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Enhancement of *in situ* Remediation of Hydrocarbon Contaminated Soil

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ABSTRACT

Approximately 750 000 sites of contaminated land exist across Europe. The harmful chemicals found in Finnish soils include heavy metals, oil products, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorophenols, and pesticides. Petroleum and petroleum products enter soil from ruptured oil pipelines, land disposal of refinery products, leaking storage tanks and through accidents. PAH contamination is caused by the spills of coal tar and creosote from coal gasification and wood treatment sites in addition to oil spills. Cleanup of soil by bioremediation is cheaper than by chemical and physical processes. However, the cleaning capacity of natural attenuation and *in situ* bioremediation is limited. The purpose of this thesis was to find feasible options to enhance *in situ* remediation of hydrocarbon contaminants. The aims were to increase the bioavailability of the contaminants and microbial activity at the subsurface in order to achieve higher contaminant removal efficiency than by intrinsic biodegradation alone. Enhancement of microbial activity and decrease of soil toxicity during remediation were estimated by using several biological assays. The performance of these assays was compared in order to find suitable indicators to follow the progress of remediation.

Phytoremediation and chemical oxidation are promising *in situ* techniques to increase the degradation of hydrocarbons in soil. Phytoremediation is plant-enhanced decontamination of soil and water. Degradation of hydrocarbons is enhanced in the root zone by increased microbial activity and through the detoxifying enzymes of plants themselves. Chemical oxidation of contaminants by Fenton's reaction can produce degradation products which are more biodegradable than the parent compounds. Fenton's reaction and its modifications apply solutions of hydrogen peroxide and iron for the oxidation of organic chemicals. The cost of oxidation can be reduced by aiming at partial instead of full oxidation of contaminants and by integrating the process to biological treatment, in which the formed degradation products can be biodegraded.

Phytoremediation was used to remove fresh and aged petroleum hydrocarbons from soil, and modified Fenton's reaction combined with biodegradation was used to remove aged creosote oil from soil. The effects of hydrocarbon aging, different plant species and soil amendments on the removal efficiency were studied in phytoremediation experiments. Lab-scale experiments were made with fresh diesel fuel, and a field study was made with aged hydrocarbons deriving from diesel fuel and lubricants. The used plant species were pine, poplar, a grass mixture and a legume mixture. The experiments with modified Fenton's treatment were carried out in soil columns, to which concentrated H_2O_2 was added simulating *in situ* injection. Iron was not added since the soil was rich in iron. After Fenton's treatment, the soil was incubated in serum bottles to determine the effects on bioavailability of PAHs by modified Fenton's oxidation and to simulate the potential of intrinsic remediation. In addition to hydrocarbon analyses, the effects of both methods on soil microbial activities and toxicity were determined.

In the presence of white clover and green pea, pine or poplar, 89 to 98 % of diesel fuel was removed, whereas the presence of grasses did not increase diesel fuel removal compared to treatment without plants, where up to 86 % of diesel fuel was removed. When diesel was applied to the trees for a second time, reduction in one month was 9 to 25 % higher than what was achieved after first month of first application. During the four growing season study with soil contaminated with aged hydrocarbon contaminants, the presence of vegetation did not increase hydrocarbon removal in unfertilised soil. Vegetation cover was denser in amended soil than in unfertilised soil. The addition of compost or NPK fertiliser enhanced hydrocarbon removal. However, the toxicity of aged hydrocarbon contaminated soil to *Vibrio fischeri* (a luminescent bacterium) and *Enchytraeus albidus* (enchytraeid) was low, and thus these toxicity indicators do not reliably reflect the progress of remediation of this aged hydrocarbon contaminated soil. The utilisation of carbon sources, as measured using Biolog®-plates, was influenced by the type of vegetation and the additions of

hydrocarbons, but the latter did not result in permanent changes of carbon source utilisation patterns. Multiwell plate method to follow the utilisation of volatile diesel fuel by soil bacteria showed that presence of trees enhanced diesel fuel utilisation. Microbes in aged hydrocarbon contaminated soil utilised diesel fuel as a carbon source, although the soil did not contain volatile hydrocarbons. The activities of extracellular hydrolytic enzymes did not correlate with hydrocarbon removals.

Chemical oxidation removed more PAHs (up to 50 %) than incubation of aged creosote contaminated soil (up to 34 %). Combined use of modified Fenton's reagent and incubation slightly improved PAH removal from the aged creosote oil contaminated soil, resulting in PAH removal of up to 59 %. Part of the removal during incubation was due to oxidation by residual H_2O_2 remaining in the soil. Hydrogen peroxide addition increased the toxicity of soil and leachate to *Vibrio fischeri* and decreased the number of bacterial cells in soil. The number of intact cells increased during incubation. The removal of PAHs was independent of whether the initial hydrogen peroxide concentration was 12 % or 30 %. However, bacterial survival was higher and regrowth faster at 12 % H_2O_2 than at 30 % H_2O_2. Carbon dioxide was produced and oxygen was consumed, even in soil treated with 30 % H_2O_2. The ability of soil bacteria to utilise PAHs as a carbon source was decreased, but not completely removed, by modified Fenton's treatment. The activity of extracellular acetate esterase decreased with the addition of H_2O_2, but it increased again during incubation to levels preceeding H_2O_2 addition or higher. Based on toxicity and microbial activity results, the initial hydrogen peroxide concentration of 12 % is recommended instead of 30 %. This study shows that the bioaugmentation often applied in other studies after oxidative treatment step is unnecessary.

All studied hydrocarbon contaminated soils contained metabolically active bacteria capable of degrading hydrocarbons. Both phytoremediation and modified Fenton's oxidation enhanced removal of hydrocarbons. However, the performance of these *in situ* remediation processes was case-dependent, the removal of aged hydrocarbon contaminants being more difficult than that of fresh hydrocarbon contaminants. Therefore, phytoremediation can be recommended to remove fresh diesel fuel contamination, but the removal of aged hydrocarbons requires other treatment methods, especially if remediation has to be achieved fast.

Contaminant utilisation assays were suitable indicators to monitor the progress of phytoremediation and modified Fenton's treatement combined to intrinsic remediation. Suitability of toxicity indicators need to be determined for each soil prior to using them to monitor remediation. Soil metabolic activity and microbial number assays are secondary to contaminant utilisation assays in the monitoring of remediation process. However, they should be used to compare soil microbiological activity before and after soil remediation.

TIIVISTELMÄ

Euroopassa on noin 750 000 pilaantunutta maa-aluetta, Yleisimpiä Suomen maaperää pilaannuttavia haitta-aineita ovat raskasmetallit, öljy-hiilivedyt, polyaromaattiset hiilivedyt (PAH-yhdisteet), polyklooratut bifenyylit, kloorifenolit ja torjunta-aineet. Raakaöljyä ja öljytuotteita vuotaa maaperään putkista ja säiliöistä sekä maahan kaadetuista öljyjätteistä. Maaperän saastumista PAH-yhdisteillä aiheuttavat öljy-yhdisteiden käsittelyn lisäksi kivihiilitervan ja kreosoottiöljyn käsittely voimalaitoksissa ja puunkyllästämöissä. Maaperän biologinen puhdistus on kustannuksiltaan edullisempaa kuin kemialliset ja fysikaaliset menetelmät, mutta maaperän luontainen puhdistuskyky voi olla rajallinen. Tämän väitöstutkimuksen tarkoituksena oli tutkia käyttökelpoisia vaihtoehtoja hiilivedyillä saastuneen maaperän *in situ* –kunnostukseen. Tavoitteena oli lisätä haitta-aineiden biosaatavuutta ja maaperän mikrobiaktiivisuutta ja siten parantaa puhdistustulosta verrattuna pelkällä luontaisella biologisella hajoamisella saavutettavaan. Mikrobiaktiivisuuden tehostumista ja maan toksisuuden vähenemistä kunnostuksen aikana arvioitiin useita biologisia menetelmiä käyttäen. Näiden menetelmien soveltuvuutta kunnostusprosessin seuraamisen indikaattoreiksi verrattiin.

Fytoremediaatio ja kemiallinen hapetus ovat lupaavia *in situ* –menetelmiä hiilivetyjen hajoamisen tehostamiseen. Fytoremediaatio on kasveilla tehostettavaa maaperän ja veden puhdistusta. Hiilivetyjen hajotus tehostuu juurivyöhykkeessä lisääntyvän mikrobiaktiivisuuden seurauksena. Lisäksi kasvit tuottavat hiilivetyjä hajottavia entsyymejä. Haitta-aineiden kemiallinen hapetus Fentonin reaktiolla voi tuottaa hajoamistuotteita, jotka ovat biohajoavampia kuin alkuperäiset yhdisteet. Fentonin reaktio ja sen sovellukset käyttävät vetyperoksidiliuoksia ja rautaa orgaanisten kemikaalien hapetukseen. Hapetuskäsittelyn kustannuksia voidaan alentaa yhdistämällä se biologiseen käsittelyyn, jossa esikäsittelyn hajoamistuotteet biohajoavat.

Fytoremediaatiokokeet tehtiin sekä tuoreilla että ikääntyneillä öljyhiilivedyillä, ja sovellettua Fentonin reaktiota käytettiin kreosoottiöljyllä saastuneen maan kunnostukseen. Fytoremediaatiokokeissa tutkittiin hiilivetyjen ikääntymisen, eri kasvilajien ja maaperän lannoituksen vaikutuksia kunnostustulokseen. Laboratoriomittakaavan kokeet tehtiin tuoreella dieselöljyllä ja kenttäkoe ikääntyneillä hiilivedyillä, jotka olivat peräisin diesel- ja voiteluöljystä. Käytetyt kasvilajit olivat mänty, haapa, valkoapilan ja herneen yhdistelmä ja ruohoseos. Sovellettua Fentonin menetelmää tutkittiin maakolonneissa, joihin lisättiin vahvaa vetyperoksidiliuosta *in situ* -injektiota simuloiden. Kokeet tehtiin ilman raudan lisäystä, koska maa sisälsi runsaasti rautaa. Kokeiden jälkeen maata inkuboitiin seerumipulloissa, jotta voitaisiin selvittää Fentonin hapetuksen vaikutus PAH-yhdisteiden biosaatavuuteen ja maan PAH:ien luontainen hajoamispotentiaali. Hiilivetyanalyysien lisäksi seurattiin molempien käsittelyjen vaikutuksia maan mikrobiaktiivisuuksiin ja maan myrkyllisyyteen.

Valkoapilan ja herneen yhdistelmä-, mänty- tai haapakäsittelyissä poistui 89-98 % tuoreesta dieselöljystä. Ruohokäsittely ei tehostanut dieselöljyn vähenemistä verrattuna kasvittomaan maahan, josta poistui maksimissaan 86 % dieselöljystä. Kun dieselöljyä lisättiin puilla käsiteltyyn maahan toisen kerran seuraavana vuonna, reduktio kuukauden kokeen jälkeen oli 9-25 % korkeampi kuin ensimmäisessä kokeessa. Kasvillisuus edisti ikääntyneiden hiilivetyjen hajoamista kompostilla tai NPK-seoksella lannoitetussa maassa, mutta kasvit eivät kyenneet peittämään lannoittamatonta maata kokonaan ja lannoittamattoman maan hiilivetypitoisuus ei muuttunut neljän kasvukauden kenttäkokeen aikana tilastollisesti merkittävästi. Maan toksisuus valobakteerille *Vibrio fischeri* ja änkyrämadolle *Enchytraeus albidus* oli alhainen, joten näitä toksisuusindikaattoreita ei voida suositella ikääntyneillä hiilivedyillä saastuneen maan kunnostuksen edistymisen arviointiin. Biolog-levyjen hiilen- ja energianlähteiden käyttöön vaikuttivat kasvillisuuden laji ja hiilivetylisäykset, mutta hiilivetylisäysten aikaansaamat muutokset eivät olleet pysyviä. Kuoppalevymenetelmä haihtuvaa dieselöljyä hiilenlähteenään käyttävien

bakteerien seuraamiseen osoitti, että puiden läsnäolo lisäsi dieselöljyn käyttöä. Myös ikääntyneillä hiilivedyillä kasvaneessa maassa oli mikrobeja, jotka pystyivät käyttämään dieselöljyä hiilenlähteenä, vaikka maa ei sisältänyt haihtuvia hiilivetyjä. Solunulkopuolisten entsymien aktiivisuudet eivät korreloineet maan hiilivetypitoisuuksien kanssa.

Kemiallinen hapetus poisti maksimissaan 50 % ja maan inkubointi enintään 34 % PAH-yhdisteistä ikääntyneellä kreosoottiöljyllä pilaantuneesta maasta. Menetelmien yhdistäminen paransi puhdistustulosta hieman johtaen parhaimmillaan 59 % reduktioon. Osa inkuboinnin aikana poistuneista PAH-yhdisteistä saattoi tosin hapettua kemiallisesti maahan jääneen H_2O_2:n takia. Vetyperoksidin alkupitoisuudet 12 ja 30 % eivät vaikuttaneet PAH-yhdisteiden hajoamiseen. Vetyperoksidin lisäys lisäsi maan ja suotovesien myrkyllisyyttä *Vibrio fischerille* ja vähensi maa-aineksen mikrobien solumäärää, mutta ehjien solujen osuus kasvoi inkuboinnin aikana. Käytettäessä 12 %:n vetyperoksidipitoisuutta ehjien solujen osuus oli suurempi ja niiden määrä lisääntyi enemmän inkuboinnin aikana kuin 30 % vetyperoksidipitoisuutta käytettäessä. Hiilidioksidia muodostui ja happea kului myös korkeammalla vetyperoksidipitoisuudella käsitellyssä maassa. Sovellettu Fentonin reaktio heikensi mutta ei kokonaan poistanut maaperäbakteerien kykyä hajottaa PAH-yhdisteitä. Vetyperoksidin lisäys alensi maan solunulkopuolisia asetaattiesteraasiaktiivisuuksia, mutta inkuboinnin aikana ne nousivat vetyperoksidilisäystä edeltäneelle tasolle tai sitä korkeammalle. Toksisuus- ja mikrobiaktiivisuustulosten mukaan vetyperoksidin alkupitoisuus 12 % on suositeltavampi kuin 30 %. Tutkimus osoitti, että mikrobien lisäys hapetuskäsittelyn jälkeen on tarpeetonta, vaikka se on yleistä yhdistetyn kemiallis-biologisen käsittelyn tutkimuksissa.

Kaikissa hiilivedyillä pilaantuneissa maissa oli metabolisesti aktiivisia mikrobeja, jotka kykenivät hajottamaan hiilivetyjä. Sekä fytoremediaatio että sovellettu Fentonin hapetus tehostivat hiilivetyjen hajoamista, mutta näiden menetelmien tehokkuus riippui tutkittavasta maasta ja haitta-aineista. Riippumatta puhdistusmenetelmästä, ikääntyiden hiilivetyjen poistaminen on vaikeampaa kuin hiilivetyjen poistaminen heti pilaantumisen jälkeen. Fytoremediaatio soveltuu äskettäin hiilivedyillä pilaantuneen maan puhdistamiseen, mutta sitä ei voida suositella ikääntyneiden hiilivetyjen poistamiseen, ainakaan maa halutaan kunnostaa nopealla aikataululla.

Menetelmät, joissa seurattiin haitta-aineen käyttöä hiilen- ja energianlähteenä, soveltuivat fytoremediaation, sovelletun Fentonin hapetuksen sekä luontaisen hajoamisen yhdistelmän seuraamiseen. Toksisuusindikaattorien soveltuvuus kullekin maalle on tutkittava ennen niiden käyttöä kunnostuksen seuraamiseen. Maan yleisen metabolisen aktiivisuuden ja mikrobimäärien selvittäminen on toisarvoista haitta-aineiden hajoamista seuraaviin menetelmiin verrattuna, mutta niitä tulisi käyttää maan mikrobiaktiivisuuksien vertaamiseen ennen maan kunnostusta ja sen jälkeen.

PREFACE AND ACKNOWLEDGEWENTS

The thesis is based on the work carried out at the Institute of Environmental Engineering and Biotechnology, Tampere University of Technology.

I would like to thank my supervisors at Tampere University of Technology, Professors Jaakko Puhakka and Tuula Tuhkanen for giving me the opportunity to start doctoral studies and for encouragement in scientific work. I appreciate the support and advice from Professor John Pichtel (Ball State University) who worked with me in the Phytoremediation project, and the support and advice from Dr. Jörg Landwaldt in the Modified Fenton's oxidation project. I thank Dr. Uwe Münster for advice and discussions on microbial extracellular enzymes. I thank Dr. Eeva-Liisa Viskari for introducing me plant phytotoxicity determination, MSc Pasi Niskanen for helping with soil analysis, Dr Kirsi-Maarit Lehto for help with mass selective detector and gas chromatograph, Dr Kati Vaajasaari for discussions on oil contaminated soil and toxicity and Tero Lehti from Tampere Regional Solid Waste Management Ltd. for help with phytoremediation field site.

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ABBREVIATIONS

AMC	7-amino-4-methylcoumarin
AOP	advanced oxidation process
BTEX	benzene, toluene, ethyl benzene and xylene
cfu	colony forming unit
CLPP	community level physiological profile
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DOC	dissolved organic carbon
EC	equivalent carbon number
EU	European Union
GC-MS	gas chromatography-mass spectrometry
GC-MSD	gas chromatograph equipped with mass selective detector
H	Henry's law constant
ICP-AES	inductively coupled plasma-atomic emission spectrometry
K_{OC}	soil organic matter / water partition coefficient
$\log K_{OW}$	octanol-water partition coefficient
MEE	microbial extracellular enzyme
μ_m	maximum specific growth rate
MUF	methylumbelliferone
O_3	ozone
PAH	polyaromatic hydrocarbon
PCA	principal component analysis
PCB	polychlorinated biphenyl
PLFA	phospholipid fatty acid
SIR	substrate induced respiration
SVOC	semivolatile organic compound
TiO_2	titanium dioxide
TPH	total petroleum hydrocarbons
US	ultrasound
UV	ultraviolet light
v_{max}	Michaelis-Menten constant, maximum rate
VOC	volatile organic compound
WST-1	2-(4-iodophyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2-H-tetrazolium monosodium salt

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LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers referred to, in this thesis, by the Roman numerals (I to V). The papers are attached as appendices at the end of this thesis and they are published with kind permission of Elsevier (Paper I), Springer Science and Business Media (Papers II and IV), Ecomed Publishers (Paper III) and Society of Chemical Industry (Paper V, first published by John Wiley & Sons Ltd.). The specific aims of each paper, the methods used and the main results are summarised in a table attached as Appendix 1.

I Palmroth, M., Pichtel, J., Puhakka, J. 2002. Phytoremediation of subarctic soil contaminated with diesel fuel. Bioresource Technology 84:221-228

II Palmroth, M.R.T., Münster, U., Pichtel, J. and Puhakka, J.A. 2005. Metabolic responses of microbiota to diesel fuel addition in vegetated soil. Biodegradation 16:91-101

III Palmroth, M.R.T., Koskinen, P.E.P., Pichtel, J., Vaajasaari, K., Joutti A., Tuhkanen, T.A. and Puhakka, J.A. 2006. Field-scale assessment of phytoremediation of soil contaminated with weathered hydrocarbons and heavy metals. Manuscript submitted to Journal of Soils and Sediments.

IV Palmroth, M.R.T., Langwaldt, J.H., Aunola, T.A., Goi, A., Münster, U., Puhakka, J.A. and Tuhkanen, T.A. 2006. Effect of modified Fenton's reaction on microbial activity and removal of PAHs in creosote oil contaminated soil. Biodegradation (Published Online)

V Palmroth, M.R.T., Langwaldt, J.H., Aunola, T.A., Goi, A., Puhakka, J.A. and Tuhkanen, T.A. 2006. Treatment of PAH-contaminated soil by combination of Fenton's reaction and biodegradation. Journal of Chemical Technology and Biotechnology 81:598-607

THE AUTHOR'S CONTRIBUTION

Paper I: Marja Palmroth wrote the paper and is the corresponding author. She planned and performed the experimental work together with John Pichtel. She interpreted all the results.

Paper II: Marja Palmroth wrote the paper and is the corresponding author. She planned the experimental work and interpreted the results.

Paper III: Marja Palmroth wrote the paper and is the corresponding author. She planned the experimental work together with John Pichtel. She performed the experimental work during years 2000 and 2001. In 2002 and 2003 Perttu Koskinen participated in the experimental work. Marja Palmroth interpreted all the results.

Paper IV: Marja Palmroth wrote the paper and is the corresponding author. She planned the biodegradation experiments and performed the microbiological and toxicological analyses as well as gas monitoring. Tuomo Aunola and Anna Goi planned and performed oxidation experiments as well as PAH analysis. Jörg Langwaldt constructed two graphs for the paper. Marja Palmroth interpreted the results.

Paper V: Marja Palmroth wrote the paper and is the corresponding author. She planned the biodegradation experiments and performed the microbiological and toxicological analyses as well as gas monitoring. Tuomo Aunola and Anna Goi planned and performed oxidation experiments as well as PAH analysis. Marja Palmroth interpreted the results.

1 Introduction

Contaminated land has elevated concentrations of chemicals or other substances deriving from man's use of the land. Soil contaminants can influence human health, surface and groundwater quality, the nature and viability of ecosystems, condition of buildings and other materials and archaeological artefacts within the ground as well as the visual amenity of an area (Vegter et al., 2002). Therefore, regulatory agencies have set acceptable limits to concentrations of many soil contaminants depending on the intended use of the land. Concentrations allowed in residential areas are lower than those allowed in industrial areas. Approximately 750 000 sites across Europe are estimated to be contaminated by past and present human activities. Some of them may pose a threat to water resources, ecosystems and/or human health (Ferguson et al., 1998). The number of contaminated sites in Finland has been evaluated to be approximately 25 000 (Sarkkila et al., 2004). About 3 000 of these sites are located at important groundwater zones (Sorvari and Antikainen, 2004). The contaminated sites in Finland are a result of distribution and storage of fuels, activities at sawmills, impregnation plants, industry, depots and repair shops, greenhouses and shooting ranges. The harmful chemicals found in Finnish soils include heavy metals, oil products, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorophenols, and pesticides (Ympäristöministeriö, 1994; Järvinen and Salonen, 2004).

Hydrocarbons are widespread in the environment. Their major source is petroleum but they are also formed by synthetic processes and by biological processes of bacteria, plants (Weisman, 1998) and fungi (Marseille et al., 1999). Petroleum and petroleum products enter soil from ruptured crude oil pipelines, land disposal of refinery products and leaking storage tanks (Schwab and Banks, 1999). Spills of petroleum hydrocarbons were more common in the last few decades than today. PAH contamination is caused by leakage of crude oil and their refined products and the spills of coal tar and creosote from coal gasification and wood treatment sites (Mueller et al., 1989). Four-, five- and six-ring PAHs are produced in incomplete combustion of organic fuels and are more abundant in coal tars and creosote than in oils (Prince and Drake, 1999).

