 97.3	52.3 ± 6.8	53.3± 30.3		
DO	mg L^{-1}	8.23 ± 0.1	7.26 ± 0.3	8.3 ± 0.1	7.7 ± 0.5		
COD		10106.6 ± 1570.1[a]	1660 ± 245.5[b]	1940 ± 343.3[b]	694 ± 84.1[b]	***	
TOC		1100 ± 96[a]	79.4 ± 5.92[b]	786 ± 83[ab]	48.3 ± 20[b]	***	
N		2.2 ± 0.1[b]	1.9 ± 0.01[b]	5.2 ± 0.7[a]	1.9± 0.1[b]	***	
P		3.1 ± 0.1	2.8 ± 0.6	2.5 ± 0.3	1.8± 0.5		

[a]Swedish limit in sensitive land(Swedish Environmental Protection Agency 2009)

[b] Water-extractable (1: 5; 25 °C). PHC C10-50, petroleum hydrocarbons C10-C50; EC, Electrical conductivity; DO, dissolved oxygen; COD, chemical oxygen demand; TOC, total organic carbon; N, soluble nitrogen and P, soluble phosphorus in (mg L^{-1})

[c]PAH-L: naphthalene, acenaphthene and acenaphthylene

[d]PAH-M: fluorene, phenanthrene, anthracene, fluoranthene and pyrene

[e]PAH-H: benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene,benzo (a) pyrene, dibenz (ah) anthracene, benzo (ghi) perylene and indeno (123cd) pyrene.

[f] *p* value of the interaction compost**M. sativa*

M. sativa was expected to contribute to Pb and Co removal by phytoextraction after a 5-month cultivation in the contaminated soil since it accumulated up to eight fold more Pb in its aerial parts at a Pb-contaminated site compared to the common values in aerial plant parts (Marchand *et al.* 2015). Our phytoremediation trial however did not lead to significant removal for total soil Co, As and Pb (Table 5.2), although a trend towards decreasing concentrations was observed. Cobalt strongly binds with PAH (Mahmoodinia *et al.* 2015) and may be sequestrated

in our soil by the high PAH amount. In addition, the soil TE content is distributed between solid and aqueous phase (Kim *et al.* 2015). The aqueous phase is hosting the most mobile and bioavailable TE species which is highly variable and strongly depending on environmental factors such as soil pH, the content of organic matter, hydroxides of Fe, Mn, and clay minerals (Sheoran *et al.* 2016; Zhang *et al.* 2014). In this studied soil, several variables are favor TE sorption and immobilization processes; the neutral soils at which the mobility of most metals is low (Table 5.2; (Kim *et al.* 2015; Sauve *et al.* 1998); the high content of organic matter (>24%), which may provide a good sorption potential to organic molecules (Minkina *et al.* 2006; Park *et al.* 2011); and the content of Mn (>259mg/kg dw) also indicates substantial presence of Mn oxides, which may scavenge Co and Pb ions from the soil solution (Jalali and Moharami 2013; Roulier *et al.* 2010). Hydrous Fe/Mn oxides may contribute to Pb and Co immobilization since they are a key factor involved in both Pb and Co sorption in soils (Bradl 2004; Roulier *et al.* 2010). Soil Pb and As could be immobilized through sorption reactions with the soil OM fraction since its surface functional groups have high affinity for Pb and As (Hashimoto *et al.* 2011; Redman *et al.* 2002). In overall these mechanisms likely hindered efficient Pb, As and Co removal by *M. sativa* from this mixed contaminated soil.

Aided-phytoremediation of a mixed contaminated soil using compost and *M. sativa*

After the 5-month growth period, survival rate of *M. sativa* planted in the compost-amended soil was significantly higher than in the unamended one (Fig. 5.1a). The compost amendment significantly increased the water content of *M. sativa* shoots resulting in higher plant vigor (Fig. 5.1b). Similar pattern was widely reported in the literature (Chen *et al.* 2015; Farrell and Jones 2010). Compost application into the studied soil may have indirectly promoted plant growth through soil TE reactions with mineral components of the compost, the formation of stable complexes with organic ligands, nutrient supply and microbial inoculation (Kumpiene *et al.* 2008). Brown *et al.* (2009) outlined that humic and fulvic acids react as ligands and form insoluble complexes with Pb. Trace element (e.g. Cu and Pb) interactions with soil OM may have reduced their leaching and bioavailability, even though the dissolved OM derived from the compost may sometimes increase these processes (Sauve *et al.* 1998).

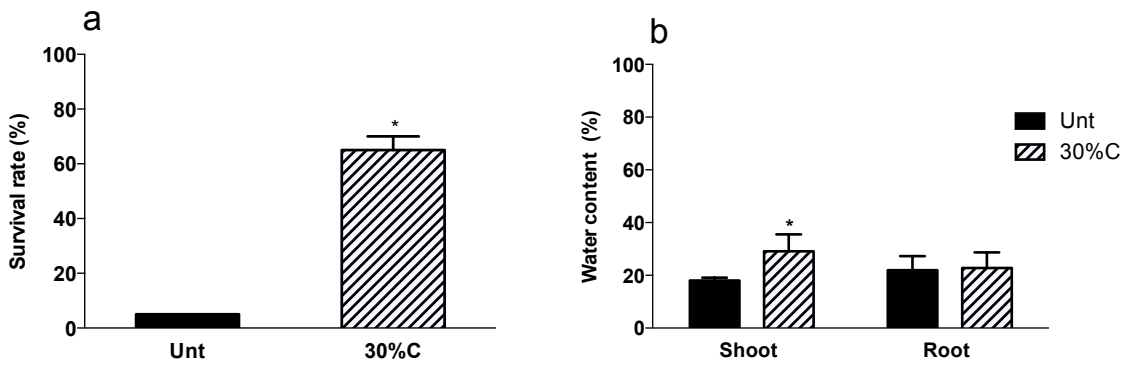

Figure 5.1. Survival rate (%) and water content (%) in shoots and roots of *Medicago sativa* after a five-month growth in the untreated (Unt) and compost-amended soils (30%C) (n=3, mean values are presented with standard deviation). Asterisks (*) denote significant differences between the compost amended and the unamended treatments at the 0.05 level using a Student-T-test.

M. sativa cultivated in compost-amended soils numerically tended to increase PHC and PAH (M and H) removal as compared to other treatments (Table 5.2). Such results agreed with Wang *et al.* (2012b) who reported higher pyrene degradation in quartz sand, red soils, and alluvial soils spiked with pyrene, planted with ryegrass and *M. sativa* and amended with compost as compared to the unamended ones. Authors attributed this dissipation to the beneficial interactions between the plant rhizodeposition, microbial communities, and compost in the rhizosphere, turning in faster pyrene rhizodegradation. *M. sativa* cultivated in the compost-amended soil significantly promoted Pb removal as compared to other treatments (Table 5.2). This was due to the cumulative impacts of both the dilution effect related to compost addition and a better Pb phytoextraction by *M. sativa*, which was more vigorous in the amended soil. In the compost-amended soil, shoot Pb concentrations of *M. sativa* reached up to 3.8 mg kg^{-1} (8 fold higher than the common values in aerial plant parts, Cf supra). Unlike for Pb, total Co did not differ across treatments (Table 5.2). Cobalt concentration in the compost was close to that in the Unt soil, thus there was no Co dilution after compost application (Table 5.2). Beside, such weak Co removal matched with its low TF, ECR and concentrations in *M. sativa* shoots and roots, which were similar to the common values (Fig. 2 and Table 2, Blum *et al*. 2012), suggesting no added value of the *M. sativa* cultivation in the compost-amended soil in terms of

Co removal. The remediation of the studied soils is limited by different factors like the low TF and ECR of TE, the molecular weight of PAH, and the limited growing period (5 months) for *M. sativa*. As reported by Chirakkara *et al.* (2016a) phytoremediation is rather slow compared to other remediation technologies because the technique is related to the metabolic activity of the plant which is related to climate and seasonal cycles. Furthermore, several harvests (more than one year treatment) are often require for the phytoremediation (Vangronsveld *et al.* 2009).

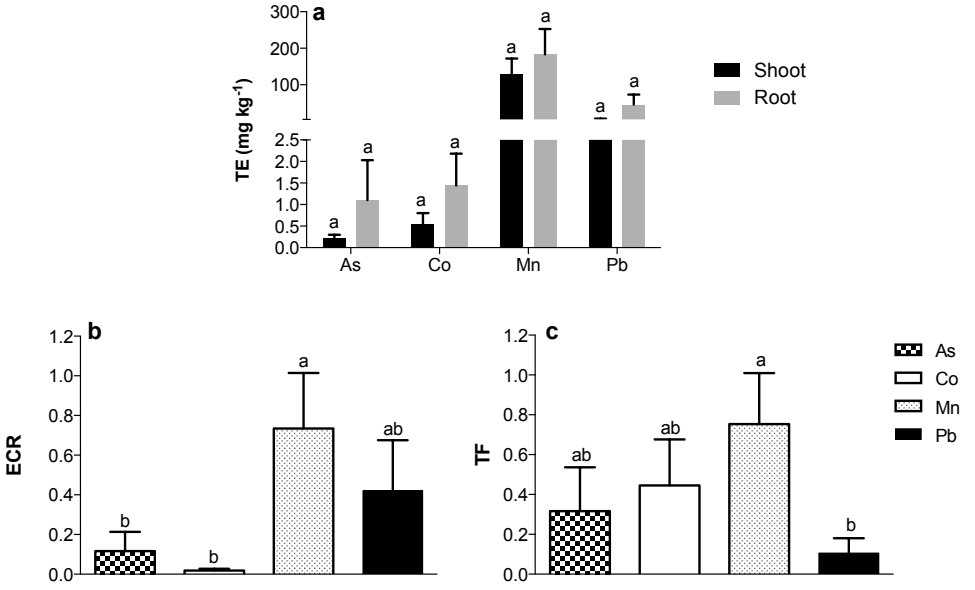

Figure 5.2. (a) Trace element (As, Co, Mn, and Pb) concentrations (mg kg^{-1}) in shoots and roots of *Medicago sativa* cultivated during the 5-month phytoremediation trial in the compost-amended soil (30%C), (b) enrichment coefficient for roots (ECR) and (c) translocation factor (TF) (n=3, mean values are presented with standard deviation and the different letters stand for statistical significance between the treatments at the 0.05 level with a Tukey's test).

Ecotoxicological analysis of soils after the phytoremediation trial

L. sativum and *Z. mays* shoot and root DW biomasses after the 21-day test were similar in both the untreated soil and the one previously planted with *M. sativa* during the phytoremediation trial ($p > 0.05$, Fig. 5.3). Earthworm growth is a sensitive indicator of exposure to contaminants (Geissen *et al.* 2008). Similarly to *L. sativum* and *Z. mays*, the

earthworm mass gains after the 14-day growth in our soils did not significantly differ between the untreated soil and the one previously cultivated with *M. sativa* ($p > 0.05$, Fig. 5.4). The absence of pollutant (PHC) linkage breakdown in unamended soils planted with *M. sativa* was related to its low survival rate (5%). Such results agreed with slow plant growth in PHC-contaminated soils (Peng *et al.* 2009). They also confirmed the negative impact of PHC (Kede *et al.* 2014) and Pb (Roy *et al.* 2014) on earthworms. The absence of positive effect of *M. sativa*–cultivation on *L. sativum*, *Z. mays* and earthworm development could also be due to the lack of total soil Mn, Co, As and Pb removal by this treatment. Although Mn and Co are essential for plants while As and Pb are not (Hanumanth Kumar and Pramoda Kumari 2015; Pourrut *et al.* 2011), previous studies showed that essential and non essential TE, when exceed the threshold limits can induce various morphological, physiological and biochemical dysfunctions in plants. Li *et al.* (2009a) reported significantly inhibition of the shoots biomass of barley, oilseed rape and tomato by Co in concentrations ranging from 7 to 1708 mg kg^{-1}. This toxicity symptom of Co^{2+} in plants can be caused by competition between Co and Fe for the same physiological binding sites (Chatterjee and Chatterjee 2003; Sree *et al.* 2015). Conversely, the shoot DW biomass of *Z. mays* was significantly higher in the compost-amended soil than in the unamended one (Fig. 6.3c). In parallel, earthworm mass gain after a 14-day growth in the compost amended soil was also higher compared to that for the untreated soil, and reached similar value as for the standard LUFA uncontaminated soil (Fig. 5.4). Such results confirmed the beneficial effect of compost addition to our soil. Ahmad *et al.* (2015) reported similar positive effect of organic amendments on crop growth and cadmium remediation.

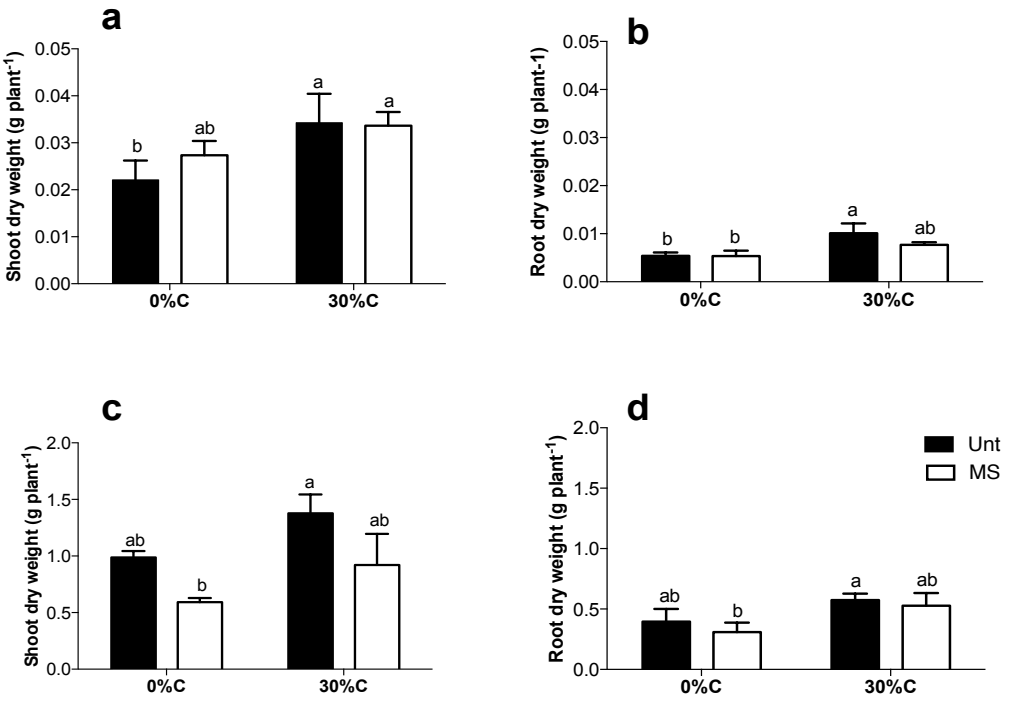

Figure 5.3. Shoot and root biomasses (g plant^{-1} DW) produced by *Lepidium sativum* (a, b) and *Zea mays* (c, d) after a 21-day growth period in the four soils (Unt, 30%C, MS, and MS-30%C) resulting from the 5-month phytoremediation trial (n=3, mean values are presented with standard deviation and the different letters stand for statistical significance between the treatments at the 0.05 level with a Tukey's test).

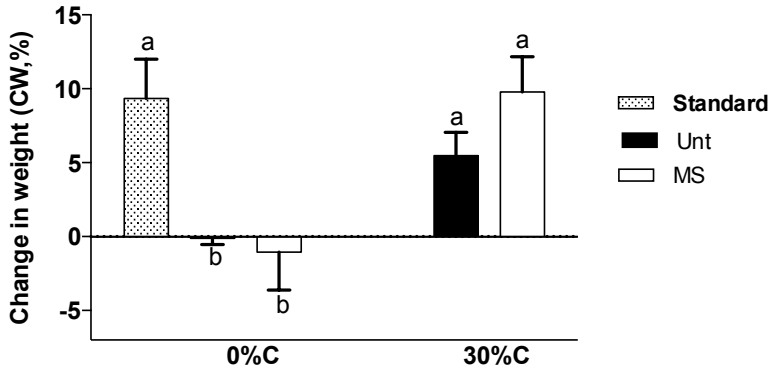

Figure 5.4. Earthworm's change in weight (CW%) after a 14-day growth in a standard uncontaminated soil and in the four studied soils (Unt, 30%C, MS, and MS-30%C) resulting from a 5-month phytoremediation trial (initially 10 individuals pot^{-1}, n=3, mean values are

presented with standard deviation and the different letters stand for statistical significance between the treatments at the 0.05 level with a Tukey's test).

Ecotoxicological effects of TE and PHC after phytoremediation were assessed by soil microorganism's respiration measurements (Fig. 5.5). The maximum initial respiration rate in untreated soils (3.79 mg O_2/L^{-1} h^{-1} ± 0.24) was higher as compared to that of the compost-amended soils (1.89 mg O_2/L^{-1} h^{-1} ± 0.24). Mukherjee *et al.* (2014) reported similar pattern when they measured microbial activity in an aged creosote-contaminated soil. They found that the basal respiration rate was positively correlated with total PHC and PAH concentrations in soils. In contrast to the bacterial diversity, the total microbial activity increased in soils with high contaminant exposure (Mukherjee *et al.* 2014). Similar increased biological respiration was reported by Labud *et al.* (2007) in the presence of diesel and resulted from high microbial activity for toxicity compensations or adaptations, degradation and/or mineralization of readily OM and organic pollutants. But, this is not the general rule, since in earlier studies, soil respiration decreased with increasing soil TE content (Kızılkaya *et al.* 2004; Doelman and Haanstra 1984). Decreasing microbial respiration after compost application agreed with Montserrat *et al.* (2006) who reported that polluted-sewage sludge added to the soil first promoted soil respiration, then when the easily OM was exhausted, the soil respiration decreased. As it can be observed in Fig.5.5 when plants were added in the pots, the respiration rate of microorganisms in the soil is much lower than in those unplanted treatment. As previously observed by Wang *et al.* (2008) this result suggests that plant rhizosphere and the coercion influence of petroleum changed the species and activity of microorganisms. Here, the respirometry results showed different ability and behavior of the PHC-contaminated soils: firstly, the growth promoting effects of readily biodegradable organic compounds and secondly the adverse effects on respiration due to the presence of soil contaminants.

