Degradation of aged creosote and diesel contaminated soils by phytoremediation or biostimulation (nutrients)

Nguyen Khoi Nghia

Photo: Nguyen Khoi Nghia

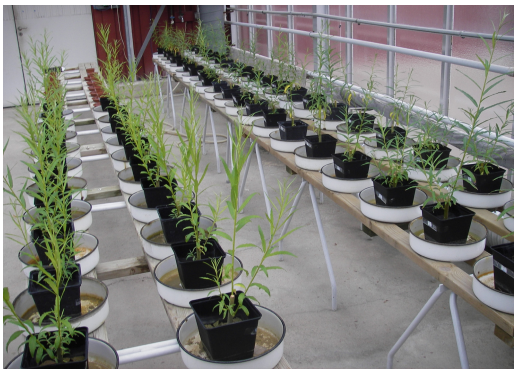

Photo: Leticia Pizzul

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ABSTRACT

Aged creosote and diesel fuel polluted soil was collected from Resecentrum, Uppsala (deposited at Hovgården). The aims of this study were: 1) to compare the growth and degradation of PAHs and diesel by five *Salix* clones and 2) to study the effect of nutrients on biodegradation of PAHs and diesel. Three kinds of soil were included in this study: creosote and diesel contaminated soil (undiluted); mixed soil 1:1 (diluted with not contaminated soil) and control soil (not contaminated). The initial total concentration of PAHs in the creosote soil (undiluted) was 10 mg/kg and 1,150 mg/kg of aliphatic and aromatic hydrocarbons (diesel components). The *Salix* clones used were Tora, Björn, Orm, 78138 and 78112 and Blomstra was used as nutrients. During four months, no obvious difference was found between the five willow clones regarding shoot growth except that Orm grew less well in creosote and diesel contaminated soils. Root biomass increased and the Shoot/Root ratio (S/R) decreased for most clones at higher concentrations of creosote and diesel contaminants. Between clones, 78112 and Orm had lower PAH degradation capacities as compared to others. The presence of plants in the creosote and diesel contaminated soil retarded the degradation of most PAHs as compared to the treatment without plants. It was probably due to the interaction between root exudates and diesel in our study since the latter hydrocarbons could act both as carbon sources and co-substrates that are needed in the co-metabolic degradation of PAHs whereas many microbes preferred the former more easily metabolisable compounds. High degradation of PAHs in the creosote soil occurred in treatments without plants, but even more so in treatment with nutrients especially for high molecular weight (HMW) PAHs, such as benzo(b)fluoranthene (94.7%) and benzo(a)pyrene (100%) as compared to the initial values. The bacterial counts were significantly higher in both the treatment with plants and the unplanted treatment with nutrients, as compared to the initial soil and the control treatment. Eight bacterial strains with the ability to degrade phenanthrene were isolated from the undiluted creosote and diesel contaminated soil.

CONTENTS

1. INTRODUCTION

Polluted soils have become a very important environmental problem all over the world in recent years, influencing health, economic and political issues (Myriam *et al*., 2005). There are many kinds of pollutants in soil, but we can classify them into two kinds: organic and inorganic (e.g. heavy metal). The organic pollution of soil environments comes from many sources such as fossil fuel burning, disposal of sewage, pesticides, wood conservation treatments, etc. and has increased dramatically since the onset of industrial revolution. Many strategies including chemistry, physics and biology (bioremediation and phytoremediation) are employed to remove contaminants from such soils and making them suitable for agricultural use and urban development. Many aspects have been studied, such as identification and range of pollutants, reduction of the total soil disturbance and optimisation of the conditions of the clean-up technique (Douglas *et al*., 1994).

One of the most common organic contaminants in soil is creosote. Creosote, is a complex mixture of over 200 chemical compounds, dominated by polycyclic aromatic hydrocarbons (PAHs), phenolic and aromatic nitrogen and sulfur compounds. Many countries have problems with creosote-contaminated soil and the sources of contamination are often the wood treatment facilities. In addition, creosotes affect not only human beings by the toxicity but also the environments. They can enter terrestrial and water environments through both natural process and anthropogenic activities by long-term exposure with low concentrations. In Sweden, unacceptable high creosote concentrations are detected in aquatic and soil environments (RECIEL, 2000).

Bioremediation, which is one of several useful methodologies for pollutant removal regarding effectiveness, costs and safety, utilizes plants or microorganisms to degrade organic contaminants to ultimately form carbon dioxide, water and other inorganic compounds (Alexander, 1994; Roberts, 1998). Phytoremediation, one of the biological remediation methods, uses green plants to remove, contain, or render harmless environmental contaminants (Sylvia *et al*., 2005). Inorganic plant nutrients (e.g. NO_3^-), organic solvents, PCBs, heavy metals, poly-aromatic hydrocarbons and radioactive elements are contaminants that are effectively cleaned up by phytoremediation. Furthermore, the role of nutrients in biodegradation of organic contaminants is very important, especially nitrogen and phosphorous. These nutrients are used by microorganisms to build up their cells, so the microbial growth and activity increase when applying nutrients and consequently the degradation rate of organic pollutants becomes higher (Roberts, 1998).

2. AIM

The aim of this thesis was to compare five different willow clones regarding growth and ability to degrade aged creosote (in combination with diesel spills) in differently contaminated soils from a former wood impregnation site (Resecentrum, Uppsala) and to test the role of nutrients on the biodegradation of PAHs.

3. BACKGROUND

3.1. Physical and chemical properties of Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are hazardous non polar hydrophobic organic chemicals consisting of two or more fused benzene rings in linear, angular or clustered arrangements (Cerniglia, 1992) and contain by definition only carbon and hydrogen atoms (Lundstedt, 2003; Karlsson, 2005). They have high solubility in organic solvents but the concentration in the soil water phase is quite low (Roberts, 1998). Generally, an increase of the number of fused benzene rings leads to a decrease in water solubility and hydrophobicity of the PAHs (Wilson *et al*., 1993). Carbon in the benzene rings may be substituted by nitrogen, sulfur and oxygen atoms to form heterocyclic aromatic compounds, which are common PAH groups. Furthermore, PAHs substituted with alkyl groups are normally found together with the PAHs in the environment (Lundstedt, 2003). The outlines of physical-chemical properties of PAHs are presented in Fig. 1 and Table. 1.

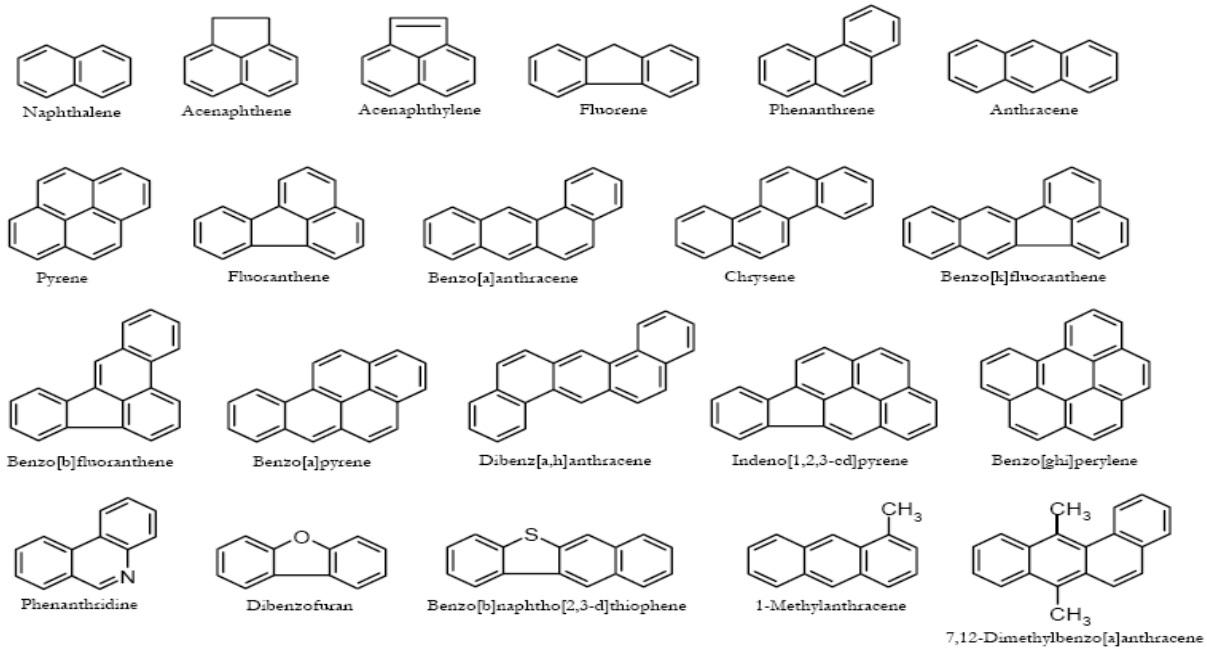

Figure 1. *Chemical structures of some PAHs, alkyl-PAHs and heterocyclic compounds from Lundstedt, (2003).*

Table 1. *Structure and physical-chemical properties of some two, three-, four-, five- and six-ring polycyclic aromatic hydrocarbons.*

PAHs	No. of rings*	Molecular weight*	mp[a] (°C)**	bp[b] (°C)**	Sol[c] (mg/l)*	Vapor press. (Pa)*	Log K_p[d]**	Log K_{ow}*
Naphthalene ($C_{10}H_8$)	2	128	-	-	31	$1.0x10^{2}$	-	3.37
Acenaphthylene ($C_{12}H_{10}$)	3	152	-	-	16	$9.0x10^{-1}$	-	4.00
Acenaphthene ($C_{12}H_8$)	3	154	-	-	3.8	$3.0x10^{-1}$	-	3.92
Fluorene ($C_{13}H_{10}$)	3	166	-	-	1.9	$9.0x10^{-2}$	-	4.18
Phenanthrene ($C_{14}H_{10}$)	3	178	101	340	1.1	$2.0x10^{-2}$	4.46	4.57
Anthracene ($C_{14}H_{10}$)	3	178	216	340	0.045	$1.0x10^{-3}$	4.45	4.54
Pyrene ($C_{16}H_{10}$)	4	202	149	360	0.13	$6.0x10^{-4}$	5.32	5.18
Fluoranthene ($C_{16}H_{10}$)	4	202	111	250	0.26	$1.2x10^{-3}$	5.33	5.22
Benzo[*a*]anthracene ($C_{18}H_{12}$)	4	228	158	400	0.011	$2.8x10^{-5}$	5.61	5.91
Chrysene ($C_{18}H_{12}$)	4	228	255	488	0.006	$5.7x10^{-7}$	5.61	5.91
Benzo[*b*]fluoranthene ($C_{20}H_{12}$)	5	252	-	-	0.0015	-	-	5.80
Benzo[*k*]fluoranthene ($C_{20}H_{12}$)	5	252	-	-	0.0008	$5.2x10^{-8}$	-	6.00
Benzo[*a*]pyrene ($C_{20}H_{12}$)	5	252	179	496	0.0038	$7.0x10^{-7}$	6.04	5.91
Dibenzo[*a,h*]anthracene ($C_{22}H_{14}$)	6	278	262	524	0.0006	$3.7x10^{-10}$	5.97	6.75
Indeno[1,2,3-*cd*]pyrene ($C_{22}H_{12}$)	6	276	163	536	0.00019	-	7.66	6.50
Benzo[*g,h,i*]perylene ($C_{22}H_{12}$)	6	276	222	-	0.00026	$1.4x10^{-8}$	7.23	6.50

*[a]mp: melting point; [b]bp: boiling point; [c]Sol: aqueous solubility; [d]log K_p: logarithm of the octanol:water partitioning coefficient; * from Karlsson., (2005); ** from Juhasz et al, (2000).*

3.2. The toxicity of PAHs

PAH compounds can have adverse effects on human health when they enter the food-chain. They are toxic, carcinogenic and mutagenic to organisms including microorganisms, terrestrial plants, aquatic biota, amphibians, reptiles, birds and terrestrial mammals etc. (Samanta *et al.,* 2002; Parrish *et al.,* 2005). The hazardous effects of PAHs have been reported on survival, growth, metabolism and tumor formation, i.e. acute toxicity, developmental and reproductive toxicity, cytotoxicity, genotoxicity and carcinogenicity. Research has focused so far on genotoxicity and carcinogenicity (Lundstedt, 2003). According to the US Environmental Protection Agency, there are sixteen PAHs considered as priority pollutants, seven of them are known carcinogens. They cause damage to DNA and mutations that lead to cancer. However, the unsubstituted PAHs are not the original compounds that react with DNA. They need metabolic activation and conversion to show their genotoxic and carcinogenic properties. This happens only when PAHs are metabolised in higher organisms by conversion to more water-soluble forms facilitating their subsequent excretion from the organism. This leads to formation of intermediates reacting with DNA to form adducts, preventing the gene involved from functioning normally. Subsequent damage of DNA may include cancer if mutations affect different functions of a cell (Juhasz *et al.,* 2000). PAHs can be highly potential carcinogens that produce tumors in organisms at single doses, but other non-cancer-causing effects are not well understood (US EPA, 2006).

3.3. Sources, environmental fate and distribution of PAHs in soils

The main sources of severe PAH contamination in soil come from fossil fuels, i.e. production or use of fossil fuels or products derived from fossil fuels, such as coal tar and creosote. This includes the following industrial activities (Recetox, 2006; Wilson *et al*., 1993):

* Gasification/liquification of fossil fuels (gasworks)
* Coke production
* Coal-tar production

* Wood-treatment processes
* Asphalt production
* Fuel processing

PAHs are ubiquitous in the environment (Karlsson, 2005). The chemical properties, and the environmental fate of PAH molecules depend on part upon both molecular size, i.e. the number of aromatic rings, and molecule topology or pattern of ring linkage (Kanaly *et al.,* 2000). However, individual PAHs differ substantially in their physico-chemical properties. For instance, properties of aqueous solubility and vapour pressure range within five to twelve orders of magnitude, respectively, moving from two to six benzene rings in the PAH-molecule (Table. 1). An increase in the size and angularity of PAH molecules leads to increase in hydrophobicity and electrochemical stability, which are two important factors deciding the persistence of high molecular weight (HMW) PAHs in the environment (Kanaly *et al.,* 2000). The octanol-water partitioning coefficient reflects the difference in hydrophobicity. The turnover of low molecular weight (LMW) PAHs will be more rapid than for HMW compounds (Recetox, 2006; Lundstedt, 2003). PAHs are found in many environments including indoor and ambient air, soil and diet (Mucha *et al*., 2006). Organic media including natural soil organic matter and its primary (celluloses, lignin, lipids and waxes) and secondary (humified organic matter) components directly absorb PAHs in soil (US EPA, 2006). The persistence of PAHs in the environment is mainly due to their low water solubility and bioavailability and strong absorption to soil particles and organic matter and trapping inside micropores where microorganisms can not reach them and they are not degraded (Cerniglia, 1992). There are different possible fates of PAHs after they have entered the soil such as degradation, volatilisation, leaching, bioaccumulation and sequestration. In short, several factors that control the fate and behaviour of PAHs in the soil include soil type (minerals and organic matter) and physico-chemical properties (e.g. aqueous solubility, polarity, hydrophobicity, lipophilicity and molecular structure) of the contaminants (Semple *et al*., 2003). The environmental fate of PAHs can be illustrated by the schematic representation in Fig. 2.