Partitioning of hydrocarbons spilled to soil depends on both the properties of soil and the contaminants themselves. Soil properties to be considered include soil bulk density, water and air contents in vadose zone as well as soil organic carbon content (Gustafson et al., 1997). When hydrocarbons are spilled to soils, they can be removed by photo-oxidation, evaporation, dissolution or biodegradation (Prince and Drake, 1999) or be sequestered within the soil's mineral and organic matter fractions. Photodegradation of contaminants in soil is of minor importance, since solar light can penetrate only a thin layer of surface soil (McBride, 1994). Significant amounts of contaminants are retained in soils, since most organic contaminants follow a biphasic decay pattern in soil. In this pattern, degradation of contaminants is initially fast and diminishes with time (Semple et al., 2003). According to contaminant sequestration hypothesis, contaminants become less extractable and less bioavailable by sequestration within the soil matrix during aging (Alexander, 2000 and Semple et al., 2003). Thus the contaminants remaining in soil do not threaten environment. However, the limited biodegradation of 4- to 6-ring PAHs might be due to the absence of specific PAH degraders and cometabolic substrates instead of reduced bioavailability (Huesemann et al., 2003. Thus, aged contaminants would pose a greater risk to the environment than estimated in the sequestration hypothesis.

Enhancing the degradation of hydrocarbons would enable bioremediation to reach the soil quality objectives set by regulatory agencies. The degradation of organic contaminants may be enhanced by adding surfactants or solubilisers to increase bioavailability of the contaminants (Carmichael and Pfaender, 1997; Tiehm et al., 1997; Soeder et al., 1996) or oxidising the contaminants to less recalcitrant form (Meulenberg et al., 1997; Lee and Hosomi, 2001). Dissolved humic matter can also increase the solubility of contaminants (Chiou et al., 1986; Johnson and Amy, 1995).

Furthermore, soil microbial activity can be stimulated by adding inorganic and organic nutrients (Prince and Drake, 1999; Carmichael and Pfaender, 1997) or by aerating, watering, tilling (Prince and Drake, 1999) and vegetation (Wenzel et al., 1999; Anderson et al., 1999). These processes target all soil micro-organisms, but supplements, specific carbon sources or inducers of genetic operons, can also be added to stimulate a specific group of micro-organisms (Carmichael and Pfaender, 1997; Ogunseitan et al, 1991). In addition, bioaugmentation with specific strains or consortia can be used to increase the degradation of contaminants (El Fantroussi and Agathos, 2005). However, all the available methods used to enhance hydrocarbon degradation are not suitable for *in situ* treatment. Furthermore, the removal of aged soil contaminants may be difficult (Alexander, 2000).

The purpose of this thesis research was to study feasible options to enhance *in situ* remediation of hydrocarbon contaminants. The aims were to increase the bioavailability of the contaminants and microbial activity at the soil subsurface in order to achieve higher contaminant removal efficiency than by biodegradation alone and to find suitable indicators to monitor microbial activity and toxicity during remediation. The remediation methods chosen were phytoremediation and chemical oxidation. They are promising *in situ* techniques to increase the degradation of hydrocarbons in soil without excavation. Phytoremediation enhances the degradation of hydrocarbons in the root zone by increasing microbial activity (Anderson et al., 1993) and through the detoxifying enzymes of plants themselves (Schäffner et al., 2002; Newman and Reynolds, 2004). Chemical oxidation of contaminants can produce degradation products which are more biodegradable than the parent compounds (Meulenberg et al., 1997; Lee and Hosomi, 2001).

1.1 Treatment technologies for contaminated soil

Remediation of contaminated sites can take place without moving the soil by *in situ* remediation, above the contaminated site by on site remediation, or *ex situ*, in which the excavated soil is treated elsewhere (Arctander and Bardos, 2002). *In situ* remediation of contaminated soil includes chemical and biological methods (Doelman and Breedveld, 1999). *In situ* remediation by intrinsic bioremediation, also called natural attenuation, relies on natural chemical, biological or physical subsurface processes to remove contaminants (EPA, 1999). However, some natural attenuation processes have a finite capacity, and the feasibility of natural attenuation has to be investigated at each site (Rittmann, 2001; Rittmann 2004). Although no engineering systems are built on natural attenuation, the site must be surveyed for its hydrology, geochemistry and microbiology, and monitored (Renner, 1998). Natural attenuation alone may not be able to remediate contaminated soil in reasonable time. Therefore, it can be used as pre- or post-treatment step or be enhanced by engineered processes (Mulligan and Yong, 2004). Most common currently available full-scale *in situ* processes are listed in Table 1, and they can remove most soil contaminants. *In situ* technologies used in this study to enhance the removal of hydrocarbons, i.e., phytoremediation and chemical oxidation, are described later in detail.

Ex situ physical and chemical treatment methods include 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). Available biological *ex* situ treatment processes include treatment in biopiles that can be aerated and watered, composting with supplemental material, landfarming, where soil is tilled regularly, or slurry phase treatment in reactors, ponds or lagoons. Addition of nutrients and inocula is possible in all these processes (FRTR, 2001 and Blackburn and Hafker, 1993). Bioremediation by *ex situ* and *in situ* methods usually costs less than chemical and physical processes. Bioremediation may, however, not always achieve stringent site clean-up goals, despite significant removal of contaminants (Bollag et al., 1994).

Table 1. Full-scale *in situ* treatment methods for contaminated soil. Cost estimate information from Arctander and Bardos (2002, in €) and FRTR (2005, in $).

Method	Description	Costs	Contaminants treated	Reference
Intrinsic remediation/ Monitored natural attenuation	Relies on natural subsurface processes, it includes monitoring of the site	Depends on duration of monitoring	Benzene, toluene, ethyl benzene and xylene (BTEX), chlorinated and petroleum hydrocarbons	Renner, 1998; Mulligan and Yong, 2004; Salminen et al., 2004
Biosparging	Oxygen/air is added below groundwater surface to stimulate microbial activity and degradation.	50-110 €/ton	Organic contaminants	Doelman and Breedveld, 1999; EPA 2005a
Bioventing	Oxygen/air is added to soil vapour phase to stimulate aerobic degradation	25-120 €/ton	Petroleum hydrocarbons, nonchlorinated solvents, some	FRTR, 2005; EPA 2005a
Enhanced bioremediation/ Biorestauration	Carbon sources and/or nutrients and/or electron acceptors and/or fungi/bacteria (bioaugmentation) are added through injection wells or by spraying, depending of required soil depth	15-160 €/ton	Petroleum hydrocarbons, solvents, pesticides, wood preservatives, and other organic chemicals as well as munition	Doelman and Breedveld, 1999; EPA 2005a and FRTR, 2005
Phytoremediation	Plants are used to remove, transfer, stabilize, and destroy contaminants in soil and sediment	depends on method	Organic or inorganic contaminants	FRTR, 2005 and Adams et al., 2000
Chemical oxidation	Hazardous contaminants are oxidised to non-hazardous or less toxic compounds	70-400 €/ton	Many toxic organic chemicals	FRTR, 2005 and EPA, 2005a
Chemical reduction	Reduction of contaminants by zero-valent iron powder or sodium polythiocarbonate		Chlorinated solvents and metals	EPA, 2005a
Electrokinetic separation	Separation and removal of contaminants from low permeability soil by electrochemical and	50-170 $/m^3	Metals and polar organics; in field-scale heavy metals,	FRTR, 2005; EPA, 2005a and 2005b
Fracturing	Cracks are developed to soil by blast, pneumatically or hydraulically in low permeability	9-200 $/m^3	Fracturing is used to enhance other *in situ* methods	FRTR, 2005; EPA 2005a
Soil flushing	Contaminants are extracted from the soil with water or other suitable aqueous solutions, the	19-190 $/m^3	Volatile organic contaminants (VOCs) and semivolatile	FRTR, 2005; EPA 2005a
Soil vapor extraction/ Dual	Vacuum is applied to unsaturated soil to induce the controlled flow of air and to remove contaminants	15-160 €/ton	VOCs and some SVOCs, some fuels as well as light non-	FRTR, 2005 EPA, 2005a;
Thermal treatment	Soil is heated with warmed gas, with electric current or electromagnetically to increase	30-130 $/m^3	VOCs and some SVOCs, some pesticides and fuels	FRTR, 2005
Solidification	Physically bounding or enclosing contaminants within stabilised mass	50-130 €/ton	Inorganic and some organic contaminants	FRTR, 2005
Stabilization	Added stabilising agent reacts chemically with contaminants and reduces their mobility	50-130 €/ton	Inorganic and some organic contaminants	FRTR, 2005; EPA 2005a

The estimated annual cost of contaminated soil cleanup in Finland is 50 to 70 million € for the next two decades. Approximately 300 to 350 sites are remediated per year. In 2003, the remediation costs per ton of soil varied from 35 to 90 €, not including *in situ* remediation of contaminated soil (Järvinen and Salonen, 2004). Whether *in situ* or *ex situ* treatment will be more cost-effective, depends on the quantity of soil to be treated as well as site and contaminant characteristics. *Ex situ* treatment of soil by bioremediation costs 20 to 40 € per ton, soil washing costs 20-200 €/t, stabilisation/solidification costs 80-150 €/t, incineration treatment costs 170-350 €/t and thermal treatment costs 30-100 € per ton of soil (Arctander and Bardos, 2002). *In situ* treatment costs are more site-specific (Table 1). They depend on on the site size, the nature and amount of contamination, and the hydrogeological settings of the site as well as on the *in situ* method chosen (FRTR, 2005; Arctander and Bardos, 2002). Monitored natural attenuation and phytoremediation have low maintenance, cost and energy requirements compared to other *in situ* methods, but they require a long treatment time compared to the so called intensive *in situ* technologies (Arctander and Bardos, 2002; Adams et. al., 2000). Both phytoremediation (Frick et al., 1999; Schnoor, 1997), and monitored natural attenuation (FRTR, 2005) require several years to achieve treatment goals.

In Europe, *ex situ* methods are more common than *in situ* methods, since applying of *in situ* methods into European soils is still at an early stage of development (Arctander and Bardos, 2002). Further, off site and on site methods may be preferred, since the site can be cleaned faster and no further legal and financial liabilities remain (Arctander and Bardos, 2002; Sorvari and Antikainen, 2004). In political or economical perspective, the *in situ* remediation should remove contaminants in four to six years time (Doelman and Breedveld, 1999). In reality, *in situ* bioremediation may require longer treatment times than this. However, transportation and excavation may not always be possible due to high cost or problems involved, such as risks related or buildings on top of the contaminated site (Arctander and Bardos, 2002). In Finland, excavation of soil and taking to a hazardous waste landfill is seen as safer alternative than *in situ* treatment in the perspective of risk assessment (Sorvari and Antikainen, 2004). The number of available *in situ* treatment methods in Europe is more than 20 (Table 1), but they are used in larger scale in only few countries (Arctander and Bardos, 2002). For example, only five sites were treated by *in* situ remediation in Finland in 2003 (Järvinen and Salonen, 2004). The most common methods used include soil vapour extraction, air sparging, bioventing, biostimulation and encapsulation (Arctander and Bardos, 2002). In the United States, *in situ* methods for contaminated soil treatment are more widely used (EPA, 2005a and 2005b) than in Europe, when technology feasibilities are often doubted despite successful applications in other countries (Arctander and Bardos, 2002).

The most-applied contaminated soil treatment methods in Europe are excavation and related materials handling, disposal of contaminated soil, infilling void, cover systems and vertical barriers. The scale of applying *ex situ* biological treatment of soil in Europe varies greatly from country to country, except that composting is applied in all European Union (EU) member states. *Ex situ* physical and chemical treatment of soil in EU countries consists mostly of soil washing, soil stabilisation, soil solidification and soil immobilisation. In Finland also *ex situ* soil venting is used. In most EU countries, except in Ireland, where treatment units are lacking, soil can be treated thermally or incinerated (Arctander and Bardos, 2002).

Risk assessment is evaluation of human health hazards and potential adverse consequences related to environmental or occupational exposures (Teaf et al., 2003).The most common practice used in remediation decision making is simple comparing of measured concentrations in soil and groundwater against predetermined guideline values (Ferguson et al., 1998). This approach is now applied in Finland (Ympäristöministeriö, 1994). This practice is less expensive than more elaborate site-specific assessment methods. On the other hand, remediation may amount to higher costs than investing on a more detailed site-specific risk assessment, which may prove remediation unnecessary (Ferguson et al., 1998). Development of contaminated soil risk assessment practices is

part of European soil protection strategy (Van-Camp et al., 2005) and risks to both humans and ecosystem are being assessed (Ferguson et al., 1998; Anon, 2006). In Finland, legislation is changing in spring 2006 and risk based remediation goals are being accepted in the new law (Anon, 2006). This gives the possibility to make site-specific risk estimates rather than relying on fixed guideline values, which can overestimate risks in some cases. Risk assessment of contaminated sites has already increased in Finland during the last few years (Sorvari and Antikainen, 2004). Defining site-specific remediation targets could increase the use of *in situ* soil treatment.

1.1.1 Phytoremediation

Phytoremediation can be defined as plant-enhanced decontamination of soil and water. Contaminants may be degraded, extracted from soil or contained in the plant. Sometimes contaminants are removed from soil by using a combination of these methods. Phytoremediation methods applicable for soil are presented in Table 2. Plants can furthermore be used to hydraulically control contaminated plumes or as a cover for contaminated material to prevent wind and water erosion (Adams et. al*.,* 2000). Naturally, plants used in phytoremediation must tolerate the pollutants at concentrations present in contaminated environments (Wenzel et al., 1999). Phytoremediation research dealing with organic contaminants is focused to the root zone, since more than half of the biomass of most plants is located in roots. Phytoremediation of soil is not feasible if contaminants have spread deep into the soil profile. Contamination must be confined to surface or near-surface soil, because plant roots reach only a limited depth (Adams et. al*.*, 2000). Vegetation can be used for final soil polishing when other technologies have been used to treat the "hot spots" (Schnoor et. al*.*, 1995). The phytoremediation costs consist of the addition of plants, site monitoring and possible maintenance measures such as irrigation, fertilisation, as well as harvesting in phytoextraction. Phytoremediation does not require an external energy source other than sunlight, if no artificial irrigation is used. The difference between natural attenuation and phytoremediation is sometimes difficult to define, because many natural attenuation sites are vegetated.

Phytoremediation mechanisms active in hydrocarbon decontamination include plant uptake and phytodegradation, which can lead to volatilisation, and rhizodegradation. Both aerobic and anaerobic degradation pathways must be considered in rhizodegradation, because microhabitats exist for both aerobic and anaerobic micro-organisms (Walton et. al., 1994). Plants can take up hydrocarbons to their roots (Trapp et al., 1990) and aboveground biomass (Chaineau et al., 1997). Phytotoxicity limits the use of phytoremediation at high contaminant concentrations. However, the toxic concentrations are plant species and contaminant dependent. Especially low molecular weight (Chaineau et al., 1997) and aromatic hydrocarbons (Chaineau et al., 1997 and Pivetz et al, 1997) can decrease germination and growth of plants. Thus, phytoremediation should be used only at low and moderate levels of contamination. Moderately hydrophobic organic compounds, with octanol-water partition coefficients ($\log K_{OW}$) of 0.5 to 3.0, such as chlorinated solvents, benzene, toluene and ethylbenzene and short chain aliphatics, are most readily taken up and translocated within plants (Schnoor et. al., 1995, Adams et. al., 2000). Hydrophobic chemicals with $\log K_{OW}$ greater than 3.0 are bound to the surface of roots (Schnoor et. al., 1995). However, plant uptake of organic chemicals is plant species dependent (Wenzel et al., 1999). Hydrophobic contaminants can also be unavailable due to attachment to soil colloids or to organic matter, both solid and dissolved matter (e.g. Pignatello and Xing, 1995; Totsche et. al., 1997). Table 3 lists examples of phytoremediation studies of hydrocarbon contaminated soil. Field-scale experiments are less numerous than lab-scale studies, but phytoremediation is used in full-scale in the United States (EPA, 2005a). Most of these studies showed that plants enhanced removal of hydrocarbons, although removal efficiency of aged contaminants was lower than that of freshly spilled contaminants. However, many of the studies indicate that similar hydrocarbon removal can be obtained without vegetation in a longer period of time.

Table 2. Description of phytoremediation methods.

Method	Description	Details	Effective soil depth	Cost estimate	Scale	Target group
Phytoextraction	Plants extract heavy metals from soil, the aboveground plant tissue is harvested[1]	Several heavy metals occur either as plant nutrients or nutrient analogs[2], depends on metal solubility, which can be enhanced with chelating agents[3]	30 cm (Indian mustard), limited by root depth[4]	29-50 $/ton[5]	Full-scale[6]	Heavy metals[4]
Phytostabilisation/ Phytoimmobilisation/ Vegetative cover	Vegetation reduces the mobility and bioavailability of contaminants[7]	Absorption/adsorbtion to roots or organic compounds produced by plant as well as decreasing leaching to groundwater[7]	Plants native to contaminated site are often used [8]	0.02-1 $/m^3 [9]	Field-scale[4]	Metals and hydrophobic organic chemicals [10]
Rhizodegradation	microbial biomass[11,12] and microbial degradation rates are higher in the root zone[3], mechanisms include improved aeration [13], addition of substrates such as plant debris[14]and root exudates[12]	Root exudates, small molecular weight organic compounds, such as amino acids, sugars organic acids, and salts [12] act as substrates for soil micro-organisms and stimulate cometabolic transformations of organic pollutants[15] Nitrogen-fixing bacteria and mycorrhiza found in many plant roots provide nutrients to the plants and improve drought resistance[16]	120 cm (grasses), 4 to 5 m (poplar)[4]	3 $/m^3 with deep-rooted plants[17] normal croppic practices 0.02-1 $/m^3 [9] 10-35$/ton[10]	Field-scale[4]	Organic contaminants[4]
Phytodegradation	Plants metabolise contaminants within the plant or they are degraded by compounds produced and exuded by plants[4,12]	Plant enzymes include e.g. cytochrome P450, peroxidases and laccases[18]	4 to 5 m (poplar)[4]	3 $/m^3 with deep-rooted plants[17]	Field-scale[4]	both nonpolar and highly polar pollutants[19]
Phytovolatilisation	Plants take up and transpire contaminants or their metabolites to atmosphere [20,4,13]	Metabolism more important mechanism than transpiration, 9 % of trichloroethene uptaken to poplars was transpired from poplars to atmosphere[21]	4 to 5 m (poplar)[4]	38 % of the costs of pump and treat + reverse osmosis[22]	Field-scale[4]	Organic and inorganic pollutants[20]

[1]Cunningham and Berti (1993); Black (1995); Salt et al. (1995); Cunningham and Ow (1996) [2] Reeves and Baker (2000) [3] Blaylock et al. (1997) [4] Adams et al. (2000) [5]Salt et al. (1995) [6] FRTR (2005) [7]Vangronsveld et al. (1995); Salt et al. (1998); Pulford and Watson (2003); Wenzel et al. (1999). [8]Kremer (2005). [9]Cunningham et al. (1995) [10]Schnoor, (1997)[11]Romantschuk et al. (2000), [12]Anderson et al. (1993) [13]Schnoor et al. (1995) [14]Shann and Boyle (1994) [15] Schwab et al. (1995); Burken and Schnoor (1996); Soeder et al. (1996); Hedge and Fletcher (1996); Fletcher et al. (1995); April and Sims (1990) [16] Whipps (1990). [17] Tsao (1999), cited in Frick et al. (1999) [18] Gramms et al.(1999) and Chroma et al.(2002). [19] Schäffner et al. (2002); Newman and Reynolds (2004) [20] Wenzel et al. (1999) [21] Newman et al. (1999). [22] Gatliff (1996), cited in Schnoor (1997)

Table 3. Examples of plants utilised in the phytoremediation of aromatic and petroleum hydrocarbons and results. TPH = total petroleum hydrocarbons.

Plants utilised	Contaminant and concentration	Result	Note	Reference
Alfalfa, sorghum, bermuda grass	Phenanthrene; 100 mg/kg	The production of radiolabelled CO_2 was 3 to 4 times higher with sorghum and bermuda grass than with alfalfa, which yielded similar production as sterile control	Radiolabelled phenanthrene was degraded rapidly within 28 days, but the majority of the degraded phenanthrene stayed in soil	Schwab et. al. (1995)
8 prairie grasses	Chrysene, benzo(a)pyrene, benz(a)anthracene and dibenz(a,h)anthracene, total PAHs 10 mg/kg	Removal of PAHs higher in vegetated soil. Removal of PAHs correlated with their water solubilities	Manure was added to all treatments. Study lasted for 219 days	April and Sims (1990)
Ryegrass	n-alkanes, pristine, hexadecane and PAHs spiked to soil, total hydrocarbons 5000 mg/kg	97 % of hydrocarbons were removed in planted soil in 22 weeks, while 82 % were removed in unplanted soil	Microbial numbers were higher in vegetated soil. n-alkanes disappeared faster with ryegrass, but removal of PAHs similar with and without plants	Guenther et al. (1996)
20 alfalfa genotypes	Crude oil;20 g/kg	33 to 56 % of crude oil was degraded with different alfalfa genotypes and 46 % was degraded without plants	Crude oil addition decreased forage yield. Study lasted for one year,	Wiltse et. al. (1998)
Alfalfa and horseradish	Kerosene-based jet fuel; 1700 mg/kg	57 to 90 % of jet fuel was removed in 5 months in watered mesocosms	52 % of jet fuel was removed in air-dried soil without plants	Karthikeyan et. al. (1999)
Alfalfa	Diesel; 7300 mg TPH /kg, 10 weeks later spiking to 12,400 mg TPH/kg	80 % degradation was achieved in 8 weeks, 90 % degradation was achieved in 2nd phase of 2 weeks	Unvegetated treatments achieved similar diesel degradation 2 to 4 weeks later than with alfalfa.	Komisar and Park (1997)
Ditch reed and alfalfa	Liquid bitumen (mainly paraffins and naphtenes) 79.7 g/kg and soil containing PAHs 80 mg/kg	82 % removal was achieved in 27 months with both plants. Most of the hydrocarbons were removed during the first 18 months	Approximately same removal without plants (74 %), but slower than with plants	Muratova et. al. (2003)
Tall fescue, field scale	PAHs (acenaphtene, fluorene, phenanthrene, fluoranthene, pyrene and chrysene) in creosote-contaminated soil	Removal of acenaphtene and fluorene in 36 months was slightly higher in the presence of tall fescue than in unvegetated soil	Removal results and microbial enumeration indicate that rhizodegradation was the main PAH removal mechanism	Robinson et al. (2003)
Tall fescue, annual ryegrass, yellow sweet clover	Aged PAHs from manufactured gas plant	PAH removal in 12 months was higher in the presence of plants, 9 % to 24 %, compared to 5 % without plants	Soil was pretreated by composting	Parrish et al. (2004)
Maize	Fuel oil; 3300 mg/kg	70 to 80 % removal of fuel oil was obtained in fertilised soil in 120 days, with and without maize	Degradation of saturated hydrocarbons faster with maize	Chaîneau et. al. (2000)

1.1.2 Chemical oxidation

Highly potent chemical oxidants such as hydroxyl radical (OH•) are generated in advanced oxidation processes (AOP) (Glaze et al., 1987). Table 4 provides examples of chemical oxidation methods developed for treatment of soil. However, some of these methods require excavation of soil and some of them can be implement in slurry phase only. *In situ* chemical oxidation can be applied to a variety of soil types and sizes (silt and clay) (EPA, 1998). Oxidation processes have been applied to most organic contaminants found in soil. However, only Fenton's reaction and its modifications, potassium permanganate, ozone (EPA, 1998) and persulfate oxidation (EPA, 2005a) have been applied in *in situ* soil remediation. Commonly, acids are added to soil to adjust soil pH to optimal conditions of the technology chosen (EPA, 2005a). The advantage of Fenton's reagent is that it can be generated *in* situ (EPA, 1998). Fenton and Fenton like treatment of soil are more cost-efficient than ozonation (Goi et al., 2005a).