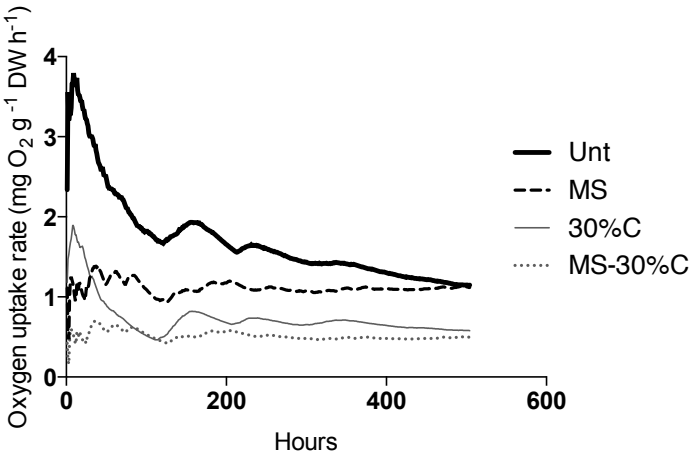

Figure 5.5. Oxygen uptake rate (mg O_2 g^{-1} DW h^{-1}; OUR) related to microbial activity in the untreated soil (Unt), the unamended soil planted with *Medicago sativa* (MS), the compost-amended soil (30%C) and the compost-amended soil planted with *M. sativa* (MS-30%C) after a 5-month phytoremediation trial (n=2).

Conclusion

During this five-month phytoremediation trial, total soil Pb significantly decreased in the composted-amended soil cultivated with *M. sativa*. Compost addition to the soil showed positive effects on *M. sativa* growth, survival rate and shoot Pb concentrations. *M. sativa* cultivation combined with compost amendment numerically increased PHC degradation but this degradation rate was lower for high molecular weight PAHs. Two hypotheses are suggested: (1) hydrophobic compounds were strongly bound to the soil solid-phase after almost a thirty-year aging process and (2) the limited growing period (5 months) for *M. sativa*. Ecotoxicological tests after the phytoremediation trial showed a positive effect of compost incorporation into the contaminated soil (as compared on the unplanted, untreated soil) on both *L. sativum* growth and earthworm development. The O_2 uptake by soil microorganisms was higher in the untreated soils compared to the compost-amended and/or *M. sativa*-planted ones, suggesting the increase of microbial activity by energy needs for toxicity compensation, adaptations and/or degradation of organics. Generally, *M. sativa* cultivation did not affect the plant (except for *Z. mays* shoots)

and earthworm development between unamended and compost-amended treatments, but based on respirometry it may decrease adverse effects on soil microbial activity in comparison to the unplanted treatment. Results from this greenhouse experiment favor the use of combined chemical and toxicological analyses for a soil-specific management of contaminated sites. Furthermore, the information obtained in this laboratory scale brings important information for further studies on pilot and even full scale. However, the process of *in situ* aided-phytoremediation on such mixed contaminated soils is considerable complex due to many external factors. It is recommended that parameters such as different combinations amendment/plant species and the relevant amendment dose for reducing the pollutant linkages are better understood.

Acknowledgment

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Preface (Chapter 6)

In previous chapters, laboratory and greenhouse studies were performed to select the suitable microorganisms or/and plant and to predict the degradation rates. After five months' greenhouse trial, *M. sativa* combined with compost promoted the degradation of PHC and the phytoextraction of Pb. Despite numerous studies which proposed phytoremediation as a realistic method to clean up contaminated soil, this technology has a number of yet unsolved problems and lacks credible demonstration on a field scale. Therefore, a pilot field scale phytoremediation experiment was carried out to investigate the efficiency of ecopiling using a *M. sativa* in monoculture and co-planting with *H. annus* to remediate a PHC and metals co-contaminated soil. The residual soil and soil leachate ecotoxicities using plants and earthworm was also studied.

Chapter 6: Pilot scale ecopiling of a soil contaminated by petroleum hydrocarbons and metals

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Abstract

A pilot scale experiment was conducted to investigate the performance of ecopiling as a remediation technology for the metal phytoextraction and phytodegradation of petroleum hydrocarbons (PHC) in a co-contaminated soil. First, this soil was amended with compost (10% w/w) and used to construct passive biopiles (Unp-10%C). Then, a phyto-cap of *Medicago sativa* L. either in monoculture (MS-10%C) or co-cropped with *Helianthus annuus* L. as companion planting (MSHA-10%C) was sown on the topsoil to complete the Ecopile. Physico-chemical parameters and contaminants in the soil and its leachates were measured at the beginning and the end of the first growth season (after five months). In parallel, residual soil ecotoxicity was assessed using the plant species *Lepidium sativum* L. and the earthworm *Eisenia fetida* Savigny, 1826, while the leachate ecotoxicity was assessed using *Lemna minor* L. After 5 months, PH C10-C40, PAH-L, PAH-M PAH-H, Pb and Cu concentrations in the MS-10%C soil were significantly reduced as compared to the Unp-10%C soil. Metal uptake by alfalfa was low but their translocation to shoots was high for Mn, Cr, Co and Zn (transfer factor (TF) >1), except for Cu and Pb. Alfalfa in monoculture reduced electrical conductivity, total organic C and Cu concentration in the leachate while pH and dissolved oxygen increased. Alfalfa co-planting with sunflower did not affect the extraction of inorganic contaminants from the soil, the PAH (M and H) degradation and had lower treatment performance for PH C10-C40 and PAH-L as compared to alfalfa monoculture. The co-planting reduced shoot and root Pb concentrations. The residual risk assessment after 5 months showed a positive effect of co-planting on *L. sativum* shoot dry weight (DW) yield. However, high contaminant concentrations in soil and elutriate still inhibited the *L. sativum* root DW yield, earthworm development, and *L. minor* growth rate. Generally, the ecopiling could be a promising remediation technology to mitigate pollutant linkages due to metals and PHC in this co-contaminated soil.

Keywords: Ecopile, Phytoremediation, Toxicity test, Compost, Phytotechnologies.

Introduction

Over decades, anthropogenic activities have left about 2.5 million of contaminated sites in European Union (EU) (European Environment Agency 2014)Most frequent contaminants in soils at these sites are metals (37%), mineral oil (20%) and hydrocarbons (22%) (Evangelou *et al.* 2012). Metals, e.g. Cu, Co, Pb, and Hg, and petroleum hydrocarbons (PHC), such as the 16 polycyclic aromatic hydrocarbons (PAHs) prioritized by the United States Environmental Protection Agency (USEPA), are of great concern due to their persistence in the environment, and their potentially serious health consequences (Khan *et al.* 2015b; Fu *et al.* 2012). In Sweden, about 80 000 sites are potentially contaminated, due to more than two hundred years of industrialization, and roughly 60 000 out of these have been risk-assessed (Swedish Environmental Protection Agency 2016). Open dumpsites and landfills are the most widespread methods for municipal solid waste (MSW) disposal due to relatively low initial investments and operational (Kaczala *et al.* 2015; Xiaoli *et al.* 2007). The member countries of the EU have consequently implemented a range of legislation such as the landfill directives (Council Directive 1999/31/EC 1999) and the waste directives (Directive 2008/98/EC 2008) to enforce the remediation of contaminated land and to minimize the negative impact on the environment and human health. To provide alternatives to conventional methods of MSW treatment (e.g. disposal to landfill, isolation, soil washing, and pump-and-treat), several methods rely on the use of plants and associated microorganisms and have been alternatively used for remediating polluted soils (Marchand *et al.* 2016b; Nagendran *et al.* 2006). Gentle soil remediation options (GRO), including in situ contaminant stabilization ("inactivation" using biological or chemical processes) and plant-based options (i.e. phytomanagement) are gaining social acceptance and have the advantages of being non-destructive, less disruptive to the soil and low-cost (Marchand *et al.* 2016a; Cundy *et al.* 2013; Kumpiene *et al.* 2014; Cundy *et al.* 2016). Biopiling, also known as bioheaps, biocells or biomounds, is a GRO that involves the assembling of PHC contaminated soils into piles and stimulates the biodegradation activity of microbial populations through the addition of oxygen, pH and moisture level adjustment, addition of nutrients and organic matter (Whelan *et al.* 2015b; Benyahia and Embaby 2016a; Coulon *et al.* 2010). Compost originated from the organic fraction of MSW is increasingly used as soil conditioner as well as a fertilizer for meeting both nitrogen and organic matter addition (Cesaro *et al.* 2015). This stimulation of

microbial activity enhances organic compound degradation within the pile (Park *et al.* 2011). Therefore, many studies reported the successful use of biopiling for remediating PHC contaminants in soils (Gomez and Sartaj 2014; Kriipsalu and Nammari 2010; Baldan *et al.* 2015). Compost amendment has been also effectively used for the phytostabilisation of metal-contaminated soil (Ruttens *et al.* 2006; Park *et al.* 2011; Ogundiran *et al.* 2015), the rhizodegradation of PHC in contaminated soils (Zhang *et al.* 2012; Ghanem *et al.* 2013; Wang *et al.* 2012b) and remediation of metal and PHC co-contaminated soil (Marchand *et al.* 2016a; Chirakkara *et al.* 2016a).

A major challenge of GRO for co-contaminated soils is the simultaneous removal or/and control of multiple contaminants. Therefore, a combination of different set of technologies is often required to achieve effective performance on soil remediation. Ecopiling process, i.e. the combination of phytoremediation and biopiling, was successfully used for the first time by Germaine *et al.* (2014) for remediating PHC-contaminated soils at a field scale. Ecopiling is a modification of conventional passive biopiling: instead of enclosing the biopile with black plastic, the pile is planted with suitable phytoremediation plants (Germaine *et al.* 2014). The selection of appropriate plant species is critical to optimize the phytoremediation and co-planting are often used for a simultaneous removal of multiple contaminants (Wang *et al.* 2013). *Medicago sativa* (alfalfa) is widely used for phytoremediation of organic (Wei and Pan 2010; Hechmi *et al.* 2014) and inorganic pollutants (Zaefarian *et al.* 2013; Vamerali *et al.* 2011; Bonfranceschi *et al.* 2009). This plant species has a fibrous root system suitable for the PHC rhizodegradation (Wang *et al.* 2012b) and can contribute to TE phytostabilisation (Zribi *et al.* 2015). Alfalfa in combination with compost was selected on the basis of previous results at mesoscale level (Marchand *et al.* 2016a). *Helianthus annuus* (sunflower) is also used to remediate metal(loid)-contaminated soils (Kolbas *et al.* 2011; Kidd *et al.* 2015a) and facilitation/intercropping is claimed to promote the efficiency of several phytotechnologies for remediating contaminated soils (Brooker *et al.* 2008; Wang *et al.* 2014; Kidd *et al.* 2015b). Co-planting of alfalfa with sunflower may be a promising option to optimize the remediation of metal and PHC co-contaminated soils.

This study aimed at investigating: (1) the efficiency of ecopiling using alfalfa either in monoculture or co-planting with sunflower to remediate a PHC and metal co-contaminated soil in a pilot scale plant, (2) the residual soil ecotoxicity using *Lepidium sativum* L. and earthworm

(*Eisenia fetida* Savigny, 1826) and (3) the residual soil leachate ecotoxicity using *Lemna minor* L.. The study also aimed to verify the hypothesis that alfalfa co-planting with sunflower would increase PHC degradation and metal removal as compared to alfalfa monoculture, thus decreasing soil and soil leachate ecotoxicity.

Materials and Methods

Site and ecopile construction

The studied area is a 40-year old MSW landfill at Moskogen, in southern Sweden (56°41'26" N; 16°10'49" E). This landfill receives approximately 65,000 tons of wastes per year from three communities with a total population of 90 000 inhabitants. Among these wastes, 3.5% are hazardous wastes including the oil-contaminated soil used in this study. The climate is typically inland but seasonally affected by the Baltic sea, with an annual rainfall of 650–700 mm $year^{-1}$ (30-year average 470 mm). The landfill area has a facility for treating about 150 000 m^3 $year^{-1}$ of leachate. This facility, described by Thorneby *et al.* (2006), consists of three consecutive ponds and a constructed wetland used to collect and treat the water. This water was used in this study as well as the compost obtained from the food waste of the whole municipality. Contaminated soil was amended with compost at 10% (w/w, fresh soil FW).

Nine ecopiles of 9 m^2 and 0.7 m in height were constructed on May 2015 at the Moskogen facility (Fig. 6.1: ecopile design). The biopile was lined with a polyethylene waterproof membrane to prevent the leachate from draining into the ground and ensure the correct inflow-outflow water balance. A 10 cm drainage layer made of gravels and sand was laid manually on the bottom. The ecotextile was placed over a 50 mm perforated drainage pipe and then ecopile was raised with compost-amended soil (10%C). The Ecopiles were constructed with 15^{o} of inclination to allow adequate flow/drainage of leachate that was further collected in 1000 L underground tanks.

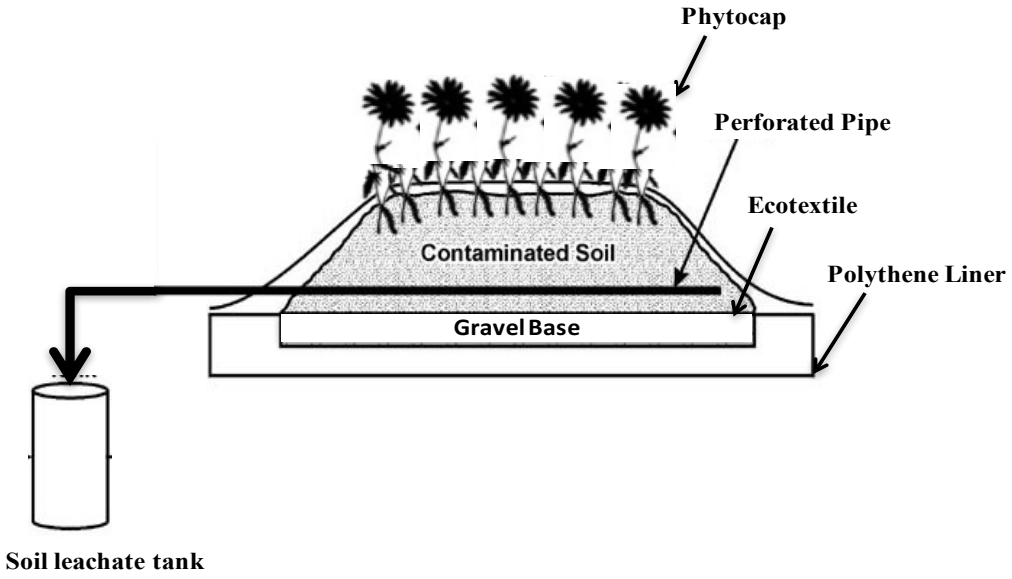

Figure 6.1: Ecopile design

Experimental set up

The experimental set-up was a full randomized design of three treatments with three replicates: unplanted ecopile (Unp-10%C), ecopile planted with *M. sativa* alone (MS-10%C) and ecopile co-planted with *M. sativa* and *H. annuus* (MSHA-10%C) (Fig. 6.2). Seeds of alfalfa and sunflower were obtained from Weibulls Seed Company, Sweden. Growth was allowed for 5 months (week 23 to week 44). During dry days, Ecopiles were automatically irrigated every two days with water from the landfill leachate treatment.

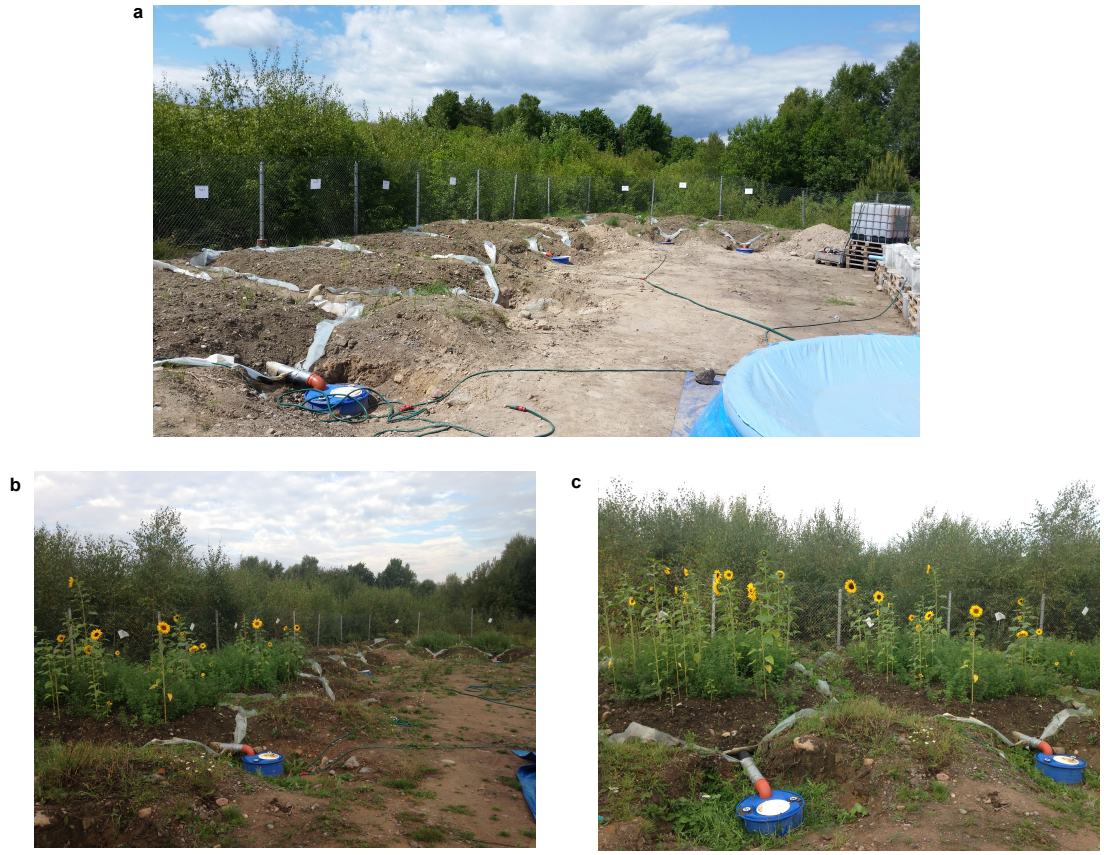

Figure 6.2: Ecopiles (a) after construction in May 2015 and (b, c) four months later in September 2015.

