Bioremediation of diesel oil contaminated soil and water

Sari Kauppi

Department of Environmental Sciences
Faculty of Biological and Environmental Sciences
University of Helsinki, Lahti
Finland

Academic dissertation in Environmental Ecology

To be presented, with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, for public examination, in the Auditorium of Lahti Science and Business Park, Niemenkatu 73, Lahti
on September 16th 2011 at 12 o'clock noon.

Lahti 2011

Supervisors: Professor Martin Romantschuk
Department of Environmental Sciences
University of Helsinki
Lahti, Finland

Professor Rauni Strömmer
Department of Environmental Sciences
University of Helsinki
Lahti, Finland

Dr. Aki Sinkkonen
Department of Environmental Sciences
University of Helsinki
Lahti, Finland

Reviewers: Professor Matti Karp
Tampere University of Technology
Department of Chemistry and Bioengineering
Tampere, Finland

Dr. Marja Tuomela
University of Helsinki
Department of food and environmental sciences
Helsinki, Finland

Opponent: Professor Jan Sørensen
Faculty of Life Sciences
University of Copenhagen
Denmark

Custos: Professor Timo Kairesalo
Department of Environmental Sciences
University of Helsinki
Lahti, Finland

ISBN 978-952-10-7118-8 (paperback)
ISBN 978-952-10-7119-5 (PDF, http://ethesis.helsinki.fi)
ISSN 1799-0580

University press Helsinki 2011

CONTENTS

ABSTRACT
LIST OF ORIGINAL ARTICLES
AUTHOR'S CONTRIBUTION
ABBREVIATIONS AND CONCEPTS
1. INTRODUCTION 7
1.1. Diesel hydrocarbons 8
1.1.1. Diesel oil and the risk of spills for the environment and human health 8
1.1.2. Threat of diesel contamination in the Baltic Sea 10
1.1.3. Diesel hydrocarbon contamination in aquatic environments 11
1.1.4. Diesel hydrocarbon contamination in terrestrial environments 11
1.2. Legislation and guidelines by authorities 12
1.3. Microbes 13
1.3.1. Detection of microbes in soil and water, and the ability to degrade oil hydrocarbons 13
1.3.2. Bacterial adaptation and effect of previous contaminations 15
1.3.3. Bioremediation and natural attenuation 16
1.3.4. The effect of soil type on bioremediation potential 18
1.3.5. Enhanced bioremediation with bioaugmentation and biostimulation 19
2. AIMS OF THE STUDY 21
3. MATERIALS AND METHODS 22
3.1. Origin of studied water and soil and experimental set-ups 22
3.2. Mesocosms experiment of natural attenuation in three natural boreal soil types 22
3.3. Measurements of volatile organic compounds (VOCs) 22
3.4. Biological and chemical analyses 23
4. RESULTS 26
4.1. Diminishing diesel concentration in the Baltic Sea water mesocosms 26
4.2. Enhancing diesel biodegradation 27
4.3. Natural attenuation in various Finnish soils 28
4.4. VOC analyses 30
4.5. Bacteria in soil and water 31
5. DISCUSSION 33
5.1. Diesel hydrocarbon degradation by microbes 33
5.2. Enhancing bioremediation 34
5.3. Influence of soil type on decreased diesel oil hydrocarbon concentrations 35
5.4. Managing natural attenuation and conifer trees in urban structural design 36
5.5. Oil concentration and toxicity of diesel 37
5.6. Future prospects 38
6. CONCLUSIONS 40
7. ACKNOWLEDGEMENTS 41
8. REFERENCES 42

ABSTRACT

Diesel spills contaminate aquatic and terrestrial environments. To prevent the environmental and health risks, the remediation needs to be advanced. Bioremediation, i.e., degradation by microbes, is one of the suitable methods for cleaning diesel contamination. In monitored natural attenuation technique are natural processes *in situ* combined, including bioremediation, volatilization, sorption, dilution and dispersion. Soil bacteria are capable of adapting to degrade environmental pollutants, but in addition, some soil types may have indigenous bacteria that are naturally suitable for degradation.

The objectives for this work were (1) to find a feasible and economical technique to remediate oil spilled into Baltic Sea water and (2) to bioremediate soil contaminated by diesel oil. Moreover, the aim was (3) to study the potential for natural attenuation and the indigenous bacteria in soil, and possible adaptation to degrade diesel hydrocarbons. In the aquatic environment, the study concentrated on diesel oil sorption to cotton grass fiber, a natural by-product of peat harvesting. The impact of diesel pollution was followed in bacteria, phytoplankton and mussels. In a terrestrial environment, the focus was to compare the methods of enhanced biodegradation (biostimulation and bioaugmentation), and to study natural attenuation of oil hydrocarbons in different soil types and the effect that a history of previous contamination may have on the bioremediation potential.

(1) In the aquatic environment, rapid removal of diesel oil was significant for survival of tested species and thereby diversity maintained. Cotton grass not only absorbed the diesel but also benefited the bacterial growth by providing a large colonizable surface area and hence oil-microbe contact area. Therefore use of this method would enhance bioremediation of diesel spills. (2) Biostimulation enhances bioremediation, and (3) indigenous diesel-degrading bacteria are present in boreal environments, so microbial inocula are not always needed. In the terrestrial environment experiments, the combination of aeration and addition of slowly released nitrogen advanced the oil hydrocarbon degradation. Previous contamination of soil gives the bacterial community the potential for rapid adaptation and efficient degradation of the same type of contaminant. When the freshly contaminated site needs addition of diesel degraders, previously contaminated and remediated soil could be used as a bacterial inoculum. Another choice of inoculum could be conifer forest soil, which provides a plentiful population of degraders, and based on the present results, could be considered as a safe “non-polluted” inoculum.

According to the findings in this thesis, bioremediation (microbial degradation) and monitored natural attenuation (microbial, physical and chemical degradation) are both suitable techniques for remediation of diesel-contaminated sites in Finland.

LIST OF ORIGINAL ARTICLES

The thesis is based on the following articles, to which the text refers by their Roman numerals.

I Sonja Suni, Kaisa Koskinen, Sari Kauppi, Emilia Hannula, Tuukka Ryynänen, Annika Aalto, Jenni Jäänheimo, Johanna Ikävalko, Martin Romantschuk. 2007. Removal by sorption and *in situ* biodegradation of oil spills limits damage to marine biota – a laboratory simulation. Ambio 36: 173-179.

II Sari Kauppi, Aki Sinkkonen, Martin Romantschuk. 2011. Enhancing bioremediation of diesel-fuel contaminated soil in a boreal climate; comparison of biostimulation and bioaugmentation. International Biodeterioration and Biodegradation 65: 359-368.

III Sari Kauppi, Martin Romantschuk, Rauni Strömmer, Aki Sinkkonen. Natural attenuation is enhanced in previously contaminated and coniferous forest soils. 2011. Environmental Science and Pollution Research, DOI 10.1007/s11356-011-0528-y.

THE AUTHOR'S CONTRIBUTION

I SK participated in the experimental work (growing enrichment cultures, sampling, analyzing chlorophyll and oil concentration) and participated in the writing of the manuscript.

II SK performed all the experimental work and wrote the manuscript assisted by the coauthors.

III SK participated in setting up the experiment, was responsible for experimental work, molecular work and analyzing of oil hydrocarbon concentrations. SK wrote the manuscript assisted by the coauthors.

Original data in the thesis:

SK performed an additional experiment using natural Finnish soils; peat, organic forest humus and clay, in a similar set-up as the mesocosm experiment in III (without similar previously contaminated soils). Analyses of these soils included determination of oil hydrocarbon concentration as well as measurements of volatile organic compounds (VOC), organic matter and pH.

VOC analyses were measured during the experiments presented in article III, and the results are presented in this thesis.

ABBREVIATIONS

API gravity	arbitrary scale, where API gravity increases inversely to density; the API gravity at 15.6°C for No. 2 diesel fuel is between 30 and 42 (for more information see Bacha et al. 2007)
CFU	colony forming unit
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dw	dry weight
LC50	lethal concentration for 50% of test organisms
LD50	lethal dose for 50% of test organisms
log Kow	octanol-water partition coefficient
MNA	monitored natural attenuation
NA	natural attenuation
PAH	polycyclic aromatic hydrocarbons
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
SE	standard error
SOM	soil organic matter
16S rRNA	small subunit ribosomal ribonucleic acid
US EPA	Environmental protection agency, United States of America
VOC	volatile organic compound

CONCEPTS

bioaugmentation	adding specific degrading microbes to enhance degradation of target compounds or pollutants
bioremediation	biological, mostly microbial, degradation, also applies to plant-assisted or animal-assisted degradation
biostimulation	adding nutrients, mixing, increasing temperature etc. to stimulate biological (microbial) degradation
enhanced bioremediation	stimulated biological (microbial) degradation by biostimulation or bioaugmentation
heavy (crude) oil	low API gravity (high density) crude oils
light (crude) oil	high API gravity (low density) crude oils
monitored natural attenuation	bioremediation technique, where a specific schedule is set for monitoring of decrease in contaminant concentration
Napthene	term used in the petroleum industry for cycloalkanes and cycloparaffins (Bacha et al. 2007)
natural attenuation	degradation by bioremediation, volatilization, sorption, dilution and dispersion

1. INTRODUCTION

Anthropogenic oil hydrocarbon spills create a serious threat to the ecology of aquatic and terrestrial environments. Oil hydrocarbons disrupt ecosystem functions, such as respiration and the nitrogen (N) cycle (Schafer et al. 2009). In addition, oils contain ingredients that are toxic to flora and fauna as well as to human health (Dorn et al. 1998, Wong et al. 1999, van Gestel et al. 2001). Moreover, oil damages and destroys infrastructure and contaminates the landscape.

Diesel oil hydrocarbons are derived from crude oil refining (Mälkönen 1995), and diesel is a complex mixture of saturated and aromatic hydrocarbons (Eriksson et al. 2001, Zanaroli et al. 2010). For the most part, diesel comprises aliphatic hydrocarbons, but it also contains polycyclic aromatic hydrocarbons such as naphthalene, fluorene and phenanthrene (Williams et al. 1986, Mälkönen 1995, Eriksson et al. 2001). These aromatic compounds represent 5-30% of diesel oil (TTL 2011).

Diesel oil contains low molecular weight compounds that are usually more toxic than long-chained hydrocarbons, because long-chained ones are less soluble and less bioavailable (Dorn et al. 2000). Light oils contain a relatively high proportion of saturated hydrocarbons, hence these can be more toxic than heavy oils (Dorn et al. 1998).

Cold regions have been considered to be especially sensitive to oil pollution, because of the prolonged degradation time of oil hydrocarbons (Horel and Schiewer 2009). Nevertheless, oil hydrocarbons are also degraded by bacteria in cold environments (Margesin and Schinner 1999). Thus, several psychrotolerant bacteria capable of degradation of oil hydrocarbons are known. Such microbial degradation enables bioremediation either without intervention (monitored natural attenuation) or with intervention (e.g. biostimulation).

Bioremediation of oil hydrocarbon spills is, in many cases, an adequate method for remediation (Plaza et al. 2005), but improvements and new methods are needed to guarantee an acceptable renovation result. The total remediation time can be long in cold climates. Excavation and transportation is the mostly used method in soil remediation in Finland (Mroueh 2004). Excavation is typically a more costly method than bioremediation *in situ* and also releases more greenhouse gases because of transportation (Penn et al. 2002), so bioremediation is considered a more sustainable method than excavation (Stenuit et al. 2008).

From the regulatory point of view, the possibility of natural attenuation shall be considered when decisions on remediation are made, as presented in EU directive 2004/35/CE. Nevertheless, natural attenuation and bioremediation are seldom chosen options. Sustainable methods should be considered and chosen more often if we wish to remediate all polluted sites in the future. Regardless of the increase in environmental knowledge and

hence the enhanced attempts to prevent pollution, the number of contaminated sites is continually growing (US EPA 2011, FEA 2007). All accidents can not be prevented even when manufacturing and transportation are carefully planned. In addition, new, previously polluted sites are found for example when the purposes of the land use changes at the sites.

1.1. Diesel hydrocarbons

1.1.1. Diesel oil and the risk of spills for the environment and human health

Diesel fuels vary according to their origin and method of production (ATSDR 1995). In general, they are similar to heating oil, consisting of aliphatic (mostly paraffins including *n, iso* and cycloparaffins) and aromatic hydrocarbons (Table 1, Fig. 1), including small amounts of organometal constituents such as vanadium and nickel (ATSDR 1995, Van Hamme et al. 2003, Bacha et al. 2007, Zanaroli et al. 2010). Some oils contain heavy residues from distillation and thermal cracking along with a variety of additives (organic nitrates, amines, phenols and polymeric substances) (IARC 1998). The main purpose of the additives is the performance of the engine and delivery system (e.g., cetane number improvers and lubricity improvers), fuel handling (e.g., antifoam and de-icing additives), fuel stability, and contaminant control (e.g., biocides, demulsifiers and corrosion inhibitors) (Bacha et al. 2007).

The colour of diesel fuels varies from colourless to brown, and the water solubility in 20°C is about 5 mg L^{-1} and Log K_{ow} 3.3 – 7.06 (ATSDR 1995). Diesel fuels are therefore partly soluble in water and possibly accumulative in tissues (Table 1).

Table 1. Selected diesel fuel hydrocarbons and some chemical properties of them (Gustafson et al. 1997, Bacha et al. 2007). B = boiling point, F = Freezing point

Compound	Chemical formula	Group	Density 20°C, g/cm^3	loc K_{ow}	Water solubility mg/L	B °C	F °C
Naphthalene	$C_{10}H_8$	Aromatic	1.175	3.37	31	218	80
n-Butylcyclohexane	$C_{10}H_{20}$	Naphthene	0.7992	5.46	-	181	-75
n-Decane	$C_{10}H_{22}$	n-Paraffin	0.7301	6.25	0.052	174	-30
Anthracene	$C_{14}H_{10}$	Aromatic	1.251	8.00	8	341	215
n-Pentadecane	$C_{15}H_{32}$	n-Paraffin	0.7684	8.63	-	271	10
Eicosane	$C_{20}H_{42}$	n-Paraffin	0.7843	11.27	3E -07	344	36

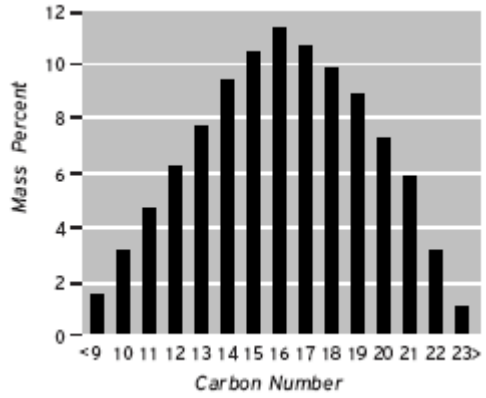

Figure 1. Typical carbon (C) number distribution of No. 2-D diesel fuel (Bacha et al. 2007, published by the permission of Chevron Corporation, © 2007 Chevron U.S.A. Inc.).

Diesel fuels have been observed to cause skin irritation and tumorigenic responses in mice, especially if the fuel contains cracked material (Mckee et al. 1994, Nessel 1999). The marine luminescent bacterium *Vibrio fischeri* is an indicator species that has been used in environmental toxicity testing of diesel in cold environments, but no specific LD50 or LC50 values were set in those studies (Margesin and Schinner 2001b, Coulon et al. 2005, Delille et al. 2008b). In warmer temperatures (>20°C) and when testing oil hydrocarbons other than diesel (such as petroleum or crude oil) earthworms, nematodes, *Daphnia magna*, and plants such as *Lactuca sativa*, *Brassica alba, Lolium perenne* and *Lemna minor* have been used for ecotoxicological testing (Table 2, Dorn et al. 1998, Dorn et al. 2000, Molina-Barahona et al. 2005, Dawson et al. 2007, Hubalek et al. 2007, Wang et al. 2010). Rats are often used for estimating toxicity to mammals (Table 2). Diesel causes eye and skin irritation in humans, but otherwise its effects on humans are considered to be poorly investigated (Muzyka et al. 2002). Diesel is considered to be harmful and possibly carcinogenic to humans (TTL 2011) and it contains PAHs that create a risk for human health because of their carcinogenic, mutagenic and teratogenic properties (Bamforth and Singleton 2005, Grant et al. 2007).

