The use of plants, including trees, to remediate oil contaminated soils: a review and empirical study

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Tiivistelmä — Referat — Abstract

Soil contamination can result in soil degradation, bring great loss to agricultural production and pose threat to human health. Many of the soil contaminants are petroleum hydrocarbons (PHCs) derived from crude oil or refined petroleum products. Phytoremediation which relies on plants and their associated microorganisms to remove contaminants is cost-effective and applicable to treat a wide variety of soil contaminants. Besides trees, herbaceous plants are widely and effectively used in the remediation of PHC contaminated soils. Greenhouse studies have found that *Galega orientalis* co-inoculated with *Rhizobium galegae* and plant growth promoting bacteria (PGPB*)* benefiting soil with nitrogen fixation is able to remediate PHC contaminated soils. The FP7 ‘‘Legume-Futures’’ remediation field experiment was established at Viikki experimental farm, University of Helsinki in 2009 in order to test the practical applicability of the greenhouse results in a field scale.

In a split-plot design, crop (*Galega orientalis*, *Bromus inermis*, *Galega orientalis* + *Bromus inermis*, bare soil control) treatments were designated the main factor, oil (±) and PGPB (±) the sub-factors in factorial combination with four replicates. Soil samples were taken at four time points from July 2009 to May 2011. Soil total solvent extractable material (TSEM) was extracted and measured by the gravimetrical method as a direct indicator of oil content. Physiochemical properties (pH, EC, total C and N and C/N ratio) of soil samples (taken in July 2009 and Nov. 2010) were determined. The losses of total C and TSEM between July 2009 and Nov. 2010 were calculated to estimate the differences crops and PGPB brought in oil treated plots. Crop dry matter yields were determined. The changes of soil microbial population, bacterial diversity and community structures were studied by the 16S rRNA gene based community fingerprinting method LH-PCR.

Bioremediation and physical removal were the main processes of oil removal in our experiment. Climate factors (e.g. temperature and precipitation) had an overriding influence on the removal of oil in our study. Soil condition with a neutral pH and C/N ratio in our field was optimal for biodegradation of hydrocarbons. The changes in soil microbial total DNA, diversity and community structure were sensitive indicators of soil contamination and recovery. Crop (*Galega orientalis* and *Bromus inermis*) and PGPB treatment had no significant effect on soil physiochemical and microbiological properties nor on the removal of oil in our experiment, which largely differed from our hypothesis. Resource competition between crops and microorganisms might have resulted in the better oil remediation in bare soils than in vegetated soils. Nevertheless, crops were found to have a high tolerance to oil contamination and surprisingly, the oil contamination seemed to increase the growth of both crop species. Bromus in mixture plots (without commercial nitrogen fertilization) had better yield than in pure plots (with commercial nitrogen fertilization) as a result of biological nitrogen fixation of *Galega orientalis* and *Rhizobium galegae*. Therefore the mixture of galega and bromus can be suggested to be applied in future phytoremediation projects.

Avainsanat — Nyckelord — Keywords
PHC contamination, soil, phytoremediation, legume-cropping system, microbial ecology, LH-PCR

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ABBREVIATIONS

BTEX	Benzene-toluene-ethylbenzene-xylene
CE	Capillary electrophoresis
LH-PCR	Length heterogeneity polymerase chain reaction
MTBE	Methyl tertiary butyl ether
PAHs	Polyaromatic hydrocarbons
PCBs	Polychlorinated biphenyl
PGPB	Plant growth-promoting bacteria
PHCs	Petroleum hydrocarbons
SOM	Soil organic matter
TPHs	Total petroleum hydrocarbons
TSEM	Total solvent extractable material
VOCs	Volatile organic compounds

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INTRODUCTION

Soil is a fundamental and irreplaceable natural resource which provides a variety of ecosystem services and is the essential link between the components air, bedrock, water and biota that make up our environment. The components interact with each other to constantly provide several goods (e.g., food, fuel and fiber) to support organisms (DEFRA 2009).

More and more goods and services are being demanded from the land due to population growth, resulting in increasing land degradation such as soil erosion, landslides, organic matter decline, salinization and contamination. Contaminated land is defined as sites having levels of contaminants present in the soil that pose a significant possibility of harm to the ecosystem (DEFRA 2009). The European Commission (2002; 2006a; 2006b) has identified soil contamination as one of eight major threats to European soils. Soil contaminants include heavy metals, mineral pollutants, monocyclic aromatic hydrocarbons, phenolic compounds, polycyclic aromatic hydrocarbons (PAHs), chlorinated hydrocarbons, pesticides and other pollutants such as mineral oils and gasoline (Beyer 1990). Contaminants can enter the soil from points (local) and diffuse sources (DEFRA 2009). In Europe, 3.5 million sites are estimated to be potentially contaminated (European Commission 2006b). It is not easy to estimate the costs of the soil contamination in terms of rehabilitating and restoring due to the lack of sufficient quantitative and qualitative data, but studies have pointed out that soil contamination results in great costs to society (European Commission 2006c).

Soil microbes as well as plants and biota are effective indicators to reflect the levels of soil contamination. They are capable of degrading or retaining more than 99% of all the types of soil pollutants (EA 2006) and preventing them from entering the wider environment. However, when the amount of contaminants exceeds the buffering capacity of a soil, it leads to a long-term negative impact on soil quality and biodiversity, and also damages to its functions as a producer of fiber, fuel and food. Once the contaminants enter the food chain, they can become a threat to human health.

Growing awareness of the harm that pollutants do to the soil as well as to the whole ecological chain has led to more research into how to clean up contaminated sites. Due to the great diversity of pollutants, however, there is no common solution to solve all types of soil contamination. Nevertheless, many of the pollutants mentioned above are petroleum hydrocarbons (PHCs) that originate from crude oil or refined petroleum products (EEA 2007). Therefore studies related to petroleum hydrocarbon contamination of soil and its biological cleanup is of great importance.

This study consists of two parts. The first part is a literature review on the current situation of petroleum hydrocarbon contamination and its remediation methods: physical, chemical and biological methods. The emphasis is put on phytoremediation, an effective method which relies on plants to remove contaminants from soil. Successful cases of using trees in the remediation of petroleum hydrocarbon contaminated soils are presented. The second part is an empirical field study focusing on the use of two crop species, a legume (*Galega orientalis*) and a grass (*Bromus inermis*), as well as plant growth promoting bacteria (PGPB) to remediate petroleum hydrocarbon contaminated soil.

The overall objectives of this thesis were to study the environmental fate of petroleum hydrocarbons in soil, introduce the phytoremediation mechanisms, test the PHC remediation potential of two crops *Galega orientalis* and *Bromus inermis* in oil-contaminated soils and detect the changes of soil bacteria population and community structure under crop, oil and PGPB treatments through a split-plot design field experiment.

LITERATURE REVIEW ON PETROLEUM HYDRCARBON CONTAMINATION AND ITS REMEDIATION METHODS

1 Petroleum hydrocarbon (PHC) contamination in soil

1.1 Introduction on oil and petroleum hydrocarbons

Oil is defined as a broad range of hydrocarbon-based substances, which are of two types: mineral oil such as different specific distillates of crude oil, and organic oil such as animal fats and vegetable oils. Natural gas, crude oil, tars and asphalts are types of petroleum hydrocarbons (Frick et al. 1999). The word "petroleum" means "rock oil" or "oil from the earth" (USEPA 2011b). Petroleum widely used in our daily life plays a vital role in our modern economy. We are dependent on it in many ways as it provides great benefits to society. It is not only the main energy source for heating, transportation and manufacturing, but also acts as a raw material for plastics and synthetic rubber.