Table 4. Chemical oxidation processes developed to treat soil contaminants. Information given in different references separated by semicolon.

Oxidation process	Contaminants treated	Type of soil treatment	Reference
Ozone (O_3)	organic contaminants	*in situ* soil	EPA, 2005a
O_3/acid at high pH (8 to 10)	organic contaminants	*in situ* soil	EPA, 2005a
O_3/H_2O_2	organic contaminants; VOCs	slurry, *in situ* soil	Gordon et al. 1994, Kasa, 2001; EPA, 2005a
O_3/H_2O_2/ultrasound (US)	endocrine disrupting chemicals	excavated soil	Kono et al., 2003
Ferrous iron (Fe^{2+})/ H_2O_2 (Fenton)	organic contaminants	*in situ* soil	EPA, 2005a
Electro-Fenton	phenols, trichloroethylene, diesel fuel, phenanthrene	*in situ* soil	Yang and Long, 1999, Yang and Liu, 2000 and 2000, Kim et al, 2005
Electron beam irradiation	recalcitrant organic matter; dioxins and PCBs	excavated soil	Jung and Kim, 2002; Hakoda et al., 2002
H_2SO_4/ electron beam irradiation	chlorinated organic contaminants	excavated soil	Yoshimura et al., 2002
Potassium permanganate	PAHs	soil slurry; *in situ* soil	Brown et al., 2003, Clayton et al., 2002
Persulfate	chlorinated solvents, petroleum hydrocarbons, MGP wastes, pesticides and energetic compounds	*in situ* soil	EPA, 2005a
Potassium permanganate/ H_2O_2	VOCs	soil slurry	Gates-Anderson et al., 2001
Ultrasound	para-chlorobenzoic acid	soil slurry	Neppolian and Choi, 2001
US/potassium permanganate	trichloroethene	excavated soil	Balba et al., 2002
Sodium silicate/ H_2O_2/US	oil	*in situ* soil	Yen, 1998
O_3/ultraviolet light (UV)		excavated soil	Tazawa and Suzuki, 1997
O_3/ H_2O_2/UV	pollutants; endocrine disrupting chemicals	Thin layers of soil; excavated soil	Liesenfeld and Winkellman, 1993; Kono et al., 2001
H_2O_2/ Fe^{2+}/UV (photo-Fenton)	dioxins	Thin layers of soil; excavated soil	Isosaari et al., 1998
UV/titanium dioxide (TiO_2)	organic compounds; p,p'-DDT, dioxins	excavated soil; soil surfaces	Zhao et al., 2002; Isosaari et al., 1998
Solar light / TiO_2	organic compounds	excavated soil	Higarashi and Jardim., 2000
UV/US	volatile organic halogen compounds	excavated soil	Kawakami and Kato, 2003

The reaction of dilute hydrogen peroxide (H_2O_2) with dissolved ferrous iron (Fe^{2+}) in an acidic aqueous solution was first described by Fenton (1894). Hydroxyl radicals (OH•) capable of oxidising organic chemicals by hydrogen abstraction are formed as follows (Haber and Weiss, 1934): $H_2O_2 + Fe^{2+} \rightarrow OH\bullet + OH^- + Fe^{3+}$. However, the presence of free radicals in Fenton's reaction has been questioned by Kremer (2000) and Dunford (2002). Intermediates of Fenton's reaction include also perhydroxyl radical ($HO_2\bullet$) (Watts and Dilly, 1996), superoxide (O_2^-) and ferryl ion ($FeIVO^{2+}$) (Kremer, 1999; Miller and Valentine, 1999; Buda et al., 2001; Ensing et al., 2002). Enzymatic (Madigan et al., 2003) and non-enzymatic (Barcelona and Holm, 1991; Watts et al., 1999a; Kremer, 2003) decomposition of H_2O_2 to O_2 and H_2O competes with the Fenton's reaction (Watts and Dilly, 1996) and thus increases the dose of H_2O_2 required.

In Fenton-like reactions Fe(III) is used instead of Fe(II) (Watts and Dilly, 1996). Modified Fenton's reactions apply concentrated H_2O_2 solutions (Watts et al., 2003), iron chelating agents (Sun and Pignatello, 1992; Balmer and Sultzberger, 1999; Nam et al., 2001), H_2O_2 stabilising agents (Kakarla and Watts, 1997) or iron containing minerals (Tyre et al., 1991; Watts et al., 1993 and 1999; Miller and Valentine, 1995; Kwan and Voelker, 2003). Besides iron, other transition metal ions, e.g. Co(II), Cr(II), Cu(I), Mn(II), Ni(II) and Ti(III), and their complexes are utilized in Fenton-like reactions (Goldstein and Meyerstein 1999; Strlič et al., 2003). H_2O_2 can be catalysed also at surfaces and the mechanism is more important at pH above 4 (Kwan and Voelker, 2002). Recent studies have shown the existence of reductive mechanisms in modified Fenton's treatment (Watts et al., 1999b). Fenton's reagent treatment cannot be generally used if more than 15 cm layer of non-aqueous phase liquid is present, if the soil organic carbon content is high, or the contaminants are sorbed to organic-rich materials or the site pH is greater than 8 (US DOE, 1999). Soil organic matter and anions present in soil compete with contaminants for hydroxyl radicals and catalyse hydrogen peroxide decomposition (Watts and Teel, 2005). Organic matter is released from soil during Fenton's oxidation (Miller et al., 1996; Kuei-Jyum Yeh et al., 2002). Dissolved organic matter increases the water solubility of hydrophobic pollutants (e.g. Chiou et al., 1986; Kögel-Knabner et al., 2000; Raber et al., 1998), and release of chlorophenols sorbed to soil matrix and their oxidation in the liquid phase has been shown during modified Fenton's reaction (Kuei-Jyum Yeh et al., 2002). Thus, release of organic matter during oxidation may improve removal of contaminants.

Many contaminants, such as pentachlorophenol (Watts et al., 1990), polychlorinated dioxins (Watts et al., 1991), explosives (Schrader and Hess, 2004) and polyaromatic hydrocarbons (Aunola et al., 2002; Nam et al., 2001; Lee & Hosomi, 2001) have been treated with Fenton's reagent or its modifications. Mineralisation of pentachlorophenol (Tyre et al., 1991), 2,4,6-trinitrotoluene (Schrader and Hess, 2004), PAHs (Watts et al., 2002), and PCBs (Aronstine and Rice, 1995) have been shown in soil slurries treated with Fenton's reaction or modified Fenton's reaction. The cost of chemical oxidation can be reduced by aiming at partial oxidation of contaminants instead of full oxidation and by integrating the process to biological treatment, in which the formed oxidation products can be biodegraded. A short-term chemical oxidation process combined with biodegradation may enhance PAH removal more than natural processes, because partially oxidized degradation products of the PAH are more water-soluble and thus more bioavailable (Matscheko et al., 2002). This has been shown with ozone and Fenton's reagent (Table 5). Modified Fenton's treatment at circum-neutral pH is more suitable for combined oxidation and biological treatment due to the low pH requirement, pH of 2 to 3, of Fenton's reaction. This low pH may be incompatible with biological treatment (Nam et al., 2001) and thus pH is often neutralised prior to soil incubation (Table 5). Microbial inoculum or sediment is often added prior to incubation of soil which has undergone Fenton's reaction, since the survival of the viable contaminant degraders is doubted.

Table 5. Examples of combined chemical and biological treatment of soil contaminated with hydrocarbons.

Oxidant	Contaminant	Study setup	Biological treatment	Result	Note	Reference
O_3, H_2O_2 and H_2O_2 + Fe^{2+}	11 PAHs spiked to sand and peat	Slurry, no addition of enrichment	H_2O_2 treated samples were neutralised to pH of 6.5 – 7.0, 30 days incubation	PAH removal was 49 % by ozonation and 66 % by Fenton in sand. Removal increased during incubation by 43 % and 10 %, in ozonated and Fenton treated sand, respectively	Fenton treatment of peat resulted in poor PAH removal, 27 %, with ozone results were similar to those with sand	Goi et al., 2005b
O_3, 180 to 900 min	Diesel fuel, 2651 mg/kg	Soil columns, O_3 (30 mg/l) introduced from the bottom of column	9 weeks incubation	Up to 48 % of diesel fuel was removed by ozonation and up to 25 % by incubation alone	Highest total removal (55 %) was achieved by 180 or 300 min ozonation combined with incubation	Ahn et al., 2005
30 % H_2O_2 + Fe^{2+}	Benz(a)anthracene 500 mg/kg spiked to simulated soil	0 to 0.35 ml H_2O_2/g soil for 24 h. Ethanol was added to enhance removal	Microbial enrichment, river bottom sediment 30 g per 10 g of soil, addition of 40 ml nutrient solution, incubation 63 days	97 % removal of benz(a)anthracene by Fenton enhanced by ethanol and 12 % removal by microbial treatment alone	Benz(a)anthracene degraded to benz(a)anthracene-7,12-dione in Fenton's treatment. 98 % of benz(a)anthracene-7,12-dione could be microbially degraded	Lee and Hosomi, 2001
O_3, H_2O_2 and H_2O_2 + Fe^{2+}	Diesel fuel, sand and peat, respectively 10.4 g/kg and 12.5 g/kg	Slurry, no addition of enrichment	H_2O_2 treated samples neutralised to pH of 6.5 – 7.0, 30 days incubation	By ozonation of sand and peat (respectively), 48 % and 38 % removal of diesel. By Fenton's treatment of sand and peat (respectively), 60 to 80% and 40 % removal of diesel. Incubation alone removed 49 % of diesel from sand and 64 % from peat	Ozonation combined to incubation removed 70 to 80 % of diesel from peat and 63.5 to 67 % from sand. Fenton's treatment combined to incubation removed 43 to 47 % of diesel from peat and 77 to 85 % from sand.	Goi et al., 2006
5 % H_2O_2 + Fe^{2+} , 80 hours	PCBs (Aroclor 1242)	Slurry, addition of PCB-degrading bacteria	H_2O_2 treated samples neutralised, buffered and 30 days incubation	98 % of PCBs were degraded with Fenton's treatment and 50 % of PCBs were degraded by biological treatment alone.	Fenton's treatment combined to 39 days incubation mineralised 72 % of PCBs	Manzano et al., 2003
O_3	Soil spiked with 1000 mg/kg anthracene	Soil was continuously mixed during ozonation	Biodegradation in slurry with Sphingomonas. yanoikuyae	Preoxidation did not enhance phenanthrene removal	Oxidation products were toxic to S. yanoikuyae	Stehr et al. 2001
0.1 to 0.7 M H_2O_2 + Fe^{2+} at ratio 28:1	Radiolabelled PAHs spiked to PAH contaminated soil	Reaction at soil water holding capacity of 60 %, radiolabelled PAHs added to microplate	170 days incubation	44 % phenanthrene and 61 of pyrene were removed by Fenton's reaction and 32 % phenanthrene and 27 % of pyrene were removed by incubation alone.	The role of chemical treatment alone was not determined	Piskonen and Itävaara, 2004

Table 5. Continued

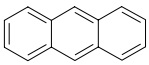
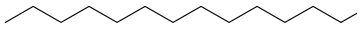
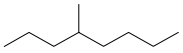
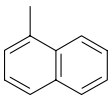
10 % H_2O_2 + Fe^{2+} at pH of 2-3 or 6-6.5 with chelating agents	Aged PAH contaminated soil	Slurry, addition of microbial enrichment culture	Addition of inorganic salts solution, incubation 4 weeks at rotary shaker	Up to 84 % of the individual PAHs were removed from aged PAH contaminated soil with Fenton's reaction. Biodegradation followed by Fenton's reaction removed 85 to 89 % of the individual PAHs, Fenton's reaction followed by biodegradation removed 12 to 39 % for the individual PAHs.	Treatment by modified Fenton with chelating agents not as efficient as by Fenton	Nam et al. 2001
H_2O_2 + Fe^{2+}	4,4'-dichlorobiphenyl and 2,2',4,4',6,6'-hexachlorobiphenyl	Addition PCB-acclimatised cell culture	Incubation after H_2O_2 disappearance at pH of 3 or 6.5	36 % and 22 % of 4,4'-dichlorobiphenyl and 2,2',4,4',6,6'-hexachlorobiphenyl, respectively, were removed by Fenton's oxidation.	65 % and 37 % of 4,4'-dichlorobiphenyl and 2,2',4,4',6,6'-hexachlorobiphenyl, respectively, were removed by Fenton's reaction combined with incubation	Carberry and Yang 1994

1.2 Characteristics and biodegradation of hydrocarbons

Crude oil consists mainly of hydrocarbons, and there are hundreds of individual hydrocarbon components, both cyclic and aliphatic. Crude oil is refined and blended to yield hydrocarbon mixtures, such as gasoline, diesel fuel and lubrication oils, that have desidered properties. Aliphatic hydrocarbons consist of alkanes and alkenes. Cyclic hydrocarbons can be both unsaturated, such as aromatic hydrocarbons, and saturated, such as cycloalkanes. Aromatic hydrocarbons are divided to mono-, di- and polyaromatic hydrocarbons (Potter and Simmons, 1998). Polyaromatic hydrocarbons have two or more conjugated rings. Creosote contains more than 200 compounds, of which more than 85 % are PAHs (Mueller et al., 1989). Alkyl-substituted PAHs are more abundant in petroleum hydrocarbon mixtures than the nonsubstituted ones (Potter and Simmons, 1998). The composition of petroleum mixture affects the degradability of individual components (Atlas, 1981). Some of the hydrocarbons, such as benzene and part of the PAHs, are considered carcinogenic (IRIS, 2006).

The chemical properties defining the fate of hydrocarbons in soil include water solubility, volatility, Henry's law constant and organic carbon partition coefficient, as well as biodegradability (Gustafson et al., 1997). Table 6 shows structures and chemical properties of some hydrocarbons found in petroleum hydrocarbon mixtures and creosote oil. In general, aromatic hydrocarbons are more water-soluble than aliphatic hydrocarbons with same equivalent carbon number (EC). EC refers to the boiling point of hydrocarbon normalised to the boiling point of equivalent straight-chained alkane (Gustafson et al., 1997).However, aliphatic hydrocarbons are more volatile than aromatic hydrocarbons with similar ECs. Solubility and volatility of hydrocarbons decreases with increasing molecular weight (Potter and Simmons, 1998; Gustafson et al., 1997). However, Henry's law constant (H), which describes water-air partitioning of a chemical, is only valid in dilute solutions where water concentrations are lower than water solubility and, therefore, does not apply to many of the petroleum contamination cases. Organic carbon partition coefficient (K_{OC}) is often substituted by octanol–water partition coefficient ($\log K_{OW}$), since due to variation in soil properties, partitioning into soil organic matter is soil-dependent. Furthermore reference values are hard to find. Aliphatic hydrocarbons are more strongly sorbed to organic matter than aromatic hydrocarbons (Gustafson et al., 1997).

Table 6. Examples of chemical structure and properties of hydrocarbons found in petroleum and creosote oil (Chemnetbase, 2006; Gustafson et al., 1997). S water solubility (mass % at 25 ºC), B boiling point as ºC, ρ density (g/cm^3), H Henry's law constant, logK_{OW} octanol / water partition coefficient, K_{OC} soil organic carbon / water partition coefficient (estimated from logK_{OW}, Gustafson et al., 1998).

Chemical and its molecular structure	**Group**	**S**	**B**	**ρ**	**H**	**logK_{OW}**	**K_{OC}**
 Cyclohexane	Cyclic	$5.8\cdot10^{-3}$	80.7	0.7739 (25ºC)	19.4 kPa m^3 mol^{-1}	3.44	$9.63\cdot10^{2}$
 Benzene	Aromatic	0.178	80	0.8765 (20ºC)	0.557 kPa m^3 mol^{-1}	2.13	$8.12\cdot10^{1}$
 Toluene	Alkyl-substituted aromatic	0.053	110.6	0.8668 (20ºC)	0.660 kPa m^3 mol^{-1}	2.73	$2.34\cdot10^{1}$
 Anthracene	Polyaromatic	$4.5\cdot10^{-6}$	339.9	1.28 (25ºC)	$3.96\cdot10^{-3}$ kPa m^3 mol^{-1}	4.56	$7.69\cdot10^{3}$
 Tetradecane	Aliphatic	$1.2\cdot10^{-5}$	253.6	0.7596 (20ºC)	$1.56\cdot10^{2}$ cm^3/cm^3	7.20	$1.17\cdot10^{6}$
 4-methyloctane	Branched aliphatic	$1.2\cdot10^{-7}$	142.4	0.7199 (20ºC)	$4.06\cdot10^{2}$ cm^3/cm^3	5.32	$3.35\cdot10^{4}$
 1-methylnaphthalene	Alkyl-substituted polyaromatic	$2.8\cdot10^{-5}$	244.6	1.022 (20ºC)	$1.81\cdot10^{2}$ cm^3/cm^3	3.87	$2.17\cdot10^{3}$

Environmental factors controlling biodegradation of hydrocarbons are pH, temperature, concentration and availability of suitable electron acceptors. Biodegradation in cold climate tends to be slow. However, the effects of temperature on biodegradation are specific to contaminant and physiology of the micro-organisms at the site (Alexander, 1994), and thus, some micro-organisms living at a cold climate can degrade hydrocarbons as efficiently as their counterparts in a warm climate (Margesin and Schninner, 1997a). However, biodegradation of hydrocarbons may not always work, since the contaminant concentration may be too high for degrading micro-organisms, or the bioavailability of the contaminant may be too low. Many micro-organisms can use only specific electron acceptors. Thus, the availability of electron acceptors determines which type of micro-organisms persist (Bouwer and Zehnder, 1993). In soil, hydrophobic contaminants are often heterogeneously distributed and may be inaccessible to bacteria, which cannot reach pores smaller than 0.2 to 0.8 µm. Furthermore, PAHs are often found in tar droplets, which have a low surface to volume ratio, limiting access of bacteria (Johnsen et al., 2005).

The complete oxidation of aromatic compounds and hydrocarbons to carbon dioxide is difficult in the absence of molecular oxygen due to the great stability of C-H and C-C bonds (Boll et al., 2002). Virtually all hydrocarbons are biodegradable under aerobic conditions. Bacteria, archaea, fungi and algae all have genera capable of degrading polyaromatic hydrocarbons (Prince and Drake, 1999; Mueller et al., 1996). Occurrence of hydrocarbon-utilising micro-organisms in soil is based on previous exposure to hydrocarbons (For a review see Atlas, 1981). Bacteria and fungi have mono- and dioxygenases, which incorporate hydroxyl groups derived from molecular oxygen into the aliphatic chain or the aromatic ring (Heider et al., 1999). In general, *n*-alkanes are easier to degrade than other compounds. Aerobically alkanes are converted into fatty acids, which are further oxidised by ß-oxidation. Methyl branching increases the recalcitrant nature of alkanes, and cycloalkanes are considered especially recalcitrant (For review see Atlas, 1981). Unsaturated

aliphatic compounds can be degraded similarly to alkanes in the presence of oxygen. Aerobic biodegradation of many aromatic compounds proceeds through formation of catechol both for eucaryotic and procaryotic organisms (Bouwer and Zehnder, 1993). Dioxygenases oxidise PAHs to *cis*-hydrodiols, and PAHs can be oxidised by cytochrome P-450 to arene oxides (Mueller et al., 1996). White-rot fungi (Bogan and Lamar, 1996) and litter-decomposing fungi (Steffen et al., 2002) produce lignolytic enzymes that oxidise PAHs to PAH-quinones.

Research in the past two decades has shown possibilities to degrade hydrocarbons under anaerobic conditions (Heider et al., 1999). Anaerobic hydrocarbon degradation requires the presence of electron acceptor, such as nitrate, ferric iron or sulphate (Fathepure and Tiedje, 1999). Hydrocarbons that can be degraded anaerobically include aliphatic alkenes and alkanes with chain lengths of 6-20 carbon atoms, monocyclic alkylbenzenes, such as toluene, ethylbenzene, propylbenzene, p-cymene, xylene- and ethyltoluene-isomers, as well as benzene and naphthalene (Heider et al., 1999). There are several initiation mechanisms of anaerobic alkane degradation (Aeckersberg, 1998). Benzoate (or its CoA-thioester) is a key intermediate in anaerobic mineralization of numerous aromatic compounds (Heider et al., 1999; Boll et al., 2002). Anaerobically, PAHs containing up to four rings can be transformed. Two-ring PAHs can be used as sole source of carbon and energy, but the transformation of 3- and 4-ring PAHs may be a result of cometabolism. Anaerobic PAH degradation has not been shown under methanogenic conditions, but anaerobic degration using external electron acceptors, such as sulphate and iron, has been shown. The pathways of anaerobic PAH degradation can vary depending on the electron acceptor used (Meckenstock et al., 2004).

Horizontal genetic transfer enables bacteria to adapt to xenobiotic compounds fast by genetic exchange and assembly of new pathways. Mechanisms of horizontal gene transfer are taking up cell-free DNA and incorporating it to genome, gene transfer by bacteriophages and gene transfer by plasmids or transposons in cell-to-cell contact (for review see Top and Springael, 2004). However, microbial communities present at a polluted site are sometimes not capable of degrading the pollutants. Bioaugmentation with specific strains or consortiums may improve the removal of pollutants from soil. Even if the added bacteria do not persist in soil, they may improve pollutant removal through genetic transfer. In order to survive in soil, the added bacteria must fill an unutilized metabolic niche in the microbial community and be protected by encapsulation or in a biofilm (for review see El Fantroussi and Agathos, 2005).

Plants can also uptake and degrade hydrocarbons, such as PAHs (Edwards et al., 1982; Chroma et al., 2002). The degradative enzymes include laccases, peroxidases and cytochrome P450.