Diesel spills usually take place during manufacturing, storage or transportation. Major spills, such as pipeline, tanker or storage tank accidents, create an acute problem of pollution. On the other hand, continuous low-level inputs are rarely noticed, and may pose a serious threat to the environment as contamination accumulates. Therefore, diesel hydrocarbons create a world-wide problem of contaminated water and soil that require decontamination.

Table 2. Examples of toxicity of hydrocarbons found in diesel (www.merc-chemicals.se 7.6.2011).

	Naphthalene	**n-Decane**	**Anthracene**
Ratt			
LD50 Oral	>2000 mg/kg	5000 mg/kg	16000 mg/kg
LC50 Inhalation	>100 mg/L (4 h)	8.1 mg/L (8 h)	-
LD50 dermal	>2500 mg/kg	<2000 mg/kg	-
Daphnia magna 48h	2.19 mg/L	18 mg/L	0.095 mg/L

1.1.2. Threat of diesel contamination in the Baltic Sea

The Baltic Sea is a shallow brackish water basin with a mean depth of only 54 meters (Baltic Sea Portal 2011). The salinity changes are notable, because of the saline water flow from the Danish Straits and fresh water flow from the rivers of the catchment area. Water in the Baltic Sea exchanges extremely slowly; the retention time is about 30 years, that is, on average, it takes that long to replace all water. A large but varying part of the water surface freezes during the winter time. The International Maritime Organization (IMO) has ranked the Baltic Sea Area as a Particularly Sensitive Sea Area (NAS 2003). The effect of oil contamination on the Baltic Sea can be severe, because of the low volume of water and slow water replacement. In addition, the cold climate and long shorelines in the archipelago create challenges for cleaning up oil contamination.

Mussels are considered as a key species in the Baltic Sea, because they are naturally present at the benthos. Sessile and long-living blue mussels are commonly used for monitoring the effects of pollution, because they accumulate contaminants from the water phase and from plankton. Low molecular weight oil hydrocarbons are highly toxic to marine species (Calder and Lader 1976, Nayar et al. 2005).

Oil transportation from the harbours around the Baltic Sea is voluminous and therefore the Baltic Sea confronts the risk of a major oil spill. The total volume of oil transportation has increased in the Baltic Sea since the 1990s (Fig. 2). A large proportion of transported oil product across the Baltic Sea is diesel (Lindgren and Lindblom 2004). The largest port for exports of oil from Russia is Primorsk Commercial Sea Port, where 79 million tonnes of oil and diesel fuel was shipped in 2009 (Hietala 2011). In 2010, the amount of exported diesel from Primorsk was over 500 000 tons in one month (Hietala 2011). Since the 1970s, many action plans and manuals have been created for co-operative management of oil spills in the Baltic Sea (HELCOM 2011), but a major oil spill would still be disastrous to the Baltic Sea and the bordering countries. The combating equipment is inadequate, despite recent acquisitions of new oil combating vessels in Finland, so new inventions for remediation techniques are essential. Along with an increase in maritime vessel traffic, small leaks, drifts and spills have also become more common. Even without a major tanker accident, about 70 000 tons of oil hydrocarbons end up annually in the Baltic Sea (Hänninen and Rytkönen 2004). These contaminations are mostly caused by oil transport, port activities, illegal discharges of bilge waters, and surface run-offs (NAS 2003). Most of the smaller spills take place in harbour areas (K. Laakso, pers. comm. 26.1.2011). The authorities have analysed the oil spills and most of the illegal spills are gas oils, such as diesel oils (N. Viitala, pers. comm. 24.1.2011). Compared to previous years, the number of airborne observed oil spills decreased in 2008, but the total volume of the spills increased (Baltic Sea Portal 2011).

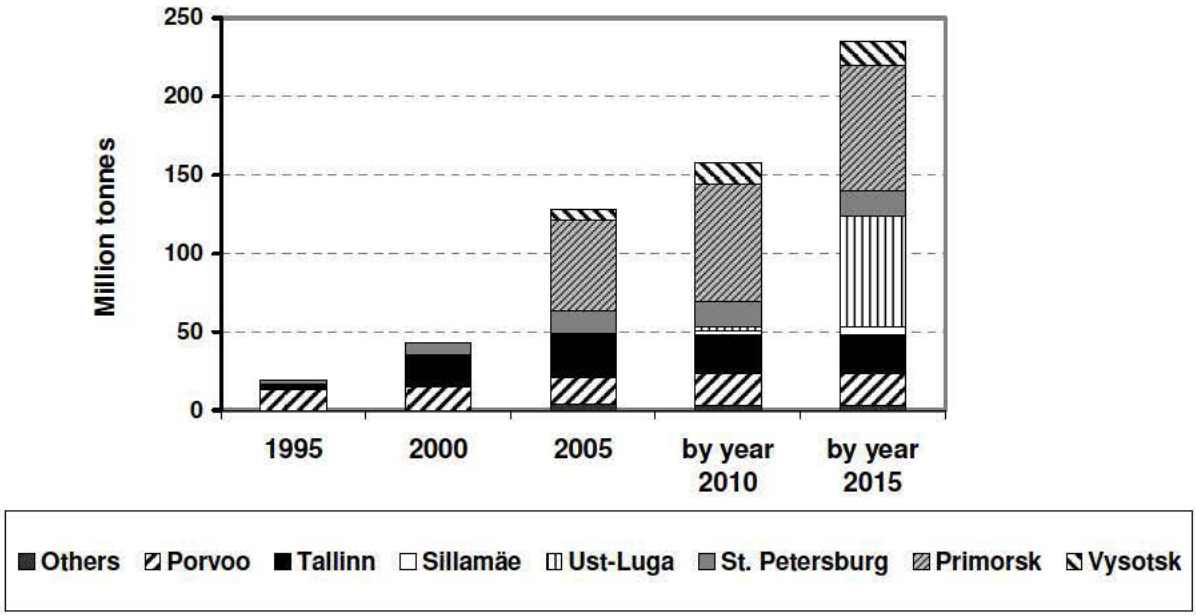

Figure 2. Oil transportation in the Gulf of Finland through main oil ports. Oil transportation 1995-2009 and estimated development until 2015 (Hietala 2011).

1.1.3. Diesel hydrocarbon contamination in aquatic environments

When released, oil spreads in water. Spreading expands according to viscosity of oil, water temperature, wind speed and sea conditions (Annunciado et al. 2005). The methods developed for oil spill remediation include physical (such as sorption), chemical (such as dispersion) and biological (bioremediation) methods (Bayat et al. 2005). According to some sources, combinations of all these methods should be used for decontamination of major accidents (Bayat et al. 2005), but for small-scale oil spills the use of a physical method such as sorption is a good operating option (Annunciado et al. 2005). Cotton grass fibre is a natural sorption material that is a low cost by-product of peat harvesting (Suni et al. 2004). Natural sorbent materials are considered as the most promising sorbent materials for further investigations (Carmody et al. 2007). Although use of sorption material is not the main method for remediation of larger spills, it can be a component of the treatment.

1.1.4. Diesel hydrocarbon contamination in terrestrial environments

Oil hydrocarbons contaminate a terrestrial environment as a result of leakages during storage or accidents in the transportation. The contamination is usually a local problem. The consequence can increase, especially due to mobility of light oil hydrocarbons, because drifting to ground water is then a serious threat.

Biological, chemical, hydraulic and sorption characteristics vary greatly between organic compounds, and these factors affect the speed of drifting to ground water (Azadpour-Keeley et al. 2001a). Organic compounds such as diesel oil hydrocarbons are degraded in soil, but some components can be recalcitrant. Lighter fractions of diesel evaporate, but some compounds bind to soil particles (Reinikainen 2007). In mineral sandy soil, drifting of diesel is faster than in humic soil so the threat of ground water contamination is greater (Leinonen et al. 2007). The spreading of contamination to ground water causes an immediate threat at a mineral soil area, and therefore excavation is a fast and safe method for use in ground-water areas. In other situations, biological remediation is considered as the best practice for remediating oil hydrocarbon contaminations (Plaza et al. 2005).

High concentration of contaminant can be toxic to microbes and inhibit degradation, so bacterial degradation is possible when the concentration of contaminant is below the threshold of toxicity (Yang et al. 2009). On the other hand, a low concentration of contaminant might not provide enough carbon for efficient degradation (Boopathy 2000). This is evident especially if the contaminant provides the only carbon source for bacteria or other microbial population. Simply, the degradation is not efficient if the bacteria population is not able to grow. When a bacterial population gains carbon and energy from the degradation process, it gains a competitive advantage, so a broad degradation capacity gives a selective advantage in contaminated environments.

Light compounds of oil are highly volatile (Schafer et al. 2009), so contaminated soil may partly be cleaned by volatilization. This can be harmful, because volatile compounds of contaminants can travel into the atmosphere and into the indoor air of buildings (FEA 2007). Therefore rapid microbial degradation is a safer method than enhanced evaporation.

1.2. Legislation and guidelines by authorities

The European parliament and the European council have stated in the directive 2004/35/CE (Annex II, Remedying of environmental damage) that the natural recovery option shall be considered for remediation of land damage. The regulators specify natural recovery as an option in which no direct human intervention in the recovery process would be taken. This defines bioremediation as it relies on natural biological degradation and also natural attenuation, which includes biological remediation and natural chemical processes. Natural attenuation combines natural processes *in situ*, including bioremediation, volatilization, sorption, dilution and dispersion (Jørgensen et al. 2000, Azadpour-Keeley et al. 2001a).

Ecological and environmental risk assessments are usually performed at the contaminated site. Oil products are complex mixtures and therefore setting specific safety limits is considered

impossible (Reinikainen 2007). Consequently, Finnish regulators have not assessed a specific *threshold limit* for oil hydrocarbon fractions, but the lower limit is set for medium fractions (C12-C21) at 300 mg kg^{-1}. If the lower limit is exceeded, the remediation of soil has to be assessed and the case by case decisions have to be made based on the risk assessment. If the land is used for industrial purposes and not as a residential area, the higher limit of 1000 mg kg^{-1} determines the need for risk assessment and remediation. Risk assessments are required by the government decree about soil contamination and evaluation of the need for remediation (214/2007). The evaluation of contaminant characteristics, namely solubility, toxicity and fate, are considered in the risk assessments. Even though the limits that are set in the decree for oil fractions are not based on either ecological or health risks, the evaluation of risks must be performed by analyzing several soil samples (Reinikainen 2007).

In environmental permit decisions, the method mostly used for remediation is an *ex-situ* one of excavation and soil replacement. Excavation was the only method in 91% of the remediated sites in 2008 (FEA 2009). In a further 4%, excavation was part of the remediation process and in only 5% remediation relied entirely on techniques other than excavation. Among these were isolation, chemical oxidation, natural attenuation and pore air collection of VOCs. In only one out of the 318 cases granted soil remediation permission, natural attenuation was used as the only remediation method (T. Haavisto, Finnish Environment Institute, pers. comm. 11.2.2011). The authorities consider natural attenuation as a fairly new and experimental method. The lack of specific instructions about natural attenuation or enhanced bioremediation diminishes the trust and therefore the use of these methods (O. Valo. Centre for Economic Development, Transport and the Environment, Finland, pers. comm. 18.4.2011).

1.3. Microbes

1.3.1. Detection of microbes in soil and water, and the ability to degrade oil hydrocarbons

Biodiversity can be defined as the habitat's animal and plant species and the ecosystems they belong to, and include both species and gene diversity (Ohtonen et al. 1997). In a wide perspective view, this would also include soil microbial biota. Soil microbial biodiversity can be analyzed by cultivating and non-cultivating methods (Vogel 1996). Rates of oxygen consumption and CO_2 evolution can be used as a measure of cellular respiratory activity (Atlas & Bartha 1997). The dilution plate count method estimates the quantity of microbes, where only viable cells form a colony, but only 0.1 - 10% of microbes are detected by this method (Torsvik et al. 1998). Molecular techniques detect more detailed information about the microbes in an environmental sample (Muyzer 1999, Romantschuk et al. 2000, Saul et al. 2005, Scow & Hicks 2005). Together, these methods allow the broad investigation of microbes.

When several molecular techniques are used to detect the bacterial community composition, the outcomes may differ because of the different targets of these methods. The DGGE method, that separates amplified fragments (e.g. portions of the 16S rRNA gene) based on differences in the sequence of the amplicon, detects the most abundant bacterial species, while cloning and sequencing of partial 16S rRNA genes may discover rare species, although the most abundant species are more likely to be cloned. Costa et al. (2006) observed that when using the DGGE method in bacterial detection, it is possible for DNA fragments with differences in the sequence to travel to the same position, since movement depends on GC (Guanine-Cytosine) content, not on order of sequence. Similarly, Ishii et al. (2000) observed that when the DNA bands were not clear, tightly confined lines on the gel, it was challenging to cut only one fragment from the gel for sequencing. Furthermore, PCR primers used for DNA amplification for DGGE gels contain a GC-glamp that inhibits amplification, so use of nested PCR is needed, even though it multiplies the possible chimeric sequences that are common in PCR amplification.

The results of bacterial detection by the molecular techniques are affected by the database information to which the studied sequences are compared. In the databases the Proteobacteria are strongly represented, and this could contribute to the apparently high abundance of Proteobacteria in the results of the soil bacterial community studies (Jansen et al. 2006).

The microbial loop in aquatic environments consist of bacteria and picoplankton, which is plankton in the size range 0.2 – 2.0 µm. Most of the primary production in the Baltic Sea is channeled through the microbial loop, and therefore it is a critically important part of the aquatic food web (Sommer et al. 2002). Bacteria in the Baltic Sea are adapted to salinity fluctuations and therefore are under stress that can be observed as physiological changes (Kaartokallio et al. 2005).

Fungi have much potential for degrading organic compounds, because of their spreading mode of growth and symbiotic association with plants (Sarand et al. 2000, Hosokawa et al. 2009). However, bacteria are considered more important colonizers in oil-contaminated soil than fungi (Aislabie et al. 1998). Fungi and bacteria can have a mutualistic relationship, so the presence of fungi can support bacterial growth by the structures of hyphae and mycorrhizae, and some bacteria can improve mycelial growth and so improve the growth conditions of fungi (Sarand et al. 2000).

Soil bacterial diversity and quantity is enormous. The total population of bacteria in 1 g of soil has been estimated to be 10^8 - 10^{10} (Sylvia et al. 2005, Roesch et al. 2007). Pollution often diminishes the total microbial diversity in soil (Lynch et al. 2004). Moreover, the community composition may be altered by environmental contamination (Lynch et al. 2004).

Several bacterial species are capable of oil hydrocarbon degradation because

hydrocarbons are naturally produced by plants and microbes (Sylvia et al. 2005). Various pathways are known for the bacterial degradation of different hydrocarbons (Van Hamme et al. 2003, Sylvia et al. 2005). *Pseudomonas* bacteria were shown to be important degraders of oil hydrocarbons, especially in cold climates (Aislabie et al. 2000, Margesin and Schinner 2001a, Belousova et al. 2002, Stallwood et al. 2005, Zhang et al. 2010). Among other oil degrading bacteria genera are *Rhodococcus* (Peressutti et al. 2003, Aislabie et al. 2006) and *Sphingomonas* (Margesin and Schinner 2001a, Aislabie et al. 2006).

1.3.2. Bacterial adaptation and effect of previous contaminations

Evolution has provided bacteria with different strategies for survival. Adaptation is an important strategy for survival in toxic environments and in competition for food resources (Poole et al. 2003, Kivisaar 2003). When bacteria are exposed to a contaminant, they must tolerate the environmental toxicity either endogenously or by adaptation. Genetic adaptation may occur by mutations, horizontal gene transfer and DNA rearrangements (Springael and Top 2004). Degradation genes are often located in plasmids that can be transferred between phylogenetically different members of the bacterial community (Johnsen et al. 2005, Sarand et al. 2000).