Large areas of soils and sediments have been contaminated due to the increase of industrial activities (Densy *et al.,* 2006). Carcinogenic PAHs appear in all surface soils. Typical concentrations of PAHs in forest soil vary between 5 µg and 100 µg/kg due to litter accumulation. In rural soil, the levels of PAHs vary between 10-100 µg/kg and originate mainly from atmospheric deposition. For both forest and rural soil values as high as 1000 µg/kg are not common. The concentrations of PAHs in metropolitan areas are higher than that in forest or agricultural soils because of sources such as fossil fuel combustion. In these areas, high concentrations of PAHs can be found varying between 600 and 3000 µg/kg. However, higher values of 1000-3000 µg/kg and 8000-336000 µg/kg were found in areas near heavy transportation and industrialization areas, respectively (WHO-Denmark, 2000; Menzie *et al*., 1992). The average increase in PAHs in the plough layer in The Rothamsted plots in England over the century since 1890 varied between 0.01 and 0.67 $mg/m^2/year^1$. By 1987 the surface soils at Rothamsted had an increase in all PAHs compounds measured by a factor of between 1.3 mg/m^2 (acenaphthalene) to more than 20 mg/m^2 (benzo(a)pyrene). The concentrations of PAHs also increased with increase in the subsurface layer from plough layer with average values ranging from 0.01-0.14 mg/m^2/year (Jones *et al*., 1989). Soil samples from rural and urban areas in Hong Kong analysed for 16 priority PAHs showed total concentrations of PAHs ranging from 7.0-410 µg/kg (dry weight). The concentrations of PAHs in the urban soils were higher than those in the rural soils. The dominant PAHs in rural soils were fluoranthene, naphthalene and pyrene, while fluoranthene, naphthalene and benzo(b+k)fluoranthene dominated in urban soils (Zhang, 2006). Concentrations of PAHs are generally higher near the emission sources, although

PAHs are thought as ubiquitous environmental pollutants. Extremely high concentrations (>10 000mg/kg soil) have been reported for many contaminated sites all over the world (Fismes *et al*., 2002; Lundstedt, 2003; Densy *et al*., 2006).

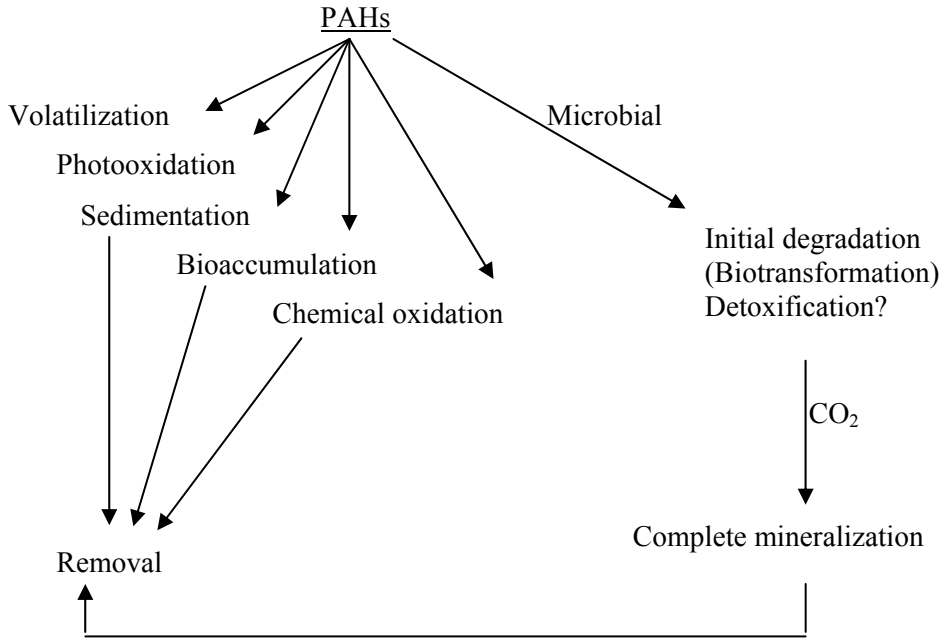

Figure 2. *Schematic representation of the environmental fate of PAHs (Cerniglia, 1992).*

3.4. Creosote pollution

Creosote is used as a wood preservation for railway ties, bridge timbers, pilling and large-sized lumber. It consists mainly of PAHs, phenol and cresol compounds that cause harmful health effects (Roberts, 1998). Coal tar creosote is a thick liquid that is typically amber to black in color. It does not dissolve easily in water. It can move through the soil to reach and enter the groundwater where it is degraded very slowly. Small amounts of creosote that remain in the soil or water for a long time can still be toxic to animal and humans. Creosote is released to water and soil mainly as a result of the wood preservation. This leads to a creosote-contaminated sludge, sediments, etc. (ATSDR, 2002).

In Sweden, the use of creosote has been forbidden since 1980s. PAH contaminated soils are almost always influenced by former wood impregnation or a former gas work. The total concentration of PAHs in soil that was found at a former gas work site in Stockholm, Sweden was around 300 mg/kg soil (Eriksson *et al.,* 2000), while the concentration of PAH in soil from a former wood impregnation site at Krylbo was much higher, i.e. up to 2 829 mg/kg soil (Önneby, 2005).

3.5. PAH remediation methods

3.5.1. Physical and chemical methods

There are many physical and chemical methods that are used to treat creosote (PAHs) contaminated soils including chemical extraction, chemical reduction or oxidation, dehalogenation, separation, soil washing, solidification, thermal treatment by hot gas, incineration, open burn, open detonation, pyrolysis or desorption or capping in a landfill or other

facility (FRTR, 2005). Soil washing and incineration are methods used for remediation of acute contaminations, such as industrial waste sites and from accidental leaks and spills of organic chemicals. Typical off-site remediation involves excavating huge quantities of contaminated soil, then treating the excavated material in a "soil washer" that remove the toxic organic chemical by means of solvents. Finally, the pollutants are recovered and destroyed by incineration or placed in a secured toxic-waste landfill. Sometimes the contaminated soil is placed in landfills without attempting to clean it. These approaches to remediation are extremely expensive and rarely result in a truly recovered soil that resembles a productive natural soil (Brady, 1996).

Chemical oxidation is a promising method for *in situ* remediation techniques of PAHs. The oxidative chemical reaction can destroy or change organic contaminants, such as PAHs, to non-toxic or less hazardous compounds which are further degraded by microorganisms. However, only Fenton reaction and its modifications have been applied in *in situ* soil remediation (Palmroth, 2006). In the original Fenton's reaction, radicals are formed during the reaction of dilute H_2O_2 solution with Fe(II) called Fenton's reagents. A combination of the short-term chemical oxidation with biodegradation may increase PAH removal more than natural processes owing to enhancement of the water solubility of the PAHs that thus become more bioavailable.

In short, chemical and physical methods are conventional techniques for decontaminating the contaminated soil. These methods are effective but are not cost effective. New techniques or methods for PAH remediation may concern not only complete removal of contamination, but also the safety for the ecosystem and cost effectiveness as well.

3.5.2. Bioremediation

According to Sylvia *et al.* (2005), "the term bioremediation can be used for any process that uses microorganisms, green plants or their enzymes to return the environment altered by contaminants to its original condition". Bioremediation may be employed to decontaminate or degrade organic pollutants in soil, water and sediments into harmless metabolic products. The process is sometimes carried out *in situ* (on-site), thus avoiding the high cost and disruptive effects of excavation and hauling large quantities of soil (Brady, 1996). Depending on the approach used, bioremediation may result in varying degrees of transformation or degradation of contaminants: **Biotransformation** is a general term that expresses the transformation of a parent compounds to daughter compounds. **Mineralization**, or complete biodegradation, is the complete conversion of an organic contaminant to its inorganic constituents, generally carbon dioxide and water, and possibly other constituents such as chloride. **Cometabolism** refers to the transformation of a contaminant without the contaminant providing of carbon or energy for the degrading microorganisms (additional carbon source needed).

Most approaches to bioremediation seek to reduce the limitations of "natural" bioremediation. This can be done in a number of ways, but they are all based on one or both of the following general approaches: **Biostimulation** is the addition of nutrients, such as nitrogen and phosphorous or air or other amendments (carbon) to stimulate indigenous microorganisms in soil. Adding small amounts of the contaminant or an analogue can also act as a stimulant by encouraging production of degradative enzymes. **Bioaugmentation** is the inoculation of a contaminated site with microorganisms to faciliate biodegradation. Bacteria are the organisms most commonly used for bioaugmentation. A single species or group (**consortium**) of microorganisms can be responsible for biodegradation. **Passive or intrinsic bioremediation** is the bioremediation that happens naturally by the decontamination of indigenous microorganisms

although the rate of degradation is usually too low for practical benefit. **Monitored natural remediation (MNR)** is the natural remediation of contaminated sites by indigenous microorganisms and possibly a biotic process. In this method, it is similar to passive or intrinsic bioremediation but includes an agreed-upon monitoring plan to confirm that remediation processes are occurring.

3.6. Environmental factors affecting the success of bioremediation

Several factors have been determined as being important to the landtreatment process such as the composition of the organic fraction of the material to be treated, temperature, soil moisture, availability of nutrients, soil pH, and oxygen availability (Sprehe *et al.,* 1985).

+ **Temperature:** For bioremediation technologies, temperature affects the rate of biological activity and the rate of organic matter decomposition. Bioremediation decline with temperature due to reduced microbial growth and metabolic rates. The biodegradation rate reduces to zero at the freezing point (Roberts, 1998). Generally, raising the temperature increases the rate of degradation of organic compounds in soil. When increasing the temperature, the adsorption of organics will be reduced, so this makes more organics available for the microorganisms to degrade (Roberts, 1998). Conversely, a decreasing of the temperature is associated with a slowing of the microbial growth rate. Low temperature can lengthen the acclimation period and delay onset of bioremediation. Low temperature can also decrease microbial enzymatic activity (Zhou *et al.,* 1995; Roberts, 1998).

+ **Moisture**: soil moisture is essential for growth and multiplication of microbes (Broberts, 1998) and a major control parameter in the land treatment process (Sprehe *et al*., 1985). The optimum moisture content for the highest degradation rate for land treatment of refinery waste was found to be 18%. Biodegradation of waste chemicals in soil needs water not only for microorganisms growth but also for diffusion of nutrients and by-products during the breakdown process. Extremes of very wet or very dry soil moisture markedly reduce waste biodegradation rate. Under saturated soil moisture conditions, the aerobic waste hydrocarbon decomposition is diminished because of low oxygen supply, whereas under very dry conditions, microbial activity is hindered (Roberts, 1998).

+ **Nutrients:** the availability of nutrients, especially nitrogen, is important to increase the biological processes. Three factors control the availability of nitrogen (N) and phosphorous (P) for soil microbial growth: (1) the amount of N and P in the soil and rate at which they are mineralised (become available for use), (2) amount of biodegradable carbon and available N and P in the added waste, and (3) rate at which the waste organic carbon is assimilated in the soil environment. Overfertilization can lead to groundwater or surface water problems. Microbial degradation of hazardous compounds requires the presence of nitrogen, phosphorous and potassium and a small amount of zinc, calcium, manganese, magnesium, iron, sodium and sulfur to optimise the biological growth (Arora *et al*., 1982; Roberts, 1998).

Feeding nutrient solutions containing inorganic nutrients, such as nitrogen, phosphorous and sulfur, to natural soil bacteria often enhances the microorganisms to degrade organic molecules into carbon dioxide and water. In contaminated wastes there is the presence of some nutrients, but maybe they are not readily available for microbes. Determination of soil organic matter, organic carbon, organic nitrogen and organic phosphorous will reveal the ratio between C:N:P and an evaluation of nutrient availability (Roberts, 1998). Bacteria generally need carbon, nitrogen and phosphorous with a ratio of 100:15:3 to build up their cells (Zitrides,

1983). If the ratio of organic C:N:P is higher than about 300:15:1 and available inorganic forms of nitrogen and phosphorous do not narrow the ratio to within these limits, supplemental nitrogen and or phosphorous should be added (Roberts, 1998). The optimal C:N ratio differs from different soils. For a clay/loam soil the C:N and C:P ratios should be around 50-60:1 and 800:1, respectively (Roberts, 1998). However, overloading of nitrogen (e.g. C:N = 1.8:1) can impair biodegradation, possibly due to ammonia toxicity (Zhou *et al*., 1995).

+ **Soil pH:** The optimum pH for biodegradation lies between 6 and 8. However, effective biodegradation can be also found outside this range. Soil pH may affect the solubility, mobility, and ionized forms of contaminants. Microbial activity in the soil is greatly affected by pH, through the availability of nutrients and toxicants and the tolerance of organisms to pH variations (Roberts, 1998). Soil pH can affect the solubility or availability of macro- and micronutrients, the mobility of potentially toxic materials, and the reactivity of minerals (Parr *et al.,* 1983). Hydrocarbon contaminants and soil nutrients can often reduce the pH of the soil. During aerobic degradation of organic molecules, carbonic acid, organic acid intermediates, and nitrate and sulfate may accumulate and this can lower the soil pH and inhibit biological activity (Zitrides, 1983).

+ **Oxygen:** heterotrophic bacteria get their energy requirements from the available carbon source substrate through electron transport pathways. In aerobic metabolism, oxygen is the terminal electron (hydrogen) acceptor. The reaction creates both carbon dioxide (carbon is oxidized) and water (oxygen is reduced). When something other than oxygen is the terminal electron acceptor, the reaction is called anaerobic. In general, aerobic degradation proceeds at a faster rate than does anaerobic degradation (Roberts, 1998). Oxygen availability throughout the zone of incorporation is essential for bio-oxidation of the organic materials (Sprehe *et al*., 1985). Oxygen can be quickly depleted by active microbial metabolism (Roberts, 1998). Anaerobic degradation still happens in soil, but must be limited for effective landtreatment applications because (1) anaerobic biodegradation results in noxious products, such as hydrogen sulfide, ammonia, amines and mercaptants; (2) anaerobic biodegradation is slower and less complete; and (3) in a reduce state, most hazardous metals are more water soluble. Measurements of redox potential (Eh) and oxygen diffusion rate (ODR) can be used to monitor biodegradation of hazardous wastes in soil and determine the effectiveness of landtreatment operations (Shaikh *et al*., 1985).

3.7. Phytoremediation of PAH contaminated soils

Phytoremediation is a broad term expressing the use of plants (e.g. *Salix*) to remove, contain, or transform contaminants. The role of microbes is considerable in the success of phytoremediation that involves the use of plant and their associated microbes in the rhizosphere for environmental cleanup, as a cost-effective, non-invasive alternative or complementary technology (Sylvia *et al*., 2005). **Phytoextraction** is the uptake of contaminants by plants. Plants are used to remove excess nutrients from soils. Some plants accumulate compounds, such as metals, to a degree greater than the concentration in the soil solution. And then plants can be harvested and disposed of or recovery of metals. Little is known about uptake of organic pollutants by plants. The amount of uptake by plants varies significantly and appears to be a function of many factors, such as plant species, initial concentration of contaminants and microbial population. There are many mechanisms that can explain for the transfer of organic contaminants from soil to plant tissue, including uptake in transpiration stream, volatilization and subsequent redeposition on leaves, and sorption from direct contact with soil particles (Chaudhry *et al.,* 2005). **Phytodegradation** is the term that describes the use of plants that are also capable

of taking up and degrading relatively water soluble organic contaminants, such as TCE. In some plants, such as hybrid poplars, TCE can be degraded by enzyme systems in the plant. **Phytovolatilization** is the uptake of contaminants by plants and then those contaminants may be volatilized from plant tissues by transpiration. **Hydraulic control:** some plants can transpire sufficient water to influence the flow of shallow groundwater. This method can reduce the build up of contaminants into the groundwater and can be coupled with phytoextraction and phytoremediation. **Phytostabilization:** plants and microorganisms in soil also influence the turnover and net accumulation of organic matter into the soil. This process is called mineralization–immobilization turnover (MIT). Some contaminants (e.g. As) or their transformation products can be chemically bound or incorporated into soil, or organic matter, a process known as humification, or physically trapped in the soil humic or mineral fractions, a process known as sequestration.