Petroleum hydrocarbons (PHCs) are used to describe mixtures of organic compounds found in or derived from geological substances such as oil, bitumen and coal (CCME 2001a). They are composed of two categories: 1) gasoline range organics refers to small chain alkanes (C6-C10), e.g. methane, ethane, propane, volatile aromatic compounds (e.g. BTEX) and common oxygenates (e.g. MTBE and ethanol); and 2) diesel range organics are the alkanes with longer chains (C10-C40) or non-halogenated semi-volatile organic compounds and hydrophobic chemicals such as PAHs (e.g. naphthalene, phenanthrene, anthracene, benzo[a]pyrene) (Kamath et al, 2007; Frick et al. 1999). The small chain alkanes, such as isopentane, always have low boiling point between 60 and 170^{0}C (Kamath et al. 2007).

1.2 Impact of PHC chemicals on soil environment

When PHCs are released into ecosystems, they are threatened in various ways. According to U.S. Environmental Protection Agency (USEPA) (2007), “oil releases threaten public health and safety by contaminating drinking water, causing fire and explosion hazards, diminishing air and water quality, compromising agriculture, destroying recreational areas, and wasting nonrenewable resources. Oil spills also

have a severe environmental impact on ecosystems by harming or killing wildlife and plants, and destroying habitats and food". They can influence on ecosystems directly or indirectly.

Each type of petroleum hydrocarbon has its distinct physical and chemical properties that affect the way of its spreading and breaking-down (USEPA 2011a). The degree of harm that oil can do to an ecosystem and human life varies with the amount and constitutions of the oil source spilled (e.g. gasoline vs. crude oil), site factors (e.g. terrain, vegetation, soil texture and climate), time since release and management (CCME 2008). Although the pollution is always caused by a mixture of hydrocarbons, aromatic compounds tend to be more toxic than aliphatic compounds (Epps 2006).

Even at low rates of contamination the residual hydrocarbons may cause serious pertubations to the cellular metabolisms for plants (Chaineau et al. 2003). When sorghum was grown in a high concentration of phenanthrene (e.g. 100 mg/kg), the total amount of root-exuded compounds decreased by 78% due to the root damage (Kawasaki et al. 2011). Therefore, food production and safety in the contaminated area can hardly be guaranteed.

Once soil is polluted by PHCs, the recovery may take several years. For instance, Wang et al. (2000) reported that four to five years was needed for the pasture to restore the dominance of *Aneurolopidium chinensis* in oil drilling sites of chernozem and Aeolian sand soils.

PHC-contamination of soil is a concern for a number of reasons (Figure 1). First of all, once released into soil, the volatility of PHC can pose a fire or even explosion hazard, especially when vapors enter confined spaces. Secondly, contaminants can interfere with the nutrients and water transmission and thus lead to land degradation. Thirdly, weathered petroleum residuals may stay bound to soil particles and be retained in soil for years. Fourthly, although these contaminants may benefit the oil degraders as a carbon source, they are still toxic to the majority of soil biota. PHC pollutants can strongly alter the ecology and the physiology of bacteria and fungi (Nardini et al. 2010). Fifthly, PHCs may destroy the aesthetic by inducing offensive odor, taste or appearance in environmental media. Last but not least, PHC contamination of soil is not only a concern for the soil itself, but is also a potential

threat to other ecosystems. If the PHC release is persistent in one place, it will probably extend its impacts to adjacent areas as individual compounds continue to separate and migrate away from the spill area via air or groundwater (ATSDR 1999). PHCs and other chemicals can also find their way to drinking water and so pose a threat to human health.

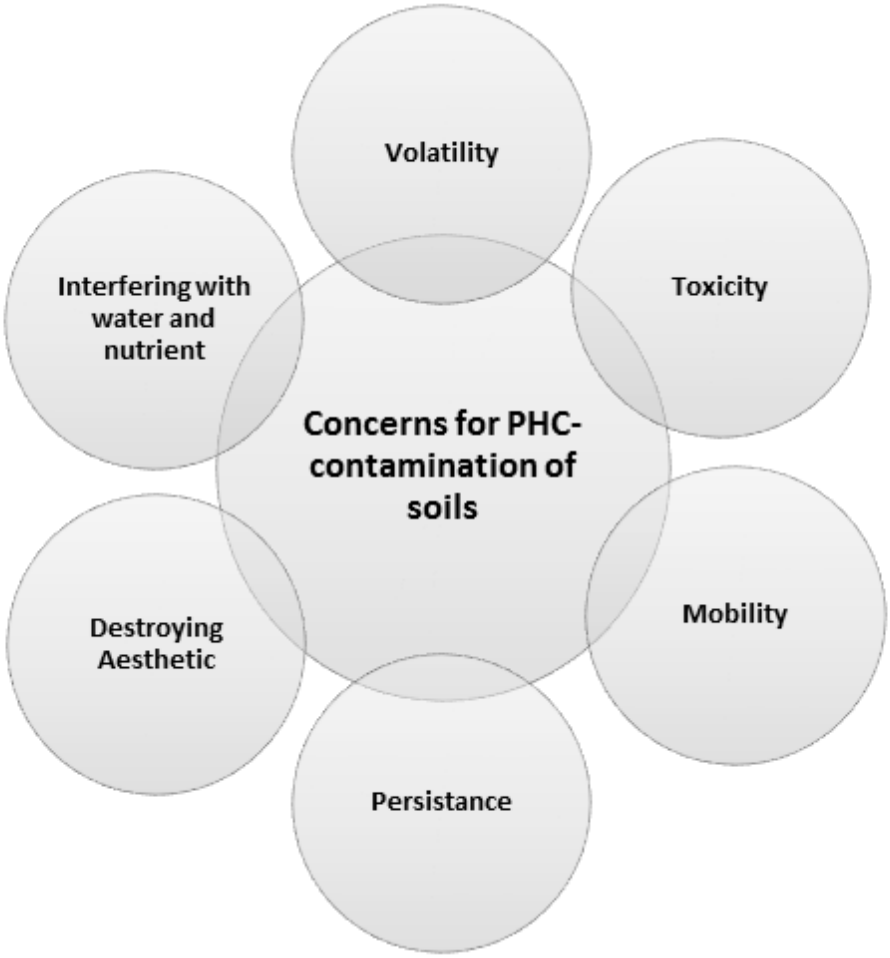

Figure 1. Concerns for PHC-contamination of soils, modified from Canada-wide standards for petroleum hydrocarbons (PHC) in soil (CCME 2001a).

1.3 Sources of PHC contamination

With the development of the oil industry, it is difficult to avoid oil contamination of soil through crude oil exploration, transportation, processing and consumption. Most of the PHCs generated from crude oil or refined oil products released to the soil environment are through anthropogenic accidents, as releases from industries or as byproducts from commercial or private uses.

In different countries, the main sources and polluting activities may vary from each other. However, industrial and commercial activities as well as the treatment and disposal of waste are reported to be the most important sources of oil contamination in Europe (EEA 2007). In Nordic countries where the climatic condition only can support a few coniferous and broadleaf tree genera (Burton et al. 2003), petroleum hydrocarbons still can find their way into the boreal ecosystem by surface spills or leaks from pipelines or storage tanks (Robertson et al. 2007).

1.4 Extent of land PHC contamination

1.4.1 US, Canada and EU

The extent of PHC contaminated sites is most well-known in the developed world, North America and Western Europe.

In the United States, oil spills caused by land-based facilities take up the bulk of the whole spillage on land (Etkin 2001). From the production, storage, transport, and use of oil, estimated 18,000 - 24,000 oil spills are reported annually in the United States (USEPA 2007). These spills correspond to 10-25 million gallons of oil spilled annually. Oil spills from pipelines are greater than spills from tankers and barges since 1985 (Etkin 2001). However, the extent of petroleum hydrocarbon contamination throughout the United States is often reflected by the numerous ‘’Superfund’’ sites that are abandoned hazardous waste sites and ‘’Leaking Underground Storage Tanks (LUST)’’ sites that contain high concentrations of PHC contamination (Kamath et al. 2007).