A new, toxic organic compound in the environment places stress on indigenous bacteria and the stress reaction results in the evolution of mutations and adaptation (Lynch et al. 2004, Sarand et al. 2001, Vogel 1996, Wright 2004). Evolution selects the fittest and therefore the ability to metabolize a new organic compound is a vital strategy for bacteria (Wright 2004). Soil is often a carbon limited environment for bacteria, and environmental stress such as starvation leads to an elevated mutation rate (Kivisaar 2003). Bacteria that are responding to stress by forming a new catabolic pathway, have an opportunity to survive (Wright 2004). Therefore degradation ability provides niche opportunities. Some degradation genes can also be found in a pristine environment and it is suggested that these genes are involved with degradation of naturally occurring organic compounds (Springael and Top 2004).

Contamination along with other physical and chemical changes in soil can cause adaptive changes in the composition of the bacterial community (Saul et al. 2005, Grant et al. 2007). Competition within and between species influences the bacterial community composition and hence degradation of contaminants. Soil pore size influences degradation by providing surface to colonize and because of predation that reduces bacterial biomass especially in pores that are larger than 2 µm (Johnsen et al. 2005). Therefore in soils where the pore size is large, there are fewer microbes available for degradation than in soils with small pore size.

Release of biosurfactants is one strategy of bacterial degradation. Biosurfactants are detergent-like molecules with a hydrophilic head and lipophilic tail (Johnsen et al. 2005), which exhibit high

surface activity that may increase the solubility of lipophilic substances and thereby improve the bioavailability of water-insoluble contaminants, which in turn leads to accelerated contaminant degradation (Bento et al. 2005a). Such an effect was observed by Bento et al. (2005a) who reported that when biosurfactants were added to soil contaminated with diesel oil, the degradation of the contaminant was enhanced. Biosurfactant-producing bacteria have been characterized in diesel-contaminated soils (Bento et al. 2005a). Bento and coworkers (2005a) considered biosurfactant-producing bacteria important for enhanced bioremediation of petroleum hydrocarbon contaminated soil.

Bioavailability is the key issue in bacterial degradation. Thus, a bioavailable organic compound is accessible for microbial uptake. Organic compounds are normally in a soluble form for substrate uptake while sorption onto soil particles creates a barrier for degradation. Some specific bacterial species adapt to interact with humic-substance-sorbed compounds, though this ability is considered rare (Vacca et al. 2005). Degradation may then take place even if the organic compound is bound to humic substances. On the other hand, a cold environment can reduce the bioavailability of oil products and therefore biodegradation in the Boreal zone can be difficult (Brakstad and Bonaunet 2006). At low temperatures, viscosity of oil increases and volatilization of short-chained alkanes decreases, resulting in increased water solubility and hence toxicity (Margesin and Schinner 2001a). This delays the start of biodegradation.

1.3.3. Bioremediation and natural attenuation

To consider bioremediation and natural attenuation as a first option is applicable, because bacteria and other microbes degrade organic compounds (Atlas 1981). Indigenous bacteria may be naturally capable of degradation or they can become degraders by adaptation. Bacteria may use an organic contaminant as an energy source, or degradation may happen by a co-metabolic pathway (Van Hamme et al. 2003) where the contaminant resembles a natural substrate but does not provide energy for bacteria (Howarth 1972). Degradation is then a by-product of the normal enzymatic activity of the bacteria.

Bioremediation and natural attenuation are sustainable and inexpensive remediation methods for contaminated soil (Aislabie et al. 1998, Margesin and Schinner 1999, Plaza et al. 2005, Delille and Coulon 2008a). Although bioremediation or natural attenuation do not require as much labour as excavation processes, they are not “no action” or passive methods. Careful measurements, validations and schedules for monitoring the process are required. If natural attenuation is estimated to be successful within a suitable time limit, no further action for purification may be necessary. Decisions regarding natural attenuation should always be based on a thorough risk assessment, and the activity itself should be backed by sufficient monitoring of the site.

Bioremediation of organic compounds in many cases may be the best choice, even if the process requires stimulation. It is

considered as one of the most important decontamination methods for diesel oil hydrocarbons (Bento et al. 2005b). Whenever the composition of the contaminant can be clearly established, and it is known that this type of contaminant can be biodegraded efficiently, a decision for bioremediation is relatively easy to reach. In contrast, a mixture of various contaminants in the soil presents the most challenging situation for bioremediation.

When planning soil bioremediation at a recently contaminated site, there are several factors to take into account, such as 1) soil type, with regard to drifting and binding to soil particles, 2) previous contamination history, including contaminants, concentrations and possible microbial adaptation, and 3) possibility for natural attenuation and whether stimulation is needed (Fig. 3). The degradation process can be enhanced by bioaugmentation or biostimulation. In bioaugmentation, an efficient degrader population (pure culture or consortium) is introduced into the contaminated site (Bento et al. 2005b, El Fantroussi and Agathos 2005). In biostimulation, various amendments or mixing procedures are used to enhance degradation (Margesin and Schinner 1999, Seklemova et al. 2001). Different combinations of nutrient addition, improvement in oxygen level, and increase of temperature can be chosen to stimulate the degradation process (Jørgensen et al. 2000).

Predicting the remediation time and efficiency is not easy, though mathematical models have been created (Alvim-Ferraz et al. 2006). Bioremediation is considered more advantageous from an economic aspect than from the point of view of time efficiency (Alvim-Ferraz et al. 2006). Knowledge of soil type can indicate if bioremediation is possible, considering site-specific biological, physical and chemical facts that are listed above. The need for time estimation is obvious when remediation of a contaminated site is required.

Background information of the site is not always accurate. Successful bioremediation requires best available historical knowledge of the site, and this knowledge should always be backed by thorough pre-analyses of the soil. Contaminants are most likely spread unevenly in the soil (Muckian et al. 2007), which makes remediation work even more challenging.

Cost efficiency is highly valued when remediation methods for total restoration of a contaminated site are compared. Additions and mixing are costly but the most expensive method usually is soil replacement, along with excavation of contaminated soil, transportation, and storage in piles for years (Azadpour-Keeley et al. 2001b, Welander 2005, Alvarez and Illman 2006, Muckian et al. 2007).

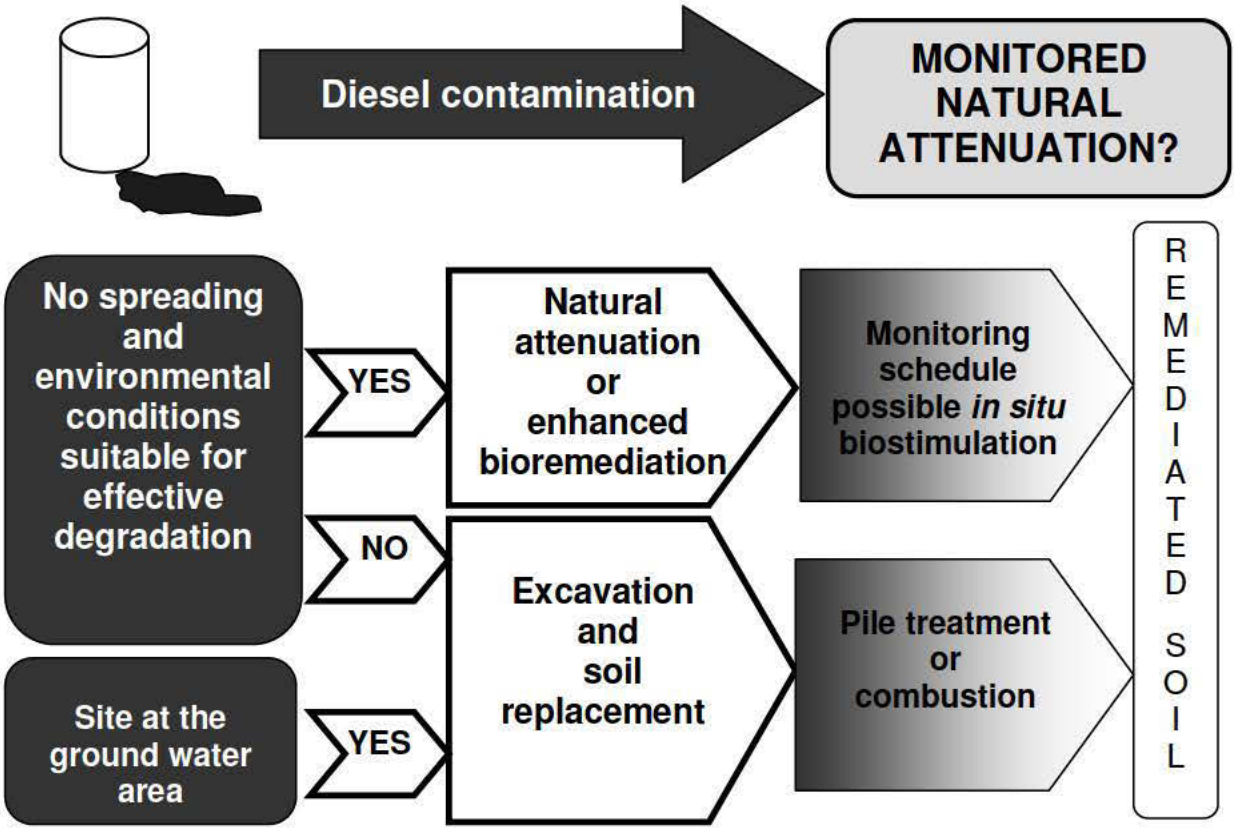

Figure 3. Monitored natural attenuation and enhanced bioremediation are possible methods for remediation of diesel-contaminated soil. Soil replacement is not needed if these techniques are used *in situ*.

1.3.4.The effect of soil type on bioremediation potential

Soil is a heterogeneous environment and creates different environments for plant and microbial survival from the micro scale to the landscape level. The soil type defines the environment of microbes (Jangid et al. 2008). Organic matter content (SOM), particle size, pH, water holding capacity, available oxygen, nutrient content, and redox potential vary between soil types (Margesin and Schinner 2001a). These factors are important to control physical and chemical degradation and microbial activity along with soil temperature, humidity and climate conditions. Conversely, some soil types bind organic compounds tightly, thus making contaminants less bioavailable for bacterial degradation (Theng et al. 2001). This means that soil may immobilize contaminants due to strong sorption (Reichenberg et al. 2010), especially if the contaminant is hydrophobic (Yang et al. 2010). Because organic compounds have strong affinity to naturally occurring organic matter, bioavailability of organic compounds is strongly affected by SOM (Yang et al. 2010).

Nutrient content has an effect on bacterial community composition (Leckie et al. 2004) and the amount of nitrogen (N) and phosphorus (P) often limit degradation efficiency (Welander 2005). Soil type

defines the vegetation on the site and the vegetation defines the nutrient concentrations and pH in soil, therefore vegetation has an effect on degradation potential of a soil. Conifer forest soil has previously been suggested to have capacities of enhanced degradation (Sarand et al. 1999, Penet et al. 2006). A natural degrading bacterial population is expected to be present at a site where organic compounds are formed naturally. Aromatic structures are often found in humic organic soils (Grandy et al. 2007), so in these soils, microbes capable of degrading such structures are expected.

Microbial communities respond to differences of soil pH (Jangid et al. 2008). Soil pH in Finnish forest soils is in the range 3.6 - 5.5 in humus and 3.6 - 5.4 in mineral soils, at the depth of 0 – 5 cm (Kähkönen 2003). Soils in Finland are typically acidic compared to Antarctic soils, or limestone-rich soils such as those in Estonia or Denmark, which are normally highly alkaline (Moores et al. 1997, Aislabie et al. 2000). These sites are often located in a similar climate (Northern Europe) or environmental condition (soil freezing in winter) as the climate and environmental conditions in Finland. Because of large differences in soil pH compared to these sites, the degradation ability needs to be tested using Finnish soil types.

1.3.5. Enhanced bioremediation with bioaugmentation and biostimulation

Bioremediation can be enhanced using bioaugmentation or biostimulation. In bioaugmentation, laboratory grown bacteria are added to a contaminated site to enhance degradation. These laboratory-grown pure cultures or consortia have the desired ability to be able to degrade certain organic compounds present as contaminants in the target site. Consortia are considered to be more efficient because full degradation and mineralization of an organic compound or group of compounds is often a cooperation project; one strain degrades a compound only partly or influences the microbial community in a way that stimulates degradation performed by the other strains (El Fantroussi and Agathos 2005). In general, laboratory-grown strains do not compete well with the indigenous bacterial population when exposed to natural environmental conditions, and this is the major flaw in bioaugmentation. The growth of the introduced microbes is inhibited by biotic and abiotic stressors (Margesin and Schinner 1999). Therefore the introduced bacteria may have a better change for survival if the addition is made within a suitable environment. New methods have been tested and soil has been used as inocula in order to import the desired degradation ability (Gentry et al. 2004). Soil addition may allow long-term survival of introduced bacteria, and may also facilitate gene transfer from degraders to indigenous bacteria (Greenwood et al. 2009). Therefore knowledge of indigenous bacteria in different soil types is valuable. On the other hand, the degradation ability of soil microbes is mostly enhanced by changing the functional bottlenecks in microbial degradation, such as nutrient availability and temperature. These can be influenced by biostimulation.

In practice, stimulation of the bioremediation process often means improving the conditions for bacterial activity, in the hope that this will lead to accelerated contaminant degradation with a sufficiently cleaned site as the end result. In biostimulation, the physical and / or chemical conditions are changed to achieve the desired degradation. Bacteria require suitable nutritional conditions, and are often limited by N and P concentrations, so improvements in N and P concentrations may increase degradation of organic contaminant (Mohn and Stewart 2000, Peng et al. 2008). An increase in N concentration enhances degradation, especially at diesel-contaminated sites, where the contaminant provides high hydrocarbon concentrations and thereby a too high C:N ratio (Peltola et al. 2006). However, too high concentrations of fertilizers can inhibit degradation (Mohn and Stewart 2000, Peltola et al. 2006, Walecka-Hutchison and Walworth 2007). Moreover, the fertilization might have only temporary effects on the remediation by increasing the bacterial growth that was limited by N (Schafer et al. 2009). Although the toxicity could be reduced in soil, the increase of bacterial abundance would be only short-term and the population of bacteria would later diminish without continuous nutrient addition.

In general, increase of soil temperature is considered to improve bacterial degradation activity. In a cold climate, however, soil bacteria are adapted to cold temperatures, and the optimum temperature for degradation, according to Margesin et al. (2003), is most likely under 20°C. Therefore a moderate temperature increase (up to 16°C) to accommodate the indigenous psychrotolerant bacteria, may be better than higher temperatures (Margesin and Schinner 1997a, Margesin and Schinner 1997b, Coulon et al. 2005). Alternatively, in some cases better results have been accomplished in degradation of organic compounds by heating soil to 25°C (Suni et al. 2007). Temperature increase can then be a stimulation that is needed for efficient oil degradation.

2. AIMS OF THE STUDY

In order to prevent significant health risks and the loss of biodiversity, and to prevent further contamination, enhanced remediation methods are necessary. Remediation can result in a speedy recovery of the environment from diesel pollution. Therefore this study aimed at new, cost-efficient inventions for enhancing bioremediation and to increase the knowledge of diesel oil remediation in water and soil.

The main objectives of this thesis were to design sustainable, low-cost tools to remediate diesel-contaminated water and soil, and to create methods to prevent spreading of harmful and toxic diesel oil hydrocarbons. The advantages of biostimulation and bioaugmentation in diesel oil degradation and the possibility of natural attenuation were tested in Finnish environments, representing boreal climate conditions.

The hypotheses tested in this thesis were:

1. The toxic effects of an oil spill in an aquatic environment can be eliminated by rapid absorption of the oil in cotton grass fibre.
2. Diesel-contaminated soil can be remediated by biostimulation relying on the indigenous bacterial population.
3. Additional remediation efficiency can be achieved by bioaugmentation, i.e., amending the soil with oil-degrading bacteria.
4. Soil with a history of oil contamination will be remediated faster when re-contaminated than pristine soil when contaminated first time, and this effect is linked to the bacterial community structure in the soil.
5. Natural attenuation and bioremediation are suitable methods for soil cleaning in Finnish soil types and, in addition, diesel degradation efficiency is affected by soil type.