Rhizodegradation: carbon exudations and secretions from roots (from e.g. *Salix*) can stimulate microorganisms in the rhizosphere. The enhanced microbial activity in the rhizosphere in turn can enhance degradation of contaminants (e.g. PAHs) (Bowen *et al.,* 1991; Sylvia *et al.,* 2005). Besides, rhizosphere microbes can promote plant health by stimulating root growth, enhancing water and mineral uptake, and thus stimulating the growth of soil microbes (Smits, 2005). Because about 20% of the carbon fixed by the plant may be released from its roots, microbial densities are 1-4 orders of magnitude higher in rhizosphere soil than in bulk soil (Salt *et al*., 1998). The number of active growing microorganisms in the rhizosphere is significantly greater than that in the nonvegetated soil and these microorganisms can enhance biodegradation of PAHs (Önneby, 2005).

Several researches have shown that plants can enhance the rate and extent of degradation of PAHs and hydrocarbons in contaminated soil (Cerniglia, 1997; Muratova *et al.,* 2003). The mechanisms for removing PAHs can be explained by involving direct incorporation into humic material, increased abiotic incorporation of biologically generated intermediate metabolites, or increased microbial interaction resulting from the effects of the rhizosphere (Roberts, 1998).

Microorganisms and plants can also enhance pollutant bioavailability. Some bacteria release biosurfactants (e.g. rhamnolipids) that make hydrophobic substances more water soluble (Smits, 2005). Plant exudates or lysates also contain lipophilic compounds that increase the water solubility of pollutants or promote biosurfactant-producing microbial densities (Siciliano *et al.,* 1998). In addition, the solubility and the bioavailability of organic pollutants consequently can be affected by plant-and microbe-derived enzymes.

Organic pollutants can be directly degraded by root-released plant enzymes or indirectly by phytostimulation of microbial degradation in rhizosphere of both PAHs, and petroleum hydrocarbons (Smits, 2005). Muratova *et al*. (2003) showed that alfalfa enhanced both total number of microorganisms and the rate of the PAH degradation in rhizosphere. The total hydrocarbons in diesel contaminated soil decreased more in the nutrient and vegetated treatment than in non vegetated or non-fertilised soils. The degradation of diesel compounds in the rhizospheres of grass and legume species increased by addition of fertilizers (Pichtel *et al.,* 2001). The removal rate of aliphatic hydrocarbons in the presence of ryegrass (*Lolium perenne*) was higher and was associated with an increase in microbial numbers and activities in the rhizosphere as compared to the non-planted treatment (Chaudhry *et al*., 2005).

Plants with a demonstrated potential to phytoremediate petroleum hydrocarbons, have been reported by Frick *et al*. (1999). A part from many cereals and grasses also a woody species (*Populus deltoides x nigra*) related to *Salix* is included. *Salix* (Orm) degraded more mineral oils than PAHs and much more as compared to the control treatment (Vervaeke *et al*., 2003).

Among the many mechanisms of phytoremediation (Fig. 3), rhizodegradation is the most important mechanisms for decontamination of petroleum contaminated soils due to the relationship between roots and rhizosphere microorganisms on degradation of organic pollutants. Phytodegradation of PAHs have many similarities with that of petroleum hydrocarbons but is less studied with regard to the direct role of the plants.

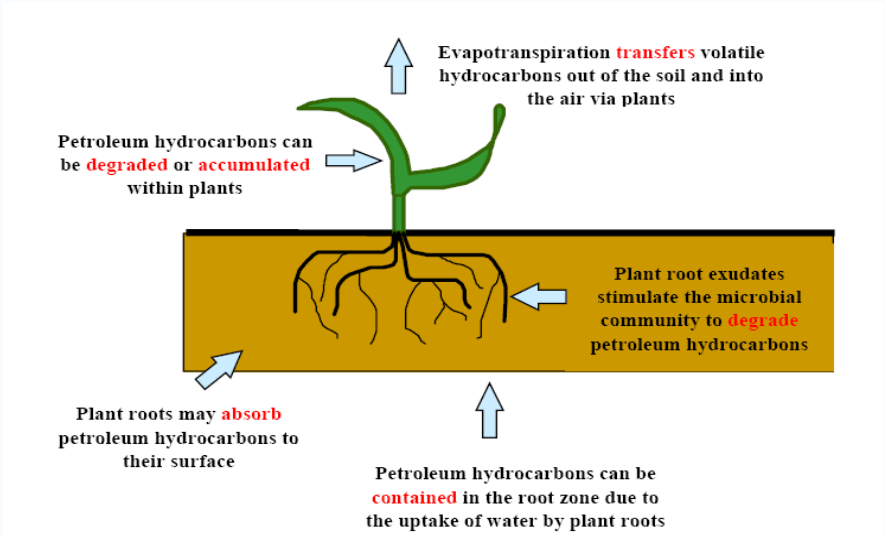

Figure 3. *Phytoremediation mechanisms: degradation, containment, or transfer of petroleum hydrocarbons in soil via interactions with plants and microorganisms. Based on Frick et al, (1999).*

3.8. PAH metabolic pathways and intermediates

The biochemical pathways were revealed by Roberts, (1989) for biodegradation of aromatic compounds. Bacteria generally use the PAHs as a carbon and energy source and play a role in the first step of aerobic catabolism of a PAH molecular via oxidation of the PAH by dihydroxylation with the company of a multi-component enzyme system. The dihydroxylated intermediates are processed by either an *ortho* or a *meta* cleavage type of pathway, coming to central intermediates e.g., protocatechuates and catechols. Those compounds are further changed to tricarboxylic acid cycle intermediates. Microorganisms also use dioxygenase enzymes to incorporate both atoms of molecular oxygen into the aromatic nucleus to form *cis*-dihydrodiols, then these forms are stereoselectively dehydrogenated by *cis*-dihydrodiol dehydrogenases, which rearomatize the benzene nucleus to form dihydroxylated intermediates (Cerniglia, 1992). Besides, the need of methane monooxygenases and lignin peroxidases is also important in the processes of PAH catabolism.

Filamentous fungi can prelude to detoxificate PAHs by a process of hydroxylation. They also mono-oxygenate PAH molecules by using the multifunctional oxidase (MFO) system whose membrane-bound variant includes cytochrome P-450, NADPA-cytochrome P-450 reductase and the phospholipid of endoplasmic reticulum membrane of the eukaryotic cell. Then phenols that may be transformed to the less toxic and more water-soluble, O-glucoside, -glucuronide,

sulphate, -xyloside, and –methyl conjugates by transferases will be formed from the result of disproportionation of arene oxides. They may also be metabolized to trans-dihydrodiols by fungal epoxide hydrolase in the presence of H_2O (Roberts, 1998). However, to get success of bioremediation technology for the decontamination of PAH contaminated sites, we need to know and understand more about the microorganisms, enzymatic processes and the environmetal conditions to optimize the degradation of PAH contaminants (Cerniglia, 1992). The pathways for microbial catabolism of PAHs are expressed in Fig. 4.

A vast number of bacteria, fungi and algae are able to metabolize PAHs (Table. 2). The biochemical pathways of naphthalene, phenanthrene, anthracene and acenaphthene by microbial degradation have been shown and elucidated. But there are few documents that show the capacity of microorganisms in using high molecular weight PAHs as sole sources of carbon and energy and about the genetic and regulatory mechanisms attending in the bacterial catabolism of high molecular weight PAHs (Cerniglia, 1992).

Table 2. *Genera of hydrocarbon and/or PAHs degrading microorganisms isolated from soil, from Frick et al, (1999).*

Bacteria		**Fungi**	
Acidovorax (phenanthrene, anthracene)	*Alcaligenes* (phenanthrene, fluorene, fluoranthene)	*Cunninghamella* (benzo[*a*]pyrene)	*Fusarium* (*n*-alkanes (C_{10} to C_{40}), benzene, naphthalene, phenanthrene)
Arthrobacter (*n*-alkanes (C_{10} to C_{40}), benzene, naphthalene, phenanthrene)	*Mycobacterium* (2-methylnaphthalene, phenanthrene, pyrene, benzo[*a*]pyrene, carbazole)	*Penicillium* (*n*-alkanes (C_{10} to C_{40}), benzene, naphthalene, phenanthrene)	*Phanerochaete* (benzo[*a*]pyrene)
Pseudomonas (phenanthrene, fluoranthene, fluorene, benzo[*a*]pyrene)	*Rhodococcus* (pyrene and benzo[*a*]pyrene)		
Sphingomonas (phenanthrene, fluoranthene, anthracene)	*Xanthomonas* (carbazole)		
Achromobacter	*Micrococcus*	*Acremonium*	*Monilia*
Acinetobacter	*Norcardia*	*Aspergillus*	*Mortierella*
Bacillus	*Proteus*	*Aureobasidium*	*Paecilomyces*
Brevibacterium	*Sarcina*	*Beauveria*	*Phoma*
Chromobacterium	*Serratia*	*Botrytis*	*Rhodotorula*
Corynebacterium	*Spirillum*	*Candida*	*Saccharamyces*
Cytophaga	*Streptomyces*	*Chrysosporium*	*Scolecobasidium*
Erwinia	*Vibrio*	*Cladosporium*	*Sporobolomyces*
Flavobacterium		*Cochliobolus*	*Sprotrichum*
		Cylindrocarpon	*Spicaria*
		Debaryomyces	*Syncephalastrum*
		Geotrichum	*Tolypocladium*
		Gliocladium	*Torulopsis*
		Graphium	*Trichoderma*
		Humicola	*Verticillum*

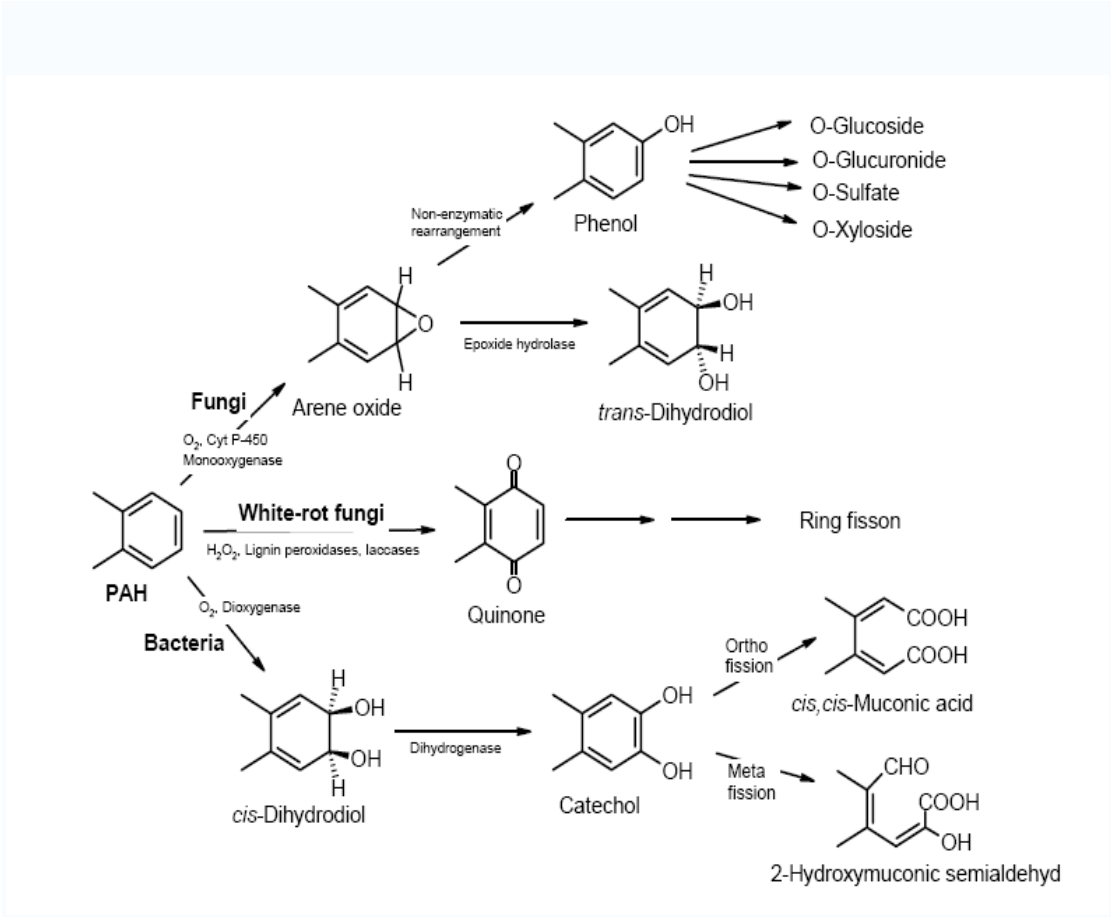

Figure 4. *General pathways for the microbial degradation of PAHs, from Bamforth et al, (2005).*

4. MATERIALS & METHODS

4.1. Soils

Creosote soil was collected at Hovgården, a waste disposal site outside Uppsala, Sweden. The soil originated from an urban site (Resecentrum, Uppsala) with aged creosote and diesel contamination and had been transported and deposited some weeks before sampling. The control soil (uncontaminated) was taken near the contaminated site in Uppsala city (with no apparent industrial activity).

Before experimentation, both contaminated and control soils were air-dried and homogenized by passing through a 5-mm sieve. In this study we tested three kinds of soil: contaminated soil, mixed soil and control soil. The mixed soil consisted of creosote and control soil, in a proportion of 1:1 was based on the soil dry weights. Some characteristics of the soils are shown in Table 3. The soils were stored in a deep freezer at -20 ^{0}C until used.

Table. 3: *Some soil characteristics at the beginning of the experiment. These parameters were analyzed by conventional methods.*

	Water content (%)	Water holding capacity (WHC) (%)	pH_{-water}	Total organic carbon (%)
Creosote soil	8.4	71.4	7.8	5.4
Mixed soil	9.1	67.2	8.2	4.3
Control soil	10.3	65.7	8.5	3.2

4.2. Plants

The plant species used in this study were five *Salix* clones (*Björn (SW910006)*, *Orm (SW870082) Tora (SW910007)*, *78112* and *78183).* They grew in the field of the Department of Crop Production Ecology, SLU. The cuttings were taken at the same time, selected to be of the same size (15 cm long), age and weight and kept in a cool room at + 4 ^{0}C. Before planting, they were put into a bucket with tap water for 4 days to develop roots in a room at + 20 ^{0}C.

4.3. Greenhouse study

The experiment was carried out in a greenhouse at the Soil Sciences Department for fifteen weeks (from 9th-May 2006 to 30th-September 2006). Five clones of Salix were tested and compared for their growth and the ability to degrade PAHs. The effect of the addition of nutrients in the soils without plants was also tested.