Soil sampling and analysis

On May 2015, three independent composite samples (10 kg fresh weight; FW each, made of ten subsamples) of the compost (C) and the untreated soil (Unt) were randomly collected from the storage area in the landfill. Similarly, three independent composite samples (10 kg FW each, made of ten subsamples) of the compost-amended soil (10%C) were randomly collected after the automatic mixing of the soil and compost. These samples were stored in plastic bags, transported to the laboratory at Linnaeus University (Kalmar, Sweden) and kept at 4 ± 1^{o}C prior analyses. All analyses were performed in triplicate for each composite and compost sample. Total PHC concentrations (aliphatic hydrocarbon compounds with chain lengths of C10-C40 and 16 PAHs prioritized by the USEPA) were quantified by gas chromatography coupled to mass spectrometry (GC-MS) using a commercial service provided by Eurofins Laboratories Saverne, France. The 16 PAHs were classified according to the Swedish EPA (2009) in (i) low

molecular weight PAHs (PAH-L): naphthalene, acenaphthene and acenaphthylene; in (ii) medium molecular weight PAHs (PAH-M): fluorene, phenanthrene, anthracene, fluoranthene and pyrene; and in (iii) high molecular weight PAHs (PAH-H): benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene,benzo (a) pyrene, dibenz (ah) anthracene, benzo (ghi) perylene and indeno (123cd) pyrene. Metal concentrations were measured using X-Ray fluorescence (XRF) equipment (XRF model Olympus DS-4000, Innov-X Systems, Inc. USA). The XRF method is applicable for heavier elements with a typical detection limit of 0.01% (w/w) (Kaartinen *et al.* 2013). However, this study focused only on metals found in concentrations above those established by Swedish quality standards (Cu, Co, Hg and Zn) and the background metal concentrations in soils (Pb and Cr). Manganese and Fe were also included since they are key players in Zn, Cu, Pb and Co sorption in soils (Roulier *et al.* 2010; Jalali and Moharami 2013). Certified reference material NIST 2709-San Joaquin Soil was used to validate the results.

The soil texture was determined using the protocol described by the Colorado Master Gardener Notes (Whiting *et al.* 2011). The dry weight (DW) of soil samples was determined in a ventilated oven at 105 ± 5 °C until constant mass (ISO 11465 1993). Organic matter (OM) was determined as sample weight loss (previously oven-dried at 105°C) upon ashing at 550°C for 16h in a muffle furnace (ASTM D 2974, n=6) (Marchand *et al.* 2016a). To measure soil pH, dissolved oxygen (DO) and electrical conductivity (EC) 500 mL of milli-Q water was mixed to 100 g of air-dried soil and the mixture was allowed to react for 1h before measurements. Soil water extracts pH, DO and EC were measured with an HQ11d portable pH meter (Hach Company, USA).

At the end of the first growing season in October (week 44), soil samples were collected from each ecopile with an unpainted steel spade in two soil layers: 0-20 cm (L1) and 20-50 cm (L2). A total of 35 individual samples per layer were taken from each ecopile. These samples were mixed to form 6 composite samples per layer for each ecopile. Thus, for each of the three treatments (Unp-10%C, MS-10%C and MSHA-10%C) there were n=18 composite samples per layer for the three replicates. Both PHC and metal concentrations in the soils, soil texture, soil moisture, OM content, and pH, DO and EC in the soil were measured in the same way as at the beginning of the experiment.

Plant growth

After 5 months (week 44), three plants of alfalfa and all plants of sunflower at each ecopile were collected. Shoots were harvested, washed with deionized water and blotted with filter paper. Roots were harvested, cleaned with deionized water to remove soil particles adhering to the surface and blotted. Shoot and root FW yields were determined. Shoots and roots were then oven dried for 48h at 80°C and their DW biomass were determined. Water content of plant parts (%WC) was determined by the weight loss (Novo *et al.* 2013).

Metal concentrations in plant parts

Metals in the contaminated soil with concentrations exceeding the Swedish threshold values for residential areas and for non-sensitive land (Swedish EPA, 2009) (i.e. Cu, Co and Zn), the background metal concentrations in soils (i.e. Pb and Cr) and with high concentration in the studied soils (i.e. Mn) were analyzed in plant tissues using the commercial service provided by CACEN, University of Montréal Laboratories, QC, Canada. Total metal concentrations in shoot and root samples were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) after digestion of air-dried samples with HNO_3, following Wilson *et al.* (2005). Translocation factor (TF) of metals from roots-to-shoot was expressed as the ratio between the shoot and root metal concentrations (on a dry weight basis (Novo *et al.* 2013). Root bioconcentration factor (BCF), a proxy of the root uptake of metals was computed as the ratio between metal concentrations (on a DW basis) in roots and the soil (Boechat *et al.* 2016). Shoot and root metal removal (mg per plant) were calculated by multiplying biomass with metal concentration in each plant tissues. For each element, the amounts (mg per plant) of metals taken up by the whole plants was expressed as the sum of shoot and root metal removals.

Analysis of ecopile leachate

Soil leachates were analyzed twice, in the month 1 and 5 of the trial. Dissolved oxygen, pH and EC were analyzed with an HQ11d portable meter (Hach Company, USA). Total organic carbon (TOC) and chemical oxygen demand (COD) were analyzed using Dr. Lange's cuvette tests (Dr. Bruno Lange, GmbH& CO. KG, Dusseldorf, Germany). Cuvettes were measured spectrophotometrically with a HACH XION 500 spectrophotometer. Lange methods were validated according to ISO 8466-1 (1990), DIN 32645 (1996) and DIN 38402 A51 (1986). Total

metal concentrations in soil leachates were analyzed by ICP-MS using the commercial service provided by Eurofins Laboratories Saverne, France.

Ecotoxicological tests

Toxicity tests were conducted on soils and soil leachates at the beginning and after the 5-month phytoremediation trial. Soil toxicity tests were conducted using the plant *L. sativum* and the earthworm *E. fetida* while soil leachate toxicity tests were conducted using the aquatic plant *L. minor*. These plant species and the earthworm are widely used in ecotoxicological tests due to their relatively high sensitivity to metals and PHC (Masakorala *et al.* 2013; Visioli *et al.* 2016; Gouider *et al.* 2010).

***Lepidum. sativum* toxicity test**

Lepidum sativum seed germination and seedling growth was performed based on the procedures described in the ISO 11269-2 (ISO, 1995). Seeds were obtained from Weibulls Seed Company (Sweden). The standardized field soil LUFA 2.3 (Landwirtschaftliche Untersuchungs- und Forschungs-Anstalt, Speryer, Germany), which is a sandy loam, was used as an uncontaminated control soil. Aliquots (500g FW) of L1 and L2 soils were placed in 0.5 L plastic pots and 15 *L. sativum* seeds were sowed on the surface of each wetted soils. Growth was allowed for a 21-day period under greenhouse-controlled conditions: the temperature was set to 25 ± 2 °C, the relative air humidity to 65 ± 5%, and a photoperiod of light:darkness of 16:8 (h) was chosen. A photosynthesis active radiation of 80 µmol m^{-2} s^{-1} was provided during light hours using Lu400W/PSL/T40 (Lucalox^{TM}). Deionized water was manually added every day (50 % water holding capacity), with no leaching from the potted soils. After 3 days of seed germination, *L. sativum* seedlings were thinned and only five of the most uniform plants per pot were left for further measurement of plant growth at harvest. Roots and shoots of each individual plant were harvested, washed with deionized water, blotted and the fresh biomass was immediately weighed. Dry biomass was determined after oven drying at 80°C for 48h. All experiments were carried out with six replicates for each soil treatment.

***Eisenia fetida* toxicity test**

Acute toxicity test with *E. fetida* was carried out according to the OECD 207 method, "Earthworm, Acute Toxicity Tests". Earthworms were obtained from an earthworm-culturing farm located in Ljungby (Sweden). Before the experiment, all earthworms were rinsed with distilled water, and maintained on Whatman No1 filter in the dark at room temperature for 24 h to allow for the voiding of gut contents. Ten adult earthworms (0.3 ± 0.05g) were washed and weighed before being transferred into 250 mL glass vessel containing the soil (400g DW) to be tested, for the four soil treatments after the 5-month phytoremediation trial. The standardized field soil LUFA 2.3 (Landwirtschaftliche Untersuchungs- und Forschungs-Anstalt, Speryer, Germany) was also used as an uncontaminated control soil. The experiments were carried out with six replicates for each soil treatment, with loose lids placed over the test vessels. After 14 days, the mortality was registered, the surviving worms were washed and weighed and the change in body weight (CW, %) was determined.

***Lemna minor* toxicity test**

The effects of ecopiling on *L. minor* growth were assessed using a static, non-renewal assay where plants were exposed to soil leachates during 21 days. To prevent changes in the initial composition of soil leachates, samples were tested as they were collected, i.e. without filtration. The *L. minor* population was collected from a small lake located in Ingelstorp Smedby in Kalmar municipality, Sweden. The test set up was established as previously reported by Marchand *et al.* (2011). In brief, duckweed fronds (2 colonies with 3 fronds and 2 colonies with 4 fronds = 14 fronds in all), randomly selected were cultivated in 250 mL Erlenmeyer flasks containing 150 mL of either soil leachate from each soil treatment or the freshwater from the Smedby river (n=6 for each treatment). Fronds were counted after 5, 9, 13, 17 and 21 days of experimentation. The number of fronds has been used by Radic *et al.* (2010) as a relevant surrogate for biomass to assess the phytotoxicity of the growth media. Relative growth rate (RGR) was then determined using the following equation: $RGR = [\ln(\text{final frond number}) - \ln(\text{initial frond number})] / \text{elapsed time between initial and final measurements}$, the initial number of fronds being n = 14 at the beginning of the experiment.

Statistical analysis

One-way ANOVAs were performed to compare differences in total ecopile PHC, N, P, moisture and OM content and also DO, EC and pH in soil water extracts between the three soil treatments. Two-way ANOVAs were used to compare the concentration of each metal (Cr, Co, Cu, Pb, Zn and Mn) in *M. sativa* shoots and roots from the MS-10%C and MSHA-10%C treatments. The DW biomasses, water content and metal concentrations in roots and shoots of *M. sativa* and *H. annuus* were compared using a two-way ANOVAs and Student-T-test, respectively. Additionally, differences in metal TF and ERC for *M. sativa* and *H. annuus* as well as *L. minor* RGR, shoot and root DW biomasses of *L. sativum* and changes in earthworm body weight developing on ecopile soils after 5 months were compared across treatments using two-way ANOVAs. ANOVAs were completed by post-hoc Tukey HSD tests to assess multi-comparison of means between treatments. Differences in chemical parameters and metal concentrations of leachates were evaluated using a Student-T-test for each parameter. Differences were considered statistically significant at $p<0.05$. Statistical analyses were performed with GraphPad Prism (version 6.0 for mac OS, San Diego, USA).

Results and Discussion

Initial characterization of the soil and compost before ecopiling

The studied soil was characterized as sandy loam texture with high total metal and PAH concentrations. The total average concentrations (in mg kg^{-1} DW) of Cu (256; 253), Co (256; 248), Hg (14.5; 14.3) and Zn (602; 562) in Unt and Unt-10%C soils respectively exceeded the Swedish threshold values established for residential areas and land used for industries (Table 6.1, Swedish EPA, 2009). The total concentrations of PHC C10-40 (alkanes) also exceeded the Swedish EPA threshold values (Table 6.1). Furthermore, total soil Pb and Cr were higher when compared to their common ranges in soils, i.e. 10-30 and 10-50 mg kg^{-1} DW respectively (Blum *et al.* 2012). The OM percentage, and total N and P were higher in compost compared to Unt and Unt-10%C (Table 6.1). Regarding the soil pore water, DO contents were lower in the Unt (1.15 mg L^{-1}) and Unt-10%C (3.5 mg L^{-1}) treatments as compared to compost (6.7 mg L^{-1}, Table 6.1) that may be attributed to the amount of organic compounds. The EC of the soil pore water indicates the relative water-soluble salt content of the soil (Sheppard *et al.* 2000). The EC for compost was lower than its values for Unt and Unt-10%C which can be an indication of lower

content of soluble salts. The pH values of 6.8-6.9 denoted roughly a neutral condition of the soil and compost. After the addition of compost at 25, 50 and 100 Mg ha^{-1} (dry weight basis) into a metal (Cd, Cr, Cu, Ni, Pb and Zn)-contaminated soil, Alvarenga *et al.* (2009) reported a raise of the soil OM following by the increase of the soil pH and EC. However, the addition of 10% of compost (w/w, FW) in the Unt soil did not significantly change the soil OM, pH and EC (Table 6.1).

Table 6.1: Physicochemical parameters and contaminant concentrations (mg kg^{-1}) of the compost (C), the untreated soil (Unt) and the compost-amended soil (Unp-10%C) before the ecopiling trial (n=9 for each parameter except for PHC: n=6, mean values are presented with standard deviations). Values in bold *italic* exceeded the Swedish threshold values established for residential areas and land used for industries (Swedish EPA 2009).

		C	Unt	Unp-10%C	SL KM; MKM[a]	BS[b]
		Solid phase				
Texture			Sandy Loam	Sandy Loam		
Sand	%		58.3 ± 3.9	60.3 ± 1.4		
Slit	%		37.7 ± 6.2	35.7 ± 2.6		
Clay	%		3.8 ± 2.3	3.8 ± 4		
Moisture	%	75.4 ± 2.6	73.6 ± 0.9	76.1 ± 0.1		
OM	%	48.8± 2	35.2 ± 1.4	30.83 ± 2.4		
N	mg kg^{-1}	2330 ± 136.6	2283 ± 910.9	2283 ± 534.5		
P	mg kg^{-1}	1573 ± 44.1	1323 ± 67.1	1380 ± 218.7		
Copper	mg kg^{-1}	28.33 ± 13	***256.7 ± 27.1***	***253.3 ± 27.1***	80; 200	10-40
Manganese	mg kg^{-1}	407.8 ± 34.1	743.2 ± 147.7	688.8 ± 84.8	-	300-1000
Cobalt	mg kg^{-1}	Nd	***256.3 ± 27.7***	***248 ± 28***	15; 35	1.0-10
Lead	mg kg^{-1}	26.1 ± 1.8	248 ± 28.3	236.5 ± 38.5	50; 400	10-30
Mercury	mg kg^{-1}	Nd	14.5 ± 2.2	14.3 ± 1.8	0.25; 2.5	0.05-0.5
Zinc	mg kg^{-1}	17.1 ± 3.1	***602 ± 126.8***	***562.7 ± 75.1***	250: 500	20-200
Chromium	mg kg^{-1}	23.5 ± 5.6	***98.3 ± 10.8***	***96.6 ± 10.6***	80; 150	10-50
Iron	mg kg^{-1}	7739 ± 471.6	35264 ± 2507	34266 ± 1106	-	10000-50000
PHC C10-40[c]	mg kg^{-1}	1540 ± 80.42	***16975 ± 3553***	***10233 ± 465***	3; 1000	
PAH-L[d]	mg kg^{-1}	0.01 ± 0.0	1.25 ± 0.4	0.82 ± 0.3	3; 15	
PAH-M[e]	mg kg^{-1}	0.37 ± 0.1	2.78 ± 0.8	1.53 ± 0.1	3; 20	
PAH-H[f]	mg kg^{-1}	0.4 ± 0.0	2.08 ± 0.7	1.57 ± 0.5	1; 10	
		Soil water extracts[g]				
pH		6.8 ± 0.2	6.9 ± 0.02	6.9 ± 0.2		
EC	µS cm^{-1}	1471 ± 259	2740 ± 10	1815 ± 161		
DO	mg L^{-1}	6.7 ± 0.0	1.15 ± 0.0	3.5 ± 0.5		

[a]Swedish threshold values for residential areas (KM) and for non-sensitive land e.g. infrastructure or industries (MKM) (Swedish Environmental Protection Agency 2009).

[b] Background metal concentration in soils (Blum *et al.*, 2012)

[c]PHC C10-40, petroleum hydrocarbons C10-C40.

[d]PAH-L, low molecular weight PAHs: naphthalene, acenaphthene and acenaphthylene.

[e]PAH-M, medium molecular weight PAHs: fluorene, phenanthrene, anthracene, fluoranthene and pyrene.

[f]PAH-H, high molecular weight PAHs: benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene,benzo (a) pyrene, dibenz (ah) anthracene, benzo (ghi) perylene and indeno (123cd) pyrene.

EC, Electrical conductivity; DO, dissolved oxygen; N, total Kjeldahl nitrogen and P, total phosphorus.

[g] soil:water,1:5,w/v

Ecopiling using *M. sativa* in monoculture

After 5 months, the PH C10-C40, PAH-L, PAH-M and PAH-H concentrations were respectively reduced in average by 40%, 42%, 56% and 36% in the Unp-10%C as compared to the Unt-10%C soil at the beginning of the experiment (Table 6.1 and Fig. 6.3). This may be due to enhanced microbial activity promoting the degradation of PHC compounds after compost application and to potential leaching with dissolved OM. Doni *et al.* (2015) reported the effectiveness of OM (compost) application in supporting the growth and metabolic activities of microorganisms capable of degrading PHC. Other studies have extensively reported this positive influence of compost in PHC degradation (Ghanem *et al.* 2013; Wang *et al.* 2012b; Gandolfi *et al.* 2010).