1.3 Indicators of soil quality

The European Commission is currently preparing a thematic strategy for soil protection. Information is needed about the response of soil to environmental changes (EC, 2002). Soil protection policy is required to avoid soil deterioration (Winding et al., 2005). The creation of sites where soil properties are continuously followed is suggested in order to obtain information about soil quality to assist policy makers in decision making. Information should be obtained about soil profile, soil geological classification, characteristics of sites including their land management history, soil bulk density, soil pH, soil cation exchange capacity and water holding capacity. Specific sites need to be selected for monitoring soil organic matter and biodiversity, soil erosion, soil contamination, soil sealing, floods and landslides (Van-Camp et al., 2002).

1.3.1 Soil microbial activity

Micro-organisms are sensitive to changes and environmental stress since they are in close contact with their surroundings due to high surface-to-volume ratio (Winding et al., 2005). Enzymatic activities, toxicity to different organisms, number of contaminant degraders and number of bioluminescent marker bacteria can be used as bioindicators to follow remediation of contaminated soil (Maila and Cloete, 2005). Using methods that determine microbial metabolic activity is important in assessing changes in land management (Engelen et al., 1998). Microbial diversity in soil can be investigated with assays of microbial metabolic activity, analysis of phospholipid fatty acids and molecular methods based on extraction of deoxyribonucleic acid (DNA) (Torsvik et al., 1990). Nucleic acid or lipid based methods target conserved cell components of bacterial groups. However, similarity in taxonomy is not directly linked to physiology and nutrition features (Becker, 1999; Degens and Harris, 1997), and the extraction of DNA can affect the measured diversity (de Liphtay et al., 2004). Therefore, results with different extraction procedures cannot be compared. Microbial indicators currently in use in European monitoring programmes include soil respiration, microbial biomass, soil enzymes, C and N mineralization, bacterial growth rates, bacterial diversity, arbuscular mycorrhiza, frequency and diversity of genus *Rhizobium* and organic matter decomposition (for review see Winding et al., 2005). Common methods used to monitor microbial metabolic activity and microbial biomass are presented in Table 7 together with their benefits and drawbacks. Often they are used in combination with methods describing microbial diversity such as determination of phospholipid fatty acids (PLFA), fatty acid methyl esters (FAME) or methods based on DNA extraction.

Table 7. Common methods to follow microbial metabolic activity and microbial biomass in soil.

Method	Description	Benefits	Drawbacks
Enumeration of culturable micro-organisms	Soil is diluted and dilutions are spread at plates or placed in tubes	Easy to use, culturable bacteria may be more active than unculturable[1]	Not all bacteria are culturable on general laboratory media[2]
Soil enzymatic activities are determined at specific substrate concentration	Soil is diluted and a substrate, usually *p*-nitrophenol substrates[3], is added. The formation of product is determined after a fixed incubation time	Easy to use, relatively fast, low cost assay[4]	The reaction needs to be stopped, thus only single reading can be obtained from one suspension[4]; substrate concentrations are higher than in natural environments[5]
PLFA as a measure of microbial biomass[6]	PLFA are extracted from soil and analysed with gas chromatography	Individual PLFAs are specific to subgroups of micro-organisms[7]	Demands expertise in fatty acid identification
Kinetic determination of soil enzymatic activities[4]	Soil is diluted and pipetted to micro-plates with fluorogenic substrates[4], the formation of product is followed during incubation	Substrate concentrations can be optimised for each soil, reaction can be measured over time[4]	The preparation of plates is time-demanding and needs expensive equipment, method sensitivity is higher than with *p*-nitrophenol substrates[4]
Thymidine incorporation[8,9]	[3H]thymidine addition to soil slurry/ centrifuged suspension containing soil bacteria, incubation, measurement of incorporated radioactivity	Fast method to measure bacterial growth rates as bacterial DNA synthesis	The use of centrifugation may eliminate differences in soil bacterial growth rates[10]; culturable bacteria are responsible for thymidine incorporation[11]
Microbial biomass by fumigation extraction[12,13]	$CHCl_3$-fumigation of soil, extraction of carbon	Easy to use and common method[5]	Dangerous solvent is used, the sensitivity of the method to soil contamination may be low[14]
Leucine incorporation[15]	Radiolabelled leucine is added to soil slurry, slurry is incubated, incorporated radioactivity is measured	Fast method to measure bacterial growth rates	
Soil respiration[16,17]	Soil is incubated and CO_2 evolution/O_2 consumption is recorded	Easy to use, widely used parameter[5]	Respiration is influenced by many soil parameters[5]
Substrate induced respiration [18,19,20]	Soil slurry is incubated with added carbon source and increase in carbon dioxide production is compared to soil respiration without substrate	Utilisation can be measured within a few hours[18]	Preparation of solutions is time-demanding
Direct enumeration of viable cells	Cells are stained with fluorochrome and enumerated by epifluorescence microscope	Relatively fast method	Cell counting is time-demanding, autofluorescence of soil particles must be taken into consideration
N mineralization[21]	Measurement of NH_4^+ accumulation in soil slurry	Commonly used in soil monitoring[5]	May not be sensitive to soil contamination[5]
Determination of soil DNA content[22,23]	DNA is extracted, DNA concentration is measured, DNA:cell ratio is used to calculte bacterial numbers[24]	Can be used in combination with microbial diversity analysis	Extraction may not be able to release all soil DNA[25]
Carbon source utilisation with microplates[26]	Soil is diluted and pipetted to microplates, substrate utilisation is measured during incubation by a colorimetric assay in which formazan is formed from tetrazolium violet of the microplates	Easy to use, substrate utilisation patterns can be used to classify microbial communities and characterise their community level physiological profiles[27]	The concentration of carbon sources in the microplates is relatively high and only culturable bacteria can grow in the microplates, soil extraction might not extract all micro-organisms, thus the patterns may not reflect bacteria in original inoculum

[1]Bakken and Olsen, 1987; Becker, 1999 [2]Staley and Konopka, 1985; Torsvik et al., 1990 [3]Tabatabai, 1994 [4]Marx et al., 2001 [5]Winding et al., 2005 [6]Frostegård et al., 1991 [7]Zelles, 1999 [8]Bååth, 1990 [9]Bååth et al., 2001 [10]Uhlirova and Santruckova, 2003 [11]Bååth, 1994 [12]Vance et al., 1987 [13]ISO, 1997a [14]Barajas-Aceves, 2005 [15]Michel and Bloem, 1993 [16]ISO 2002a [17]ISO 2002b [18]Anderson and Domsch, 1978 [19]Degens and Harris 1997 [20]ISO, 1997b [21] ISO, 1997c [22]Marstorp et al., 2000 [23]Taylor et al., 2002 [24]Torsvik et al., 1990 [25]Martin-Laurent et al., 2001 [26]Garland and Mills, 1991 [27]Lehman et al., 1995 [28]Preston-Mafham et al., 2002

Single-substrate utilisation patterns of microbial communities with Biolog® GN and ECO plates have been used to monitor the effects of soil contamination with hydrocarbons (Bundy et al., 2002; Dobler et. al., 2000; Wünsche et. al., 1995), pesticides (El Fantroussi et. al. 1999), and heavy metals (Dobler et. al., 2000). ECO plates were specially designed for microbial community analyses in microbial ecology studies (Biolog, 2001), while GN plates were developed for identification of bacterial strains based on their carbon source utilisation (Biolog, 2005). Carbon source utilisation patterns of contaminated soil have been often found to differ from uncontaminated soils. The use of rhizosphere carbon sources (Campbell et. al., 1997) or carbon sources relevant to hydrocarbon degradation (Bundy et al., 2002) might increase the ability of carbon source utilisation patterns to detect changes in soil microbial communities. Recently, new methods of carbon source utilisation have been developed. Campbell et al. (2003) described a microtiter plate method measuring carbon dioxide production with whole soil instead of soil extracts and Garland et al. (2003) developed a microtiter plate, in which oxygen consumption is measured with fluorescence. The Biolog® method has also been used to differentiate soil fungal communities (Dobler et al., 2000 and Buyer et al., 2001) and to measure pollution-induced community tolerance of heavy metals in contaminated soil. Inhibition of pollutants to soil metabolic activities can be measured by adding different amounts of pollutants to Biolog GN plates (Rutgers et. al., 1999).

Plants and micro-organisms release extracellular enzymes to soil. Soil enzymes are involved in the decomposition of organic inputs and the detoxification of xenobiotics (Margesin et al., 1999). Extracellular enzymes reach substrates in small pores, hydrolyse them and transform polymers to smaller compounds which can be taken up by membrane transport systems. Some microbial enzymes are specific to only a single substrate, whereas some are not highly specific and can therefore utilise a group of structurally similar substrates. The substrates of hydrolases in soil are found mainly in polymerised form, which cannot be directly taken up by micro-organisms (Quiquampoix et al, 2002). Land management (Kandeler et al., 1999; Naseby and Lynch, 2002), pollution (Margesin et al., 1999; Brohon et al., 2001) and vegetation (Broughton and Gross 2000) influence soil enzymatic activities. Activities of the lipases (Margesin et al., 1999), dehydrogenases (Frankenberger and Johanson, 1982), catalases and ureases (Margesin et al., 2000) have been used to monitor hydrocarbon removal in soil.

Biodegradation potential should be determined in order to understand the biogeochemistry of contaminated sites (Madsen et al., 1995). The bioremediation potential of soil can be investigated by following the biodegradation of the pollutants or radiolabelled compounds (Madsen et al., 1995), screening genes coding for pollutant degradation (for review see Galvao et al., 2005), microbial enumeration of contaminant degraders (Wrenn and Venosa, 1996; Stieber et al., 1994) or isolation of contaminant degrading bacteria and fungi and screening their ability to degrade contaminants (Chaineau et al., 1999)**.**

1.3.2 Contaminated soil toxicity

Due to the complexity of soil ecosystems, the impacts of pollutants vary and range from direct toxicity symptoms to effects on reproduction of organisms and indirect effects to predator-prey relationships as well as changes in landscape. Pollutants impact on all levels: organism, population, community, ecosystem and landscape level (Edwards, 2002). Soil ecotoxicity tests were developed in order to quantify the toxicological impact of chemicals on ecological receptors, such as bacteria, earthworms and plants (Saterbak et al., 1999). Currently available tests inlude single species laboratory tests as well as a few multi-species assays and integrated soil microcosms and terrestrial model ecosystems (Edwards, 2002). Toxicity of soil can be determined directly from the soil, from the leachate produced in a soil leaching test or from soil extracts.

Table 8 describes direct contact assays available for determining toxicity of hydrocarbon contaminated soil. According to these studies, remediation can decrease the ecotoxicity of hydrocarbon contaminated soil. The toxicity of soil leachates and soil extracts can also be determined with other methods such as algal growth inhibition test, water flea immobilisation test, bacterial growth inhibition test, reverse electron transport assay, plant root elongation test, plant growth inhibition test and commercial microbial enzyme assays. Ecotoxicity tests can reveal the presence of hazardous chemicals, which have not been analysed from soil (Vaajasaari, 2005).

Table 8. Examples of ecotoxicological tests used for studying hydrocarbon contaminated soil. Methods in which soil is in direct contact with organisms.

Species	Contaminant studied	Description	Result
Luminescent marine bacterium *Vibrio fischeri*	PAHs (Aicheson et al., 2004; Ahtiainen et al., 2002) and oil hydrocarbons (Juvonen et al., 2000;)	direct contact assay with soil suspension in contact with *Vibrio fischeri*, luminescence measured	toxicity depends on contaminant aging and oil type; remediation decreased soil toxicity
Plant-seed germination test (barley, corn, wheat, oat, mustard, lettuce)	Oil hydrocarbons (Siddiqui and Adams, 2002; Plaza et al., 2005; Salanitro et al., 1997, Saterbak et al., 1999; Vaajasaari et al., 2002)	Seeds are germinated in contaminated soil, plants are counted	remediation decreased soil toxicity, heavy crude oil not toxic to plants, some hydrocarbon contaminated soils non-toxic to plants
Earthworm *Eisenia foetida*	Phenanthrene (Shin and Kim, 2001), creosote oil and diesel fuel (Shin and Kim, 2001), oil hydrocarbons (Dorn and Salanitro, 2000; Dorn et al., 1998, Salanitro et al., 1997; Saterbak et al., 1999)	Earthworms placed in soil and soil dilutions, their survival followed	Remediation decreased soil toxicity, toxicity was dependent on type of hydrocarbon contamination
Enchtraeid *Enchytraeus albidus*	crude oil (Filiminova and Pokarzhevskii, 2000), aged petroleum hydrocarbons (Vaajasaari et al., 2002) and oily wastes (Juvonen et al., 2000).	Enchytraeid worms placed in soil and soil dilutions, their survival followed	crude oil toxic to enchytraieds, less toxic to oily wastes, oil analyses cannot always predict soil ecotoxicity

Luminescent bacterium *Vibrio fischeri* is often used in ecotoxicological studies. It is more sensitive to organic than to inorganic chemicals (Vaajasaari et al., 2002). However, *Vibrio fischeri* is a marine bacterium, and thus, luminescence tests with terrestrial bacteria have been developed (Shaw et al., 2000; Bundy et al., 2004). *Vibrio fischeri* test describes only acute toxicity and not the effects of long-term exposure. Long-term exposure effects can be determined with earthworms and enchtraeids. Earthworms have been used as test-organisms in soil ecotoxicological tests, because they are common in a wide range of soils. They represent 60 to 80% of the total soil animal/invertebrate biomass (Loureiro et al., 2005). Enchytraeids are also sensitive to contamination and changes in land use (Römbke, 2003). The *Enchytraeus albidus* reproduction test has been found to be more sensitive than *Enchytraeus albidus* survival test for toxicity assessment of oily waste (Juvonen *et al.,* 2000). Performance of avoidance tests in addition to survival and chronic tests has been suggested as a fast soil screening tool for both earthworms and enchytraeids (Loureiro et al., 2005).

However, no single test is significant to assess risk to entire ecosystem (Ferguson et al., 1998). Recently, Fernandez et al. (2005) described a soil column ecotoxicity assessment system, in which toxicity to earthworms, plants and micro-organisms are monitored simultaneously. The leachate toxicity and chemical concentrations in the leachate are also analysed. This type of toxicity test enables determination of species interactions.

2 Aims of the present work

The purpose of this thesis research was to study feasible options to enhance *in situ* remediation of hydrocarbon contaminants. The aims were to increase the bioavailability of contaminants and microbial activity at the subsurface in order to achieve higher contaminant removal efficiency than by intrinsic biodegradation alone. Two approaches, phytoremediation and oxidation with modified Fenton's reagent combined with biodegradation, were chosen to achieve these goals. Phytoremediation was used to remove fresh and aged petroleum hydrocarbons from soil, and modified Fenton's reaction combined with soil incubation was used to remove aged creosote oil from soil. Furthermore, enhancement of microbial activity and decrease of soil toxicity during remediation were estimated by using several biological assays. The performance of these assays was compared in order to find suitable indicators to monitor the progress of remediation.

The specific aims of this thesis were

1. to compare the performance of different plant species in the removal of hydrocarbons (Papers I, III)
2. to study whether the removal of hydrocarbons from soil was affected by the aging of contaminants and repeated exposure to hydrocarbons (Papers I, II, III)
3. to study if treatment by modified Fenton's oxidation combined to incubation increased PAH removal during incubation compared to the treatments alone (Papers IV, V) and how hydrogen peroxide concentration influenced the PAH removal
4. to determine how phytoremediation influences microbial activities (Papers II, III) and soil toxicity (Paper III)
5. to determine soil toxicity during phytoremediation (Paper III) and modified Fenton's reaction (Papers IV, V)
6. to study the effects of hydrogen peroxide concentration on microbial activity and toxicity of soil and leachates (Papers IV, V)
7. to compare the suitability of biological indicators for monitoring microbial activity and toxicity during remediation process

3 Materials and methods

3.1 Phytoremediation

The setup of laboratory phytoremediation experiments is explained in detail in Papers I and III. The removal of fresh diesel fuel (Papers I and II) and the removal of aged hydrocarbons (Paper III) were studied in order to determine the effect of hydrocarbon aging on the removal. Further, the removal of hydrocarbons in the presence of different combinations of plant species and soil amendments were compared in order to find suitable conditions for fast hydrocarbon removal (Papers I and III). Changes in soil microbial metabolic and biocatalytic activities in the presence of contaminants and plants were compared in Papers II and III. The suitability of plant species to phytoremediation studies was determined following plant physical appearance (Papers I and III) and plant uptake of hydrocarbons (Paper I).

Briefly, lab-scale phytoremediation study was made with soil artificially contaminated with diesel fuel (Papers I and II). 0.5 weight-% of diesel fuel was mixed thoroughly with spoon to the soil taken from A and B horizons of mixed forest. The soil was allowed to stabilise for five days before adding seeds of grasses or white clover and green pea or planting with year-old Scots pine or poplar. Hydrocarbon removal in the presence of different plant species and soil amendments, and physical appearance of the plants were followed for a maximum of one year. After one year, the effect of soil recontamination with diesel fuel was studied with pine and poplar (Figure 1). In this application, diesel fuel was applied on top of the soil.

Figure 1. Pines (in front) and poplars (at back) used in the lab-scale experiment with fresh diesel fuel. Photo taken after addition of second dose of diesel fuel.

In phytoremediation field study (Paper III), contaminated soil from a bus depot was used for a single circular field plot measuring 6 m in diameter. The soil contained aged lubrication oil and diesel fuel and lead, copper and zinc exceeding the Finnish limit values in soil (Mroueh *et al.* 1996). Volatile organic compounds were not present in the soil. Composted sieved biowaste was added to two quarters and NPK-fertiliser to one quarter. No nutrients were added to the fourth quarter (Figure 2). Pines and poplars were planted to three quarters of the field plot and a grass mixture and white clover were sown over the entire plot (Figure 2). The field plot soil, plants and leachate were monitored for four growing seasons.

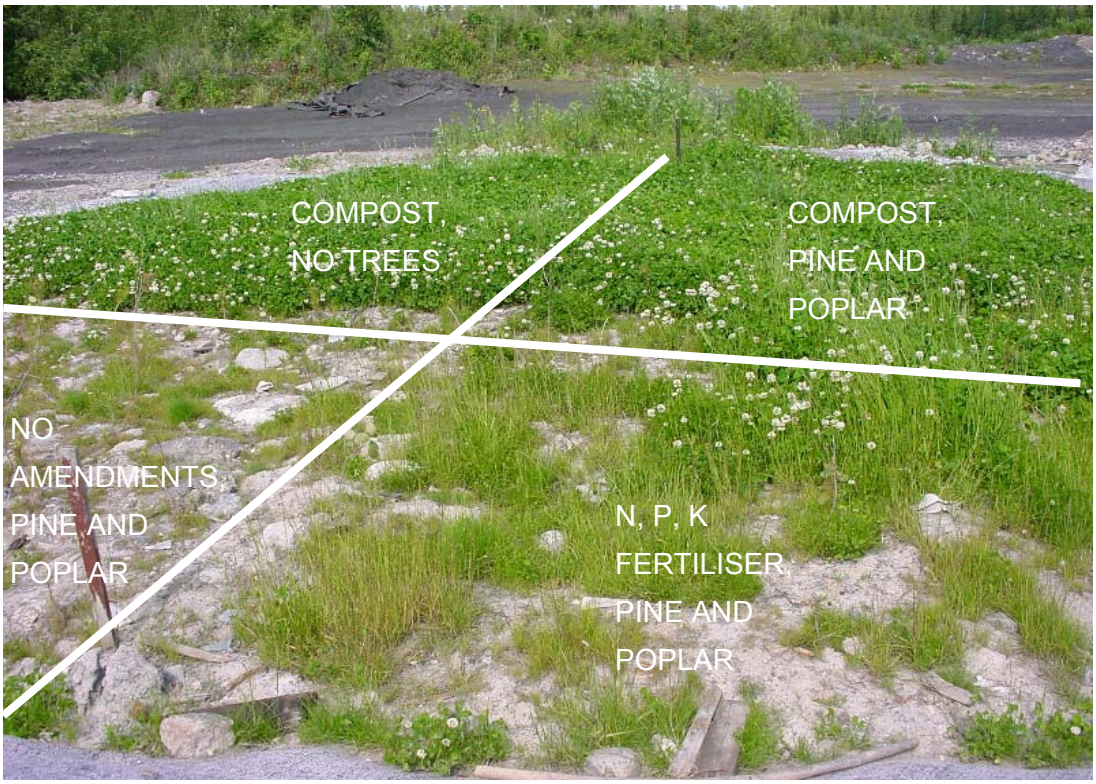


Figure 2. Field site layout, fertilisation and distribution of trees in field plot quarters.

3.2 Modified Fenton's treatment

Modified Fenton's treatment was combined to soil incubation to determine if the bioavailability of PAHs increased during modified Fenton's oxidation and to determine the effects of hydrogen peroxide addition on the microbial activity and toxicity of the soil and leachate (Papers IV and V). The laboratory-scale experiments were carried out in packed soil columns with an H_2O_2 delivery system simulating *in situ* injection (Figure 3). The columns were filled with sandy soil, which had been contaminated with creosote oil at a wood impregnation site in the 1960s. The contamination was due to a leak in a storage tank and uncontrolled operating procedures. Because the studied soil was rich in iron, no iron was added in the experiments.

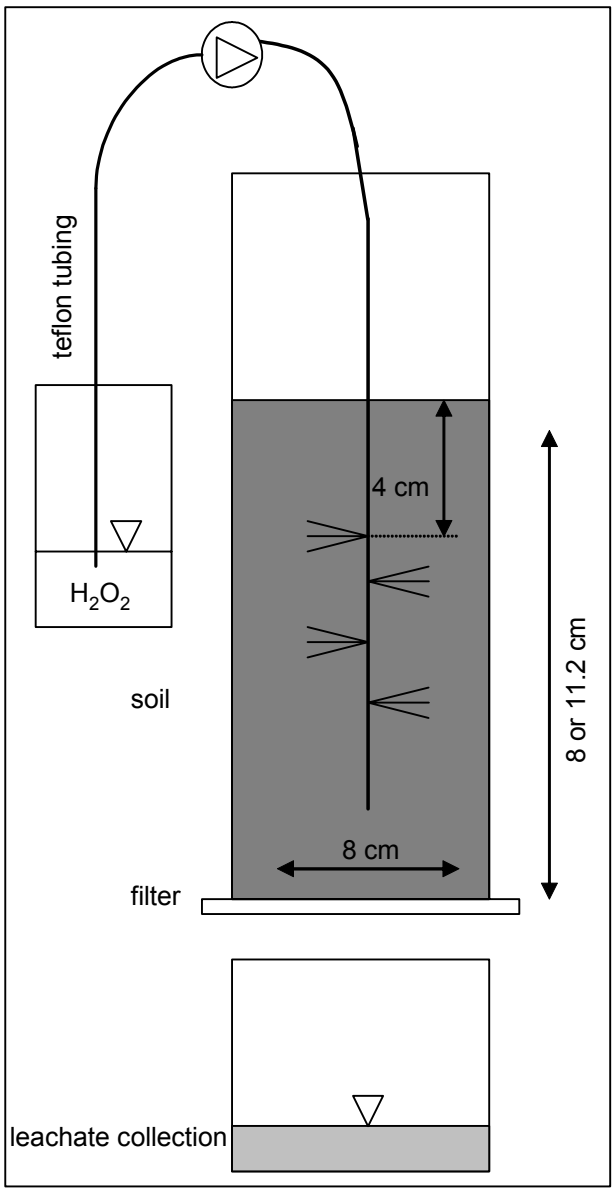


Figure 3. Set-up of column experiments. Hydrogen peroxide solution was injected to packed (1.25 kg/dm^3) creosote contaminated soil via perforated tubes. Filter mat was installed to the bottom of the column to retain the solids. Figure modified from Aunola et al. (2006).

