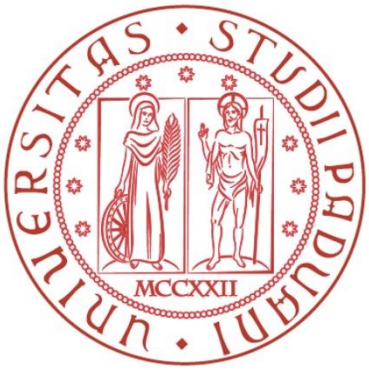

STUDY ON BIOREMEDIATION OF HYDROCARBONS CONTAMINATED SOIL: THE USE OF MEAT AND BONE MEAL AND CYCLODEXTRINE TO ENHANCE THE PROCESS. MONITORING THE DYNAMICS OF SOME RELEVANT METALS IN THE SOIL.

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ABSTRACT

There is increasing awareness throughout the world of how human activities of various kinds have been contaminating the Earth. All environmental compartments are affected: air, water, soil and sediments. In this thesis work we focused on the problem concerning soil contamination, in particular on hydrocarbon contamination. Global scientific investigations, conducted by authoritative subjects such as the European Environment Agency (EEA), the US Environmental Protection Agency (EPA) and the Food and Agriculture Organization, (FAO) identify the hydrocarbon compounds derived from oil among the major contaminants of soil and groundwater, together with heavy metals (HM). As some of these pollutants are considered toxic and possibly carcinogenic (International Agency for Research on Cancer, IARC), environmental remediation in such circumstances is strictly necessary. In this experimental study, a biological treatment method of contaminated soils was used, called biostimulation. The latter exploits the innate abilities of soil microorganisms in degrading organic compounds, such as hydrocarbons, providing them with the oxygen and nutrients needed to optimize metabolic processes. Based on the encouraging results obtained in previous studies, Meat and Bone Meal (MBM, 1% w / w) was used as a bio-stimulant agent for soil organisms. A natural surfactant, cyclodextrin, was also used in some of the tests to assess its ability to improve the removal of hydrocarbon contaminants from the soil (this effect is well known in literature). The pilot study and related experiments were conducted in the laboratory, trying to create optimal microcosms for biodegradation reactions (temperature, pH and water content were monitored, and the aeration of the soil was favoured by turning it over). Two different experimental tests were prepared: one for the analysis of hydrocarbons in the soil, the other for the monitoring of some metals of particular interest. Concerning hydrocarbon test, two fractions of hydrocarbons were separately quantified by GC-FID technique, i.e. Diesel (C_{10}-C_{21}) and Lubricant Oil (C_{22}-C_{40}). It was therefore possible to note the different behaviour of the two fractions over time. The analyses of these hydrocarbons were made every two weeks for three consecutive months. The final results showed that the treatments with MBM had faster kinetic in hydrocarbons removal, although the final concentrations are comparable to those of the control treatments (Co, soil only). Finally, in the last experimental period the effect of cyclodextrin seemed to be relevant, as it can increase the bioavailability of some contaminants and can be an additional source of carbon.

As regards the study of the dynamics of metals, both soil and leachate samples were analysed, collected specifically to quantify stored and leached metals. As reported in the literature, even in our case the tests in which MBM is present influence the mobility of some elements, in particular the lead is held for a short time in the soil before being released, which is not the case in control treatments. This effect can be related to the cationic adsorption carried out by the phosphates present in the MBM product. Cyclodextrin, on the other hand, seems to help to solubilize some metals, which pass into the aqueous phase and are leached.

Purified DNA extracts were then obtained from soil samples in three different periods, namely beginning, middle and end of experiments. These soil DNA extracts were subsequently sequenced to define which microbial community is present in the system and what changes take place over time as the concentration of organic contaminants in the soil decreases.

AIM OF THE THESIS

The aim of the thesis is to test whether the product defined MBM can be used as a biostimulant agent for the indigenous microbial community, in a soil contaminated by hydrocarbons. At the same time, the effects induced by the addition of the natural cyclodextrin surfactant in the system are evaluated. The behaviours of the metals in the various treatments will also be monitored, to eventually prove the immobilization capacity of some metals by MBM. In this case the cyclodextrin could instead favour leaching.

Finally, it may be interesting to define the microbial community present in the contaminated soil and its evolution over time. The study takes place in the laboratory, but the purpose of the research is to define optimal methods for the bioremediation of real contaminated sites, using natural, environmental non-invasive and economically sustainable products.

1. Introduction

1.2. Soil Ecosystem

Soil is an essential component of natural environment. Citing a sentence written by the Food and Agriculture Organization of United Nation (FAO, 2015): "soils deliver Ecosystem Services that enable life on Earth". Among the many soil functions, gas exchange and carbon sequestration, water purification, soil contaminant reduction and detoxification, climate regulation, nutrient cycling, food fibre and fuel provision, habitat for organisms, flood regulation. Moreover, it constitutes an important element of our cultural heritage as it preserves the traces of our past (European Environment Agency, 2019).

Those vital functions are mostly implemented by soil organisms [1–5]. A typical healthy soil might contain thousands of species of bacteria and actinomycetes, hundreds of species of fungi, many different species of insects and mites, and then nematodes, earth worms and vertebrates (fao.org/soils-2015). Soil organisms, plants and animals interact with each other, forming a complex web of ecological activity [6].

Soil is a matrix that can contain all the states of matter, solid liquid and gas. It is a mixture of different materials that are present in different proportions. Soil average composition can be identified with 5% Organic Matter, 25% Air, 25% of water and 45% Mineral, expressed in volumes (fao.org/soils-2015). Those percentages are generics: soils can be very diverse and characterized by different compositions [2]. Types of soil are strictly dependent to the geographical position on Earth and they are a key element of every landscape.

Soil formation is influenced by many factors, i.e. parent material, topography, climate, organisms and time. It is a very long and complex process that can take up to 1000 years to form just 1 cm of new soil layer (fao.org/soils-2015, Land Development Department of Thailand). For this reason, it can be considered a non-renewable resource [7,8].

Many processes and reactions take place in the soil ecosystem, such as transformations of various compounds and translocations and loss of elements, for example by percolation. Then, additional material coming from external environment and losses from the soil itself change its composition.

The rate and the effectiveness of those processes in the soil are mostly connected with (micro)biological activity [2,9,10] and thus dependent on chemical-physical parameters, like type of soil, Organic Matter, nutrients, light, pH, temperature, air (O_2 in particular) availability, water content [1–4,11–15].

The soil ecosystem is an environmental compartment strictly related to the atmosphere and groundwater [16–19]. For this reason, energy and matter are constantly transferred and transformed within the soil system, creating biological and chemical-physical dynamics that are subject of study for numerous scientific fields[20–23]. In this master thesis work, attention will be given to organic compounds remove from a hydrocarbon contaminated soil, sampled in a disposal centre in Finland (https://www.phj.fi/in-english/). The research is based along the lines of previous studies [10,11,24,25].

Experiments and studies, both theorical and empirical, have been done to understand environmental dynamics and transport processes, which cause the fate of chemicals in the environment. Particular attention has been paid to toxic and hazardous compounds [16,19,20,22,26–29]. Nowadays, environmental pollution problem caused by different contaminants and sources around the world is well known, even if real actions aimed at solving certain situation are proceeding slowly and more often against the interests of important international markets [18,30–33].

1.2. Soil and Groundwater contamination

1.2.1. Soil contaminants

Many different kind of compounds (chemicals) can be considered soil pollutants. They can be described according to their reactivity and biodegradative properties: in this last case, a particular molecule can be biodegradable, bioaccumulative and/or persistent (hazardous compounds in this class are POPs or Persistent Organic Pollutants, i.e. dioxins, DDT and PCBs, most of the case manmade molecules and bioaccumulative [34,35]). Pollutants can also be described according to their toxicity towards environment and human being: in Europe, REACH regulations [36]require the Registration, Evaluation, Authorisation and Restriction of Chemicals (European Chemicals Agency), while CLP regulation [37] rules the Classification, Labelling and Packaging of the chemical substances and mixtures. Another way to classify a pollutant substance is to focus on its physical characteristics: it can therefore be defined by its density, volatility, solubility, miscibility and physical state. Those parameters have been also used to model the dynamics of pollutants in the environment and possible interactions with living beings [16,19,20,27,33].One last way to define a pollutant substance is to look at its chemical class, inasmuch organic or inorganic molecules. Inorganic substances are the one without C, even if carbonates, CO and CO2 are considered inorganic too[27]. Usually in this class attention is paid on Heavy Metals (HM) elements such as As, Cr, Hg, Pb, Cu, Cd, Se among others. Those elements are characterized by high density (> 5g/cm^3), positively charged, aptitude to form complex and affinity to sulphides, many of them form oxides, hydroxides and hydrates compounds with low solubility and salts. As they are particularly reactive, they are often used as catalysts. Most Heavy Metals are transition elements in periodic table. HM can have toxic effects when bioavailable in the environment. Indeed, when organisms are exposed to harmful HM in their bioavailable speciation, toxicological effects can occur, and human health can be compromised too[33,38,39]. HM can be bioaccumulative and they are not biodegradable: the only way to reduce their threat is to change their redox state (speciation) and induce removal, adsorb or immobilize them[17,40–49].

Organic contaminants can be categorized according to their chemical structure and functional groups: they can be volatile, semi-volatile or non-volatile substances (VOC, SVOC & NVOC respectively), persistent in the environments thus very difficult to biodegrade (POPs), halogenated or not, they can also be just nutrients[27]. In the organic compounds class fall aliphatic and aromatic hydrocarbons, as well as polycyclic aromatic hydrocarbons (PAHs).

A further group of contaminants, recently monitored due to their potential ecotoxicity, is that of the so-called Emerging Pollutants [33,51]. Chemicals such as pharmaceuticals and personal care products (PPCPs), endocrine disrupters as bisphenol A and phthalates have been defined, hormones and toxins fall into this category. Two other large EP groups are manmade nanoparticles (MNPs) and treatment by-products [33]. The behaviour of these compounds in the various environmental compartments depends on their chemical-physical and possibly biological characteristics, such as volatility, polarity and adsorbent capacity among many [51].

1.2.2. Hydrocarbons and PAHs contamination

Hydrocarbons are the main components of petroleum or crude oil. They are usually present mostly as linear and saturated compounds, while branched structures, such as isobutane or 2-methylpropane, cyclical rings

i.e. cyclohexane and cyclopentane, are present in trace. This mixture of components present different structural isomers [19,27].

Crude oil is extracted from oil fields, through pumping and the use of technologies designed to reduce surface tension and viscosity of the fluid. The subsequent refining process takes place in the fractionation towers. It provides for the use of high temperatures (360-400 ° C) for the separation of homogeneous fractions of similar compounds. Thus solidified (coke) and vaporized states are obtained. The latter will be subject to condensation at appropriate temperatures. In this way different fractions of the initial oil will be obtained, separable in light crude oil (C-C_4 gas, petrol [52] and kerosene) and heavy crude oil ("naphthenes" , PAH and lubricant oils). in addition to oil fields, other reserves defined as unconventional are the bituminous areas. The extraction of crude oil from these systems is expensive and environmentally impacting [27,31].

When organic compounds such as petroleum are found to be in the soil, here they undergo "attack" by microorganisms, in particular fungi and bacteria [2,5,9,53–58]. The efficiency and results obtainable thanks to these natural processes vary depending on the environmental conditions and the microbial strains involved. The work of these microorganisms consists in the production of surface-active compounds which allow the emulsion of the organic petroleum molecules and their solubilization. Once the oil is brought into solution (in the aqueous phases contained in the soil) the microorganisms can use it as a source of carbon and / or electron acceptor for their metabolic cycles [15,53].

Fossil fuels biodegradation is influenced by chemical-physical factors such as the presence of oxygen, pH, temperature, humidity and nutrients. Oil-contaminated soil remediation techniques seek to control these parameters to maximize biodegradation processes, creating optimal conditions for microbial activity. Furthermore, the degradability of hydrocarbon molecules is closely linked to their molecular weight and to their structure (cyclic, branched).

PAHs are a group of persistent, semi-volatile organic compounds, made of two or more unsubstituted benzene rings fused together when a pair of carbon atoms is shared between them. The most common PAHs are anthracene, fluoranthene, naphthalene, pyrene, phenantrene and benzopyrene [33,50,59]. Due to their chemical properties, most of these compounds are POPs and they are widespread in the environment. Low-molecular weight PAHs are volatile and they mainly occur in the atmosphere, whereas medium and high molecular ones are partitioned between atmosphere and particles [27,60,61]. PAHs compounds are produced by reactions of incomplete oxidation of fossil fuels and garbage, pyrolysis of organic materials by industries, agriculture and traffic [33,62–64]. Traffic emissions and fossil fuel combustion are the main identified sources of PAHs in urban areas [65]. A recent PhD research on the effect of living environment and environmental exposure on the composition of microbial community in soil, on human skin and in the gut [66] conclude that densely built areas and high pollution level can affect microbes community composition in the environment, and this can result in qualitative and quantitative changes in skin and gut microbiota in humans. PAHs have long been studied because of their potential toxicity and carcinogenicity towards organisms [62,67–69]. Particular attention has been paid on 16 of those molecules that have been identified as carcinogenic [70,71].

1.2.3. Heavy Metal contamination

Even if these elements naturally occur at low concentrations in soil, many years of human activities significantly increased their presence in the environment [72]. The main causes of dissemination are industrial

processes, mine tailings (i.e. Acid Rock Drainage effect[73]), waste disposal, leaded gasoline and paints, phytopharmaceuticals and fertilizers, animal manures, sewage sludge, wastewater irrigation, residues of coal combustion, petrochemicals spillage and atmospheric deposition from different sources [33,74,75].

As an example, is the map produced by the European Environment Agency in 2005 [76], which represents the European situation regarding Lead contamination in topsoil (0-25 cm). It is clear that the concentration of this heavy metal is high in areas of high industrial activity, as in the north-eastern part of Italy.

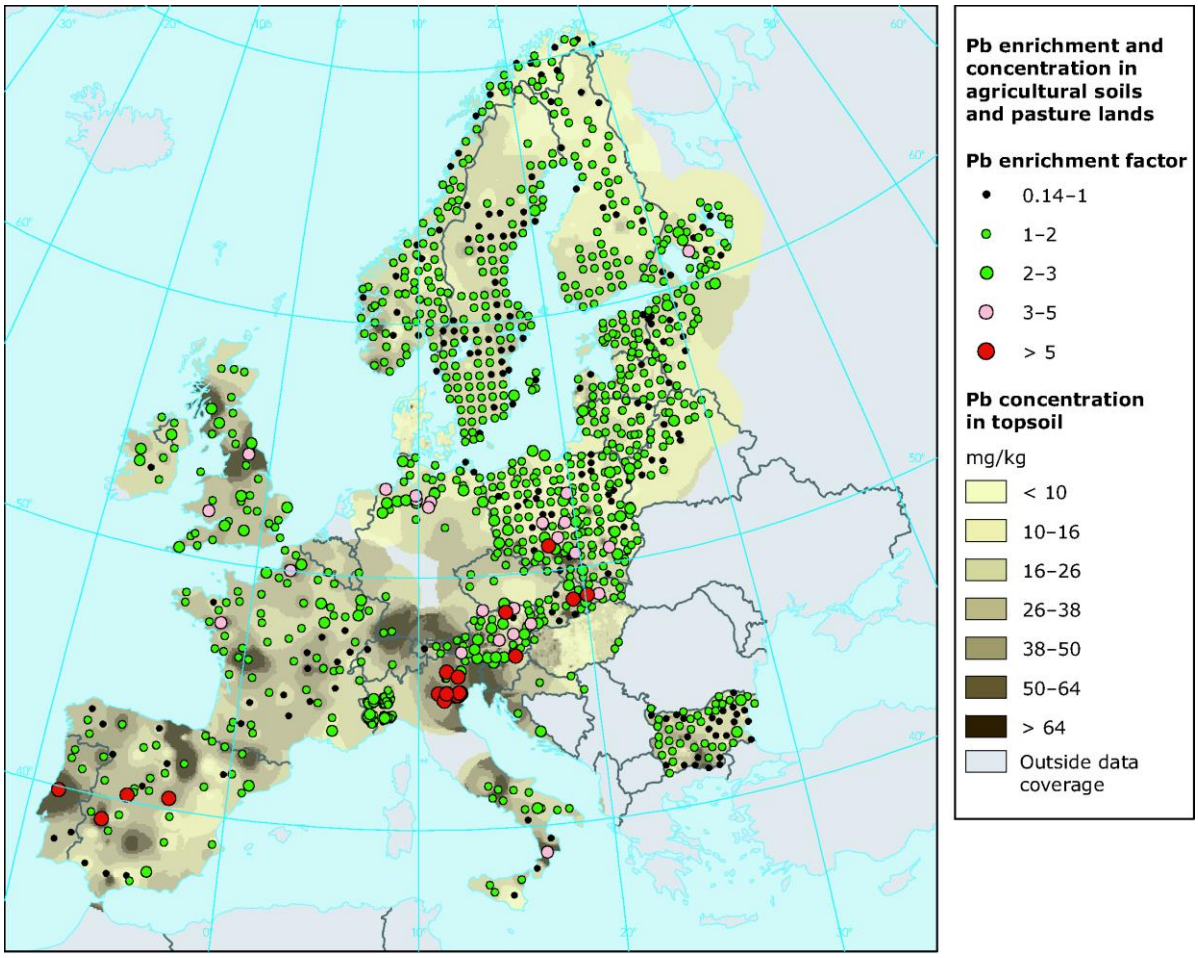

Fig.1 Lead presence in Europe, European Environment Agency in 2005

Heavy metals degrade the quality of water bodies, soil and atmosphere, and they accumulate in the tissue of living organisms, threatening the health and well-being of animals and plants. They are not subject to metabolic breakdown [33]. Among others, it was found that Zn, Ni, Co, and Cu are relatively more toxic to plants, and As, Cd, Pb, Cr and Hg are relatively more toxic to higher animals [38,50,71]. The World Health Organization and the International Agency Research for Cancer have compiled a list of the most dangerous heavy metals for humans and living beings [38,71]. Heavy metals can interact with soil system in different ways, according to the chemical nature of the element and the environmental condition, such as pH and pE (E_h, redox potential)[20,23,27]. They can be subject to adsorption phenomena, for example on colloids and other negatively charged particles. Usually such interactions are physical and therefore temporary. Metals can establish covalent bonds with soil organic compounds, producing more or less volatile and soluble molecules. Still they can complex suitable compounds, crystallize and / or precipitate.

It is interesting to see how organism response when they are in contact with heavy metals [15,26,77]. Plants and bacteria can accumulate certain amounts of toxic metals in specific cellular areas, such as vacuole or membranes. Plants can also help the complexation of metals in the soil, thanks to the production of molecules as radical exudates that induce bonding [26,48,78,79]. Dead vegetable biomass can adsorb metal cations. Fungi belonging to the Penicillium species perform particular actions of extracellular detoxification, inducing the precipitation of heavy metals such as copper. The marine bacteria Shewanella is studied for its ability to precipitate Uranium chains, decontaminating the surrounding water. Finally, the metals can undergo methylation by microorganisms, increasing volatility and hydrophobicity.

Given the numerous actions that plants and microorganisms can carry out against potentially toxic heavy metals, they are often used in the bioremediation of contaminated soils. Typically, appropriate systems are created to maximize remediation operations (bioreactors, permeable reactive barriers, wetland and the like).

1.2.4. A look at the contaminants in Europe

Figure 2 report the most common contaminants that are affecting soil and groundwaters in Europe, according to the results obtained from Joint Research Centre (EEA) during a Network study that include European countries:

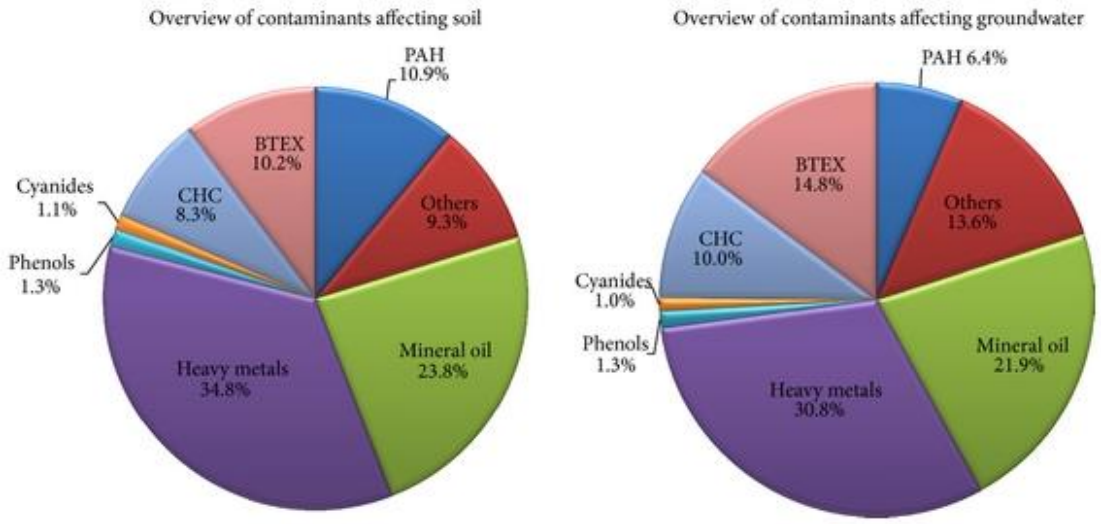

Fig.2 Distribution of contaminants affecting soil and groundwater in Europe (European Commission, Joint Research Centre, Institute for Environment and Sustainability, 2013 [28,74]

It is clearly visible that both environmental compartments are mainly contaminated by heavy metals and what is called Mineral oil, which is a light mixture of higher alkanes derived from petroleum or its refined by-products [74,80,81]. BTEX, which is the acronym for Benzene, Toluene, Ethylbenzene and Xylene, and PAHs are affecting soil and groundwater too [70,74,82,83]. CHC (chlorinated hydrocarbon or organochloride), used in many applications such as vinyl chloride production and phytopharmaceutical synthesis [84], are a common environmental contaminants and some of them are considered hazardous chemicals [85]. Same thing applies to PAHs compounds.

1.2.5. Legislation on soil contamination

Even if the soil is a key element in the various human activities, to date there is still no European legislation on the subject (EEA, 2019). Soil protection, unlike water and air, is indirectly contemplated or within sectoral policies such as agriculture and forestry, energy, water, climate change, nature protection, waste and chemicals. For this reason, in Europe as well as internationally, it is still not easy to produce harmonized data (on legislation) to define the state of the soil. Recently, policies aimed at soil conservation and related data production are increasing. For example, in 2006 the European Commission proposed the "thematic strategy for soil protection" which highlights the need to protect the functioning of the soil as an essential element of sustainable development (Commission communication of 22 September 2006: "Thematic strategy for soil protection". Proposal for a European Parliament and Council directive of 22 September 2006 defining a framework for soil protection and amending Directive 2004/35 / EC). This strategy specifically involves identifying risk areas and polluted sites, as well as restoring degraded soils. At the global level, the concept of soil degradation (limited for now to arid areas) is used, defined by the United Nations Convention against Desertification (UNCCD). In 2015, the United Nations General Assembly updates this concept with "soil degradation neutrality", in which the notion of preserving soil functions is integrated, in order to guarantee sustainable development. Objectives such as soil quality, management of soil contamination, chemicals and waste are therefore brought together. Once the regulations on soil protection are well defined, the production of standardized and harmonized data and information is also possible.

In Europe, the EEA uses indicators such as land use, hardiness, management of contaminated sites, soil moisture, soil erosion and soil organic carbon, to evaluate and manage this important resource (LSI, Land and Soil Indicators). The Copernicus land monitoring service, promoted by the European Union, facilitates regular updates of many of these indicators. Specific assessments are also produced on soil-related topics, such as thematic maps on the efficiency of use of this resource, or maps showing soil contamination by heavy metals (HM). The European Environment Agency works with European Commission colleagues [Joint Research Centre (JRC) and Environment Directorate-General] and other experts at European level, but also global partners such as the United Nations Environment Program.