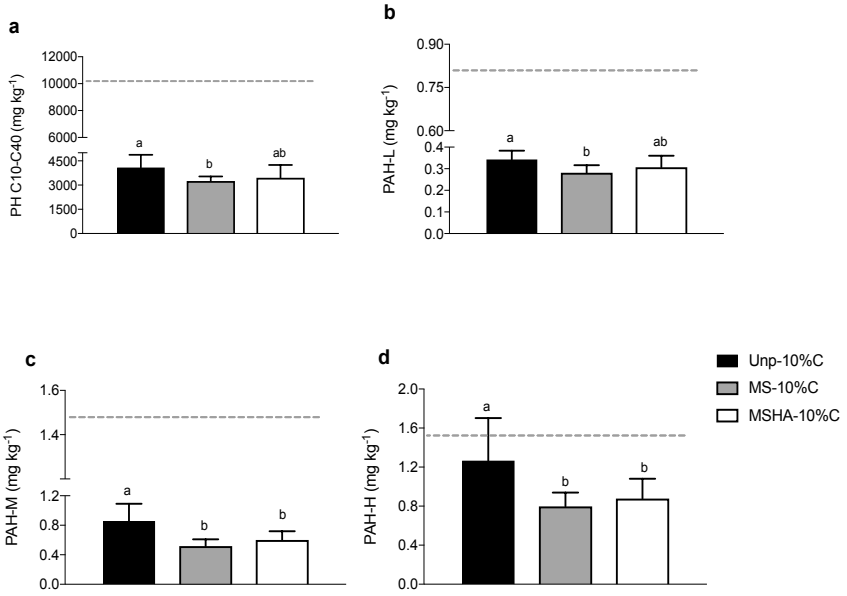

Figure 6.3: Total aliphatic hydrocarbon compounds with (a) chain lengths of C10-C40 (PH C10-C40), (b) low molecular weight PAHs (PAH-L[*]), (c) medium molecular weight PAHs (PAH-

M[**]) and (d) high molecular weight PAHs (PAH-H[***]) after five months in the unplanted ecopile (Unp-10%C), the ecopile planted with *M. sativa* (MS-10%C) and the ecopile co-planted with *M. sativa* and *H. annuus* (MSHA-10%C). Mean values are represented with standard deviations, n= 12 for each parameter. The different letters stand for statistical significance between the modalities at the 0.05 level with a Tukey's test. Discontinuous line represents the concentration at the beginning of the experiment (Unt-10%C).

[*]PAH-L: naphthalene, acenaphthene and acenaphthylene.

[**]PAH-M: fluorene, phenanthrene, anthracene, fluoranthene and pyrene.

[***]PAH-H: benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene,benzo (a) pyrene, dibenz (ah) anthracene, benzo (ghi) perylene and indeno (123cd) pyrene.

After 5 months, PH C10-C40, PAH-L, PAH-M and PAH-H concentrations were significantly reduced in average by 20%, 18%, 40% and 38% in the MS-10%C as compared to the Unp-10%C (Fig. 6.3a-d). Marchand *et al.* (2016a) found similar results after five months for a greenhouse-scale aided-phytoremediation of a mixed PHC (alkanes and PAHs) and metal (Co and Pb) contaminated soil using compost and alfalfa. They reported that alfalfa cultivated in the compost-amended soil promoted Pb removal but not Co removal. These findings are in line with this trial using alfalfa (Fig. 6.4a-h). The decrease of total soil Pb and Cu in this study was mainly due to the cumulative effect of both alfalfa cultivation (which contributes to the metal removals from soil and the rhizodeposition) and the compost addition (which can promote soil structural stability, microbial activity and dissolved organic matter (DOM)). A compost amendment can improve soil nutrients, water provision and favors soil porosity (Jones *et al.* 2016; Mench *et al.* 2010a).

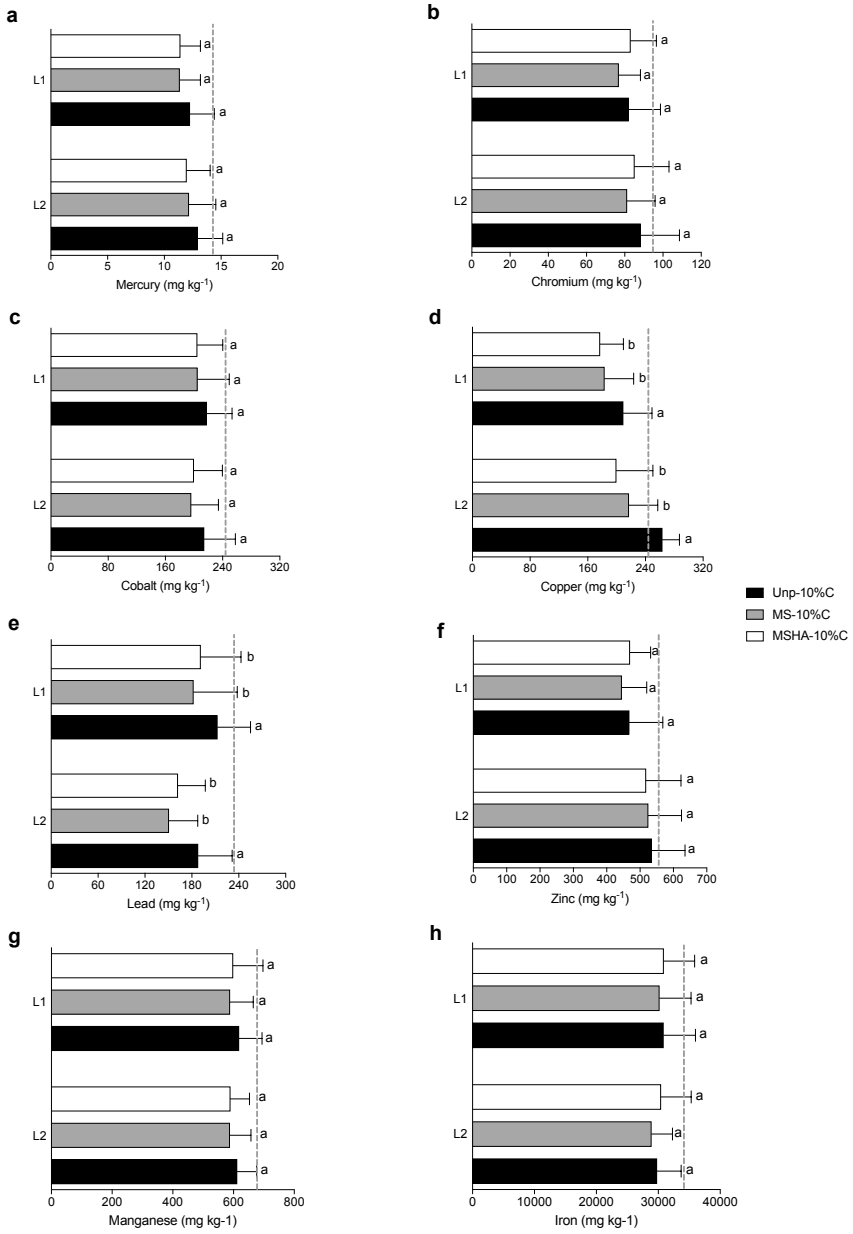

Figure 6.4: Total soil metals: (a) Mercury, (b) Chromium, (c) Cobalt, (d) Copper, (e) Lead, (f) Zinc, (g) Manganese and (h) Iron after five months in the unplanted ecopile (Unp-10%C), the ecopile planted with *M. sativa* (MS-10%C) and the ecopile planted with *M. sativa* and *H. annuus* (MSHA-10%C) in the two soil layers: 0-20 cm (L1) and 20-50 cm (L2). Mean values are represented with standard deviations, n= 18 for each parameter. The different letters stand for statistical significance between the modalities at the 0.05 level with a Tukey's test. Discontinuous line represents the concentration at the beginning of the experiment (Unt-10%C).

Both plant species grew well in the ecopiles without any visible toxicity symptoms (Fig. 6.2). At harvest, alfalfa biomass and water content in shoots and roots were similar in both planted ecopiles (Fig. 6.5). These results agreed with Jones *et al.* (2016) who reported that compost amendment improved sunflower growth in Cu-contaminated soil. After 5 months, the ability of alfalfa and sunflower plants to uptake metals from soil to root and translocate them to shoots was characterized using the BCF and the TF, respectively (Meeinkuirt *et al.* 2012; Novo *et al.* 2013). Metal uptake from soil by alfalfa was low as showed by all BCF values bellow 1 (Fig. 6.6a). This may firstly result from several soil factors including the pH, the content of OM and Mn/Fe (hydr)oxides, which controlled metal reactions (adsorption/desorption, complexation, dissolution, etc.) with soil components. The pH is a crucial parameter of metal sorption. At soil pH 6.5-6.7 (Table 6.2), metals, e.g Co, Pb, are less soluble. Soil OM is also pivotal for controlling metal bioavailability. Some metals, e.g. Fe, Cu, and Pb, form strong organo-metallic complexes and OM content is high (>30%) in the studied soils (Table 6.1). Increase in soil OM after compost amendment can reduce the mobile soil Cu, Pb and Zn fraction (Alvarenga *et al.* 2009). In addition, high total soil Mn (>688 mg kg^{-1} dw) and Fe (>34266 mg kg^{-1} dw) suggest the presence of Mn/Fe (hydr)oxides favoring the sorption to organic molecules. Several metals such as Pb, Cd, and Zn are immobilized with Mn and Fe (hydr)oxides (Doni *et al.* 2015). After five months, soil chemical parameters did not differ between the MS-10%C and Unp-10%C soils, except EC in the soil pore water (Table 6.2). This question about the influence of the vegetation cover in reducing soluble salts.

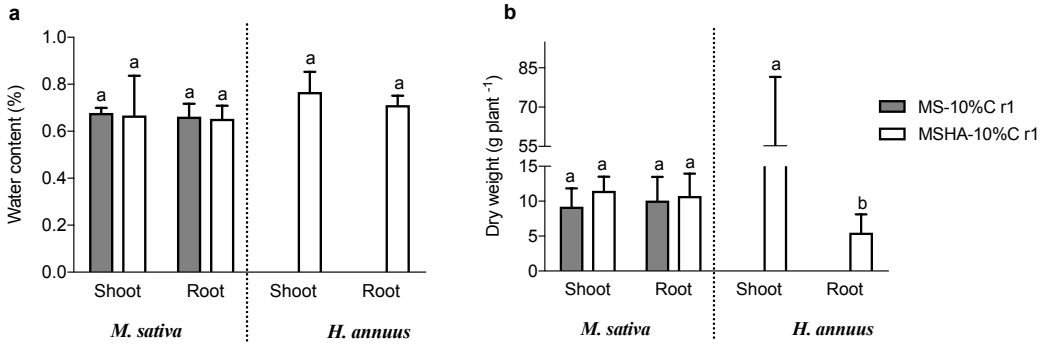

Figure 6.5: Water content (%) and shoot and root DW yields (g $plant^{-1}$) of *M. sativa* and *H. annuus* cultivated on ecopiles planted with *M. sativa* (MS-10%C) and ecopiles planted with *M.*

sativa and *H. annuus* (MSHA-10%C). Mean values are presented with standard deviation (n=6) and the different letters stand for statistical significance between the treatments at the 0.05 level with a Tukey's test (*M. sativa*) and Student-T-test (*H. annuus*).

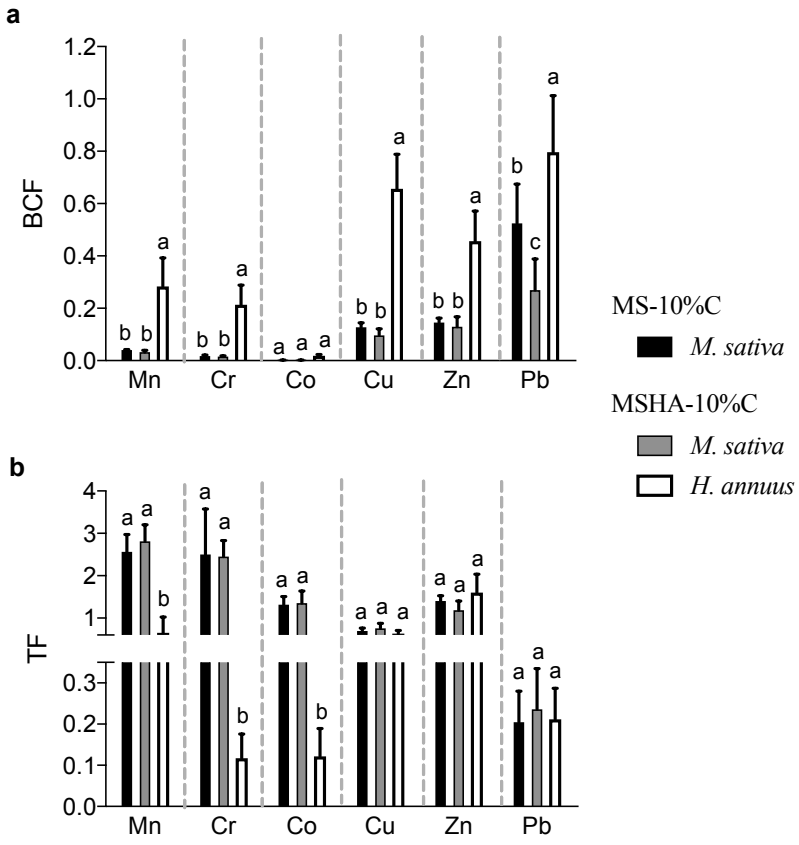

Figure 6.6: (a) Root bioconcentration factor (BCF) and (b) translocation factor (TF) of *M. sativa* and *H. annuus* cultivated on ecopiles planted with *M. sativa* (MS-10%C) and ecopiles planted with *M. sativa* and *H. annuus* (MSHA-10%C). Mean values are presented with standard deviation (n=6) and the different letters stand for statistical significance between the treatments at the 0.05 level with a Tukey's test.

Table 6.2: Chemical parameters of the unplanted Ecopile (Unp-10%C), the Ecopile planted with *M. sativa* (MS-10%C) and the Ecopile planted with *M. sativa* and *H. annuus* (MSHA-10%C) after five-months (n=10 for each parameter except for pH, EC and DO: n=6, mean values are presented with standard deviations). The different letters stand for statistical significance between the modalities at the 0.05 level with a Tukey's test.

	soil	Unp-10%C	MS-10%C	MSHA5%C
		Solid phase		
N	mg kg^{-1}	1673 ± 155.5a	1592 ± 412.2a	1808 ± 431.6a
P		1087 ± 166.3a	1103 ± 149.4a	1113 ± 110.5a
Moisture	%	78.25 ± 3.4a	75.8 ± 1.8a	75.7± 2a
OM		26.8 ± 4.7a	25.1 ± 4.7a	19.64 ± 4.9a
		Soil pore water		
pH (1:1,w/v)		6.5 ± 0.1a	6.7 ± 0.0a	6.6 ± 0.0a
EC	µs cm^{-1}	2385 ± 81.7a	1746 ± 378.8b	1710 ± 147.7b
DO	mg L^{-1}	6.48 ± 0.7a	6.8 ± 0.2a	6.9 ± 0.2a

EC, Electrical conductivity; DO, dissolved oxygen; N, total Kjeldahl nitrogen and P, total phosphorus

Chemical parameters and total metal concentrations of leachates from Ecopiles were analyzed twice, after one and five months (Table 6.3). Parameters such as COD and TOC, which are part of the EU regulations (Directive 91/271/EEC on Urban Wastewater Treatment), are generally used to monitor toxic compounds in water (Aydin *et al.* 2015). The EC, TOC and Cu concentration in leachates were reduced while pH and DO increased in month 5 as compared to month 1 (Table 6.3). These results confirmed findings of Alvarenga *et al.* (2009) who showed a reduction of mobile Cu concentration as pH increased. Here, Mn, Co, Cr and Zn taken up by alfalfa were translocated to shoots as shown by their TF values >1 (Fig.6.6b), in contrast to Cu and Pb. Such high TF values for Mn, Co and Zn are probably due to their micronutrient role at low concentrations for plants growth. Shoot Cr concentration for alfalfa exceeded its common values (Table 6.4) (Brunner *et al.* 2008). This is usually observed in soil after OM amendment (Hattab *et al.* 2014). Lead concentration in alfalfa shoots was above its common values (up to

12.53 mg kg^{-1}; Table 6.4) and higher than values found by Marchand *et al.* (2016a) for the same plant species growing on a soil less contaminated by Pb.

Table 6.3: Chemical parameters and metal concentrations (µg L^{-1}) of leachates from the landfill leachate treatment (WLLT), the unplanted Ecopile (Unp-10%C), the Ecopile planted with *M. sativa* (MS-10%C) and the Ecopile planted with *M. sativa* and *H. annuus* (MSHA-10%C) in month 1 (t1) and month 5 (t5) (n=3 for each parameter, mean values are presented with standard deviations). Asterisk stand for statistical significance between t1 and t5 at the 0.05 level with a Sidak test.