3. MATERIALS AND METHODS

3.1. Origin of studied water and soil and experimental set-ups

Water for the mesocosms experiment (I, Table 3) was collected from an approximate depth of 1 meter from the Gulf of Finland, near the city of Porvoo. The unprocessed cotton grass fibers were from a peat harvesting company, VAPO, Ltd, Finland (density 22 kg m^{-3}, water holding capacity of 5.2%).

Soil for the bioaugmentation and biostimulation experiment (II, Table 3) was clay loam soil from Päijät-Häme Waste Disposal Ltd, Lahti, Finland. The soil originated from several locations in Southern Finland.

All soils for the mesocosms experiment (III, Table 3) were gathered from Southern Finland; mineral soil from two industrial sites and conifer forest humus from a Norway spruce (*Picea abies* L.) dominated forest near the city of Lahti. To examine the influence of previous contamination, similar soil type of both contaminated and pristine soils were excavated from nearby locations.

3.2. Mesocosms experiment of natural attenuation in three natural boreal soil types

An additional mesocosm experiment of natural attenuation (not included in I, II and III) was conducted using three natural Finnish soils, the first gathered from a pine (*Pinus sylvestris* L.) forest in Hollola (61°0'N 25°E), the second a clay soil beneath herbaceous perennials from Orimattila (60°52'7N 25°41'4E), and the third a peat soil from the Haapasuo peat production area, Leivonmäki (61°54'N 26°4'E). Only a thin organic soil layer was excavated beneath the forest litter layer. In each area, about 20 L of soil was collected from three points 5 – 10 m apart, mixed immediately and divided into two polyethene mesocosms (10 L each). Six replicates represented each soil type and half of these were artificially contaminated with diesel oil. Oil additions were performed as described in III, adding the diesel oil to sand and mixing the sand with the mesocosm soil. The amounts of diesel additions were calculated to reach equal concentrations per unit of organic matter in the soil. Diesel oil hydrocarbon concentration was analyzed as described in II and III, according to method ISO 16703. Organic matter and pH were analyzed using the same methods as in III (Table 4). Sampling was performed as described in III at four time points, at the beginning of the experiment, and on days 56, 84 and 140 of the experiment.

3.3. Measurements of volatile organic compounds (VOCs)

VOCs were expected to be diesel origin, and were measured in the mesocosms experiment III (data not included in III) when sampling. A hand-held air monitor and photoionization detector, portable

VOC-detector (Photovac Microtip MP-100) measured the amount of VOCs in the air close to soil.

3.4. Biological and chemical analyses

A general survey of materials and methods is listed in Tables 5 - 7; the precise description of each experimental set-up and method was given in the original articles (I, II and III). The microbiological and molecular techniques and their purposes are collected in Table 5, the physical and chemical methods in Table 6, and ecotoxicological tests (only in I) in Table 7.

Table 3. A summary of experimental set-ups. BS = biostimulation, BA = Bioaugmentation, MNA = monitored natural attenuation

Set up	Article	Sorption	BS	BA	MNA
Laboratory, water mesocosms	I	+	+		+
Laboratory, soil columns	II		+	+	
Field, pile treatment	II		+		+
Laboratory, soil mesocosm	III				+

Table 4. Soil parameters in the additional natural attenuation experiment: pH, organic matter (OM) content (g g^{-1} dw) and the amount of added diesel (per mesocosm).

	OM		pH		
Soil type	**Mean**	**SE**	**Mean**	**SE**	**Diesel addition**
Clay	0.54	0.00	4.8	0.1	10 - 21 ml
Peat	0.98	0.00	3.5	0.0	14 – 18 ml
Organic forest soil	0.54	0.12	3.6	0.2	10 – 16 ml

Table 5. Microbiological and molecular techniques used in this thesis. PCR = polymerase chain reaction, DGGE = Denaturing gradient gel electrophoresis. More precise descriptions of methods are presented in I-III.

Analysis	Article	Purpose of the method	Reference/ Manufacturer
DNA-extraction	II, III	To extract DNA from soil or bacterial culture as a first step in determining bacterial presence in samples	II: FastDNA® SPIN kit (Q-BIOgene Inc, Carlsbad, U.S.A.) III: MoBio PowerSoil™ DNA-extraction kit (soil) Ultra-Clean Microbial DNA Isolation kit (culture) (MoBio, USA)
PCR	II, III	To amplify bacterial DNA	Muyzer et al. 1993, modified, Muyzer and Smalla 1998, Ishii et al. 2000, modified
Nested PCR	III	To amplify the DNA of *Pseudomonas* bacteria	Widmer et al. 1998
Cloning of bacterial 16S rRNA genes	II, III	To isolate marker DNA products in order to identify bacterial species or groups	QIAGEN PCR Cloning plus Kit, Qiagen Inc., USA
DGGE	II, III	To divide the DNA PCR products according to sequence in variable regions, and to enable comparison of bacterial communities	Muyzer and coworkers (1993, 1998, 1999) DCode ™ System (Bio-Rad Inc., Hercules, U.S.A.)
Enrichment cultures	I, II, III	To grow degrading microbes in liquid when given specific growth environment	M9-salts (Sambrook 2001) and diesel as substrate
Plating	I, II	To grow bacteria on agar plates from dilution series, to determine the amount of colony-forming bacteria present (CFU)	Hernesmaa et al. 2005 Laine et al. 1997
CO_2-production measurement	II	To measure microbial activity as seen by formation of CO_2	Geotechnical Instruments GA45 (Set Point Technology) – meter
Sequencing and sequence analyses	II, III	To identify the bacteria based on marker regions in the rRNA genes	sequencing (Institute of Biotechnology, University of Helsinki) Staden Package software

Table 6. Physical and chemical analyses used in this thesis. More precise descriptions of methods are presented in I-III.

Analysis	Article	Purpose of the method	Reference/ Manufacturer
oxygen (O_2) concentration	I, II	To determine oxygen concentration in water or soil	Water: YSI 52 Soil: Geotechnical Instruments GA45 (Set Point Technology) – meter
pH	I, II, III	To determine acidity or alkalinity of water or soil	Water: Scientific Instruments, IQ150 Soil: Metler Toledo MP 220 pH meter
Nutrients C, N, P, silicates N, P	 I II, III	To determine soil or water nutrient levels and / or specify the type of nutrients present	N: SFS 5505 and SFS-EN 13342 P: with ISO 11885 (1996)
moisture content	I, II, III	To determine soil water content	I: HR73 Halogen Moisture Analyzer II,III: oven heated
loss on ignition	II, III	To determine the amount of C in the soil	UMEGA Heating system oven
oil hydrocarbon concentration	I, II, III	To determine the oil hydrocarbon concentration (C9 – C40)	standard ISO16703
temperature	I, II, III	To determine the temperature in indoor or outdoor experiment	
VOC measurements	thesis	To determine the volatilization of organic compounds in different soil types	Photovac Microtip MP-100, see chapter 3.3

Table 7. Ecotoxicological tests for the effects of diesel spill in the Baltic Sea water mesocosm experiment. More precise descriptions of analyses are presented in I.

Analysis	Article	Purpose of the method	Reference/ Manufacturer
mussel	I	To observe the changes in mussels after diesel spill	Howard et al. 2004 Light microscope
chlorophyll-*a*	I	To determine the biomass of phytoplankton	SFS 5772, 1993

4. RESULTS

4.1. Diminishing diesel concentration in the Baltic Sea water mesocosms (I)

The changes in oil concentration were measured from water samples after artificial diesel oil spill in three sets of Baltic Sea water mesocosms experiments (set of experiments A, B and C). Oil hydrocarbon concentrations were influenced by different treatments (1 - 4), where in treatment 1, diesel and cotton grass were added to water, and in treatment 2, diesel and cotton grass were added, but cotton grass was removed after 24 hours. In experiment B, supplementary N and microbial consortium inoculum were added in treatment 2, but these were not added in experiments A and C. Oil concentrations diminished radically when cotton grass was added (Fig. 4). In treatment 3, where diesel was added but cotton grass was not used as a sorbent material, oil concentrations remained high through the whole experiment time. In treatment 4, the control, oil concentrations remained at the natural low level throughout the experiment. The efficiency of the cotton grass oil sorption was determined on the basis of the oil concentrations remaining in the water. The sorption was clearly fast, because the oil concentration diminished strongly in mesocosms where cotton grass was removed after 24 hours. Moreover, cotton grass could hold the oil even if it was not collected from the basins, as shown by the reduction of oil content in treatment 1, where cotton grass remained in the basins but the oil had no toxic effect on the indicator organisms, the mussels. Additionally, cotton grass not only absorbed the diesel but also provided a large colonizable surface area for bacteria as well as a large oil-microbe contact area that benefited the bacterial growth.

Part of the diesel evaporated from the water of the mesocosms. Water circulation was slow and therefore a thin oil layer remained on the surface during the experiment. This film was clearly visible in treatment 3, diesel addition with no removal. In this treatment, diesel oil toxicity was demonstrated by the 100% mortality of the mussels (Fig. 3). Toxicity and oil concentration decreased in water if diesel oil was absorbed to cotton grass fiber (Fig. 3), and 0% mortality of mussels was observed.

Phytoplankton biomass was determined by chlorophyll *a* concentration. In general, the biomass of phytoplankton decreased in all basins (also in control treatments), apparently as a result of altered conditions, such as lower light intensity in the indoor mesocosm laboratory, compared to natural conditions in the Gulf of Finland. In the cotton grass treatments, both phytoplankton and microbial levels remained higher than in other treatments. Growth of phytoplankton was even more abundant when microbial inocula were added. Algal growth increased after diesel additions.

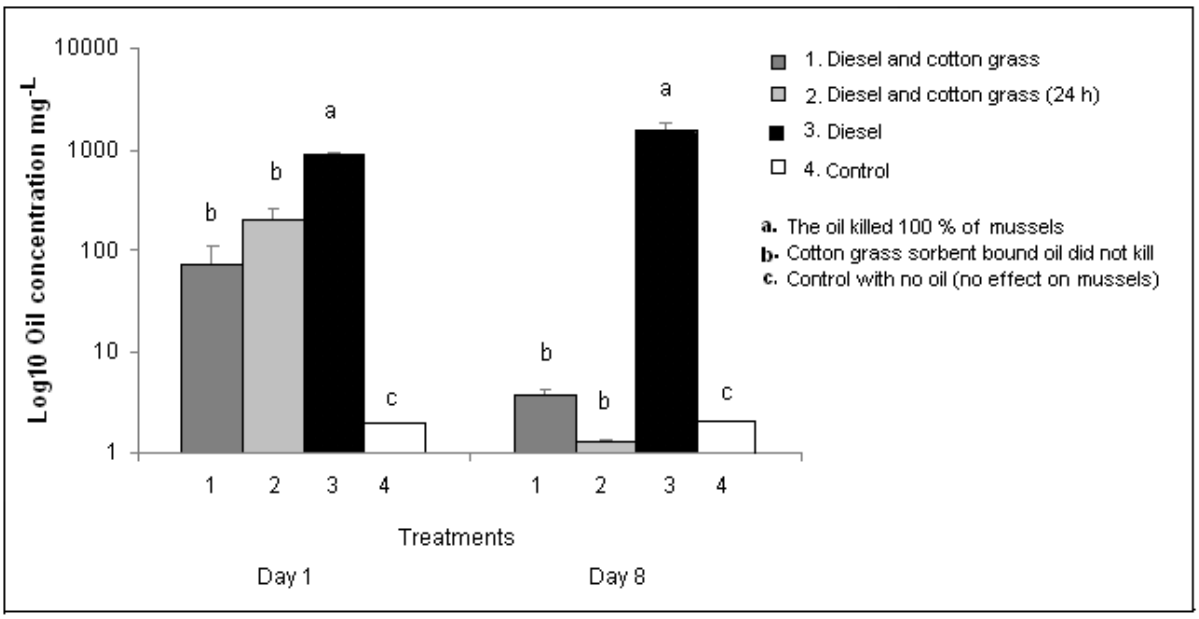

Figure 4. Results of the Baltic Sea water experiment. Oil concentration in the water mesocosms in experiment set A on days 1 and 8. Oil killed the mussels (a) in the basins where oil was added, treatment 3, but not if oil was removed using cotton grass as a sorbent material (b) in treatments 1 and 2. No oil was added to control treatment 4 (c). (Based on I)

4.2. Enhancing diesel biodegradation (I and II)

Addition of enrichment cultures of diesel degraders did not significantly enhance oil hydrocarbon degradation when sea water (I) or soil (II) was bioaugmented. Instead, an increase in phytoplankton and microbial CFU:s in diesel treated water indicated that a limiting resource was carbon, rather than a lack of oil degrading strains (I).

In the treatments where a consortium of microbes was added into soil columns, the concentration of oil hydrocarbons in the soil reached slightly lower levels than in treatments without microbial addition (II, Fig. 5). The difference, however, was not significant. In general, the combination of addition of slowly released N (methylene urea) and improvement of aeration was beneficial for degradation. When a bulking agent was not added, the soil was very dense, and nutrient or microbial additions did not enhance oil degradation. Forced air created channels in the soil and travelled through them, when soil permeability was not improved by adding a bulking agent. In dense soil, aeration did not equally reach the whole soil column.

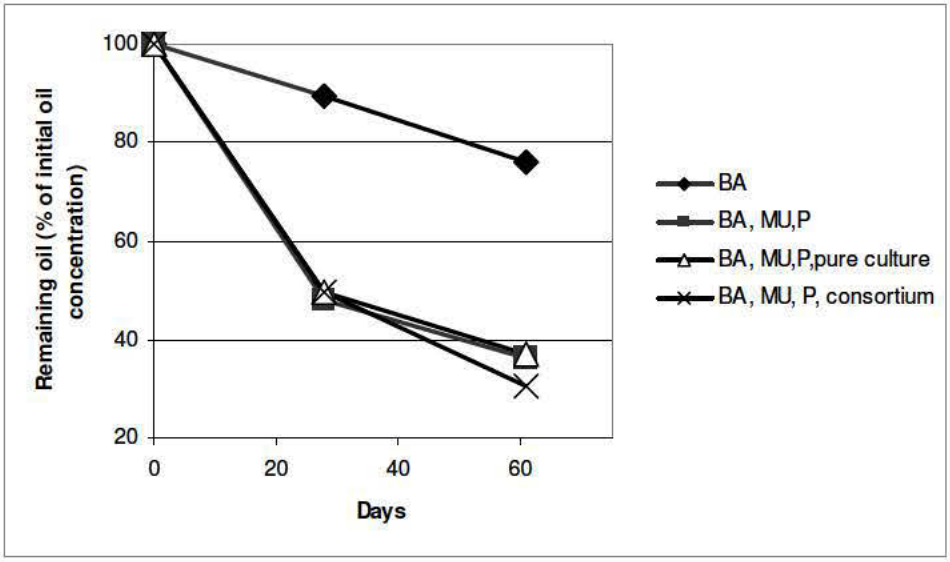

Figure 5. Oil concentration of soil in the laboratory experiment of biostimulation and bioaugmentation. Oil hydrocarbon concentration as a percentage of initial concentration in diesel-contaminated soil treated by adding wood chips as bulking agent (BA); by adding methlylene urea (MU) and phosphorus (P) as nutrients; and microbial inoculum from a pure culture or consortium. All treatments had forced aeration. SE's of original oil concentrations were 187 mg kg^{-1} dw (day 28) and 88 (day 61) in control and 14-38 (day 28) and 30-47 (day 61) in treated soil. (Based on II)

4.3. Natural attenuation in various Finnish soils (II, III and data from additional experiment)

In the original data presented in this thesis (not included in III), on the additional mesocosm experiment of natural attenuation in diesel treated boreal soils, degradation of added diesel oil was observed in all tested soil types (Fig. 6). The diesel oil was added similarly as in III, except the amount of addition was done according to OM concentration in soil, which caused a substantial variation in oil hydrocarbon concentrations (peat 10 200 mg kg^{-1} dw, forest humus 12 000 mg kg^{-1} dw, clay 2250 mg kg^{-1} dw on average of oil concentration) when calculated on a dry weight basis at the beginning of the incubation. Considerable heterogeneity of oil dispersion in the soil was also observed when oil was analyzed twice in a short time period, as shown by the existence of some increases in oil concentration between sampling times. The variations in the concentrations did not allow reliable statistical testing. Peat samples without diesel addition also showed relatively high oil concentrations of oil or oil-like substances as compared to forest humus or clay (2100 mg kg^{-1} dw, 280 mg kg^{-1} dw, 10 mg kg^{-1} dw, respectively). Oil concentrations diminished in all soils in a similar way, but apparently faster in clay than in forest humus or peat. Analysis of oil hydrocarbon concentrations gave naturally high readings in peat soil samples. None of the soils reached the oil hydrocarbon concentration 300 mg kg^{-1}, which is the lower limit for light oil

hydrocarbon concentration in contaminated soil according to Finnish decree (214/2007).