In Italy, the first legislation that deals with issues concerning soil contamination is Law n.349 of 1986 (regulation of areas at high risk of environmental crisis). Numerous changes, updates and additions have been made over time, both nationally and regionally. The Ministerial Decree 471/99, implementing regulation of article 17 of Legislative Decree n.22 of 1997 ("Ronchi Decree") can be defined as the first national organic law on contaminated sites. It establishes a first definition of a contaminated site, or site where the concentrations of the contaminants exceed the limit values. The contamination is therefore based on criteria of a table type: if the concentration in the soil (for industrial / commercial and green / residential uses) or in the groundwater of a certain contaminant exceeds the limit values (called Contamination Threshold Concentrations, CSC), then the site is contaminated. With the entry into force of Legislative Decree 152/2006, which brings together the rules concerning environmental protection in Italy (it is in fact defined as "environmental framework law"), the technical approach for the identification and management of contaminated sites changes. In particular, they are now defined Risk Threshold Concentrations (CSR), or remediation objectives determined by site-specific risk characterization. The tabular values defined by DM 471/99 are maintained as values of screening (CSC), upon passing of which the site can be considered potentially contaminated. In this regulatory context it is important to determine the natural background concentration values for soil and soil groundwater, so as to be able to define the extent of site-specific contamination. It should be emphasized that the risk analysis for the definition of CSR only considers the effects of contamination on human health, while the assessment of ecological risk is not, to date, required by law [86]. The Minister for the Environment and the Protection of the Territory and the Sea, in collaboration with the Environmental Protection and Research Institute (ISPRA) and regional agencies for environmental protection, deals with problems related to contamination and land conservation.

With regard to the remediation of soil and groundwater in Finland there are no separate legislation on soil protection, and the relevant controls are included in several different statutes (Ministry of the Environment, Finland). The Environmental Protection Act No. 527 of 2014 is the most important legislative instrument concerning soil contamination and environmental restoration. The environmental sustainability linked to the protection and management of the soil resource is also part of the specific regulations of the various human activities (such as construction, earth extraction, farming and forestry), but also in some rules of nature conservation and landscape protection. Waste legislation deals with waste coming from reclaimed soils.

1.3. Source of pollutants

Contamination can naturally occur on Earth. For instance, heavy metal concentration in a place can be high, according to the pedo-geochemical fraction specific of the location [87]. Several soil parent materials are natural sources of certain heavy metals and perhaps radionuclide. For example, a major environmental problem is Arsenic released from volcanic activity and weathering of As-containing minerals and ores. Radioactive gas Radon diffusion, from deeper layer to the Earth surface, can also be considered natural source of pollutants. Natural events such as volcanic eruptions or forest fires can release many toxic elements into the environment: polycyclic aromatic hydrocarbons and dioxin-like molecules are some of these [33,82]. Another natural source of contamination can be asbestos, which poses a high risk to human health from inhalation if exposure occur.

Besides the natural sources of contamination, which are usually well localized and known, there are anthropogenic sources. The major institutions and organizations that deal with the environment on a world level agree in affirming that centuries of human activity have led to a diffused contamination and deterioration of the soil ecosystem [28,50,83,88].

The main products that impact on the soil natural composition, function and activity are manmade chemical compounds or by-products of those. They originate from industrial activities, domestic and municipal wastes releasing, including wastewater, agrochemicals, and petrol-derived products.

The graphs below show which activities cause major contamination in some EU countries (Soil data collection on contaminated sites provided by Joint Research Centre, EEA, 2006)

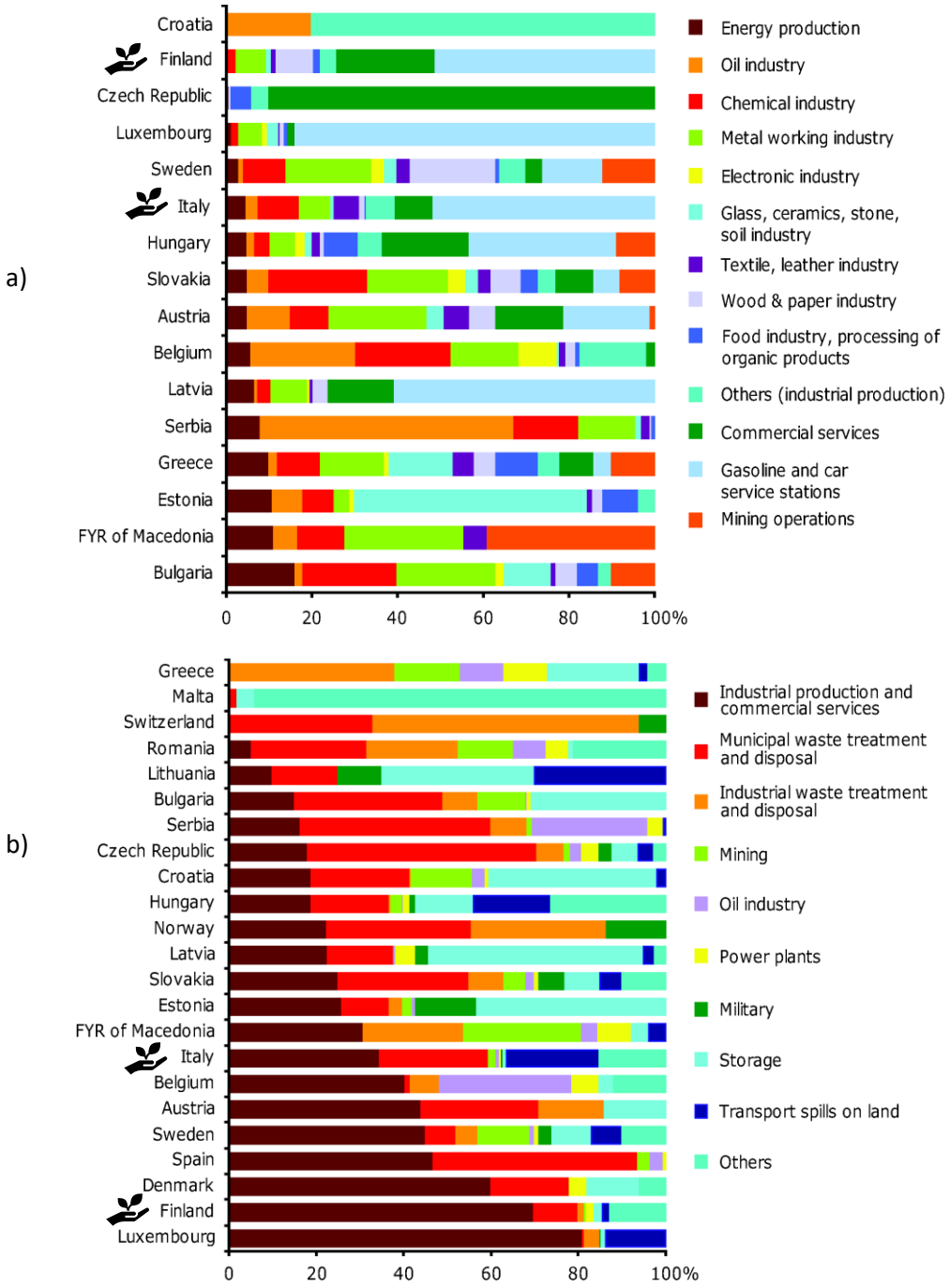

Fig.3 a) detailed analysis of industrial and commercial activities causing soil contamination by country and b) breakdown of activities causing local soil contamination

Pollutants are released to the environment accidentally or intentionally, and the consequent contamination can be diffused, for example from vehicle traffic emission, or point-like, as is the case of single tank of gasoline spill on the soil and leaching through it.

In the Review Article on the situation of contaminated sites in Europe[74], JRC define which activities are the main contributor to soil contamination, focusing on industrial and commercial sectors because they are particularly impacting. Results are reported in Fig.4.

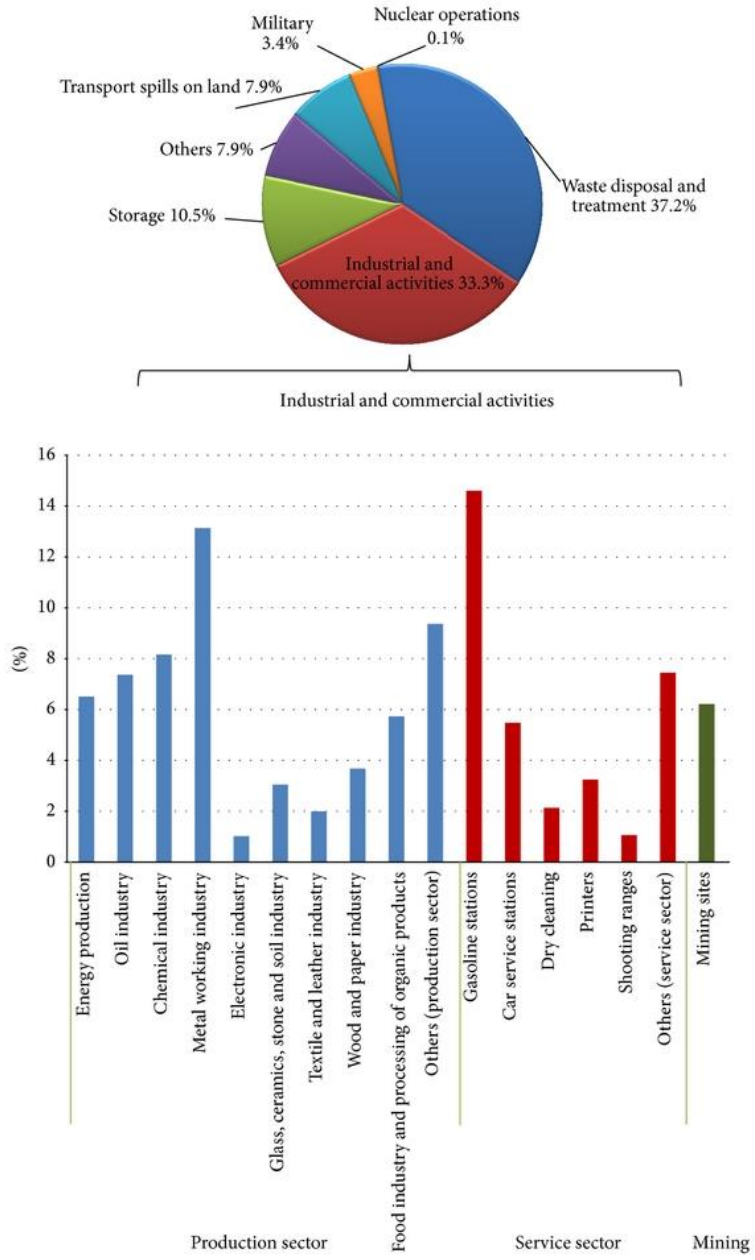

Fig.4 Distribution of sectors contributing to soil contamination in Europe, 2013

According to the results obtained by European Network survey on the average distribution of contaminants in the European countries, it can be seen that most common contaminant in the environment are mainly released by metal working industries and gasoline station [19,33,74].

1.4. Thermodynamic properties of contaminants and interactions with the soil matrix

The identification of the thermodynamic properties of a certain contaminant is important for predicting and estimating its behaviour in the environment, identifying its dynamics and analysing the possible level of risk associated with the compound (considering toxicity, exposure of subjects and transport factors). These properties reflect the equilibrium constants involved in the various exchange processes between the contaminant and air - water - soil phases [16,20,23], thus including vapor pressure, tendency to volatilize, solubility in water, lipophilicity, as well as ebullioscopy and melting point.

Vapour pressure is defined as the pressure at which the pure liquid is in equilibrium with its gas phase and is closely linked to the volatility of a substance. Volatile, semi-volatile and non-volatile organic compounds are

identified which, respectively, have vapor pressures > 10^{-4} atm, between 10^{-4} and 10^{-11} atm, and < 10^{-11} atm (VOC, SVOC and NVOC).

Volatility is defined as the tendency of a substance to be distributed in the air phase or in the gas phase and is identifiable by Henry Law and its related K_H. In the soil, for example, it may happen that there is a balance of distribution in the interstitial areas of the medium. The volatility depends on the vapor pressure of the pure compound, its solubility and molecular weight. For certain pollutants with adequate volatility, there are direct, fast and economic sampling techniques, which concentrate on the portion of contaminant passed in the gas phase (see SPME techniques and adsorbent surfaces).

The solubility (in the aqueous phase) of a compound is defined as the maximum concentration beyond which there will be precipitation phenomena. It is characterized by the so-called solubility product, and by the connected K_{sp} constant. Influential factors on this property are in the first place the temperature, but also the pH, the presence of competing ions and the ionic strength of the compound (salinity).

The lipophilicity of a compound indicates its tendency to stay in the polar aqueous phase or in the non-polar organic one. In the soil environment there are various apolar phases, for example fats of organisms, rather than organic matter of the soil. Living cell membranes are in part apolar in nature, as they are composed of phospholipids with hydrophilic heads and hydrophobic tails. To simulate the model for the division of a compound between aqueous phase and cell membrane, n-octanol was chosen as the apolar organic phase. In this distribution equilibrium, the equilibrium constant is the K_{ow}, which is the higher the more the compound is hydrophobic. K_{ow} (for convenience, its logarithm) is used to identify the tendency of a contaminant to bioaccumulate.

The U.S. Environmental Protection Agency has published several Hazard Characterization Documents regarding the risk associated with log $_{Kow}$ - toxicity and bioaccumulation in biota. Numerous software are used for the modelling of these phenomena.

K_{ow} is related to K_{oc}, which is the constant that measures the division between the aqueous phase and the organic carbon of the soil. The greater the quantity of organic substance (OM or Organic Matter) present in the soil, the greater the distribution of the compound in this phase. The value of this constant can be an index of contaminant mobility in the soil: low K_{oc} values will identify a compound that has little affinity for the OM of the soil and will (eventually) be transported with water. K_{oc} is also linked to the soil-water partition coefficient, K_d.

The contaminant particles can also be subject to molecular diffusion, a phenomenon described mainly by Fick's laws. These equations include concentration gradient and diffusion coefficient, the latter depending on temperature, type of substance and characteristics of the medium, such as geometry, in which the compound is spreading. Advection and reactions occur as well. In the theoretical study of contamination dynamics, some researchers define the ADR equation (advection-diffusion-reaction) to summarize the mix of processes that occur in the system [23].

In addition to the thermodynamic characteristics of the contaminants, also the hydrogeological parameters, such as the permeability of the soil and the hydraulic gradient, as well as the biotic (aerobic and anaerobic) and abiotic processes influence the fate of the compounds in the soil. Abiotic processes include sorption, ion exchange, precipitation, hydrolysis and volatilization. The characteristics of the contaminants and the environmental ones contribute in determining the phenomena of transport and mobility of the compounds in the environment[19,20,23,27].

When pollutants enter the soil, they undergo physical - chemical, microbiological and biochemical processes that retain, reduce or degrade them[33]. In the soil matrix a compound can be dissolved as a solute in water,

or it can behave like steam and move in interstitial areas together with other gases. It can also be adsorbed on soil particles such as colloids and humus [27,33]. Particular liquid substances defined as NAPL (Non-Aqueous Phase Liquid) are immiscible with water. They are subdivided into LNAPL (Light), as they are less dense than water and therefore fluctuating on it, and dense (DNAPL), or heavier than water and therefore sink into it.

Sorption phenomena involve the particles of a fluid that interacting with a solid are retained for a certain time [17,33,89]. This interaction can be of a chemical nature, for example with the formation of H and ionic bonds, or properly physical, as with van der Waals forces. Soil particles such as clays and organic matter are negatively charged and therefore cation exchange sites for positively charged ions or molecules. The cation exchange capacity (CEC) varies widely depending on the type of soil constituents and the OM content. Furthermore, the absorption of some ions or molecules can be pH-dependent as: i) the oxidation state can change from cationic to neutral to anionic, in response to changes in pH; ii) sorption of molecules with acid or basic behaviour varies with pH. For example, in the case of metals, absorption is higher in less acidic soils, while acidic condition favour desorption and release charged ions (and molecules) in the soil. The quantity of water and the availability of oxygen also influence the oxidation state of the contaminants, contributing to their mobility. The addition of inorganic and organic amendments increases the number of binding sites, thus making pH levels vary and increasing sorption processes. Compost, sewage sludge, manure and by-product of industrial activities are part of these amendments [4,11,90–92]. They can have a positive effect on the soil and at the same time reduce the amount of waste.

1.5. The role of microorganisms in the degradation of contaminants in the soil

Bacteria and fungi, but also algae and plants, are able to degrade, transform and immobilize some contaminants in the soil [15,27]. These activities can release by-products into the environment, as in the case of the dehalogenation reaction (usually reductive dehalorespiration) of trichloroethylene into dichloroethylene. Microorganisms can use the contaminant as a metabolic substrate, from which to derive energy. The limiting factors for metabolic reactions include the presence of nutrients (especially N and P), trace elements, adequate environmental conditions and the presence of oxygen [53]. The latter is generally the main limiting factor, which acts as an oxidizing agent, acceptor of electrons. In the absence of oxygen (anaerobic conditions) other chemical species can act as electron acceptors, such as NO_3^-, Fe^{3+}, SO_4^{2-}, CO_2. The microorganisms responsible for the degradation of the contaminants can be native, in the sense that they are already naturally present and selected in the contaminated soil, or some strains can be artificially inoculated as they are particularly adept at breaking down specific compounds[10,58,93,94].

1.6. Exposure to contaminants, risk definition and health effects

Environmental problems and carcinogenic effects of some substances on living organisms have been studied and proved widely by ecotoxicological and epidemiological research [18,38,71,83,95], by authoritative subjects such as The International Agency for Research on Cancer (IARC), The World Health Organization of the United Nation (WHO) and The Food and Agriculture organization (FAO).

Living organisms can come into contact with contaminants in the soil through different exposure routes. Generally, it is about the ingestion of the soil itself or consumption of plants and animals that have accumulated significant amounts of contaminant (s). There is also dermal exposure to contact with the ground, usually in areas such as parks and gardens. A further possible way of exposure is the inhalation of compounds that have passed from the ground into the air phase, or powders, which are then breathed. Finally, polluted soil can be a source of secondary environmental contamination, adversely affecting the health of human beings and organisms.

The WHO's International Program on Chemical Safety has identified ten chemical compounds or groups found in the soil that have particularly important effects on human health: among these are heavy metals such as Cd, Pb and Hg, dioxins and similar molecules, phytopharmaceutical compounds defined as highly hazardous pesticides (HHP). Furthermore, pathogenic microorganisms and antimicrobial resistant bacteria and genes (AMR) present in the soil can pose risks to human health [33,66].

The effects of contaminating substances on organisms can be acute and / or chronic: this distinction is necessary for the choice of remediation actions (such as site safety, restoration). There are many contaminants in the soil, and they have different ecotoxicological effects on organisms [33]. Cadmium taken with food has been shown to penetrate the placenta during pregnancy and damage membranes and DNA. It is also considered an endocrine disruptor and can induce renal, hepatic and bone damage. Lead-induced biochemical imbalances affect several internal organs such as the liver, kidneys, spleen and lungs. Pb also causes neurotoxicity, especially in the more sensitive areas such as new-borns and children. Mercury in organic form, in particular methylmercury, is considered highly toxic. It can induce mutation of the neuronal and gastric systems of the human being, even leading to the death of the individual. Arsenic can be taken orally or inhaled. It is bioaccumulated mainly in the liver, kidneys, heart and lungs, but also in muscle and nervous tissue. As has been called a carcinogen, capable of inducing disorders of the nervous system, hepatic and renal insufficiency, as well as anaemia and skin cancer [33,38,71]. As for the intake of persistent organic compounds called POPs (such as PCDD, PCDF and PCB), they tend to circulate in the human body. Being these liposoluble, they are found for example in breast milk, representing a high risk for the health of new-borns and foetuses. PAHs are molecules affine to cell membranes (high $\log_{Kow}$) through which they can pass, subsequently covalently binding to DNA molecules, where they can cause mutations and cancer [64,66,68,69,81].

Generally, in the presence of a contamination event, such as oil spill and nuclear accident, the safety and remediation operations start promptly, even if there are not always specific protocols to implement. In some states or regions of the world there are agencies or bodies in charge of identifying a possible contamination and subsequent remediation works, if necessary. For example, in Italy the Ministry of the Environment and the Protection of the Territory and the Sea deals with these actions, with the help of the Higher Institute for Environmental Protection and Research (ISPRA) and other regional agencies [33,96].

In the past, there was a tendency to consider standard limits that defined the state of soil contamination: for example, in Italy it referred to contamination threshold concentrations (CSC)[19,29]. Today we also try to define and characterize the risk associated with the event of contamination and the possible problems related to it such as human health, environmental pollution and food safety [33,97]. This analysis is not an easy task, especially due to the complexity of the soil system, the dynamics of the pollutants in it and the lack of ecotoxicological studies and results [59,82,85,98]. The risk assessment for human health and for the environment can be carried out in different ways and in order to achieve different goals, such as the derivation of soil quality standards, site specific risk assessment, the development of remediation objectives and to classify contaminated sites by priority of intervention.

Risk analysis can follow two different methods, defined as forward and backward [99]. The first defines the risk starting from the source of contamination, passing through transport phenomena and considering the exposure of the subjects to contaminants. The second focuses instead on the tolerable risk (exposure) downstream, defining an acceptable concentration of the contaminant at the source.

Nowadays there are numerous scientific tools for the characterization of the risk deriving from human exposure to contaminated soil [33]. These tools use specific site environmental parameters and ecotoxicological

data of the contaminant to define the risk related to such contamination. However, these analyses are most often complicated, especially when more contaminants are studied and modelled in the system [100].

The risk assessment process in any case follows similar steps worldwide: identifying and evaluating the endogenous or exogenous substances that have or are contaminating the soil, and what is the impact that such contamination has on human health and environment [59,85]. Contaminated soil characterization is often differentiated according to their specific intended use, for example industrial areas and public green areas [29]. Soil, subsoil and groundwater are analysed, using direct (sampling and drilling, with subsequent chemical analysis) and indirect (geophysical methods) investigation techniques.

Soil screening values are based on generic soil quality standards, such as routes of human exposure to contaminants and contamination scenarios. These screening values are adopted in various countries around the world to identify, manage and monitor polluted soils [33,83]. Even if their objective is the same, they can be defined differently: trigger values, reference values, target values, intervention values, cleaning values, cut-off values and others [85,97]. These values are to be considered as screening, as they rarely take into account important site-specific parameters, such as the real properties of the soil.

When the contamination is linked to the presence of heavy metals, the total quantity of these in the soil does not provide an estimate of the associated risk. In these cases, the attention is usually placed on the bioavailability of the toxic elements present in the soil and an attempt is made to define the speciation of metals under these specific conditions [72,98,101]. Biological tests, pH analysis and quantity of organic matter are then conducted to define the potential ecotoxicity of the metal (loid) s present in the soil.

Many countries have developed benchmarks for the content of heavy metals in soil and food, these being particularly important for the protection of human health. The reference values produced by the US EPA Environmental Protection Agency are particularly important as they are used by many countries. These standards are based on risk assessment policies and define the acceptable basic levels in the study of human and environmental toxicity. FAO instead produced the Codex Standard, which shows the values for contaminants and toxins (including heavy metals) allowed in food products. These tables are constantly updated and revised, also in collaboration with the WHO, based on the best scientific knowledge of the moment.

1.7. Environmental Restoration Techniques

There are numerous methods for treating and reclaiming the soil or groundwater of a contaminated site [27,33,102]. Those methods are generally site-specific [82,98], as researchers (academic or governmental) in charge of choosing the best management practices must consider combinations of parameters including pollutants, soil properties, land use , responsibility and technical and economic reality of the site or area. There will also be the need to carry out specific risk characterization and assessment plans for the contaminated site [33,59]. The definitive conceptual model resulting from these studies will be used for choosing the best reclamation technology.

Remediation techniques are generally divided into in-situ technologies (i.e. remediation actions carried out on the contaminated site) and ex situ (post-excavation of the contaminated soil, which is transferred to specific sites and subsequently treated). The latter are usually the most used but also the most expensive and impacting from the environmental point of view [10,26,79,102], as they require the transfer of large quantities of soil (or water) with the use of vehicles and appropriate instruments.