		WLLT*	Unp-10%C	MS-10%C	MSHA5%C	WHO**
	ST[a]	Chemical parameters				
pH	t1	6.4 ± 0.2	6.9 ± 0.05	6.9 ± 0.02	6.7 ± 0.07	
	t5	6.8 ± 0.0*	7.2 ± 0.1*	7.3 ± 0.1*	7 ± 0.1	
DO (mg L^{-1})	t1	10.2 ± 0.6	4.2 ± 0.5	4.3 ± 0.4	3.6 ± 0.2	
	t5	9.4 ± 0.4	8.6 ± 0.6*	9.6 ± 0.2*	9.7 ± 0.4*	
EC (us cm^{-1})	t1	267 ± 5	2903 ± 206	2996 ± 265	2926 ± 250	
	t5	287 ± 26	2270 ± 568*	2175 ± 186*	1787 ± 67*	
TOC (mg L^{-1})	t1	17.2 ± 0.8	152.3 ± 2.5	150.6 ± 2	150.3 ± 1.5	
	t5	17.8 ± 1	129 ± 8.5*	120 ± 5.2*	54.1 ± 1.1*	
COD (mg L^{-1})	t1	24.1 ± 1	411.6 ± 56	345 ± 8,8	424.3 ± 5.6	
	t5	23.7 ± 0.8	360.6 ± 12.2*	121.3 ± 17*	155 ± 4.3*	
		Metals				
Cu	t1	3.2 ± 1.3	43.6 ± 4.9	45.1 ± 1.1	62.7 ± 22.6	2000
	t5	3.8 ± 1.8	32.7 ± 4	27.8 ± 5.5*	28 ± 5.9*	
Mn	t1	0.3 ± 0.0	6.9 ± 4.2	3.4 ±2.5	19.4 ± 7.8	
	t5	0.3 ± 0.0	1.4 ± 0.7	1.8 ±1.6	1.7 ± 2.1*	
Co	t1	0.1 ± 0.0	1.7 ± 0.1	1.8 ± 0.6	1.5 ± 0.7	
	t5	0.1 ± 0.0	1.5 ± 0.2	1.5 ± 0.4	1.2 ± 0.5	
Pb	t1	0.1 ± 0.0	1.4 ± 0.3	1.1 ± 0.2	2.9 ± 0.6	10
	t5	0.1 ± 0.0	1 ± 0.1	0.5 ± 0.1	0.9 ± 0.7*	
Zn	t1	9.6 ± 0.1	30.2 ± 10.6	23.2 ± 7.6	21 ± 2.8	
	t5	6.1 ± 0.0	21.1 ± 4.1	18.1 ± 4.6	21.6 ± 1.8	
Cr	t1	0.2 ± 0.1	2.1 ± 0.5	1.6 ± 1	2.4 ± 0.9	50
	t5	0.2 ± 0.0	1.1 ± 0.2	0.7 ± 0.2	1.1 ± 0.3	
Fe	t1	6.7 ± 2.9	9.1 ± 1.4	7.8 ± 1.9	8.6 ± 2.1	
	t5	6.3 ± 1.9	4.9 ±1.1	7 ± 2	5.6 ± 1.8	

[a]ST, sampling time

*Water from the landfill leachate treatment

**Drinking water criteria (WHO 2011)

Table 6.4: Metal concentration (mg kg^{-1}) in the shoots and roots of *M. sativa* and *H. annuus* grow on the Ecopile planted with *M. sativa* (MS-10%C) and the Ecopile planted with *M. sativa* and *H. annuus* (MSHA-10%C) after five-months (n=12 for each parameter except for the shoots of *M. sativa*: n=4, mean values are presented with standard deviations). The different letters stand for statistical significance between the modalities at the 0.05 level with a Tukey's test (*M. sativa*) and Student-T-test (*H. annuus*).

TE		MS-10%C		MSHA-10%C		CVP[a]
mg kg^{-1}		Shoots	Roots	Shoots	Roots	
Cu	*M. sativa*	11.58 ± 1.62b	17.97 ± 0.51a	11.44 ± 1.49b	16.51 ± 3.47a	3-12
	H. annuus	-	-	72.92 ± 14.41b	120.06 ± 27.07a	
Mn	*M. sativa*	53.07 ± 8.58a	21.91 ±1.02b	55.91 ± 5.59a	19.15 ± 5.34b	20-400
	H. annuus	-	-	88.72 ± 41.43b	186.07 ± 70.24a	
Co	*M. sativa*	0.36 ± 0.06a	0.29 ± 0.03ab	0.31 ± 0.04ab	0.26 ± 0.07b	0.02-0.5
	H. annuus	-	-	0.32 ± 0.13b	3.44 ± 0.92a	
Pb	*M. sativa*	12.53 ± 3.44c	72.50 ± 17.53a	9.56 ± 2.25c	42.18 ± 14.01b	0.1-0.5
	H. annuus	-	-	23.21 ± 8.12b	127.55 ± 29.20a	
Zn	*M. sativa*	76.54 ± 10.57a	58.50 ± 5.38b	69.74 ± 9.67a	65.16 ± 14.47a	20-100
	H. annuus	-	-	327.64 ± 60.02a	234.89 ± 53.23b	
Cr	*M. sativa*	2.88 ± 0.98a	1.30 ± 0.28b	2.76 ± 0.56a	1.25 ± 0.12b	0.1-0.5
	H. annuus	-	-	1.72 ± 0.56b	20.31 ± 7.09a	

[a] Common values of TE in aboveground plant parts (Blum *et al.*, 2012)

Ecopiling using *M. sativa* co-planting with *H. annuus*

After 5 months, PH C10-C40 and PAH-L concentrations did not differ between unplanted ecopiles and the co-planted ones, but PAH (M and H) concentrations were significantly decreased in the co-planted ecopile (Fig. 6.3). The fates of PAH-M and H are mainly related to their sorption or degradation while the dominant lose mechanism for PH C10-C40 and PAH-L is the volatilization (Howard *et al.* 2005; Serrano *et al.* 2006). Whelan *et al.* (2015a) showed that the volatilization is more pronounced in the biopile during the summertime

with the increase of the temperature. Here, the lack of differences in PH C10-C40 and PAH-L concentrations between unplanted ecopiles and the co-planted ones which could be the result of their similar volatilization process. Moreover, alfalfa co-planting with sunflower did not affect PAH (M and H) degradation but was less efficient for PH C10-C40 and PAH-L as compared to *M. sativa* in monoculture (Fig. 6.3cd, ab). This may be related to sunflower canopy which helps to contain easily volatile contaminants by reducing their volatilization. It may be also related to change in conditions or/and microbial community in their shared rhizosphere. As highlighted by (Ashrafi *et al.* 2015), rhizosphere is a competitive environment where roots of different adjacent plants and microorganisms compete for available resources. Under some circumstances, the effect of contaminant on the species' performance can be modified by the presence of a co-occurring species (Koelbener *et al.* 2008). In this study, similar to the monoculture, the results presented in Fig. 6.4 did not show significant reduction of the total Hg, Cr, Co, Zn, Mn and Fe concentrations in soils while total Pb and Cu concentrations were significant decreased after five months using alfalfa and sunflower as compared to the Unt10%C soil (Fig. 6.4a-h). Co-planting is a common agronomic practice to increase productivity and decrease diseases (Wang *et al.* 2013). We expected that the total biomass production by alfalfa would be increased by the use of different niches. The absence of changes in the alfalfa biomass and water content in shoots and roots co-planted treatment compared to alfalfa in monoculture indicated that sunflower did not modify its resource allocation pattern (Fig. 6.5). Shoot metals (Mn, Cu and Zn) removals were significantly higher in co-planting than in monoculture due to the high concentration of these metals in sunflower shoots (Fig. 6.7). Shoot Cu, Pb, Zn and Cr concentrations for sunflower exceeded their common values (Table 6.2) (Brunner *et al.* 2008). After 2 and 3 years of aided phytoextraction, Hattab-Hambli *et al.* (2016) also found that shoot Cu and Cr concentrations for sunflower exceeded their common values. The co-cropping did not alter the metal partitioning in alfalfa as showed by insignificant changes in BCF and TF values, except for Pb as its alfalfa BCF value significantly decreased in the co-planting treatment as compared to that in monoculture (Fig. 6.6). This result is consistent with the shoot and root metal concentrations of alfalfa presented in Table 6.2. Except for Pb, the metal removals in the co-planted treatment were not affected by the planting pattern: shoot and root Pb concentrations of alfalfa were significantly higher in monoculture than in co-planting (Table 6.2). However,

metal removals (Mn, Cr, Co, Cu, Zn and Pb) by each alfalfa plant were similar between mono and co-planting treatments (Fig. 6.7).

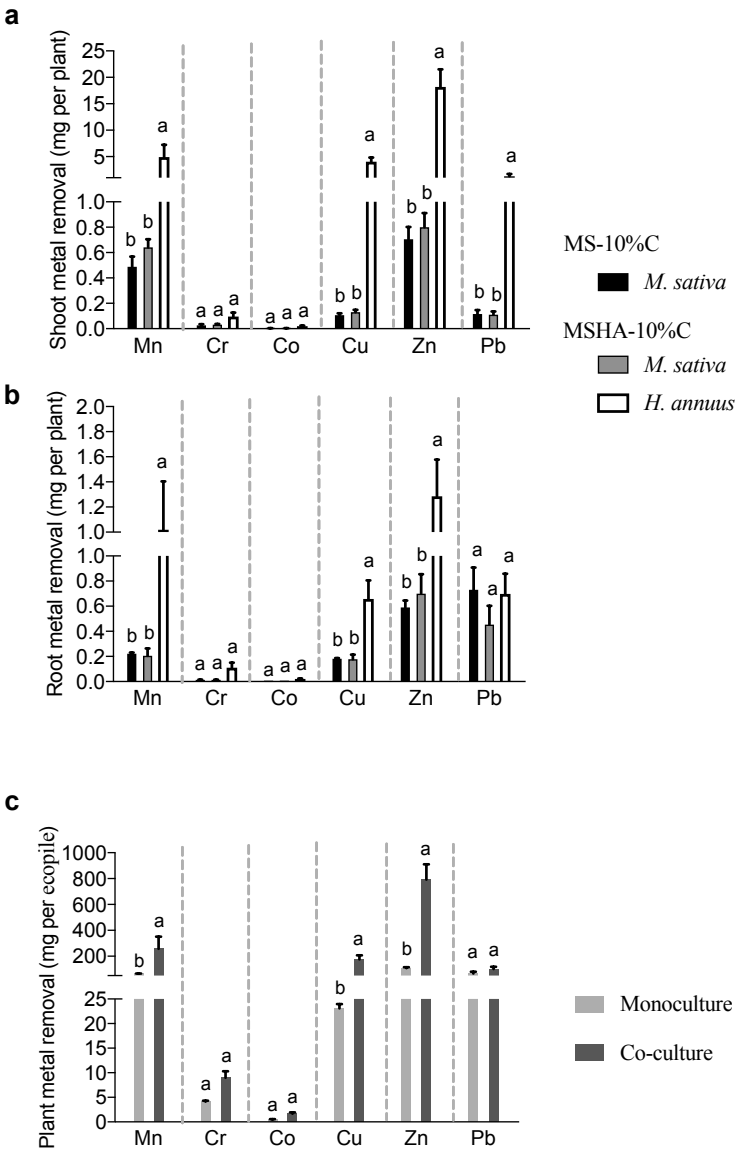

Figure 6.7: (a) Shoot metal removal (mg per plant), (b) root metal removal, and (c) metal removal by the whole plant of *M. sativa* and *H. annuus* cultivated on ecopiles planted with *M. sativa* (MS-10%C) and ecopiles co-planted with *M. sativa* and *H. annuus* (MSHA-10%C). Mean values are presented with standard deviation (n=6) and the different letters stand for statistical significance between the treatments at the 0.05 level with a Tukey's test.

Although intercropping generally increases shoot removal in phytomanagement of contaminated soils, it may also change metal removal by adjacent crops (Koelbener *et al.* 2008; Ashrafi *et al.* 2015). For all the studied metals, sunflower BCF was significantly higher as compared to alfalfa in mono and co-culture (Fig. 6.6a). Both plants showed different ability for metals accumulation, with alfalfa better in both mono and co-culture for the allocation of Mn, Cr and Co to the shoots as showed by the higher TF values of these metals in alfalfa (Fig. 6.6b). Sunflower was more efficient to remove Mn, Cu and Zn from this contaminated soil as showed by the higher shoot metal removals (Fig. 6.7). Alfalfa co-planting with sunflower significantly reduced Mn and Pb percolation (Table 6.4). As previously found, plants and associated microorganisms exhibit both mobilization and immobilization effect on metals in the root zone depending on parameters such as the plant species, metal(loid)s and soil properties (Doni *et al.* 2015; Nowack *et al.* 2010).

Toxicity tests

As organisms differ in sensitivity to various substances and earthworms and plants are pivotal in EU ecosystems, *L. sativum* and *E. fetida* were used for the residual risk assessment after 5 months. Shoot DW yield of *L. sativum* was significantly higher for the monoculture treatment as compared to the untreated and the unplanted ones (Fig. 6.8a). The mixed stand of sunflower and alfalfa reduced the soil phytotoxicity to the highest extent as showed by the similar shoot DW yields of *L. sativum* in both co-planted and uncontaminated standard soils (Fig. 6.8a). Both L1 and L2 soil layers displayed similar shoot and root DW yields for *L. sativum,* but both root biomass was inhibited by all contaminated soils, with and without phytomanagement, relative to uncontaminated soil (Fig. 6.8a,b). Roots are directly exposed to the contaminated soils, which may explain their negative response as compared to shoots. Higher N and P supply (and therefore less need for extension of the root system) as well as high EC in the ecopile soils can contribute to reduce root DW yield of *L. sativum*.

After 7 days and for the L1 soil layer, the mixed stand of alfalfa and sunflower best promoted the earthworm body weight as compared to other ecopile treatments albeit not significantly relative to the monoculture (Fig. 6.9). Unexpectedly, at day 14, the L1 soil ecotoxicity was lowest for the unplanted and the mixed stand-cultivated ecopiles. For the L2

soil layer at day 7, all ecopile treatments similarly decreased its toxicity for the earthworms. At day 14, the toxicity of the L2 soil was lower for the unplanted and monoculture treatments as compared to the untreated soil. As *L. sativum*, toxicity test with earthworms showed that these organisms are relevant bio-indicators of soil toxicity because of their continuous exposure to soil contaminants by roots and dermal contact respectively. After the 5-month trial, the residual soil leachate ecotoxicity based on *L. minor* relative growth rate (RGR) was similar for the monoculture and co-culture during the 21-day growth (Fig. 6.10). The leachate of both the monoculture and the mixed stand did not show a reduction of the toxic effect during the 21-day test as compared the water from the small lake located in Ingelstorp, Smedby (Fig. 6.10). This was consistent with the high COD and metal (Cu, Mn, Co, Pb, Zn, Cr and Fe) concentrations in the leachates (Table 6.4). Yu *et al.* (2014) reported a positive correlation between the toxicity indicators (*Scenedesmus obliquus* and *Vibrio fischeri*) and the COD levels of the wastewater. However, due to the complexity of contaminated soils and leachates, chemical parameters alone are not sufficient to estimate the real risks (Aydin *et al.* 2015).

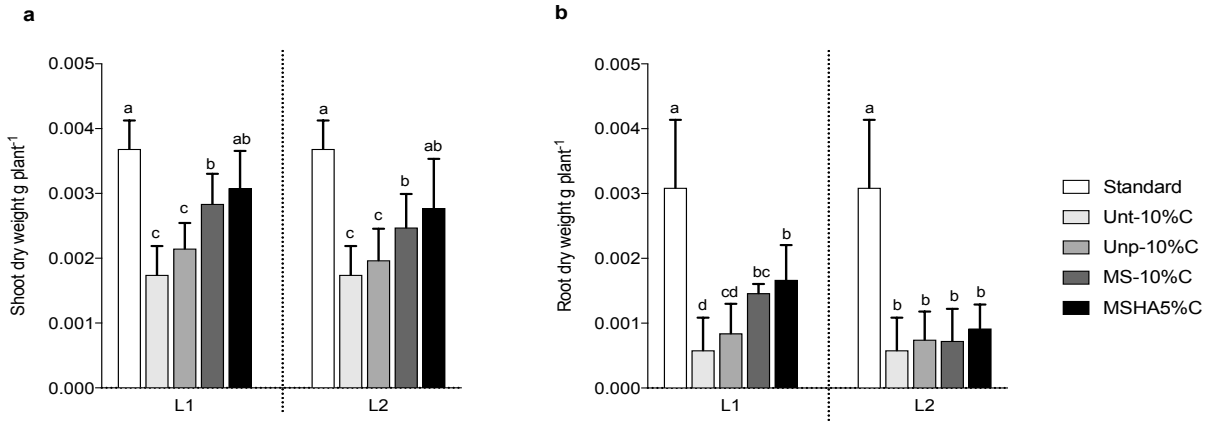

Figure 6.8: (a) Shoot and (b) root DW yields (g plant^{-1} DW) of *Lepidium sativum* cultivated (i.e. 21-day growth period) in a standard uncontaminated soil, the untreated soil (Unt), the unplanted soil (Unp-10%C), the soil planted with *M. sativa* (MS-10%C) and the soil co-planted with *M. sativa* and *H. annuus* (MSHA-10%C) after 5 months of trial: 0-20 cm (L1) and 20-50 cm (L2). Mean values are presented with standard deviation (n=6) and the different letters stand for statistical significance between the treatments at the 0.05 level with a Tukey's test).

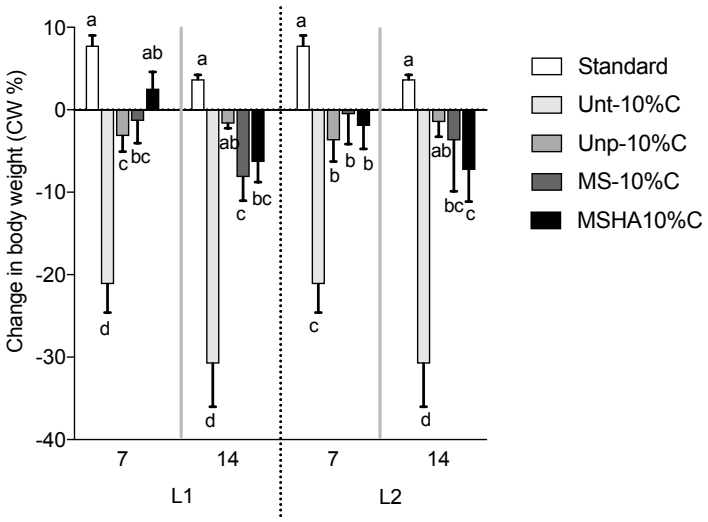

Figure 6.9: Earthworm's change in body weight (CW%) after a 14-day growth period in the standard uncontaminated soil, the untreated soil (Unt), the unplanted soil (Unp-10%C), the soil planted with *M. sativa* (MS-10%C) and the soil planted with *M. sativa* and *H. annuus* (MSHA-10%C), after the 5-month trial, for both soil layers: 0-20 cm (L1) and 20-50 cm (L2). Mean values are presented with standard deviation (n=6) and the different letters stand for statistical significance between the treatments at the 0.05 level with a Tukey's test).