In the field experiment, oil concentration diminished in loamy clay soil with no additions, i.e., as a result of natural attenuation (II). When pristine and previously contaminated soils were tested in the mesocosm experiment (III), the results revealed that a history of previous contamination was an advantage for the oil degradation efficiency, i.e., that the oil hydrocarbon concentration decreased faster in previously contaminated soil than in pristine soil (Fig. 7). The decrease in diesel oil hydrocarbon concentrations of both the previously contaminated soils tested (mineral and organic forest humus) was significantly faster compared to comparable soils that had no previous oil exposure. Additionally, in pristine organic conifer forest humus soil, the oil concentrations decreased faster than in pristine mineral soil.

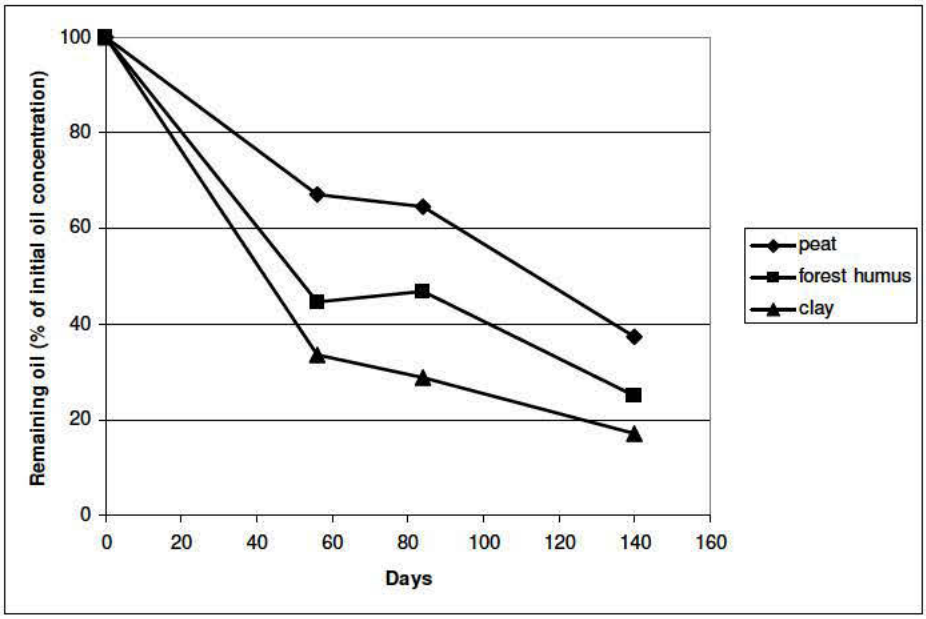

Figure 6. Oil concentration of soil in the additional mesocosms of NA experiment. Oil hydrocarbon degradation in peat, forest humus and clay soil during 140 days of incubation at 16°C following artificial contamination with diesel. Oil concentrations are presented as percentages of the initial value. SE's (mg kg^{-1} dw) of the concentrations were in peat 118-1985, in forest humus 430-577 and in clay 128-761 (Data presented in this thesis).

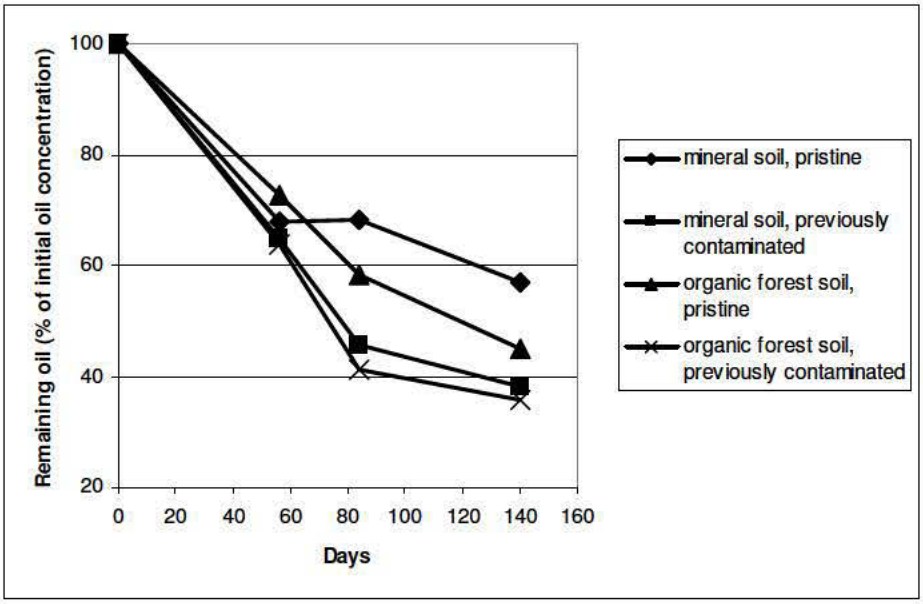

Figure 7. Oil concentration of soil in the mesocosms of NA experiment. Oil hydrocarbon degradation in mineral and organic forest humus soils, both pristine and previously contaminated during 140 days of incubation at 16℃, after artificial contamination with diesel. Oil concentrations are presented as percentages of the initial value. SE's (mg kg^{-1} dw) of original concentrations were 54-150 in mineral pristine, 93-155 in mineral previously contaminated, 143-434 in forest pristine and 49-178 in forest previously contaminated soil. (Adapted from III).

4.4. VOC analyses

VOCs were measured six times from the mesocosms experiment soils. In mineral soil, evaporation of VOCs was higher than in forest humus soil in the experiment presented in III (data not included in III, Fig. 8). Evaporation was greater at the beginning of the experiment in all soil types. The lowest concentrations of VOCs were observed in previously contaminated organic forest humus soil.

VOCs were measured only once from the natural boreal soils experiment (data presented in this thesis) and the means (±SE) were of peat 42 (±3.5), forest humus 50 (±6.4) and clay 31 (±1.0).

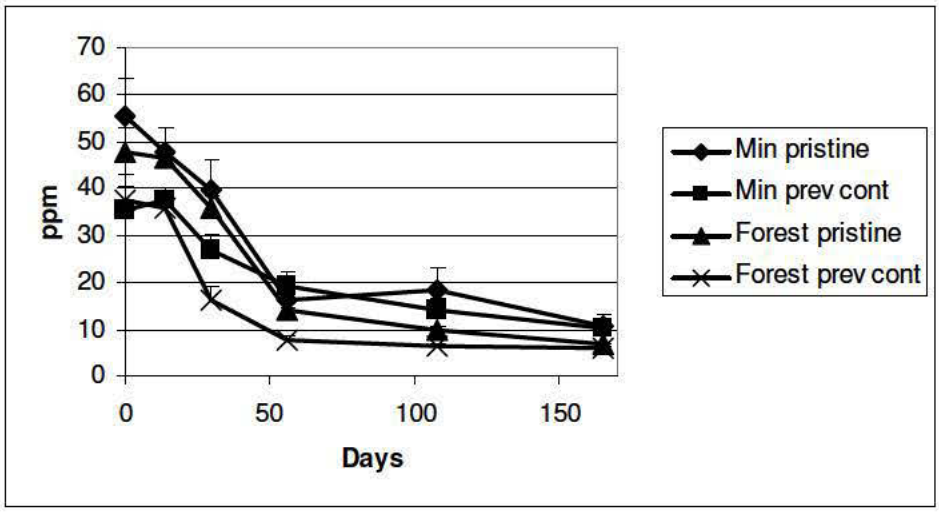

Figure 8. Mesocosms experiment VOC analyses. Evaporation of VOCs in diesel added mesocosms during incubation at 16°C (additional analyses for III), mean values and SE's (ppm).

4.5. Bacteria in soil and water (I, II and III)

The banding patterns on DGGE gels differed between the samples of different treatments (III). These banding patterns, used as an indicator of community composition, showed that contamination drove the selection of bacterial genera in a specific direction, whereas the pristine soils showed a rather random pattern of the specific group (III). Soil heterogeneity was observed in unique banding patterns of the replicates and studied soils, in pristine soils and when diesel was added. That is, diesel addition did not increase the similarity of banding patterns between different soil types (III). Furthermore, diesel addition increased the number of noticeable bands in DGGE-gels in pristine soil samples, which indicates that a few bacterial groups grew in size. On the other hand, some bacterial groups could have been sensitive to diesel, so their abundance diminished.

Oil-degrading bacteria were found in all soils used in the experiments (II, III). DGGE focuses on dominant species, and detected *Proteobacteria* as dominant species in all soil types (II, III). Cloning of 16S rRNA genes revealed some rare species and confirmed the dominance of the *Proteobacteria* species (II).

Bacterial density was highest in diesel-treated water mesocosms (I). Addition of nutrients (N) and microbes (consortium) did not significantly increase the abundance of bacteria in water.

In soil experiments, the maximum abundance of bacteria was found on plates when both nutrients and diesel were available (II, Fig. 9). The abundance of bacteria started to decrease sooner, when rapidly released N was used instead of slowly released N (II, Fig. 9). Bacteria did not benefit from nutrient addition or bioaugmentation if bulking agent was not added to soil (II, Fig. 9).

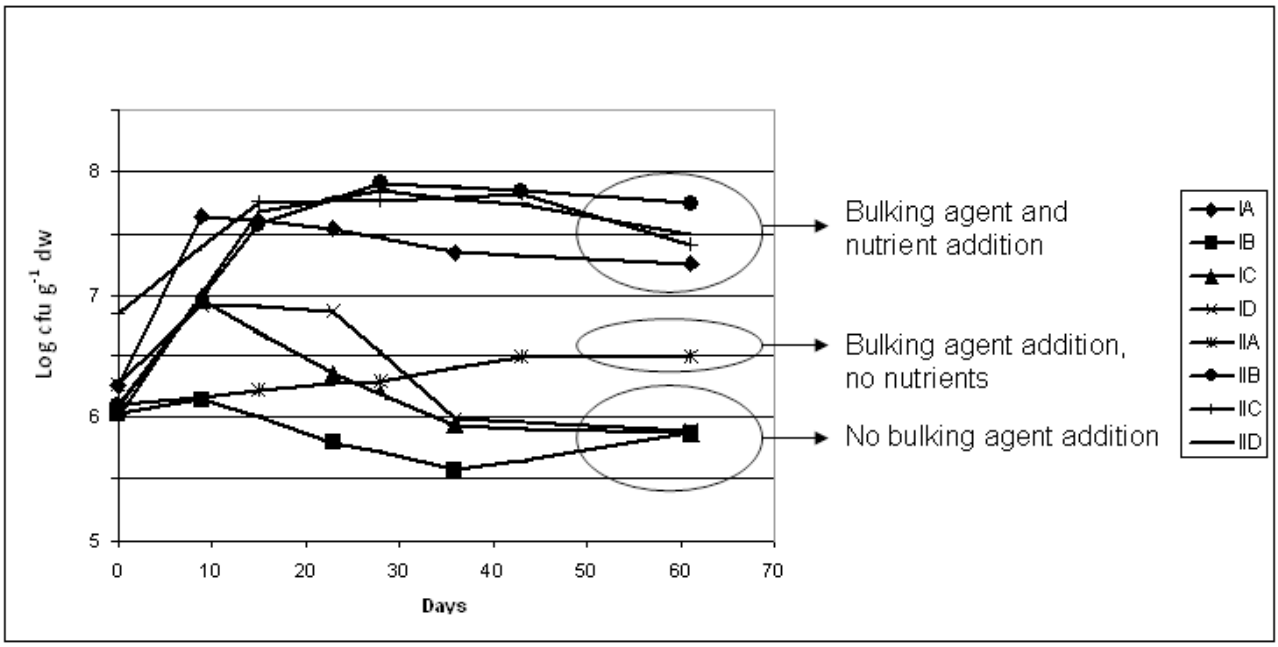

Figure 9. Bacterial density (plating results) in soil treated with bulking agent (IA, IIA-IID), nutrients (IA, IC, ID, IIB-IID), and enrichment cultures (ID, IIC, IID). In nutrient-treated soils, rapidly released N was added in treatment IA, and slowly released nitrogen in the others. Treatment IB had no amendments. Treatements: IA; BA+U+P, IB; no amendments, IC; MU+P, ID; MU+P+consortium, IIA; BA, IIB; BA+MU+P, IIC; BA+MU+P+ pure culture, IID; BA+MU+P+ consortium. Abbreviations: BA = bulking agent, MU = methylene urea, slowly released nitrogen, P = phosphorus, U = urea, rapidly released N (Based on II).

5. DISCUSSION

5.1. Diesel hydrocarbon degradation by microbes

Microbial ability to degrade diesel hydrocarbons was observed in soil and water. Diesel degraders were found in all tested soil types and increase of bacterial growth was discovered in diesel amended Baltic Sea water (I, II and III). Therefore, the conclusion could be drawn that oil-degrading bacteria are ubiquitous in Finnish environments, representing boreal climates. Similarly, Margesin et al. (2003) suggested the ubiquity of hydrocarbon-degrading microbes in a cold climate, because oil-degrading bacteria and yeast were found in alpine, Siberian and Antarctic environments and some of these were from pristine soils. Moreover, diesel oil served as the sole carbon source for bacteria enriched from the terrestrial environments (I, II and III). Therefore, a diesel oil spill enhanced growth of diesel-degrading bacteria and thus caused changes in bacterial community composition (I, III).

Soil is a very heterogeneous environment, which was reflected by the high variability in bacterial community composition between replicates of pristine soils and also in the variation of bacterial quantities between the replicates and samples (III). Environmental factors, especially availability of C, N and P had a great influence on bacterial growth in both water and soil (I, II).

Bacterial community structure in soil changed after contamination, and the communities in different soil types differed, as suggested by Bundy et al (2002). Thus, diesel oil contamination does not define the bacterial community composition, but it does influence the change in it. It can be concluded that the resulting community originates from the indigenous bacteria present in soil (III). Each soil type had a different bacterial population and hence different responses to contamination (III).

It was not possible to identify individual bacterial species as being particularly important for diesel degradation (I, II). The DGGE method revealed the changes in the bacterial community composition but when using universal bacterial primers, only few of the sequences could be identified, because of the double sequences in one band in the gel. Notably, the DGGE method is not quantitative. Use of qPCR along with plating could provide better information on the relative numbers of bacteria of each species.