The cuttings (one per pot) were planted in 2 L pots containing the creosote, the mixed or the control soil as shown in Table 4. Soil water content was kept at 60-70% of the WHC by watering with distilled water all treatments. During the experiment when nutrient depletion was observed, Blomstra® (5 ml in 1 L distilled water was added) a commercial liquid fertilizer that contains 3.1 g NO_3^-; 2.0 g NH_4^+; 1.0 g P; 4.3 g K; 0.4 g S; 0.3 g Ca; 0.4 g Mg; 35 mg Fe; 20 mg Mn; 10 mg B; 3.0 mg Zn; 1.5 mg Cu and 0.4 mg Mo in 100 ml was used. The amount of Blomstra was later increased to 10 ml per 1 L for every watering time.

Table. 4: *Description of treatments of the second experiment*

Björn+control soil	Björn+mixed soil	Björn+creosote soil
Orm+control soil	Orm+mixed soil	Orm+creosote soil
Tora+control soil	Tora+mixed soil	Tora+creosote soil
78112+control soil	78112+mixed soil	78112+creosote soil
78183+control soil	7818+mixed soil	78183+creosote soil

The soils (creosote, mixed 1:1 and control) without plants were incubated in plastic 0.2 L pots. Soil water content was kept at 60-70 % of the WHC by watering with distilled water in all treatments. Blomstra was added to the soils at the same time and the same concentrations as the soils with plants giving following combinations:

1. Control soil + only distilled water
2. Control soil + nutrients
3. Mixed soil + only distilled water
4. Mixed soil + nutrients
5. Creosote soil + only distilled water
6. Creosote soil + nutrients

The treatments with plants were run in four replicates and the treatments without plants were run in duplicates.

Immediately before harvesting, the chlorophyll in leaves was measured by a chlorophyll meter (Rexolix Tracer). All leaves were separated from the stem. Chlorophyll intensity is a value related to nitrogen concentrations in leaves. Nitrogen is an integral part of chlorophyll, which converts light into chemical energy needed for photosynthesis. An adequate supply of N is associated with high photosynthetic activity, vigorous vegetative growth, and a

dark green color (Havlin *et al.,* 2005). The positive correlation between leaf nitrogen concentration (%) and chlorophyll intensity for willows is shown by the formulation: Leaf Nitrogen Concentration (LNC) = SPAD * 0.064 – 0.29 (Weih, 2006 pers.com) (Note: SPAD is chlorophyll intensity value).

At the end of the experiment, the soil in treatments with plants was separated from the roots by hand. As the root density was very high and covered all the soil volume, we considered all soil to be rhizosphere soil. The soil was harvested, thoroughly mixed, put into plastic bags and kept in deep freezer (-20 ^{0}C) until analysis.

All leaves were separated from the stem. Roots were rinsed several times with tap water and distilled water (the last time). All plant material was dried in oven at 75 ^{0}C for 48 h for biomass determination and put into paper envelopes and kept in a suitable place.

The content of remaining PAHs in the soils was analysed at Microbiology Department, SLU while duplicate of soil samples in the control, nutrient and Tora treatment were sent to a certified laboratory (AlControl laboratories, Linköping) to determine the diesel (aliphatic and aromatic) concentrations. The soils were analysed for both chemical (PAH concentrations, surface tension, total organic carbon (TOC), pH and water content) and microbial parameters, such as the number of extracted and cultivable bacteria (CFU/g soil), dominating bacteria (gram, fluorescence and oxidize reactions) as well ability to degrade PAH (phenanthrene). **Note**: after the experiment Tora was used as reference clone to compare with the control and the nutrient addition without plants.

4.4. PAH analysis

PAHs in soil were extracted by adding 10 ml of toluene and 10 ml of 0.05 M sodium pyrophosphate to tubes containing 10 g soil and shaking vigorously for 16 hours on a shaking table (Karstensen, 1997). The supernatant was centrifuged for 10 min at 492 x *g* and a portion of the toluene layer was cleaned in an alumina column (Isolute®). PAHs in liquid medium were extracted by adding 10 ml toluene to each tube and shaking for 1 h. after 10 min centrifugation at 492 x *g*. A sample of the supernatant was analysed directly by GC-MS (Pizzul *et al*., 2005).

GC-MS analysis was performed using a HP 6890 Series GC-system equipped with a HP 5971 Mass Selective Detector and HP 19091S-433 capillary column (0.25 mm inner diameter, 30 m length, 0.25 μm thickness). The oven programme was: 80 ^{0}C for 4 min followed by ramping at 7 ^{0}C min $^{-1}$ up to 310 ^{0}C maintained for 4 min. The injector temperature was 250 ^{0}C. Quantification was performed using external standards for 16 PAHs.

4.5. Surface tension measurements

Ten ml of distilled water was added to a tube containing 5 gram of soil sample and shaking for 1 hour. The mixture was centrifuged for 5 min at 358 x *g*. The surface tension of the supernatant was measured by the du Nouy method adapted from Pizzul *et al*. (2005) by using an Educational Tensiometer K6 (Kruss GmbH, Germany).

4.6. Isolation and identification of bacteria from soil

Soil bacteria were extracted from the soil by mixing 1 g of soil with 99 ml of phosphate buffer containing (per L) 23.99 g NaH_2PO_4 and 15.59 g Na_2HPO_4 and 1 ml 10 % Calgon (KEBO) in a 200 ml jar and shaking vigorously for 1 hour. The soil particles were allowed to sediment for a while and 1 ml of the supernatant was transferred to 9 ml of phosphate buffer for further dilutions and spread on TSB agar media (Tryptone Soya Broth (TSB) 10%) including 3 g Tryptone Soya Broth, 15 g agar and 1 g of the anti-fungal Delvocid® in 1 L of distilled water. The number of colony forming units (CFU) was determined after three days of incubation at 25^0C.

After counting, single colonies from TSB agar plates were streaked on agar plates with Kings B medium for Gram- and fluorescence tests or agar plates with nutrient agar (30 g nutrient agar in 1 L of distilled water) for oxidase tests. Kings medium contained 1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 20 g proteose, 10 g glycerol and 15 g agar in 1 L of distilled water (King *et al*., 1954).

Gram reaction was tested by mixing a loop of bacterial colonies with 1 drop of 3% KOH on a microscope slide by a toothpick. Bacterial strain was considered as Gram negative when a thread was formed between the toothpick and the microscope slide (Suslow *et al.,* 1982). The fluorescent properties were examinated by observing the King B media plates with bacteria on a CROMATO-VUE® (ultra-violet products, inc.) instrument at the wavelength 365 nm (Kovacs, 1956). Oxidase test was performed with a commercial kit (Bactident® Oxidase, MERCK). Bacteria were described according to bacterial colony morphology (Gerhardt *et al.,* 1994) and microscopic observations to identify the shape of bacteria.

4.7. Screening of PAH (phenanthrene) degraders

Individual strains were tested for their ability to degrade phenanthrene in the presence of glucose in liquid culture.

4.7.1. Preparation of the inoculum

Each strain was cultivated in 100-ml Erlenmeyer flask containing 50 ml of GYE medium (10 g of glucose and 10 g of yeast extract in 1 L of distilled water) for 4 days on a shaker (150 rpm) at 25 ^{0}C. The culture was centrifuged (8 min, 10 000 x *g*), the supernatant discarded and the pellet was washed twice with 20 ml of sterile tap water to make sure no carbon sources remain on it. The pellet was re-suspended in sterile tap water to an OD_{600} of 0.700, and used as inoculum.

4.7.2. Degradation of phenanthrene in liquid medium

0.5 ml of phenanthrene in acetone (500 µg/ml) was aseptically added into 50-ml tubes with 20 g of glass beads previously autoclaved. The acetone was allowed to evaporate. After acetone evaporated, 8 ml of minimal salt medium (9 ml in the control treatment), 1 ml of glucose solution (1% w/v) and 1 ml of the inoculum were put into 50 ml tubes (not in the control treatment). The minimal salt media contained 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4$ x $7H_2O$, 0.1 g NaCl, 0.026 g $CaCl_2$ x $2H_2O$, 1.0 g NH_4NO_3 in 1 L of distilled water. The pH after autoclaving was adjusted to 7.3, and 1 ml salt solution and 1 ml vitamin solution added.

The tubes were placed on a horizontal shaking table at 40 rev/min with inclination of about 10^{o} to the horizontal. The incubation time was 7 days at 30 ^{0}C. Duplicates were run for each treatment. Samples were taken at the seventh day of incubation to analyse the phenanthrene concentrations by GC-MS as described in 4.4.

4.8. Statistical analysis

The mean values of each treatment were used in analyses. Reported levels of variance were mainly based on 95% confidence limits (2xSE). A two-tailed Student t-test was used to determine the statistically significant differences ($p<0.05$).

5. RESULTS

5.1. Plant growth

During the first six weeks of the experiment, most plants showed healthy growth with no phytotoxicity symptoms regardless of the PAH or diesel concentrations in both mixed and creosote soil. Plants in the control soil (uncontaminated) seemed to grow better than plants in the creosote and mixed soil during this period. Appearance of chlorosis near midribs and some yellow leaves indicated nutrient deficiency during the first two weeks. This phenomenon was more pronounced in the clones 78183, Tora, and Orm compared to the clones Björn and 78112 in both creosote soil and mixed soil. In addition, there were sporadic attacks of aphids on young leaves and shoots, mostly in the clone Björn. However, nutrients were added and after the second month, there was a complete change. All plants in the control soil grew less well compared to plants in both mixed soil and creosote soil. Bacterial disease signs appeared in some plants in all soils. Plants in control soil showed disease signs in both the main cutting and shoots, whereas in plants in mixed and creosote soil such symptoms happened only in the main cuttings. The frequency of symptoms was also higher in control soil plants than that in either mixed soil or creosote soil plants. Two weeks before ending the experiment, two plants in the control soil had died. They belonged to the 78183 and 78112 clones.

The biomasses of the five Salix clones in all soils are shown in Table 5. In general, shoot and root biomass was above 14.0 and 3.0 g dry matter/plant, respectively for all soils. No significant differences between Salix clones regarding shoot and root biomass were found in the creosote soil, except for 78112, which had a lower root biomass. In the mixed soil, Orm showed lower shoot biomass than the clones Tora and 78183. Orm and 78112 had a lower root biomass than Tora.

Table 5: *Shoot and, root biomass and shoot/root ratio of plants within soils after 15 weeks. All values in parentheses are 95% confidence limits. The lower case letters following numbers express the statistical differences between Salix clones within a soil whereas the upper case letters present the statistical differences between soils within a clone, n=4, p<0.05.*

		Shoot (g)	*Root (g)*	*Shoot/Root*
Creosote soil	78183	22.6 (5.8)a AB	9.7 (3.8) a A	2.6 (1.0) b AB
	Tora	20.0 (0.3) a B	8.6 (0.9) a B	2.4 (0.3) b B
	Björn	17.0 (2.4) a A	8.2 (1.1) a A	2.1 (0.6) b B
	Orm	16.5 (3.6) a A	5.4 (2.4) ab A	3.3 (0.7) b B
	78112	19.8 (3.7) a A	3.7 (1.0) b A	5.5 (0.6) a A
Mixed soil	Tora	26.5 (3.4) a A	12.1 (1.6) a A	2.2 (0.1) b B
	78183	24.8 (1.7) a A	9.3 (2.1) ab A	2.8 (0.5) ab B
	Björn	21.6 (5.2) abA	7.8 (3.8) ab AB	3.3 (1.4) ab AB
	78112	21.6 (2.3) abA	5.2 (2.9) b A	5.3 (3.0) ab A
	Orm	13.9 (6.2) bA	4.2 (2.3) b A	3.4 (0.7) a B
Control soil	Björn	17.4 (1.9) abA	5.1 (0.9) a B	3.4 (0.5) b A
	78112	19.6 (1.0) a A	4.8 (0.7) a A	4.1 (0.8) ab A
	Orm	20.3 (2.1) a A	3.4 (1.3) ab A	6.6 (2.2) a A
	Tora	17.7 (2.5) ab B	3.4 (0.2) b C	5.3 (1.1) a A
	78183	16.0 (0.9) b B	3.3 (1.1) ab B	5.2 (1.7) ab A

Leaf nitrogen concentration between the Salix clones was also compared. In the creosote soil, Björn had the highest value of leaf nitrogen concentration (2.8 %), while Orm had the lowest value (2.0 %). The leaf nitrogen content of Björn in the mixed soil was higher than that of Orm, 78112 and 78183 (Table. 6). The value of Orm in the control soil was higher than that of either Tora or Björn. This indicates that Orm grew well only in the control soil as compared to the mixed and the creosote soils. The differences in leaf nitrogen content of 78183 and 78112 were not significant between soils. This confirms that 78183 and 78112 were not affected by low levels of PAH and diesel contaminants. Björn had higher leaf nitrogen content in both the creosote and the mixed soils than in the control soil. Contrary to Björn, the leaf nitrogen value of Orm was lowest in the creosote soil. The nitrogen content of Tora was highest in the mixed soil.

Table 6: *The leaf nitrogen concentration and chlorophyll intensity of plants within soils after 15 weeks. All values in parentheses are 95% confidence limits. The lower case letters following numbers express the statistical differences between clones within a soil whereas the upper case letters present the statistical differences between soil within a clone, n=4, p<0.05.*

		Chlorophyll intensity	*Leaf nitrogen concentration (%)*
Creosote soil	Björn	48.1 (1.1) a A	2.8 (0.1) a A
	78183	38.6 (1.2) b A	2.2 (0.1) b A
	Tora	36.9 (1.3) bc B	2.1 (0.1) bc B
	78112	36.4 (2.1) bcA	2.1 (0.1) bcA
	Orm	36.1 (1.1) c B	2.0 (0.1) c B
Mixed soil	Björn	48.4 (3.8) a A	2.8 (0.2) a A
	Tora	43.4 (3.0) ab A	2.5 (0.2) ab A
	Orm	40.6 (1.0) b A	2.3 (0.1) b A
	78112	40.0 (4.0) b A	2.3 (0.3) b A
	78183	38.3 (2.5) b A	2.2 (0.2) b A
Control soil	78183	43.7 (5.6) ab A	2.5 (0.4) ab A
	Orm	43.4 (2.1) a A	2.5 (0.1) a A
	78112	40.8 (3.0) ab A	2.3 (0.2) ab A
	Tora	35.8 (3.6) b B	2.0 (0.2) b B
	Björn	34.8 (2.6) b B	1.9 (0.2) b B

5.2. PAH degradation

The initial total concentration of PAHs in the undiluted creosote soil was low, around 10 mg/kg (Table A in the appendix). Acenapthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene and benzo(a)pyrene were detected initially. PAHs with two, three or four aromatic rings were the most abundant components in the concentration range 0.4 - 2.3 mg/kg soil. As expected, the concentrations of PAHs in the mixed soil were almost half (4.5 mg/kg) of the PAH concentrations in the creosote soil. All compounds that were present in the creosote soil were also detected in the mixed soil.

The percentage of degradation after 4 months in the creosote soil is shown in Fig. 5. The disappearance rate of most of the compounds was quite high and generally decreased with increasing molecular weight. Fluorene, phenanthrene, anthracene, benzo(a)anthracene and chrysene were almost completely degraded in all treatments. Phenanthrene and benzo(a)anthracene completely disappeared in the soils without plants regardless of the addition of nutrients. In the presence of plants the degradation (%) was lower, approximately 90% for phenanthrene and down to less than 60% for benzo(a)anthracene.

In the absence of plants, the dissipation of some PAHs was significantly higher in the soil with nutrient addition treatment than in soils where no nutrients were added. These PAHs were acenaphthene, benzo(b)fluoranthene and benzo(a)pyrene.