The Canadian Council of Ministers of the Environment (2001b) reported that petroleum hydrocarbon is one of the most widespread types of soil contamination in Canada. There are tens of thousands of contaminated sites across Canada, about 60% of which involve PHC contamination (CCME 2001a; 2001b).

Due to the use of dangerous substances and lack of effective management practice in the past, the whole Europe is faced with soil contamination problems as a result of industrialization (European Commission 2006c). Heavy metals and mineral oil are the main contaminants in soil (EEA 2007). However, the level and range of soil contamination depend on local conditions and source of contaminants. The European Environmental Agency (2007) has estimated that there are approximately 250 000

contaminated sites that require immediate remediation in the EEA member countries and this number is growing. Although some measures have been taken, it may still need several decades until they are totally cleaned up.

Legal frameworks have been made to identify and remediate soil contamination in most developed countries. For example, Canada developed the Canada-Wide Standard for Petroleum Hydrocarbons in Soil (PHC CWS) under the Harmonization Sub-Agreement on Standards and was endorsed (with the exception of Quebec) in May 2001 (CCME 2001a). Its supporting technical document (CCME 2008) provides a consistent approach to managing PHC-contaminated sites across the country.

1.4.2 Developing countries e.g. China and Nigeria

There is little awareness of oil contamination in the developing countries and the challenge for soil remediation lies in the developing countries.

Soil contamination has been a problem for local authorities in China since the rapid development of the economy from the last 40 years. Although more concern has been given to heavy metal contamination, some 600, 000 tons of petroleum is released into to the environment every year, resulting in the pollution of soil, ground water and the sea (Lu et al. 2003). Oilfields, oil transmission stations, oil and wastewater tanks, and oily sludge are the main sources of soil PHC contamination in China (Liu et al. 2007).

Currently, there are more than 400 oil fields and oil gas fields under exploration and development distributed in 25 provinces in China. These oil fields cover an area of 320, 000 km^2, which is 3% of the whole territory of China. In the exploiting and manufacturing process of petroleum products, it is estimated that there are 4.8 million hectares of land where the petroleum content exceeds the safety threshold for crop growth (Liu et al. 2007).

Liu et al. (2003) showed that the average oil content in soils in Shengli oil field, the second largest oil field in China, increased between 1986 and 2003. The oil content in most of soils within 100 m distance of the four largest oilfields (Daqing, Shengli, Jianghan and Jiangsu) was higher than the threshold value (500 mg/kg) (Liu et al.

2007; Liu et al. 2003). This problem could be even worse as the exploitation activities are still going on, if no effective measures are taken.

Nigeria is now the largest oil producer in Africa and the sixth largest in the world (Nwilo & Badejo 2006). The Nigerian economy is heavily dependent on the oil sector. Of the total oil spilled in the Niger Delta area during the period of 1976 to 1996, 6% was on land, 25% in swamps and 69% in offshore environments (Nwilo & Badejo 2006). These crude oil activities especially occur dominantly in the southern states of Nigeria, where it belongs to the humid tropical forest zone with most of forest trees (Agbogidi & Dolor 2007).

The lack of scientific basis often results in over- and under- management of oil-contaminated soils in these countries. Technology and money may be additional barriers for these countries to improve the environment. It is likely that contamination problems will be left to the next generation to find and solve. Although some figures and facts on PHC contamination have been made by the authorized environmental institutions in these countries, the public is not aware of the problems.

1.5 Fate of PHCs in soil

Chemical pollution is the diversion of chemical elements from their natural cycle (Bohn et al. 1985). Soil is able to degrade most chemicals quickly and make the components back to their natural cycles, in which way it can minimize the environmental disturbance brought by contamination.

However, the interactions between PHCs and soil are extremely complex. PHC contamination affects soil properties and in turn, soil properties also have significant impacts on PHC degradation. Petroleum hydrocarbons are extremely complex mixtures of hundreds of compounds (Epps 2006). The major hydrocarbon fractions have differing environmental fates (ATSDR 1999). Once oil is spilled into the environment, thousands of compounds, mainly hydrocarbons with a small amount of nitrogen, sulfur and oxygen, in different proportions are produced (CCME 2001b). With the interaction between mixtures of chemicals, soil and soil biota, the environmental fate of chemicals in soil may be different from that of individual PHC chemicals (ATSDR 1999). Generally, the degradability of simple hydrocarbons and

petroleum fuels decreases as molecular weight and degree of branching increase (Shukla et al. 2010).

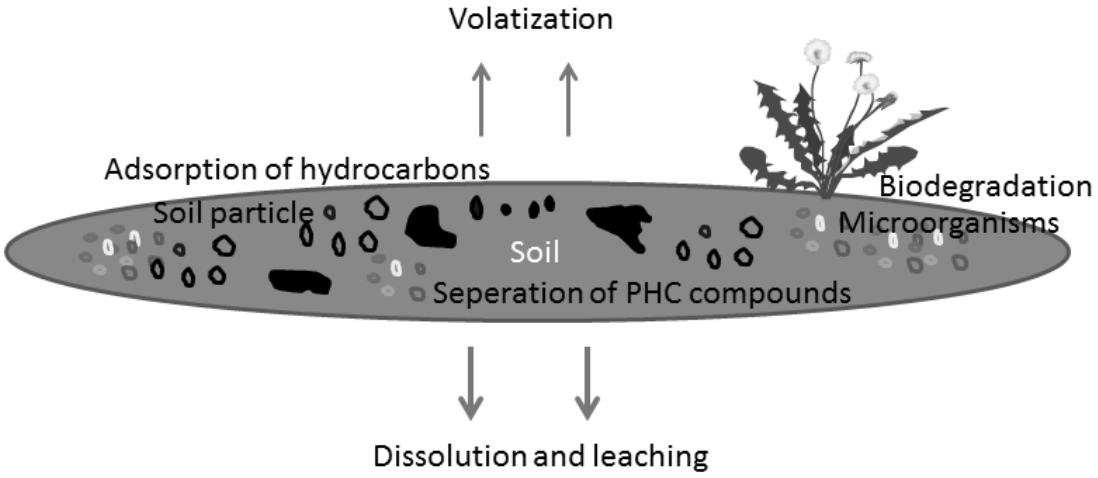

Figure 2. Physical and biochemical behavior of petroleum hydrocarbons in soil, modified from ATSDR (1999)

The fate of PHCs following an oil spill is summarized in Figure 2. Once released to soil, the complex of PHCs mixture may separate into individual compounds, depending on their chemical properties. Compounds of lower molecular weight, e.g. BTEX compounds (benzene, toluene, ethylbenzene, and xylenes), are highly mobile in the environment and more likely to volatilize to the air, or leach to the groundwater than PHCs of higher molecular weight (ATSDR 1999; Kamath et al. 2007). Thus gasoline, which has lighter compounds, tends to break down and volatilize more easily compared to motor oil. The amount of the oil spills also has an impact on the leaching of the PHCs to the ground water. Apart from the product-specific factors (e.g. the complexity of PHCs and the extreme variability of sources), the site-specific factors (e.g. terrain, climate, vegetation and soil inherent properties) can affect the rate of bulk oil infiltration (CCME 2001b; ATSDR 1999). PHC compounds can be broken down and degraded by soil biota, especially by soil microorganisms. The biodegradation process mainly depends on the properties of the pollutants and the activity of oil degraders. Generally, hydrocarbons with straight and few chains degrade more readily than those with highly condensed ring structures (ATSDR 1999). The final products of microbial degradation are carbon dioxide, water and microbial biomass (ATSDR 1999; Nichols et al. 1997). Compounds with longer chains, such as PAHs and aromatics tend to bind strongly to soil particles and

remain relative immobile at the location where they are spilled until they separate into smaller fractions and are degraded by plants and microorganisms (ATSDR 1999; Kamath et al 2007). Soil organic matter and clay particles play a vital role in the binding of PHCs, reducing their bioavailability to microorganisms.