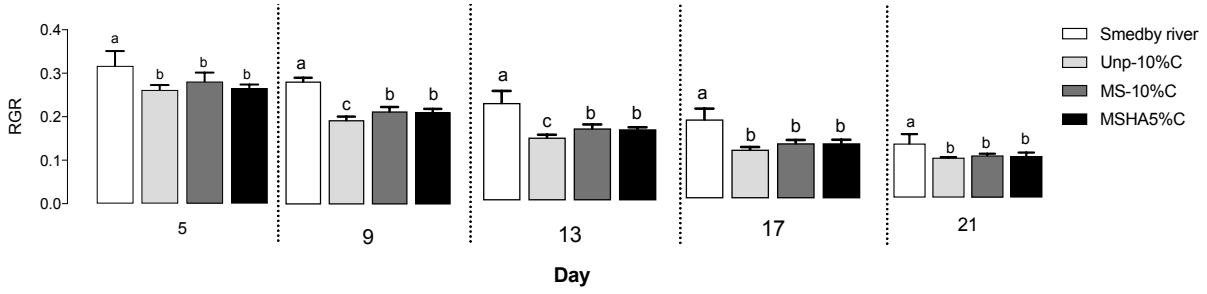

Figure 6.10: Relative growth rate (RGR) after a 5, 9, 13, 17 and 21-day growth period in the water from a small lake located in Ingelstorp, Smedby (Kalmar komun, Sweden), and the leachates from the unplanted soil (Unp-10%C), soil planted with *M. sativa* (MS-10%C) and soil planted with *M. sativa* and *H. annuus* (MSHA-10%C) after the 5-month trial (n=6, mean values are presented with standard deviation and the different letters stand for statistical significance between the treatments at the 0.05 level with a Tukey's test).

Conclusion

This pilot scale experiment was conducted for investigating the performance of ecopile as a remediation option for the phytoextraction of metals and phytodegradation of petroleum hydrocarbons (PHC) in a co-contaminated soil. The selected plant species (*M. sativa* and *H. annuus*) were growing on the ecopile soil, amended with compost, without visible phytotoxicity symptoms. The alfalfa cultivation stimulated the degradation of organic contaminants (e.g. PAH-M, and PAH-H); shoot metal (Pb, Cu, Mn, and Zn) removals were increased in the co-cropping treatment as amounts were higher for sunflower than for alfalfa. Based on *L. sativum* shoot DW yield, after 5 months, phytomanaged soils were less toxic than the unplanted soil, but they remained ecotoxic according to *L. sativum* root DW yield in comparison to the uncontaminated soil. Further investigations will focus on testing the long term ability of ecopiling with alfalfa and sunflower to remediate such PHC and metal-contaminated soil.

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Chapter 7: General highlights of the thesis and conclusion

In my thesis, I focused on understanding and improving of gentle remediation technologies for the reclamation of contaminated soils. To achieve this goal, I integrated the results of soil and microorganism characterization, greenhouse and pilot field scale phytoremediation studies.

7.1 Contaminated soil characterization

In my work, the characterization of soils samples from a former auto scrap yards in which oil spill tank leakage occurred was performed. This first step aimed to understanding the physicochemical and biological properties of soil. It is necessary because GRO involve complex processes and interaction between soil particles, contaminants and biological materials. The investigated soils were characterized in term of physico-chemical properties, total TE and PHC content as well as toxicity assessment. After thirty-two years, the concentration of TE (Co and Pb), PHC C10-C40 (alkanes), medium (M) and high (H) molecular weight PAH in the investigated soils were higher than the threshold limits established by the Swedish EPA and the Canadian Ministry of the Environment. This result showed the potent persistence of these contaminant in soil. This persistence could be also related to the high percentage of OM in soil which favour the sorption reaction. In contrary to previous authors who reported an increase of pH in crude oil contaminated soil, we found an almost neutral pH in the investigated soils. We attributed this to the chemical interaction between PHC, TE and soil elements.

As a consequence of the high amount of contaminant in soil, results regarding the water extract from those contaminated soils showed a high content of COD and TOC. These physicochemical analyses complemented with eco-toxicological analyses revealed a negatives effects of those contaminants on earthworm development and *L. sativum* biomass. Despite the decrease of the bioavailability of contaminant in soil related to the factor such as the aging process or the organic matter content, these results showed that the remediation of this contaminated soil is necessary. In contrary to seed germination, our results showed that plant

growth and earthworm development were suitable to assess the toxicity of PHC and TE due to their sensitivity for those contaminants. Thus, these bioassays were used as a screening tools to ass soil remediation in the next experiments. Moreover, results obtained during the respirometric tests showed an inhibition effect of TE and a stimulatory effect of PHC on soil respiration. This high respiration of microbial community in PHC contaminated soil could be attributed to the mineralization of OM or/and organic contaminants by the viable indigenous microbial community. Thus, both the stimulation and the cultured of these indigenous hydrocarbon degraders have been studied.

7.2 Microorganisms characterization

The cultured of hydrocarbon degraders that can be used *in situ* to enhance the bioremediation of PHC contaminated soil was study is the chapter 4. First, both nutrient-rich (standard media) and impoverished media supplemented with various types and concentration of PHC (selective media) have been used in order to isolated and to identify indigenous microorganisms which have the metabolic capacity to degrade PHC.

Total of 781 bacterial strains belonging to 88 OTUs and 279 fungal strains belonging to 49 OTUs were isolated and sequenced from slightly contaminated (SC), contaminated (C), and highly contaminated (HC) using seven standard and selective culture media per microbial group. Although cultivation allow to isolated a small proportion of microorganisms which even represented rare taxa in CI dataset, these microbial communities could be more efficient for the PHC degradation than the most abundant one. Thus screening assays were performed on 95 bacterial and 160 fungal strains in order to evaluate their petroleum biodegradation efficiency. We looked at how soil contaminant concentration, stain phylogeny and isolation culture media related to PHC bioremediation potential. Results showed that fungal and bacterial isolated from soil that was lightly, moderately and highly contaminated with PHC was equally efficient and the type of culture medium did not impact the biodegradation. But the taxonomy had a strong effect on PHC biodegradation. Three strains: *Trichoderma tomentosum*, *Fusarium oxysporum* and *Rhodococcus sp* were identify due to their high efficiency to degrade PHC. Since my hypothesis that microorganisms indigenous from highly PHC contaminated soil would more

efficiently degrade PHC than those isolated from lightly contaminated soil was reject, I decided to focus more on the the stimulation of indigenous hydrocarbon degraders. This have been done in the greenhouse experiment by supplying limiting nutrients and by improving the physico-chemical properties of the contaminated soil.

7.3 Greenhouse phytoremediation

During a 5-mounth greenhouse trial, the efficiency of *M. sativa* singly and combined with compost have been used for the degradation of organic pollutants and the phytostabilization or extraction of TE. *M. sativa* cultivated in this contaminated soil without compost did not lead to the rhizodegradation of high molecular weight PAH and the removal of soil Co, As, and Pb. In addition to the difficulty related to the use of aged co-contaminated soils and the complexity of these pollutants, soil characteristics such as the high content of organic matter are favor the the sorption to soil particles. However, aided-phytoremediation of this contaminated soil using compost and *M. sativa* promoted Pb removal, PHC degradation, plant growth and survival. We attributed this to the cumulative impact of the nutrient supply, the dilution effect related to compost addition as well as a better phytoextraction and phytodegradation by *M. sativa*, which was more vigorous in the amended soil. Ecotoxicological tests after phytoremediation also showed a positive compost incorporation into contaminated soil. However, the remediation in this study was limited by different factor like the low TF and ECR of TE, molecular weight of PAH and the limiting growing period (5 months). These results bring important information that have been used on pilot field scale experiment.

7.4 Pilot field scale experiment

A pilot field scale experiment was conducted to investigate the application of ecopile as suitable remediation technology for the phytoextraction of heavy metals and phytodegradation of petroleum hydrocarbons (PHC) in co-contaminated soil. Results presented in this thesis were obtained during the two growing seasons: 5 months (Paper V) and 17 months (Paper VI).

After 5 months, The PH C10-C40 and PAH-L concentrations did not differ between unplanted ecopiles and the co-planted ones, but PAH (M and H) concentrations were significantly decreased in the co-planted ecopile. This lack of differences could be the result of their similar volatilization process. Moreover, alfalfa co-planting with sunflower did not affect PAH (M and H) degradation but was less efficient for PH C10-C40 and PAH-L as compared to *M. sativa* in monoculture. In this study, similar to the monoculture, the results did not show significant reduction of the total Hg, Cr, Co, Zn, Mn and Fe concentrations in soils while total Pb and Cu concentrations were significant decreased after five months using alfalfa and sunflower. Moreover, metal uptake from soil by alfalfa was low as showed by the general BCF values <1. Here, Mn, Co, Cr and Zn taken up by alfalfa were translocated to shoots as shown by their TF values >1, in contrast to Cu and Pb. Metal removals (Mn, Cr, Co, Cu, Zn and Pb) by alfalfa were similar between mono and co-planting treatments. Sunflower and alfalfa showed different ability for metals accumulation, with alfalfa better in both mono and co-culture for the allocation of Mn, Cr and Co to the shoots. However, sunflower was more efficient to remove Mn, Cu and Zn from this contaminated soil. After 17 months, PH C10-C40, PAH-L, PAH-M and PAH-H concentrations were reduced in average by 80%, 60%, 50% and 40% in the study treatments as compared to the soil at the beginning of the experiment (Paper VI). This remediation process also allows the removal of 20% of the study metals after 17 Months (Paper VI).

7.5 Conclusion

My thesis focused on the understanding and the improving of gentle remediation technologies for the local treatment of polluted land, without need of transportation of the pollutants though the city. To achieve this goal, plants (alfalfa and sunflower) and their associated microbial communities have been used to remediate aged co-contaminated soils at different scales. The processes involved are complex and imply interactions between soil characteristics, plants, microorganisms and environmental factors. Major results and findings are presented below:

1. The main contaminants found in the studied soils are PHC (alkanes, PAH-M and PAH-H) and TE (Zn, Co, Cr, and Pb). (Papers I-VI) This finding are in agreement with the

most common environmental contaminants found at contaminated sites, in order of abundance.

2. The combined approach using physico-chemical and ecotoxicological analysis allow a better characterization of these contaminated soil (Papers I, IV-VI).
3. Soil microorganism's respiration, earthworm's development and *L. sadivum* growth were highly sensitive biological indicators of soil quality. However, the two last toxicity tests were more adequate to obtain fast answers with low costs. The respirometer needs constant maintenance and frequent calibration (Papers I, III and IV).
4. PHC and TE containing in these soils had a negative effect on *L. sativum* shoot and root dry biomass as well as on earthworm's development (Papers I, IV-VI). These two groups of contaminants have different effect on soil microorganisms: PHC increase the total microbial activities in soils due to the transformation/mineralization of readily organic pollutants while TE inhibit soil microorganism respiration.
5. The nutrient-rich (standard culture media) and impoverished media, supplemented with various hydrocarbons form and concentration (selective culture media) did not influence the ability of microorganism isolates to effectively degrade PHC (Paper III).
6. The long-term exposure of soil microorganisms to high PHC concentration did not also influence the ability of isolates to effectively degrade PHC. However, phylogenetic affiliation had a significant effect on the biodegradation of PHC. In laboratory conditions, *Rhodococcus* sp., *Trichoderma tomentosum,* and *Fusarium oxysporum* effectively enhance the biodegradation of PAH compounds (Paper III).
7. Compost used at the rate of 10-30% (w/w, fresh soil FW) have a positive effect on *M. sativa* growth, survival rate, shoot Pb concentrations and PHC degradation due to the increase of microbial activity (Paper IV-V).
8. The selected plant species (*M. sativa* and *H. annuus*) grew well on the contaminated soil, amended with compost, without visible phytotoxicity symptoms and modification of the resource allocation pattern of one plant by the other ones (Paper IV-VI). The alfalfa cultivation stimulated the degradation of organic contaminants (e.g. alkanes, PAH) and the phytoextraction of Pb and Cu. Co-planting was less efficient than alfalfa in monoculture regarding PH C10-C40 and PAH-L degradation but shoot metal (Pb, Cu, Mn, and Zn) removals were increased in the co-cropping treatment (Paper IV-VI). Both

plants showed different ability for metals accumulation, with alfalfa better for the allocation of Mn, Cr and Co to the shoots and sunflower more efficient to remove Mn, Cu and Zn from contaminated soil.

9. The phytomanaged soils were less toxic than the unplanted soil, but they remained ecotoxic in comparison to the uncontaminated soil. The remediation of these contaminated soils was limited by different factors like the molecular weight of PAH, the metabolic activity of the plant and the limited growing period (5 months in the greenhouse and 5 to 17 months in the pilot field scale).

7.6 Recommendations

Even though phytoremediation is beneficially used for the remediation of co-contaminated soil as in this thesis, this technology is far from being optimize and there are still aspects to be improved and considered:

1. A better attention needs to be addressed to the characterization of the soil before and after the phytoremediation for a better management of the environmental problems.
2. The used of *E. fetida* and *L. sadivum* as a screening tool to assess soil contamination or its remediation are recommended.
3. Compost at 10% of soil (v/v) as source of microbial inoculants can be used as source of microbial inoculants in alternative to the isolation, the cultivation and the characterization of the best performing microorganism to degrade PHC.
4. Since the process of *in situ* aided-phytoremediation of mixed contaminated soils is complex, the investigation of parameters such as different combinations amendment/plant for reducing the pollutant linkages are recommended.
5. Results presented in this thesis were obtained during the two-growing season: 5 months (Paper V) and 17 months (Paper VI) and several more harvests are strongly recommended for the fully remediation of this contaminated soil.

7.7 Future Research

My overall research results can be used as ground information for further studies examining the long-term ability of phytoremediation with alfalfa and sunflower to remediate such PHC and metal-contaminated soil. The results obtained in my thesis brings important information and it documented that biological toxicity tests are important in soil characterization along with physico-chemical analyses. My thesis shed light for further studies on full scale and can be used to scale-up phytoremediation using different plant species. Thus, future researches are need and they should include long-term trials with different combinations of amendment/plant species for a better remediation of contaminated soil. In order to contribute to the sustainability of phytoremediation, a special attention should be addressed to the soft re-use of brownfields by the creation of public green space and the management of the generated biomass (reduced by compaction, composting or thermal treatments for disposal; or used to metals recovery).

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Annexe

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RESEARCH ARTICLE

Culture-Dependent and -Independent Methods Capture Different Microbial Community Fractions in Hydrocarbon-Contaminated Soils

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Abstract

Bioremediation is a cost-effective and sustainable approach for treating polluted soils, but our ability to improve on current bioremediation strategies depends on our ability to isolate microorganisms from these soils. Although culturing is widely used in bioremediation research and applications, it is unknown whether the composition of cultured isolates closely mirrors the indigenous microbial community from contaminated soils. To assess this, we paired culture-independent (454-pyrosequencing of total soil DNA) with culture-dependent (isolation using seven different growth media) techniques to analyse the bacterial and fungal communities from hydrocarbon-contaminated soils. Although bacterial and fungal rarefaction curves were saturated for both methods, only 2.4% and 8.2% of the bacterial and fungal OTUs, respectively, were shared between datasets. Isolated taxa increased the total recovered species richness by only 2% for bacteria and 5% for fungi. Interestingly, none of the bacteria that we isolated were representative of the major bacterial OTUs recovered by 454-pyrosequencing. Isolation of fungi was moderately more effective at capturing the dominant OTUs observed by culture-independent analysis, as 3 of 31 cultured fungal strains ranked among the 20 most abundant fungal OTUs in the 454-pyrosequencing dataset. This study is one of the most comprehensive comparisons of microbial communities from hydrocarbon-contaminated soils using both isolation and high-throughput sequencing methods.

Introduction

Over the past few decades, human activities related to petroleum consumption have led to massive releases of both aliphatic and aromatic hydrocarbons, making these compounds some of the most ubiquitous environmental pollutants on Earth [1–3]. Among these are the polycyclic aromatic hydrocarbons (PAHs), which are of particular concern, given their persistence in the environment (especially in soil) and the potent carcinogenic, mutagenic, and teratogenic effects

that these compounds have on living organisms [4–6]. Many isolated strains of bacteria and fungi can degrade at least some components of hydrocarbon contaminants in culture [7–11], which makes these two major soil microbial groups promising reservoirs of hydrocarbon-degrading activity.

A number of studies have shown that bioremediation, the use of living organisms to decontaminate polluted sites, is likely a feasible solution for treating these contaminants [11–13]. One approach to enhancing hydrocarbon bioremediation is the stimulation of indigenous hydrocarbon degraders, by supplying limiting nutrients, oxygen, and/or improving the physicochemical conditions of the polluted soil [13–15]. Alternatively, cultured hydrocarbon degraders can be used to degrade contaminants *ex situ*, or spiked into contaminated soils *in situ*, a process known as bioaugmentation [16–18]. Regardless of the approach taken, effective bioremediation hinges on our ability to study microbes that are indigenous to polluted sites. Culture-independent and-dependent methods for microbial community analysis have both been used frequently to describe microorganisms from hydrocarbon-contaminated environments.

For culture-independent analysis, high-throughput sequencing techniques [19,20] have revolutionized our view of microbial ecology, revealing hyperdiverse communities that are extremely responsive to hydrocarbon contaminants across a variety of environments [21–25]. Although culture-dependent methods generally recover a small portion of the diversity from soil environments, they are still a critical component of bioremediation development and research [26–30]. In addition to the potential *in situ* and *ex situ* applications of cultured isolates, microbial isolation allows *in vitro* assessments of isolate physiology and hydrocarbon degradation pathways and performance, providing a basis for annotating extensive metagenomic datasets, and helping to identify genes and/or organisms that could be useful in land reclamation.

Hydrocarbon-contaminated soils may be more amenable to comprehensive culture-dependent sampling than other soil environments, since hydrocarbon contamination often leads to a decline in microbial diversity [22,24,31,32], meaning that a lower sampling effort may be required to isolate a representative proportion of the active community. Hydrocarbon contaminants may also suppress certain sensitive groups [33] and tend to select primarily for subgroups of the *Actinobacteria* and *Proteobacteria* in affected soils [22,23]. Although these phyla are extremely diverse, they are some of the best represented among cultured isolates [34,35]. However, although the gap between culture-independent and culture-dependent analyses of soil microbial communities is often mentioned, few studies [36–38] have directly compared these approaches, and none has specifically investigated the biases associated with culturing both bacteria and fungi in a bioremediation context, despite the critical role of culturing in this field.