Indigenous bacteria in conifer forest humus soil were capable of degrading diesel oil hydrocarbons faster than bacteria in mineral soil (III). This result is in agreement with earlier findings on biodegradation of organic compounds in conifer forest soil (Koivula et al. 2004, Penet et al. 2006, Vauramo et al. 2011). It was suggested that soil bacteria adapt to

degrade organic contaminants in humus soil, possibly because of high concentrations of natural organic compounds in humus (Sarand et al. 2001). Thus, faster oil hydrocarbon degradation in conifer forest humus soil could be a result of bacteria being already adapted to degradation of aliphatic and aromatic compounds structurally or chemically similar to those found in oil. Considering the driving force for adaptation, Wright (2004) reviewed the mechanisms of adaptive mutations including stress-directed mutagenesis, which may provide advantages for bacteria capable of utilizing such mechanisms. Mutations could be directed to specific genes if mutations are directed by stress (Wright 2004). In this way indigenous bacteria may be capable of adapting their catabolic capacity towards degradation and utilization of diesel oil as a normal growth substrate (Chaineau et al. 1999, Romantschuk et al. 2000, Margesin et al. 2003, Aislabie et al. 2006). Furthermore, the Baltic Sea water microbes adapted to degrade oil hydrocarbons and this could be explained by stress related adaptation. Since microbes suffer from starvation, and salinity changes create a stressful environment for them, elevated mutation rates of bacteria could lead to adaptation (Bonsdorff and Pearson 1999, Kivisaar 2003). Therefore, bacterial adaptive evolution can be accelerated, by stress reactions caused by contaminants, the result being that bacteria adapt to degrade also organic compounds from an anthropogenic source (Poole et al. 2003, Gianfreda and Rao 2008).

5.2. Enhancing bioremediation

The availability of oxygen and inorganic nitrogen are important for bioremediation in terrestrial habitats (Allard and Neilson 1997). Addition of a bulking agent improves the aeration in soil, thus enabling higher oxygen levels throughout the soil and hence initiating efficient bacterial activity (II, Gea et al. 2007, Jørgensen et al. 2000). Forced aeration and bulking agent together can dry the soil, decreasing its bacterial activity (II). Bulking agent alone could be sufficient for increasing the oxygen level enough to stimulate bacterial activity and enhance degradation. The best results in biostimulation of diesel oil contaminated soil were achieved by combination of slowly released N and improved aeration (II).

Diesel oil was degraded in all tested soil types, so bioaugmentation might be unnecessary, as also noted by Herwijnen et al. (2005) and Margesin and Schinner (1999). In addition, Sanchez et al. (2004) noticed that even good, laboratory-grown degraders seldom survive in soil for long after inoculation. Oil hydrocarbon degradation was not significantly improved by microbial addition, even though the oil concentrations were slightly lower in environments where bacterial consortium were added (I, II). Therefore it is concluded that in larger-scale treatments, the benefits of microbial addition would not repay the financial input. Bioaugmentation often gives poor results in enhanced bioremediation, because of the lack of niche fitness of introduced bacteria (Thomassin-Lacroix et al. 2002, Yergeau et al. 2009). It is not necessary to introduce

new laboratory-grown species to remediate soil. For successful bioremediation, the aim is to maximize the degradation ability of the bacteria already present in soil and this can be accomplished by improving their growth conditions. In certain circumstances, bacteria are able to transfer genes horizontally, and the possibilities for this can be enhanced by adding soil containing bacteria that are already capable of oil degradation, either innately or previously adapted by earlier exposure. Therefore, if inoculation is needed, the best choice would be to use soil as an inoculum, as also suggested by Gentry et al. (2004). The introduced bacteria are then more likely to survive long enough for gene transfer.

A history of previous contamination enhanced the rate of diesel oil degradation (III). This indicates that earlier adaptation of the bacterial community towards diesel oil utilization capacity was still present in the soil. When pristine soil is freshly contaminated with diesel oil, bacterial adaptation to degrade the oil hydrocarbon compounds may be time-consuming (Romantschuk et al. 2000). Therefore addition of previously contaminated and remediated soil could enhance the bacterial adaptation and the degradation process (III, Greenwood et al. 2009). Bacteria that are already adapted to utilization of oil hydrocarbons can start the degradation immediately. Since the indigenous microbes in organic conifer forest humus soil were able to degrade diesel oil hydrocarbons rapidly (III), and since an inoculum could enable horizontal gene transfer to bacteria present in the contaminated site, addition of conifer forest humus soil could enhance the degradation.

Survival of indigenous species present in the soil used as an inoculum is likely to be better than using microbial pure cultures as an inoculum. The degrading bacteria present in the introduced soil may either themselves perform well in the contaminated site, or transfer their contaminant degradation genes (residing on plasmids or transposons) to bacteria present in the contaminated soil, thereby generating efficient, locally adapted degraders. In practice, the pathway does not matter, as the desired result is to introduce the degradation ability, not a certain bacterial strain. The use of conifer forest humus soil as a potential inoculum would have the additional advantage that there would be no risk of introducing residual quantities of contaminants, as might be the case if previously contaminated soil was used (Laine and Jørgensen 1996, Greenwood et al. 2009). Atlas et al. (1981) stated that genes involved in the degradation process of hydrocarbons are often plasmid-borne. Since plasmid DNA in many cases can be transformed between bacterial cells by conjugation, the rate of spreading of such plasmid-encoded degradation-gene clusters may be quite efficient (Sarand et al. 2001).

5.3. Influence of soil type on decreased diesel oil hydrocarbon concentrations

Soil type *per se* had an influence on bacterial community composition (III). Each soil type harboured indigenous

bacteria, as shown by the unique banding patterns in DGGE gels (III). Soil pH, nutrient level, pore size and other physical and chemical factors influence microbial populations and chemical processes in soil (Margesin and Schinner 1999). Interactions between microbes, such as mutualism and antagonism, affect populations and biodegradation of organic compounds (Sarand et al. 2000). In terrestrial environments, evaporation of VOCs was observed from all soil types, especially at the freshly polluted site (III and data presented in this thesis). Later, the decrease in oil concentration was likely to be more a result of microbial degradation than volatilization. Binding to particles was assumed to play a role, but could not be measured. Nevertheless, it is assumed that at least 20 – 30% of the loss of diesel from soil is abiotic (Margesin et al. 2007). In conclusion, microbial degradation and various chemical processes affect the decrease of diesel oil hydrocarbon concentrations in soil. Evaporation of VOCs seemed to be fastest in mineral soil, possibly because of the larger pore size and smaller surface area than in other soils. Clay, on the other hand, binds organic compounds (Gianotti et al. 2008), so bioavailability may be lower in clay than in organic soil. In clay, diesel concentration decreased effectively (data presented in this thesis). Nevertheless, part of this decrease could be caused by binding to soil particles, preventing extraction. The amount of VOCs was also lowest in clay, which indicates that hydrocarbons could have bound to soil particles rather than evaporating.

Diesel oil hydrocarbon concentration diminished naturally in Finnish soil types, which was statistically demonstrated in mineral and in organic forest soils and indicated in peat and clay soils (III and data presented in this thesis). Concentrations of oil hydrocarbons, or compounds chemically close to oil hydrocarbons, were naturally high in peat soil. Nevertheless, the decrease of added diesel oil hydrocarbon concentration was slow in peat (data presented in this thesis). Ghaly et al. (1999) used peat addition as a method to absorb diesel oil from soil and combusted it afterwards. Perhaps peat could be more useful as a sorption material for diesel oil contamination than for advancing biological degradation. The amount of VOCs in peat samples was quite high, so not all of the decrease in oil concentration was due to microbial degradation (data presented in this thesis). A low degradation rate in peat soil could be explained by low soil pH, which apparently inhibits the biological degradation (data presented in this thesis). Notably, the conclusions of the additional natural attenuation experiment using natural pristine soils are only indicative, because the results of these oil hydrocarbon analyses were not tested statistically.

5.4. Managing natural attenuation and conifer trees in urban structural design

When the decision of a suitable remediation method is made, the possibility to utilize natural attenuation should be considered, as suggested by EU

directive (2004/35/CE), although in practice natural attenuation and bioremediation are seldom chosen options. Nevertheless, natural attenuation or enhanced bioremediation are sustainable and economical methods for cleaning oil hydrocarbon contamination *in situ*. These methods have been used *in situ* to remediate oil contaminated soil, mostly in experimental cases such as the SOILI-program in Finland (Öljyalan keskusliitto 2011). The knowledge about sensible use of natural attenuation could lower the costs of remediation of oil-contaminated soil. The local authorities and consulting companies, who are dealing with polluted soils, could benefit from more specific instructions on how natural attenuation and enhanced bioremediation can be used as remediation methods. Finland's environmental administration is processing a web page information sheet of various remediation techniques (A.-M. Pajukallio, Ministry of the environment, pers. comm. 28.2.2011, J. Reinikainen, Finnish Environment Institute, pers. comm. 4.3.2011). This information should help the local environmental authorities to make valid case-by-case decisions based on data regarding each specific site. The information will also assist authorities in setting the requirements for the procedures and permits. Using such an approach, the best available remediation technique can be chosen for each specific site.

The need to manage an increasing amount of contaminated sites in urban areas is inevitable in the future. Notably, conifer forest humus soil microbes possess an excellent ability for degradation of organic compounds (III, Sarand et al. 1999, Sarand et al. 2000, Koivula et al. 2004, Penet et al. 2006, Vauramo et al. 2011). Therefore, possibly in order to ensure the availability for degradation activating soils, conifer trees could be used more frequently in urban structural design. Soil beneath the conifer trees could hypothetically build a barrier against spreading of diesel oil and other organic soil contaminants. Conifer trees could surround the yard, for example at the sites where low-level surface drifting is possible, such as fuel stations. In case of larger spills, however, major remediation actions would still be required. In addition, conifer trees would have esthetic value all year around in urban areas.

5.5. Oil concentration and toxicity of diesel

Toxicity of the environmental contaminant is one of the primary reasons for remediation work. Diesel spills constitute a world-wide problem because of the toxic compounds in the oil. In the Baltic Sea water mesocosms experiment, water circulation caused weathering of diesel oil and mixed it with the water, even though the spill was still visible on the surface (I). The water toxicity after the diesel spill was evident according to the results of the mussel survival test, when all survived when there was no oil or when it was absorbed into cotton grass fibre immediately after the spill (I). Since sessile benthic animals cannot escape the toxicity of diesel, rapid removal of the oil is necessary to protect them as also stated by Roth and Baltz (2009).

Oil addition is known to inhibit algal growth (Fiala and Delille 1999), but phytoplankton growth can also be limited by nutrients (Wersal and Madsen 2011). Phosphorus is one of the limiting nutrients in the Baltic Sea waters (Sandberg et al. 2000). This could explain the decrease of phytoplankton in all basins, because no P was input during the experiments (I). Microbial activity was enhanced by adding diesel and the growth of microbial abundance was observed in the plating results (I). Nayar et al. (2005) concluded similar results and observed the increased abundance of heterotrophic bacteria after an oil spill. Bacterial activity depends on carbon and nutrient (N and P) availability and limitations of these affect bacterial community composition (Teira et al. 2010). In conclusion, diesel oil was not toxic to all algal or bacterial species in the Baltic Sea water. On the other hand, oils can remain in phytoplankton cells and cause them to sink to the bottom (Nayar et al. 2005). Moreover, oil could diminish the growth of algal species, because the thin layer of oil on the water surface decreases the light in water (Gonzales et al. 2009). Added cotton grass had most likely the same effect. Consequently, a diesel spill changes the community composition of algae and bacteria, and diesel served as a C source for some microbes and this possibly enhanced the growth of algal species.

Aged spills contain less light, short-chain, low molecular weight hydrocarbons, because of their volatility, while long-chain hydrocarbons are less soluble and hence less bioavailable (Dorn and Salanitro 2000, Schafer et al. 2009). Moreover, because light oil contains a relatively high proportion of saturated hydrocarbons, it can be more toxic than heavy oils (Dorn et al. 1998). Therefore fresh diesel spills are the most threatening for water and soil ecosystems, and rapid actions for clean-up are necessary. Cotton grass absorbed the diesel oil and benefitted the bacterial growth by providing a large colonizable surface area, so bioremediation of diesel oil spill could be enhanced when cotton grass was used as a sorption material.

Cotton grass fiber is an excellent sorbent material for small diesel oil spills, and could limit the impact of oil spills if used before the oil spill meets the shoreline. The cotton grass fiber is most likely easy to collect with the absorbed oil, thereby avoiding contamination of the shoreline. Nevertheless, this method requires more research and should be tested in scale-up experiment at field conditions in order to demonstrate the benefits of it in Baltic Sea conditions.

5.6. Future prospects

The state of the Baltic Sea remains a constant concern, partly because of the strong growth in oil transportation. The sensitive nature of the Baltic Sea needs protection by international co-operation in planning the transport routes and in remediation after an oil spill. Joint action of the catchment-area countries and the responsible partners in oil transportation is essential, along with governmental guidance. More research is needed, in order that rapid action would be possible in the event of a major oil spill. Cotton grass fibre sorption needs to be tested on a field

scale. It would be especially important to study how to spread cotton grass fibre onto oil-polluted water and how to collect this kind of sorption material from the water or shoreline.

Natural attenuation and bioremediation are both possible techniques for remediation of diesel-contaminated soil in Finland. To diminish the costs of the remediation, and therefore possibly enable more sites to be cleaned, these sustainable methods could be used more often in the future. Using these methods instead of excavation and mass change could diminish the emission of CO_2 during remediation.

Diesel is naturally degraded in boreal soil by bacteria, but no specific strains can be identified as responsible for degradation. Future research could focus more on degrading ability than on bacterial species, and qPCR would be the tool for this, although primer development will be a challenge. In addition, research on fungal populations and their degradation abilities should be studied, especially considering conifer forest soils, which are typically acidic and therefore favourable for fungal species.

6. CONCLUSIONS

According to the findings in this thesis, bioremediation and monitored natural attenuation are both suitable techniques for remediating diesel oil contaminated sites in Finland.

In conclusion:

- *(a) remediation of diesel oil hydrocarbons diminishes the harmful effect to species and biodiversity*; the use of cotton grass fibre as a sorbent material decreased toxicity of diesel oil hydrocarbons, which is especially important in the Baltic Sea, where the relatively small volume of water limits the dilution of toxins;
- *(b) biostimulation enhances bioremediation;* particularly, using slowly released N in aerobic conditions increased bioremediation in soil;
- *(c) diesel-degrading bacteria are found in various environments and soil types in the boreal zon*e, so microbial inocula are not needed;
- (d) when microbial inocula are necessary to initiate the degradation of diesel, *previously contaminated and remediated soil or conifer forest humus soil could be used as inocula.*

7. ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to my supervisors Professor Martin Romantschuk, Professor Rauni Strömmer and Dr. Aki Sinkkonen. Especially, I thank Martin for creating inspirational working environment and developing my interest in research during my Master thesis studies. I am deeply thankful that he provided an opportunity for me to participate in several interesting research programs. Discussions about research questions, possibilities or problems of a current task have always widened my understanding. I thank Rauni for having the patience to read and comment on my writing and giving new viewpoints. I thank Aki for employing me on his project and teaching me how things are managed. I thank all co-authors for successful teamwork. I deeply thank all my friends and colleagues in Lahti for creating a wonderful working environment in joint activity of finding something new in each research project and understanding more about our environment, or simply managing the daily issues. You helped me whenever I needed help. I really enjoyed the fellowship of the PhD students in Lahti. I wish to acknowledge each specialist who kindly answered to my questions about oil pollution or remediation and gave me permission to refer our conversations in this thesis. Thank you, Kirsi Niemissalo for your assistance in presenting my thesis in Finnish. I thank Dr. Fred Stoddard, for commenting my thesis. The EnSTe graduate school provided me the best knowledge at their courses and good opportunities to meet many interesting people. The Marjatta and Eino Kolli Foundation, Finnish Cultural Foundation, Kymin osakeyhtiön 100-vuotissäätiö and University of Helsinki are acknowledged for the financial support of my thesis.

I thank my friends and family for the interest in my work and for filling my life outside the university with something totally different. I thank Virpi Ruusunen, because of our journey to the world of novels and music has given me much entertainment during last years and I really hope to continue it with you in the future. Thank you, Taina Meriläinen, for giving me your advice and your time whenever I have asked for any. Päivi Sippu, thank you for including me in the social activities that you create in our lives.

My dear children, Janne, Sonja, and Joonas, I have continued with my studies to encourage you to do the same; to study something you enjoy. You have been the anchor in my life, even it might have seem that I have sometimes lost myself in typing more words and calculating more results for the research papers or the thesis. You are always my top priority. Jouko, your encouragement and belief in me have always been the most important foundation for any goal I reach or work I finish. All that I do, I do for you.