As microorganisms in soil are many and not easy to monitor and control, the assessment and risk management are difficult. After a few decades of intensive research, many cleanup methods have been developed. However, more trials and methods are still needed since the nature of contamination varies in different sites and countries.

2 Methods of remediation of oil-contaminated soils

Commission of the European Communities (2006) suggests ‘’Remediation shall consist of actions on the soil aimed at the removal, control, containment or reduction of contaminants so that the contaminated site, taking account of its current use and approved future use, no longer poses any significant risk to human health or the environment’’. The contaminants can be treated either *in situ* or *ex situ*. *In situ* remediation requires treating the contaminated material in place, while *ex situ* involves the physical removal of the soil and contaminants from the polluted site to be treated elsewhere (Kapley & Purohi 2009; Boopathy 2000).

Conventional oil remediation methods can be broadly divided into physical, chemical and biological methods, besides the natural attenuation (USEPA 2011a; Zhu et al. 2004).

2.1 Physical and chemical remediation

Mechanical and chemical methods are common traditional technologies used in treating contaminated soils. Zhu et al. *(*2004) listed the commonly used mechanical methods as follows: booming and skimming, manual removal (wiping), mechanical removal, water flushing, sediment relocation and tilling. Chemical methods such as the use of dispersants are most widely applied in oil-contaminated water bodies. These physical and chemical methods relying on incineration, volatilization or immobilization of pollutants are often adopted when the oil concentration is quite high at the contaminated sites. The cost of removing 1 m^3 soil from a 1-acre contaminated site is estimated at 0.6-2.5 million USD (Mclntyre 2003). These methods are often very effective to remove the original contaminants. However, there is increasing debate about the use of these methods as they have the potential to transfer the pollutants or produce secondary pollution as incineration residues which might pose long-term threat to the environment (Zhu et al. 2004; Shukla et al. 2010).

2.2 Biological remediation

Biological remediation is often used in the moderate oil-contaminated soils. Compared to the mechanical and chemical remediation, biological remediation is regarded as a better remedial technology in soil contamination.

Defined as the elimination, attenuation or transformation of polluting or contaminating substances by the use of biological processes, biological remediation presents a potentially low-technology, cheaper option to physicochemical technologies (Obuekwe & Al-Muttawa 2001; Shukla et al. 2010). Firstly, they are more cost effective without much investment in labor. Secondly, the risk of causing secondary pollution is lowered. Thirdly, they are applicable for treating a wide variety of environmental contaminants. Last but not least, they can give an aesthetical value by providing pleasant landscape by phytoremediation (Kamath et al. 2007).

There are four types of the biological tools that can be used in soil remediation: 1) use of microorganisms (e.g. fungi or bacteria) to decompose the organic pollutants, 2) use of plants, especially the fast growing plants with large biomass and 3) soil animals (e.g. earthworms) to accumulate or stabilize the non-degradable contaminants in their body or in the soil; 4) the combinative use of the above all organisms or even the merge of both physicochemical and biological methods.

Here I will give an introduction on narrowly-defined bioremediation and phytoremediation, however, the broad concepts of these two terms nowadays have somehow overlapped or merged.

2.2.1 Bioremediation

Bioremediation is the use of biological agents, mainly microorganisms (e.g., yeast, fungi or bacteria) or their enzymes to destroy or reduce the concentration of hazardous wastes on a contaminated site (Kapley &Purohi 2009; Shukla et al. 2010; Boopathy 2000). It has been widely used in organic and inorganic contaminated sites. In the organic contaminated sites, microbes are able to use the contaminants as their source of energy, resulting in the degradation of the contaminants.

Boopathy (2000) summarized three factors affecting bioremediation: 1) energy and nutrient sources, 2) bio-activity of microorganisms and biochemistry of enzymes, and 3) the bioavailability of a contaminant. The bioavailability of a pollutant in soil depends on the mass transport towards the sites where degrader populations are abundant, besides the solubility of the pollutant in soil (Wenzel 2009). For a successful remediation, we should have the right microbes, e.g., bacteria or fungi, which have the physiological and metabolic abilities to decompose the contaminants,

in the right place with the right environmental factors for degradation to occur. It is also suggested that temperature and oxidation-reduction (redox potential) are important parameters of bioremediation since most bioremediation systems run under aerobic conditions (Shukla et al. 2010; USEPA 1994).This method which encourages the biodegradation of hydrocarbons by providing oxygen and in necessary nutrients to microorganisms into the soil unsaturated zone is called bioventing (Shukla et al. 2010).

Indigenous microbes are capable of adapting and responding rapidly to contamination under favorable nutrient conditions and degrading the contamination (ATSDR 1999; Margesin & Schinner 2001). Even in extreme conditions or environments (e.g. deserts), microbes have been found capable of dealing with hydrocarbon contamination (Margesin & Schinner 2001). The method of using indigenous microorganisms to degrade soil contaminants is called biosparging and is regarded most cost efficient and noninvasive (Shukla et al. 2010). Some bacteria such as *Pseudomonas (*e.g. *P. cepacea, P. fluorenscens* and *P. putida), Rhodococcus, Acinetobacter, Mycobacterium, Arthrobacter, Acaligenes* and *Acidobacteria* species and some fungi such as *Pencillium*, *Mucor* and *Aspergillus* have been found able to degrade hydrocarbons (Johnsen et al. 2005 & Ellis 1994).

2.2.2 Phytoremediation

Plants can well adapt to different environmental conditions and can also modify conditions of the environment to some extent (Susarla et al. 2002). Phytoremediation (*phyto* – Greek for plants) is a general term to describe the ways how plants can be used to remove contaminants from soil and water (USEPA 1998; USDA 2000). More specifically, phytoremediation is a term applied to a group of technologies that use plants to reduce, remove, degrade or immobilize environmental toxins with the aim of restoring a site to a condition useable for private or public applications (Peer et al. 2006). Phytoremediation has been widely applied to the remediation sites contaminated by metals, pesticides, solvents, explosives, crude oil, PAHs and landfill leachates (FRTR 2012). The advantages and constraints of phytoremediation are shown in Table 1.

Table 1. Advantages and constraints of phytoremediation (Susarla et al. 2002).

Advantages	Constraints
In situ and faster than natural attenuation	Application is limited to shallow ground water, soils and sediments and is dependent on soil and climate conditions of site
In moderate and low levels of contamination	Not applicable in high concentrations of contaminants
Low-cost and beneficial for breaking down organic pollutants with a wide range	Slower than physiochemical treatments and often in need of supplementary treatments such as nutrient supply
High public acceptance	Toxicity and bioavailability of biodegradation products are not known

3 SUMMARY OF PHYTOREMEDIATION RESEARCH (INCLUDING USE OF TREES)

3.1 Mechanisms of phytoremediation

As mentioned, phytoremediation relies on the ability of plants to remove, degrade, transform or stabilize contaminants within soil and groundwater through physical, chemical and biological processes (Peer et al. 2006). Plants are also capable to change the composition or the amount of root exudation as to stimulate bioremediation by altering the microbial community structure, stimulating the growth of microorganisms, or increasing microbial catabolic activities (Kawasaki et al. 2011).

Mechanisms involved include hydraulic control, volatilization, stabilization, transformation, degradation and rhizodegradation (Figure 3 and Table 2).