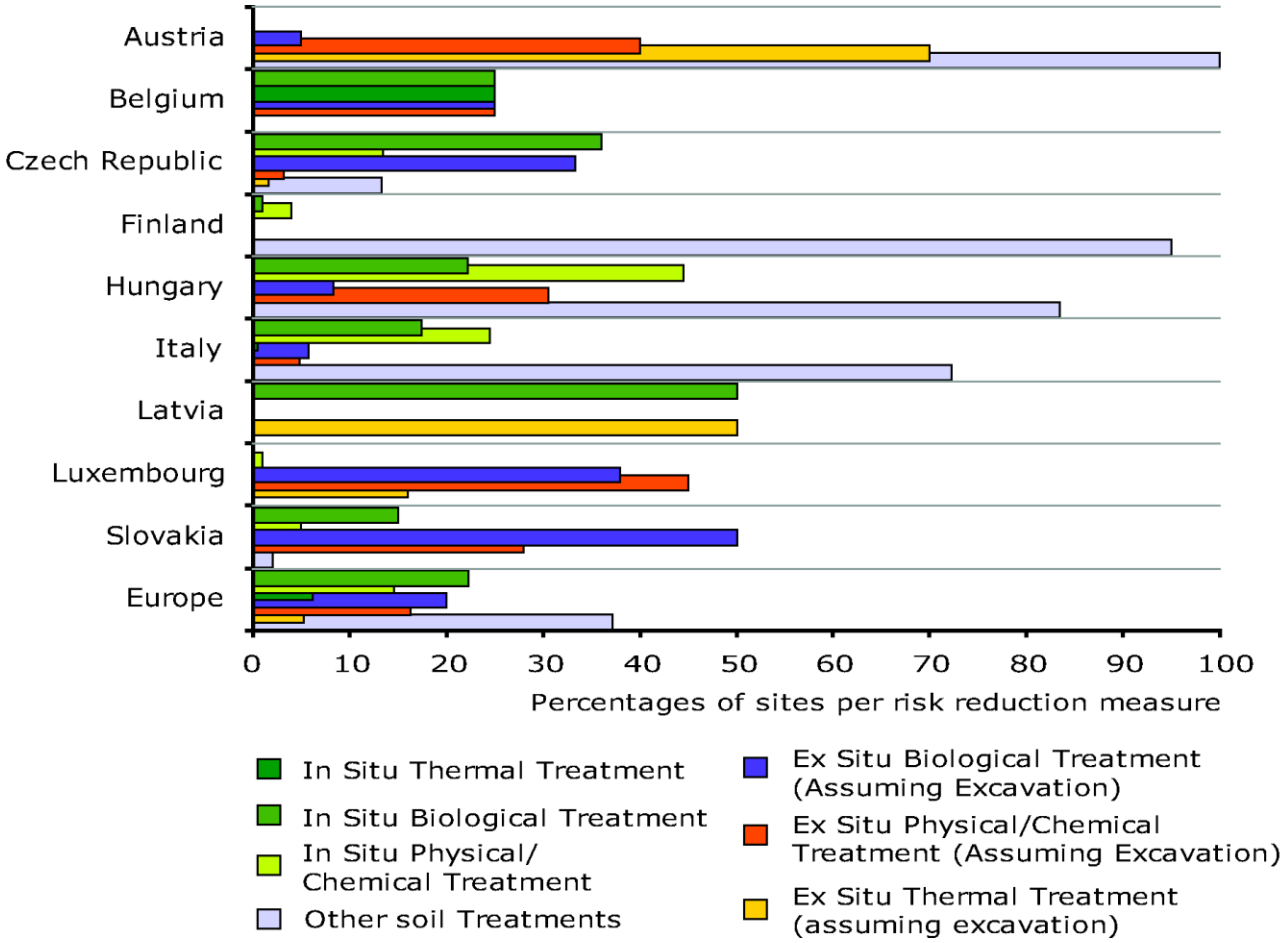

Fig.5 The graph was produced by the European Environment Agency in 2014 and shows which are the most used technologies in some of the member countries.

Remediation techniques can be biological, chemical-physical or purely thermal. The matrix of restoration technologies [103] provided by the Italian Institute for Environmental Protection and Research (ISPRA) is shown below. The table shows the main contaminants found in the soil and in the groundwater at the top, while on the left there are the most commonly used remediation techniques. It is possible to identify the effectiveness of the technique with respect to the particular contaminant by crossing these tabular parameters.

		INORGANIC COMPOUNDS							ORGANIC COMPOUNDS																		
		As	Cd	Cr	Pb	Hg	Zn	Other metals and inorganic compounds	Aromatic Hydrocarbons	Policyclic Aromatic Hydrocarbons	Carcinogenic Chlorinated Aliphatic Hydrocarbons	Non-Carcinogenic Chlorinated Aliphatic Hydrocarbons	Carcinogenic Halogenated Aliphatic Hydrocarbons	Nitrobenzenes	Chlorobenzenes	Non-Chlorinated Phenols	Chlorinated Phenols	Aromatic amines	Phytopharmaceuticals	Dioxins and Furans	Timing	Need for long-term maintenance / monitoring	Short and long term impacts on natural resources	
SOIL	BIOLOGICAL METHODS																										
	Bioventing	X	X	X	X	X	X	X	V V	V V	V V	V V	V V	X	V V	V V	X	V V	X	X	V	V V	V				
	Bioremediation (aerobic)	X	X	X	X	X	X	V	V V	V V	V	V	V V	V V	X	V V	V V	V V	V	V	V	X	V				
	Bioremediation (anaerobic)	X	X	X	X	X	X	V	X	X	V V	V V	V V	V V	X	V V	V V	V V	V	V	V	X	V				
	Phytoremediation	V V	V V	V	V	V V	V V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	X	V				
	CHEMICAL-PHYSICAL METHODS																										
	Chemical Oxidation	X	X	V	X	X	X	/	X	X	V V	V V	V	V	V	X	V	X	V	V	V	V V	V				
	Electrochemical Oxidation	X	X	V	X	X	X	/	X	X	V V	V V	V	V	V	X	V	X	V	V	V	V V	V				
	Electrokinetic Separation	X	V V	V V	V V	X	V V	V V	X	X	V	V	V	V	X	X	V	V	V	V	V	X	V				
	Soil Flushing	V V	V V	V V	V V	V V	V V	V V	V	V	V V	V V	V V	V	V	V	V	V	V	V	V	X	V				
	Soil Vapour Extraction	X	X	X	X	X	X	V V	V V	V V	V V	V V	V V	X	X	V V	V	V	V	V	V	X	V				
	Solidification / Stabilization	V V	V V	V V	V V	V V	V V	V V	X	X	X	X	X	X	V	V	V	V	V	V	V	X	V				
	THERMAL METHODS																										
	Thermic Treatment	X	X	X	X	X	X	X	V V	V V	V V	V V	V V	X	V V	V V	V V	V V	V V	V V	V V	X	X				
	BIOLOGICAL METHODS																										
	Bioplle	X	X	X	X	X	X	/	V V	V V	V V	V V	V V	V V	V	V V	V V	V	V	V	V	X	V				
	Composting	X	X	X	X	X	X	X	V V	V V	V	V	V	V V	V	V	V	V	V	V	V	X	V				
	Landfarming	X	X	X	X	X	X	X	V V	V V	V	V	V	V V	/	V	V V	V	V	V	V	X	V				
	Bioreactors	X	X	X	X	X	X	/	V V	V V	V V	V V	V V	V V	V	V	V V	V	V	V	V	X	V				
	CHEMICAL-PHYSICAL METHODS																										
	Chemical Extraction	V V	V V	V V	V V	V V	V V	V	V	V	V	V	V	V	X	V	V V	V	V V	V V	V V	X	V				
	Chemical Oxidation / Reduction	X	X	V V	X	X	X	X	V	V	V	V	V	V	V	V	V	V	V	V	V	X	V V				
	Dehalogenation	X	X	X	X	X	X	X	X	X	V V	V V	V V	X	V	V V	V	V	V	V	V	X	V				
	Separation (Gravity, Magnetic,Physic)	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	X	V V				
	Soil Washing	V V	V V	V V	V V	V V	V V	V	V	V	V	V	V	V	X	V	V	V	V	V	V	X	V V				
	Solidification / Stabilization	V V	V V	V V	V V	V V	V V	V V	X	X	X	X	X	X	V	V	V	V	V	V	V	X	V				
	THERMAL METHODS																										
	Incineration / Pyrolisis	X	X	X	X	X	X	X	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	X	X				
	Thermic Desorption	X	X	X	X	V V	X	X	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	X	X				
	Capping	V V	V V	V V	V V	V V	V V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	X	V				
	Excavation and Landfill Disposal	V V	V V	V V	V V	V V	V V	V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	X	V				
OTHER																											
GROUNDWATER	BIOLOGICAL METHODS																										
	Bioremediation	X	X	X	X	X	X	/	V V	V V	V V	V V	V V	V	V	V V	V	V	V	V	V	/	X	V V			
	Monitored Natural Attenuation	X	X	X	X	X	X	X	V V	V	V	V	V	X	X	V	V	V	V	V	V	/	X	V V			
	Phytoremediation	V V	V	V	V	V V	V V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	X	V V	V V			
	CHEMICAL-PHYSICAL METHODS																										
	Air Sparging	X	X	X	X	X	X	X	V V	V V	V	V	V	X	V	V V	V	V	V	V	V	V	V V	V			
	Chemical Oxidation	X	X	V	X	X	X	/	X	X	V	V	V	X	V	V	V	V	V	V	V	V V	V V	V V			
	Electrochemical Oxidation	X	X	V	X	X	X	/	X	X	V V	V V	V	X	V	X	V	V	V	V	V	V	X	V			
	In-Well Air Stripping	X	X	X	X	X	X	X	V	V	V	V	V	X	V	X	V	X	V	V	V	V	X	V			
	Dual/Multi Phase Extraction	X	X	X	X	X	X	X	V V	V V	V V	V V	V V	X	V V	V V	V V	V V	V V	V V	V V	V	X	V			
	Permeable Reactive Barriers	X	X	V V	V V	X	V V	/	V	V	V V	V V	V V	V V	V	V	V	V	V	V	V	X	V	V V			
	BIOLOGICAL METHODS																										
	Bioreactors	X	X	X	X	X	X	X	V V	V V	V V	V V	V V	V V	V	V V	V	V	V	V	V	X	V	V			
	Lagoons	V V	V V	V	V V	V	V V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	/	V	V			
	CHEMICAL-PHYSICAL METHODS (water extraction and transfer to a suitable plant)																										
	Advanced Oxidation Processes	X	X	X	X	X	X	/	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V	X	X	V			
	Air Stripping	X	X	X	X	X	X	X	V V	V V	V V	V V	V V	X	X	V V	V V	V V	V V	V V	V V	X	X	V			
	Active Carbons	V	V	V	V	V	V	/	V V	V V	V V	V V	V V	/	V V	V V	V V	V V	V V	V V	V V	X	X	V			
	Pump and Treat	V V	V V	V V	V V	V	V V	V	X	V	V	V	V	V	V	V	V	V	V	V	V	X	X	V			
	Ionic Exchange	V V	V V	V V	V V	V V	V V	V V	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	V			

Tab. 1 Summary of environmental restoration techniques, based on ISPRA's Remediation Technologies matrix, 2018. The classification is subdivided into contaminated compartment (soil or groundwater), applied method (biological, chemical-physical, thermal) and types of techniques (in situ, ex-situ, other). The effectiveness of the technique in relation to the specific contaminant is also reported: V good, VV very good, X inefficient.

In bioremediation technologies, biological organisms and in particular microorganisms, already present in the soil or inoculated from external sources, implement particular biochemical processes that destroy or render harmless the contaminants they come into contact with [33,104].

The FAO book on Soil Pollution [33] describes the requirements for having biodegradation of contaminants in the soil (a study carried out by Alexander, 1999). First the organism must be present in the contaminated soil; this organism must have the necessary enzymes to be able to carry out biodegradation processes; moreover, the contaminant must be accessible to the organism. The enzymes involved in the various biochemical catalysis reactions must be adequate, both extra and intra-cellular. Finally, the conditions in the soil must be optimized to allow proliferation of the potentially active microorganisms, as these are generally few due to contamination.

Christopher Chibueze Azubuike *et al*. [105] have produced a diagram that shows the recovery methods based on bioremediation technologies, differentiating those that can be implemented in-situ and those that need to be excavated (or pumped) and transferred elsewhere:

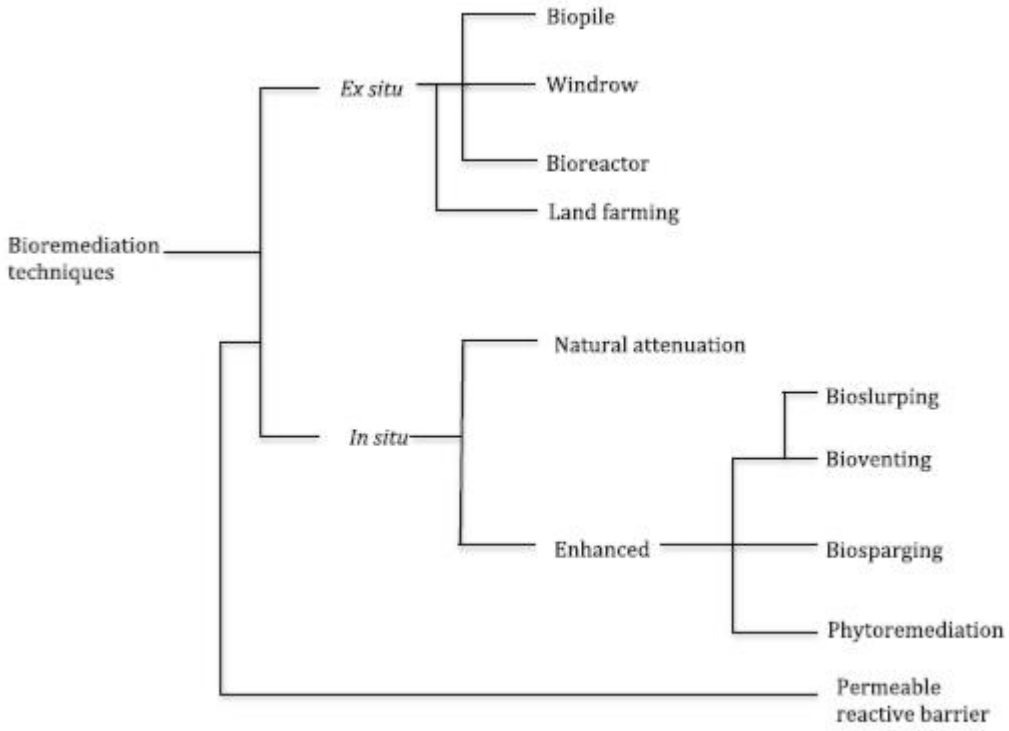

Fig.6 Bioremediation techniques, Christopher Chibueze Azubuike et al.*2016*

The technology defined as Bioventing involves pumping or in any case introducing and circulating oxygen (air) into the contaminated environment. This methodology also includes the contribution of nutrients to better promote the biological processes of contaminant removal: it is called biostimulation and concerns the addition of natural or synthetical fertilizers to provide essential nutrients such as nitrogen, phosphorus, sulfur and trace essential elements. Biostimulation was the methodology applied in this thesis work and in previous studies [11] to treat a soil heavily contaminated by hydrocarbons. As a stimulating agent, meat and bone meal (MBM), a compound obtained from meat industry residues, was used. MBM is rich in carbon (≈ 30%), nitrogen (≈ 8%), phosphorous (≈ 5%) and calcium (≈ 10%) mainly in slow-soluble form. Magnesium, sodium and potassium, as well as oxygen are also found [11,106]. The N:P ratio of this product ranges from 0.5 to 2 and

C:N ratio from 3 to 4 [11]. Given the considerable nutrient content, MBM is used as a fertilizer for agriculture and as an alternative source of phosphorus for the industry (for example for the production of phosphoric acid and phosphates) [106]. Recently the researchers are studying and evaluating a possible use of MBM in the remediation of contaminated soils and waters. In particular, its use as a stimulator of indigenous soil microorganisms, which operate the biodegradation of organic contaminants such as hydrocarbons [11,14,90], but also as an adsorbent material for ionic contaminants in solution, such as Lead [43,44]. In fact, it is thought that having high content in phosphate groups gives the MBM adsorbent properties, for example against heavy metals. From the studies previously conducted on the use of MBM as a soil fertilizer and also considering the results obtained in this thesis, it can be stated that it does not involve a significant variation in soil pH and therefore does not negatively interfere with microbial activity.

As can be seen also in the table shown before (Table 1: ISPRA matrix, aerobic bioremediation), Bioventing (combined with Biostimulation) techniques are particularly efficient for organic compounds such as hydrocarbons [1,25,55,57,107–109].

Other natural soil improvers include sawdust, wood chips, bark, straw, plant waste and food waste, manure and sewage sludge. The use of some of these products is particularly important from the point of view of the circular economy, as their use as micro-organism biostimulators also overcomes their disposal problem [33,91,92].

New technologies to treat contaminated land effectively and conveniently are currently a subject of study. For example, it is of great interest the use of biochar to remove contaminants, improve environmental conditions for the microbial community and reduce greenhouse gas emissions [110,111].

Other techniques involve the use of nanoparticles, such as nZVI (nano-Zero-Valent-Iron), carbon nanotubes and magnetic attractors [33,47,112]. Restoration is also employed through the use of electrokinetic techniques [26,107,113], especially for metal ions, polar and water-soluble compounds.

Phytodepuration can be used for the remediation of contaminated sites. Some plants act on the contaminants present in the soil (or in water) either directly, as in the case of phyto-extraction of metall(oid)s through the roots, but also indirectly, for example by producing particular compounds that improve the bioavailability of the contaminating molecules. Furthermore, the presence of plants in a soil optimizes the environmental conditions for microbial life [15,78].

To induce the removal of contaminants in the soil, surfactants (**surf**ace **ac**tive **a**ge**nts**) compounds are also added [12]. They are characterized by wide ranging properties including the lowering of surface and interfacial tensions of liquids. Their presence can lead to an increase in concentration of hydrophobic compounds in solution thanks to the emulsion and solubilization phenomenon those compounds produce. As the water solubility of contaminants is a rate-limiting factor for microbial activities in the soil, surfactants would be able to improve the bioremediation processes, especially in the case of less soluble organic molecules, as in the case of PAHs [12,42,114].

These compounds can be produced synthetically or biologically by various living organisms, such as microorganisms, plants and animals, including humans. Surfactants are molecules with polar hydrophilic portion, usually named “head”, and hydrophobic non-polar portion, called “tale” (they are amphipathic compounds). This feature is particularly important in the soil system, especially for the effects at the water / oil interface [12]. Surfactants are classified according to the residual ionic charge present on the polar part of the molecule: therefore, cationic, anionic, non-ionic and zwitterionic (which have both cationic and anionic charges) surfactants can be distinguished.

In the experiments conducted and reported in the present thesis work, a surfactant compound was added in half of the experimental tests. In particular, cyclodextrin (CD) was used, which is an oligosaccharide formed by bacteria during the enzymatic degradation of starch [24].

The most used cyclodextrins are the α-, β-, and γ-CD, which contain 6, 7, and 8 monomeric glucopyranose units, respectively. These molecules are torus-shaped: the hydrophilic part is external, which provides them with high aqueous solubility, while the internal cavity is hydrophobic and, in these sites, the organic contaminants can be chelating [12,42,114,115]. Cyclodextrins are biodegradable, non-toxic, and relatively stable in a wide range of physico-chemical conditions.

β-CDs are particularly interesting in the use of soil remediation as they are the most economical and are structurally suitable for complexing environmentally relevant organic compounds, such as PAHs [116]. Furthermore, this type of cyclodextrin can be doped to increase its solubilization capacities.

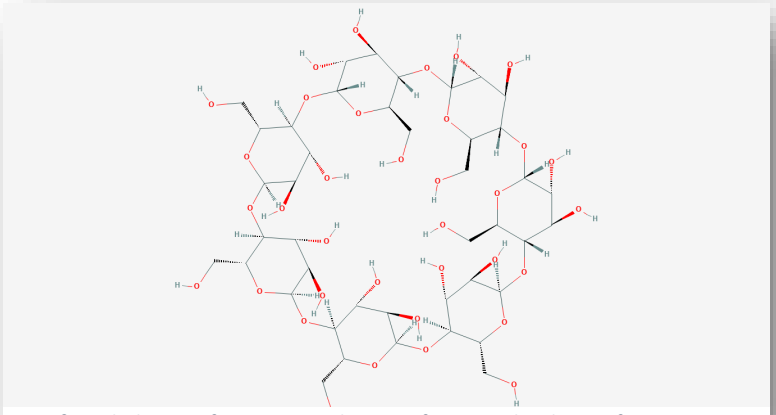

Fig.7 β-Cyclodextrin, from National Centre for Biotechnology Information. PubChem Database. beta-CYCLODEXTRIN

2. Materials and Methods

2.1. Soil

The soil used for experiments was collected in Päijät-Häme Waste Disposal Ltd (PHJ), the Kujala Waste Center (https://www.phj.fi/in-english/), and it was in detail analysed by Eurofins (Eurofins Environment Testing, Finland Oy, Lahti) in October 2018.

Consistent amount of soil was sampled on 10.10.18 and stocked in Lahti University garage through the winter. The soil has been defrozen on 06.02.19 in University laboratory for a week, for further use in our experiments.

2.2. Meat and Bone Meal:

Physical and chemical data obtained from the meat and bone meal (MBM) analysis are shown below: Name: Remsoil®, Remrapid
Form: Grain, grainsize 1-3mm
Color: Brown, fawn
Moisture content <3%
Organic matter 66%
Raw material and nutrients: In accordance with Regulation (EC) No 1069/2009 of the European Parliament. Nitrogen (N), Phosphorus (P), Calcium (Ca), Magnesium (Mg), Sodium (Na), Sulphur (S), Potassium (K), Boron (B), Zinc (Zn) Lead (Pb) < 0.30 mg/kg; Copper (Cu) < 4.00 mg/kg; Cadmium (Cd) < 0.10 mg/kg; Iron (Fe) < 60,0 mg/kg; Mercury (Hg) < 0,01 mg/kg.

Because of its composition, it was found to be a good stimulator for the bioremediation of organic hydrocarbon polluted soils [11,14,90]. MBM nutrients are slowly released and used by microorganisms, therefore they are not able to leach to the surrounding area and possibly affect negatively the environment. It has been proven that MBM product does not change the pH-level of the soil. It is reported in literature that it can be used as HM immobilizer due to his chemical composition [43,44]. At last, it doesn't contain any substances or pathogens which are harmful to the humans or animals.

Previous studies on MBM application as microbe booster for the biodegradation processes in the soil [11] showed that, if it is used in too high concentration, it gives too much competing substrates for the microbes. In this case the limiting factor, oxygen, is consumed to degrade the meat rather than the oil.
Considering this effect and according to the previous studies and results [11,90], we used an MBM concentration in our treatments 1% in soil (v/v), which should be an efficient proportion to be used as N and P source, and maybe a little C-source as well.

2.3. Cyclodextrin

We used a 50% (w/v) aqueous stock solution of methyl-β-cyclodextrin (Cawasol W7MTL, Wacker Chemie AG, Germany). 1% v/v solution of cyclodextrin (CD) in tap water was prepared and used for the chosen treatments. Previous test [24] showed that high concentration of CD (i.e.5% v/v) dissolves oil compounds more, even too fast, so that microbes are not able to degrade the oil that become bioavailable. In the study, 1% CD was enhancing the biodegradation in a good way, while 5 % was not. Authors of the experiments concluded that different levels should be tested in each new case because many factors can affect the bioavailability process in every specific kind of soil.

2.4. Reagents and solvents:

clean and sterilize: ETAX Aa, 99.5% pure ethanol, Altia Oyj

oil extraction: clean-up column, Florisil ® 60 – 100 mesh, for chromatography, VWR chemicals

water removal: Na_2SO_4 anhydrous VWR chemicals prolabo, ANALAR NORMAPUR

solvents: Acetone Hipersolv Chromanorm for HPLC, VWR chemicals

n-hexane ≥ 95% HiPerSolv Chromanorm ® for HPLC, VWR chemicals

Suprapure nitric acid 65% Merck

standards: oil analysis (Diesel and Lubricant), Bam Calibration Standards. Internal standard for chromatography integration window: nonane Merck VWR International, tetracontane 98% Acros organic

HM analysis: internal standard pureplus AA standard Pekinelmer, standard solution XIII ICP multielement Merk

Ultra pure water (UP): PureLab Ultra, ELGA

2.5. Instruments

Gas Chromatography GC-FID: 6890N Network GC System Agilent Technology

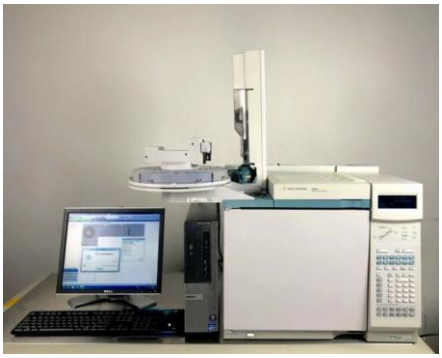

Fig.8 GC-FID: *6890N Network GC System Agilent Technology*

Chromatographic column is a Zebron ZB-5HT Inferno™ capillary column (length 15 m, inner diameter 320 µm and phase thickness 0.1. µm).

Flame ionization detector (GC-FID, Agilent 6890N).

Air generator connected with GC-FID: Zero Airgenerator mzAGC1L Labgas Instrument Company

GC oven temperature programme was started from 50 °C (hold 2 min), increased at rate 20 °C/min to 320 °C and hold for 10 min. The injection port and detector temperature were 320 and 340°C respectively. Hydrogen gas flow ratewas set at 35 mL/min and air flow at 350 mL/min. Helium makeup gas was delivered at a rate of 25 mL/min.

Parameters were chosen on results obtained from previous study, to allow the most effective detection of analytes.