The details of H_2O_2 injection are explained in Table 9. The flushing of contaminants out of the column due to liquid injection was studied in a control experiment, where deionised water was added to the soil for 10 days. Column leachates were collected after each injection of H_2O_2. Non-treated soil and soil collected from the columns after the modified Fenton's reaction, i.e. from the upper and lower part of the columns, were analysed for PAHs, microbial activity and toxicity. Residual H_2O_2 was not removed from the soil. Biodegradation of PAHs in the soil during incubation was studied in serum bottles. Soil samples, including control, were incubated in sealed serum bottles at 20 °C (Papers IV and V) or 6 ºC (Paper V) for 5 weeks to 16 weeks.

Table 9. Set-up of H_2O_2 injection. Aimed H_2O_2 dose as 100% hydrogen was 0.4 g H_2O_2 /g soil and liquid was injected three times a day, but applied doses varied slightly. In control experiment, deionised water was applied instead of hydrogen peroxide.

Paper	H_2O_2 (%)	Treatment time (d)	Volume per injection (ml)	Total volume injected to each column (ml)	Soil content (g)	H_2O_2 dose as 100% H_2O_2 (g H_2O_2 /g soil)
IV	30	4	50	600	500	0.40
IV	30	10	25	750	700	0.37
IV	0	10	25	750	700	0
V	12	10	77	2310	700	0.40

3.3 Analyses

This chapter describes the methods used in the analyses of Papers I to V. The methods developed during this thesis are described in detail, while other methods are described briefly.

3.3.1 Physico-chemical analyses

The physico-chemical analyses carried out in this thesis research are summarised in Table 10. The following analyses were made in external laboratories, either at TUT, Ball State University or in commercial laboratories: particle size distribution, total phosphorus and sulphur in soil, cation exchange capacity of soil, concentrations of soluble nitrate, phosphate and sulphate in soil and metal concentrations in soil and leachate. Results of soil chemical and physical properties are discussed only in the Papers I to V. Results of soil, leachate and plant metal concentrations (Paper III) are not included in the scope of this thesis summary. They are discussed only in Paper III.

Table 10. Summary of the physico-chemical analyses carried out in this study.

Parameter	Sample	Method	Reference and in which Paper(s) used
pH	Soil	Electrode measurement from soil:deionized water slurry	Paper I, ASTM, 2001
Moisture content	Soil	oven-drying at 105^{o}C	Papers I to V
Particle size	Soil	Dry and wet sieving; Sedigraph	Paper I
Organic carbon content	Soil	Loss at ignition	Nelson and Sommers, 1982; Paper I, III and V
total P	Soil	Bray-1 method	Olsen and Sommers, 1982; Paper I
Cation exchange capacity	Soil	Ba replacement method	Rhoades, 1982; Paper I
Soluble NO_3^-, PO_4^{3-} and SO_4^{2-}	Soil	With ion chromatograph from 10:1 soil:water extract	Paper I
Metals	Soil	Microwave assisted acid digestion, ICP-AES (inductively coupled plasma-atomic emission spectrometry) using EPA method 3051	EPA, 1994, Papers I and III
Diesel fuel concentration	Soil and plant roots	Field-moist soil samples from growth chamber as well as washed and dried pine, poplar, grass and legume roots were hexane-extracted in ultrasonic bath, extract analysed with gas chromatograph equipped with mass selective detector (GC-MSD)	Paper I; Papers I and II
Plant-available metals	Soil	1 M ammonium acetate extraction at pH of 4.5, ICP-AES	Internal method of Finnish Geological survey, Paper III
Mineral oil concentration	Soil	ISO committee draft method, extraction twice (instead of once) with heptane/acetone, clean-up with sodium sulphate and Florisil, GC/MS	ISO, 2000a; Paper III
Concentration of fourteen aliphatic hydrocarbons ranging from nonane to hexatriacontane	Soil	Hexane extraction of field-moist soil samples in ultrasonic bath, extract analysed with gas chromatograph equipped with mass selective detector	Paper I
C- and N- concentrations	Soil	CN-analyser	Paper IV; Paper IV and V
Metal, P- and S concentrations	Soil	Extraction at 90 ºC in HCl-HNO3 mixture, 3:1 ratio	Paper IV; Paper IV and V
Gas formation and O_2, CO_2 and CH_4 in the gas phase	Serum bottles containing soil	Gas formation was measured with gas-tight syringe and gas sample was taken with syringe and analysed with gas chromatograph equipped with thermal conductivity detector	Paper IV
Polyaromatic hydrocarbon concentrations	Soil	Dichloromethane extraction with Soxhlet apparatus and analysis with GC-MSD	Paper IV; Papers IV and V
Polyaromatic hydrocarbon concentrations	Column leachate	Dichloromethane extraction, concentration with N2 stream and analysis with GC-MSD	Paper IV
The dissolved organic carbon (DOC) content	Column leachate	Leachate filtered (0.45µm) and DOC measured with total organic carbon analyzer	Paper V
Content of total iron	Column leachate	Photometric method	SFS, 1976; Paper V
Hydrogen peroxide concentration	Soil	fluorometric method from diluted soil	Paper V

The method to extract hydrocarbons from soil has to be chosen based on the type of the hydrocarbon studied. Petroleum hydrocarbon concentrations were determined from field-moist samples (Papers I to III) and PAH concentrations were determined from soil dried with anhydrous sodium sulphate (Papers IV and V). Diesel fuel contaminated soil was extracted with hexane in an ultrasonic bath (Papers I and II). Extraction efficiency was determined by extracting 16 samples of soil freshly spiked with diesel fuel and was found to be 93±12 % (Paper I). However, extraction

efficiency of aged hydrocarbons (Papers III to V) cannot be determined, since properties of spiked samples are different from those of aged samples.

The hexane extraction in ultrasonic bath was not suitable to extract aged hydrocarbon contaminated soil (Paper III). Thus, ISO committee draft method (ISO, 2000) i.e. extraction with acetone/heptane and washing with water, drying with sodium sulphate and cleanup with Florisil was used for aged hydrocarbon contaminated soil (Paper III). Soil was extracted twice, because the yield with one extraction was found to be substantially lower (data not shown), possibly due to the presence of clay, resulting in low permeability, and weathered hydrocarbons. The petroleum hydrocarbon contaminated soil extracts were analysed for diesel fuel and/or lubricant oil using gas chromatography with a mass-selective detector (GC/MSD) in scan mode. The GC was equipped with cross-linked 5 % phenyl methyl siloxane capillary column, HP-5MS. Helium was used as carrier gas. The temperature program was started at 40 ºC and raised by 10 ºC/min until 300 ºC, which was maintained for 8 min. In the study with two year-old trees, the temperature program was started at 60 ºC, and continued as mentioned above (Papers I and II). The concentrations in soil are reported on a dry weight basis (Papers I; II and III). The total diesel fuel concentrations were measured as the area of total ion chromatogram from the retention time of nonane to the retention time of octacosane (Papers I and II). Total area between decane and octacosane, which were spiked into n-heptane used in extraction, was determined from extracts of aged hydrocarbon contaminated soil (Paper III). External standards were prepared by dissolving commercial diesel fuel and/or lubrication oil in n-hexane. Stock solutions contained 100 g of diesel or lubrication oil per one liter of n-hexane. A volumetric mixture of one part diesel fuel and two parts lubrication oil was used to prepare standards of aged hydrocarbon contaminated soil, since the chromatogram shape was close to that of aged hydrocarbon contaminated soil extracts (Paper III).

The PAHs were extracted from soil samples dried with anhydrous Na_2SO_4 with dichloromethane (60 ml) in a Soxhlet apparatus for 16 h at 105 °C. The extracts were diluted with dichloromethane and filtered (0.2 µm) prior to analysis. Internal standard (d10-phenanthrene) was added into the vials. A solution containing 16 PAHs (Z-0313-17, Accustandard) was used as external standard. The extracts were analysed with Hewlett Packard 6890 GC-MSD using selected ion monitoring. Further, filtered (0.45 µm) leachate samples the PAHs were extracted with dichloromethane. Dichloromethane extracts were concentrated under nitrogen stream to final volume and the PAHs were analysed as described above.

Hydrogen peroxide concentration in soil after Fenton's treatment was determined with fluorometric method modified from the method for water phase (Lazrus et al., 1985). The method allows determination of concentrations below 10^{-3} M. All the solutions were prepared with glassware washed with 30 % nitric acid and rinsed several times with deionised, distilled water. First the soil samples were diluted 1:50 in autoclaved deionised, distilled water. Further dilutions were made in a buffer containing tetrasodium EDTA, 8.4 10^{-4} M and potassium hydrogen phthalate,0.35 M. The final pH of the buffer was set to 5.5. The samples (150 µL) and hydrogen peroxide standards of 5.0 10^{-6} M to 5.0 10^{-3} M (150 µL) prepared in the buffer were added to black microtiter plate wells in duplicates. Buffer (30 µL) was added to one sample well, whereas catalase (0.490 units of catalase L^{-1} buffer) (30 µL) was added to the second well to produce the analytical blank. Fluorescence reagent, 8.0 10^{-3} M 4-hydroxyphenylacetic acid in 0.35 M potassium hydrogen phtalate (pH to 5.5 with 10 M NaOH) containing horseradish peroxidase (8 purpurogallin units of peroxidase/ ml reagent), 30 µL, was added to each well. The plate was allowed to stand for 5 min and thereafter 30 µl of 0.4 M NaOH was added. The fluorescence in microtiter plate wells was measured with Fluoroskan FL (Thermobio Labsystems) at 320 nm/ 405 nm. The analytical blanks of both samples and standards were subtracted before calibration.

3.3.2 Microbiological analyses

The microbiological analyses carried out in this thesis are summarised in Table 11.

Table 11. Summary of the microbiological analyses carried out in this study.

Parameter	Method	Reference and in which Paper(s) used
Viable plate counts of bacteria	Spread plate method	Burlage et al. (1998); Paper II
Viable plate counts of actinomycetes	Spread plate method	Wellington and Toth (1994); Paper II
Utilisation of diesel	Incubation in Biolog MT2 plates, measurement of absorbance at 590 nm	Paper II
Utilisation of ECO plate carbon sources	Incubation in Biolog ECO plates, measurement of absorbance at 590 nm	Paper II; Papers II and III
Microbial extracellular enzymatic activities	Incubation with fluorogenic substrates, measurement of fluorescence at excitation 355 nm/emission 460 nm	Papers II and IV
Enumeration and viability of bacteria	BacLightTM live/dead assay from soil suspension collected to filter	Paper III
Utilisation of PAHs	Microplate assay with 2-(4-iodophyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2-H-tetrazolium monosodium salt (WST-1)	Paper V

All the microbiological parameters, except PAH utilisation, were measured from soil suspension prepared in sterile deionised water (Papers II to V). The soil suspension was not buffered to better simulate enzyme activity in field conditions.

BacLight™live/dead assay with SYTO®9 and propidium iodide stains (Viability Kit L-7012, Molecular Probes) was used to determine the viability based on cell wall integrity of bacteria in treated and non-treated soil. Soil (1 g) was mixed with 50 ml sterile deionised water (Milli-Q®) and the suspension was sonicated (5 × 1 min). The suspension (50 or 100 µl) was mixed with 0.01 M tetrasodium pyrophosphate to achieve final volume of 1 ml in an Eppendorf tube. BacLight™ reagent (5 µl) was added to the mixture, vortexed for 10 s and incubated for 15 min in the dark. The final suspension and Eppendorf tube rinsing liquid (0.1 M tetra sodium pyrophosphate) was filtered with polycarbonate membrane filter (0.2 µm), supported by cellulose acetate filter (0.45 µm). At least 130 cells per filter were enumerated with an epifluorescence microscope (Zeiss Axioskop 2).

Carbon source utilisation of soil microbial communities (Papers II and III) were assessed using Biolog ECO (Biolog, 2000) and MT2 plates (Biolog Inc., Hayward). ECO plates contain 30 carbon sources with three replicas, redox dye and buffered nutrient medium, while MT2 plates contain only the redox dye and buffered nutrient medium. None of the Biolog ECO plate carbon sources are contaminant relevant, and thus, creating a method to account for contaminants was seen necessary. The MT2 plate assay can only characterize soil microbiota capable of utilising volatile hydrocarbons. This fraction disappears fast (Chaineau et al. and Paper I). The use of less-volatile hydrocarbons as substrate could better differentiate treatments. However, during method development, no growth was observed with diesel fuel applied directly to the MT2 plate wells or with diesel fuel dissolved in fast volatilising dichloromethane (Unpublished results).

A 90 µl aliquot of soil suspension was injected into each of the 96 wells of the plates. The utilisation of the volatile fraction of diesel fuel was used as an indicator of diesel fuel degradation. Diesel fuel was allowed to evaporate into a desiccator atmosphere and the Biolog MT2 plates without a lid were incubated in the closed desiccator, thus exposing the plates to the maximum vapour pressure of diesel fuel volatile compounds. Introduction of volatile carbon source to MT2 plates has been used to screen the potential of microbial isolates to degrade toluene, carbon tetrachloride and o-xylene (Strong-Gunderson and Palumbo, 1994). The incubation with diesel fuel

is shown in Figure 4. The suitability of the assay for hydrocarbons found in diesel fuel was tested with two known hydrocarbon degrading strains (*Pseudomonas oleovorans* possessing an alkane monooxygenase gene and *Pseudomonas putida* G7 possessing a naphthalene dioxygenase gene) and decane and naphthalene as carbon sources. However, *Pseudomonas oleovorans* could not grow in the MT2 plates on decane, although the colour development of soil suspension grown on decane was at the same magnitude as with diesel fuel and naphthalene. Furthermore, inoculation density of *Pseudomonas oleovorans* was similar to inoculation density of *Pseudomonas putida* G7 (Unpublished results). Thus, not all the bacteria may be able to grow in MT2 plates with volatile hydrocarbons. According to the producer of the diesel fuel, its water solubility is below 50 mg/l, but as diesel fuel is consumed, more is solubilised to achieve equilibrium state between water and the gaseous phase.

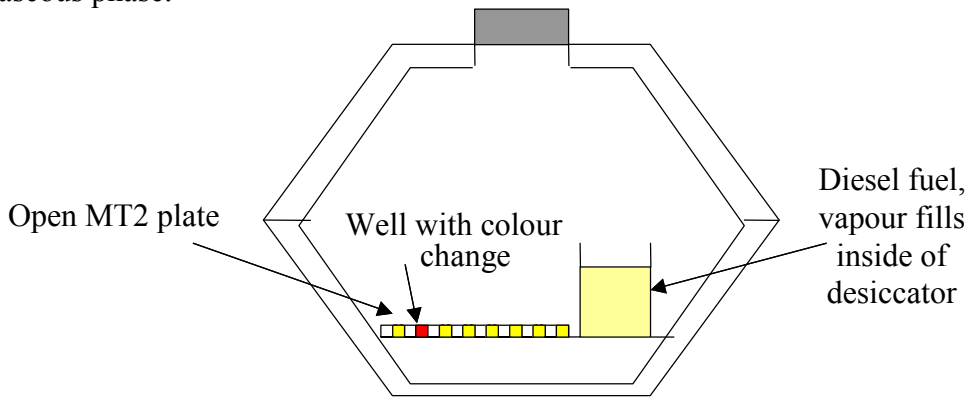


Figure 4. Experimental set-up of diesel fuel utilisation by soil micro-organisms.

ECO plates were incubated with closed lids and the sterility of the ECO plate batch was tested by inoculating several plates with autoclaved deionised water. MT2 control plates filled with soil suspension and sterile water controls were incubated in a desiccator without carbon sources. Both Biolog plate types were incubated at room temperature (20-22 °C) in the dark. ECO plates were measured until day 6. MT2 plates were measured at least for two weeks or until the absorbance reached 2.0 in some of the wells. Absorbance was measured at 590 nm with a Victor™ Multilabel reader or Multiskan Ascent FL. The values obtained with these two instruments were comparable, but the standard deviation was higher in Multiskan measurements (data not shown). The absorbances were statistically analysed (MATLAB Release 12) with principal component analysis (PCA) and analysis of variance. Inoculum densities of Biolog plates were not standardised with the average well colour development method (Garland and Mills, 1991), because this standardisation method decreases inter-sample distances in PCA (Howard, 1997). Statistical analyses of absorbances were carried out at 2 and 3 day incubations. Natural logarithms of MT2 plate absorbance divided by MT2 plate absorbance at time zero in a time series were used to calculate the lag time (λ), asymptote (A) and maximum specific diesel utilisation rate (μ_m). A modified Gompertz equation $y = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t)+1\right]\right\}$ (Zwietering et al., 1990) and nonlinear least squares data fitting in MATLAB Release 12 by the Gaussian-Newton algorithm with Levenberg-Marquardt modifications for global convergence were used in the data analysis.

2-(4-iodophyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2-H-tetrazolium monosodium salt (WST-1) has been used to detect microbial growth on PAHs together with an additional substrate (Johnsen et al., 2002). However, in our assay the PAHs act as the sole source of carbon, like in the microplate assay of Stieber et al. (1994), who measured the absorbance increase in PAH microplates incubated with dilutions of soil or mixed bacterial culture. Since PAHs are not as volatile as diesel fuel, the PAHs were applied directly to the multiwell plates. The chosen PAHs (anthracene, fluorene, naphthalene and phenanthrene) were dissolved (1000 mg L^{-1}) in n-hexane and added to 96-well plates with

following final concentrations: 100, 50, 25 and 5 mg L^{-1}. Eight replicate wells were used for each concentration. The same volumes of pure hexane were added to control wells. The PAH solutions and hexane were allowed to evaporate under filtered N_2 stream in a desiccator before the plates were filled. The evaporation resulted in the formation of PAH crystals on the surface of the wells. A solution of 1-methoxy-5-methylphenazinium methyl sulphate (Gerbu Biochemicals, Germany), 0.1 mmol L^{-1}, and WST-1, 1 mmol L^{-1}, was prepared in autoclaved deionised water. It was added in 10 µL aliquots to each of the wells, except the corner wells to minimise edge effects. Contaminated soil was removed from columns or incubation bottles and mixed. Aliquot of each soil sample (1 g) was added to 50 mL of mineral medium at pH of 7.9. Soil suspension (190 µL) was added to each well, including the corner wells, and the plate was shaken prior to the first measurement. Absorbance at 450 nm was measured with a multiwell plate reader (Multiskan, Thermo Labsystems) periodically up to one month. The highest specific growth rates for each PAH concentration were calculated from specific growth rates determined during one week of incubation. Specific growth rate was determined as ln(absorbance at time t) subtracted by ln (absorbance at previous measurement time) and the difference was divided by time elapsed between two measurements. Specific growth rates derived from background absorbance of hexane and soil suspension itself in the control wells were calculated in addition to growth rates obtained from utilisation of PAHs. The specific growth rate of control was subtracted from that of PAH containing soil suspension to confirm that growth was caused by utilisation of PAH only. A preliminary experiment testing redox-indicators showed that the redox reaction measured by absorbance increase (450 nm) at pH 7.9, close to optimum of WST-1, was much higher than that at pH 5.4, close to soil natural pH and that the redox reaction with WST-1 was faster than using Biolog MT2 plates with another tetrazolium salt (Unpublished results).

The stock and working solutions of enzyme substrates and the model fluorogenic molecules, 4-methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (AMC), were prepared in dimethyl sulfoxide, due to the low water solubility of the compounds. The activity of 14 microbial extracellular enzymes taking part in the hydrolysis of C, N, and P compounds was determined in a 96-well assay to determine the effects of remediation on microbial metabolic activity in soil. The enzyme substrates and MUF and AMC were purchased from Sigma-Aldrich. The substrate concentrations are shown in Table 13. Standards of MUF and AMC (0.001 to 50 µM) were prepared in duplicate in soil suspensions i.e. standard curves were prepared separately for each sample. Soil suspension density and its effect on autofluorescence were evaluated for the different types of soil. Soil suspension 1:100 (mass to volume ratio) was chosen for fresh diesel fuel contaminated soil, Papers II, and aged hydrocarbon contaminated soil (Paper III), while soil suspension 1:50 was used for creosote contaminated soil (Papers IV and V).

Table 13. Enzymes, substrates and the substrate concentrations (μM) used in the experiments (Papers II to V).

Enzyme	Fluorogenic substrate	Substrate concentration	
		Papers II, III and IV	Paper V
Phosphatemonoesterase	4-MUF phosphate, free acid	0.5…200	0.5…500
Butyrate-Esterase	4-MUF butyrate	1…500	10…2000
Acetate-Esterase	4-MUF acetate	2…1000	10…2000
Arylsulphatase	4-MUF-sulfate	0.5…200	
α-Glucosidase	4-MUF a glucoside	1…500	
β-Glucosidase	4-MUF b glucoside	1…500	
β-Xylosidase	4-MUF b xyloside	0.5…200	
Stearate-esterase	4-MUF stearate	1…500	
Leucine aminopeptidase	L-leucine-7-AMC	1…500	
Alanine aminopeptidase	L-alanine-7-AMC	1…500	1…500
Serine aminopeptidase	L-Serine-AMC	1…500	
Cellulase	4-MUF-cellobiose	0.5…200	
Chitinase	MUF-β-D-N-acetyl-glucosamide	0.5…200	
Galactosidase	4-MUF-β-galactose	1…500	
Lipase	4-MUF-palmiate	1…500	

The fluorescence of the hydrolysed model substrates and standards was measured at excitation 355 nm/ emission 460 nm with a fluorometer (Victor™ Multilabel reader or Fluoroskan Ascent FL), immediately after the addition of soil suspension and at least once during incubation. The rates of hydrolysed enzyme substrate [μM MUF/h/g] or [μM AMC/h/g] at 3 h incubation time were calculated for dry soil to enable comparison of different soil samples. The potential maximum rate of microbial extracellular enzyme hydrolysis is referred in text as v_{max}. The term potential is used in this context due to the interference of the soil matrix to the assay. The v_{max} and K_m values were calculated from Michaelis-Menten plots with SigmaPlot™ Enzyme Kinetics Package 1.1.