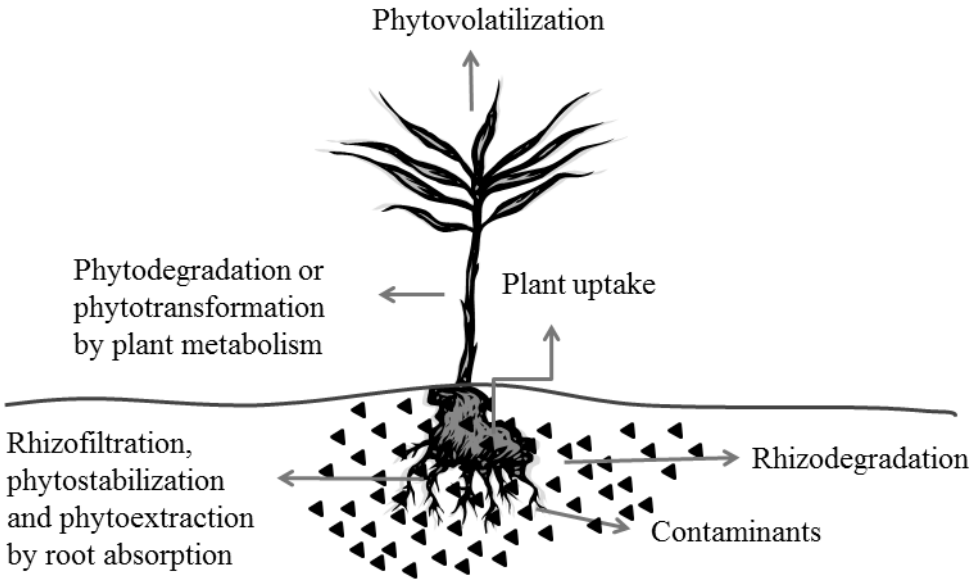

Figure 3. Mechanisms of phytoremediation, modified from Kamath et al. (2007)

Table 2. Phytoremediation mechanisms (USEPA 1999; Epps 2006; USEPA 2010; Jussila 2006; Kamath et al. 2007 & ITRC 2009)

Location	Mechanisms	Definition	Contaminants and media	Cleanup goals
Plant shoots	Phytoextraction or phytoaccumulation	Plants absorb and accumulate contaminants, especially heavy metals, from soil into aboveground part of t plants	Metals and other toxic inorganics in soil, sediment and surface water	Remediation by removal of plants containing the contaminant
	Phytodegradation or phytotransformation	Breakdown of contaminants through metabolic processes with enzymes	Mobile organics: herbicides, TNT, MTBE, TCE in soil, sediment and surface water	Remediation by destruction
	Phytohydraulics	Plants act as 'pumps' to pull-in large volumes of contaminated water as part of transpiration process, resulting in reduced migration of contaminants	Organics and inorganics in ground and surface water	Containment by controlling hydrology
	Phytovolatilization	Uptake and release of contaminants to atmosphere which always happens along with transpiration	VOCs such as TCE and MTBE, and volatile inorganics such as Se and Hg in surface water or surface soil	Remediation by removal through plant
Rhizosphere	Rhizofiltration	Use of plants to clean up water by absorbing or precipitating contaminants onto or into their roots	Organics and inorganics such as heavy metals in surface water	Containment
	Phytostabilization or phytosequestration	Certain plant species immobilize contaminants through absorption and accumulation to prevent contaminants from migrating to the groundwater or air	Organics and inorganics in soil and water	Containment
	Rhizodegradation	Plant-assisted bioremediation which mainly relies on breakdown of contaminants through metabolic activity of microorganisms (fungi, yeast, or bacteria) in soil	Hydrophobic organics such as PCBs and PAHs, and other petroleum hydrocarbons in soil and water	Remediation by destruction

3.1.1 Hydraulic control and plant uptake

Hydraulic control, also called phytohydraulics, is the ability of plants to capture and evaporate water off the plant and thus prevent migration of contaminants to the groundwater (ITRC 2009). Deep-rooted, high-transpiring, water-loving phreatophytes are particularly useful. Trees in the *Salicaceae* family, such as cottonwood, hybrid poplars and willows are often used (Kamath et al. 2007; ITRC 2009).

In this mechanism, water as well as contaminants from soils and aquifers is drawn upwards and either oxidized into harmless or volatile forms in aerobic soil or taken up and modified into volatile forms in plants, preventing further dispersion and migration (Cook et al. 2007; Kamath et al. 2007).

The contaminants must be dissolved in the soil water before they can be extracted by the plant roots through the transpiration stream. The rate of contaminant removal is highly associated with transpiration rate, contaminant concentration and uptake efficiency in soil water (Kamath et al. 2007). Factors that affect the potential uptake of organic chemicals into plants through the transpiration stream include hydrophobicity, polarity, sorption properties and solubility (ITRC 2009). For instance, hydrophobic hydrocarbons, such as PAHs, strongly sorb to soil and therefore are poorly taken up by plants (Kamath et al. 2007).

3.1.2 Phytovolatilization

Once taken up by plants, the contaminants are modified or broken down into volatile forms and thus diffuse from the plants to the atmosphere through open stomata on leaves together with a small amount of redial diffusion through stem tissues and bark (Kamath et al. 2007; ITRC 2009).

Studies have shown that trees, especially poplars (*Populus* spp.) and willows (*Salix* spp.), can successfully dissipate or attenuate fuel contaminants such as benzene-toluene-ethylbenzene-xylene (BTEX) and methyl tertiary-butyl ether (MTBE) in contaminated groundwater and soils, because their half-life in aerobic environment is relatively short compared to saturated anaerobic conditions (Cook et al. 2007; Kamath et al. 2007). Jordahl et al. (1997) reported that hybrid poplar trees (*Populus deltoids x nigra*) had 5 times more benzene-toluene-xylene (BTX) degraders in rhizosphere soil compared to bulk soil. Compounds with double-bonds such as

trichloroethylene (TCE) and perchloroethylene (PCE) can also be rapidly oxidized in the atmosphere by hydroxyl radicals following the emission from plant leaves (Kamath et al. 2007).

Nevertheless, phytovolatilizaiton is not a terminal solution, especially under circumstances when the air circulation is poor. Some volatile organic compounds such as MTBE can exist in atmosphere for a long period of time (Kamath et al. 2007), and pose a threat to the ecosystem as they do in soil and water. However, the emission rate of VOCs from plant tissues is rather small and it is a potentially viable remediation strategy for many VOCs.

3.1.3 Phytostabilization or phytosequestration

According to ITRC (2009), phytochemical complexation in the root zone, transport protein inhibition and vacuolar storage in the root cells are the three mechanisms of phytostablization, reducing the mobility of the contaminants and preventing migration to soil, water, and air. Grasses, sedges, forage plants and reeds with high transpiration rates are widely used in phytostabilization (Peer et al. 2006). Peer et al. (2006) suggest that the combinations of hardy, perennial, dense rooted plants or deep rooting trees (e.g. poplar, cottonwoods) have a particularly positive effect on the remediation of soil contamination.

3.1.4 Phytotransformation and phytodegradation

Plants have a series of detoxification mechanisms that transform parent chemicals into non-phytotoxic metabolites. Once taken up into plant system, contaminants are detoxified through a number of reactions in three phases: conversion, conjugation and compartmentation (Kamath et al. 2007).

Unlike microbes which metabolize organic contaminants to carbon dioxide and water, phytodegradation relies on plant enzymes to metabolize or mineralize chemicals completely into carbon dioxide and water (ITRC 2009). For example, dehalogenase, an enzyme produced by hybrid poplars (*Populus* spp.), algae and parrot feather (*Myriophyllum aquaticum*) is able to degrade organic compounds (e.g. dehalogenates chlorinated solvents) (Susarla et al. 2002), which poses potential use of these plants in remediating oil-contaminated soils.

3.1.5 Rhizoremediation

The rhizosphere is the zone around plant roots. It is mainly influenced by root secretion and by the root-associated soil microorganisms. The term rhizoremediation refers to the combined use of plants and rhizosphere microorganisms to improve the bioremediation capacity of plants (Pajuelo et al. 2011). As a combination of two methodologies (bioremediation and phytoremediation), rhizoremediation is called as *in situ* plant-assisted bioremediation or generalized phytoremediation (Pajuelo et al. 2011).