Mass spectrometer coupled with plasma source

The instrument used for heavy metals analysis was Elan 6000 ICP-MS (Perkin Elmer SCIEX)

Fig. 9 Elan 6000 ICP-MS, Perkin Elmer SCIEX

Microwave digestor:

The treatment to analyse the HM in the soil started with the mineralization of the solid matter (almost everything was digested, except of silicates compounds contained in the soil). MARS 6 One Touch Technology CEM Corporation was used, equipped with PTFA containers, turntable and optical thermometer.

Temperature was hold for 20 min at 200°C.

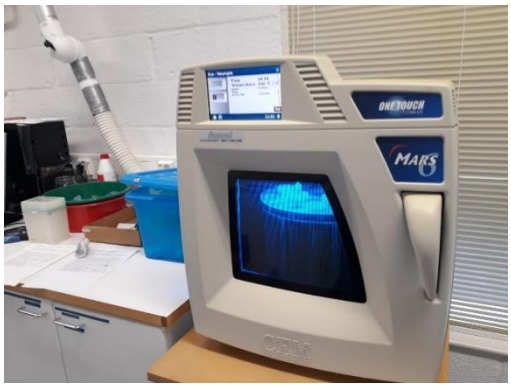

Fig. 10 MARS 6 One Touch Technology, CEM Corporation

Microbial community analysis:

AGEP: Thermo Electron Corporation Minicell^{R} Primo^{TM} Electrophoretic Gel System, Bio-Rad Gel Doc XR

Total DNA quantitation with picogreen: fluorometer Wallac Victor^{3} 1420 multilabel counter, PerkinElmer.

PCR: SimplyAmp^{TM} Thermal Cycler, Applied Biosystem^{R}

Fig.11 SimplyAmp ™ Thermal Cycler, Applied Biosystem ᴿ

pH measurement: pH-meter WTW inoLab series pH 720. The instrument has been washed with UltraPure water and calibrated before every use, according to the instruction given by the laboratory technicians

Centrifuge: Biofuge primo R Heraeus

Sonicator: Branson 8510

Microscopes: VWR BI500, LEICA S6E, Highlight 2001 Olympus Europe.

Washing mashine: DEKO 260 with 15 NF Deconex alkaline washing solution

Shaker: mechanical shaker for oil test tubes KUHNER shaker Lab-Therm

Vortex for plastic test tubes (HM final samples & DNA extraction) Heidolf Reax 2000

Oven: 80/105/160°C: Memmert Gmbh modell 100-800

550°C: Umega Snol 30/1100 Omron E5CK

Dessicator: vakuumfest schott duran glass (borosilicate 3.3)

Scale: Denver Institute Company TL403, Mettler AE 260 Deltarange

Incubators: -80°C UltraLow freezer CFC free Newbrunwick scientific C66085, -20°C whirlpool AFG 6592 B, -4 °C Rosenlew vahavirtanen eko system

2.6. Setup of the experiment

Experimental microcosm:

Two experiments have been set up: one batch for the oil test and one for heavy metals test. In both cases 20 glasses (450 mL) have been prepared, 4 different treatments, 5 replicates each. The treatments are: 1) Co = control 2) M = Meat and Bone Meal 3) C = cyclodextrin 4) CM = cyclodextrin and MBM.

The experimental setup oil and heavy metals tests is the same; the only difference concern the amount of water that has been added, as it will be explained later.

Plastic straws have been inserted in HM glasses test only, to allow the sampling of the leached water from the soil. About 100g of granite gravel has been washed with UP water and positioned at the bottom of the glass to enhance aeration and make the water sampling easier. A circular piece of geomembrane has been placed on the top of the gravels, to separate soil and rocks and avoid packing of the soil through the rocks.

Fig.12 A photograph of the prepared tests is reported: on the left there are the treatments for degradation of the hydrocarbons, while on the right the metals test. The beakers of the latter also contain a straw, which allows the suction of water percolated through the soil (about 20 mL each sampling), of which the metal content will then be analyzed.

The soil has been defrosted for 1 week. Later, the soil has been sieved and homogenised. In a certain amount of soil meat and bone meal has been added in quantity of 1% w/w at the beginning of the experiment. This is the soil that has been used in treatments M and CM, for both oil and HM tests.

Approximative weights of materials used: glass 350g, soil 250g, gravels 100g, brown fabric 0.83g

Watering schedule:

The importance of a proper content of water in the soil to allow the microorganism to work as efficiently as possible is well known [56,94]. We kept the amount of water in the soil around 60% of the water hold capacity, like it is suggested in literature and in previous study of our research group [11].

We have been watering once a week. The water used during the experiment was normal tap water of the Department, because deionized water would be harmful for the living organisms.

For the HM test, when the leachate was needed, we overloaded the system with water, to get about 20 mL of leachate at the bottom of the glass, that would be later taken out, treated and analysed with ICP-MS instrument, according to Almalab protocol (University of Helsinki, Faculty of Biological and Environmental Science, Lahti).

Cyclodextrin has been added in C and CM treatments (cyclodextrin and cyclodextrin/meat and bones meal) every time we water the glasses. The concentration of the solution used is 1% $V_{cyclodextrin}$ / $V_{tap\ water}$. This concentration has been used according to the previous study [24] where cyclodextrin was tested with different concentration of 1% and 5%. According to the results obtained, the 1% V/V concentration showed the best

biodegradation enhancement, probably due to the solvation effect on hydrocarbon molecules given by cyclodextrin [42,114,116].

Sampling schedule:

Oil hydrocarbon samples were taken every two weeks (T2, T4, T6, T8, T10, T12). About 2 g of soil was sampled from each glass and transferred in a test tube (Kimax 15 mL). Subsequently, slightly modified "International Standard protocol ISO 16703:2004(E)" for oil extraction and analysis with GC-FID has been done, as it will be explained in detail later.

HM water extraction and analysis has been done with a different schedule compared to the oil. This is because we expected that washing out of the available heavy metal would be immediately effective and significant in the first period, while in time the concentration released would be less, if physico-chemical parameters are steady. Thus, we sampled washout leachate every week for the first three weeks, and then at the week n° 6 (half time of the experiment) and week n° 12 (end of experimental period): T1, T2, T3, T6, T12. The pH of the leachate samples has been measured every time. The method used to collect and treat those samples will be explained later. The analysis and quantification were carried out using ICP-MS instrument, according to the protocol set up by Almalab analytical laboratory, University of Helsinki, in Lahti.

Fig.13 A test tube containing the acidified aqueous solution is shown, ready to be analysed with ICP-MS

HM content in the soil has been analysed at the beginning, half and at the end of the experiment, as well as the pH of the soil in that period. Before the analysis with ICP-MS, soil samples have been digested using microwave digestor [47].

2.7. Basic parameters monitored: WHC, moisture, OM, T, pH

The Water Hold Capacity of the utilised soil has been measured.

The formula applied was: $WHC = \frac{mwet\ soil - mdry\ soil}{mwet\ soil}$ [110]

To completely wet the soil, we kept soaking about 50g of soil, 5 replicates, in a funnel with a thick paper filter for 16h. Then we collected the wet soil and note the weight. After this the soil has been dried in the oven at 105°C for 16h and the weights were taken.

To obtain the Organic Matter amount, we moved the dry soil in the oven (550°C, 16h) and we weighed the material left, which is the Inorganic Matter in the samples. The relation between dry soil and inorganic material content gives the organic amount in the soil.

For the oil test, the water content of the soil has been kept about 60% of the WHC, which amount has been used in previous laboratory study and it was found to be optimal for microbial biodegradation [11,24,56]

Concerning the HM test, the amount of water added was the same as in oil test, with different amount when we needed to collect the leachate sample. In that moment, 100% of the WHC was reached, plus circa 20 mL of water, that would be washing out through the soil, ready to be sampled.

The temperature has been monitored with a thermometer placed in laboratory.

The pH of the soil for both oil and HM test has been measured at the beginning, half time and at the end of the experimental period, using a pH-meter for liquid solution. Therefore, we diluted one part of soil with 5 parts of UltraPure (UP) water, i.e. transferring in 100 mL flask 10 g of soil and 50 mL UP water, stirring for 1h and let it settle down 1h. After this procedure the pH can be measured. This method is part of AlmaLab protocol for pH measure using VWR 720 pH-meter (University of Helsinki, Lahti, spring 2019).

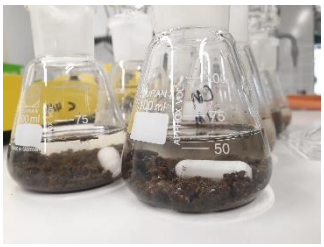

Fig.14 Ultrapure water and soil samples were transferred in volumetric flasks, the latter provided with magnetic stirrer.

The instrument report the temperature of each solution, that has been noted every time we measured.

The pH of the leachate (HM test) has been measured whenever we collect the samples. Of the total 20 mL of leachate, collected in plastic test tubes, about 5 mL were transferred in another test tube and treated for the HM analysis, and circa 15mL were used as such for the pH analysis.

2.8. Procedure for oil quantification, HM analysis and microbial community study

2.8.1. Oil hydrocarbons

The procedure to extract and purify the oil contained in the soil samples and the consequent quantification analysis is based on the International Standard protocol ISO 16703:2004(E): Soil quality – Determination of content of hydrocarbon in the range C_{10} – C_{40} by gas chromatography. The protocol has been slightly modified to be the most suitable for our experimental purpose.

The method leads to the quantification of oil hydrocarbon content in field-moist soil samples by gas chromatography. It is applicable with oil mass fraction between 100mg/kg and 10000 mg/kg soil (dry weight). According to the chemical results produced by Eurofins (https://www.eurofins.com/), the soil we used contained up to 9000 mg/kg of oil, which concentration is therefore within the method limits.

The method is not applicable for the quantitative determination of hydrocarbon $<C_{10}$ from gasoline.

Qualitative information is given by the peaks pattern of the chromatogram, and of the boiling point of the different n-alkanes reported in attached table B of ISO protocol.

First, the retention-time window (RTW) standard solution has been prepared. To do so, 15 µL of n-nonane and 15 mg of n-tetracontane were dissolved in 500 mL of n-Hexane and sonicated 30 min. This solution has been used as range-defining solution, as extraction solution for soil samples and for all the dilution steps of the hydrocarbon standard.

The hydrocarbon standard solutions for the calibration have been prepared combining proper amount of two different stock standard solution, one of diesel and one of lubricant oil, which concentration were 10000 µg/mL (ppm) in 10 mL volumetric flask. The table below report the amount transferred from the stock solution to six volumetric flasks, 10 mL, to prepare the standard solutions with incremental concentrations, used in the calibration. Standard solutions were diluted with defined volumes of extraction solution (RTW), until the concentrations sought were reached:

C [µg/mL]	10	50	250	500	750	1000
V [µL]	10+10	50+50	250+250	500+500	750+750	1000+1000

Tab.2 The concentrations of Diesel and Lubricant Oil in the standard solutions are reported, as well as the volumes taken from the stock solutions for their preparation.

The amount of volume transferred refers to both stock solutions (i.e. 10 µL withdraw from diesel stock solution + 10 µL from lubricant oil stock solution).

Extraction and clean-up procedure:

2 g of homogenized field-moist soil has been sampled into a glass test tube (11 g) and 4 mL of acetone were added. After a gentle shaking by hand, 2 mL of RTW-extraction solution were added. The test tubes were closed and shaken for 1 h using mechanical shaking, 200 rpm.

Fig. 15 A snapshot showing soil samples added with extraction solution (n-hexane) and acetone

After the solid material were settled down, the supernatant part was transferred into a new test tube.

(At this point, the solid part left was placed in the oven, 105 °C for 16h, and scaled, to have an exact amount of moisture in each soil samples and related dry weight needed to express the final oil concentration in the samples).

Then, to clean the solution from acetone, which could interfere with the determination (non-polar and weakly polar compounds, or high content of polar compounds can be interferent) and to remove the water originally present in the sample, 2.5 mL of UP water were added, and the test tubes were shaken by hand thoroughly for 5 min. This step has been done twice.

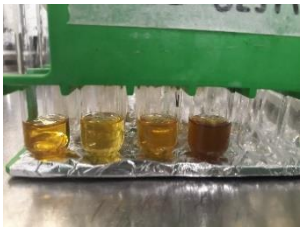

Fig.16 The samples obtained from the first extraction procedures are shown. These organic extracts still show colorations.

The organic layer obtained were collected in a new test tube. Enough activated sodium sulphate (160°C for 16 h), about 1/4 of teaspoon, was added. This reagent is needed to remove any water from the solution which could be harmful in subsequent steps. If no lumps were formed, it means that the water was removed from the organic solution.

The clean-up column to purify the organic solution from polar compounds and PAHs has been prepared immediately before use. Pasteur pipet were filled with 0.5 g of activated Florisil ® (160°C, 16 h), with small amount of cotton placed at the bottom of the column to avoid the loss of Florisil ®. The extracted solution was scrolled through the clean-up column, and the entire eluate was collected in a GC-vial and closed immediately.

Fig. 17 The column prepared to purify the organic extracts

Dilution of the extracted samples were needed to fit the calibration curve: the first three extractions request a dilution 1:10 (eluate: extraction solution). After, the concentration of oil in soil decreased, so a dilution 1:5 was enough.

The purified extract was analysed with GC-FID.

Determination by gas chromatography:

the analytical quantification of hydrocarbon fraction C_{10} – C_{40} was done with Agilent Tech 6890N GC-FID instrument. The chromatographic column is a Zebron Inferno ZB-5HT.

The measurement of purified oil extraction samples, blank, control solution (RTW-solution) and calibration standards were done under identical gas chromatographic conditions. The method utilized in the analysis has been reported in paragraph 5.5.1. Appropriate instrumental analysis sequence has been setup before the run.

An external calibration has been performed every time we used the GC-FID instrument, analysing six different dilution of hydrocarbon standard solution, plus the zero concentration (only RTW-solution analysed). The standards covered the working range. By linear regression analysis of the signal results, we obtained the equation of the calibration function which has been used to quantify the oil concentration in each sample.

Using the instrumental software Chemstation, the chromatogram peaks obtained were integrated separating the diesel fraction (C_{10} – C_{21}) from that of lubricant oil (C_{22} – C_{40}). The integration started at the retention time just after the end of n-nonane peak, which is at the signal level in front of the solvent peak (n-hexane). The integration ended just before the beginning of n-tetracontane peak. All chromatograms have been checked visually for correct integration.

Fig. 18 The purified extracts are shown, ready for GC-FID analysis

The mineral oil content of the soil samples has been calculated using the equation:

$$Csoil = Cextract * \frac{V}{m} * f$$

$$Csoil\ is\ the\ hydrocarbon\ mass\ fraction\ of\ the\ soil\ sample, in \left[\frac{mg}{Kgdw}\right]$$

Cextract $is\ the\ hydrocabon\ mass\ concentration\ of\ the\ extract\ calculated\ from\ the\ calibration\ function, in\ [\frac{mg}{L}]$

$$V\ is\ the\ volume\ of\ extraction\ solution\ used, RTW - solution, in\ [mL]$$

$$m\ is\ the\ dry\ mass\ content\ of\ the\ soil\ sample$$

$$f\ is\ the\ dilution\ factor$$

Comparation of oil concentration between different tests and differences in time, together with statistical analysis of the data, has been done, as it will be said later in the result.

2.8.2. Heavy Metals analysis:

Heavy metal behaviour has been monitored during the experiment. Leachate and soil concentrations were obtained using ICP-MS instrument. The protocol followed for samples preparation is set up by Almalab laboratory manager, Helsinki University, Lahti.

Leachate analysis:

About 20 mL of water leachate through the contaminated soil were collected using Hanke Sass Wolf 100 mL syringe and transferred in a plastic test tube. Centrifuge (1449 x g) was made, for 5 min at room temperature of 20 °C. 5 mL was then poured in a 10 mL NORM-JECT ® HSW PP syringe where a 0.2 µm PES filter has been attached. The sample was filtered and collected in a 15 mL PP tube. 1 mL was then transferred in a new test tube and diluted 1:5 with UP water. Concentrated suprapure nitric acid (65%) was added to reach a concentration of 2% v/v (100 µL). 50 µL of internal standard solution (Indium) was added. The PP tube was closed and vortex to mix the sample.

Blank sample was prepared in the same way, without leachate solution.

Soil analysis:

Soil samples were digested in concentrated HNO_3 in MARS 6 microwave unit to extract the total metals contain. 0.1g of soil were placed in PTFA container and 10 mL of nitric acid were added. The tubes were closed and placed into the instrument. An optical thermometer was placed in a real sample to sense and control the temperature.

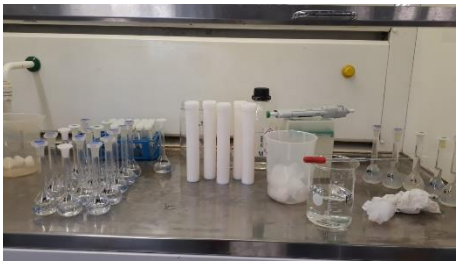

Fig.19 Material used to contain soil and nitric acid samples during MW digestion

The instrumental program used was optimized by the laboratory to specifically digest soil samples (200 °C, hold time 20 min). When cooling stage was over, we carefully transferred quantitatively the sample in a 50 mL volumetric flask inside fume hood (pressurized NOx and acidic fumes could be released). The volume of the flask was then filled up with UP water.

If the solution presented dissolved particles, centrifuging was done. 0.5 mL of the sample were transferred in PP test tube and dilution 1:10 with purified water was done to obtain a concentration of nitric acid 2% v/v, requested for ICM-MS analysis. 50 µL Internal standard was added, and the PP test tube vortex briefly.

Blank and control sample (multielement metalloorganic standard) were prepared.

The analytical quantification with ICP-MS Elan 6000 was done by the laboratory technician, who processed the instrumental data and gave us the results.

2.8.3. Microbial community analysis:

Soil sampling and storing:

approximately 1g of soil has been sampled from each glass, both oil and HM tests, and placed in a plastic test tube. Particular attention has been paid for the sampling procedure: 70% ethanol diluted solution was used twice to clean the sampling spoon for every different glass, avoiding cross contamination between soil community.

Once the samples were named, they've been stored in -80°C freezer, ready to be processed for the study of the microbial community

B10-DNA extraction-140321:

DNeasy ® PowerSoil ® Kit Handbook, for the isolation of microbial genomic DNA from all soil types, QIAGEN. The Kit is intended for molecular biology application and experiments with recombinant DNA, certified by ISO Quality Management System.

The method uses patented Inhibitor Removal Technology ® for isolating genomic DNA from environmental samples, even with high humic acid content, such as compost, sediment and manure. A wide variety of organism can be detected from the isolated high-level purified DNA including bacteria, fungi, algae and actinomycetes.

0.25 g soil samples were lysed and homogenized. The use of a buffer help soil particles to be dispersed, humic acid dissolved and nucleic acid being protected from degradation.

SDS (sodium dodecyl sulphate) and other disruption agents were added to complete cell lysis and break down fatty acid and lipids associated with the cell membrane of several organism.

Samples were vortexed at maximum speed for complete homogenization and cell lysis, by a combination of chemical agents' reaction and mechanical shaking. After the vortex step, centrifuge 10,000 x g was done to separate the suspended fraction.

Supernatant was transferred in a clean collection tube. A reagent that can precipitate non-DNA organic and inorganic material, including humic substances, cell debris and proteins, was added. Those reagents are part of patented Inhibitor Removal Technology (IRT) and are important to remove contaminating matter that may reduce DNA purity. The solution was then centrifuged, and the supernatant collected in a new and clean tube, avoiding the transfer of any pellet.

At this point, a high-concentration salt solution was added to allow DNA binding tightly to silica (present at the bottom of the specific collection tube called MB Spin Column), but not non-DNA organic and inorganic material that may still be present at low level. With centrifuging, contaminant pass through the filter, leaving only DNA bound to the silica membrane.

Ethanol based wash solution was added to further clean the DNA bounded to the silica filter membrane, removing residual salt, humic acid and others eventual contaminant. Centrifuge was done to collect and discharge the flow-through.

Sterile elution buffer (which lacks salt) was placed in the centre of the silica membrane, allowing the DNA to be released after centrifuge and collected in a clean collection tube.

Obtained DNA has been stored frozen at -20°C, until further analysis: agarose gel electrophoresis, quantitation of total DNA and qPCR standard using picogreen method, PCR Amplification of bacterial 16S rRNA using overhang primers, sequencing (in another location, Meilahti Miseq).

B11-AGE-140324. Agarose gel electrophoresis:

The aim of the technic is to check if DNA extraction procedure gave a pure product and separate the different length of double stranded DNA. In electric field, DNA and RNA move towards the positive pole because nuclei acids are negatively charged. Short fragments move faster and further than the long one through the porous gel. Bands are made visible using ethidium bromide dye and UV light.

Fig. 20 Purified DNA samples are loaded onto agarose gel for subsequent electrophoresis

The agarose gel (1.5%) has been prepared weighting 3 g agarose to 500 mL Erlenmeyer flask. 200 mL of 1xTAE (tris-acetate EDTA) buffer were added and mixed. The solution has been heated in MW oven and cooled down. 10 µL EtBr 10 mg/mL added to obtain a final concentration of 0.5 µg/mL. The solution has been poured to the tray, air bubbles removed with a sterile pipet tip, and cooled down until the gel is formed. The tray is placed into the electrophoresis tank, and 1xTAE is added to submerge the gel in the buffer solution before AGE run.

6x loading dye has been mixed with the DNA-extract sample and loaded into a well of the gel. Ladders (GeneRuler 100 bp Plus, Thermo Scientific) were loaded in different well of the gel. Electrophoresis has been done at 100V for 1 h, and photo of the gel under UV light was taken (UV-illuminator BioRad).

B19-Quantitation of total DNA using picogreen method-150211:

The Quantitation of total DNA has been done according to "Quant-iT™ PicoGreen® dsDNA reagent kit" (Life Technology) manual by Almalab technicians, who gave us the results of the analysis.

The volume of the colour reagents for the working solution are based on the protocol by Jukka Ekman (Department of Applied Chemistry and Microbiology, University of Helsinki). Standards are made by Almalab.

Quant-iT™ PicoGreen® dsDNA reagent is an ultra-sensitive fluorescent nucleic acid dye for quantitating double-stranded DNA in a solution. The instrument used for the quantification is fluorometer. Measurement is based on the study of $\lambda_{excitation}$ and $\lambda_{emission}$ of the double-stranded DNA and Picogreen reagents complex. (Picogreen: $\lambda_{excitation}$ = 480 nm, $\lambda_{emission}$ = 520 nm). The linear detection range of the Quant-iT™ PicoGreen® assay in a standard fluorometer extends from 25 pg/mL to 1,500 ng/mL.

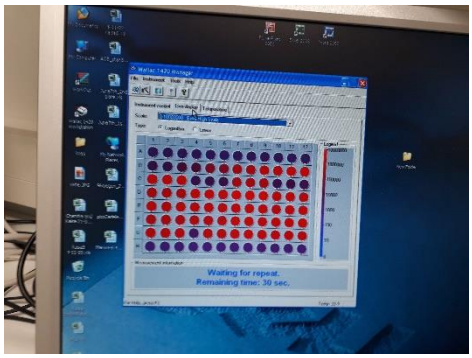

Fig. 21 Purified and Picogreen-treated DNA samples are subjected to fluorometric analysis. Red represents large amounts of DNA in the sample, while blue represents low amounts

B12.1-PCR Amplification of bacterial 16S rRNA with regular PCR using overhang primers:

Chosen region of bacterial DNA was amplified using primers which hybridize to the boundaries of 16S rRNA molecules. The polymerase chain reaction was done by Almalab laboratory technicians according to the protocol B12.1 of the Department of Biological and Environmental Science, University of Helsinki, Lahti.

A typical amplification program for the Thermal Cycler used in the laboratory is below.

Initial denaturation at 98°C for 5 min followed by 25 cycles with denaturation 94°C for 1 min, annealing for 10 s at 50°C, extension for 1 min at 72°C, final extension for 10 min. Cooling at 4°C.

The evaluation of the PCR products is done with method B11-AGE-140324, Almalab.

After the soil sample preparations, 20 µL of the final products will be pipetted in 96-plate well and sent to Meilahti Miseq for sequencing.

2.9. Statistical analysis:

As regards the biodegradation test of hydrocarbon oils, the Univariate Analysis of Variance (two-way ANOVA) was performed using the SPSS Statistics software package (IBM). Meat and Bone Meal and Cyclodextrin have been set as factors. To check the normality of the data, Kolmogorov-Smirnov and Shapiro-Wilk tests were done. Levene's Test was used to check the Equality of Error Variances.