8. REFERENCES

Aislabie J., D. J. Saul, and J. M. Foght. 2006. Bioremediation of hydrocarbon-contaminated polar soils. Extremophiles **10**:171-179.

Aislabie J., J. Foght, and D. Saul. 2000. Aromatic hydrocarbon-degrading bacteria from soil near Scott Base, Antarctica. Polar Biology **23**:183-188.

Aislabie J., M. McLeod, and R. Fraser. 1998. Potential for biodegradation of hydrocarbons in soil from the Ross Dependency, Antarctica. Applied Microbiology and Biotechnology **49**:210-214.

Allard A. S., A. H. Neilson. 1997. Bioremediation of organic waste sites: A critical review of microbiological aspects. International Biodeterioration & Biodegradation **39**:253-285.

Alvarez, P. J.J., and W.A. Illman. 2006. Bioremediation and natural attenuation - Process fundamentals and mathematical models. Hoboken, NJ, USA. ISBN 978-0-471-65043-0.

Alvim-Ferraz M. C. M., J. Albergaria, and C. Delerue-Matos. 2006. Soil remediation time to achieve clean-up goals II: Influence of natural organic matter and water contents. Chemosphere **64**:817-825.

Annunciado T. R., T. H. D. Sydenstricker, and S. C. Amico. 2005. Experimental investigation of various vegetable fibers as sorbent materials for oil spills. Marine Pollution Bulletin **50**:1340-1346.

Atlas R. M. 1981. Microbial-degradation of petroleum-hydrocarbons - an environmental perspective. Microbiological Reviews **45**:180-209.

Atlas R., R. Bartha. 1997. Microbial ecology, fundamentals and applications. Benjamin/Cummins Science Publishing, California, U.S.A., 4th edition.

ATSDR. 1995. Fuel oils, chemical and physical information. The agency for toxic substances and disease registry, U. S. Department of health and human services.

Azadpour-Keeley A., J. W. Keeley, H. H. Russell, and G. W. Sewell. 2001a. Monitored natural attenuation of contaminants in the subsurface: Processes. Ground Water Monitoring and Remediation **21**:97-107.

Azadpour-Keeley A., J. W. Keeley, H. H. Russell, and G. W. Sewell. 2001b. Monitored natural attenuation of contaminants in the subsurface: Applications. Ground Water Monitoring and Remediation **21**:136-143.

Bacha, J., J. Freel, A. Gibbs, L. Gibbs, G. Hemighaus, K. Hoekman, J. Horn, A. Gibbs, M. Ingham, L. Jossens, D. Kohler, D. Lesnini, J. McGeehan, M. Nikanjam, E. Olsen, R. Organ, B. Scott, M. Sztenderowicz, A. Tiedemann, C. Walker, J. Lind, J. Jones, D. Scott, and J. Mills. 2007. Diesel fuels technical review. Chevron Corporation.

Baltic Sea Portal. 2011. http://www.itameriportaali.fi/en_GB/. http://www.itameriportaali.fi/en/ajankohtaista/itameri-tiedotteet/2009/en_GB/oljypaastot/ 3.1.2011.

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

Bayat A., S. F. Aghamiri, A. Moheb, and G. R. Vakili-Nezhaad. 2005. Oil spill cleanup from sea water by sorbent materials. Chemical Engineering & Technology **28**:1525-1528.

Belousova N. I., L. M. Baryshnikova, and A. N. Shkidchenko. 2002. Selection of microorganisms capable of degrading petroleum and its products at low temperatures. Applied Biochemistry and Microbiology **38**:437-440.

Bento F. M., F. A. D. Camargo, B. C. Okeke, and W. T. Frankenberger. 2005a. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiological Research **160**:249-255.

Bento F. M., F. A. O. Camargo, B. C. Okeke, and W. T. Frankenberger. 2005b. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresource Technology **96**:1049-1055.

Bonsdorff E., T. Pearson. 1999. Variation in the sublittoral macrozoobenthos of the Baltic Sea along environmental gradients: A functional-

group approach. Australian Journal of Ecology **24**: 312-326.

Boopathy R. 2000. Factors limiting bioremediation technologies. Bioresource Technology **74**:63-67.

Brakstad O. G., K. Bonaunet. 2006. Biodegradation of petroleum hydrocarbons in seawater at low temperatures (0-5 degrees C) and bacterial communities associated with degradation. Biodegradation **17**:71-82.

Bundy J. G., G. I. Paton, and C. D. Campbell. 2002. Microbial communities in different soil types do not converge after diesel contamination. Journal of Applied Microbiology **92**:276-288.

Calder J. A., J. H. Lader. 1976. Effect of dissolved aromatic-hydrocarbons on growth of marine-bacteria in batch culture. Applied and Environmental Microbiology **32**:95-101.

Carmody O., R. Frost, Y. Xi, and S. Kokot. 2007. Surface characterisation of selected sorbent materials for common hydrocarbon fuels. Surface Science **601**:2066-2076.

Chaineau C. H., J. Morel, J. Dupont, E. Bury, and J. Oudot. 1999. Comparison of the fuel oil biodegradation potential of hydrocarbon-assimilating microorganisms isolated from a temperate agricultural soil. Science of the Total Environment **227**:237-247.

Costa R., M. Götz, N. Mrotzek, J. Lottmann, G. Berg and K. Smalla. 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial quilds. FEMS Microbiology Ecology **56**: 236-249.

Coulon F., E. Pelletier, L. Gourhant, and D. Delille. 2005. Effects of nutrient and temperature on degradation of petroleum hydrocarbons in contaminated sub-Antarctic soil. Chemosphere **58**:1439-1448.

Dawson J. J., E. J. Godsiffe, I.P. Thompson, T. K. Ralebitso-Senior, K S. Killham, and G. I. Paton. 2007. Application of biological indicators to assess recovery of hydrocarbon impacted soils. Soil Biology & Biochemistry **39**: 164-177.

Delille D., F. Coulon. 2008a. Comparative mesocosm study of biostimulation efficiency in two different oil-amended sub-Antarctic soils. Microbial ecology **56**:243-252.

Delille D., A. Duval, and E. Pelletier. 2008b. Highly efficient pilot biopiles for on-site fertilization treatment of diesel oil-contaminated sub-Antarctic soil. Cold Regions Science and Technology **54**:7-18

Dorn P. B., J. P. Salanitro. 2000. Temporal ecological assessment of oil contaminated soils before and after bioremediation. Chemosphere **40**:419-426.

Dorn P. B., T. E. Vipond, J. P. Salanitro, and H. L. Wisniewski. 1998. Assessment of the acute toxicity of crude oils in soils using earthworms, Microtox (R) and plants. Chemosphere **37**:845-860.

El Fantroussi S., S. N. Agathos. 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Current Opinion in Microbiology **8**:268-275.

Eriksson M., J. O. Ka, and W. W. Mohn. 2001. Effects of low temperature and freeze-thaw cycles on hydrocarbon biodegradation in Arctic tundra soil. Applied and Environmental Microbiology **67**:5107-5112.

European parliament and the European council. 2004/35/CE, Annex II, Remedying of environmental damage.

FEA. 2007. Finland's environmental administration, Ministry of the environment, Maaperän pilaantuneisuuden ja puhdistustarpeen arviointi, ympäristöhallinon ohjeita 2/2007.

FEA. 2009. Finland's environmental administration. Pilaantuneilla alueilla tehdyt kunnostukset. http://www.ymparisto.fi/default.asp?contentid=342926&lan=FI (updated 10.12.2009) 4.2.2011

Fiala M., D. Delille. 1999. Annual changes of microalgae biomass in Antarctic sea ice contaminated by crude oil and diesel fuel. Polar Biology **21**:391-396.

Finnish law 214/2007. Valtioneuvoston asetus maaperän pilaantuneisuuden ja puhdistustarpeen arvioinnista.

Gea T., R. Barrena, A. Artola, A Sanchez. 2007. Optimal bulking agent particle size and usage for heat retention and disinfection in domestic wastewater sludge composting. Waste Management **27**: 1108-116.

Gentry T. J., C. Rensing, and I. L. Pepper. 2004. New approaches for bioaugmentation as a remediation technology. Critical Reviews in

Environmental Science and Technology **34**:447-494.

Ghaly R., J. Pyke, A. Ghaly, and V. Ugursal. 1999. Remediation of diesel-oil-contaminated soil using peat. Energy Sources **21**:785-799.

Gianfreda L., M. A. Rao. 2008. Interactions between xenobiotics and microbial and enzymatic soil activity. Critical Reviews in Environmental Science and Technology **38**:269-310.

Gianotti V., M. Benzi, G. Croce, P. Frascarolo, F. Gosetti, E. Mazzucco, M. Bottaro, and M. C. Gennaro. 2008. The use of clays to sequestrate organic pollutants. Leaching experiments. Chemosphere **73**:1731-1736.

Gonzales J., F. Figueiras, M. Aranguren-Gassis, B. Crespo, E. Fernandez, X. Moran, M. Nieto-Cid. 2009. Effect of a simulated oil spill on natural assemblages of marine phytoplankton enclosed in microcosms. Estuarine, Coastal and Shelf Science **83**:265-276.

Grandy A., and J. Neff, M. Wintraub. 2007. Carbon structure and enzyme activities in alpine and forest ecosystems. Soil Biology & Biochemistry **39**:2701-2711.

Grant R. J., L. M. Muckian, N. J. W. Clipson, and E. M. Doyle. 2007. Microbial community changes during the bioremediation of creosote-contaminated soil. Letters in Applied Microbiology **44**:293-300.

Greenwood P. F., S. Wibrow, S. J. George, and M. Tibbett. 2009. Hydrocarbon biodegradation and soil microbial community response to repeated oil exposure. Organic Geochemistry **40**:293-300.

Gustafson, J., J. Tell, and D. Orem. 1997. Selection of representative TPH fractions base on fate and transport. Total Petroleum Hydrocarbon Criteria Working Group Series, Volume 3. Massachusetts, USA. ISBN 1-884-940-12-9.

Haavisto T., Finnish Environment Institute. Personal communications 11.2.2011.

Hernesmaa A., K. Bjorklof, O. Kiikkila, H. Fritze, K. Haahtela, and M. Romantschuk. 2005. Structure and function of microbial communities in the rhizosphere of Scots pine after tree-felling. Soil Biology & Biochemistry **37**:777-785.

HELCOM. 2011. Helsinki commission, Baltic Marine Environment Protection Commission, www.helcom.fi 3.2.2011

Hietala M. 2011. Finnish Environmental Institute, News about the development of the Ports of Gulf of Finland 19.10.2010. Sea Center, Preventing environmental damages. Personal communication 24.1.2011.

Horel A., S. Schiewer. 2009. Investigation of the physical and chemical parameters affecting biodegradation of diesel and synthetic diesel fuel contaminating Alaskan soils. Cold Regions Science and Technology **58**:113-119.

Hosokawa R., M. Nagai, M. Morikawa, and H. Okuyama. 2009. Autochthonous bioaugmentation and its possible application to oil spills. World Journal of Microbiology & Biotechnology **25**:1519-1528.

Howard D., E. Lewis, B. Keller, C. Smith. 2004. Histological techniques for marine bivalve mollusks and crustaceans. NO AA Technical Memorandum NOS NCCOS 5, p. 218.

Howarth R. 1972. Microbial co-metabolism and the degradation of organic compounds in nature. Bacteriological Reviews **36**:146-155.

Hubalek T., S. Vosahlova, V. Mateju, N. Kovacova, and C. Novotny. Ecotoxicity monitoring of hydrocarbon-contaminated soil during bioremediation: A case study. Archives of Environmental Contamination and Toxicology **52**:1-7.

Hänninen S., and J. Rytkönen. 2004. Oil transportation and terminal development in the Gulf of Finland. VTT Publications 547.

IARC. 1998. International agency for research on cancer. IARC Monographs on Evaluation of Carcinogenic Risks to Humans, Volume 45, Occupational Exposures in Petroleum Refining; Crude Oil and Major Petroleum Fuels.

Ishii K., M. Fukui, and S. Takii. 2000. Microbial succession during a composting process as evaluated by denaturing gradient gel electrophoresis analysis. Journal of Applied Microbiology **89**:768-777.

Jangid K., M. A. Williams, A. J. Franzluebbers, J. S. Sanderlin, J. H. Reeves, M. B. Jenkins, D. M. Endale, D. C. Coleman, and W. B. Whitman. 2008. Relative impacts of land-use, management intensity and fertilization upon

soil microbial community structure in agricultural systems. Soil Biology & Biochemistry **40**:2843-2853.

Jansen, P. 2006. Minireviews; identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA Genes. Applied and Environmental Microbiology **72**: 1719-1728.

Johnsen A. R., L. Y. Wick, and H. Harms. 2005. Principles of microbial PAH-degradation in soil. Environmental Pollution **133**:71-84.

Jørgensen K. S., J. Puustinen, and A. M. Suortti. 2000. Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environmental Pollution **107**:245-254.

Kaartokallio H., M. Laamanen, and K. Sivonen. 2005. Responses of Baltic Sea ice and open-water natural bacterial communities to salinity change. Applied and Environmental Microbiology **71**:4364-4371.

Kivisaar M. 2003. Stationary phase mutagenesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. Environmental Microbiology **5**:814-827.

Koivula T. T., M. Salkinoja-Salonen, R. Peltola, and M. Romantschuk. 2004. Pyrene degradation in forest humus microcosms with or without pine and its mycorrhizal fungus. Journal of environmental quality **33**:45-53.

Kähkönen M. 2003. Biodegradation in coniferous forest soils and freshwater sediments. Academic dissertation in microbiology. University of Helsinki. Finland. ISBN 1239-9469.

Laakso K. 2011. Crime Prevention, Oil Spill Charges, West Finland Coast Guard District Operations. Personal communications 26.1.2011

Laine M., H. Haario, K.S. Jørgensen. 1997. Microbial functional activity during composting of chlorophenol contaminated sawmill soil. Journal of Microbiological Methods 30: 21-32.

Laine M. M., K. S. Jørgensen. 1996. Straw compost and bioremediated soil as inocula for the bioremediation of chlorophenol-contaminated soil. Applied and Environmental Microbiology **62**:1507-1513.

Leckie S. E., C. E. Prescott, S. J. Grayston, J. D. Neufeld, and W. W. Mohn. 2004. Characterization of humus microbial communities in adjacent forest types that differ in nitrogen availability. Microbial Ecology **48**:29-40.

Leinonen E., M. Orava, K. Raatikainen. 2007. Kenttäopas tiekuljetusöljyvahingon hallintaan. Suomen ympäristöopisto, SYKLI (Finnish environmental institute) www.sykli.fi

Lindgren, C. and Lindblom, E., 2004. Short-term effects of accidental oil pollution in waters of the Nordic Countries. IVL Swedish Environmental Research Institute Ltd, Arkivnr U964.

Lynch J. M., A. Benedetti, H. Insam, M. P. Nuti, K. Smalla, V. Torsvik, and P. Nannipieri. 2004. Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms. Biology and Fertility of Soils **40**:363-385.

Margesin R., F. Schinner. 2001a. Biodegradation and bioremediation of hydrocarbons in extreme environments. Applied Microbiology and Biotechnology **56**:650-663.

Margesin R., F. Schinner. 2001b. Bioremediation (natural attenuation and biostimulation) of diesel-oil-contaminated soil in an alpine glacier skiing area. Applied and Environmental Microbiology **67**:3127-3133.

Margesin R., F. Schinner. 1999. Biological decontamination of oil spills in cold environments. Journal of Chemical Technology and Biotechnology **74**:381-389.

Margesin R., F. Schinner. 1997a. Effect of temperature on oil degradation by a psychrotrophic yeast in liquid culture and in soil. FEMS Microbiology Ecology **24**:243-249.

Margesin R., F. Schinner. 1997b. Efficiency of indigenous and inoculated cold-adapted soil microorganisms for biodegradation of diesel oil in Alpine soils. Applied and Environmental Microbiology **63**:2660-2664.

Margesin R., S. Gander, G. Zacke, A. M. Gounot, and F. Schinner. 2003. Hydrocarbon degradation and enzyme activities of cold-adapted bacteria and yeasts. Extremophiles **7**:451-458.