Plant roots perform a vital role in this process. Roots favor microbial growth by providing habitats, oxygen, nutrients and enzymes. Plant roots provide a large surface area for microbes to colonize and allow them to move to deeper soil layers (Anderson et al. 1993). Roots promote the contact of detoxifying microbes and soil contaminants (Pajuelo et al. 2011). Roots also help with oxygen availability either by transporting oxygen or by creating void spaces in the subsurface that allow for greater oxygen diffusion from the atmosphere (Epps 2006), accelerating the bioremediation process. ATSDR (1999) highlighted the importance of oxygen in enhancing the biodegradation of PHCs whereas anaerobic conditions lead to extremely low rates of PHC degradation. A higher microbial density (10^2–10^4 folds) was observed in the surface soil than in deeper bulk soil or unplanted soil (Hinsinger et al. 2005; Epps 2006). As much as 20% of carbon released by roots is into the rhizosphere in an organic form, which serves as an energy source for microbes. Soil organic matter (SOM) serves as a nutrient and energy reservoir. Higher SOM content in the surface soil is always associated with higher microbial numbers, diversity and bioactivity (Boopathy 2000). Roots also release ‘’allelopathic chemicals’’ or so-called ‘’degradative enzymes’’, enhancing rhizodegradation of PHCs (Wenzel 2009).

The number or population of bio-degraders is an indicator of the remediation potential microbes in the soil (Mikkonen 2008). Several studies have revealed that organic pollutants generally increase bacteria populations in the rhizosphere soil by ‘’most-probable-number’’ (MPN) method, a technique used to estimate a microbial population size based on a process-related attribute (Chaineau et al. 2003; Mikkonen et al. 2011a; Chaudhary et al. 2012; Wallenius et al. 2012). In turn, a greater microbial numbers benefit the plant growth by degrading soil pollutants. Chiapusio et al. (2007), for example, demonstrated that the increase of red clover biomass and

its establishment in soils was due to the ability of rhizobium to adapt and metabolize the chemicals.

Phytoremediation holds promise for the *in situ* treatment of PHC polluted soils. *In situ* treatments may be more difficult to control than *ex situ* treatments, e.g. treating the excavated soil from the refinery waste. Nevertheless, *in situ* remediation treatments are widely used nowadays since they offer cost advantages and avoid disruption to the contaminated site. The intensity of biodegradation is influenced by a number of environmental factors: soil parameters (e.g. nutrients, oxygen, moisture content and pH), contaminants characters (e.g. composition, concentration and bioavailability), and contamination history of the environment (Margesin & Schinner 2001 & ATSDR 1999). How to control those factors to optimize the biological activity in this process would be of great importance. The effectiveness of this process also depends on the plant species used in phytoremediation process.

3.2 Species selection (including forest trees) in phytoremediation

3.2.1 Plant selection

Plant species for phytoremediation should be selected to ensure that the roots can expand throughout the entire contaminated zone. A number of criteria for selecting plants were identified for phytoremediation (Kamath et al. 2007). The selection of plant species in principle has to follow the needs of the application, the contaminants of concern and their potential to thrive on contaminated sites. Preferably they should be native plants to avoid the introduction of invasive species. For instance, two indigenous plants, kenaf (*Hibiscus Cannabinus*) and vetiver (V*etivera Zizanioides*) have proven to be very effective in cleaning crude oil contamination in Nigeria (MERCK 2002).

Grasses, herbs, shrubs as well as deciduous and coniferous trees are candidate plant species (Frick et al. 1999), depending on the local situation and the pollutants. Legumes (e.g. alfalfa, clover, peas and reed canary grass), grasses (e.g. ryegrass, wheatgrass and sunflowers) and trees (e.g. *Populus* sp., *Salix* sp., *Cordia subcordata, Thespesia populnea, Prosopis pallida* and *Scaevola serica*) have been proven to be tolerant of PHC contaminated soil (Frick et al. 1999; Kamath et al. 2007). Tolerance

is defined as the ability of a plant to grow in hydrocarbon contaminated soil but it does not necessarily mean the plant is healthy (Frick et al. 1999).

1) Grasses in phytoremediation

Grasses, often planted with trees are widely used as a primary remediation species in oil-contaminated sites as they provide tremendous fine roots in the surface soil. Grass species are effective at binding and transforming hydrophobic contaminants such as BTEX and PAHs due to large fine root biomass that can hold a higher microbial population than other species of a comparable size (Kamath et al. 2007; Chiapusio et al. 2007).

2) Legume – rhizobium symbiosis in phytoremediation

Nutrient deficiency, particularly that of nitrogen and phosphorus is common in contaminated soils (Wenzel 2009). In addition, resource competition among soil biota makes nutrients a limiting factor of bioremediation. Under extreme conditions, especially when soil temperature or moisture content is low, N deficiency is exacerbated due to poor nutrient transportation and restricted enzymes and microbial activities (Wenzel 2009). Chaineau et al. (2003) suggested that adequate fertilization and periodical tillage are helpful in PHC degradation as compared to untreated soil. In their experiment, 70% to 81% of the initial PHCs were removed through bioremediation in fertilized soils compared to 56% through natural attenuation without nutrient addition.

However, excessive use of nitrogenous fertilizers can result in environment problems. To avoid this problem, nitrogen fixing plants, such as legumes, can be used instead (Miller & Cramer 2004). Rhizobia are able to penetrate the roots of leguminous plants and form symbiotic associations, nodules, which are able to fix atmospheric nitrogen into the plant as ammonia (Suominen et al. 2000). Azotobacter, azospirillum, rhizobium, actinomycete, frankia, blue-green algae and anabaena are commonly used N-fixing microorganisms in soils (Havlin et al. 2010). This capacity of biological N-fixation is substantial, often exceeding 100 kg ha^{-1} y^{-1} (Vitosek et al. 2002). The rhizobia have also been found to increase the uptake of K and P by plants (Vershinina 2012).

The interaction between rhizobia and legume plants has been proved to be successful in remediating PHC and heavy metal contaminated soils (Pajuelo et al. 2011).

Legumes such as alfalfa (*Medicago sativa*), Fescue (*Vulpia myuros*), rye (*Elymus sp.*), clover (Trifolium *sp.*) and reed canary grass (*Phalaris arundinacea*) have been successfully used to remediate contaminated sites, especially petrochemical waste contaminated soils (Kamath et al. 2007; Chiapusio et al. 2007). The use of woody legumes in tropical regions reflects their abundance there *(*Vitosek et al. 2002). Legumes are also associated with different microbial populations. Like grass, legumes can create an aerobic soil environment and stimulates microbial activity, resulting in an enhancement in oxidation of organic chemical residues (Peer et al. 2006).

3) Trees and their hybrids in phytoremediation

Trees are widely used in the remediation of PHC contaminated soils. As recorded in the CLUIN phytoremediation database, most of the successful phytoremediation projects were carried out using trees (Table 3).

Fast growing plant hybrids with desirable characteristics (e.g. resistant from diseases, pests, contaminants, harsh climates and soil conditions) have been selected as prospective candidates in phytoremediation (ITRC 2009). For instance, hybrid trees such as from poplars and willows have been successfully and widely used in the phytoremediation of both organic and inorganic polluted soils. However, attention should be paid to avoid the risks of using genetically modified or engineered species (ITRC 2009).