3. Results and discussion

Some basic soil parameters were initially measured, such as the water content and the Water Hold Capacity, the amount of Organic Matter (OM), the initial soil pH and the temperature of the room in which the experiment was performed. Over twelve weeks, or the time span of the experiments, aliquots of soil were systematically sampled for the analysis of hydrocarbons, metals (soil and leachate) and pH, as described in the Materials and Methods chapter. Furthermore, about one gram of soil was sampled from the beaker of each treatment and stored in a freezer (- 80 ° C). From these samples the total purified DNA present in the soil was subsequently extracted: the extraction product was then subjected to Agarose Gel Electrophoresis (AGE) and to the total DNA quantification, to check if and how much DNA was actually present. Once the presence of DNA was confirmed, amplification and subsequent sequencing were performed. The results of the sequencing have recently been produced and are currently being processed. When this will be completed it will be interesting to see if and how the native microbial community of the soil in question (prokaryotic cells as r-RNA 16 S was analysed) varies over time, depending on the quantity of hydrocarbon contaminants.

3.1. Measurement of basic soil parameters: moisture and WHC, Organic Matter and pH

The water content in the soil is one of the fundamental parameters influencing the biodegradation reactions of hydrocarbons in the soil. Initial soil moisture was measured by placing some soil samples in the oven at 105 ° C for 16 hours. The weight of the dried soil was removed from that of the initial moist soil to obtain the quantity of water present. Below is the results table for the analysis of the water content.

#	1	2	3	WEIGHT [g]
beaker	53.05	48.6	46.64	
beaker and soil	70.01	70.47	69.94	
Soil	16.96	21.87	23.3	
beaker and dry soil	66.78	66.11	65.33	
dry soil	13.73	17.51	18.69	
moisture II %	19	20	20	20

Tab.3 Moisture calculated on day zero soil (beginning of the experiment): 105°C oven was used to dehydrate soil samples.

The starting soil contain about 20% of moisture (water), means that the rest 80% is dry matter. The soil samples used for the analysis of hydrocarbons have also been dried to correlate the exact weight of dry soil to the relative soil sample, since the final hydrocarbon concentration (Diesel or Lubricant Oil) is expressed in $mg_{oil}/g_{dry\ weight\ of\ soil}$.

Throughout the experimental period all treatments were moistened weekly for the duration of the experiment, immediately after turning the soil into the beaker to favour the supply of oxygen, another key element in aerobic degradation reactions. In particular, the amount of water calculated to reach 60% of the Water Hold Capacity (WHC) has been added. As far as the metal test is concerned, 100% of the WHC value was exceeded of about 20mL of water whenever a leachate sample was obtained, i.e. the first, second, third, sixth and twelfth week. BIOHIT m5000 pipettes have been used to transfer the necessary water quantities.

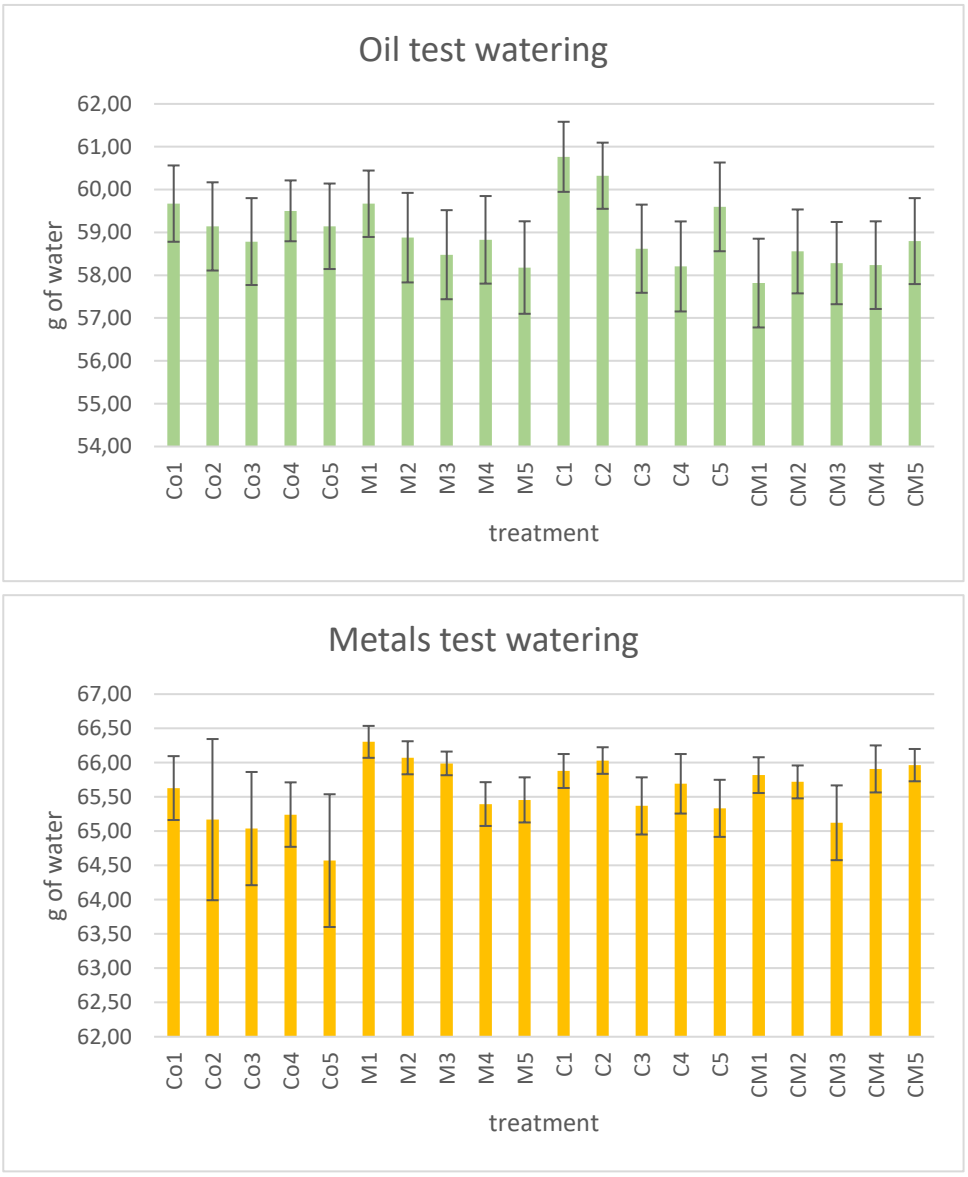

Fig.22 Average amount of water, in grams, contained in each treatment during the experiment, and the related fluctuations in time (±SD). The amount of water contained in each beaker was kept around 60% of the WHC.

Previous studies on the biodegradation of hydrocarbons have shown that a water content in the soil equal to 60% of the WHC value favours the metabolic activity of microorganisms [11]. The table below shows the result we obtained for WHC calculation:

Tab.3 The weights of initial soil, dry soil and wet soil after soaking 16h are reported. The average value, given by the average of the values obtained by applying the formula for the calculation of WHC, is highlighted in yellow.

#	1	2	3	4	5	WEIGHT [g]
cup	2.2	2.19	2.17	2.17	2.18	
cup and soil	48.42	48.91	48.51	48.4	47.17	
soil	46.22	46.72	46.34	46.23	44.99	
cup and dry soil	36.84	37.2	35.98	37.09	35.46	
dry soil	34.64	35.01	33.81	34.92	33.28	
wet soil	54.54	55.41	51.56	53.13	53.11	
WHC	0.36	0.37	0.34	0.34	0.37	0.36

This parameter reflects the amount of water, in grams, that the soil can keep in certain condition, i.e. temperature of the room. In our case, WHC of 0.36 (36%) means that the soil can keep almost half of its weight of water, for example 200g of dry soil can ideally hold 72 mL of water. The moisture in the soil has been kept around 60% of the WHC during the whole experiment period, which amount should be a favourable quantity for microbial biodegradation of hydrocarbons. In the previous example, if 72 mL is the maximal capacity of the soil to hold water, circa 43 mL would be the optimal amount calculated. We used those relation for the whole watering work during the three months experiment.

The Organic Matter present in the soil (OM) at the beginning of the experiment and that contained after 3 months was quantified. This is an important factor for metabolic reactions of soil organisms, but also for chemical-physical reactions that occur in this matrix: in fact, the OM content is linked, for example, to adsorption, complexing, chelation and soil acidity. Eight samples of the starting soil have been dried in oven 105°C and later at 550°C for Loss On Ignition (LOI). OM contained in the soil was calculated by subtraction (1 – IM), and the average result for the day zero soil was 12.25%. The amount of Organic Matter present in the soil at the end of the experimental period is reported in the table below, as average values of five replicates for each treatment.

Tab.4 The average values, expressed in percentage, of Inorganic and Organic Matter are reported. Soil samples were taken at the end of the experiment, that is, after a period of three months.

#	IM %	OM %
Co	86.49	13.51
M	87.86	12.14
C	86.29	13.71
CM	86.40	13.60

In almost all treatments the amount of organic matter in the soil increased comparing day zero and endpoint of the experiment. The amount of Inorganic Matter in M treatment (Meat and Bone Meal) is higher compare to others, probably because of the chemical composition of the product itself (rich in phosphate groups and essential elements) that affect the relative C proportions in the samples [14,43,44,106].

It must be considered that no additional fertilizer has been added after the beginning of the experiment: it is therefore probable that most of useful nutrient for microorganism were uptake after 3 months. More Meat and Bone Meal could be added periodically to supply nutrients such as N and P throughout the experiment.

Further studies in this sense are necessary to verify if the biodegradative activity of microorganisms can be favoured by a constant intake of MBM in the soil.

As described earlier in the introductory paragraph, the pH value of the soil can be related to the metabolic dynamics of microorganisms. Moreover, the pH (together with the pE) affects the release and mobility of the elements present in the soil, such as metals [38,40,44,77]. Soil pH values were therefore measured before the beginning of the experiments (day zero soil). The protocol followed, developed by Almalab technicians with the use of VWR 720 pH-meter, involves mixing a part of soil with five parts of water (dilution 1:5). The pH result of the day zero soil was 6.29 and room temperature was T=22°C (on 26/02/2019).

The soil pH analyses done in the middle of the experiment T6weeks, and those made at the end of the experimental period T12weeks for both hydrocarbons oil and heavy metal test are reported below, as average values.

Tab.5 Average values obtained from soil pH measurements after 6 and 12 weeks from the start of the experiment. The soil of both tests was analysed: hydrocarbon oils (oil) and heavy metals (HM).

T6weeks 08/04			T12weeks 13/06		
Treatment	Oil	hm	Treatment	oil	hm
Co	6.57	6.64	C	6.80	6.82
M	6.66	6.69	M	6.78	6.75
C	6.53	6.72	C	6.73	6.91
CM	6.70	6.63	CM	6.85	6.98

Furthermore, pH hasn't changed that much during the experiment, at least at the time we measured it. It has slightly increased as the experiment progresses, probably because the microbial activity has decreased over time and consequently also the quantity of hydrogen ions (H^+) present in the soil matrix. As reported in literature, soil pH values may reflect the intensity of microbial activity [10,11,13,90].

In addition to those of the soil, the pH trends in the leachate collected for the analysis of HM were measured, immediately after sampling. The pH of these aqueous samples may also reflect the pH of the soil, since this is washed away and possibly even some ions present therein. It is well known that pH and redox conditions define mobility and possibly bioavailability of elements such as heavy metals: it is therefore important to measure the pH of the solution (correlated to the temperature) and then compare the concentration of the HM present. pH results of the water leachate (HM test) are reported below as average data.

Tab.6 Average values of pH and temperature of the solutions percolated by the various HM treatments are reported. pH I, II, III, IV and V respectively represent the first, second, third, sixth and twelfth week pH leachate analysed. The standard deviation from the mean value is shown in brackets

Treatment	pH I, T = 20.5°C, 01/03	pH II, T = 23°C, 08/03	pH III, T = 23°C, 22/03	pH IV, T = 22°C, 17/04	pH V, T = 24.5°C, 06/06
Co	6.95 (±0.07)	7.36 (±0.08)	7.62 (±0.08)	7.28 (±0.04)	6.95 (±0.05)
M	7.16 (±0.11)	7.60 (±0.24)	7.67 (±0.06)	7.29 (±0.11)	7.06 (±0.09)
C	6.97 (±0.09)	7.36 (±0.07)	7.69 (±0.14)	7.29 (±0.07)	7.02 (±0.03)
CM	7.08 (±0.08)	7.48 (±0.10)	7.58 (±0.21)	7.21 (±0.10)	7.05 (±0.10)

pH values are quite stable. The amount of Meat and Bone Meal and Cyclodextrin, added to enhance biodegradation of hydrocarbon compounds, didn't affect the pH significantly during the experimental period. It looks like there is a slight increment of the washout water pH at the beginning of the experiment. In the last period, those pH levels are decreasing. This effect could be due to the higher temperature values of the percolated aqueous solution, since the last measure coincides with the summer season (June), during which the laboratory room was also slightly heated.

3.2. Oil hydrocarbons degradation

The main objective of the thesis work was to evaluate the degradation and removal efficiency of hydrocarbon compounds present in the soil. In particular, two fractions were monitored: Diesel C_{10}-C_{21} and the Lubricant Oil C_{22}-C_{40}. As reported in literature, these organic compounds are used by some microorganisms naturally present in the soil (mostly bacteria and fungi) as an energy and carbon source. Hydrocarbons concentration trend in time was monitored in five different treatments of contaminated soil: control soil with no addition (Co), soil with 1% by weight of Meat and Bone Meal (M), soil and cyclodextrin (C) and finally soil with both cyclodextrin and MBM (CM). Previous studies have shown that the addition of the natural MBM fertilizer, as well as the use of biocompatible cyclodextrin surfactant, can improve the removal processes of hydrocarbon contaminants catalysed by microorganisms. Oil extraction and quantification were done every two weeks for twelve weeks in total, applying a slightly modified procedure based on the International Standard protocol ISO 16703:2004(E): Soil quality – Determination of content of hydrocarbon in the range C_{10} – C_{40} by GC-FID. Six standards with increasing concentration of Diesel and Lubricant Oil (10, 50, 250, 500, 750 and 1000 mg / L or ppm) were also analysed for calibration purpose.

Below is a typical calibration curve obtained from standards analysis plotted in graph. The equations resulting from linear regression were used to quantify hydrocarbon oil concentration on week n° eight:

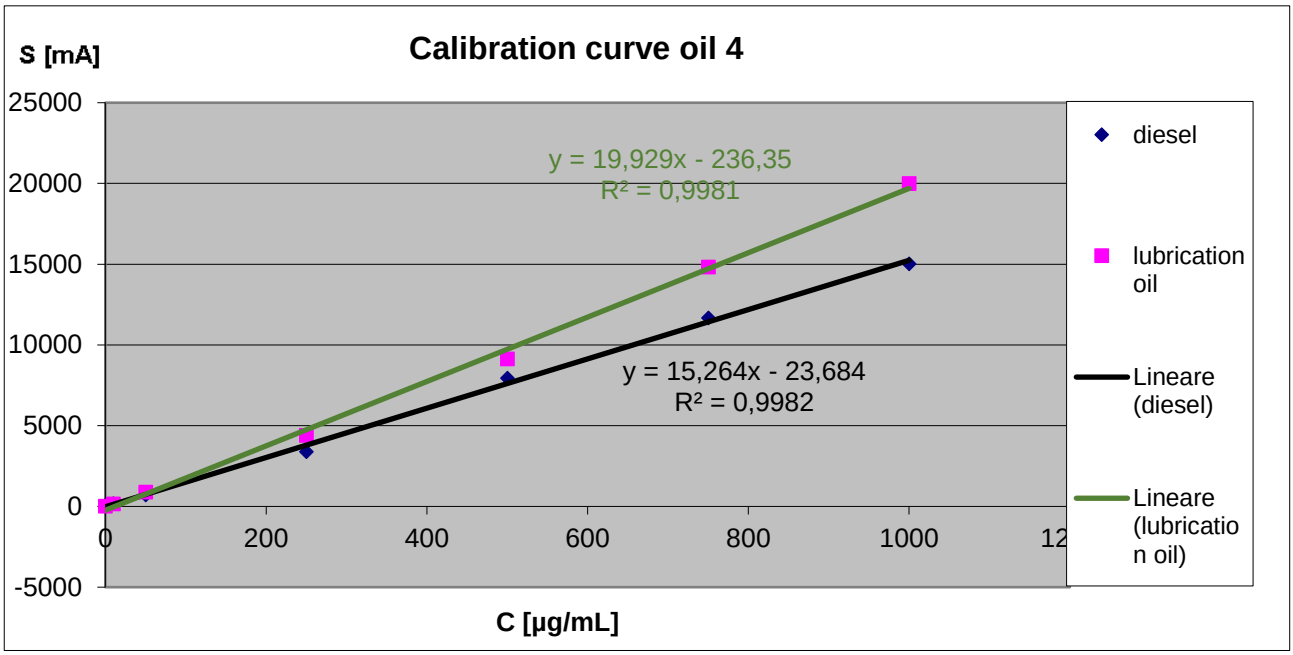

Fig.23 Calibration curves of Diesel and Lubricant Oil standards the relative instrumental signals are reported.

The GC-FID analysis of the standards, as well as for the various samples extracted from the soil, produces chromatograms in which it is possible to identify a specific pattern of the two different hydrocarbon fractions, Diesel and Lubricant Oil. In the left side of the graph, most volatile and short chain (C10 – C21) hydrocarbons, which is the diesel fraction, is recognizable. Those compounds are usually easier molecules to degrate by microbes and so it is the fraction that will decrease faster during the bioremediation processes [3,56,58,107,117]; on the right part of the chromatograph, it can be see the ubricant oil fraction which is formed by more carbon atoms (C_{22} – C_{40}), characterized by higher molecular weight and usually more recalcitrant to biodegradation [3,24,61,63,116,118,119]. In this fraction, molecules such as PAHs are included. With the help of the ISO 16703:2004(E) protocol and the advices of laboratory supervisor, signals of hydrocarbon fractions were integrated separately, so as to identify the relative concentrations.

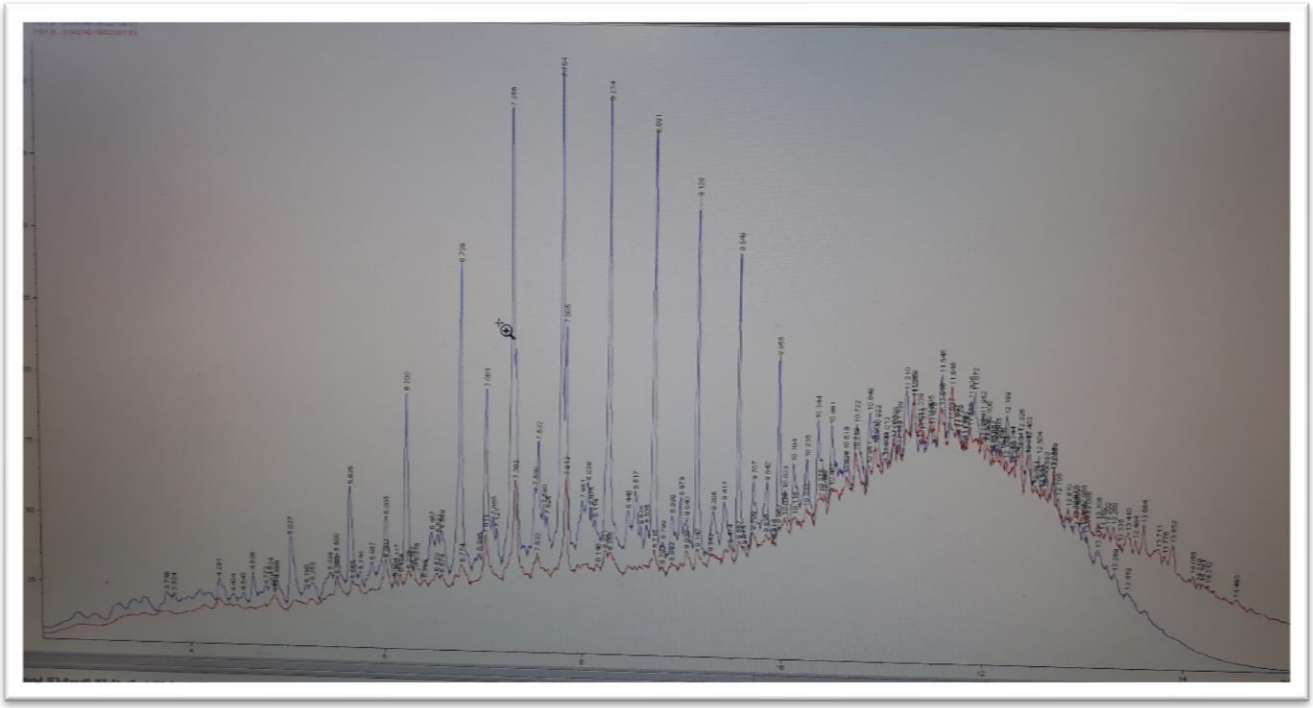

Fig.24 GC-FID chromatogram in which blue represents Standard 6 (1000 ppm) while red color identifies the concentration of Diesel and Lubricant Oil in the initial soil(T0). It is possible to note that the area of the two curves is similar, while in the 1000 mg / L standards there are high peaks falling within the Diesel fraction.

Purified oil extraction samples were analysed and similar chromatograph were obtained, where diesel fraction and lubricant oil were visible. Below the first result obtained from the analysis of day zero soil (T0 sample) toghether with the highest Standard 1000 ppm is reported. It is visible that signals for both samples are quite similar, which means that their relative concentration in solution are similar. The red curve represents T0 sample: the tale on the right of the peak is probably represented by various organic compounds which are contained in soil extract. GC-MS analysis could lead to the identification of those compounds [60,120,121].

As will be shown in detail in the following tables, two weeks since the beginning of the experiment, the concentration of the hydrocarbon oils was already decreased significantly in the various treatments, especially in the soil where MBM has been added (about 40% removal of the Diesel fraction in the first two weeks). After three months from the beginning of the experiments, most of the Diesel fraction was removed in all treatments, although this effect is more pronounced in soils added with MBM and cyclodextrin (CM). In this regard, the effects of cyclodextrin are not significant in the first experimental period, indeed it seems that the concentration of hydrocarbons, specifically the Lubricant Oil fraction, in the analysed solutions is

higher. This may be due to the fact that surfactants such as cyclodextrin are known to form complexes (inclusions) with many organics, PAH included [3,24,116], increasing the solubilization of the hydrocarbon oils, making the extraction of these more complete and therefore increasing the final concentration measured. However, soil analysis in the last experimental period shows lower concentrations of Diesel and Lubricant Oil in the CM treatments (cyclodextrin combined with MBM) than in the others, suggesting that the positive effects induced by cyclodextrin on the remediation of hydrocarbon contaminated soil may request some time [24,42,116].

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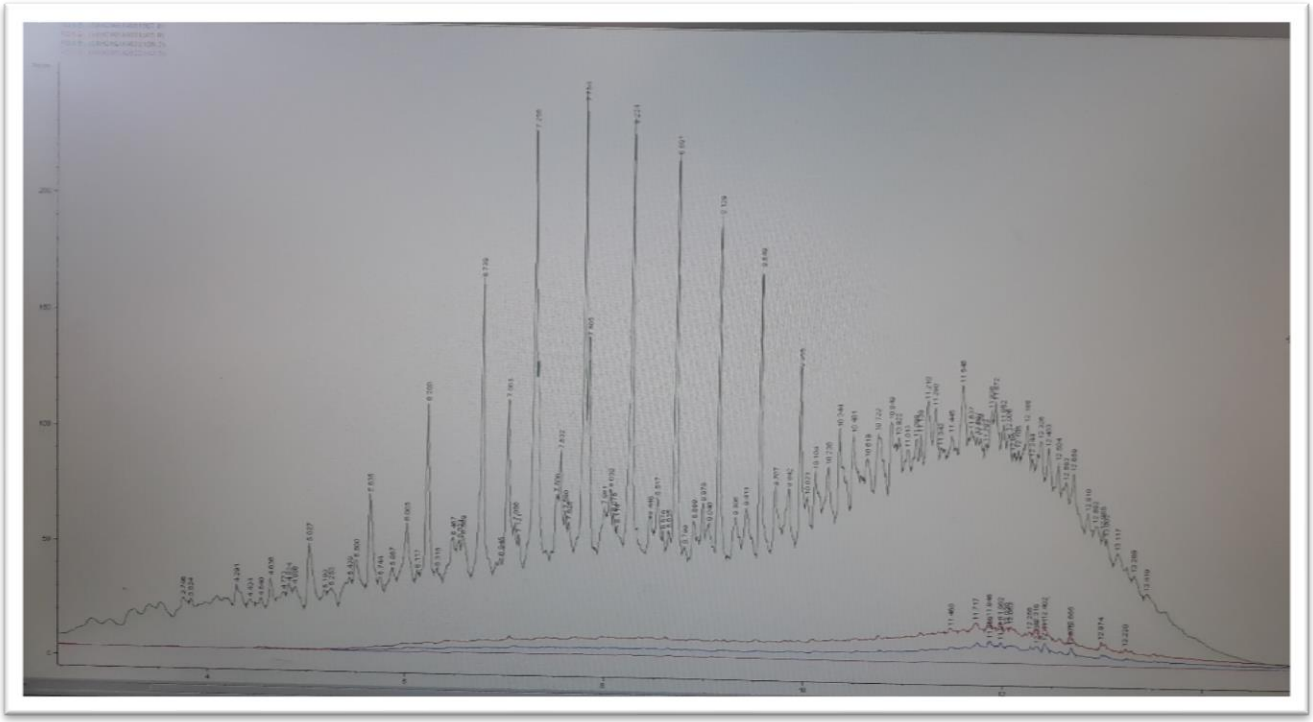

Fig.25 T10w: the green curve represents the standard 1000 ppm, while red and blue curves show respectively the signals obtained from the analysis of Co (Control soil) and M (MBM) extracts. Comparing this chromatogram with the previous one it is evident that a big amount of the hydrocarbon fractions has been removed. Fuchsia colour line is also visible, which represents the standard at 50 mg / L. We want to show that the concentrations of Diesel and Lubricant Oil at the tenth week approach the second lowest standard.