Margesin R., M. Haemmerle, and D. Tscherko. 2007. Microbial activity and community composition during bioremediation of diesel-oil-contaminated soil: Effects of hydrocarbon concentration, fertilizers, and incubation time. Microbial Ecology **53**:259-269.

Mckee R. H., M. A. Amoruso, J. J. Freeman, and R. T. Przygoda. 1994. Evaluation of the Genetic Toxicity of Middle Distillate Fuels. Environmental and Molecular Mutagenesis **23**:234-238.

Mohn W. W., G. R. Stewart. 2000. Limiting factors for hydrocarbon biodegradation at low temperature in Arctic soils. Soil Biology & Biochemistry **32**:1161-1172.

Molina-Barahona L., L. Vega-Loyo, M. Guerrero, S. Ramirez, I. Romero, C. Vega-Jarquin, and A. Albores. 2005. Ecotoxicological evaluation of diesel-contaminated soil before and after a bioremediation process. Environmental toxicology **20**:100-109.

Moores, Eldridge M. and Fairbridge, Rhodes W. 1997. Encyclopedia of European and Asian regional geology. Chapman & Hall. London, UK. ISBN 0-412740-400.

Muckian L., R. Grant, E. Doyle, and N. Clipson. 2007. Bacterial community structure in soils contaminated by polycyclic aromatic hydrocarbons. Chemosphere **68**:1535-1541.

Muyzer G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinion in Microbiology **2**:317-322.

Muyzer G., K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology **73**:127-141.

Muyzer G., E. C. Dewaal, and A. G. Uitterlinden. 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal-rna. Applied and Environmental Microbiology **59**:695-700.

Muzyka V., S. Bogovski, A. Viitak, and T. Veidebaum. 2002. Alterations of heme metabolism in lymphocytes and metal content in blood plasma as markers of diesel fuels effects on human organism. Science of the Total Environment **286**:73-81.

Mälkönen P. 1995. Orgaaninen kemia. Otava. ISBN 951-1-10574-4.

NAS. 2003. Oil in the sea III: inputs, fates, and effects. National Academy of Sciences, National Academy Press, Washington, DC, USA. ISBN 0-309-08438-5.

Nayar S., B. P. L. Goh, and L. M. Chou. 2005. Environmental impacts of diesel fuel on bacteria and phytoplankton in a tropical estuary assessed using in situ mesocosms. Ecotoxicology **14**:397-412.

Nessel C. S. 1999. A comprehensive evaluation of the carcinogenic potential of middle distillate fuels. Drug and Chemical Toxicology **22**:165-180.

Ohtonen R., S. Aikio, and H. Vare. 1997. Ecological theories in soil biology. Soil Biology & Biochemistry **29**:1613-1619.

Pajukallio A-M. 2011. Ministry of the environment, personal communications 28.2.2011.

Peltola R., M. Salkinoja-Salonen, J. Pulkkinen, M. Koivunen, A. R. Turpeinen, T. Aarnio, and M. Romantschuk. 2006. Nitrification in polluted soil fertilized with fast- and slow-releasing nitrogen: A case study at a refinery landfarming site. Environmental Pollution **143**:247-253.

Penet S., C. Vendeuvre, F. Bertoncini, R. Marchal, and F. Monot. 2006. Characterisation of biodegradation capacities of environmental microflorae for diesel oil by comprehensive two-dimensional gas chromatography. Biodegradation **17**:577-585.

Peng R., A. Xiong, Y. Xue, X. Fu, F. Gao, W. Zhao, Y. Tian, and Q. Yao. 2008. Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiology Reviews **32**:927-955.

Penn I., R. Vos, M. Schlarb, L. Mitchell. (ed.). 2002. Eco-industrial strategies and environmental justice: An agenda for healthy communities: Integrating Brownfields and Eco-Industrial Development. National Center for Eco-Industrial Development, Center for Economic Development, School of Policy, Planning and Development, University of Southern California, USA.

Peressutti S. R., H. M. Alvarez, and O. H. Pucci. 2003. Dynamics of hydrocarbon-degrading

bacteriocenosis of an experimental oil pollution in Patagonian soil. International Biodeterioration & Biodegradation **52**:21-30.

Plaza G., G. Nalecz-Jawecki, K. Ulfig, and R. L. Brigmon. 2005. The application of bioassays as indicators of petroleum-contaminated soil remediation. Chemosphere **59**:289-296.

Poole A. M., M. J. Phillips, and D. Penny. 2003. Prokaryote and eukaryote evolvability. Biosystems **69**:163-185.

Reichenberg F., U. G. Karlson, O. Gustafsson, S. M. Long, P. H. Pritchard, and P. Mayer. 2010. Low accessibility and chemical activity of PAHs restrict bioremediation and risk of exposure in a manufactured gas plant soil. Environmental Pollution **158**:1214-1220.

Reinikainen J. 2007. Maaperän kynnys- ja ohjearvojen määritysperusteet. Finland's environment 23/2007, Finland's environmental institute. ISBN 978-11-273-1.

Reinikainen J, Finland's environmental institute. Personal communications 4.3.2011.

Roesch L. F., R. R. Fulthorpe, A. Riva, G. Casella, A. K. M. Hadwin, A. D. Kent, S. H. Daroub, F. A. O. Camargo, W. G. Farmerie, and E. W. Triplett. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. Isme Journal **1**:283-290.

Roth A.-M., D. M.Baltz. 2009. Short-term effects of oil spill on marsh-edge fishes and decapod crustaceans. Estuaries and Coasts **32**:565-572

Romantschuk M., I. Sarand, T. Petanen, R. Peltola, M. Jonsson-Vihanne, T. Koivula, K. Yrjala, and K. Haahtela. 2000. Means to improve the effect of in situ bioremediation of contaminated soil: an overview of novel approaches. Environmental Pollution **107**:179-185.

Sambrook J. R. D. W. 2001. Molecular cloning, a laboratory manual, third edition.

Sanchez M., M. Vasquez, and B. Gonzalez. 2004. A previously unexposed forest soil microbial community degrades high levels of the pollutant 2,4,6-trichlorophenol. Applied and Environmental Microbiology **70**:7567-7570.

Sandberg J., R. Elmgren, F. Wulff. 2000. Carbon flows in Baltic Sea food webs - a re-evaluatuion using a mass balance approach. Journal of Marine Systems **25**: 249-260.

Sarand I., H. Haario, K. S. Jørgensen, and M. Romantschuk. 2000. Effect of inoculation of a TOL plasmid containing mycorrhizosphere bacterium on development of Scots pine seedlings, their mycorrhizosphere and the microbial flora in m-toluate-amended soil. FEMS Microbiology Ecology **31**:127-141.

Sarand I., E. Skarfstad, M. Forsman, M. Romantschuk, and V. Shingler. 2001. Role of the DmpR-mediated regulatory circuit in bacterial biodegradation properties in methylphenol-amended soils. Applied and Environmental Microbiology **67**:162-171.

Sarand I., S. Timonen, T. Koivula, R. Peltola, K. Haahtela, R. Sen, and M. Romantschuk. 1999. Tolerance and biodegradation of m-toluate by Scots pine, a mycorrhizal fungus and fluorescent pseudomonads individually and under associative conditions. Journal of Applied Microbiology **86**:817-826.

Saul D. J., J. M. Aislabie, C. E. Brown, L. Harris, and J. M. Foght. 2005. Hydrocarbon contamination changes the bacterial diversity of soil from around Scott Base, Antarctica. FEMS Microbiology Ecology **53**:141-155.

Schafer A. N., I. Snape, and S. D. Siciliano. 2009. Influence of Liquid Water and Soil Temperature on Petroleum Hydrocarbon Toxicity in Antarctic Soil. Environmental Toxicology and Chemistry **28**:1409-1415.

Scow M., and K. Hicks. 2005. Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. Current Opinion in Biotechnology **16**:246-253.

Seklemova E., A. Pavlova, and K. Kovacheva. 2001. Biostimulation-based bioremediation of diesel fuel: field demonstration. Biodegradation **12**:311-316.

Sommer U., H. Stibor, A. Katechakis, F. Sommer, and T. Hansen. 2002. Pelagic food web configurations at different levels of nutrient richness and their implications for the ratio fish production: primary production. Hydrobiologia **484**:11-20.

Springael D., E. M. Top. 2004. Horizontal gene transfer and microbial adaptation to xenobiotics: new types of mobile genetic elements and lessons from ecological studies. Trends in microbiology **12**:53-58.

Stallwood B., J. Shears, P.A. Williams, and K.A. Hughes. 2005. Low temperature bioremediation of oil-contaminated soil using biostimulation and bioaugmentation with a Pseudomonas sp. from maritime Antarctica. Jounal of Applied Microbiology **99**:794-802.

Stenuit B., L. Eyers, L. Schuler, S. N. Agathos, and I. George. 2008. Emerging high-throughput approaches to analyze bioremediation of sites contaminated with hazardous and/or recalcitrant wastes. Biotechnology Advances **26**:561-575.

Suni S., E. Malinen, J. Kosonen, H. Silvennoinen, and M. Romantschuk. 2007. Electrokinetically enhanced bioremediation of creosote-contaminated soil: Laboratory and field studies. Journal of Environmental Science and Health Part A-Toxic/hazardous Substances & Environmental Engineering **42**:277-287.

Suni S., A. L. Kosunen, M. Hautala, A. Pasila, and M. Romantschuk. 2004. Use of a by-product of peat excavation, cotton grass fibre, as a sorbent for oil-spills. Marine Pollution Bulletin **49**:916-921.

Sylvia, D., J. Furhrmann, P. Hartel, and D. Zuberer. 2005. Principles and applications of soil microbiology. Pearson Prentice Hall, Pearson Education Inc., Upper Saddle River, New Jersey 07458, USA.

Teira E., S. Martinez-Garcia, A. Calvo-Diaz, X.Anxelu, G. Moran. 2010. Effects of inorganic and organic nutrient inputs on bacterioplankton community composition along a latitudinal transect in the Atlantic Ocean. Aquatic Microbial Ecology **60**:299-313.

Theng B. K. G., J. Aislabie, and R. Fraser. 2001. Bioavailability of phenanthrene intercalated into an alkylammonium-montmorillonite clay. Soil Biology & Biochemistry **33**:845-848.

Thomassin-Lacroix E. J. M., M. Eriksson, K. J. Reimer, and W. W. Mohn. 2002. Biostimulation and bioaugmentation for on-site treatment of weathered diesel fuel in Arctic soil. Applied Microbiology and Biotechnology **59**:551-556.

Torsvik V., F. Daae, R.-A. Sandaa, and L. Øvreås. 1998. Novel techniques for analysing microbial diversity in natural and perturbed environments. Journal of Biotechnology **64**: 53-62.

TTL , Työterveyslaitos. 2011.OVA, Onnettomuuden vaaraa aiheuttavat aineet, Accident hazardous substances safety instructions (diesel 1202), http://www.ttl.fi/ova/yklista.html

US EPA. 2011. U. S. Environmental protection agency, Land revitalization and reuse. http://www.epa.gov/region4/landrevitalization /success/index.html 18.3.2011

Vacca D. J., W. F. Bleam, and W. J. Hickey. 2005. Isolation of soil bacteria adapted to degrade humic acid-sorbed phenanthrene. Applied and Environmental Microbiology **71**:3797-3805.

Valo O. Centre for Economic Development, Transport and the Environment, Finland. Personal communications 18.4.2011.

Vauramo S., V. Jääskeläinen, and H. Setälä. 2011. Environmental fate of polycyclic aromatic hydrocarbons under different plant traits in urban soil as affected by nitrogen deposition. Applied Soil Ecology **47**:167-175.

van Gestel C. A. M., J. J. van der Waarde, J. G. M. Derksen, E. E. van der Hoek, M. F. X. W. Veul, S. Bouwens, B. Rusch, R. Kronenburg, and G. N. M. Stokman. 2001. The use of acute and chronic bioassays to determine the ecological risk and bioremediation efficiency of oil-polluted soils. Environmental Toxicology and Chemistry **20**:1438-1449.

Van Hamme J. D., A. Singh, and O. P. Ward. 2003. Recent advances in petroleum microbiology. Microbiology and Molecular Biology Reviews **67**:503.

van Herwijnen R., B. Joffe, A. Ryngaert, M. Hausner, D. Springael, H. A. J. Govers, S. Wuertz, and J. R. Parsons. 2006. Effect of bioaugmentation and supplementary carbon sources on degradation of polycyclic aromatic hydrocarbons by a soil-derived culture. FEMS Microbiology Ecology **55**:122-135.

Viitala N. 2011.National Bureau of Investigation Finland, Forensic laboratory, personal communications (interview) 24.1.2011.

Vogel T. M. 1996. Bioaugmentation as a soil bioremediation approach. Current opinion in biotechnology **7**:311-316.

Walecka-Hutchison C. M., J. L. Walworth. 2007. Evaluating the effects of gross nitrogen

mineralization, immobilization, and nitrification on nitrogen fertilizer availability in soil experimentally contaminated with diesel. Biodegradation **18**:133-144.

Wang S., Z. Yan, G. Guo, G. Lu, Q. Wang, and F. Li. 2010. Ecotoxicity assessment of aged petroleum sludge using a suite of effects-based end points in earthworm *Eisenia fetida*. Environmental Monitoring Assessment **169**:417-428.

Welander U. 2005. Microbial degradation of organic pollutants in soil in a cold climate. Soil & Sediment Contamination **14**:281-291.

Wersal R., and J. Madsen. 2011. Influences of water column nutrient loading on growth characteristics of the invasive aquatic macrophyte Myriophyllum aquaticum (Vell.) Verdc. Hydrobiologia, in press.

Widmer F., R. J. Seidler, P. M. Gillevet, L. S. Watrud, and G. D. Di Giovanni. 1998. A highly selective PCR protocol for detecting 16S rRNA genes of the genus Pseudomonas (sensu stricto) in environmental samples. Applied and Environmental Microbiology **64**:2545-2553.

Williams P., K. Bartle, and G. Andrews. 1986. The relation between polycyclic aromatic compounds in diesel fuels and exhaust particulates. Fuel **65**:1150-1158.

Wong D. C. L., E. Y. Chai, K. K. Chu, and P. B. Dorn. 1999. Prediction of ecotoxicity of hydrocarbon- contaminated soils using physicochemical parameters. Environmental Toxicology and Chemistry **18**:2611-2621.

Wright B. E. 2004. Stress-directed adaptive mutations and evolution. Molecular microbiology **52**:643-650.

Yang Si-Zhong, Jin Hui-Jun, Wei Zhi, He Rui-Xia, Ji Yan-Jun, Li Xiu-Mei, and Yu Shao-Peng. 2009. Bioremediation of oil spills in cold environments: a review. Pedosphere **19**:371-381.

Yang Y., N. Zhang, M. Xue, and S. Tao. 2010. Impact of soil organic matter on the distribution of polycyclic aromatic hydrocarbons (PAHs) in soils. Environmental Pollution **158**:2170-2174.

Yergeau E., M. Arbour, R. Brousseau, D. Juck, J. R. Lawrence, L. Masson, L. G. Whyte, and C. W. Greer. 2009. Microarray and real-time PCR analyses of the responses of High-Arctic soil bacteria to hydrocarbon pollution and bioremediation treatments. Applied and Environmental Microbiology **75**:6258-6267.

Öljyalan keskusliitto, Soili-ohjelma http://www.oil.fi/index.php?m=12&id=32 21.6.2011 (in Finnish)

Zanaroli G., S. Di Toro, D. Todaro, G. C. Varese, A. Bertolotto, and F. Fava. 2010. Characterization of two diesel fuel degrading microbial consortia enriched from a non acclimated, complex source of microorganisms. Microbial Cell Factories **9**:10.

Zhang Z., Q. Shen, N. Cissoko, J. Wo, and X. Xu. 2010. Catalytic dechlorination of 2,4-dichlorophenol by Pd/Fe bimetallic nanoparticles in the presence of humic acid. Journal of Hazardous Materials **182**:252-258.