Table 3. Cases of using forest trees in phytoremediating the PHCs in soils (source: CLUIN phytoremediation database)

Project name (period)	Trees and other vegetation used	Contaminants (initial concentration) in media	Phytoremediation mechanisms	Cost of the project	Performance and lessons learned	Contact
Phytoremediation at a former fuel loading facility in Ontario (1999- 2006)	Hybrid poplar trees and associated ground cover vegetation	BTEX, Xylenes (1, 7000 µg/L) in soil	Hydraulic control	Not available	Concentrations of BTEX and xylenes in groundwater showed consistent decrease. Petroleum hydrocarbon concentrations fluctuate from year to year.	tereza.dan@jacqueswhitford.com
Phytoremediation at a gasoline release site in Georgia (1999-2002)	White willow, black willow, wooly bull rush, cattails, rush, native sedge	Gasoline in soil and ground water (Soil ave BTEX: 1,400 µg/L; ave benzene: 44 µg/L)	Phytovolatilization Rhizodegradation Phytodegradation	Not available	Soil BTEX concentrations decreased 82% and benzene decreased 80%. Approximately 90% of the planted trees survived in the first growing season, although the highest mortality was in areas with the highest gasoline concentrations. Low concentrations of BTEX were found in plant branches and leaves as was benzoic acid (a degradation product).	woniell@planteco.com
Phytoremediation at a Hydrocarbon Burn Facility at NASA Kennedy Space Center in Florida (1998-)	4,400 trees and understory grasses	TPHs (110-760 mg/kg) mixed with other organic compounds in soil	Hydraulic Control Phytoextraction Phytovolatilization Rhizodegradation	$70,000	Not able to establish phytoplantation due to competing vegetation (grasses) and drought.	lou-licht@ecolotree.com

Phytoremediation at a privately owned scrap yard in the Southeastern United States (2001-2006)	Mulberry and bermuda grass	TPHs (0.77-222 ppm) and PCBs (10-14,800 ppm) in soil	Hydraulic Control Phytoextraction, Phytovolatilization Rhizodegradation	Approximately $140,000	TPHs and PCBs were reduced to acceptable low occupancy levels at a cost that was orders of magnitude lower than offered by other conventional remedial alternatives.	flc33@sbcglobal.net
Phytoremediation at a self-serve gasoline fueling facility in California (1999-2000)	Conifers	MTBE and t-Butyl alcohol in ground water	Phytovolatilization Phytoattenuation	Not available	The mean MTBE and TBA concentrations downgradient of the stand in Wells MW-2 and MW-4 were 200 ± 240 µg/L (n = 13) and 93 ± 110 µg/L (n = 11).	carnold@waterboards.ca.gov
Phytoremediation at a solvent spill site in Iowa (2002-)	Hybrid poplar and understory grasses	Toluene and xylenes (20,000 mg/kg) in ground water	Hydraulic Control	Not available	PCE, 1,1,1-trichloroethane, toluene, and xylenes are all trending downwards in the area of highest initial concentrations. More than 93% of the trees survived, but some phytotoxicity was observed in area of highest PCE concentrations.	lou-licht@ecolotree.com
Phytoremediation at Active Retail Gas Station in Ohio (1997-)	Hybrid poplar, black Willow and maples	BETX in ground water	Hydraulic Control Phytoextraction Phytovolatilization Rhizodegradation	Design: $3,500 Installation: $12,000 Annual operations and maintenance: $8,500	There was 100% tree survival through the first four growing seasons. A groundwater drop of 4.5 to 5.5 feet was observed in the third and fourth planted area.	Wjozewicz@arcadis-us.com
Phytoremediation at an abandoned gasoline station in Denmark (1999-)	Hybrid poplar and willow	TPHs >20,000 mg/kg in soil	Hydraulic Control Phytoextraction, Phytovolatilization Rhizodegradation	Not available	Trees survived higher concentrations in the field than in the lab. Low to medium levels of hydrocarbon contamination can be treated with willows.	uka@dmu.dk

Phytoremediation at an experimental dredged sediment disposal site in Belgium (1999-2004)	Willow	PAHs (11.88 mg/kg) in sediment		Not available	In two growing seasons, mineral oil concentration declined 57% in the vegetated plot compared to 15% in the control plot. In contrast, the control plot saw a decline in total PAH concentration of 32% compared to only 13% in the vegetated plot.	jan.mertens@ugent.be
Phytoremediation at an unknown BTEX contaminated experimental field (1999-)	Hybrid poplar, English oak, European ash	Trichloroethene (100 mg/L) and BTEX in ground water	Hydraulic Control Phytoextraction Phytovolatilization Rhizodegradation	Not available	Within the plant-associated bacterial communities, strains were identified that could potentially be used to improve phytoremediation strategies e.g. by *in situ* inoculation of these bacteria.	nele.wayens@uhasselt.be
Phytoremediation at an unknown toluene-contaminated site in New Jersey (1997-1998)	Hybrid poplar	Toluene (900 mg/L) in soil and ground water	Phytovolatilization Rhizodegradation	$51,005	Trees need to be planted earlier in the spring to reduce transplanting shock.	aferro@ensr.aecom.com
Phytoremediation at AOC-539, Grand Forks Air Force Base in North Dakota (2001-)	Hybrid poplar, Eastern cottonwood, Russian olive, Carolina poplar, imperial Carolina poplar	TPHs (1400 mg/kg) in soil, (19 mg/L) in ground water and other organic compounds	Hydraulic Control Phytoextraction Phytovolatilization Rhizodegradation	1 year monitoring: approximately $320,000	To date there are no clear trends in contaminant concentrations in groundwater or soil.	larry.olderbak@grandforks.af.mil
Phytoremediation at Ashland Inc. in Wisconsin (2000-)	Hybrid poplar and understory grasses	Diesel fuel in soil. BTEX, gasoline, ethylenzene and other organic	Hydraulic Control Phytoextraction and Rhizodegradation	$80,000	Trees have tripled in height since planting. Roots observed at 10 feet depth during first growing season. Subsurface aeration has increased soil	jevondracek@ashland.com

		compounds in soil and ground water			oxygen levels from 5% to 15%.	
Phytoremediation at Bofors-Nobel Superfund Site in Michigan (1999-2002)	Hybrid poplar, bur oak, jack pine, white willow, Norway maple, hackberry, honey locust, black hills spruce, Eastern red cedar	BTEX in soil and ground water	Hydraulic Control Phytoextraction Rhizodegradation	Estimated total remedy cost can be from about $15 million up to $30 million.	Phytoremediation is not the main goal of the remedy. The main goal is containment using the underground barrier (slurry) wall, with phytotechnology as an enhancement.	fagiolo.john@epa.gov
Phytoremediation at former MGP site in New Hampshire (2005-)	Conifers and hardwoods	Residue BTEX in ground water	Hydraulic Control	Not available	To date, the system has experienced 92% survival of the plantings and early indications are that roots are extending downward toward the capillary fringe.	ctammi@ensr.aecom.com
Phytoremediation at Fort Drum Gasoline Alley (2007-)	Willow	BTEX in surface water	Hydraulic Control	$1,000,000	Survival rate is anticipated at 90%. Over 50,000 gallons of water are able to be filtered through the 22,000 planted willows daily.	canowak@esf.edu
Phytoremediation at Greiner's Lagoon in Ohio (2004-)	Hybrid poplar and switch Grass	BTEX and other contaminants (incl. Heavy metals) in soil (0.160 mg/kg) and ground water (63 µg/L)	Hydraulic Control	$719,000	Not known.	williams.thomas@epa.gov