When comparing clones, no significant difference regarding degradation of PAHs was observed for Tora, Björn and 78183. The degradation capacity for certain PAHs was found to be lower for the clone 78112 than for the other clones. Orm showed lower degradation than Tora for two PAHs.

The degradation of PAHs in the mixed soil (Fig. 6) was slightly lower but similar to that in the undiluted soil. Only Orm differed from the other clones by showing less degradation of several PAHs.

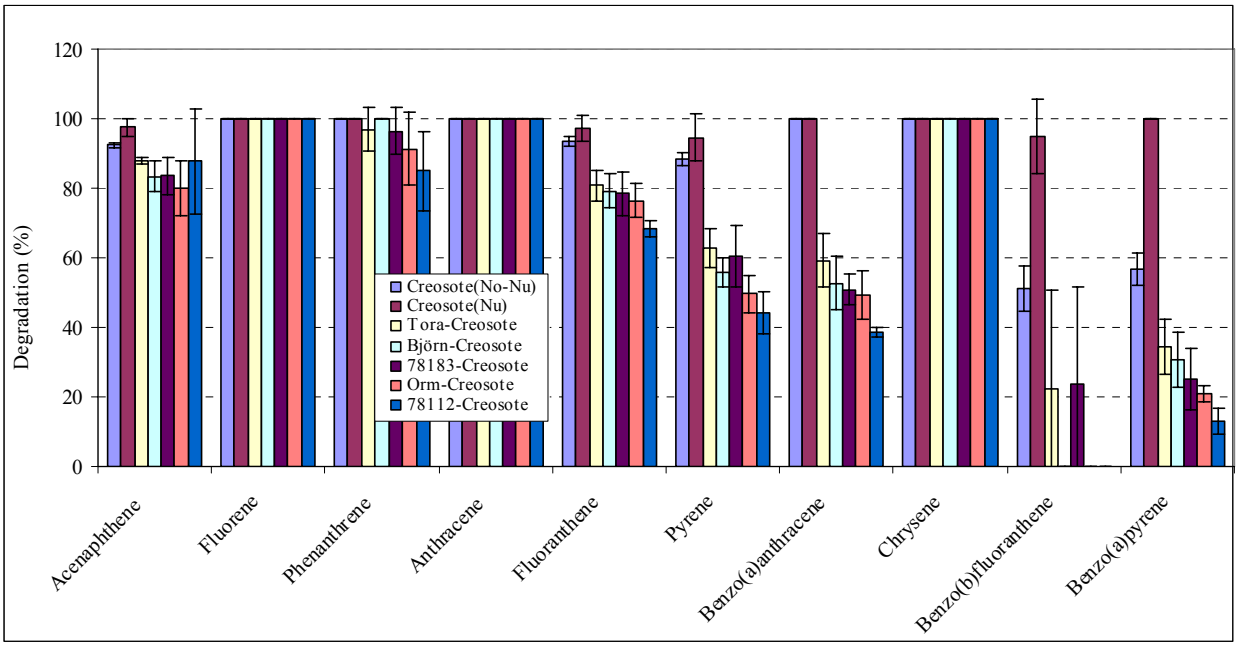

Figure 5. *PAH degradation (%) in the creosote soil after 15 weeks. Values ± 95% confidence limits, n=4 (except treatments without plants, n=2).*

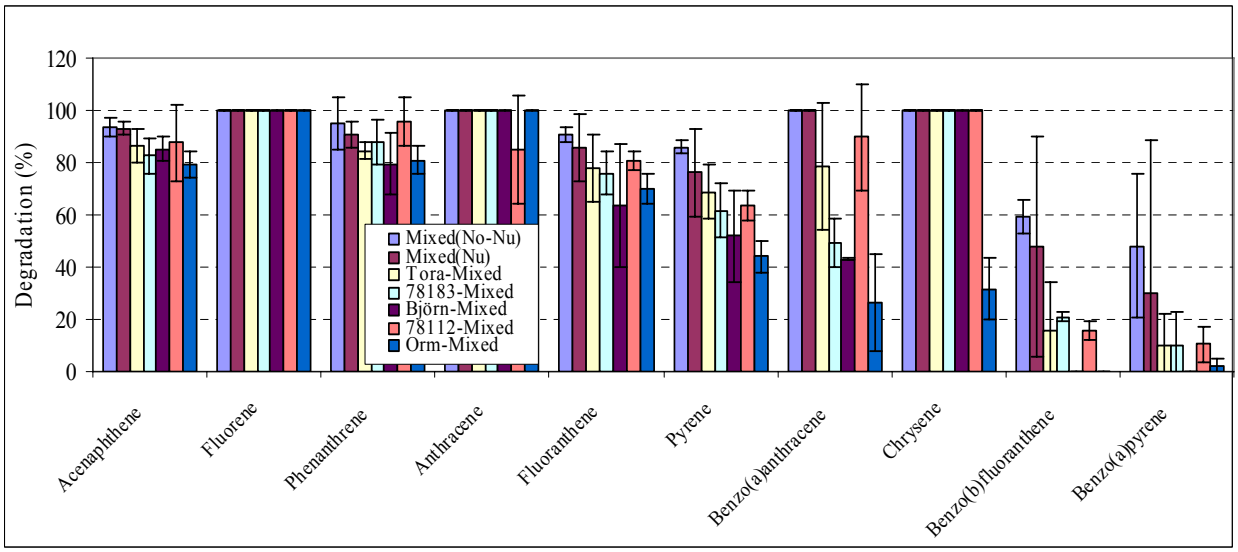

Figure 6. *PAH degradation (%) in the mixed soil after 15 weeks. Values ± 95% confidence limits, n=4 (except treatments without plants, n=2).*

5.3. Diesel (aliphatic and aromatic) concentrations in the creosote soil

The soil in this study had two mixed sources of contaminants: creosote and diesel. The diesel contamination was detected by quantifying aliphatic and aromatic hydrocarbons. The initial concentrations are shown in Table. 7. The compounds were divided into two groups according to the number of carbon atoms: C5-C16 and C16-C35 for aliphatics and C8-C10 and C10-C35 for aromatics.

After four months under greenhouse conditions, the amounts of both aliphatic and aromatic diesel dramatically dropped and became significantly lower as compared to the initial values. Aliphatics were less degraded than the aromatics (percentage of disappearance). No significant difference between the treatments regarding diesel degradation was found (although C5-C16 aliphatics and C8-C10 aromatics tended to be somewhat more degraded in the nutrient treatment and the treatment with plants, respectively).

Table 7. *Diesel concentration values at the beginning and after 4 months for creosote soils. All values are presented in mg/kg and 95% confidence limits are given in parentheses, n=2, except the initial value, n=1, p<0.05.*

		Initial value	*Control*	*+ Nutrients*	*+ Tora*
Aliphatic	C5-C16	350 a	155 (30.1) b	135 (10.0) b	160 (20.1) b
	C16-C35	750 a	395 (10.0) b	395 (90.3) b	435 (50.1) b
Aromatic	C8-C10	9.1 a	3.2 (0.9) b	3.7 (1.5) b	2.7 (0.6) b
	C10-C35	38 a	4.0 (0.8) b	3.7 (1.7) b	3.7 (1.3) b

5.4. Surface tension values in creosote soil

Surface tension was a parameter that was used in this study to evaluate whether there could be formation of biosurfactants by microorganisms. The values of surface tension significantly increased at the end of the experiment for all the treatments compared with initial values (Table 8), and the difference was highest in the soil without plants with addition of nutrients.

All treatments had the same tendency in that diesel decreased and surface tension increased. In the nutrient treatment higher diesel degradation and higher increase surface tension was found. Lower surface tension in the plant and the control treatments agrees with diesel dissipation. The conclusion from this part is that no clear indication of surfactant production by Tora or rhizosphere microorganisms was found.

Table 8: *Surface tension at the beginning and after 4 months for creosote soils. 95% confidence limits are given in parentheses, n=4, p<0.05.*

	Initial value	*Control*	*+ Nutrients*	*+ Tora*
Surface tension (*mN/m)*	59.8 (0.4) c	65.5 (0.7) b	67.5 (0.7) a	64.5 80.7) b

5.5. Microbial enumeration and composition

The number of active, viable soil microorganisms was determined in all the soils without plants, with and without nutrients, and in the presence of the clone Tora and compared to the initial numbers. The number of colony forming units per gram of soil is presented in Table 9. At the end of the experiment, in the creosote soil without plants, the bacterial count was higher in the treatment with addition of nutrients as compared to the initial value. The same behaviour was found in the mixed soil. In the control soil, however, the number of microorganisms in the soil without nutrients was slightly higher than the initial values.

Tora significantly increased the bacterial count in the control and the mixed soils as compared to either the initial values or the soil without plants. The effect of the plants was less than that of nutrients in the creosote soil.

Overall, significant differences between soils with the same treatment regarding bacterial counts were found. The highest initial bacterial count was found in the creosote soil, the second

was in the mixed soil and the lowest one was in the control soil. After 4 months under greenhouse conditions, the bacterial count in the treatment without nutrients both with and without plants was lower in the creosote soil than that of both the mixed and control soils. However, the bacterial count with the nutrient treatment in both the mixed and the control soils was much lower than that in the creosote soil. This result indicates that bacterial counts increased with increased amounts of PAH and diesel contaminants with nutrients whereas it was the other way round in the presence of plants (Tora). Plants may be assumed to have consumed most nutrients. The bacterial counts in the untreated treatment seemed to decrease more with the higher PAH and diesel contaminated levels.

Table 9: *The bacterial counts at the beginning and after 4 months. All values are presented in CFU x 10^5/g soil and 95% confidence limits are given in parentheses. The lower case letters following numbers express the statistical differences between treatments within a soil whereas the upper case letters present the statistical differences between soils within a treatment, n=2, p<0.05.*

	Initial value	*Control*	*+ Nutrients*	*+ Tora*
Creosote soil	232.5 (21.9) c A	4.4 (0.5) d B	363 (15.3) a A	285.8 (21.2) b B
Mixed soil	17.4 (6.2) c B	7.6 (2.5) d A	37.5 (4.3) b B	760.0 (119.1) a A
Control soil	6.4 (0.7) d C	8.2 (0.7) c A	51.7 (10.2) bB	911.7 (173.4) a A

After quantification, the microorganisms isolated from the creosote soil without plants and with Tora were grouped according to gram, oxidase test and fluorescence following the classification of Nejad *et al*. (2004). In general, group A (bacillii), C (enterobacteria) and D (coryneforms) were the most frequent bacterial groups in all treatments without plants (Fig. 7 to 9). The organisms isolated from this studied soil are thus primarily gram-positive bacteria. After 4 months there was a stimulation of microorganisms belonging to group E (*Xanthomonas* sp) in the soil without nutrients (Fig. 8) and to group B (*Pseudomonas spp., Sphingomonas)* in the soil with nutrients (Fig. 9). The most frequent bacteria in the soil with the clone Tora belonged mainly to the group D (coryneforms) but also to group B (*Pseudomonas spp., Sphingomonas)* and group C (enterobacteria) (Fig. 10).

A comparison between treatments regarding colony color and appearance showed that different colony types in the treatment with nutrients were slightly more abundant (9 types) than in the initial soil and the creosote soil without nutrients (7 types).

All bacteria isolated were further tested for their ability to degrade phenanthrene in liquid medium containing glucose (1%) as co-substrate. The results showed that one bacterial type belonging to group A (bacillii) and one of the group D (coryneforms) in the initial soil had the capacity to degrade phenanthrene. The first one could degrade up to 93.1% of phenanthrene in one week while the second degraded approximately 33.6%. In the creosote soil without nutrients and without plants one bacterial type belonging to the group D and one to group E, were capable to degrade up to 57.6% and 31.0% phenanthrene, respectively. In the same soil with addition of nutrients without plants, one bacterial type (group C) was capable to degrade phenanthrene, 20.4% in 7 days.

Three PAH-degrading bacteria types were found in the Tora treatment, one belonging to the group B and two to group D. They were able to degrade 53.3%; 29.0% and 12.7% of the phenanthrene, respectively. In summary, the most efficient phenanthrene degraders seemed to belong to *Bacillus* spp., followed by two coryneform strains. The latter seems most important as they together with group C constituted the only groups that were present in all treatments, but the former (group D) had more PAH-degraders (4 strains versus 1).

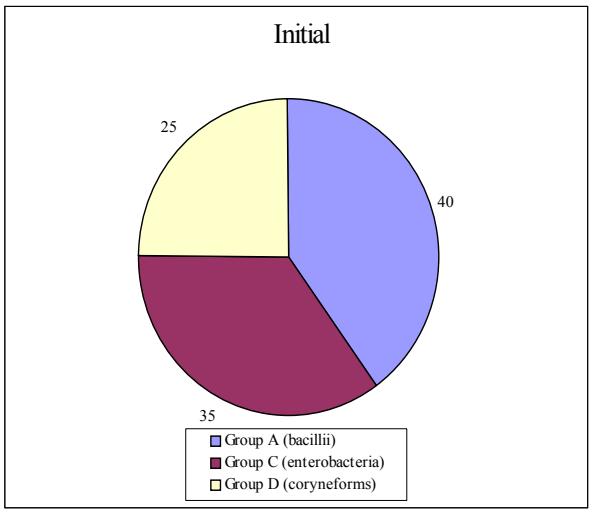

Figure 7: *Bacterial groups and their abundance (% of the total number of microorganisms/g soil) in the initial creosote soil*

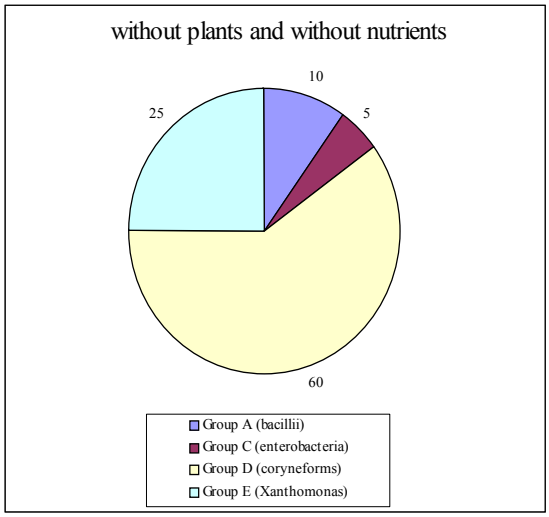

Figure 8: *Bacterial groups and their abundance (% of the total number of microorganisms/g soil) in the creosote soil without nutrients after 4 months*

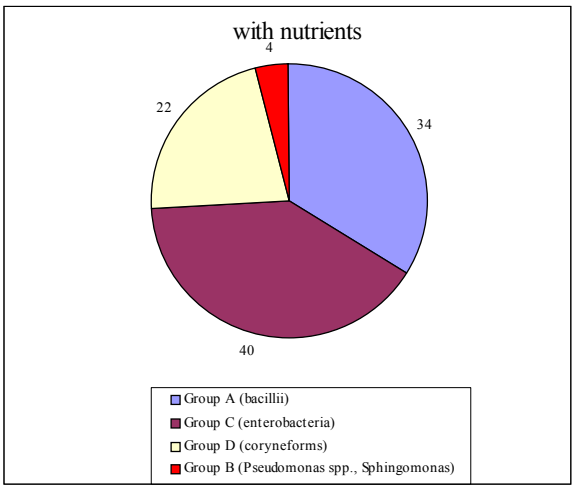

Figure 9: *Bacterial groups and their abundance (% of the total number of microorganisms/g soil) in the creosote soil with nutrients after 4 months*

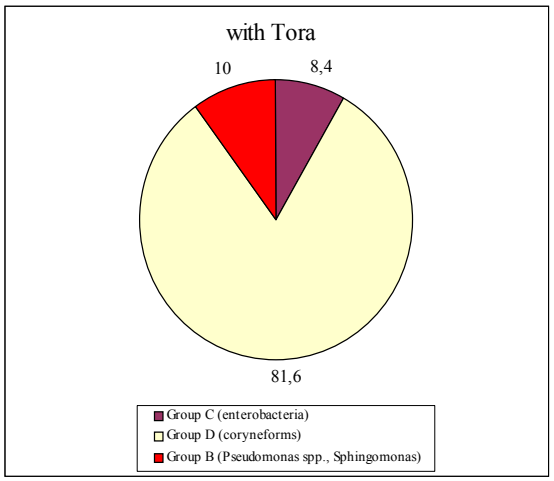

Figure 10: *Bacterial groups and their abundance (% of the total number of microorganisms/g soil) in the creosote soil with Tora after 4 months*

5.6. Soil pH

The soil pH values in the treatments without plants and with plants between soils are showed in Table 10. In general, soil pHs in all treatments were high (above 7). Some significant differences were found between treatments without plants. For all soils, pH in the control treatments remained high or increased somewhat. pHs in the nutrient treatments were lower than treatments without nutrients. Nutrients levelled out initial differences in soil pHs. Soil pHs in the treatments of plants (nutrients added) showed the same tendency but, were not significantly different from the nutrient treatments for all soils, except in the control soil where the soil pH of the plant treatment was higher than that of the nutrient treatment.