In this study, we used both nutrient-rich and impoverished media, supplemented with various types and concentrations of petroleum hydrocarbons, in order to assess the effectiveness of culture-based methods at recovering indigenous microorganisms from hydrocarbon-contaminated soils. We paired 454-pyrosequencing of bacterial 16S rDNA and the fungal ITS region with extensive culturing of bacteria and fungi, using sediment samples harvested from a basin that is highly contaminated with hydrocarbons, at the site of a former petrochemical plant. Since different substrates select for different groups of bacteria [39] and fungi [40], bacterial and fungal strains were isolated using seven different culture media to enhance the number of potential isolates. Although there are many approaches for cultivating soil bacteria and fungi [41], we chose to use basic nutrient-rich and impoverished agar plates, since these are still the most widely applied culturing method in microbiology. While we expected much lower richness in the cultured dataset, it was interesting to observe that current culturing methods do not capture most of the dominant microorganisms found in hydrocarbon-contaminated soils

through 454-pyrosequencing. This was more apparent among the bacterial isolates than the fungal isolates. Even more surprisingly, a number of the cultured microorganisms were not identified at all in the 454-pyrosequencing data.

Materials and Methods

Ethics statement

No specific permits were required for the described field study. The land on which we conducted the phytoremediation field is privately owned by ConocoPhillips. ConocoPhillips gave permission for the study to be conducted on their land. This field study did not involve endangered or protected species.

Experimental design and sampling

Sampling occurred at the site of a former petrochemical plant at Varennes, on the south shore of the St-Lawrence River near Montreal, Quebec, Canada (45°41'56"N, 73°25'43"W). The sampling site is contaminated by a variety of industrial waste products related to petrochemical processing that have been released over the last forty years. The site was permanently closed in 2008, and since 2010, it has been used extensively to study the potential of willow cultivars for the phytoremediation of hydrocarbons (for details see www.genorem.ca and [23,42]). Five plots of 300 m^2 each were set up within a contaminated area of approximately 2500 m^2. Each plot was subdivided into 12 sub-plots of 25 m^2 each. In June 2011, two soil samples were collected at depths of 25 cm and 50 cm in each of the 12 sub-plots within each plot. The 24 soil samples from each plot were then pooled to obtain representative composite soil samples. Soil samples were chilled at 4°C during transport from the field to the lab, and were stored at -20°C until isolation and DNA extraction. A portion of each composite soil sample was analyzed for F1-F4 hydrocarbons (sum of all aromatic and aliphatic hydrocarbon compounds with chain lengths of C10-C50) by Maxxam Analytics (Montreal, Quebec, Canada) on June 2011, according to the protocol set forth by The Canadian Council of Ministers of the Environment. Results from hydrocarbon analyses (S1 Table) revealed an increasing contamination gradient from plots 1 through 5, which led us to classify the plots into three discrete contaminant levels: slightly contaminated (plots SC1, SC2), contaminated (plot C3), and highly contaminated (plots HC4, HC5).

Culture-dependent (CD) sample processing

For each composite soil sample, one gram of soil was suspended in 9 ml of sterile distilled water and vortexed thoroughly. From this stock solution, serial dilutions were performed to 10^{-7}. Aliquots of 100 µl from dilutions of 10^{-3} and 10^{-4} for fungi, and of 10^{-6} and 10^{-7} for bacteria, were plated in duplicate on each of the seven culture media. Bacteria were isolated on tryptic soy agar (TSA, containing 30 g / L of tryptic soy broth (TSB)) and impoverished TSA plates (1 to 15 g /L of TSB) containing various concentrations of diesel engine oil or crude oil, or that had been coated with crude oil (Table 1). Fungal strains were isolated on potato dextrose agar (PDA, containing 24 g/L of potato dextrose broth (PDB)) and impoverished PDA plates (1 to 12 g/L of PDB) containing various concentrations of diesel engine oil or crude oil, or that had been coated with crude oil (Table 2). Petri dishes were inoculated, inverted, and incubated at 27°C for bacteria and 25°C for fungi. Bacterial colonies were checked every 48 hours for six days, and each new morphotype was subcultured on TSA for 48 hours. Fungal colonies were checked every 5 days for two weeks, and were then subcultured on PDA for three weeks. Bacterial and fungal subcultures were stored at 4°C until DNA isolation.

Table 1. Composition of the standard and selective media (based on one litre of medium) used to isolate soil bacteria. Each medium contained 100 mg / L of cycloheximide.

Medium	Agar (g)	TSB (g)	Micro-element [d] (ml)	Macro-element [e] (ml)	Diesel (ml)	Oil (ml)	Crude Oil (ml)	Acetone (ml)
1.5% TSA—1%OD [a]	15	15			5	5		5
0.1% TSA—COC [b]	15	1	1	10			0.2	0.1
0.1% TSA—COM [c]	15	1	1	10			20	10
0.1% TSA—1%OD	15	1	1	10	5	5		5
0.1% TSA—5%OD	15	1	1	10	25	25		25
0.1% TSA—10%OD	15	1	1	10	50	50		50

[a] Oil–Diesel engine oil (Rotella diesel engine oil, Shell, Montreal, QC).

[b] Crude Oil was Coated onto the medium after solidification (from Gulf of Mexico, provided by Montreal pipeline).

[c] Crude Oil was mixed with the growth medium (Montreal pipeline).

[d] $MgSO_4$ (739 mg / L), KNO_3 (76 mg / L), KCl (65 mg / L), KH_2PO_4 (4.1 mg / L).

[e] $MnSO_4*7H_2O$ (6 mg / L), $ZnSO_4*7H_2O$ (2.65 mg / L), H_3BO_3 (1.5 mg / L), $CuSO_4$ (0.13 mg / L), $Na_2MoO_4*2H_2O$ (0.002 mg / L), KI (0.75 mg / L).

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DNA isolation, amplification, and sequencing of microbial isolates

Bacterial strains were picked with a 1 μl sterile inoculation loop (Sarstedt, Montreal, Canada) and spiked directly into a PCR master mix (described below). Fungal strains were subcultured for one week in PDB before we harvested fresh mycelium for the isolation of genomic DNA (gDNA). All gDNA isolations were performed using a Freedom EVO100 extraction robot (Tecan Group, Mannedorf, Switzerland) with the NucleoMag 96 Plant kit (Macherey Nagel, Oesingen, Switzerland) according to the manufacturer's instructions. Bacterial 16S rDNA and fungal ITS sequences were amplified using the 27F [43] / 1492R [44], and the ITS1-F [45] / ITS4 [46] primer sets, respectively. PCR mixtures were made up of 1× PCR buffer, 4% DMSO, 0.5 mg BSA, 0.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.5 μM of each primer, one unit of DreamTaq DNA polymerase (Fermentas, Canada) and one μl of gDNA (or picked colony) in a total volume of 50 μl. Thermal cycling conditions for bacteria were as follows: initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final elongation at 72°C for 10 min. Thermal cycling conditions for fungi were as follows: initial denaturation at 94°C for 2 min; 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and a final elongation at 72°C for 5 min. PCR reactions were performed using an Eppendorf Mastercycler ProS (Eppendorf, Mississauga, ON), and products were visualized on GelRed-stained 1.5% agarose gels using the Gel-Doc system (Bio-Rad Laboratories, Mississauga, ON). DNA sequencing was performed on an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems, Carlsbad, CA) at the McGill University and Genome Quebec

Table 2. Composition of the standard and selective media (based on one liter of medium) used to isolate soil fungi.

Medium	Agar (g)	PDB (g)	Micro-element (ml)	Macro-element (ml)	Diesel (ml)	Oil (ml)	Crude Oil (ml)	Acetone (ml)
1.2% PDA—1%OD	15	12			5	5		5
0.1% PDA—COC	15	1	1	10			0.2	0.1
0.1% PDA—COM	15	1	1	10			20	10
0.1% PDA—1%OD	15	1	1	10	5	5		5
0.1% PDA—5%OD	15	1	1	10	25	25		25
0.1% PDA—10%OD	15	1	1	10	50	50		50

Each medium contained 200 mg / L of streptomycin, and 100 mg / L of ampicillin.

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Innovation Centre (Montreal, QC). Sanger sequencing data are deposited in GenBank under the accession numbers KP177318—KP177405 and KP177406—KP177454 for bacteria and fungi, respectively.

Culture-independent (CI) sample processing

Total soil DNA was isolated from one gram of each composite soil sample using the MoBio PowerSoil DNA Isolation Kit following the manufacturer's instructions. For each sample, triplicate PCR reactions of partial 16S rDNA and fungal ITS amplicons were performed using barcoded primers with the required 454 adapter sequences (S2A and S2B Table). Thermal cycling conditions for reactions using 16S rDNA barcoded primers were as follows: initial denaturation at 95°C for 5 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final elongation at 72°C for 7 min. Thermal cycling conditions for reactions using ITS barcoded primers were as follows: initial denaturation at 94°C for 4 min; 30 cycles at 94°C for 30 s, 50°C for 60 s, and 72°C for 90 s; and a final elongation at 72°C for 10 min. PCR reactions were performed on an Eppendorf Mastercycler ProS (Eppendorf, Mississauga, ON). PCR triplicates were combined and quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Life Technologies). 16S rDNA and ITS pools from each of the five composite samples were then combined in equimolar ratios. High-throughput sequencing was performed on the 454 GS FLX+ platform using Lib-L chemistry (Roche, Branford, CT, USA). The 16S rDNA and ITS pools were each sequenced using ¼ of a plate. The 454-pyrosequencing data generated in this study have been deposited in the NCBI Sequence Read Archive and are available under the BioSample accession numbers SAMN03199986 and SAMN03199987.

Bioinformatic analyses

Quality processing (read quality trimming, chimera check) of partial 16S rDNA and ITS1 sequences was performed in Mothur v.1.33.2 following the protocol used in Schloss et al. [47] and accessed online (http://www.mothur.org/wiki/454_SOP) in September 2014. Sequences obtained from Sanger sequencing were edited, cleaned, and assembled in Geneious Pro v.6.1.5 (Biomatters). For both 454 sequences and Sanger sequences, clustering analyses were performed in Geneious, and OTUs were defined at 97% similarity. For 454-pyrosequencing datasets, singletons were not considered, and doubletons with a nucleotide similarity <100% were excluded. OTU rarefaction curves, Venn diagrams, taxonomic identifications, relative abundance analyses, and richness and diversity indices were performed in Mothur. Taxonomic identifications for bacterial and fungal sequences were performed in Mothur using the SILVA [48] and UNITE [49] databases, respectively. Prior to performing community comparisons based on the 454-pyrosequencing datasets, each library was randomly subsampled to the minimum number of sequences observed per composite soil sample. Heatmaps and Venn diagrams were created using the R statistical language v.3.0.0 [50] with the packages gplots [51] and VennDiagram [52]. Krona charts were calculated using the KronaTools available from http://krona.sourceforge.net [53]. To partition the datasets between abundant and rare OTUs, the number of reads observed for each OTU was divided by the number of reads counted for the most abundant OTU. Rare OTUs were defined as those with a read proportion of < 5% (S1 Fig).

Results

After quality trimming, OTU filtering, and standardizing the number of sequences per sample, 22,422 16S rDNA sequences and 10,098 ITS sequences were recovered from bacterial and fungal CI datasets, respectively (Table 3). OTU richness reached saturation at each level of

Table 3. Bacterial and fungal OTU richness and diversity recovered in each library.

Method [a]	Level of contamination [b]	No. sequences	Coverage [c] (%)	Nb. OTUs	D [d]	H [e]	PD [f]
CI-Bacteria	SC	9082	98.7	1342	658.4	6.8	57.9
	C	4519	94.1	1030	385.9	6.4	47.3
	HC	8821	98.6	887	135.1	5.9	47.2
CI-Fungi	SC	4017	99.5	235	35.1	4.3	32.1
	C	2033	98.5	144	16.5	3.6	19.4
	HC	4048	99.3	153	17.1	3.5	26.5
CD-Bacteria	SC	297	91.2	56	16.8	3.3	2.46
	C	172	93.6	30	9.0	2.7	1.36
	HC	312	98.7	36	13.0	3.0	1.47
CD-Fungi	SC	223	94.2	43	19.8	3.2	5.42
	C	27	59.2	16	15.2	2.5	2.35
	HC	29	89.6	8	4.3	1.6	1.12

[a] CI: culture-independent (454-pyrosequencing); CD: culture-dependent (isolation).

[b] SC: slightly contaminated; C: contaminated; HC: highly contaminated (see S1 Table).

[c] Good's coverage.

[d] Simpson's inverse index of diversity.

[e] Shannon index.

[f] Faith's phylogenetic diversity.

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contamination (S2A and S2B Fig), with the exception of plot 3 in the fungal CD dataset (coverage = 59.2%, Table 3). Using a similarity threshold of 97% to cluster sequences within the same OTU, a total of 2047 and 360 OTUs were recovered from bacterial and fungal CI datasets, respectively. For the culture-dependent (CD) datasets, 781 bacterial strains and 279 fungal strains were isolated and sequenced from the five plots, using the seven different culture media per microbial group. A total of 88 bacterial and 49 fungal OTUs were defined based on analysis of the 16S rDNA and ITS sequences, respectively.

Bacterial and fungal communities recovered by CI and CD methods

The CI and CD methods provided two extremely divergent views of the microbial communities recorded in the five plots (Fig 1). The proportion of shared OTUs between CD and CI datasets was only 2.4% (51 OTUs) for bacteria and 8.2% (31 OTUs) for fungi (Fig 1A and 1B). This did, however, represent 58% and 63% of the bacterial and fungal strains isolated *in vitro*. Based on Bray-Curtis dissimilarity, bacterial and fungal communities from each contaminant level clustered first according to sampling method (due to the large difference in species richness between CI and CD datasets), and secondarily according to the level of hydrocarbon-contamination (Fig 1C and 1D), with the single exception of the CI HC fungal community which segregated independently from the other fungal assemblages. The heatmaps clearly show that the most abundant bacterial and fungal OTUs recovered via culturing do not correspond with those recovered by 454-pyrosequencing. *Proteobacteria* was the dominant bacterial phylum, representing 61% of the 16S rDNA reads from the 197 most abundant bacterial OTUs (Fig 2A). *Rhodocyclales* (15%), *Burkholderiales* (10%), *Actinomycetales* (10%), *Rhizobiales* (8%), *Xanthomonadales* (7%), and *Sphingomonadales* (5%) were the most frequently observed bacterial orders in the CI sequences (Fig 2A). Among the 51 bacterial OTUs shared between CI and CD methods (Fig 2B), OTU7 (*Arthrobacter* sp.) was the most frequently isolated, but represented the 61st most abundant OTU out of 2047 in the CI dataset. Most of the shared OTUs

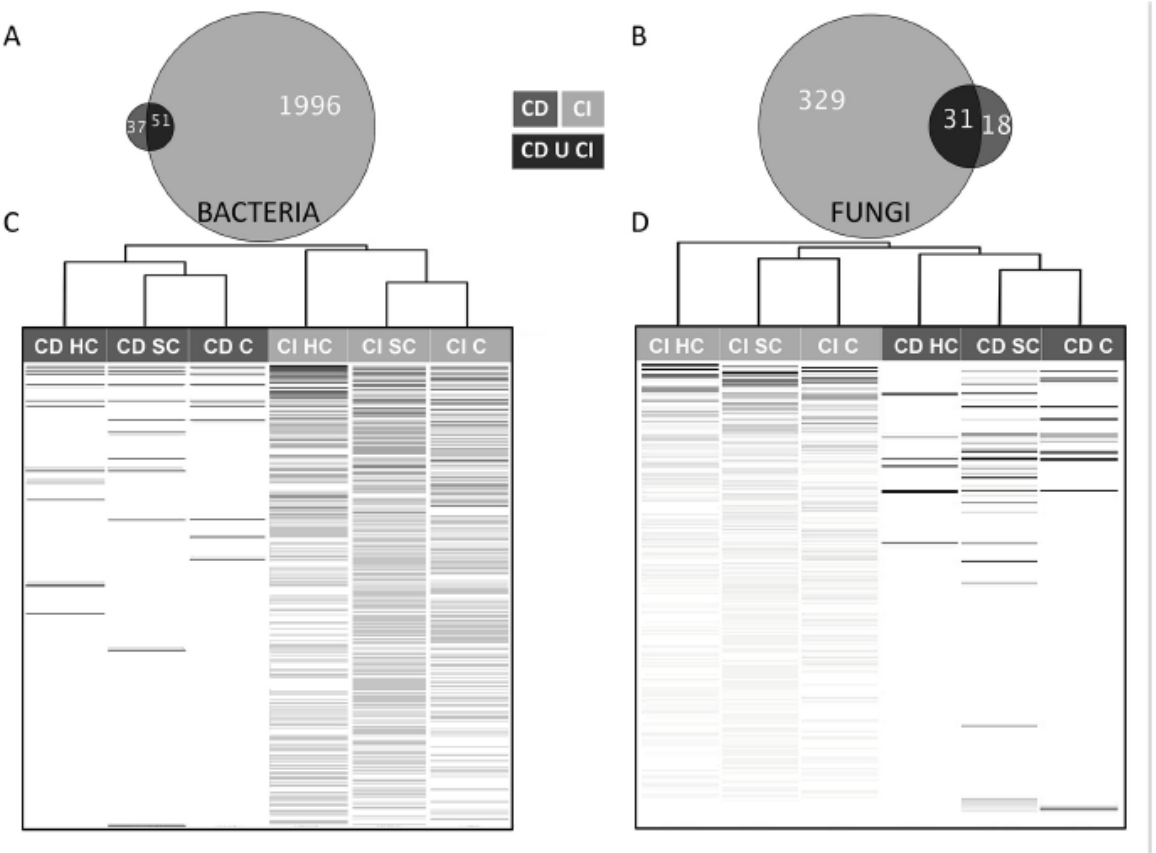

Fig 1. Proportional Venn diagram showing the distribution of bacterial (A) and fungal (B) OTUs between culture-independent (CI, light grey) and culture-dependent (CD, dark grey) methods. Heatmap distribution of the relative abundance of bacterial (C) and fungal (D) OTUs recorded with CI (light grey) and CD (dark grey) methods. OTUs are in rows and colour intensity indicates relative abundance, with black indicating the highest relative abundances observed for slightly contaminated (SC), contaminated (C) and highly contaminated (HC) composite soil sediments recovered with CI and CD methods. OTUs are presented by descending number of reads from top to bottom, based on proportion of total reads recorded in CI and CD datasets. The top dendrogram shows the hierarchical clustering of bacterial and fungal communities recovered for each PAH contamination level based on Bray-Curtis dissimilarity.