3.3.3 Toxicity analyses

The analyses of soil, leachate and plant phytotoxicity carried out in this thesis are summarised in Table 12. The analyses of toxicity to *Vibrio fischeri* in Paper III was made at Pirkanmaa Regional Environment Centre and analyses of toxicity to *Enchytraeus albidus* (Paper III) were made at Finnish Environment Institute. Results of plant phytotoxicity symptoms and plant biomass (Papers I and III) are not in included in the scope of this thesis summary.

Table 12. Summary of the soil, leachate and plants toxicity analyses carried out in this study.

Parameter	Sample	Method	Reference and in which Paper(s) used
Toxicity to *Vibrio fischeri*	Soil and column leachate	Short term incubation of samples diluted in 2 % NaCl, luminescence measurement after addition of Vibrio fischeri	Paper IV; Papers IV and V, modified from Lappalainen et al., 1999
Toxicity to *Enchytraeus albidus*	Soil	*Enchytraeus albidus* survival test	ISO, 2000b; OECD, 1984; Paper III
Needle length	Pine	Direct measurement	Paper I
Leaf length	Poplar	Direct measurement	Paper I
Yearly growth	Pine	Direct measurement	Paper I; Papers I and III
Visual appearance of plants	Pine and Poplar	Follow-up of possible chlorosis, necrosis, burns and abnormal growth	Paper I; Papers I and III
Plant dry matter	Grass and legume	Heating at 40°C for 12 h	Paper I

Toxicity of samples to *Vibrio fischeri* was determined using a short term incubation assay (flash kinetics) modified from Lappalainen et al. (2001), where the soil was in direct contact with *Vibrio fischeri* (Papers IV and V). Having the solid phase incuded in the assay (Papers III, IV and V), is better than using extracts, since mixing time influences the assay (Aicheson et al., 2004). On the other hand, soil particles may affect solid phase *Vibrio fischeri* assay, since luminescent bacteria may adhere to fine particles (Aicheson et. al. 2004. The assay accounts for the color and turbidity caused by soil particulate matter. Prior to measurement, soil and leachate were diluted with 2% NaCl solution. The pH of the final solutions was adjusted to 6-8 with 1 M NaOH. The assay was performed in 96-well plates and 150 µl of each sample dilution were added to duplicate wells. NaCl solution (2%, 150 µl) was added to 4 control wells. Lyophilised *Vibrio fischeri* was rehydrated according to Biotox™ kit instructions (BO1243-500, Aboatox). Luminometer (Fluoroskan Ascent FL, Thermo Labsystems) was used to dispense 150 µl of *Vibrio fischeri* solution to a well and to measure simultaneously the luminescence intensity in the well. The kinetic measurement, at 40 ms intervals up to 1000 measurements, was continued for 40 s and each well was measured, again, after 30 min for 1 min. Inhibition percentages (INH%) were calculated using the following formula:

$INH\% = 100 - 100 \times \frac{IT_t \times IC_0}{IT_0 \times IC_t}$, where IC_t is the luminescence intensity of the control sample after contact time t, IC_0 the maximum luminescence intensity of control sample, IT_t the luminescence intensity of the test sample after contact time t and IT_0 the maximum luminescence intensity of the test sample. EC_{50} values, i.e the dilution causing 50 % inhibition to *Vibrio fischeri*, were calculated by plotting inhibition percentages against dilution. Figure 5 shows luminescence curves (40 s) of control sample (Vibrio fischeri in 2 % NaCl) and soil suspension diluted in 2 NaCl. The lower curve (soil suspension) shows decrease of luminescence caused by soil toxicity to *Vibrio fischeri*.

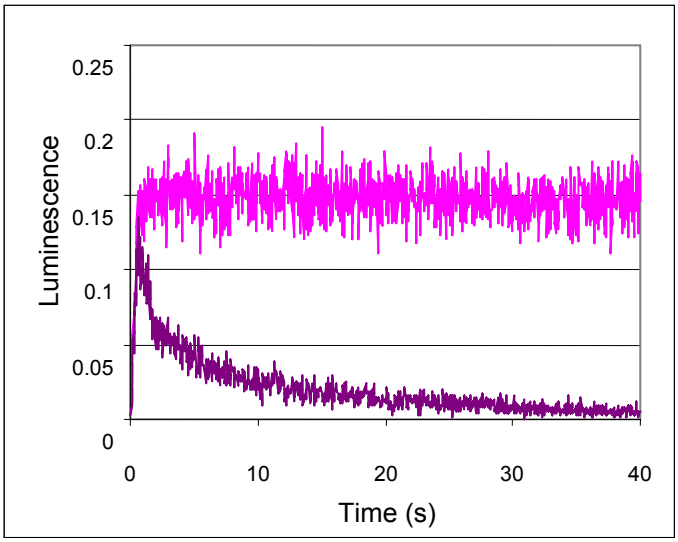


Figure 5. Luminescence of control (upper curve, 2 % NaCl and *Vibrio fischeri*) and soil suspension diluted in 2 % NaCl (lower curve).

The reproducibility of this assay and comparability to the conventional *Vibrio fischeri* luminescence inhibition assay were studied with determining toxicity of 3,5-dichlorophenol solutions. The standard deviation of INH% was low, but the toxicity to *Vibrio fischeri* was lower than toxicity to *Vibrio fischeri* in a conventional *Vibrio fischeri* luminescence inhibition assay (Unpublished results).

4 Results and discussion

4.1 Removal of hydrocarbons

In the phytoremediation study of Paper I with fresh diesel fuel contamination, most of the diesel fuel was readily removed from the soil with and without the presence of plants (Table 14). The presence of grasses did not increase diesel fuel removal compared with the unvegetated treatment, but in the other vegetative treatments the removal was statistically significant. Short-chained aliphatic hydrocarbons, nonane to dodecane, were removed faster than unresolved complex mixture, some of which could be extracted one year after the spill (Paper I). According to Chaîneau et. al. (1995), short-chained hydrocarbons below C14 (e.g. tetradecane) are lost by evaporation within a few days. Linear alkanes are easily biodegradable (e.g. Atlas, 1981).

Table 14. Hydrocarbon removal and its standard deviation (%) in the plant treatments during the first and second diesel fuel application in the laboratory scale (Papers I and II).

Plant treatment	First year				Second Year		
	30	60 days	150 days	180 days	330 days	14 days	28 days
Poplar	60±11	60±13			96±6	68±23	85±10
Pine	50±19	37±35			93±10	79±11	59±28
Legume	52±9	69±9		98±1			
Grass	31±29	29±32	65±17	78±21			
No vegetation	52±22	51±10	49±18	73±8			

The pine and poplar treatments were continued, since the roots of trees can reach more soil than the legumes studied and trees are perennial plants. The studied legume and grasses are annual plants and thus could not be monitored for more than 180 days. After the second application of fresh diesel fuel to the trees, diesel was removed considerably faster in poplar vegetated soil than in the first application. Thus, biodegration may have been enhanced in the second spill compared to the first year results, or the diesel fuel may have been volatilized more than in the first addition, since the fuel was applied on the surface of the soil instead of being mixed with it. Hydrocarbon removal at different treatment times varied (Table 14), since it was difficult to produce uniformly mixed artificially contaminated soil. Heterogeneity of contaminant concentrations can be decreased by adding a solvent with the contaminant, but that influences soil microbiota (Brinch et al., 2002). The soil was was irrigated twice a week at a moderate level, so that no leachate was produced. However, the amount of water received by the treatments varied, since leachate production was avoided. This may have impacted diesel fuel removal, since the addition of water increases the availability of contaminants.

Figure 6 shows the initial hydrocarbon concentration and the hydrocarbon concentrations at different sectors of the field site during the fourth growing season (Paper III). In unfertilised soil, hydrocarbon concentrations did not decrease significantly (Paper III). This sector also had the lowest density of vegetation (Paper III). The target values of hydrocarbons in Finland, legislation of spring 2006, vary depending on the number of carbon atoms (Anon, 2006). The hydrocarbons of the soil studied in Paper III belong to classes C10 to C20 and C21 to C40, which have upper target values of 1000 and 2000 (mg/kg) and lower target values of 300 and 600 (mg/kg), respectively. In paper III, none of the treatments met the Finnish target values for soil (Anon, 2006) during the four growing seasons studied. However, phytoremediation of fresh hydrocarbons takes less than one year to reach target levels (e.g Paper I; Guenther et al. 1996).The treatments with composted biowaste had the lowest hydrocarbon concentrations, but part of this is explained by the diluting effect of the compost supplementation, which is taken into consideration in Figure 6. The soil volume of the two quarters that received biowaste increased by 20 %. The greater vegetative coverage as a result of compost structure and nutrients provided by the compost could explain the higher mineral oils removal. Both NPK fertiliser and biowaste compost addition increased

hydrocarbon removal compared to that of unfertilised soil. The presence of pine and poplar in one of the compost-amended sectors did not enhance hydrocarbon removal significantly compared to that in the sector vegetated with grasses and clover. Hutchison et al. (2001a and 2001b) have shown that frequent irrigation and nutrient addition during phytoremediation can enhance the removal of aged hydrocarbons. However, in Paper I, fertilisation was not shown to increase the removal of fresh diesel fuel. Different hydrocarbon removal results may be due to soil and hydrocarbon properties.

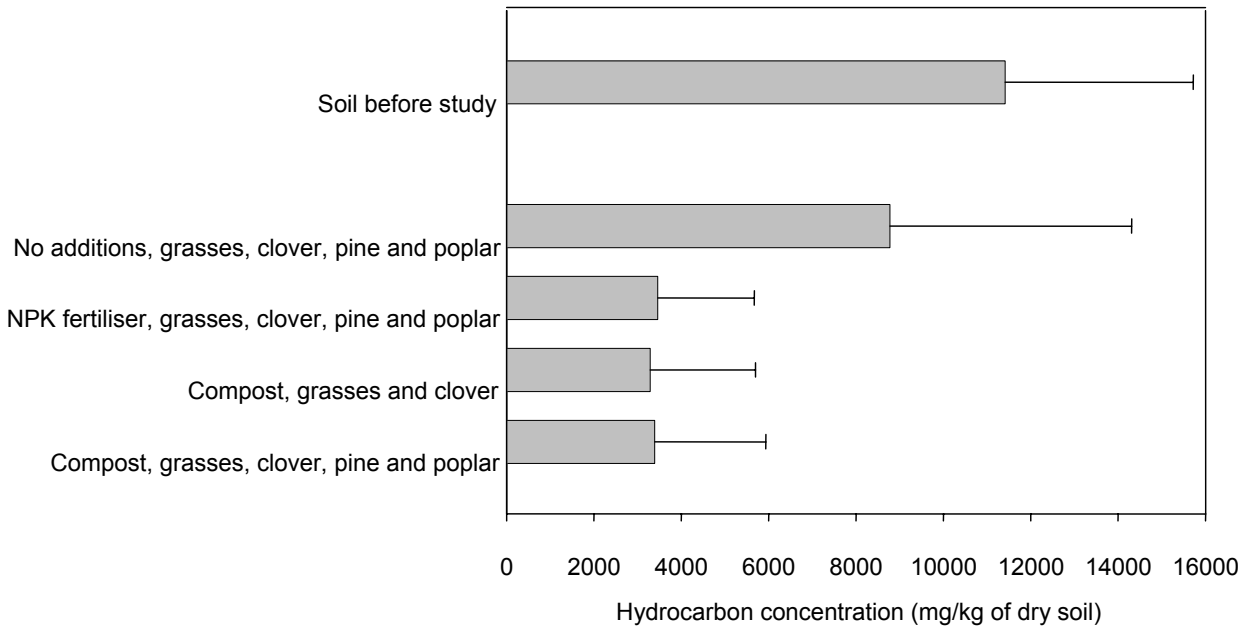


Figure 6. Average and standard deviation of hydrocarbon concentration in the soil before the study and hydrocarbon concentrations in the four phytoremediation treatment during the fourth growing season (Paper III). The hydrocarbon concentrations in the compost-amended treatments have been corrected to take into account the volume increase by biowaste compost (20%).

The transfer of hydrocarbons into plants must be taken into consideration when following hydrocarbon removal from soil, since plant aerial parts may be eaten by animals. According to the measurements of Paper I and a model developed by Trapp et al. (1990), hydrocarbons can be accumulated into roots. However, accumulation of hydrocarbons into plant aerial parts was not studied in this thesis. Results of Chaineau et al. (1997) showed no uptake of diesel fuel into roots of sunflower, maize, wheat, barley, bean, lettuce and clover at diesel fuel concentrations similar to those reported in Papers I, II and III. However, in heavily contaminated soil, plants took up hydrocarbons into their aerial parts and died of phytotoxicity symptoms (Chaineau et al., 1997). Uptake of hydrocarbons into plant aerial parts was not probable in Papers I, II and III, since the relatively high k_{ow} values of the studied hydrocarbons favour adsorption to roots (Schnoor et al., 1995). Accumulation into plant roots does not increase risks associated with contaminated soil. Plants themselves produce hydrocarbons, mainly long chained aliphatic compounds, which make the identification of petroleum hydrocarbons from plant tissue difficult (Chaineau et al., 1997). These compounds were also found in plant tissue extracts of clovers, pine and poplar (Unpublished results).

Since phytoremediation was not able to remove substantial concentrations of aged hydrocarbons, chemical oxidation with modified Fenton's treatment was attempted (Papers IV and V). For these studies, an aged creosote oil contaminated soil was chosen instead of aged petroleum hydrocarbons,

since the removal of petroleum hydrocarbons by Fenton's reaction has been studied extensively (Watts et al., 2000). Petroleum hydrocarbons studied include BTEX (Kang and Hua, 2005; Watts et al., 2000); diesel fuel (Watts and Dilly, 1996; Goi et al. 2006) and crude oil (Millioli et al., 2003). Furthermore, the studied petroleum hydrocarbons (diesel fuel in Papers I and II, and aged hydrocarbons in Paper III) both contained polyaromatic hydrocarbons and, according to Watts et al. (2000), the removal of aromatic hydrocarbons can be achieved with smaller dosage of H_2O_2 and iron than that of aliphatic hydrocarbons. Removal percentages during the combined and individual processes of modified Fenton's reaction and incubation are presented in Table 15. Chemical oxidation removed more PAHs than incubation of aged creosote contaminated soil. Removal of aged PAHs could be slightly increased by combination of modified Fenton's treatment and incubation (Papers IV and V) without the usually applied addition of bacteria (Table 5) after oxidation step, similarly to the studies of Goi et al. (2005b and 2006), where Fenton's treated and ozonated soil was incubated without addition of bacteria. However, part of the PAH removal during incubation maybe due to ongoing chemical oxidation, since residual H_2O_2 (Paper V) was left in soil and it reacted to form oxygen during incubation (Paper IV). On the other hand, increased oxygen concentration may have enhanced microbial activity and thus enhanced the PAH removal. Residual H_2O_2 was not removed from soil, since the purpose of the study was to simulate *in situ* remediation experiment. However, in future studies, soils with and without removal of H_2O_2 could be studied in parallel to determine the roles of chemical and biological oxidation.

Table 15. PAH removal percentage from creosote contaminated soil and its standard deviation in soil column treatment by modified Fenton's reaction (0.4 g H_2O_2/ g of soil) and soil incubation at 6 ºC (Paper V) and 20 ºC (Papers IV and V). Standard deviation was calculated as percentages of concentration in non-treated soil. Soil from columns was divided into upper and lower part samples.

Treatment	**Column treatment**			**Incubation time (weeks)**	**Temperature (ºC)**	**Removal (%)**
	H_2O_2 (%)	**Treatment time (days)**	**Sampled from soil column part**			
Modified Fenton's treatment	12	10	Upper	None		40±9
Modified Fenton's treatment	12	10	Lower	None		50±4
Modified Fenton's treatment	30	4	Upper	None		44±1
Modified Fenton's treatment	30	4	Lower	None		44±2
Modified Fenton's treatment	30	10	Upper	None		25±7
Modified Fenton's treatment	30	10	Lower	None		52±3
Incubation[1]	None			8-16	6	29±5
Incubation[1]	None			8-16	20	24±7
Combined treatment[2]	12	10	Upper	8-16	6	51±11
Combined treatment[2]	12	10	Lower	8-16	6	59±5
Combined treatment[2]	12	10	Upper	8-16	20	43±9
Combined treatment[2]	12	10	Lower	8-16	20	57±8
Combined treatment[2]	30	4	Upper	5-10	20	47±1
Combined treatment[2]	30	4	Lower	5-10	20	50±1
Combined treatment[2]	30	10	Upper	8-16	20	37±12
Combined treatment[2]	30	10	Lower	8-16	20	53±4

1 Soil was incubated to simulate intrinsic removal potential of PAHs 2 Modified Fenton's treatment followed by incubation to simuate intrinsic removal of PAHs

The incubation temperature did not influence the PAH removal. Reducing the initial H_2O_2 concentration from 30 % to 12 %, while maintaining the total H_2O_2 dose similar, had no significant effect on the PAH removal. Chemical oxidation was a suitable pre-treatment method for aged PAHs, but even in the combined chemical-biological treatment, the removal of PAHs was low, 50 to 60 %, compared with that of removal in slurry phase Fenton's treatment, where removal percentages of more than 90 % are achievable (Nam et al., 2001; Aunola et al., 2002). In slurries, however, mixing improves the mass transfer of contaminants. Increasing the total dose of H_2O_2 might enhance the removal of PAHs, but according to Goi (2005a) and Walling (1975), the increase of hydrogen peroxide dose may not increase the removal of contaminants, since hydroxyl radical production may be more efficient at lower concentrations of H_2O_2 in soil (Petigara et al., 2002). An explanation for the fact that the incubation in combined treatment only slightly increased the PAH removal compared with Fenton's treatment alone (Papers IV and V) could be that the modified Fenton's oxidation targeted the most available fraction of the aged PAHs. Also Cuypers et al (2000) found that the fraction of benzo(a)pyrene which could be removed by persulfate oxidation was

similar to the fraction removed by soil incubation and suggested persulfate oxidation as a method to estimate contaminant availability. Thus, similarity of fractions available for biological and chemical treatments may apply to soil oxidation in general. Nam et al. (2001) achieved higher PAH removal by performing biodegradation step prior to Fenton's reaction compared to that of Fenton's reaction before biodegradation. However, their study was performed with soil slurry at room temperature. Such practice demands excavation of soil prior to treatment and was thus not applied in Papers IV and V. One option to enhance PAH removal would be to integrate the modified Fenton's treatment to phytoremediation, since plants have enzymes capable of degrading PAHs (Edwards et al., 1982; Chroma et al., 2002). This strategy has been suggested by Li et al. (1997) for trinitrotoluene contaminated soil. However, phytotoxicity of the creosote contaminated soil was not studied and thus, the survival of plants in this soil cannot be guaranteed. Furthermore, the costs of combined treatment (see Tables 1 and 2) might be too high compared to off-site treatment.

Samples of polluted soil are often mixed to reduce heterogeneity of the soil, but this treatment creates bias. During mixing the soil is aerated, particles are broken, volatiles may be lost and the contact between substrate and micro-organisms may be enhanced. These changes may lead to improved removal of contaminants called priming effect (Joner et al. 2002, Kuzyakov et.al, 2000), which may well apply to the laboratory studies in this thesis. Thus, *in situ* removal of PAHs is likely to be less than the 21-34 % observed in soil incubation (Papers IV and V). By similar reasoning, *in situ* removal of diesel fuel by phytoremediation should be less than the ca. 90 % achieved by phytoremediation of fresh diesel fuel in the laboratory (Paper I).

When studying the composition of studied soil contaminants, removal of aliphatic and short-chained hydrocarbons could be seen from the chromatograms of aged hydrocarbon contaminated soil compared to those of lubrication oil and diesel fuel (Paper III). The original spills of hydrocarbons have most likely been diesel fuel and used lubrication oil, since the soil was contaminated by bus maintenance. In addition, the abundance of naphthalene was lower in aged PAH contaminated soil than in German creosote oil (Unpublished results, Figure 7). Thus, the composition of the studied hydrocarbons has changed from original spills, which indicates that intrinsic remediation is possible. Natural attenuation of the studied aged soils would propably take many years, since the contaminants, diesel and lubricants (Paper III) and creosote oil, were spilled decades ago, and the sites have not recovered fast.

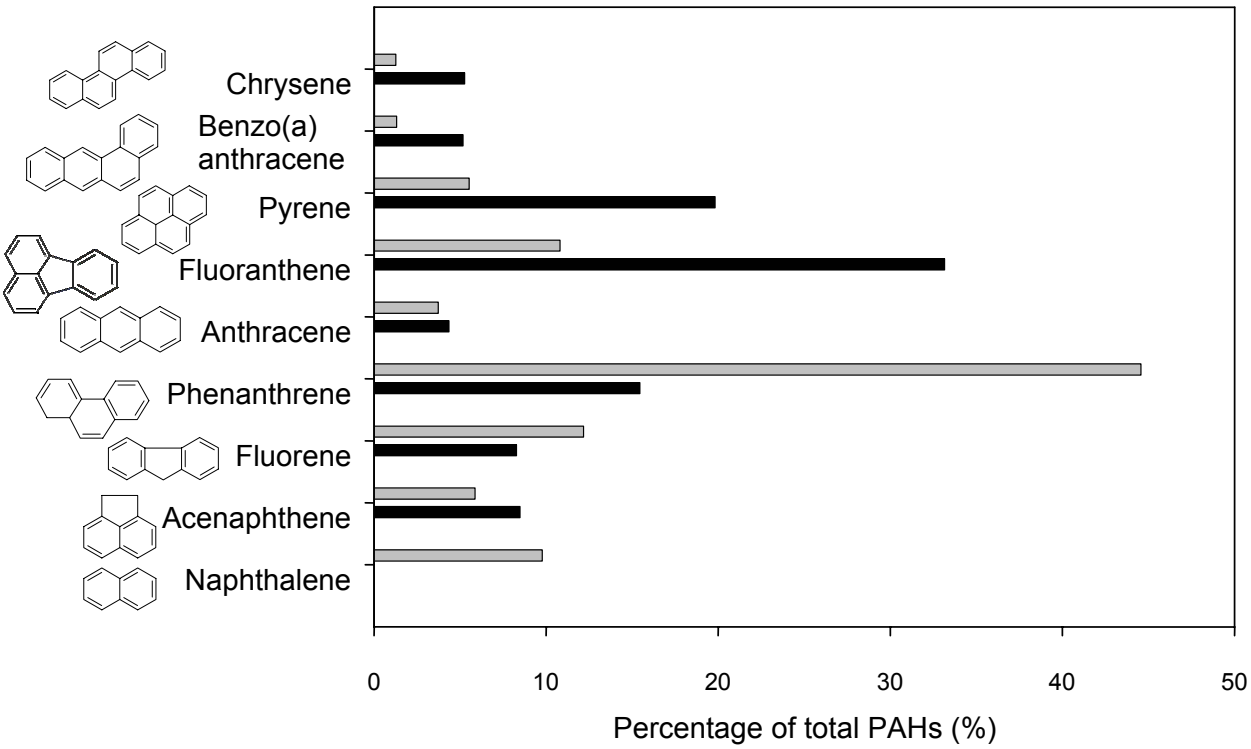


Figure 7. Molecular structures of studied PAHs and composition of PAHs as percentage (%) of total PAHs in studied soil (Paper IV, black bars) and German creosote oil (unpublished results, grey bars).