Table 10: *The soil pH values for treatments with and without plants. 95% confidence limits are given in parentheses. The lower case letters following numbers express the statistical differences between treatments within a soil whereas the upper case letters present the statistical differences between soils within a treatment, n=4, p<0.05*

	Initial value	*Control*	*+ Nutrients*	*+ Tora*
Creosote soil	7.9(0.05)bC	8.1(0.05)aC	7.8(0.12)bA	7.9(0.05)bB
Mixed soil	8.2(0.04)bB	8.3(0.05)aB	7.8(0.10)cA	7.9(0.04)cB
Control soil	8.5(0.01)aA	8.5(0.02)aA	7.7(0.05)cA	8.1(0.07)bA

6. DISCUSSION

We found that the dissipation of PAHs in the clones 78112 (the undiluted soil) and Orm (the diluted soil) was lower than that of other Salix clones. This agreed with a low root biomass. Consequently, we hypothesize that there could be a relation between root biomasses and degradation of PAHs as suggested also by Önneby (2005). Both the Tora and 78183 clones had a higher shoot biomass in the contaminated soils than in the control. Furthermore, the root biomass of 78183, Tora and Björn was significantly higher in both the mixed and the creosote soil than in the control soil and increased with higher levels of contaminants. This is logical since most plants remarkably expand their root systems when introduced to adverse environments (Cornish, 2005).

The shoot to root ratio is a good general parameter which indicates if plants exhibit a healthy growth or not. The shoot to root biomass for willow ranges between 2:1 and 3:1. Values above 3:1 could be considered as normal in fertilized soils (Granhall, 2006 pers.com). The clone 78112 had a higher shoot to root ratio (5.3-5.5) than the other Salix clones in both the mixed and the creosote soils. The ratios of all other clones were higher in the control soil than in either the mixed or the creosote soils. Taken together, this indicates that the clone 78112 can grow normally also in less ideal environments, but that the PAH and diesel contaminants caused at least a moderate stress effect in the other clones.

The PAH and diesel concentration seemed to affect the chlorophyll content (leaf nitrogen concentration (%)) and the healthy growth of certain clones. Björn did not seem negatively affected by the presence of the PAH and diesel amounts (in the mixed and the creosote soils) whereas Orm in particular showed reduced chlorophyll intensity in the presence of PAH and diesel contaminants. Chlorophyll measurements seem to correlate with growth and PAH degradation but depend on both clone type and level of contamination (cf. Önneby, 2005).

The success with which low molecular weight (LMW) PAH compounds were degraded in all treatments is attributed to their simple structures, e.g. fluorene, phenanthrene, anthracene,

containing basically three benzene rings, low molecular mass and a relatively higher solubility in water (Atagana *et al*., 2003). However, this is not a possible explanation for the degradation of benzo(a)anthracene, and chrysene because of their more complex structure, consisting of four benzene rings and much lower solubility. The disappearance of the high molecular weight (HMW) PAHs is probably due to the simultaneously occurring diesel contaminants. Diesel compounds may act both as carbon sources and co-metabolites (compounds that do not support microbial growth on their own but can be modified or degraded when another growth-supporting substrate is present) (Keck *et al.,* 1989; Cunningham *et al*., 1993). According to Kanaly *et al*. (2000) the mineralization of [14C] BaP in soil was 40% after 100 day incubation period with 0.2 (wt/wt) diesel fuels. Surprisingly, acenaphthene expected to be easy to degrade due to its two-ring structure was not completely degraded in neither the mixed nor the creosote soil (one possible explanation for this could be due to its recirculation to and from the air since acenaphthene is easily evaporated).

The degradation of acenaphthene, benzo(b)fluoranthene and benzo(a)pyrene was higher in the nutrient treatment than in the control treatment. This is attributed to the nutrient factor enhancing the ability of biodegradation by increasing the microbial population and/or stimulating the activity of special degraders. Accordingly, at the end of the experiment a larger microbial population was observed for the treatment with nutrients. Additionally, a higher density (according to the order of abundance) of PAH-degrading bacteria populations during the late stage could be linked to the higher PAH degradation rates observed in nutrient treated undiluted soil. The cultivable microbial population was stimulated more by nutrients in the creosote soil than either in the mixed or the control soils at the end of the experiment (see section 5.5). This result agrees with findings of Harris *et al.* (2006) that microbial population densities and biodegradation rates increase when either nitrogen or phosphorous is added to chemically-dispersed crude oil soil in lab-scale studies since the introduction of large amounts of hydrocarbons into the soil environment creates an imbalance in carbon to nutrient ratios because of the high amount of carbon supplied.

However, the question is why the degradation of some other PAHs was not significantly different between the two treatments and if nutrients stimulated only some special PAH degraders, such as acenaphthene, benzo(b)flouranthene and benzo(a)pyrene degraders. The control treatment had an impressive PAH degradation although the number of active bacteria was low. This is possibly explained by the role of slow-growing bacteria (e.g. coryneforms) that functioned as PAH-degrading bacteria in this soil. In severe environments bacteria have to use even recalcitrant hydrocarbons as their energy or carbon source for survival. In the control treatment slow growth bacteria (group D) that can degrade PAHs increased at the end of experiment.

In the mixed soil there was not any significant difference between the two treatments with respect to PAH degradation although there was also a considerable bacterial count increase in the nutrient treatment. This could be explained either by the much lower PAH concentrations in the mixed soil or that bacteria that were stimulated by nutrients were not much involved in the degradation of PAHs (cf. Pizzul *et al*., 2005). Other explanations could be due to sorption of PAHs to soil organic matter and clay particles since the clay content in the mixed soil seemed higher than in the creosote soil. Clay particles strongly absorb with PAH molecules and clay is normally rich in nutrients. Accordingly, the lower availability of PAHs and more nutrients could have been present in the mixed soil. A negative effect of an overload of nutrients is another explanation for this issue since according to Atagana *et al*, (2003), the highest nitrogen supplementation (C:N=5:1) did not enhance microbial growth and creosote removal.

Overall, in contrast to earlier studies (Önneby, 2005) it seemed that in both soils the Salix clones had a relatively low capacity to degrade benzo(b)fluoranthene and benzo(a)pyrene (most pronounced in the mixed soil). Although the presence of Salix clones had a considerable influence on the degradation of LMW PAHs in the creosote soil, the dissipation of HMW PAHs was lower than in the treatments without plants. The same tendency was found in the mixed soil, but not as clear as in the creosote soil.

We found that Tora, Björn and 78183 were better candidates as PAH degraders in the creosote soil as compared to the other clones. In this study a positive correlation between root biomasses of Salix and the dissipation of PAHs in the undiluted soil (creosote and diesel contaminated soil) became obvious. It is not known which plant properties that are mainly involved in the degradation of PAHs. The question if roots of Salix can take up LMW PAHs and transfer them to shoot and leaves or if they just adsorb PAHs (all kinds) on root surfaces is an important one, because known published information is available concerning the PAH uptake capacity of Salix species. For instance, Densy *et al.* (2006) found that Salix was contaminated with PAH in shoots and leaves when planted in soil amended with PAH contaminated sewage sludge, but the authors were not sure if the Salix species could take up PAHs through their roots. They thought that volatilization and atmospheric deposition were the main pathways causing the contaminations. Differences in efficiency among plants to degrade PAHs could be due to the size of the root-mass, root penetration properties, nature of root exudates and the species composition of the rhizosphere microbial community (Wang, 2006 pers.com).

The clone Orm had the lowest PAH degradation rate, especially regarding chrysene. This could partly be due to its lower root biomass. A higher shoot to root ratio, low leaf N values and an even lower root biomass of the clone 78112 in the creosote soil also coincided with a lower degradation of PAHs. However, in the mixed soil, degradation of PAHs in the clone 78112 was not less than that of Tora, although the shoot and root ratio of 78112 was higher than that of Tora. Either the 78112 clone had root exudates promoting the growth of soil microorganisms, including degraders (cf. Muratova *et al*., 2003), that were different from the other clones or that S/R ratios are not related with PAH degradation. However, further studies are necessary to solve this issue.

The higher PAH degradation rate in the nutrient treatment and even in the control treatment as compared to the Salix treatments is believed to be partly due to the competition between roots and microorganisms for nutrients, especially nitrogen and phosphorous. It could also be a competition for oxygen when nutrients were applied to soil. Thus, nitrogen and phosphorous may limit the conversion of carbon in external hydrocarbons to “cell carbon” (Rossenberg *et al*., 1983). Additionally, the quantity and quality of root exudates are important and vary with plant species (Sylvia *et al.,* 2005). Normally, in unstressed conditions most plants excrete root exudates with low amounts of amino acids (organic nitrogen) and high amounts of hydrocarbons. The amount of amino acids and/or biosurfactants increases in plants exposed to stress factors, such as pathogenic infections and hostile soil environments (Granhall, 2006 pers.com). In our studied soils, creosote levels were not so high and caused no diseases. Diesel contaminant levels were high but not obviously harmful. Consequently, plants did not suffer from inadequate conditions. The diesel components only affected the dissipation of PAHs to a small extent in the presence of Salix clones since they competed with root exudates as co-metabolites for PAH degradation. Roots can directly degrade PAHs by exoenzymes. The accumulation of metabolites resulting from oxidation of PAHs by roots may reduce the viability of certain PAH degraders in addition to inhibiting the degradation of PAHs (Vinas *et al.,* 2005). Actually, this result is quite contrary to other past results. Most articles have shown that *Salix* plants enhance the

biodegradation of either PAHs (e.g. Önneby, 2005) or mineral oils (Vervaeke *et al*., 2003). But in our studied soil, both PAH and diesel were presented. Diesel contaminants are relatively easy to degrade by microorganism as utilized carbon sources compared to PAH compounds. The presence of root exudates in the *Salix* treatments probably interacted with the specific co-metabolic degradation of HMW PAHs by certain diesel fractions (Kanaly *et al*., 2000).

According to Oleszczuk *et al*, (2005), the total PAHs that were maximally found in leaves and shoots of willows that were planted in the sewage sludge-amended soil contaminating PAHs constituted around 0.6 % of total PAHs of the initial value. But almost only light PAHs were found. Consequently, in our study we assume that if willows have capacity to take up light PAHs (with 0.6 % capacity), the amount of PAHs taken up by the willows could be regarded as negligible (0.06 mg/kg total PAHs).

As compared to PAH levels, total amounts of diesel dissipation were high. The fact that the degradation of aliphatics was faster than that of aromatics (in total amounts) is logical since shorter n-alkanes or aliphatic chains are believed to be preferentially degraded compared to aromatic compounds (Stout *et al*., 1998). According to Stronguilo *et al,* (1994) aliphatics are lost by both abiotic and biotic processes. Abiotic processes are more effective for short-chain aliphatic compounds. The n-alkanes between C10 and C25 are widely and readily utilized hydrocarbons by many microorganisms. However, the fact that both C16-C35 aliphatics and C10-C35 aromatics, considered as more recalcitrant compounds, were more degraded (90%) as compared to C5-C16 aliphatic and C8-C10 aromatic diesel, respectively is rather surprising. Slow-growing bacteria using the most abundant hydrocarbons as energy, carbon source and/or co-metabolite for PAH degradation may have had higher populations as compared to the fast-growing ones. The latter but not so much the former are counted in CFU-enumerations (favouring actively fast-growing type). May be these organisms were most active in the metabolism between diesel and PAHs linked to certain fractions of HMW diesel compounds (cf. Kanaly *et al*., 2000). The PAH-degrading bacteria in the treatment with nutrient in our case seemed to prefer C5-C16 aliphatic compounds as co-metabolite substrates compared to longer aliphatics.

The addition of limiting nutrients (e.g. N and P) would be expected to increase microbial degradation of petroleum hydrocarbons (Atlas *et al.,* 1972; Prince, 1992; Piehler *et al.*, 1996) Contrary to these expectations, the nutrient addition treatment was not clearly effective in stimulating microbial diesel degradation in our case. This could be due to the presence of other limiting factors after adding nutrients in spite of higher bacterial counts.

The initial bacterial count of the mixed soil was much lower than that of the creosote soil and lower than expected (intermediate value). This could be due to the sudden change of environmental conditions after mixing between the control and the creosote soil. It may have affected the survival of some microorganisms. The dramatical decrease of the bacterial counts in the untreated creosote soil treatment during the four months is believed be due to the lack of nutrients for long time leading to starvation of bacteria. Nutrient supplementation stimulated bacteria more in the creosote soil than either in the mixed soil or the control soil. This could be due to a response to nutrients in an unbalance nutrient situation, especially, between C, N and P in the creosote soil from the start. Nutrient addition brought about an adaptation for incorporating carbon from diesel and PAHs (creosote) into the biomass of bacteria. Actually, the C:N ratios from the start of the creosote, mixed and the control soils were 22:1; 16:1 and 14:1, respectively. It seems high in creosote soil for biodegradation since according to Atagana *et al.* (2003), the adequate C:N ratio to stimulate microbial growth and creosote degradation is 10:1.

The less stimulation of microbial numbers with nutrient treatment in both the control and mixed soils could thus be due to less need of nutrients (lower C:N ratios). In conclusion, nutrient addition stimulated bacterial counts in all soils, but most in the creosote soil.

One hypothesis was that degradation of the organic pollutants would increase with an increase of the active microbial community (biomass). Because of the high pH (see below) bacteria were probably the main candidates for decontaminating the organic pollutants in soil. Plant roots and exudates are expected to be factors that stimulate the activity and increase particularly the bacteria in organic contaminated soil. In all soils, *Salix* (Tora) also significantly increased the bacterial count more as compared either to the control treatment or the initial value due to the root exudates enhancing the active bacteria. This is in agreement with previous studies on PAH contaminated soil (Mastera, 2004; Hultgren, 2004; Önneby, 2005). In those latter cases PAH degradation, however, was also stimulated by the plants.