As regards the effects induced by the addition of MBM (in soil) on the degradation of hydrocarbons, as already noted by other authors previously [11,14,90], it can be said that this material acts as a booster in the kinetics of biochemical catalysis of organic compounds. MBM can in fact serve as a source of nutrients needed by cells, such as Nitrogen and Phosphorus, but also other important elements such as Calcium, Potassium and Magnesium.

3.2.1. C_{10}-C_{21} Diesel removal:

Averaged results obtained from the analysis of contaminated soil of the various treatments in three-month experimental period (T0 - T12 weeks) are shown below. Standard Deviation (SD) associated with the average of each value is shown in brackets. The initial Diesel concentration at time T0 weeks is the same in all treatments, since reference is made to "day zero" soil analysis. Looking down from top to bottom in the table, we note that the concentration of contaminants, expressed in mg of Diesel per kg of dry weight soil, decreases over time. The results obtained were initially calculated as µg of Diesel per g of dry soil, but this unit of measurement was transformed into mg / Kg for convenience, as the legislation usually uses this quantity. Furthermore, the values of the blank, measured and calculated at each hydrocarbon extraction procedure, was subtracted from the final figure as it influenced the results obtained in the last analyses.

Tab.7 The table shows trends over time of Diesel units in the various experimental treatments. This quantification was done using the GC-FID technique, following the extraction protocol for hydrocarbon oils described in ISO 16703:2004(E).

	Treatment			
t [weeks]	<Co>	<M>	<C>	<CM>
0	2880 (±207)	2880 (±207)	2880 (±207)	2880 (±207)
2	2150 (±185)	1410 (±528)	1930 (±221)	1600 (±373)
4	2200 (±768)	1390 (±366)	1850 (±534)	1140 (±348)
6	950 (±116)	720 (±169)	870 (±279)	510 (±114)
8	740 (±107)	570 (±109)	790 (±288)	430 (±140)
10	250 (±149)	230 (±47)	200 (±86)	150 (±16)
12	252 (±11)	250 (±46)	170 (±33)	120 (±26)
	C [mg/kg (dw)]			

The initial concentration of Diesel in T0 soil was 2880 (±207) mg/kg of dry weight. This amount is decreasing in time, faster in the first period (T0 – T6weeks) in most of the treatments, as visible in the degradation curve. In M and CM treatments (yellow and orange colours in the graph) the removal process of short hydrocarbons chain is working faster, probably due to the nutrient provided by Meat and Bone Meals mixed with the soil (1% w/w). In two weeks, Diesel concentration in soil decrease from 2880 (±207) mg/kg to 1410 (±528) mg/kg (dw) in M treatment (51.04 % of reduction), which is quite a high amount compared to Control dynamics where average concentration of Diesel was still 2150 (±185) mg/kg, (25.35 % removal). The presence of MBM is significantly important in the efficiency of degradation processes of Diesel compounds over time, as confirmed by UNIANOVA analyses (Sig <0.05 for T2, T4, T6, T8 weeks). Cyclodextrin treatments (C) do not show any enhancing in biodegradation of Diesel fraction compared to Control, at least at the beginning of the experiment. From the week n°6, concentrations in all the treatments are getting closer, and those where Meat and Bone Meal is present have still the lowest concentrations of Diesel hydrocarbons. Starting from one month after the beginning of the experiment (T4weeks) the CM treatment, which is a combination of Cyclodextrin and MBM, present the lowest concentration above all other treatments. Furthermore, at the end of experimental time, CM treatment is the one that contain less Diesel, 120 (±26) mg/kg (dw), which means total reduction of the starting amount of Diesel equal to 95.83 %. Control (Co) experiment, which represent monitored natural attenuation process, is characterized by a total removal after 12 weeks of 91.25 %, Diesel concentration 252 (±11) mg/kg (dw).
Trends in concentration described in table 6 are shown in the graph below. Biodegradation reactions of organic compounds generally follow first order exponential kinetics: this can be noticed following the yellow dot trend line (CM). As regards the results of the other treatments, tendency is not exactly of this type, especially with regard to Co and C, whose dynamics do not reflect the expected trends.

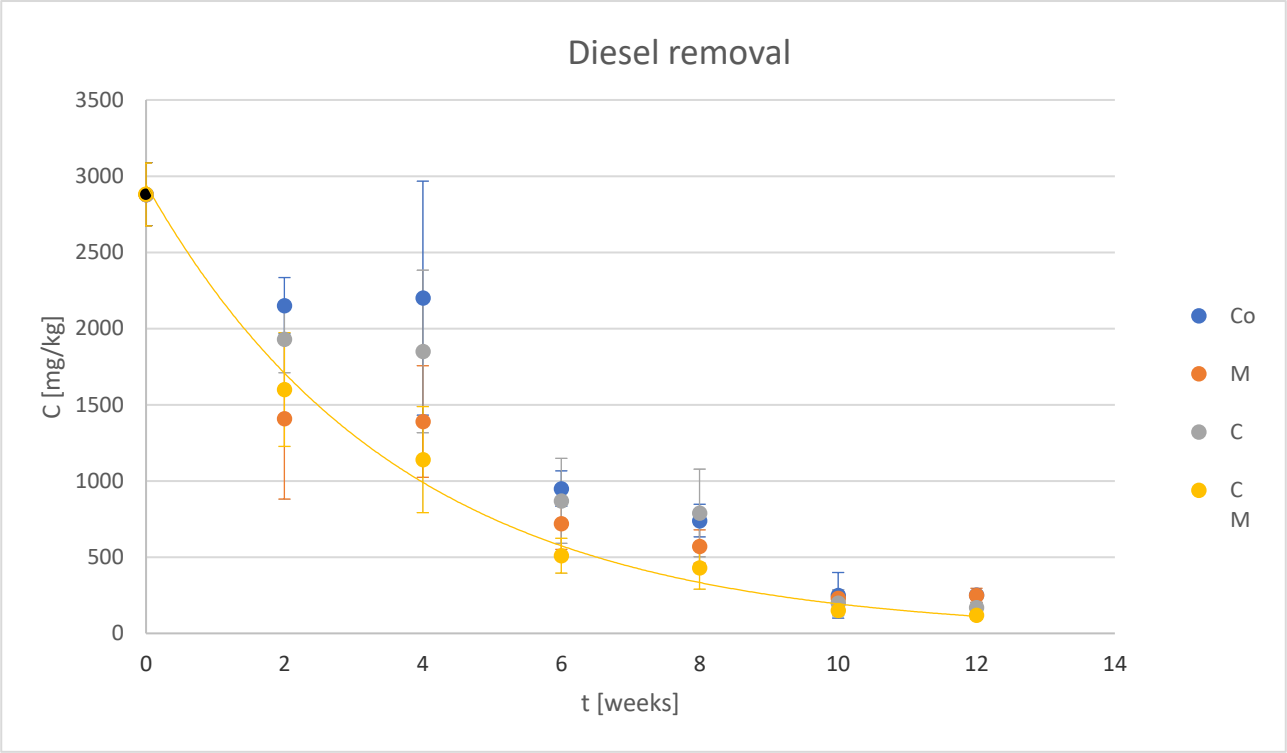

Fig.26 The results obtained from the GC-FID analysis of the organic extract of a soil contaminated by hydrocarbons are plotted. In particular, concentration of Diesel was analysed over time. There are different colours depending on the treatment applied (Control, Meat and Bone Meal, Cyclodextrin and combination of C and M). The degradation of the Diesel components (C10-C21) generally follows a first-order kinetic, which is fast initially and very stable over time.

3.2.2. C_{21}-C_{40} Lubricant Oil removal:

In addition to the Diesel fraction, the one defined as Lubricant Oil, which represents those compounds containing 21 to 40 carbon atoms, was analysed with GC – FID technique. The same procedure used for the extraction of the Diesel organic phase from the soil samples (ISO 16703:2004(E) was followed and concentration of Lubricant Oil present was quantified using the equation of the calibration line. Results are expressed, as well as for the Diesel fraction, in mg/kg of dry soil (dry weight, dw).

Tab.8 The table shows the concentrations, in ppm, of Lubricant Oil analysed in the contaminated soil of the various treatments. This hydrocarbon fraction is usually more refractory to (bio) degradation and may take longer to have a more complete removal. It is possible to see that, in general, concentration decreases over time in all treatments. There is an anomaly regarding the fourth week, where the values could be particularly high due to the sampling of an organic cluster, rich in Lubricant Oil.

	Treatment			
t [weeks]	<Co>	<M>	<C>	<CM>
0	3820 (±506)	3820 (±506)	3820 (±506)	3820 (±506)
2	3390 (±146)	2440 (±1250)	3300 (±500)	3140 (±856)
4	5090 (±1362)	2820 (±625)	4780 (±1108)	2950 (±813)
6	2460 (±339)	1670 (±505)	2720 (±819)	1490 (±282)
8	1910 (±260)	1360 (±137)	2920 (±791)	1480 (±403)
10	610 (±292)	530 (±97)	900 (±281)	720 (±113)
12	800 (±51)	720 (±57)	960 (±149)	720 (±100)
	C [mg/kg]			

Lubricant oil represents the heaviest fraction of oil we analysed in our experiment. It is known to be the most recalcitrant and complex part of hydrocarbons and it is characterized by different compounds in terms physical – chemical properties and ecotoxicological effect [56,92,94,120,121]. The breaking down process of Lubricant Oil in the soil is slower compared to the Diesel one studied before. In fact, it can be noted that the concentration between day zero (T0weeks) and T2weeks differ only of 11.3% comparing starting Lubricant Oil concentration and the one in Control after two weeks, which are 3820 and 3390 mg/kg (dw) respectively. Similarly to the analysis of the Diesel fraction, Meat and Bone Meal affected positively the hydrocarbon degradation in soil. Also, in this case, from the results of the GC-FID analysis of the purified organic soil extracts it can be seen that the removal of the hydrocarbon contaminants included in the Lubricant Oil fractions (C_{22}-C_{40}) is greater in soils treated with Meat and Bone Meal, as confirmed by the UNIvariate ANalysis Of VAriance (Sig. <0.05 for T4, T6, T8). This product seems to act mainly as a booster for microorganism, increasing degradation efficiency about 20% with Control treatments (in the first two weeks, Lubricant Oil decreased by 11.3% in Co and 36% in MBM. It should also be emphasized that the addition of MBM does not affect soil pH (see paragraph on pH measurements).

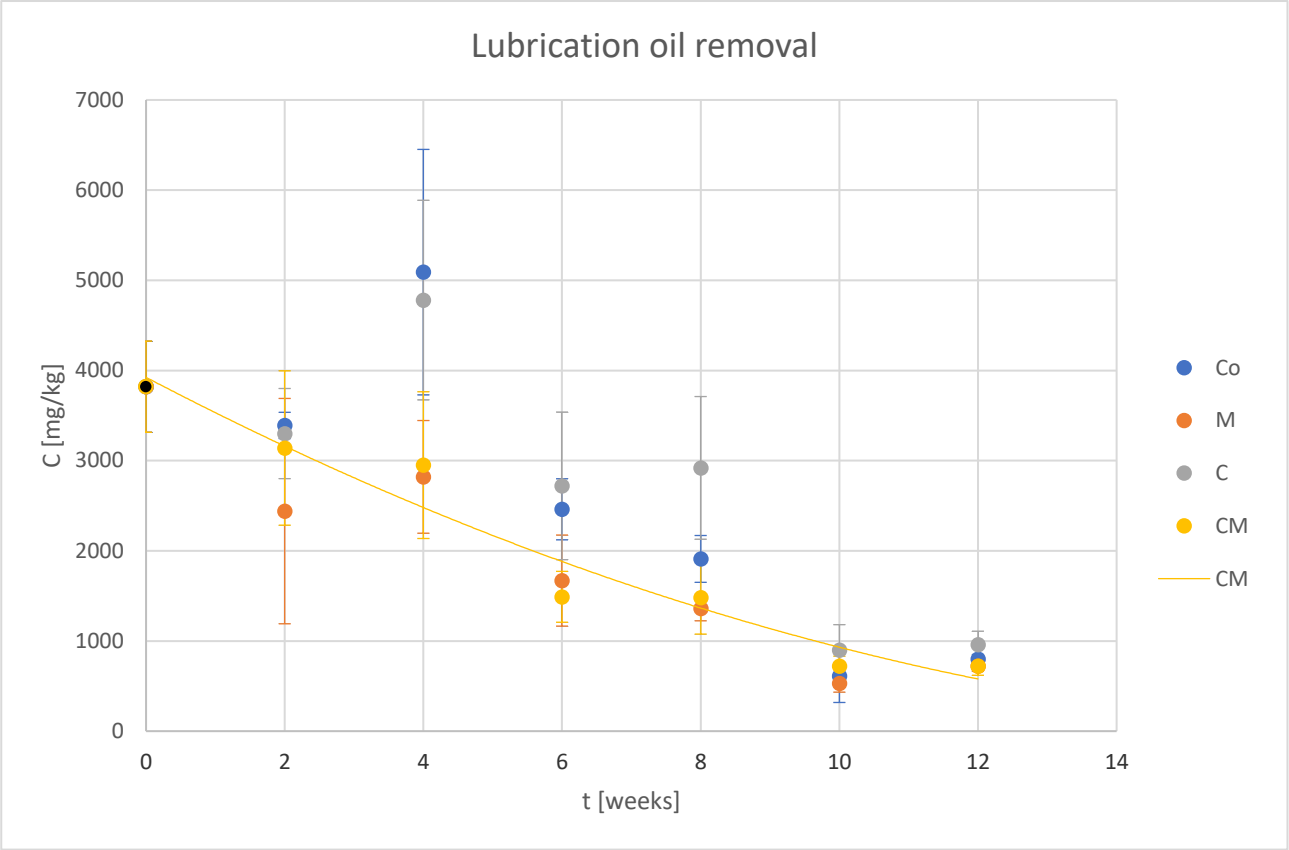

Fig.27 The decrease in concentration of Lubricant Oil in the test occurs more slowly than that of the Diesel compounds (C_{10}-C_{21}), as can be seen from the slope of the degradation curve. The example of the CM treatment is reported. Moreover, the decrease is not really regular over time: this may be due to both cluster samples particularly rich in C_{22}-C_{40} hydrocarbons, but also a possible non-uniform release of these molecules from the soil.

As can be seen from the graph above, the analysis carried out in the fourth week produced unexpected results with regard to the concentration of Lubricant Oil in the soil, especially in Co and C treatments (Control and Cyclodextrin). As mentioned in the caption of the graph, this anomaly could be bound to the fact that, even after careful sieving of the soil before the experiment started, dark clusters were still rarely present. These could be particularly rich in Lubricant Oil fraction, so sampling them with subsequent analysis could result in high concentration results. Another cause of these unexpected increases in concentration could be linked to the pH-Eh conditions that occur in the soil, even temporary. These could facilitate the release of C_{22}-C_{40} fraction molecules retained by soil particles, as in the case of PAHs. It is curious that in treatments containing Meat and Bone Meal (M and CM) this effect is not so pronounced. There may therefore be particular influences of this compound on the sorption processes, but such dynamics must be thoroughly investigated elsewhere.

3.2.3. Legal parameters in contaminated soil sites:

It may be interesting to compare the initial concentration values of Diesel and Lubricant Oil fractions and their trend over time with some national threshold or guideline values. In this thesis we focus on the Italian and the Finnish case.

The table below shows the values included in Italian legislation that regulates the concentrations of hydrocarbons admissible in soils, depending on the purpose of use of these (commercial and industrial, or public green). The reference legislation is the Legislative Decree 152/2006, Annex 5, Title V of Part Four. This Decree is defined "the Code on the Environment", which collects the various environmental regulations (on air, water, soil and waste) of both national and European origin

Tab.9 Concentration threshold of contamination (CSC) in the soil and subsoil referred to the specific use of the sites, based on Italian Legislative Decree 152/2006, Annex 5, Title V of Part Four. Light hydrocarbons are defined as those contaminants with less than 12 carbon atoms, while those that contain more are called heavy hydrocarbons. This classification differs slightly from the fractionation we have considered, namely C10-C21 for the Diesel compounds and C22-C40 for Lubricant Oil

# Substance	A Sites for public green areas, private and residential use (mg/kg expressed as dry weight).	B Sites for commercial and industrial use (mg/kg expressed as dry weight)
Light hydrocarbons with C ≤ 12	10	250
Heavy hydrocarbons with C > 12	50	750
Summation of aromatic organic products	1	100
Summation of polycyclic aromatic hydrocarbons (PAH)	10	50
Benzene	0.1	2

As regards the legislation in force in Finland concerning soil pollution, reference is made to the Government Decree on the Assessment of Soil Contamination and Remediation Needs 214/2007.

These threshold limits and the guide values, defined by the regulations in force in Italy and Finland, can be used to identify possible contamination of soils (and possibly subsoils). In particular, we can compare the results of the analyses obtained during the experiments to get an idea of the extent of soil contamination we studied. However, it must be remembered, as extensively described in the introductory chapter, that the comparison of values measured with table values is used as a screening for a possible contamination: if the contaminant concentrations are higher than the limits and guide values shown in the table, then it would be carried out a site-specific risk characterization. The latter will have to take into consideration various parameters that can determine a risk for human health, such as the intrinsic toxicity of the contaminants in question, the exposure to these and any pollutant transport factors, as well as the reactions that can the quantity of pollutants in the system.

Tab.10 Finnish legislation on limit concentrations and guide values of hydrocarbons in soil separates four fractions of these contaminants: Petrol fractions (C5 - C10), Middle distillates (> C10 - C21), Heavy petroleum fractions (> C21 - C40), Petroleum fractions (> C10-C40). The intervals in analysis in this thesis work coincide with middle distillates (in our case defined as Diesel) and heavy petroleum (Lubricant Oil) fractions.

# Substance	Threshold value (mg/kg expressed as dry weight)	lower guideline value (mg/kg expressed as dry weight)	Higher guideline value (mg/kg expressed as dry weight)
Petrol fractions (C5 - C10)	-	100	500
Middle distillates (>C10 - C21)	-	300	1000
Heavy petroleum fractions (>C21 - C40)	-	600	2000
Petroleum fractions (>C10-C40)	300	-	-

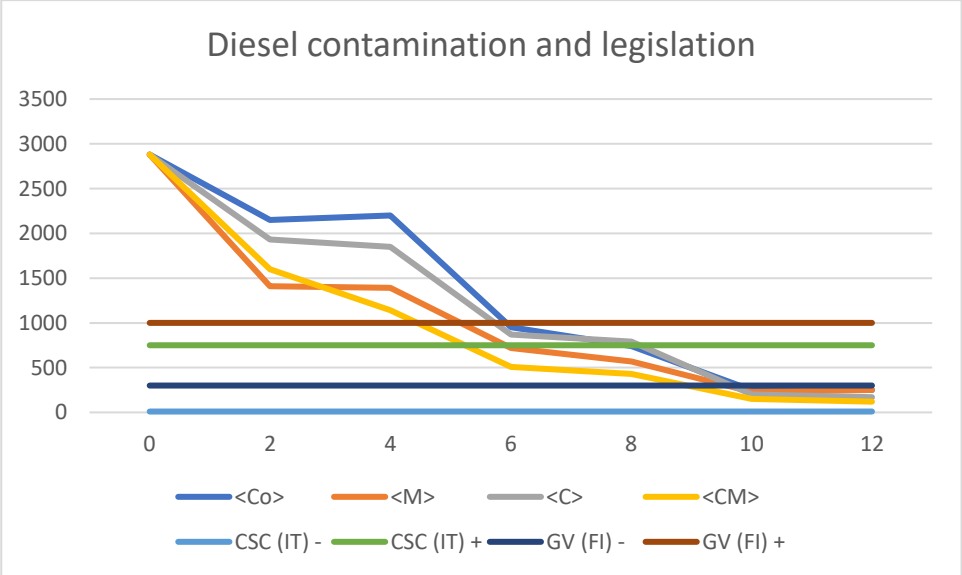

Fig.28 Average concentration values of Diesel contaminants extracted from the soil of various treatments (Co, M, C, CM) during experimental period are reported. The contamination threshold values (CSC, Italy) and the guideline values (GV, Finland), higher (+) and lower (-), defined by the respective national regulations, are then shown. It should be noted that the initial concentration of Diesel does not respect the limits defined by the law, while over time the quantity of contaminants leads to values falling within the norm. This decrease occurs more quickly in treatments containing the natural fertilizer product Meat and Bone Meal.

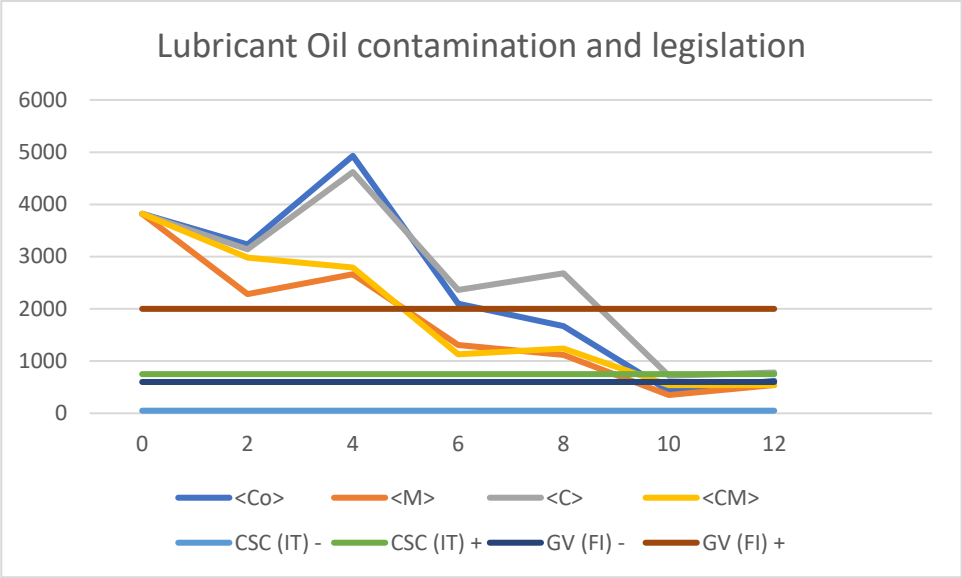

Fig.29 The trend over time of Lubricant Oil concentration in the soil of the various treatments (Co, M, C, CM) is plotted in graph. At the same time, higher (+) and lower (-) contamination threshold values (CSC, Italy) and guideline values (GV, Finland) are reported as constant lines. As in the case of the removal of Diesel compounds, even here the treatments containing soil added with MBM (M and CM) have reached concentration levels of Lubricant Oil according to the law (Italian and Finnish) up to two weeks earlier than the control soil and soil added with cyclodextrin alone (C).