Each hydrocarbon extraction result depends on the particular extraction method it was produced with. This must be taken into consideration when comparing results obtained with different extraction methods (Weisman, 1998). When comparing removal of fresh (Papers I and II) to that of aged hydrocarbon (Papers III to V) contamination, the latter was more difficult with and without vegetation. This is most probably due to the most biodegradable hydrocarbons having been lost from the studied aged soils. E.g. no naphthalene could be extracted from creosote oil contaminated soil (Paper IV), although it is one of the major constituents of fresh creosote oil (Mueller et al., 1989). Furthermore, some of the fresh diesel fuel was probably lost by volatilisation (Figure 8, Papers I and II). In contrast, the aged contaminated soil did not contain volatile hydrocarbons (Figure 8, Paper III). Also the bioavailability of contaminants decreases during aging due to sorption to soil organic matter and minerals (Alexander, 2000). Since currently used extraction methods overestimate the amount of available toxic chemicals, milder extraction methods have been developed to evaluate the amount of bioavailable contaminants (Breeveld and Karlsen, 2000; Chung and Alexander, 1999; Hickman and Reed, 2005; Tang et al., 2002). However, milder extraction was not attempted in this study.

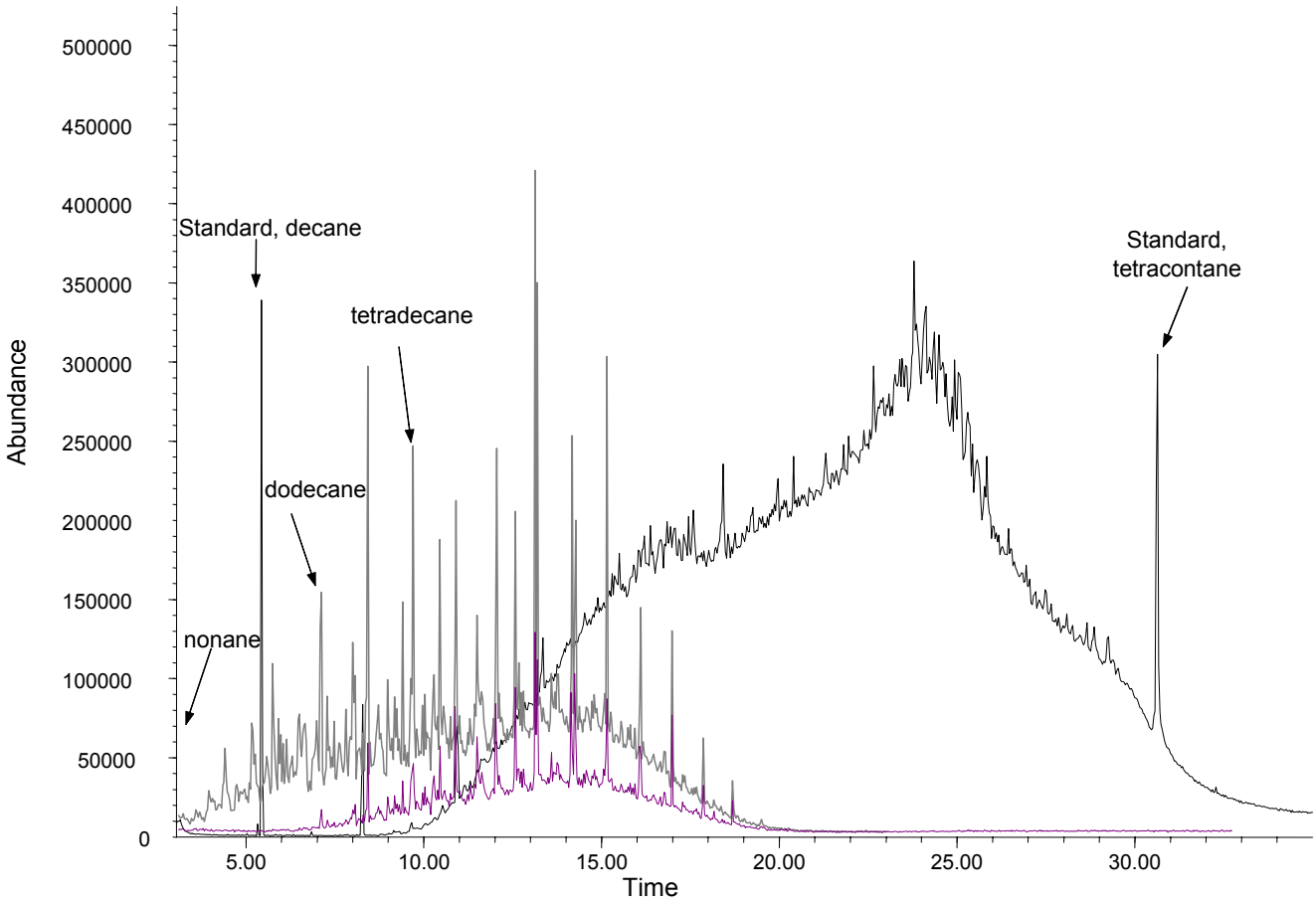


Figure 8. Chromatograms of soil extract from soil spiked with diesel fuel at time 0 (grey) and after one month (violet) and chromatogram of soil extract of weathered hydrocarbon contaminated soil (black.

The intrinsic remediation potential of soil was studied by incubating the soil (Papers I, II, IV and V) and following the hydrocarbon removal. The results of Papers I to V show that compared to natural attenuation, phytoremediation (Papers I to III) and modified Fenton's treatment can enhance the removal of hydrocarbons, although the removal was not significant in unfertilised soil undergoing phytoremediation (Paper III). However, as noted in Table 2, the final removal results of phytoremediation and natural attenuation can be in the same scale. Furthermore, the target hydrocarbons of chemical oxidation can be the most bioavailable ones (Cuypers et al., 2000). In many cases, natural attenuation should be combined with active remediation methods to reach the set of remedial goals in reasonable time (Salminen, 2005). However, obtaining representative samples from heterogeneous *in situ* soil can be a problem, which may even lead to a false interpretation of the efficiency of natural attenuation (Allard and Neilson, 1997) as well as possibly phytoremediation and chemical oxidation. According to Salminen (2005), monitoring hydrocarbon concentrations in spatially closely located soil samples is not a useful tool to follow the progress of monitored natural attenuation of boreal hydrocarbon contaminated soil, although hydrocarbon removal from the same soil was shown in lab-scale microcosms in near *in situ* conditions at 8 ºC (Salminen et al., 2004). One should be able to demonstrate that natural attenuation is occurring and that micro-organisms are active before choosing it as a sole remediation method.

4.2 Biological indicators of soil quality

4.2.1 Changes in microbial activity of soil

Changes in microbial activity during soil treatment were followed with analyses of soil respiration (Paper IV), carbon source utilisation by soil bacteria (Papers II, III and V), microbial extracellular enzymatic activities (Papers II, III, IV and V) as well as by counting viable and unviable cells (Papers IV and V).

4.2.2 Soil respiration

Concentrations of CO_2, CH_4 and O_2 were monitored during the incubation of modified Fenton's treated soil and non-treated soil to determine the levels of microbial activity in the soils and to evaluate the degradation of organic compounds. Excess volume was removed by gas-tight syringe, and its volume was recorded and used to calculate gas formation (Paper IV).

Table 16. Production and consumption of O_2 and CO_2 (nmol of gas/ g dry soil) during incubation of non-treated soil and soil with application of deionised water and modified Fenton's reaction.

Treatment	**Column treatment**			**Incubation time (weeks)**	**Consumption and production of O_2**	**Consumption and production of CO_2**
	H_2O_2 (%)	**Treatment time (days)**	**Sampled from soil column part**			
Incubation[1]	None			5-16	-49	38
Incubation[1]	None			8	-50	26
Incubation[1]	None			10	-13	18
Incubation[1]	None			16	nd	41
Combined treatment[2]	30	4	Upper	5	245	20
Combined treatment[2]	30	4	Lower	5	255	14
Combined treatment[2]	30	4	Upper	10	-8	2
Combined treatment[2]	30	4	Lower	10	12	1
Combined treatment[2]	30	10	Upper	8	72	5
Combined treatment[2]	30	10	Lower	8	-23	14
Combined treatment[2]	30	10	Upper	16	nd	3
Combined treatment[2]	30	10	Lower	16	-17	29

1 Soil was incubated to simulate intrinsic removal potential of PAHs 2 Modified Fenton's treatment followed by incubation to simuate intrinsic removal of PAHs, nd not determined

The results indicated that the incubation conditions were aerobic. Further, due to residual H_2O_2 (Paper V) decomposition, increase of oxygen concentrations above atmospheric concentration was observed in serum bottles with soil treated by modified Fenton's reaction. Consumption of CO_2 was detected in non-treated soil and soil treated by Fenton's reaction, although in the 4-day experiment with 30 % H_2O_2, O_2 consumption was lower in soil samples from the upper part of soil column. In all the serum bottles with non-treated soil and Fenton treated soil, formation of CO_2 was observed (Paper IV and unpublished results of experiment published in Paper V). CO_2 production was higher

in non-treated soil than in modified Fenton's treated soil. This indicates that microbial activity in non-treated soil was higher than in modified Fenton's treated soil, although also chemical oxidation may produce CO_2. The result is in accordance with the numbers of total and viable cell numbers determined in Papers IV and V. However, the removal of organic matter in the modified Fenton's treated soil (Paper V) may have also lowered CO_2 production.

4.2.3 Carbon source utilisation of soil bacteria

The number of utilised carbon sources in the Biolog ECO plate did not change as a result of vegetative treatment or hydrocarbon addition, once or twice (Paper II). Microbial communities in aged hydrocarbon contaminated soil were able to utilise most of the 31 carbon sources found in Biolog ECO plates, thus indicating a metabolically diverse soil microbial community despite the presence of both heavy metals and hydrocarbons (Paper III). Heavy metal contamination may, however, reduce the metabolic diversity (Dobler et al., 2000 and Rutgers et al. 1999). Also other studies with hydrocarbon contaminated soil have shown that carbon source utilisation in soil microbial communities is not affected by hydrocarbon contamination and that soil bacteria are metabolically diverse (Bundy et al.; 2002; Juck et al. 2000; Dobler et al., 2000). Microbial communities of creosote oil contaminated soil utilised 18 carbon sources of the Biolog ECO plates, which is considerably less than in the diesel fuel and aged hydrocarbon contaminated soil (Unpublished results). However, after modified Fenton's reaction, none of the carbon sources were utilised, even though plates were followed for one month (Unpublished results). This was assumed to be due to low inoculation density after chemical oxidation, since hydrogen peroxide and the radicals formed in Fenton' reaction are biocides (Büyüksönmez et al., 1998). The concentration of soil in the microplate wells cannot be increased, since it would make absorbance measurements impossible. Thus, the Biolog® method was left out from further experiments with modified Fenton's treated soil.

Principal component analysis of Biolog ECO plate carbon source utilisation indicated minor differences in microbiota in soil vegetated with pine compared to microbiota in soil vegetated with poplar in artificially contaminated soil. The microbiota in all soil samples, contaminated and uncontaminated, was metabolically diverse as indicated by the ECO plate tests. This was also the case for field site communities. Furthermore, tree rhizosphere and compost affected the carbon source utilization profiles of field communities (Papers II and III). Biolog ECO plates were not a suitable method for the follow-up of bioremediation of contaminated soil, since all the soils studied contained metabolically diverse microbial communities. Similar and contrasting results have been obtained in other studies with rhizosphere and contaminated soil, presented in Table 17.

Table 17. Biolog® plates utilised to follow microbial communities in contaminated or rhizosphere soil.

Contaminant	Plant	Type of utilised plate	Result	Note	Reference
Hydrocarbon contaminated soil	Scots pine	Biolog GN	Differences of substrate utilisation were linked to presence of pine roots	Also presence of mycorrhiza influenced carbon source utilisation	Heinonsalo et al. (2000)
None	Sweetpotato and soybean	Biolog GN	Substrate utilisation patterns depended on plant species	Quantity of root exudates affected substrate utilisation	Garland (1996)
Diesel	None	Biolog GN and 19 carbon sources relevant to hydrocarbon degradation	Soils from different sources yielded different utilisation patterns despite diesel contamination	With PLFA patterns similar result	Bundy et al. (2002)
Petroleum hydrocarbons originating from diesel and jet fuel	None	Biolog GN	Clustering patterns were based on geographic origin of samples, rather than contamination	Similar results with DGGE	Juck et al. (2000)
None	Maize	Biolog GN	Metabolic activity was different in distinct root zones and different from bulk soil	All Biolog GN carbon sources were not relevant to rhizosphere	Baudoin et al. (2001)
None	Herbaceous perennials	Biolog GN	Plant species number and amount of biomass did not correlate with carbon source utilisation	Biolog patterns did not correlate with FAME profiles, substrate-induced respiration correlated with plant productivity	Broughton and Gross (2000)
None	Corn, soybean, wheat, rye	Biolog GN	Rotation of species affected substrate utilisation	With PLFA patterns results were similar to those with Biolog patterns	Buyer and Drinkwater, (1997)
None	3 types of grassland vegetation from 9 sites	Biolog GN and 27 compounds found in plant root exudates	Microbial communities from different sites varied, but type of vegetation did not influence Biolog patterns		Campbell et al., (1997)
Hydrocarbons and metals, real and artificial contamination	None	Biolog GN and Biolog SF-N for fungi	Heavy metal contamination decreased metabolic diversity, hydrocarbon contaminated soil was metabolically diverse	Absorbance increase was slower in SF-N plates than in Biolog GN plates	Dobler et al., (2000)
Hydrocarbons, spiked 1.2 % or aged contaminated soil	None	Biolog GN	Changes in hydrocarbon content resulted in shift in carbon source utilisation	The shift correlated with the number of hydrocarbon degrading bacteria	Wünsche et. al., (1995)

In artificially contaminated soil, the utilisation of the volatile fraction of diesel fuel increased compared to the utilisation in uncontaminated soil. Community diesel fuel utilisation was highest in contaminated soil vegetated with trees (Paper II). The increased hydrocarbon utilization by trees was also seen with field communities (Paper III). Communities in tree rhizospheres had higher growth rates with volatile fraction of diesel in the MT2 plates than those in non-vegetated soil. In artificially spiked soil, the results obtained with diesel fuel utilisation assay were in accordance with the actual diesel removal (Paper II). The growth rates (Table 18) were similar with field samples (Paper III) as with laboratory samples (Paper II), indicating that the bacteria in weathered soil had maintained their ability to degrade diesel fuel, although GC/MS results indicated that short-chained hydrocarbons of diesel fuel hydrocarbon range had been removed from the soil (Paper III). However, the growth rate of a tested bacterial strain, *Pseudomonas putida* G7, on a pure chemical, naphthalene, was at same order of magnitude as those of soil suspensions from hydrocarbon contaminated soil (Unpublished results). This indicates that soil suspensions were not under carbon limitation. The microbial communities of aged hydrocarbon contaminated soil may not be able to degrade the remaining aged hydrocarbons, which are mostly present as unresolved complex matter in the gas chromatograms (Paper III). The diesel fuel utilisation method was useful in following the remediation of fresh diesel fuel contamination but not that of aged hydrocarbon contaminated soil.

Table 18. Maximum specific growth rates (μ_m, 1/h) of diesel fuel utilisation in Biolog MT2 plates inoculated with soil suspension. - Absorbance increase not statistically significant during incubation time.

Soil sample	**Average of μ_m (1/h)**	**Contamination**	**Source of soil**
no additions + trees, grasses and clover	$4.3 \cdot 10^{-4}$	Weathered hydrocarbons	Field plot
NPK fertilised+ trees, grasses and clover	$2.4 \cdot 10^{-3}$	Weathered hydrocarbons	Field plot
compost + trees, grasses and clover	$5.8 \cdot 10^{-3}$	Weathered hydrocarbons	Field plot
compost + grasses and clover	$2.7 \cdot 10^{-3}$	Weathered hydrocarbons	Field plot
Unvegetated soil	-	None	Forest
Unvegetated soil	$5.3 \cdot 10^{-3}$	Diesel spill, two weeks ago	Forest
Pine vegetated soil	$6.2 \cdot 10^{-3}$	Diesel spill one year ago	Forest
Pine vegetated soil	$7.1 \cdot 10^{-3}$	Two diesel spills, one year and two weeks ago	Forest
Poplar vegetated soil	$7.1 \cdot 10^{-3}$	Diesel spill one year ago	Forest
Poplar vegetated soil	$1.3 \cdot 10^{-2}$	Two diesel spills, one year and two weeks ago	Forest

Soil bacteria could utilise phenanthrene, naphthalene, anthracene and fluorene as carbon sources both in modified Fenton's treated and non-treated soil. PAH utilisation range and specific growth rates were in general lower in modified Fenton's treated soil (Paper V). Bioaugmentation may improve the degradation of contaminants after Fenton's reaction (Schrader and Hess, 2004), and problems may arise if the bacteria capable of degrading contaminants do not tolerate hydrogen peroxide (Fiorenza and Ward, 1997). Therefore, bioaugmentation has been applied in many of the previous studies of combined chemical-biological treatment (reviewed in Table 5). On the other hand, bioaugmentation is most successful in confined systems, such as bioreactors (El Fantroussi and Agathos, 2005). Soil respiration, as indicated by carbon dioxide production, took place even in soil treated with 30 % H_2O_2 (Paper IV) and PAH-degrading bacteria persisted in soil, thus eliminating the need to augment the soil with microbial inoculum or sludge.

4.2.4 Microbial extracellular enzymatic activities

Microbial extracellular enzyme (MEE) activities of soil microbial communities were assessed to study the effects of plants and contamination type on microbial activity (Papers II and III) and to study the effects of modified Fenton's reaction on microbial activity and estimate the release of nutrients during modified Fenton's reaction (Papers IV and V).

Phosphomonoesterase activity, indicated by v_{max}, was low in forest soil (Paper II), before and after the addition of diesel fuel and plants, and this could indicate that the soil bacteria were not undergoing phosphorus limitation. In contrast, v_{max} was high in aged hydrocarbon contaminated soil fertilised with compost (Paper III) at some sampling times, which indicates a phosphorus deficiency in soil. Thus, the addition of phosphorus might increase hydrocarbon degradation. Aminopeptidase and esterase activities, measured as v_{max}, were increased by soil fertilisation (Papers II and III) and diesel fuel addition (Paper II), although the latter did not cause long-term effects (Paper II). The minor impact of petroleum hydrocarbon removal on extracellular enzymatic activities (Papers II and III) might be due to the minor interactions of hydrophobic hydrocarbons with proteins in soil solution (Speir and Ross, 2002). However, the activities of dehydrogenase (Frankenberger and Johanson, 1982; Margesin et al., 2000, Margesin and Schinner, 1997b), lipase (Margesin et al., 1999 and Margesin et al. 2000a), catalase and urease (Margesin et al., 2000a) as well as phosphomonoesterase (Margesin et al., 2000b) have been shown to correlate with hydrocarbon content, but contradicting results have also been obtained (Margesin et al., 2000b; Guenther et al., 1996). Extracellular enzymatic activities of soil were more dependent on the type of soil than on hydrocarbon contamination (Papers II and III). Thus, general conclusions about the effects of hydrocarbon contamination and vegetation cannot be drawn from these Papers.

Aminopeptidase activities fluctuated after modified Fenton's reaction, but during incubation they returned to similar level as prior to the treatment (Papers IV and V). Phosphomonoesterase activity was high in creosote oil contaminated soil (Papers IV and V) but decreased after modified Fenton's reaction. Possible reasons for this are a higher availability of phosphorus containing compounds released during the oxidation of cells and inositol phosphates deriving from plants and decrease of microbial activity, which was also measured as microbial counts and soil respiration. With the addition of 30 % H_2O_2, acetate esterase activities were lower in chemically treated soil than in nontreated soil (Paper IV), but with 12 % H_2O_2 the esterase activities decreased only slightly (Paper V). Furthermore, with the addition of lower H_2O_2 concentration (12 %), esterase activities increased during incubation. Also other chemical oxidation methods, such as ozonation (Stehr et al., 2001) can decrease the enzymatic activities of PAH contaminated soil. However, although modified Fenton's treatment decreased the activities of extracellular esterases, it did not result in permanent changes to soil microbial enzymatic activities.

4.2.5 Changes in microbial counts

The microbial numbers were determined with viable counts (Paper II) and direct microbial counts (Paper V). In Paper II viable plate counts on soil extract agar and starch casein medium were followed to determine if culturable organisms are more abundant in vegetated or recently contaminated soil than in uncontaminated soil. However, no significant differences in viable counts were observed. In contrast, according to literature, viable counts are higher in rhizosphere soil (Anderson et al., 1993) also contaminated with hydrocarbons (Radwan et al., 1995; Komisar and Park, 1997). In petroleum contaminated soil, the numbers of colony-forming units may even be at the same scale as the numbers of total cells, since hydrocarbon degrading bacteria are in an active metabolic state (Becker, 1999). The viable counts of Paper II, 10^6 to 10^7 cfu/ g dry soil, were tenfold lower than those of oil-utilising micro-organisms observed by Radwan et al. (1995). This could be due to the type of agar used, since in this study we used soil extract agar prepared from

clean soil. Soil extract agar may allow growth of more bacteria than generally used media, but the bacteria enriched in hydrocarbon-contaminated soil may prefer other carbon sources than those found in original clean soil. However, unculturable hydrocarbon-degraders may also play an important role in hydrocarbon degradation (Zucchi et al., 2003).

The number of viable (intact cell membranes) and dead (disrupted cell walls) cells was determined from soil samples prior to modified Fenton's reaction, directly after treatment and after incubation in order to follow the effects of H_2O_2 addition (Figure 9, Papers IV and V). The addition of H_2O_2 solution to soil decreased both the number of all cells and the percentage of intact cells in soil (Paper V). Incubation increased the number of viable cells, but the numbers of all cells were still lower than in non-treated soil (Papers IV and V). However, the number of viable cells was higher in incubated soil treated with 12 % H_2O_2 (Paper V) than with 30 % H_2O_2 (Paper IV). This shows that bacteria could better survive and re-grow with lower H_2O_2 concentration (12 %). The inhibitory effects to bacterial cells are in accordance with the WST-1 microplate assay (Paper V), which showed inhibition to PAH utilising bacteria.