Soil pH is believed to be a parameter indicating biodegradation in organic contaminated soils since during the biodegradation of organic contaminants soil pH is reduced (Roberts, 1998). In our case no clear correlation between soil pH and PAH degradation in the contaminated soils was found taking all treatments into account. Both plants and nutrients lowered the pH significantly but only nutrient additions stimulated PAH degradation.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

No significant differences were found regarding shoot biomass between clones in the creosote soil. Clone 78183 and Tora showed stimulated shoot growth in the mixed soil whereas Orm did not grow well in this soil. Most clones showed better shoot growth than the old reference clone 78183 in the control soil.

Root biomass increased and the Shoot/Root ratio (S/R) decreased for most clones at higher concentrations of creosote and diesel contaminants.

Overall, there were little differences in degradation of most PAHs between the five Salix clones in both the creosote and the mixed soils. Tora, Björn and 78183 were better than 78112 and Orm in the creosote soil for certain PAHs whereas Orm was the worst clone in the mixed soil.

None of the five willow clones showed better degradation of any PAHs compared to the treatments without plants.

The presence of plants in all three soils increased the bacterial counts and stimulated group B (*Pseudomonas* spp, *Sphingomonas*) and D (coryneforms).

Addition of nutrients enhanced the degradation of certain PAHs and increased total microbial populations particularly group B (*Pseudomonas* spp, *Sphingomonas*) and C (enterobacteria) in the unplanted creosote soil, but not in the unplanted mixed soil. The C:N ratio in the creosote soil was higher than in the mixed soil. This ratio, indirectly lowered by nutrient addition, may have affected the biodegradation of certain PAHs. Further investigations on the effect of C:N ratios (unbalance of nutrients) on PAH-degradation in creosote and diesel contaminated soil is necessary.

Identifying the community of microbes and PAH-degrading bacteria in particular in the creosote soil by DNA extraction and sequencing or other genetic methods should be included in future research. The strains isolated have been preserved for such purposes.

Dynamics of PAH-degradation and microbial communities during the experiment should be tested in future studies to clarify the correlation between dissipation of PAHs and microbial populations in soil.

The role of different fractions of petroleum hydrocarbons as co-metabolites for bacteria during the biodegradation of PAHs should be further elucidated.

The function of fungi in bioremediation of PAH should be included in further studies.

We still don't know if the *Salix* clones can take up and/or adsorb certain PAH compounds or if all dissipation is due to rhizosphere degradation. Methods for analysing PAHs in roots and shoots of *Salix* clones should be elucidated in future studies.

8. REFERENCES

Alexander, M. 1994. Biodegradation and Bioremediation. Academic Press, San Diego, CA.

Arora, H.S., Cantor, R.R., Nemeth, J.C. 1982. Land treatment: available and successful methods of treating petroleum industry wastes. *Environ. Int*. 7: 285-291.

Atagana, H.I., Haynes, R.J., Wallis, F.M. 2003. Optimization of soil physical and chemical conditions for the bioremediation of creosote-contaminated soil. *Biodegradation.* 14: 297-307.

Atlas, R.M., Bartha, R. 1972. Degradation and mineralization of petroleum in sea water: Limitation by nitrogen and phosphorous. In: Piehler, M.F., Paerl, H.W (Eds). 1996. Enhanced biodegradation of diesel fuel through the addition of particulate organic carbon and inorganic nutrients in coastal marine waters. *Biodegradation.* 7: 239-247.

ATSDR. 2002. Public health statement creosote: Wood creosote, Coal tar creosote and Coal tar. http://www.atsdr.cdc.gov.

Bamforth, S. M., Singleton, I. 2005. Review bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. *Journal of Chemical Technology and Biotechnology*. 80: 723-736.

Bowen, G.C., Rovira, A.D. 1991. The rhizosphere-the hidden half of the hidden half. In: Plant Root-The Hidden Half,. Waisel, Y., Eshel, A., Kaffkafi, U (Eds). 641-669. New York: Marcel Dekker.

Brady, N.C., Weil, R.R. 1996. The Nature and Properties of Soils. Prentice Hall: Upper Saddle River, New Jersey.

Cerniglia, C.E., 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation.* 3: 351-368.

Cerniglia, C.E. 1997. Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *Journal of Microbial Biotechnology*. 19: 324-333.

Chaudhry, Q., Zandstra, M.B., Gupta, S., Joner, E. 2005. Utilizing the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment. *Environ Sci & Res.* 12: 34-48.

Cornish, P. 2005. Soil physical requirements for germination, root development, plant production and survival. Agricultural Research Institute, Wagga Wagga. NSW 2650. http://region.org.au/au/roc/1984/roc198407.htm

Cunningham, S.D., Berti, W.R. 1993. Remediation of contaminated soils with green plants: An overview. In: Frick, C.M., Farrell, R.E., Germida, J.J. 1999. Assessment of Phytoremediation as an In-Situ Technique for Cleaning Oil-Contaminated Sites. Department of Soil Sciences, University of Saskatoon, SK Canada. S7N 5A8. *In Vitro Cellular & Developmental Biology Plant*. 4: 207-212.

Densy, S., Rollin, C., Guillot, F., Baroudi, H. 2006. *In-situ* phytoremediation of PAHs contaminated soils following a bioremediation treatment. *Water, Air and Soil Pollution*. 6: 299-315.

Douglas, G.S., Prince, R.C., Buler, E.L., Steinhauer, W.G. 1994. The use of internal chemical indicators in petroleum and refined products to evaluate the extent of biodegradation. In: Remediation of Petroleum Contaminated Soils-Biological, Physical and Chemical Processes (Eds), Roberts, E.V. 1998.

Eriksson, M., Dalhammar, G., Borg-Karlson, A.K. 2000. Biological degradation of selected hydrocarbons in an old PAH/creosote contaminated soil from a gas work site. *Appl Microbiol Biotechnol.* 53, 619-626.

Fismes, J., Ganier, C.P., Bissonnet, P. E., Morel, J.L. 2002. Soil-to-root transfer and translocation of Polycyclic Aromatic Hydrocarbons by vegetables grown on industrial contaminated soils. *J. Environ. Qual*. 31: 1649-1656.

Frick, C.M., Farrell, R.E., Germida, J.J. 1999. Assessment of Phytoremediation as an In-Situ Technique for Cleaning Oil-Contaminated Sites. Department of Soil Sciences, University of Saskatoon, SK Canada. S7N 5A8.

FRTR. 2005. http://www.frtr.gov/matrix2/section4/4-2.html

Gerhardt, P., Murray, R.G.E., Wood, W.A., Krief, N.K. 1994. Methods for general and molecular bacteriology. *American Society for Microbiology*, Washington, D.C.

Harris, B.C., Bonner, J.S., McDonald, T.J., Fuller, C.B., Page, C.A., Dimitriou-Christidis, P., Sterling, M.C., Autenrieth, R.L. 2006. Nutrient effects on the biodegradation rates of chemically-dispersed crude oil. *Enviromental Resources Management*. Houston, TX-USA.

Hultgren, J. 2004. Fytosanering med *Salix viminalis* av kreosotförorenad jord-ett växthusförsök. Master thesis at the Department of Soil Sciences, SLU, Uppsala.

Juhasz, A., Naidu, R. 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. *International Biodeteration & Biodegradation*. 45: 57-88.

Kanaly, R.A., Harayama, S. 2000. Biodegradation of high molecular weight polycyclic aromatic hydrocarbons by bacteria. *Journal of Bacteriology*. 2059-2067.

Karlsson, S. 2005. Mobility of PAHs and Oxy-PAHs in contaminated soil. Master thesis work. Department of Chemistry, Umeå University, Environmental Chemistry.

Katensen, K.H. 1997. Nordic guidelines for chemical analysis of contaminated soil samples. Nt techn. Report. 329 Nordtest. Espoo. Finland.

Keck, J., Sims, R.C., Cover, M., Park, K., Symons, B. 1989. Evidence for cooxidation of polynuclear aromatic hydrocarbons in soil. *Water Research*. 23: 1467-1476.

Kings, E.O., Ward, M.K., Raney, D.E. 1954. Two simple media for demonstration of pyocyanin and fluorescence. *Journal of Laboratory and Clinical Medicine.* 44. 301-307.

Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178, 703.

Lundstedt, S. 2003. Analysis of PAHs and their transformation products in contaminated soil and remedial processes. Master thesis. Department of Chemistry- Environmental Chemistry, Umeå Unversity, Sweden.

Mastera, J. 2004. Biosanering av polyaromatiska kolväten med hjälp av *Salix viminalis*. Master thesis at the Department of Microbiology, SLU, Uppsala.

Menzie, C., Chemlsford, A. 1992. Exposure to carcinogenic PAHs in the environment. *Environmental Science and Technology*. 26: 1278-1284.

Mucha, A.P., Hryhorczuk, D., Serdyuk, A., Nakonechny, J., Zvinchuk, A., Erdal, S., Caudill, M., Scheff, P., Lukyanova, E., Nyzhnyk, Z.S., Chislovska, N. 2006. Urinary 1-Hydroxypyrene as a biomarker of PAH exposure in 3-year-old Ukrainian children. *Environmental Health Perspectives.* 114: 603-609.

Muratova, A., Hubner, T., Tischer, S., Turkaskaya, O., Moder, M., Kuschk, P. 2003. Plant-rhizophere-microflora association during phytoremediation of PAH-contaminated soil. *International Journal of Phytoremediation*, Vol. 5. 2: 137-151.

Myriam, A., Allieri, A., Lead, J.R., Vazquez, R.R. 2005. Impact of microbial activity on copper, lead and nickel mobilization during the bioremediation of soil PAHs. *Chemosphere.* 61: 484-491.

Nejad, P., Ramstedt, M., Granhall, U. 2004. Pathogenic ice-nucleation active bacteria in willows for short rotation forestry. For. Path. 34: 369-381.

Oleszczuk, P., Baran, S. 2005. Polycyclic Aromatic Hydrocarbons content in shoots and leaves of willow (*Salix viminalis*) cultivated on sewage sludge-amended soil. Water, air and Soil Pollution. 168: 91-111.

Palmroth, M.R.T., Langwaldt, J.H., Aunola, T.A., Goi, A., Munster, U., Puhakka, J.A., Tuhkanen, T.A. 2006. Effect of modified Fenton's reaction on microbial activity and removal of PAHs in creosote oil contaminated soil. *Biodegradation*. DOI. 10. 1007/s10532-005-6060-3.

Parr, J.F., Sikora, L.J., Burge, W.D. 1983. Factors affecting the degradation and inactivation of waste constituents in soils. In: Land Treatment of Hazardous Wastes. Parr, J.F., Marsh, P.B., Kla, J.M (Eds). Noyes data corp., Park Ridge, N.J. pp.20-49: 321-337.

Parrish, Z.D., Banks, M.K., Schwab, A.P. 2005. Effect of root death and decay on dissipation of Polycyclic Aromatic Hydrocarbons in the rhizosphere of yellow sweet clover and tall fescue. *Journal of Environmental Quality*. 34: 207-216.

Pichtel, J., Liskanen, P. 2001. Degradation of diesel fuel in rhizosphere soil. *Environmental Engineering Science.* 18: 145-157.

Piehler, M.F., Paerl, H.W. 1996. Enhanced biodegradation of diesel fuel through the addition of particulate organic carbon and inorganic nutrients in coastal marine waters. *Biodegradation.* 7: 239-247.

Pizzul, P., Hultgren, J., Granhall, U., Castillo, M.D.P. 2005. Phytoremediation of polycyclic aromatic hydrocarbons by means of *Salix viminalis* a greenhouse experiment with creosote contaminated soil. In: Pizzul, L. 2006. Degradation of Polycyclic Aromatic Hydrocarbons by Actinomycetes. Doctoral Thesis No. 2006:50. Faculty of Natural Resources and agricultural Sciences, Swedish University of Agricultural Sciences (SLU).

Prince, R.C. 1992. Bioremediation of oil spill, with particular reference to the spill from the Exxon Valdez. In: Piehler, M.F., Paerl, H.W. 1996. Enhanced Biodegradation of Diesel Fuel through the Addition of Particulate Organic Carbon and Inorganic Nutrients in Coastal Marine Waters. *Biodegradation*. 7: 239-247.

Recetox. 2006. Persistent, bioaccumulative and toxic chemicals in Central and Eastern European Countries-State-of-the-art report.
http://www.recetoc.muni.cz/index.php?language=en&id=4307

RECIEL. 2000. Article 95 EC treaty in practice: The European commission decisions on creosote, sulphite, nitrates and nitrites.

Roberts, E.V. 1998. Remediation of petroleum contaminated soils-biological, physical and chemical processes. Lewis Publishers. Boca Raton, London, New York Washington, D.C.

Rossenberg, E., Gottlieb, A., Rosenber, M. 1983. Inhibition of bacterial adherence to hydrocarbon and epithelial cells by emulsan. In: Atagana, H.I., Haynes, R.J., Wallis, F.M (Eds). 2003. Optimization of Soil Physical and Chemical Conditions for the Bioremediation of Creosote-contaminated Soil. *Biodegradation.* 14, 297-307.

Salt, D.E., Smith, R.D., Raskin, I. 1998. Phytoremediation. *Annu. Rev. Plant Physiol. Plant*. 49: 643-668.

Samanta, S.K., Singh, O.V., Jain, R.K. 2002. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends Biotechnol*. 20: 243-248.

Semple, K.T., Morriss, W.J., Paton, G.I. 2003. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *European Journal of Soil Science.* 54: 809-818.

Shaikh, A.U., Hawk, R.M., Sims, R.A., Scott, H.D. 1985. Redox potential and oxygen diffusion rate as parameters for monitoring biodegradation of some organic wastes in soil. *Nucl. Chem. Waste Manage.* 5: 337-343.

Siciliano, S.D., Germida, J.J. 1998. Mechanisms of phytoremediation: biochemical and ecological interactions between plants and bacteria. *Environment. Rev*. 6: 65-79.

Smits, E.P. 2005. Phytoremediation. Biology Department, Colorado State University, Fort Collins, Colarado 80523.

Sprehe, T.G., Streebin, L.E., Roberton, J.M., Bowen, P.T. 1985. Processes considerations in land treatment of refinery sludges. In proc. 40th Ind. Waste Conf. May 14-16, Purdue University, West Lafayette, In. Butterworth, Boston. P. 529-534.

Stout, S.A., Lundegard, P.D. 1998. Intrinsic biodegradation of diesel fuel in an interval of separate phase hydrocarbons. *Applied Geochemistry*. 13: 851-859.

Stronguilo, M.L., Vaquero, M.T., Comellas, L., Broto-Puig, F. 1994. The fate of petroleum aliphatic hydrocarbons in sewage sludge-amended soils. *Chemosphere.* 29: 273-281.

Sylvia, D.M., Hartel, P.G., Fuhrmann, J.J., Zuberer, D.A. 2005. Principles and Applications of Soil Microbiology. 2nd edition. Pearson Prentice Hall.

US EPA. 2006. Information on the toxic effects of various chemicals and group of chemicals. U.S. Environmental Protection Agency-Ecological Risk Assessment. http://www.epa.gov/region5superfund/ecology/html/toxprofiles.htm

Vervaeke, P., Luyssaert, S., Mertens, J., Meers, E., Tack, F.M.G., Lust, N. 2003. Phytoremediation prospects of willow stands on contaminated sediment: a field trial. *Environmental Pollution.* 126: 275-282.

Vinas, M., Sabate, J., Espuny, M.J., Solanas, A.M. 2005. Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavy creosote-contaminated soil. *Applied and Environmental Microbiology*. 7008-7018.