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belong to the genera *Sphingobium* and *Sphingomonas* (*Sphingomonodales*), *Pseudomonas* (*Pseudomonadales*) and *Arthrobacter* (*Actinomycetales*). *Sphingomonodales* and *Actinomycetales* were the most abundant orders identified within the shared OTUs, with relative abundances of 33% and 30% respectively, while accounting for 5% and 10% of reads in the CI dataset.

With respect to the fungal communities observed in the CI dataset, *Ascomycota* was the dominant phylum, representing 65% of all reads (Fig 3A), while no *Glomeromycota* sequence was recovered in the CI dataset. At the order level, *Agaricales* (13%), *Saccharomycetales* (12%), *Hypocreales* (10%), *Pleosporales* (9%), *Sordariales* (8%) and *Spizellomycetales* (6%) dominated the fungal community. Eighteen percent of the reads could not be identified. In contrast to the

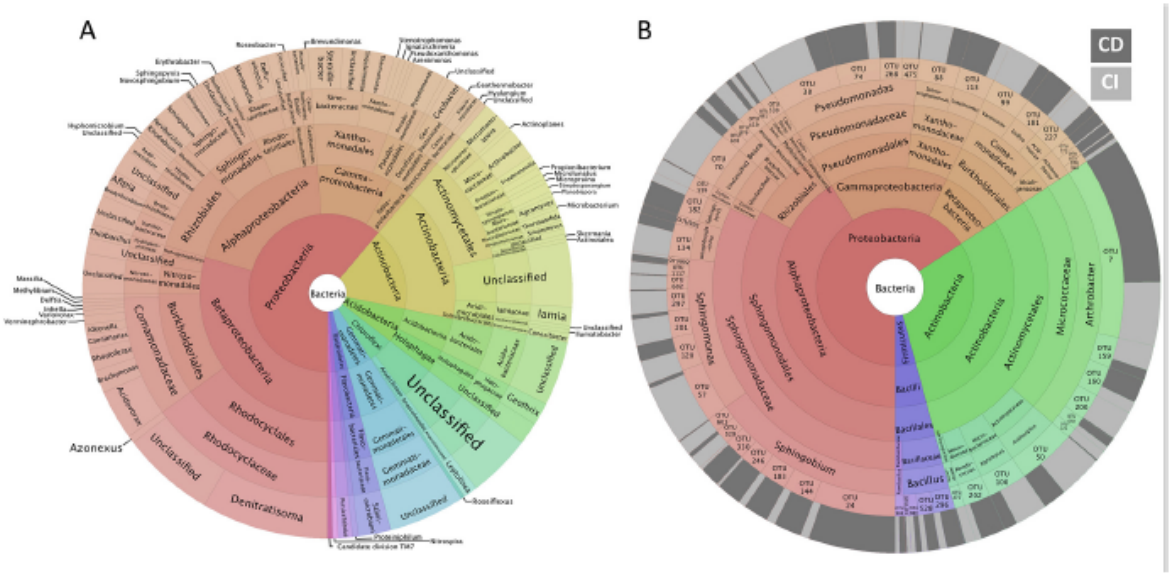

Fig 2. Krona charts showing the taxonomic identification and relative abundance of the most abundant bacterial OTUs recorded in CI datasets (A) and shared OTUs between CI and CD datasets (B). The proportion of sequences from the CI (light grey) or CD (dark grey) datasets for each shared OTU is shown in the outer ring.

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bacterial datasets, some fungal isolates ranked among the 20 most abundant OTUs in the CI dataset (Fig 3B). For example, OTU7, OTU13, and OTU15 were ranked seventh, thirteenth, and sixteenth in the CI dataset, respectively, based on read abundance. Only two isolated strains (OTU 75 and 87) were more frequently observed in the CD dataset than in the CI dataset (Fig 3B). A large proportion of the shared OTUs could not be identified (39% of all shared sequences), while *Hypocreales* (40%) and *Pleosporales* (10%) were the two prevalent fungal orders in the CD dataset. While some members of the *Basidiomycota* were recorded in the CI dataset, none were isolated.

Interestingly, some of the isolated bacterial and fungal strains could not be detected by 454-pyrosequencing (S3A and S3B Fig), despite the fact that the number of reads from the CI analysis was more than an order of magnitude higher than the number of sequences in CD datasets, and the rarefaction curves were saturated. *Sphingobium* represented 24% of the bacterial taxa that were found only in the CD dataset, followed by *Staphylococcus* (14%) and *Arthrobacter* (8%) (S3A Fig). For fungi, OTU81 and OTU108 (*Trichoderma*), and OTU97 and OTU118 (*Paraphoma*) represented 66% of the isolated strains that were not detected by 454-pyrosequencing (S3B Fig). The majority of non-shared bacterial and fungal OTUs were isolated on media containing oil-derived hydrocarbons (data not shown).

Discussion

Culturing is essential to both the study and application of microbially-mediated bioremediation, yet the proportion and identity of the microbial community in hydrocarbon-contaminated soils that is culturable has remained unclear. Our results show that not only does culturing select a small proportion of the microorganisms that can be observed through CI methods, a

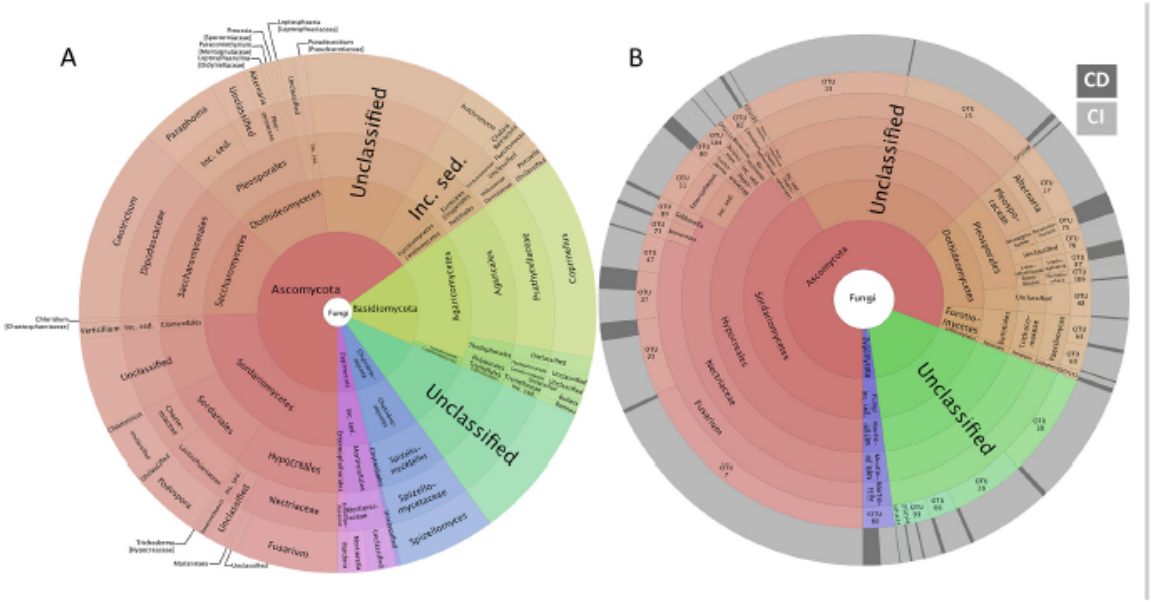

Fig 3. Krona charts showing the taxonomic identification and relative abundance of the most abundant fungal OTUs recorded in CI datasets (A) and shared OTUs between CI and CD datasets (B). The proportion of sequences from the CI (light grey) or CD (dark grey) datasets for each shared OTU is shown in the outer ring.

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fact that has been long known, but that many of the most abundant microorganisms detected using CI methods are not captured with plate-culturing techniques. This suggests that the microbes that are selected by culturing are not even representative of those that are highly competitive in the environment.

Culture-independent versus culture-dependent methods

As shown by saturated rarefaction curves, the sampling effort for both CI and CD methods was adequate for capturing most of the abundant OTUs that could be identified with these methods in the five composite sediment samples. This allows us to draw robust comparisons between these datasets. Previous studies that have compared bacterial or fungal diversity using CI and CD approaches concluded that these methods were complementary [36,38,54–57]. In this study, the number of taxa that were unique to the CD dataset was limited, despite the use of seven different culture media for isolation. Taxa identified from isolation alone increased total species richness by only 2% for bacteria and 5% for fungi, meaning that 454-pyrosequencing captured 95% or more of the microbial diversity that could be obtained using these methods, and with far less effort. The OTUs that were only identified by culturing likely represent rare taxa that could not be observed at the sequencing depth used in this study. Extremely rare taxa may in fact represent a large proportion of the OTUs in natural microbial communities [58]. The presence of hydrocarbons in six out of the seven isolation media could explain the limited number of OTUs that we recovered through isolation, since hydrocarbon contaminants can negatively affect soil bacterial and fungal diversity [22,23,59]. Alternative culturing methods, such as the use of rhizosphere isolation medium [37], micro-cultivation techniques [40,60] and

dilute nutrient media [25,61,62], have isolated a larger number of microorganisms, but the overlap with CI community analysis has been limited in all cases, and many OTUs that are highly abundant in CI datasets cannot be isolated. For instance Shade et al. [37] performed 454-pyrosequencing on total cultivated soil bacteria without colony picking, and reported that only 9.1% of bacterial OTUs were shared between CD and CI datasets, while each dataset contained 37,645 and 46,347 reads, representing a total of 4957 OTUs. As in our study, the authors demonstrated that many of the shared OTUs represented rare taxa in the CI dataset. Lagier et al. [36] investigated human gut bacteria by microbial culturomics (212 different culture conditions, yielding 32,500 colonies) and metagenomics, and in this case, only 5.1% of total OTUs were shared between sampling approaches. Furthermore, the authors identified 174 novel bacterial species in the CD dataset, demonstrating that metagenomics can be an inefficient method for detecting extremely rare bacteria.

Since many of the bacterial and fungal taxa that we isolated are not representative of the most abundant taxa *in situ*, their potential as effective *in situ* inoculants is questionable, even if some of these isolates demonstrated a high capacity for hydrocarbon degradation (unpublished data). Intermicrobial competition appears to play an important role in shaping microbial activity and abundance [63–65], and the persistence and activity of bioaugmented strains depends fundamentally on their ability to compete with indigenous microorganisms [66]. If strains isolated through culturing are only transient contributors to microbial community function, or exist permanently within the rare biosphere, they are unlikely to dominate when reinoculated, even if their abundance is maximized in the inoculum. This may be a major contributing factor to the poor efficiency of many prior attempts at bioaugmentation [66,67]. Spatial and temporal monitoring of microbial abundance in contaminated soils using CI methods appears to be a fundamental step towards determining the factors that govern the abundance of microorganisms *in situ*, as well as for selecting strains that can be successful across varied environments. However, if members of the rare biosphere are more efficient at hydrocarbon degradation than microbes that are naturally abundant, it may be desirable to develop vacant niches that allow these organisms to persist. This may be possible through the addition of plant hosts, for example [68].

Comparing fungal and bacterial isolation from hydrocarbon-contaminated soils

A greater proportion of fungal OTUs identified via CI analysis were isolated from the hydrocarbon-contaminated sediments when compared with isolation of bacteria. This is likely due in part to the lower richness of fungi than bacteria in soil. While roughly 1000 fungal OTUs were identified in 4 grams of forest soil [69], between 6000–50,000 bacterial species can potentially be recovered from a single gram of soil, depending on where the "species" cutoff is drawn [70]. In addition, fungi and bacteria may be differentially sensitive to small changes in environmental conditions. Fungal propagules, such as hyphae and spores, are generally much larger than bacteria. As a result, certain soil microenvironments that are relevant to bacterial distribution may not affect fungi, as these organisms may develop niches at different spatial scales. Fungi have also been shown to be much less sensitive to pH variation than bacteria, which might allow cultivation of a wider range of fungi than bacteria using media at only one pH level [71]. On the other hand, fungi have been shown to be more sensitive to high hydrocarbon concentrations and were more strongly influenced by the presence of plants than bacteria in this study [23].

Still, only a fraction of known fungal species can be cultured (ranging from 5 to 17% according to [72–74]), while most species that belong to the phylum *Basidiomycota* cannot yet be

isolated or grown under *in vitro* conditions. Taxa within the *Glomeromycota* cannot be cultivated at all without host plant roots. In our CI dataset, two taxa from the *Agaricomycetes* (*Basidiomycota*) ranked first and second in terms of read abundance, but were not captured by culturing efforts. These two OTUs have non-conspecific matches with ITS sequences of *Coprinellus*, a genus in the family *Psathyrellaceae*. Their absence in the CD dataset is somewhat surprising, since species of *Coprinellus* and *Coprinopsis* have been grown in Petri dishes [75]. Suhara et al. [75] reported near-complete degradation of 1 μmol of polychlorinated dibenzo-p-dioxin by *Coprinellus disseminatus* in two weeks. Minimal information is available with regards to the hydrocarbon-degrading capacity of members of this family, although *Coprinellus bisporus* was recorded, along with other fungi, from soil contaminated with phenanthrene and pyrene and treated with pea wheat straw [76]. The third most abundant OTU had a non-conspecific match with ITS sequences from the genus *Geotrichum*. *Geotrichum candidum* was previously found to increase benzo[a]pyrene degradation in mineral liquid medium [77]. Zheng & Obbard [78] isolated a strain identified as *Geotrichum* sp. in a PAH-contaminated soil, which was able to oxidize more than 50% of the phenanthrene present in liquid medium after seven days. The authors were able to isolate and cultivate this strain of *Geotrichum* sp. using generic media that were not hydrocarbon-coated, while the OTU observed at our site was not isolated, despite the use of both selective and non-selective media.

The three most abundant bacterial taxa in our CI dataset had non-conspecific matches in GenBank with environmental 16S rDNA sequences from heavy metal or oil-contaminated soils. The first two OTUs belong to the *Rhodocyclaceae* (*Denitratisoma* and unclassified taxon) while the third is a member of the *Comamonadaceae* (*Acidovorax*). PAH-degrading bacteria have been reported from both of these families [79–81]. In addition, members of the *Rhodocyclaceae* have been shown to degrade PAHs adsorbed in hydrophobic sorbents, highlighting the ability of these strains to degrade PAHs even when they are poorly bioavailable [80]. Thus, the most abundant bacterial and fungal taxa recorded by 454-pyrosequencing from this hydrocarbon-contaminated site belong to taxonomic groups that include members with the capacity to degrade hydrocarbons. Unfortunately, none of these taxa could be isolated using traditional culturing techniques, preventing comprehensive testing of the hydrocarbon-degrading potential of these strains.

Conclusions

To date, this is the most comprehensive comparison of CI and CD methods in hydrocarbon-contaminated soils, and the first to examine fungal communities. Although microbial diversity can be reduced in contaminated environments, we did not recover a large proportion of the bacterial or fungal OTUs that were identified through 454-pyrosequencing. Surprisingly, many of the most abundant OTUs *in situ* were not cultured, despite the use of several types of hydrocarbon-containing media, suggesting that factors other than hydrocarbon tolerance and metabolism are critical to *in situ* community composition. Although obligate hydrocarbonoclastic microorganisms have been identified [82], they appear to be uncommon, and many microorganisms can adapt their existing metabolic pathways to the degradation of hydrocarbon compounds. Based on this, as well as our results, hydrocarbons may be a poor selective agent for comprehensively culturing representative microorganisms from hydrocarbon-contaminated soils, despite the widespread use of hydrocarbon-coated media for isolating strains from such environments. This suggests that novel culturing techniques are essential to the progression of bioremediation research. Although next-generation sequencing has caused CI analyses to wildly outpace our ability to culture microorganisms, novel 'omics' tools will be invaluable in

developing future generations of culturing methods, as some of the environmental and ecological factors that shape microbial abundance can be identified and exploited.

Supporting Information

S1 Fig. Rank abundance distribution of bacterial and fungal OTUs recorded in CI datasets. Singletons and doubletons with a pairwise similarity not equal to 100% are omitted. The dashed lines represent the partition between abundant and rare OTUs. A total of 197 bacterial OTUs were defined as abundant, representing 39.4% of the 16S rDNA reads, while 87 fungal OTUs were recognised as abundant representing 81.4% of the ITS reads.
(DOCX)

S2 Fig. OTU rarefaction curves based on 454-pyrosequencing of bacteria (A) and fungi (B) and on Sanger sequencing of the isolated bacterial strains (C) and fungal strains (D) in the five plots sampled, combined according to hydrocarbon contamination level. The green, orange, and red curves show data observed in soil samples from the slightly contaminated, moderately contaminated, and highly contaminated plots, respectively.
(DOCX)

S3 Fig. Krona charts showing the taxonomic identification and relative abundance of bacterial (A) and fungal (B) taxa recovered by CD methods.
(DOCX)

S1 Table. Polycyclic aromatic hydrocarbons (PAHs) and C10-C50 hydrocarbons recorded in the five plots sampled.
(DOCX)

S2 Table. A. Fusion primers used to amplify bacterial taxa from the five plots sampled using 454-pyrosequencing. Adapters 1 and 2 were CCATCTCATCCCTGCGTGTCTCCGAC and CCTATCCCCTGTGTGCCTTGGCAGTC, respectively, followed by the key sequence TCAG. **B. Fusion primers used to amplify fungal communities from the five plots sampled using 454-pyrosequencing.** Adapters 1 and 2 were CCATCTCATCCCTGCGTGTCTCCGAC and CCTATCCCCTGTGTGCCTTGGCAGTC, respectively, followed by the key sequence TCAG.
(DOCX)

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Author Contributions

Conceived and designed the experiments: AEY MH MSA. Performed the experiments: CM AEY IEDLP. Analyzed the data: FS. Contributed reagents/materials/analysis tools: MH. Wrote the paper: FS THB IEDLP MSA MH.

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