The concentrations of Diesel and Lubricant Oil hydrocarbon fractions in the starting soil are higher than the legal parameters, both in Italy and in Finland. Looking at the legislation, we note that screening values according to which there is contamination (CSC in Italy and guideline values in Finland) are more restrictive in Italy, in the sense that acceptable concentrations of hydrocarbon contaminants are lower, especially for public green and residential areas. Six weeks from the beginning of bioremediation of the contaminated soil almost all the treatment analysed contain a Diesel concentration in the soil which is lower than 1000 mg/kg dw (higher guideline value, Finland). At week n° ten, all the treatment present values are lower than 750 mg/kg dw (threshold for commercial and industrial use sites, Italy), and some of them are near 300 mg/kg dw (lower guideline value, Finland). At this time, the most efficient hydrocarbon degradation has been obtained in CM treatment, where a certain amount (1% v/v added to tap water whenever treatments have been moistened) of methyl-β-cyclodextrin was added in the soil, in combination with 1% w/w of Meat and Bone Meal, which is a natural fertilizer [11,14,106,122]. At week n° twelve the best Diesel removal from the soil was still obtained in CM treatment: this can suggest that combination of methyl-β-cyclodextrin and Meat and Bone Meal could have a positive effect on hydrocarbons bioremediation. The increase in contaminants removal is possibly linked to the fact that soils treated with MBM contain more nutrients, necessary for the metabolic reactions catalysed by the indigenous microorganisms present in the soil, which use organic contaminants as carbon and energy source. MBM can supply the nutrients necessary for soil micro-organisms, in the initial moments when the need is high, as is the concentration of organic contaminants. Over time, even the effects of cyclodextrin, as this is a natural surfactant capable of increasing the bioavailability of organic molecules such as PAHs, increasing their solubility [11,24,42], seems to improve bioremediation processes.

Lubricant Oil degradation was higher in M and CM treatments, where the concentration at week n° six are lower than 2000 (higher guideline value, Finland) and at the end of the experiment lower than 750 mg/kg dw (sites for commercial and industrial use, Italy. Even if the bioremediation process removed a consistent part of organic pollutants in the studied soil, especially Diesel fraction, the final concentrations are still quite high.

The final values of petroleum fractions (C_{10}–C_{40}) measured in the soil at the end of the thesis work indicates that the soil is no more an environmental danger, but it can only be used in sites with industrial or commercial purpose, where the quality of the soil is not mandatory. Further studies could be carried out to see if an additional input of MBM into the soil, for example in the middle of the experimental period (sixth week), leads to an increase in the degradative efficiency of the contaminants present. In fact, in our study, MBM was only added at the beginning of the experiments, therefore a further addition of nutrients could again favour microbial metabolism. More time could also be needed to improve the Bioremediation of Diesel and Lubricant Oil compounds in the soil.

It has not been said that in the first two weeks of the experiments, some seeds of dicotyledonous plants have germinated (the classification has not been carried out and by not providing light to the plants, these have died in a short time). It might be interesting to see how the presence of such plants, supported in their growth i.e. by providing appropriate light, affects the biodegradation of hydrocarbons. In this sense, it is well known that plants favour the restoration of polluted soils [3,48,79].

GC-MS could be a useful tool to identify which specific organic compounds there are in the soil system, so as to be able to monitor which of these are removed faster and which ones are more persistent. Furthermore, through mass spectrometry, other interesting molecules can be identified, such as microbial biosurfactants and phytosterols.

Specific experiments should be tested to be able to detect and quantify the amount of hydrocarbons removed from contaminated soil through different mechanism than biodegradation, such as chemical degradation, volatilization of light compounds and leaching.

3.3. Heavy Metals mobility

In addition to the degradation processes of Diesel and Lubricant Oil that occurred in the soil within three months, we also tried to monitor the dynamics of some metals. In particular, the leached metals were analysed after an excessive addition of tap water (the WHC was reached, plus about 20 mL of water) at the first, second, third, sixth and twelfth week. Furthermore, the metals present in the initial soil were quantified in the middle of the experiment and at the end. The protocol used by the Almalab laboratory of the University of Helsinki (based in Lahti) has been followed, which involves the addition of Ultrapure nitric acid to a small amount of soil. The "digestion" of the soil takes place in a microwave oven. Subsequently we proceed to dilution of the digestion product, addition of internal standard (Indium) and instrumental analysis with ICP-MS. Details of the method are described in "Materials and Methods" chapter.

The metals that have been examined are chromium, cobalt, manganese, lead, arsenic, iron and zinc. Phosphorus concentration was measured as well. Some of these elements are linked to both environmental and human health issues as elements with toxic properties, defined as such by major global bodies and agencies such as IARC, FAO, EEA and EPA. As far as Phosphorus is concerned, this is not generally linked to harmful effects on human health (at least in the concentrations considered in this thesis), it could possibly cause eutrophication if present in large quantities, for example in a body water. Usually Phosphorus in the soil is added, together with other elements such as nitrogen, to increase fertility.

Given the importance of the pH in defining the form in which metals are found in the soil (in addition to the pH there is the redox potential of the system, i.e. Eh or pE), pH values were measured during the analysis of metals. In particular, pH of the leachate was measured immediately whenever sampling occurred. As regards the measurement of soil pH, this was added to a quantity of Ultrapure water (dilution 1: 5), stirred for an hour, after which the solution was measured with a pH-meter.

Values of pH and temperature are summarized in the table below and they are the same ones already shown above, in the paragraph concerning measurement of basic soil parameters. These values were automatically measured by the pH meter.

Tab.11 pH and temperature values obtained by analysing a) soil and b) leachate samples related to metal test are reported. It should be noted that these pH values do not vary significantly in the different treatments during the experiments.

a)

	t [weeks]			
#	0	6	12	
co	6.29	6.64	6.82	<pH>
m	6.29	6.69	6.75	
c	6.29	6.72	6.91	
cm	6.29	6.63	6.98	
	21	22	25	
	T [C°]			

b)

	t [weeks]					
#	1	2	3	6	12	
co	6.95	7.36	7.62	7.28	6.95	<pH>
m	7.16	7.6	7.67	7.29	7.06	
c	6.97	7.36	7.69	7.29	7.02	
cm	7.08	7.48	7.58	7.21	7.05	
	20.5	23.2	23.2	22	24.5	
	T [C°]					

From the results obtained it can be said that the pH remains almost constant in the three months in which the experiments were carried out. It is also positive that the addition of MBM fertilizer does not affect these pH values. Previous studies have concluded that other natural soil improvers, such as urea and organic waste, cause the soil pH to change, with considerable variation (pH> 8) [11,24,92]. These variations may adversely affect the microbial community present in the system and consequently the biodegradation reactions of the contaminants. Finally, the addition of cyclodextrin also does not significantly change the pH values.

The results obtained from the ICP-MS analyses of leachate and soil samples are now shown in graph. Values are expressed in mg $_{metal}$ / kg $_{soil}$ for the analysis of the metals in the soil, while in µg $_{metal}$ / L $_{solution}$ (ppb) in the samples coming from leachate of the various treatments. From the concentrations of metals measured in the different treatments, it is possible to see if and in what quantities the elements behave differently when Meat and Bone Meal is present in the system, and how cyclodextrin affects the dynamics of metals in soil, from the comparison of final values with those of the control treatment (Co, soil without any addition).

Chromium

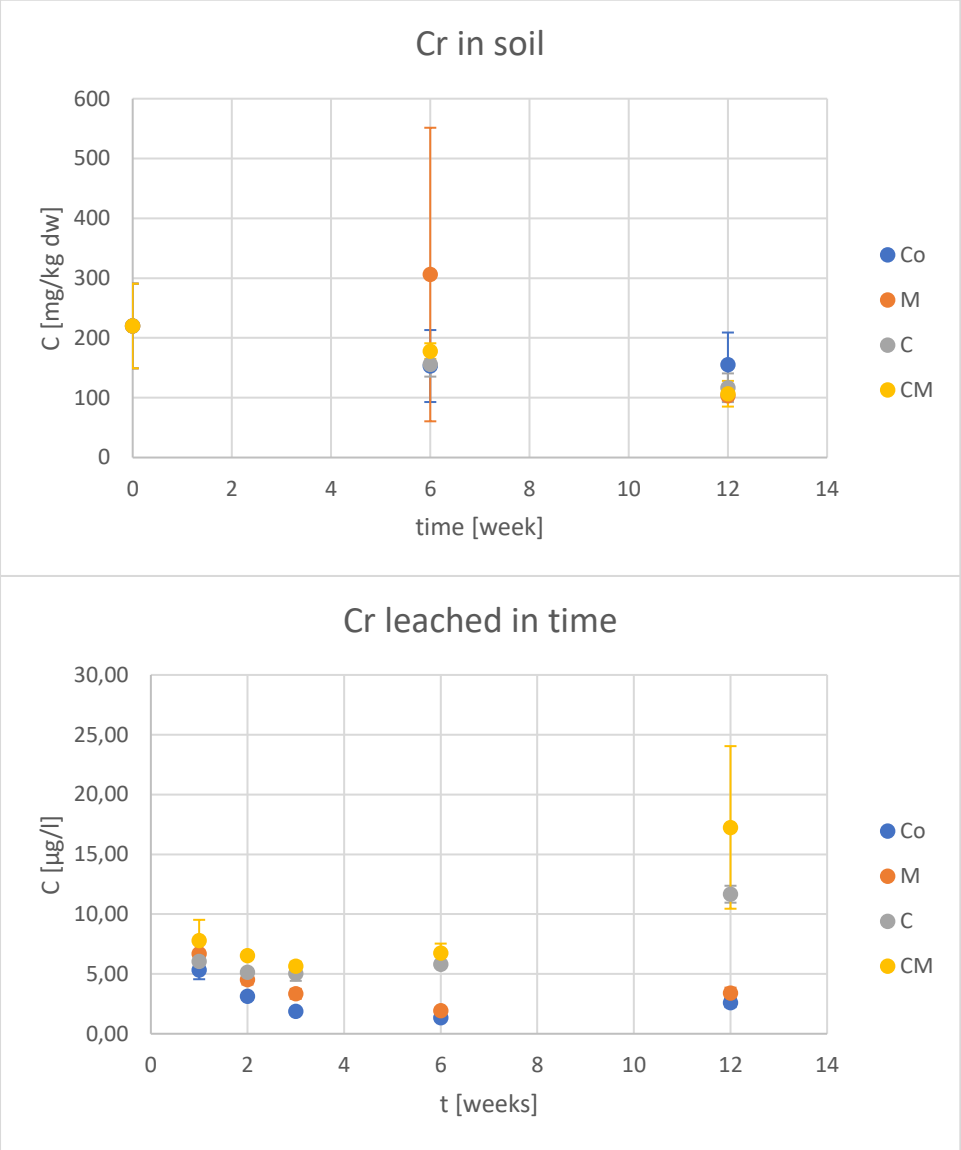

Fig.30 Concentration of chromium in the soil falls slightly within three months. In the graph representing concentrations of chromium in the leachate, we note that treatments in which cyclodextrin, and in particular methyl-β-cyclodextrin, is added weekly (C and CM) contain a slightly higher quantity than the others. This is possibly due to the fact that cyclodextrin forms inclusions with elements and molecules, increasing their solubilization.

Cobalt

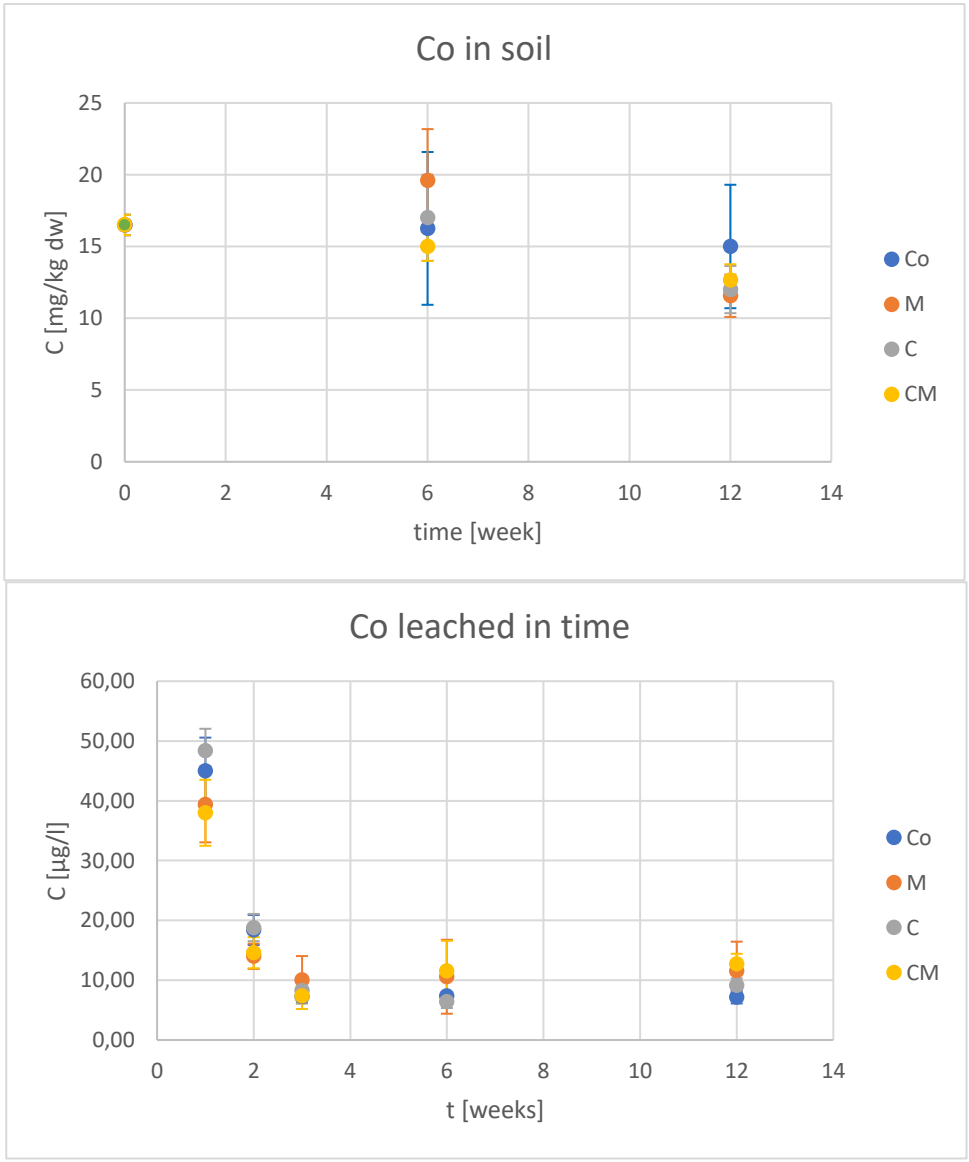

Fig.31 Also, with regard to the dynamics of cobalt in the soil, there is only a slight amount that is released from the soil within three months. Leachate analysis shows a higher concentration of Co in the very first weeks, which then decreases in all treatment

Iron

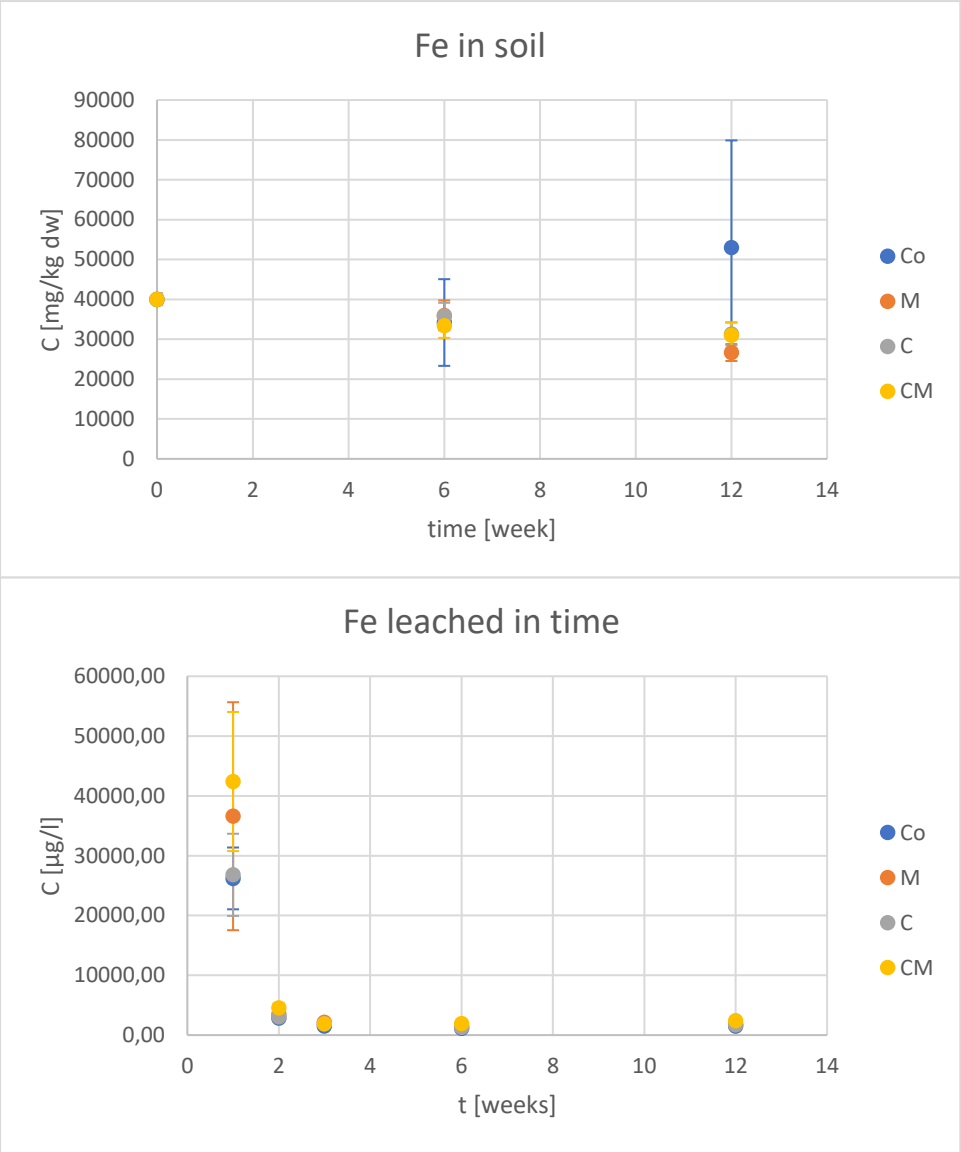

Fig.32 The analysed soil contains high concentrations of Fe (as well as aluminium, not reported in the thesis but in any case, quantified by ICP-MS). These, in the soil, drop slightly during the three months of experiments. In leachate it is possible to notice that the metal is released during the very first washing events, while over time its concentration is low and almost constant.

Zinc

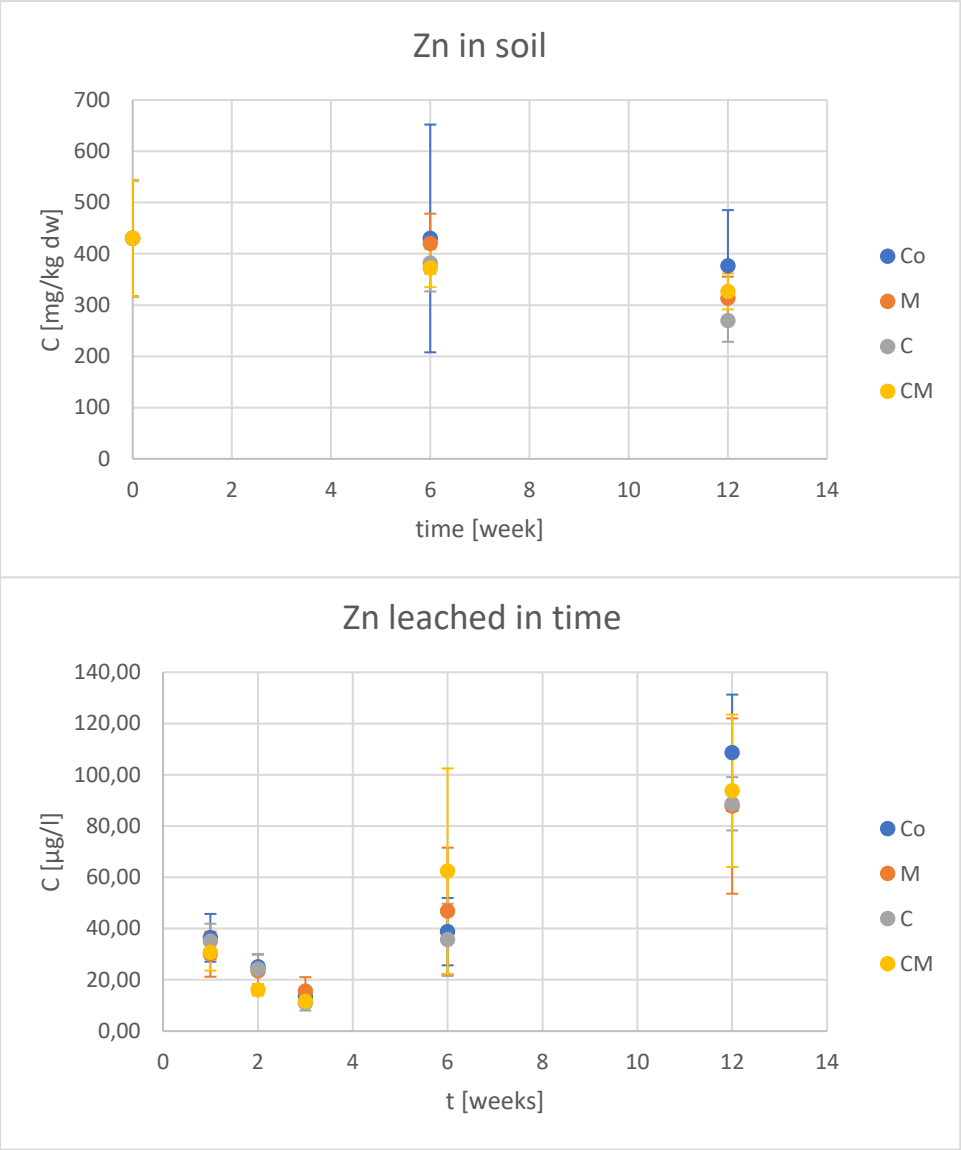

Fig.33 The concentrations of zinc found in soil and in leachate, coming from the control treatments (Co), soil with Meat and Bone Meal (M), soil wet weekly with cyclodextrin (C) and the treatment characterized by the combination of MBM and cyclodextrin (cyclodextrin (CM), are reported. The results obtained show an increase in the concentration of zinc in solution (i.e. in the leachate) over time. It must be said that these values, measured at the sixth and twelfth week, could be relatively high as they also include the sum of the quantities of zinc which was not washed away from the soil. moreover, in every single beaker used in the experiments to contain the soil, a sort of geomembrane filter was placed, which served to separate the soil from the bottom of the glass. This fabric has been stapled with a small crimping machine, which uses staples that can be made of natural steel, galvanized steel (Zn), aluminium (light alloy), Monel alloy (nickel, copper, and small parts of other metals) (balmacapoduri.it)*. These objects could also release some of the metals they are made of over.*

Phosphorous

Phosphorus is a fundamental element, together with nitrogen, for microbial growth in the soil, but in general for all living cells. Some of the fundamental molecules in the reactions of biological metabolism contain phosphorus, usually as phosphate groups (see DNA, RNA, ATP / ADP, NADP / NADPH). Furthermore, calcium phosphate is an essential component of bones. For this reason, it is expected that treatments containing the MBM product will have a higher concentration of phosphorus in the soil. This could be one of the main reasons for improving the bioremediation (biostimulation) processes of organic contaminants: the fact of having more phosphorus available for indigenous soil microorganisms could positively influence microbial activity (see hydrocarbon removal chapter).