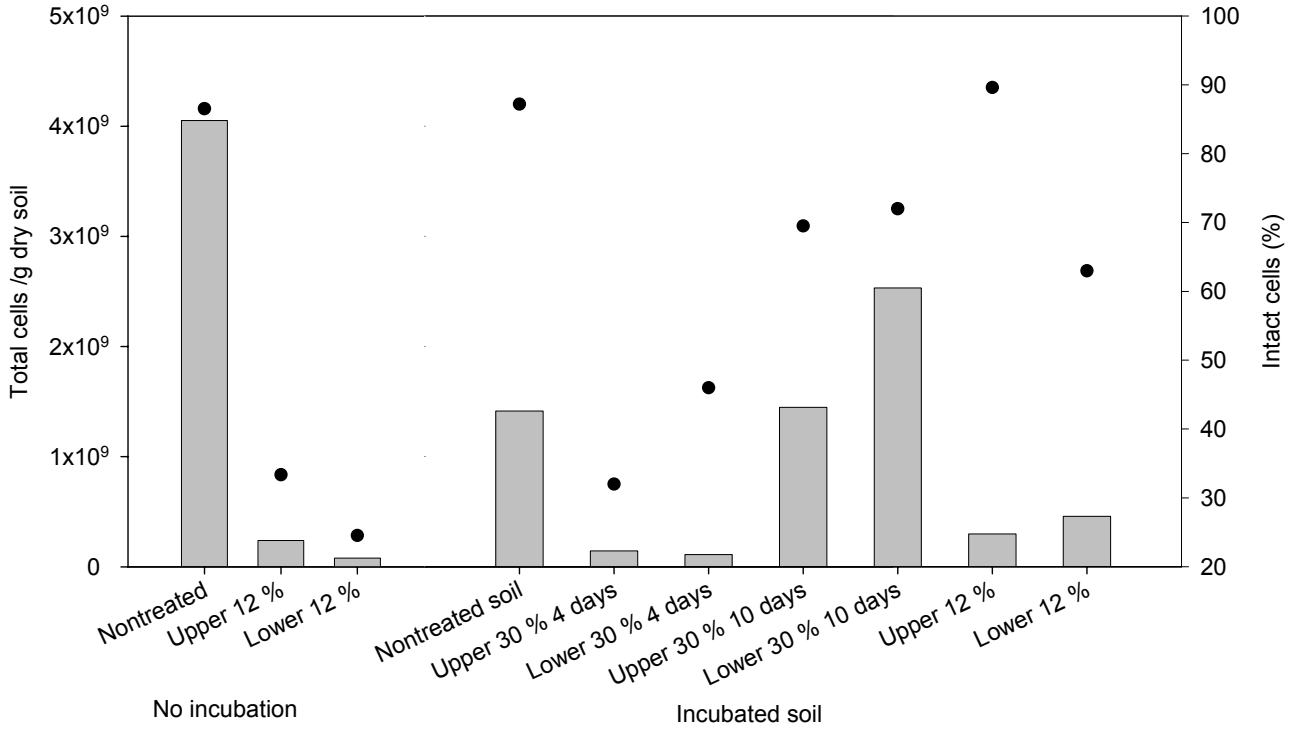


Figure 9. Numbers of total cells (bars) and percentage of viable cells (circles) in nontreated soil, modified Fenton's treated soil (upper and lower parts of soil columns) with H_2O_2 concentration of 12 or 30 % and treatment time of 4 or 10 days and during incubation simulating intrinsic removal of PAHs (Papers IV and V).

Microbial activities in oxidised soil have mostly been characterised by culture-based methods. Decrease in microbial numbers during Fenton's treatment has been found with heterotrophic plate counts (Miller et al., 1996; Schrader and Hess, 2004), but plate counts are known to underestimate the total number of bacteria (Stayley and Konopka, 1985) and thus we used bacterial staining in this study (Papers IV and V). Ahn et al. (2005) investigated the changes in petroleum hydrocarbon degradation potential after ozonation of diesel fuel contaminated soil and found that heterotrophic plate counts and counts of phenanthrene and hexadecane degraders decreased with increasing ozonation time. However, the number of colony forming units increased during soil incubation. Non-culture methods, whole cell hybridisation and slot blot hybridisation of DNA extracts, showed higher microbial abundance than culture based methods. The microbial community composition of incubated ozonated soil determined with whole cell hybridisation was slightly different from that of

unozonated soil. During the treatment of monochlorobenzene contaminated groundwater by Fenton's reaction, Bittkau et al. (2004) found that after 100 days incubation of groundwater, the total phospholipid fatty acid concentrations and the numbers of bacteria with intact cell membranes were higher in Fenton's treated groundwater than in the creosote oil contaminated control. However, phospholipid fatty acid composition indicated a less diverse community in Fenton's treated groundwater than in the control. Thus, microbial diversity may decrease as a result of modified Fenton's treatment, although microbial numbers can return to levels preceding chemical treatment. The H_2O_2 used and the radicals formed in the Fenton's reaction are biocides (Büyüksönmez et al., 1998) and, therefore, reduce the abundance of viable cells. Bacteria have been shown to adapt to treatment with 0.05 % H_2O_2 by increasing catalase and superoxide dismutase activity (Fiorenza and Ward, 1997). Most bacteria are present in biofilms, regardless of type of habitat (Costerton et al., 1995). Biofilm bacteria are more protected from environmental stress factors than free-floating bacteria. Results obtained with free-floating bacteria cannot be directly compared to biofilm bacteria, such as bacteria growing on soil surfaces. Biofilm bacteria have been shown to be more resistant to hydrogen peroxide than sessile bacteria (Hassett et al., 1999). Also PAH degrading bacteria are mostly found at soil particle surfaces (Amellal et al., 2001). Thus the relatively high numbers of viable bacteria after H_2O_2 treatment (Papers IV and V) are likely to result from the presence of bacteria attached to soil particles and organic matter.

4.3 Changes in toxicity

The toxicity of the aged hydrocarbon contaminated soils and their leachates to *Vibrio fischeri* was determined during the phytoremediation experiment (Paper III) and during the modified Fenton's treatment followed by incubation (Papers IV and V). *Vibrio fischeri* tests of acute toxicity have been used to estimate the quality of soil and sediment contaminated with PAHs (e.g. Aicheson et al. 2004; Mowat and Bundy, 2001), oil hydrocarbons (e. g. Salanitro et al., 1997, Dorn et al., 1998, Dorn and Salanitro, 2000; Plaza et al., 2005) and heavy metals (Mowat and Bundy, 2001), but *Vibrio fischeri* has been shown to be more sensitive to organic chemicals than metals (Vaajasaari, 2005).

The studied contaminated aged soils and their leachates resulted in different levels of toxicity to *Vibrio fischeri* (Table 19). The aged hydrocarbon contaminated soil and its leachate were only slightly toxic to *Vibrio fischeri,* and soil toxicity decreased slightly during the first year of phytoremediation experiment (Paper III). The aged creosote contaminated soil and its leachate were more toxic to *Vibrio fischeri* (Papers IV and V), especially after modified Fenton's treatment. With 30 % H_2O_2 (Paper IV) the toxicity of the modified Fenton's treated leachates was higher than with 12 % H_2O_2 (Paper V), but the volume of leachates was higher with 12 % H_2O_2 due to higher injection volumes (Table 9) and thus, the total toxicity might be of similar extent. Even after incubation, modified Fenton's treated soil was more toxic than non-treated soil (Papers IV and V), but with 12 % H_2O_2 the toxicity of soil reduced during incubation. The toxicity of soil from the control column, which received deionised water, remained unchanged during incubation and was similar to that of non-treated soil. The increased toxicity during modified Fenton's treatment may be due to the increased bioavailability of the PAHs, since water extract from fresh PAH contaminated soil is more toxic to *Vibrio fischeri* than water extract from aged soil with similar PAH concentrations (Weissenfels et al., 1992). Furthermore, toxic contaminant metabolites may have been formed during the treatment (Lundstedt, 2003), since the oxidized PAH metabolites have been shown to be more toxic to *Vibrio fischeri* than their parent compounds (El-Alawi et al., 2002). Furthermore, the released dissolved organic matter (Paper V) may have been toxic to *Vibrio fischeri*. However, PAH metabolites may also be more biodegradable than the parent compounds (Meulenberg et al., 1997; Lee and Hosomi, 2001). The toxicity of uncontaminated soil to *Vibrio fischeri* and the effect of modified Fenton's treatment on uncontaminated soil were not studied, since clean soil from the studied site could not be accessed. Bioremediation of PAH contaminated

soil has been shown to reduce the toxicity to *Vibrio fischeri* (Lau et al. 2003).

Table 19. Toxicity of soil and leachate to *Vibrio fischeri* (Papers III, IV and V). Numeric values of soil toxicity unpublished.

Type of sample	Origin	Treatment	Toxicity
Soil	Aged hydrocarbon contaminated soil (Paper III)[1]	Initial soil	29 %[a]
Soil	Aged hydrocarbon contaminated soil (Paper III)[1]	After first phytoremediation growing season[2]	6-26 %[b]
Soil	Aged creosote contaminated soil (Paper IV)	None	14±10 and 19±14[c]
Soil	Aged creosote contaminated soil (Paper IV)	Incubation[3]	14±7 and 22±25[c]
Soil	Aged creosote contaminated soil (Paper IV)	Modified Fenton's treatment H_2O_2 30 %, 4 days	2 and <4.5[c]
Soil	Aged creosote contaminated soil (Paper IV)	Modified Fenton's treatment H_2O_2 30 %, 10 days	<3.5 to 5 and <3.5 to 6[c]
Soil	Aged creosote contaminated soil (Paper V)	Modified Fenton's treatment H_2O_2 12 %, 10 days	<1 and <1[c]
Soil	Aged creosote contaminated soil (Paper IV)	Modified Fenton's treatment H_2O_2 30 %, 4 days + incubation[3]	3±9 and 3±20[c]
Soil	Aged creosote contaminated soil (Paper IV)	Modified Fenton's treatment H_2O_2 30 %, 10 days + incubation[3]	7±5 and 4±4[c]
Soil	Aged creosote contaminated soil (Paper V)	Modified Fenton's treatment H_2O_2 12 %, 10 days + incubation[3]	11±9 and 14±14[c]
Soil	Aged creosote contaminated soil (Paper IV)	Deionised water for 10 days	16±4 15±6[c]
Leachate	Aged hydrocarbon contaminated soil (Paper III)	First phytoremediation growing season[2]	Not toxic[d]
Leachate	Aged creosote contaminated soil (Paper IV)	Modified Fenton's treatment H_2O_2, 30 %, 4 days	< 0.3 and < 0.3[e]
Leachate	Aged creosote contaminated soil (Paper IV)	Modified Fenton's treatment H_2O_2 30 %, 10 days	< 6.4 and < 0.3[e]
Leachate	Aged creosote contaminated soil (Paper V)	Modified Fenton's treatment H_2O_2 12 %, 10 days	14±12 and 13±13[e]
Leachate	Aged creosote contaminated soil (Paper V)	Deionised water for 10 days	12±5 and 6±2[e]

[1]Aged hydrocarbon contaminated soil from bus depot [2]Aged hydrocarbon contaminated soil treated with phytoremediation, treatments as shown in Figure 2. [3]Soil was incubated to simulate the intrinsic removal potential of PAHs [2]Modified Fenton's treatment followed by incubation to simuate intrinsic removal of PAHs
[a]inhibition percentages in 50 % soil suspension (1g soil in 4 ml 2 % NaCl) [b]Average of end of first growing season and first samples of second growing season [c]Concentration (g/l) of soil diluted in 2 % NaCl which caused 50 % inhibition to *Vibrio fischeri* [d]increase in *Vibrio fischeri* luminescence, undiluted leachate [e]Concentration (%) of leachate diluted in 2 % NaCl, in which inhibition to *Vibrio fischeri* 50 % in flash and 30 min tests

Earthworm tests have been shown to be more sensitive to oil contamination than luminescent bacteria tests (Dorn and Salanitro, 2000; Dorn et al., 1998). Enchytraeids are sensitive to contamination and changes in land use (Römbke, 2003) and were thus used to follow the toxicity of aged hydrocarbon contaminated soil in the phytoremediation experiment (Paper III). The number of living enchytraeids was similar in uncontaminated soil and contaminated soil at the test concentrations 0, 12.5, 25, 75 and 100% (percentage of contaminated soil). Thus, the tested soil samples were non-toxic to *Enchytraeus albidus* (Paper III). Enchytraeids have been shown to be sensitive to crude oil at concentrations 3000 to 10000 mg/kg soil (Filiminova and Pokarzhevskii, 2000). Concentrations of weathered hydrocarbons in studied soil (Paper III) were at the same level or higher; but the enchytraeids survived well. The *Enchytraeus albidus* reproduction test has been found to be more sensitive than *Enchytraeus albidus* survival test for toxicity assessment of oily waste (Juvonen *et al.,* 2000); therefore, it may be preferable for ecotoxicological monitoring.

4.4 Suitability of the studied indicators for monitoring of remediation

The following order of assays applied in this thesis is recommended when selecting a biological indicator to monitor the remediation process:

1. Assays to follow the utilisation of contaminant
2. Indicator of soil toxicity
3. MEE activities
4. Soil respiration
5. Assays of microbial numbers with microscopy
6. Utilisation of carbon sources in the Biolog ECO plates
7. Determination of culturable bacteria

Assays to follow the utilisation of contaminant with Biolog MT2 and WST-1 plates were found to be useful in estimating the activity of degrading micro-organisms (Papers II and V). However, the method to follow the utilisation of contaminant must be designed to suit the properties of the contaminant. For example, water soluble chemicals can be added directly to microplates, and the effect of their concentration can be studied (Fulthorpe and Allen, 1994).

Ecotoxicity indicators are useful in monitoring remediation process. However, the initial toxicity of the soil must be determined with several indicators, since the soil may not be toxic to some indicators in the beginning of remediation (for example *Vibrio fischeri* and *Enchytraeus albidus* in Paper III). Such toxicity indicators are not useful in the follow-up of remediation, although they may indicate that soil has low ecotoxicity despite high contaminant concentrations present, and risk assessment may prove remediation unnecessary. However, the same toxicity test with *Vibrio fischeri* was suitable to follow the remediation of aged hydrocarbon contaminated soil (Papers IV and V).

The incubation time of Biolog ECO plates, 2 to 3 days, and the high substrate concentrations allow microbial growth and induction of genes coding for utilisation of the 31 different organic substrates. Enzyme assays are faster and report enzymatic activities that correspond more to *in situ* field conditions. Furthermore, soil enzyme activity analysis provides information about the biocatalysis cleaving different chemical bonds of biopolymers, while the Biolog® method measures the activity of the organic substrate utilisation of soil bacteria that can tolerate high substrate concentrations. However, besides contamination and oxidative treatments, the soil properties, soil tillage (Kandeler et al., 1999) and fertilisation (Marcote et al., 2001) as well as soil moisture levels (Hinojosa et al., 2004) influence enzymatic activities, including lipase activity (Margesin et al., 2000a and 2000b). Also Biolog patterns may vary mainly within soil geographic origin (Campbell et al., 1997; Juck et al., 2000). Thus, comparisons of enzymatic activity or carbon source utilisation patterns can be made only within one experiment or studied site. According to Trasar-Cepeda et al. (2000) information on enzyme activities can be useful in quantifying degradation in soil, provided that it is supplemented with information on other soil biochemical parameters, such as soil N and C contents.

Microbial numbers in soil determined with microscopy give an estimate of total microbial activity, while methods based on culturing cannot predict total bacterial numbers. However, the culturability can give an estimate of the metabolic state (Bååth, 1994) and size (Bakken and Olsen, 1987). In the remediation studies described in this thesis, microbial numbers obtained with microscopy (Papers IV and V) were more important parameter than heterotrophic plate counts obtained with culturing (Paper II). Soil respiration measurements can also be used to estimate the quantities of microbial biomass and are easy to perform compared to analysing microbial counts. However, soil respiratory activity does not necessarily reflect the numbers of micro-organisms capable of degrading the contaminants present in the soil matrix.

General physiological activity tests, such as carbon source utilisation, enzymatic activity and microbial biomass assays, are secondary to contaminant biodegradation assays when assessing bioremediation potential. Thus, general microbial activity assays alone should not be used to compare bioremediation treatments. However, at least one of the methods to follow general metabolic activity and at least one method to follow soil toxicity should be used in combination with assays of contaminant utilisation. This would allow estimating changes to soil microbial activity during the remediation process in order to make sure that the remediation does not cause adverse effects on microbiota.

5 Conclusions

Both phytoremediation (Papers I, II, III) and modified Fenton's reaction combined to incubation (Papers IV and V) were shown to enhance the removal of hydrocarbons compared to soil incubation alone. However, the performance of these *in situ* remediation processes was case-dependent.

Of the plant species studied, the presence of white clover and green pea, pine and poplar enhanced the removal of fresh diesel fuel from artificially spiked soil (Paper I), but the same plant species did not increase the removal of aged hydrocarbons (Paper III). Diesel fuel removal was faster in soil that had been contaminated with diesel fuel earlier (Paper II) than in previously uncontaminated soil (Paper I). Phytoremediation can be recommended to remove fresh diesel fuel contamination, but aged hydrocarbons require other treatment methods.

The combined use of modified Fenton's reagent and biological treatment improved PAH removal from aged creosote oil contaminated soil compared to biological treatment alone (Papers IV and V). However, part of the removal during incubation is due to oxidation by residual H_2O_2 (Paper V). PAH removal was similar, regardless the initial hydrogen peroxide concentration applied (12 % or 30 %, Papers IV and V).

Changes in microbial activity during phytoremediation, measured as carbon source utilisation patterns and extracellular enzymatic activities, were not permanent (Paper II). Bacteria in uncontaminated soil could not utilise diesel fuel in MT2 plate assay (Paper II). MT2 plate assays indicated that trees increased the diesel utilization rates of both laboratory and field site microbial communities. The activities of extracellular hydrolytic enzymes were more dependent on the soil type and fertilisation than on the hydrocarbon contamination (Papers II and III).

The toxicity of aged hydrocarbon contaminated soil to *Vibrio fischeri* and *Enchytraeus albidus* was low (Paper III) and thus not a sensitive indicator to monitor the progress of remediation of this soil. Aged creosote contaminated soil and its leachate were more toxic to *Vibrio fischeri* (Papers IV and V) than aged hydrocarbon contaminated soil (Paper III), especially after modified Fenton's treatment. Even after incubation, modified Fenton's treated soil was more toxic than non-treated soil (Papers IV and V), but with 12 % H_2O_2 the toxicity of soil reduced during incubation. Thus, hydrogen peroxide concentration of 12 % is recommended instead of 30 %.

Hydrogen peroxide addition increased the toxicity of soil and leachate from the soil columns. The percentage of intact bacterial cells decreased as a result of hydrogen peroxide addition (Paper V), but numbers of viable cells increased during incubation (Papers IV and V), especially with 12 % H_2O_2. This shows that bacteria could better survive and re-grow with lower H_2O_2 concentration (12 %). Bacteria maintained the ability to utilise PAHs as a carbon source after hydrogen peroxide addition at 12 % (Paper V).

Contaminant utilisation assays with Biolog MT2 (Papers II and III) and WST-1 microplate (Paper V) assays were suitable indicators to follow the progress of phytoremediation and modified Fenton's reaction combined to incubation. Suitability of toxicity indicators needs to be studied for each soil prior to using them to monitor remediation. Soil general metabolic activity and microbial number assays are secondary to contaminant utilisation assays in the monitoring of remediation process. However, they should be used to compare soil microbial activity before and after remediation.

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Appendix 1

Summary of the methods used, aims and results obtained in each Paper (I to V) in relation to the scope of this thesis.

Paper number and Title	Aims	Methods	Results
I: Phytoremediation of subarctic soil contaminated with diesel fuel	to study if diesel fuel removal depends on plant species, fertilisation or repeated exposure to diesel fuel to study if the plants used accumulate diesel fuel	Plants were grown in diesel fuel contaminated soil Diesel fuel concentrations in soil were analysed over time Diesel fuel concentration in the plant roots was determined at the end of the experiment	In presence of legumes, diesel fuel was removed faster than in other vegetated treatments. Vegetation with grasses did not enhance diesel removal compared to unvegetated soil Grass roots accumulated diesel fuel
II: Metabolic responses of microbiota to diesel fuel addition in vegetated soil.	to study the effects of repeated exposure of pine and poplar to diesel fuel to determine how phytoremediation influences microbial activities	Trees were grown in diesel fuel contaminated soil Diesel fuel concentrations in soil were analysed over time Microbial extracellular enzymatic activities, viable counts and carbon source utilisation of soil were determined	Diesel fuel removal faster after second diesel fuel spill than after first one (Paper I). Vegetation influenced carbon source utilisation in Biolog ECO plates. Microbial extracellular enzymatic activities were influenced by diesel fuel addition. Contaminated soil suspensions could utilise diesel fuel in MT2 plates faster than those of uncontaminated soil.
III: Phytoremediation of soil contaminated with weathered hydrocarbons and heavy metals	compare removal of aged hydrocarbons in the presence of different plant species combinations to determine soil toxicity and microbial activities during phytoremediation	Plants were grown in hydrocarbon contaminated soil for four growing seasons and hydrocarbon concentrations were monitored Microbial extracellular enzymatic activities carbon source utilisation and toxicity of soil were determined	Hydrocarbon removal was shown in compost-amended soil, but removal of hydrocarbons in unfertilised soil was not significant, despite presence of viable hydrocarbon-degrading micro-organisms. Microbial activities in compost-amended soil were different from unamended soil. Soil toxicity to test organisms was low.
IV: Effect of modified Fenton's reaction on microbial activity and removal of PAHs in creosote oil contaminated soil.	to determine if pre-treatment by modified Fenton's reaction increases PAH removal compared to incubation alone to study the effects of modified Fenton's reaction on soil and leachate toxicity and microbial activities	Modified Fenton's treatment of creosote contaminated soil was studied in lab-scale down-flow columns Microbial extracellular enzymatic activities, microbial counts and toxicity of soil and leachate were determined	The PAH removal in in combined Fenton's treatment and incubation, up to 54 %, was slightly higher than in incubation, 31 % or modified Fenton's treatment alone, 25 to 52 %. Modified Fenton's reaction increased toxicity of soil and column leachate. After incubation cells with intact cell membranes were more abundant in non-treated soil than modified Fenton's treated soil.
V: Treatment of PAH-contaminated soil by combination of Fenton's reaction and biodegradation.	to study the effects of lowering hydrogen peroxide to 12 % concentration from 30 % (Paper V) to study the effects of modified Fenton's reaction on soil and leachate toxicity, microbial counts and PAH utilisation	Modified Fenton's treatment of creosote contaminated soil was studied in lab-scale down-flow columns Microbial extracellular enzymatic activities, microbial counts, PAH utilisation and hydrogen peroxide concentration in soil as well as toxicity of soil and leachate were determined.	The PAH removal in in combined Fenton's treatment and incubation, 43 to 59 %, was slightly higher than in incubation, 22 to 30 % or modified Fenton's treatment alone, 40 to 50 %. Oxidation during incubation may account for part of PAH removal Modified Fenton's reaction increased toxicity of soil and column leachate. Microbes survived modified Fenton's treatment and were able to utilised PAHs as carbon source

I

II

III

IV

Erratum: Page 4, line 4 should read with a microplate photometer (Multiskan Ascent)

V

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