WHO-Denmark. 2000. Polycyclic aromatic hydrocarbons (PAHs). In: Air quality guidelines for Europe. Copenhagen, World Health Organization Regional Office for Europe, 2000, pp: 1-5.

Wilson, S.C., Jones, K.C. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs). *Review. Environ. Pollut*. 81: 229-249.

Zhang, H.B., Luo, Y.M., Wong, M.H., Zhao, Q.G., Zhang, G.L. 2006. Distribution and concentrations of PAHs in Hongkong Soils. *Environmental pollution*. 141: 107-114.

Zhou, E., Crawford, R.L. 1995. Effects of oxygen, nitrogen, and temperature on gasoline biodegradation in soil. *Biodegradation.* 6: 127-140.

Zitrides, T. 1983. Biodecontamination of spill sites. *Pollut. Eng*. 15: 25-27.

Önneby, K. 2005. Phytoremediation of highly creosote-contaminated soil by means of *Salix viminalis*. Master thesis at The Department of Microbiology, SLU, Uppsala.

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10. APPENDIX

Table A. *PAH concentrations at the start and after 4 months for treatments without plants with and without nutrients. 95% confidence limits are given in parentheses. The lower case letters following numbers express the statistical differences between treatments regarding PAH compounds, nd: not detected, n=2, p<0.05.*

PAH compound	*Creosote soil-initial (mg/kg)*	*After 4 months-control*	*After 4 months-nutrients*	*Mixed soil-initial (mg/kg)*	*After 4 months-control*	*After 4 months-nutrients*
Acenaphthene	1.6 (0.07) a	0.12 (0.01) b	0.04 (0.04) c	0.6 (0.08) a	0.04 (0.02) b	0.04 (0.01) b
Fluorene	1.1 (0.07)	*nd*	*nd*	0.4 (0.08)	*nd*	*nd*
Phenanthrene	2.3 (0.14)	*nd*	*nd*	1.0 (0.18) a	0.05 (0.10) b	0.09 (0.05) b
Anthracene	0.9 (0.08)	*nd*	*nd*	0.4 (0.06)	*nd*	*nd*
Fluoranthene	1.6 (0.12) a	0.10 (0.02) b	0.04 (0.05) b	0.8 (0.07) a	0.08 (0.03) b	0.12 (0.10) b
Pyrene	1.4 (0.09) a	0.16 (0.03) b	0.07 (0.09) b	0.7 (0.05) a	0.10 (0.02) b	0.17 (0.12) b
Benzo(a)anthracene	0.2 (0.02)	*nd*	*nd*	0.1 (0.01)	*nd*	*nd*
Chrysene	0.7 (0.05)	*nd*	*nd*	0.3 (0.03)	*nd*	*nd*
Benzo(b)fluoranthene	0.2 (0.12) a	0.11 (0.01) b	0.01 (0.02) c	0.1 (0.01) a	0.03 (0.00) b	0.04 (0.03) b
Benzo(a)pyrene	0.2 (0.03) a	0.09 (0.01) b	*nd*	0.1 (0.02) a	0.05 (0.03) a	0.07 (0.06) a

Table B. *The concentrations of PAHs at the beginning and after 4 months for treatments with plants in the creosote soil. 95% confidence limits are given in parentheses. The lower case letters following numbers express the statistical differences between treatments regarding PAH compounds, nd: not detected, n=4, except the control treatment (n=2), p<0.05.*

PAH compound	*Creosote soil-initial (mg/kg)*	*After 4 months-control*	*After 4 months-Tora*	*After 4 months-Björn*	*After 4 months-78183*	*After 4 months-Orm*	*After 4 months-78112*
Acenaphthene	1.6 (0.07) a	0.12 (0.01) c	0.19(0.02) b	0.26(0.07) b	0.26(0.08) b	0.31 (0.12) b	0.19 (0.22) bc
Fluorene	1.1 (0.07)	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Phenanthrene	2.3 (0.14) a	*nd*	0.07(0.00) c	*nd*	0.08(0.00) c	0.20 (0.15) bc	0.46 (0.18) b
Anthracene	0.9 (0.08)	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Fluoranthene	1.6 (0.12) a	0.10 (0.02) c	0.30(0.07) b	0.32(0.07) b	0.34(0.1) b	0.37 (0.08) b	0.78 (0.57) b
Pyrene	1.4 (0.09) a	0.16 (0.03) d	0.50(0.08) c	0.60(0.06) bc	0.54(0.12) bc	0.68 (0.07) b	0.99 (0.48) abc
Benzo(a)anthracene	0.2 (0.02)	*nd*	0.08(0.02) b	0.10(0.02) b	0.10(0.01) b	0.10 (0.01) b	0.27 (0.28) abc
Chrysene	0.7 (0.05)	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Benzo(b)fluoranthene	0.2 (0.12) ab	0.11 (0.01) b	0.19(0.09) ab	0.26(0.07) a	0.20(0.10) ab	0.28 (0.10) a	0.32 (0.11) a
Benzo(a)pyrene	0.2 (0.03) a	0.09 (0.01) d	0.15(0.04)abc	0.16(0.04)abc	0.15(0.02)abc	0.16 (0.00) b	0.13 (0.01) c

Table C. *The concentrations of PAHs at the beginning and after 4 months for treatments with plants in the mixed soil, 95% confidence limits are given in parentheses. The lower case letters following numbers express the statistical differences between treatments regarding PAH compounds, nd: not detected, n= 4, except the control treatment (n=2), p<0.05.*

PAH compound	*Mixed soil-initial (mg/kg)*	*After 4 months-control*	*After 4 months-Tora*	*After 4 months-78183*	*After 4 months-Björn*	*After 4 months-78112*	*After 4 months-Orm*
Acenaphthene	0.6 (0.08) a	0.04 (0.02) c	0.09(0.04) bc	0.11 (0.04) b	0.09 (0.03) b	0.08 (0.06) bc	0.13 (0.03) b
Fluorene	0.4 (0.08)	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Phenanthrene	1.0 (0.18) a	0.05 (0.10) b	0.16(0.03) b	0.12 (0.04) b	0.20 (0.12) b	nd c	0.19 (0.05) b
Anthracene	0.4 (0.06) a	*nd*	nd	nd	nd	0.05 (0.1) b	nd
Fluoranthene	0.8 (0.07) a	0.08 (0.03) d	0.18(0.11)bcd	0.20 (0.07) bc	0.30 (0.19) bc	0.16 (0.03) c	0.25 (0.05) b
Pyrene	0.7 (0.05) a	0.10 (0.02) d	0.22(0.07) c	0.27 (0.07) c	0.43 (0.12) bc	0.26 (0.04) c	0.40 (0.04) b
Benzo(a)anthracene	0.1 (0.01) a	*nd*	0.05(0.06)abc	0.04 (0.01) b	0.06 (0.04) ab	nd	0.06 (0.02) b
Chrysene	0.3 (0.03) a	*nd*	nd	nd	nd	nd	0.22 (0.04) b
Benzo(b)fluoranthene	0.1 (0.01) a	0.03 (0.00) d	0.07(0.07)abcd	0.07(0.03)abc	0.09(0.04)abc	0.06 (0.00) c	0.07 (0.00) b
Benzo(a)pyrene	0.1 (0.02) ab	0.05 (0.03) b	0.11(0.11) ab	0.09 (0.02) ab	0.13 (0.05) ab	0.09 (0.01) ab	0.10 (0.01) a

Table D. *The dissipation of diesel in the soil with and without plant treatments, n=2, p<0.05.*

		Dissipation (%)		
		Control	*+ Nutrients*	*+ Tora*
Aliphatic	C5-C16	55.7 (8.6)	61.4 (2.9)	54.3 (5.7)
	C16-C35	47.3 (1.3)	47.3 (12.0)	42.0 (6.7)
Aromatic	C8-C10	65.4 (9.9)	59.9 (16.5)	70.3 (6.6)
	C10-C35	89.5 (2.1)	90.4 (4.5)	90.4 (3.4)

Table E. *Biochemical and morphological characteristics of the bacteria isolated from the creosote soil at the beginning of the experiment. * Bacterial groups were adapted from Nejad et al, (2004).*

No.	Bacterial strain	Gram	Oxidase	Fluorescence	Colony morphology/cell shape	Bacterial group example*	Phenanthrene-degrading capacity	Abundance (% of the total number of bacteria
1	IC1	+	+	-	Pink, rough. Form: irregular; elevation: raised; margin: lobate. Shape: short rod	A (bacillii)	+	I (40%)
2	IC2	-	-	-	Yellow, rough. Form: irregular; elevation: raised; margin: lobate. Shape: cocci	C (enterobacteria)	-	II (35%)
3	IC3	+	-	-	Light yellow, rough. Form: irregular; elevation: flat; margin: undulate. Shape: long and big rod and slow growing.	D (coryneforms)	-	III (5%)
4	IC4	+	-	-	Transparent, small colony, rough and dry surface. Form: irregular; elevation: flat; margin: lobate. Shape: big cocci	D (coryneforms)	-	III (5%)
5	IC5	+	-	-	Pink, small colony, smooth surface. Form: circular; elevation: raised; margin: entire. Shape**:** big cocci and slow growing.	D (coryneforms)	-	III (5%)
6	IC6	+	-	-	Orange-pink, smooth surface in the center of colony, dry in margin. Form: irregular; elevation umbonate; margin: undulate. Shape: small cocci	D (coryneforms)	+	III (5%)
7	IC7	+	-	-	Orange-lighter pink, smooth in center of colony. Form: irregular; elevation: raised; margin: lobate. Shape: long and big rod, paired cells.	D (coryneforms)	-	III (5%)

Table F. *Biochemical and morphological characteristics of the bacteria isolated from the creosote soil in the untreated treatment. * Bacterial groups were adapted from Nejad et al, (2004).*

No.	Bacterial strain	Gram	Oxidase	Fluorescence	Colony morphology/cell shape	Bacterial group example*	Phenanthrene-degrading capacity	Abundance (% of the total number of bacteria
1	C-No-Nu1	+	-	-	Creamy pink, rough surface. Form: irregular; elevation: raised; margin: lobate. Shape: rod	D (coryneforms)	+	I (45%)
2	C-No-Nu2	+	-	-	Yellow, smooth. Form: irregular; elevation: umbonate; margin: undulate. Shape: cocci, paired cells	D (coryneforms)	-	II (15%)
3	C-No-Nu3	+	Weak +	-	Yellow, small colony, dry and rough surface. Form: punctiform; elevation: rasied; margin: undulate. Shape: short rod.	A (bacillii)	-	IV (5%)
4	C-No-Nu4	-	Weak +	-	Pink, small colony and smooth surface. Form: irregular; elevation: raised; margin: undulate. Shape: short rod	E (*Xanthomonas*)	-	II (15%)
5	C-No-Nu5	+	+	-	Heavy pink, small colony and smooth surface. Form: irregular; elevation: umbonate; margin: undulate. Shape: short rod, and paired cells.	A (bacillii)	-	IV (5%)
6	C-No-Nu6	-	-	-	Light brown and smooth surface. Form: irregular; elevation: raised; margin: lobate. Shape: short rod	C (enterobacteria)	-	IV (5%)
7	C-No-Nu7	-	Weak +	-	Light yellow, dry and rough surface. Form: irregular; elevation: raised; margin: lobate. Shape: long rod	E (*Xanthomonas*)	+	III (10%)

Table G. *Biochemical and morphological characteristics of the bacteria isolated from the creosote soil in the unplanted treatment with nutrients. * Bacterial groups were adapted from Nejad et al, (2004).*

No.	Bacterial strain	Gram	Oxidase	Fluorescence	Colony morphology/cell shape	Bacterial group example*	Phenanthrene-degrading capacity	Abundance (% of the total number of bacteria
1	CN1	+	-	-	Pink, small colony, smooth. Form: circular; elevation: umbonate; margin: entire. Shape: cocci	D (coryneforms)	-	II (10%)
2	CN2	+	-	-	Yellow, rough, big colony. Form: irregular; elevation: flat; margin: undulate. Shape: cocci	D (coryneforms)	-	III (4%)
3	CN3	+	+	-	Heavy brown, big colony, rough. Form: irregular; elevation: raised; margin: undulate. Shape: cocci	A (bacillii)	-	I (30%)
4	CN4	+	Weak +	-	Transparent orange, small colony, dry and rough surface. Form: punctiform; elevation: flat; margin: undate. Shape: short rod	A (bacillii)	-	III (4%)
5	CN5	-	-	-	Weak brown, smooth and shiny surface. Form: irregular; elevation: umbonate; margin: undulate. Shape: short rod	C (enterobacteria)	-	II (10%)
6	CN6	-	-	-	Yellow, big colony, smooth in center of colony and rough surround margin of colony. Form: irregular; elevation: raised; margin: lobate. Shape: cocci and paired cells	C (enterobacteria)	+	I (30%)
7	CN7	-	+	-	Pink, small colony and rough surface. Form: irregular; elevation: flat; margin: undulate. Shape: cocci	*B (Pseudomonas spp., Sphingomonas)*	-	III (4%)
8	CN8	+	-	-	Orange, small colony, smooth and shiny colony. Form: filamentous; elevation: convex; margin: erose. Shape: long and big rod.	D (coryneforms)	-	III (4%)
9	CN9	+	-	-	Light yellow, small colony, very shiny and smooth surface. Form: irregular; elevation: convex; margin: undulate. Shape: big cocci	D (coryneforms)	-	III (4%)

Table H. *Biochemical and morphological characteristics of the bacteria isolated from the creosote soil in the treatment with plants. * Bacterial groups were adapted from Nejad et al, (2004).*

No.	Bacterial strain	Gram	Oxidase	Fluorescence	Colony morphology/cell shape	Bacterial group example*	Phenanthrene-degrading capacity	Abundance (% of the total number of bacteria
1	CT1	-	+	-	Pink, smooth surface. Form: irregular; elevation: raised; margin: undulate. Shape: cocci	*B (Pseudomonas spp., Sphingomonas)*	+	II (10%)
2	CT2	+	-	-	Yellow, small colony, smooth surface. Form: lilamentous; elevation: convex, margin: undulate. Shape: cocci and paired cells	D (coryneforms)	-	I (25%)
3	CT3	+	-	-	Transparent, very small colony, rough. Form: irregular; elevation: flat; margin: undulate. Shape: cocci	D (coryneforms)	+	II (10%)
4	CT4	+	-	-	Pink, big colony, smooth and shiny colony. Form: irregular; elevation: raised; margin: lobate. Shape: big cocci and paired cells	D (coryneforms)	-	III (8.3%)
5	CT5	+	-	-	Heavy yellow, small colony, smooth and shiny colony. Form: irregular; elevation: raised; margin: undulate. Shape: small cocci and paired cells	D (coryneforms)	-	II (10%)
6	CT6	+	-	-	Weak yellow, big colony, smooth and very shiny colony. Form: irregular; elevation: convex; margin: undulate. Shape: short rod	D (coryneforms)	-	II (10%)
7	CT7	+	-	-	Dark yellow-orange, small colony, shiny and rough colony. Form: irregular; elevation: convex; margin: undulate. Shape: cocci	D (coryneforms)	-	III (8.3%)
8	CT8	-	-	-	Orange, small colony, smooth and shiny surface. Form: irregular; elevation: raised; margin: undulate. Shape: cocci	C (enterobacteria)	-	III (8.3%)
9	CT9	+	-	-	Pink, small colony, smooth and shiny colony. Form: irregular; elevation: raised; margin: undulate. Shape: long chain rod and slow growing.	D (coryneforms)	+	II (10%)