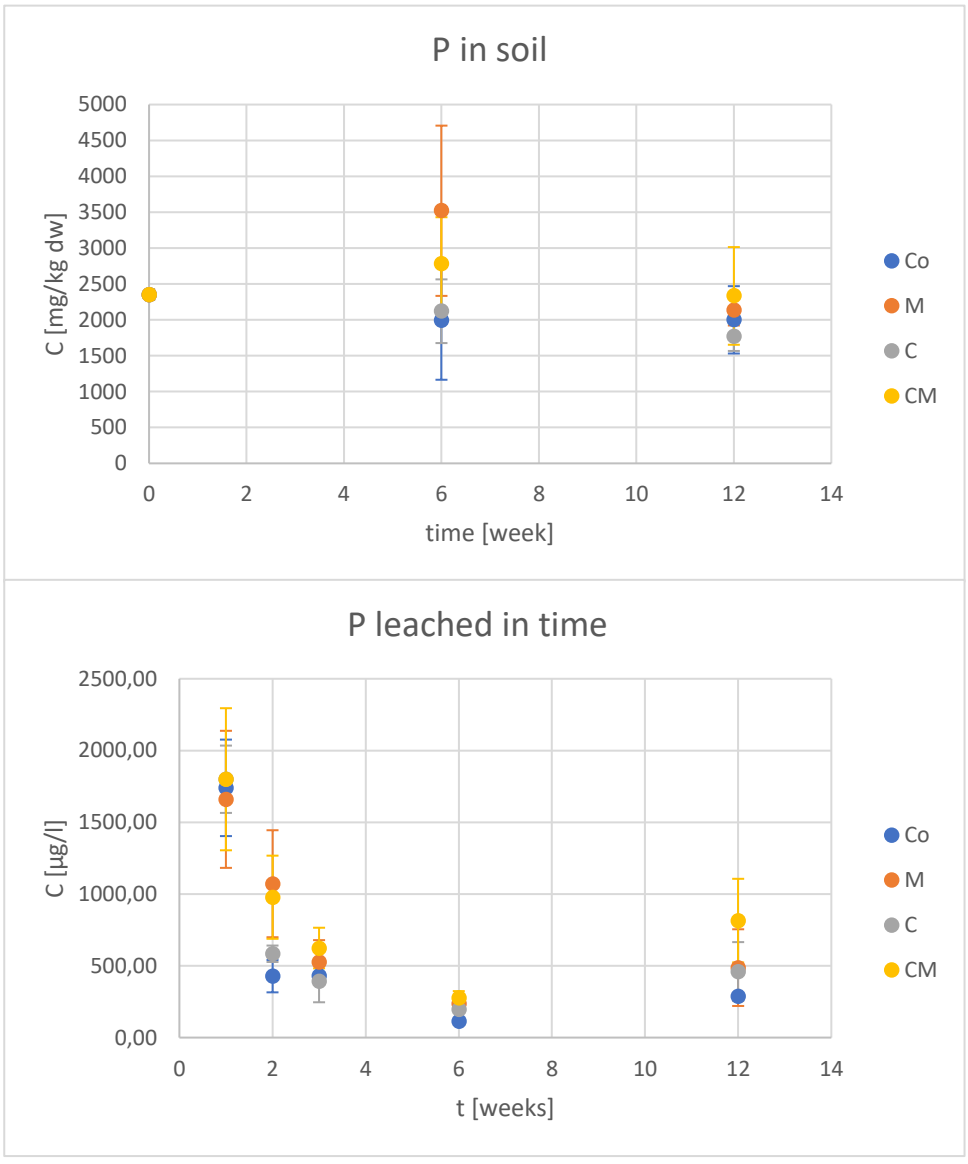

Fig.34 Phosphorous values are higher in treatments M and CM, in which MBM (1% w / w) was mixed with the soil at the beginning of the experiments. The concentration of phosphorus in the soil of M and CM treatments, six weeks after the beginning of the experiments, is even higher than that of the initial soil. This can promote soil microbial activity over time. Furthermore, from the leachate analysis, it can be noted that there is a greater release of phosphorus in water-soluble form over time: this phenomenon can also be positive for soil micro-organisms, which can encounter bio-available phosphorus, important for physiological functions. In this regard, recirculation of the percolating water could provide further phosphorus, useful to the microbial community (and other organisms possibly present, such as plants).

Lead

It can be found in literature that Meat and Bone Meal, thanks to its phosphate-rich chemical composition [46,106], has good ability to remove the lead present in water [43–45]. Ecotoxicological have also been conducted, which has come to say that MBM is able to sequester Pb from water and soil, in a very efficient manner, so that the biota does not come significantly damaged by the presence of the Lead.

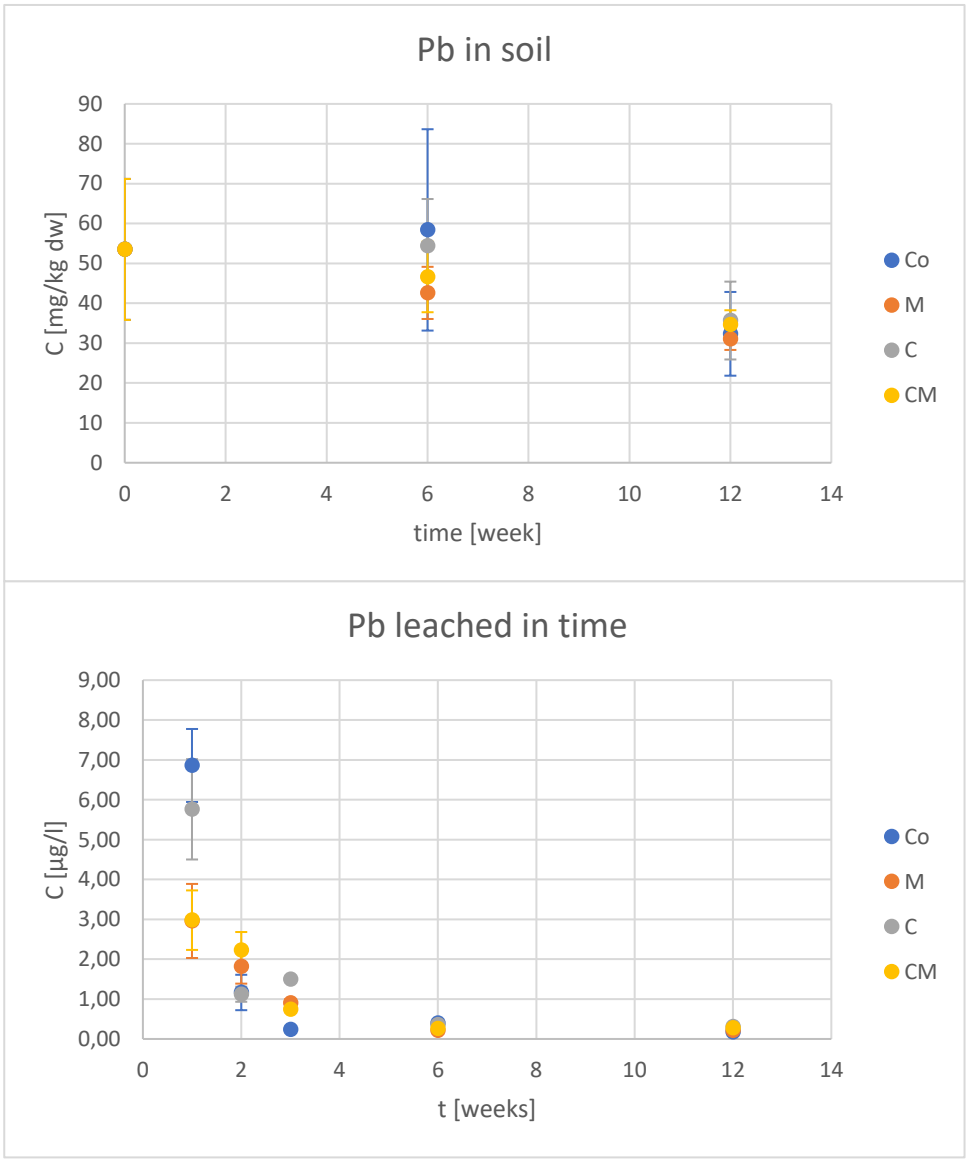

Fig.35 The graph shows the averaged values (and relative standard deviations) of the lead concentrations resulting from soil and leachate analysis in the different treatments, carried out in experimental period of three months. Soil samples were analysed three times in total (beginning, middle and end of experiments), while leachate was sampled and analysed more frequently, to monitor the dynamics of metal release. It should be noted that lead released in the very first period is much smaller in treatments containing MBM: this is probably because the metal was retained (adsorbed) by phosphates, naturally contained in MBM.

The analyses we carried out confirm that in fact Lead is retained in the soil where MBM is present (M and CM). This is visible by looking at the concentrations of Pb in the leachate: it can be seen that in treatments Co and C, without the Meat and Bone Meal product, dissolved Lead is present in larger quantities. However, this "immobilization" effect seems to come only in the first few weeks of soil washout, after which the concentrations in solution are comparable in all treatments.

Manganese

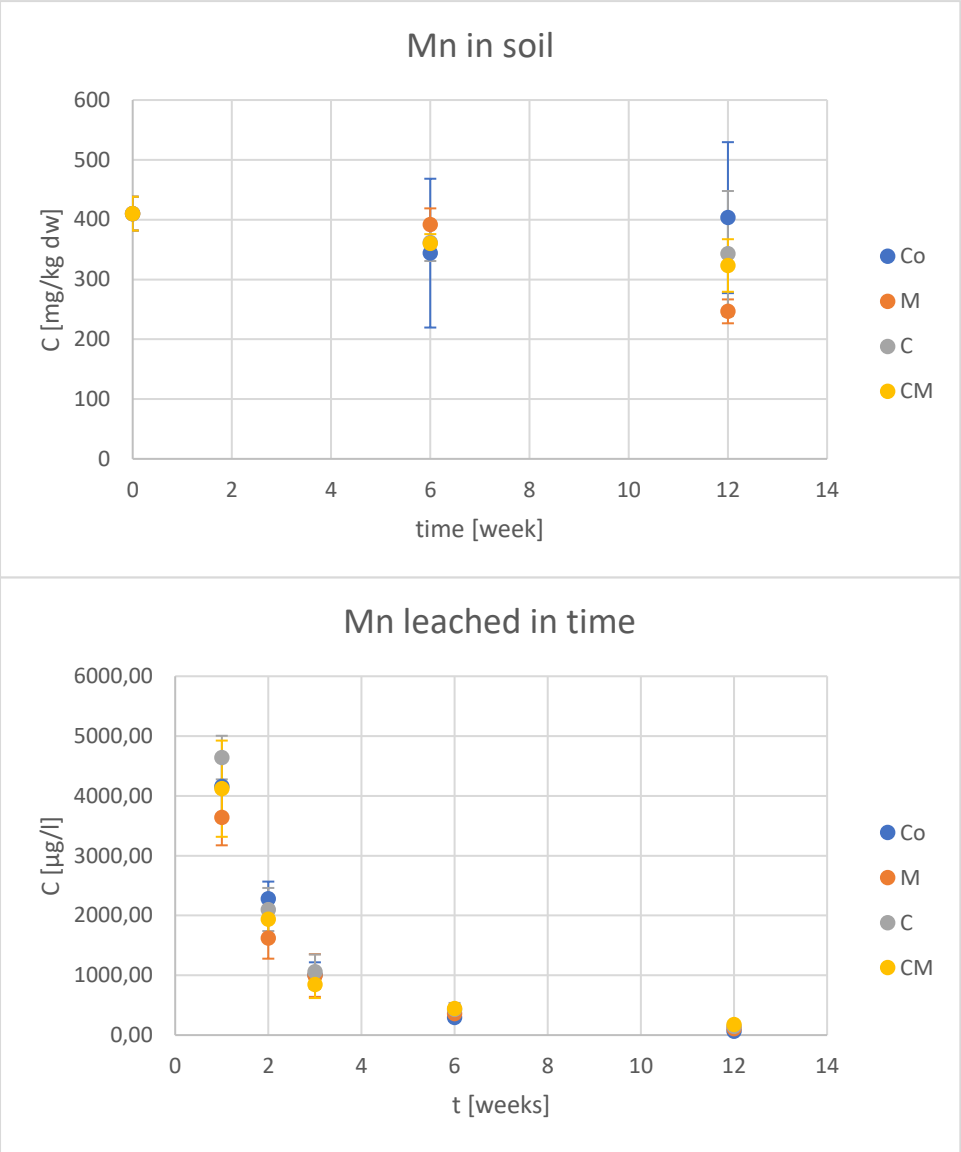

Fig.36 Manganese concentrations measured in the soil [in mg/kg] and those in the leachate [in µg/L] are reported, in the different treatments. As in the case of most metals, even Mn concentration decrease slightly in soil within three months, while leachate contains greater quantities of the metals in the first few weeks. Over time, the concentrations in water solution are low and approximately constant. From the collected data there are no particular differences comparing the behaviour of Mn in the different treatments: it may perhaps be noted that cyclodextrin (C), in the first soil washout event, seems to slightly increase the leaching of the Mn through the soil.

Arsenic

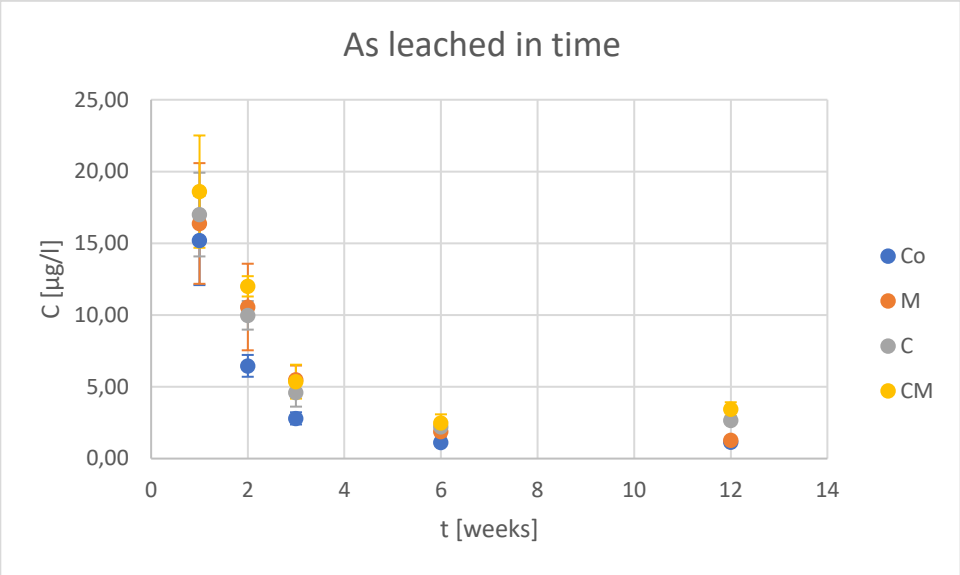

Fig.37 Arsenic analysis in the soil did not produce reliable results, as the values obtained are lower than Limit Of Quantification (LOQ) of the ICP-MS instrument used. Looking instead at the dynamics of the metal in leachate, here concentrations decrease rapidly whenever the soil is washed out. Treatments containing Meat and Bone Meal and cyclodextrin (CM) seem to release slightly larger quantities of As: this phenomenon could be linked to the cationic exchanges that occur between soil ions and those contained in MBM (i.e. Na^+, K^+). More in-depth studies should be conducted to define the possible cation exchange capacity (CEC) of Meat and Bone Meal product, detect which elements are released most and in which quantities. Given the properties of cyclodextrin, this could eventually increase the solubilization and therefore the mobility of arsenic through the soil.

3.4. Microbial community analysis:

Soil samples were collected at the beginning (day zero soil), in the middle (sixth week) and at the end (twelfth week) of the experimental period. These soil samples, about one gram for each treatment, were placed in freezer and stored at - 80 ° C. Purified products of the total DNA were extracted from the soil samples using DNeasy ® PowerSoil ® Kit Handbook for the isolation of microbial genomic DNA, QIAGEN, and stored at - 20°C. The extraction sequences were summarized in the previous chapter "Materials and Methods". To confirm the success of the extraction, Agarose Gel Electrophoresis (AGE) was carried out for all samples. Subsequently, using the technique explained previously in the thesis, based on the study of $\lambda_{excitation}$ and $\lambda_{emission}$ of the double-stranded DNA and Picogreen reagents complex, the total amount of DNA present in the purified extracts was measured and appropriate dilutions were made to obtain products containing approximately the same amount of DNA. After this, amplification was performed by Polymerase Chain Reactions (PCR) and in particular specific primers were used to amplify the 16S ribosomal RNA (or 16S rRNA), which is the component of the 30S small subunit of a prokaryotic ribosome. AGE was used a second time to confirm the amplification of PCR products for each sample. Once the searched products were obtained, these final samples were sent elsewhere for sequencing (Meilahti Miseq, https://www.fimm.fi/en/services/technology-centre/sequencing).

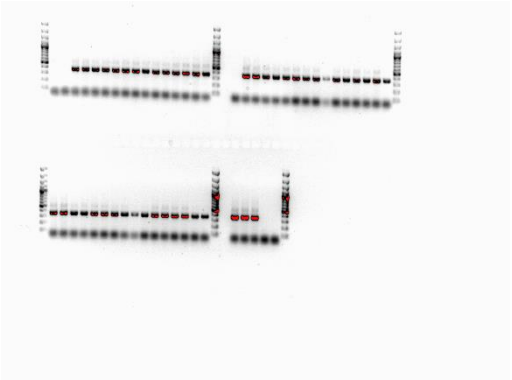

Fig.38 An example of electrophoresis on agarose gel is reported. The separated bands are visible thanks to the UV light that excites ethidium bromide, which intercalates into the major grooves of the DNA, to emit in fluorescence.

The results of the sequencing have recently arrived in the laboratories of the University of Helsinki, located in Lahti, Finland. Once processed and compared with the online databases, it will be possible to identify which are the bacterial microorganisms that populate the soil in the different treatments analysed (Co, M, C, CM). In particular, it will be interesting to see how the microbial community in the soil varies, from an initial state particularly rich in hydrocarbon contaminants, an intermediate state, in which the microbial activity could be particularly intense, to a final state in which the (eco)system may have reached a kind of balance. The dynamics of the microbial community that take place during the three months experiments could highlight particularly resistant and resilient strains to oil-polluted soils.

Optical microscope has been used, from time to time during the experiments, to look at the biggest living organisms present in the studied soil. Fungi, plants, protozoa, nematodes and mites (the latter have been seen only in the last period of the experiment) were observed. Interesting mineralization took place in treatments where cyclodextrin and Meat and Bone Meal was used. Further studies in these areas would be necessary to understand better the processes involved and the new products obtained.

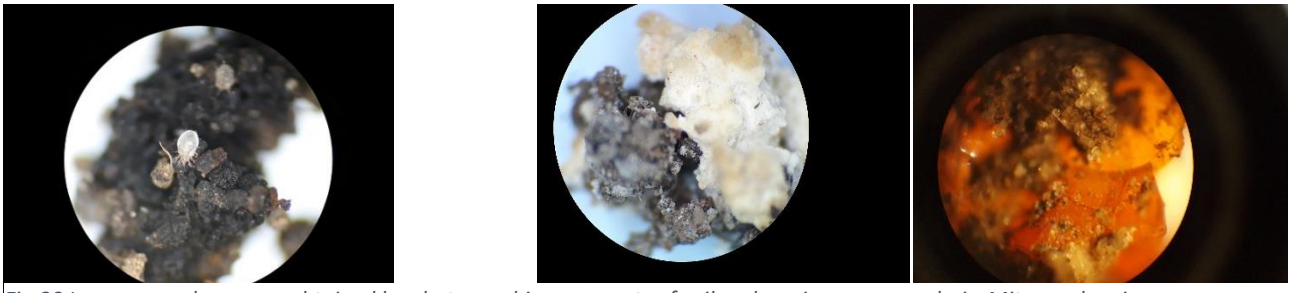

Fig.39 Images are shown are obtained by photographing moments of soil under microscope analysis. Mites and curious crystallizations are visible only in the last phases of the experiments.

4. Conclusions

A soil contaminated with hydrocarbons was collected from Kujala Waste Centre (PHJ), Lahti, Finland. This soil also contained considerable quantities of heavy metals. Analyses of some basic soil parameters were carried out: pH was slightly acidic at the beginning of the experiments (6.3), but it reached neutrality over time. Water content in the soil was 20%, with a specific soil Water Hold Capacity equal to 36% by weight. The content in Organic Matter (OM) was then measured as LOI and it turned out to be around 12.2% by weight in the initial soil, while in general it was at least one percentage point higher at the end of the experiments (i.e. after three months, twelve weeks). Two laboratory experimental tests were then set up, one for studying the degradation of hydrocarbons in the soil, the other for monitoring the mobility of metals in the soil. Control Co, M containing Meat and Bone Meal, C in which dissolved cyclodextrin in aqueous solution is added and CM, which is a combo of M and C. From previous studies it has been seen that Meat and Bone Meal, a natural sanitized product deriving from food industry waste and already used as fertilizer for agriculture and in some environmental applications (i.e. to remove lead present in aqueous solution), can be an excellent biostimulant for the microbial community in the soil and so it can improve biodegradation reactions of hydrocarbons.

The results obtained, analysing soil samples of the various treatments over time using GC-FID technique, confirm the characteristics referred to the MBM. Analysis considered hydrocarbons containing ten to forty carbon atoms, divided in two fractions: C_{10}-C_{21} Diesel and C_{22}-C_{40} Lubricant Oil. Soils added with MBM actually reached lower concentrations of organic contaminants. The decrease in these concentrations seems significantly faster in the first weeks since the start of the experiments, after which the concentrations in the soil of the various treatments tend to reach similar values. It can therefore be said, in agreement with the results obtained previously in other studies [10,11,25], that MBM product has a "booster" effect on degradation reactions of hydrocarbon contaminants in the soil (C_{10}-C_{40}) and can therefore shorten the time required to obtain acceptable concentrations in contaminated soils, also in relation to the limits set by law. Furthermore, the effect of cyclodextrin combined with MBM appears to be positive in hydrocarbon Biodegradation reactions: in fact, the so-called CM treatments have more often reported lower contaminant concentrations than all other treatments. Cyclodextrin, according to the chemical-physical properties that define it, could therefore increase the bioavailability of some molecules present in the soil, making them more easily "attackable" by soil micro-organisms. More in-depth studies could aim to identify which are the compounds that remain in the soil longer, as adsorbed and / or recalcitrant to microbial degradation. The use of the analytical GC-MS technique could help in this regard. Furthermore, a study focusing on the amount of nutrients in the soil available for microorganisms over time (such as C, N, P, S) could be interesting, possibly evaluating the effect of MBM systematically additions (for example monthly) on the microbial metabolic activity.

As regards the test on the mobility of metals, positive results have been obtained from ICP-MS analysis on the immobilization effect of lead in the soil. In fact, as demonstrated by some experiments found in literature [43,44], MBM can trigger sorption processes on some metals in solution. Of great importance are the possible mechanisms of lead sequestration since it is a metal particularly toxic to humans, biota in general and the environment. The results we obtained show essentially that lead is retained for a short time in soils containing MBM; after a few weeks, however, the metal appears to be released from the soil, together with percolation water. Metal analyses also show higher values of phosphorus in MBM-treated soils and related leachate, which could be beneficial for the development of the microbial community. In general, it can be said that soils of the treatments in which methyl-β-cyclodextrin has been added release, in the aqueous solution, slightly greater quantities of metals, probably because water-soluble complexes (inclusions) are formed between metal and surfactant.

The study of the microbial community has not yet been completed, even if procedures that led to the extraction and purification of soil DNA and subsequent amplification of microbial (prokaryotic, 16S r-RNA)

genetic material have been done. The sequencing results of these genes have recently arrived in the laboratories of the University of Helsinki (Department of Biological and Environmental Sciences based in Lahti) and will soon be processed here, so as to obtain a panorama on the type of native bacterial community present in the soil, and the evolution of this during the treatment period (soil sample analysis of the beginning, middle and end experiments) .

Experiments were performed in the laboratory and the various tests were kept under aerobic conditions. Different results, both as regards the biodegradation of hydrocarbons and as regards the dynamics of metals in the soil, could arise if the system was maintained under anaerobic conditions. Interesting products, even from the commercial and energy point of view (such as methane and ethanol), could be formed from the degradation reactions of hydrocarbons under anaerobic conditions, possibly in a controlled Bioreactor type system. Encouraging the growth of plants by exposing them to sunlight (or in any case to an alternative light source) could increase the removal of contaminants from the soil (phytoremediation).

5. Acknowledgments

I would like to thank my family and friends warmly for supporting me during my course of study, making it possible for me to train both in Italy and abroad. I also want to thank the professors I met in my educational experience at the University of Padua, some of whom taught me so much, transmitting me their passion for science and the world of nature. A great recognition goes also to the Finnish professors and researchers who helped me to carry out my thesis work during the Erasmus project: in particular, Aki Sinkkonen, who was my supervisor in Finland, professor at the head of "Nature -Based Solution "research group from the Department of Biological and Environmental Sciences of the University of Helsinki. He helped me to be accepted at the Finnish University and gave me all the tools and information needed to do my job there. Then, I want to thank Ville Selonen (REMSOIL ltd), who thanks to his knowledge in the field of soil contamination helped me to set up the experimental part of the thesis work. His advice was fundamental to the success of the experiments. I thank Professor Anna-Lea Rantalainen (Ansku), who enlightened me on chemical and analytical procedures and helped me in writing the thesis. With her I also followed a very interesting course on the chemical fate and transport of environmental pollutants, so I owe her special thanks. I also thank Martin Romantshuk for his wise advice on soil remediation processes and his help in correcting the thesis. I also thank the Almalab laboratory technicians (Jukka, Santeri and Kaisa) and all the colleagues who in one way or another have shared with me their time, their ideas and some moments of happiness, in what was a life experience as well as an educational one. The Italian professors, who gave me advice and material while I was in Finland were also very important for the success of the thesis: so, a big thank you to Professor Bogialli, who is also my Italian supervisor and who helped me to give a structure to my thesis project and was available in times of need. I also thank professors Artioli and Squartini for their support and their explanations on unclear topics, that helped produce the final result.

I sincerely hope to be able to collaborate again in the future with such exquisite people, working on equally interesting and intellectually stimulating subjects.

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