Phytoremediation at Indiana Harbors Canal in Indiana (2002-)	Hybrid poplar and willow	TPHs (250,000 mg/kg) and PAHs (4,100 mg/kg) in soil	Phytovolatilization Rhizodegradation	Not available	Commercial clones exhibited greater survival rates than experimental clones. Overall survival rate of 67% was greater than expected given the high levels of TPH contamination encountered.	rzalensy@fs.fed.us
Phytoremediation at Naples Truck Stop in Utah (1998-)	Siouxland poplar	TPHs in ground water	Phytovolatilization Rhizodegradation	Not available	The ground water concentration reductions could be attributed to rhizosphere microbially enhanced degradation	compton.harry@epa.gov
Phytoremediation at Naval Air Station Joint Reserve Base in Texas - Project 3 (1996-1998)	Eastern cottonwood	Toluene and other organic compounds (e.g. TCE) in ground water	Phytovolatilization Rhizodegradation	Total estimated cost: $641,467	Though the system did not achieve all mass flux goals, the data show a general decrease in TCE concentrations throughout the demonstration site over the course of the study.	rock.steven@epa.gov
Phytoremediation at Oneida Tie Yard Site in Tennessee (1997-)	Hybrid poplar	PAHs (17,500 µg/L) and naphthalene (18,500 µg/L) in soil	Phytovolatilization Rhizodegradation	Design:$50,000 Installation: $90,000 Annual operations and maintenance: $35,000	Concentrations of PAHs and naphthalene were 6,400 µg/L, 4,900 µg/L respectively at the end of 7-year monitoring.	mwiddows@vt.edu
Phytoremediation at Oregon Poplar Site in Oregon (1997)	Hybrid poplar	BTEX and other organic compounds in ground water	Hydraulic Control	Not available	Trichloroethylene, PCE, and/or cis-dichloroethylene were found in the tissue or transpiration gas of three of the four trees examined in 2002, indicating that the tress are utilizing ground water or soil contaminated with these compounds.	compton.harry@epa.gov

Phytoremediation at retail outlets in North Carolina (2003-)	Hybrid Poplar and willow	TPHs in ground water	Hydraulic Control	Not available	Rates of water use (seasonal averages) were higher in Area B (100 L/d) than in Area A (65 L/d), suggesting that the ground water contaminants may be somewhat inhibitory.	aferro@ensr.aecom.com
Phytoremediation at RTDF Site B in Ohio (1999-2002)	Hybrid poplar, hackberry, rye grass, legumes, fescue and willow	TPHs (average of 12,155 mg/kg) and PAHs (0 - 15 cm) (average of 52.3 mg/kg) in soil	Phytostabilization Rhizodegradation	Not available	After the first drought year, vegetation growth was good with plant cover between 60 - 95%. However, there was no evidence that vegetation enhanced degradation of petroleum hydrocarbons at this site.	kulakow@ksu.edu
Phytoremediation at RTDF Site F in New York (1999.2002)	Hybrid poplar, willow, white clover, boreal red fescue, Kentucky bluegrass, annual rye grass, perennial rye grass and volunteer re-vegetation	Soil layer (0-20 cm): TPHs (1429 mg/kg) and PAHs (361.4 mg/kg)	Phytostabilization Rhizodegradation	Not available	The concentrations of PAHs in surface soil declined, and there was trend showing that vegetated treatments were declining more than the unvegetated treatment, The grass/legume mixture and the willow/poplar treatment showed very high vegetation cover.	kulakow@ksu.edu
Phytoremediation at Solid Waste Management Unit 15 in North Carolina (2007-)	White willow, sunflower, Chinese brake fern and sea pink thrift	Benzo(a)pyrene (50 mg/kg), PAHs, Arsenic (0.19 to 52 mg/kg) and Lead (1.8 to 2,700 ppm) in soil	Phytoextraction Phytovolatilization Rhizodegradation	Not available	Not available	jmusella@ensr.aecom.com

Phytoremediation at Solvent Recovery Service New England (SRSNE) Superfund Site - Project 3 (1998-2030)	Hybrid Poplar, sweet gum, silver maple, white willow, pin oak, river birch, tulip tree, Eastern red bud, Eastern white pine	organic compounds (e.g. 1,1,1-Trichloroethane: 35 mg/kg, 1,1-Dichloroethane 25 mg/kg, Toluene :40 mg/kg) in soil	Hydraulic Control Phytoextraction Phytovolatilization Rhizodegradation	Not available	Phytoremediation appeared to reduce the volume of groundwater needing ex-situ treatment by approximately 40%. It is estimated that approximately 340 kg of VOCs were removed in one growing season.	lumino.karen@epa.gov
Phytoremediation at the Combustion Inc. Superfund site in Louisiana (2002-)	Hybrid poplar, eucalyptus and native willow	Mixture of organic compounds (e.g. BTEX, PCB etc.) and heavy metal (e.g. Lead, Nickel etc.)	Hydraulic Control Phytostabilization, Phytovolatilization Rhizodegradation	$1,859,000	Metals are phyto-sequestered in the root zone with minor uptake into leaves, while salinity is accumulated (phytoextracted) into the above ground tissues.	tsaodl@bp.com
Phytoremediation at the Edward Sears Property in New Jersey (1995-2004)	Hybrid poplar	Mixture of organic compounds (e.g. 2700 ppm of Xylenes) in soil and ground water.	Hydraulic Control Phytodegradation	$ 386,400 from 1997 to 1999.	Almost of the contaminants were reduced over the first 3 years of monitoring	prince.george@epamail.epa.gov

3.2.2 Plant growth-promoting bacteria (PGPB)

Certain rhizosphere bacteria have important consequences for plant growth. They can defend plants against pathogens, promote benefical plant-microbe symbioses, increase nutrient uptake by solubilizing phosphate and fixing nitrogen, stimulate plant growth by secreting phytohormone, exhibite antifungal activity, and induce systemic resistance (Bhattacharyya & Jha 2011; Pajuelo et al. 2011). These bacteria are called plant growth-promoting bacteria (PGPB).

PGPB such as *Pseudomonas, Acinetobacter, Achromobacter, Flavobacterium, Bacillus, Nocardia* and *Rhizobium* species have been shown to increase plant yields and SOM contents (Pajuelo et al. 2011). They have also been shown to enhance the rhizoremediation of polluted soils (Vershinina 2012; Pajuelo et al. 2011; Bhattacharyya & Jha 2011).

EMPIRICAL STUDY – THE OIL-CONTAMINATED SOIL REMEDIATION FIELD EXPERIMENT

4 Introduction about the soil remediation field experiment

This soil remediation field experiment was a part of the EU supported FP7 project ‘‘Legume-Futures’’. This project is constituted by 18 research consortiums from 13 major EU countries, aiming to ‘‘develop and assess legume-supported cropping systems that raise the economic and environmental performance of European agriculture’’ (Legume-Futures 2012). It started on 1 March 2010 and is lasting for 4 years. There are currently 13 field experiment sites distributed in different agro-environment zones in Europe (Legume-Futures 2012). Our soil remediation experimental field in Viikki experimental farm of University of Helsinki is one of them.

Fodder galega (*Galega orientalis*) (hereafter referred to as galega) is a perennial, fast-growing forage legume (Lindstrom et al. 1985). It is also suitable for the low temperature and acid soil conditions of northern regions (Lindstrom et al. 1985). Fodder galega in mixture with grasses are able to economically provide continuous and high forage production during summer season without additional N fertilization (Adamovich 2002). One important property for fodder galega is that it can survive in pure stands for 15 to 19 years without thinning compared to other legumes (Adamovich 2002).

Only *Rhizobium galegae* is found to nodulate fodder galega (Lindström 1989). Several greenhouse studies have demonstrated the potential use of *Galega orientalis* (*G. orientalis*) and its microsymbiont *Rhizobium galegae* (*R. galegae)* for rhizoremediation of oil-contaminated soils (Suominen et al. 2000; Cybulski et al. 2003; Lindström et al. 2003; Kaksonen et al. 2006; Jussila et al. 2006; Jussila et al. 2007; Mikkonen et al. 2011a). For example, Suominen et al. (2000) demonstrated that galega, inoculated with its *Rhizobium galegae*, could withstand up to ten-fold higher of toluate (a type of hydrocarbon) concentrations than non-inoculated plants.

Plant growth promoting bactera (e.g. *Pseudomon* strains*)* cannot only promote the growth of *G. orientalis*, but also enhance the capacity of *G. orientalis* and its microsymbiont *R. galegae* in rhizoremediation of oil-contaminated sites (Suominen

et al. 2000; Lindström et al. 2003; Kaksonen et al. 2006; Mikkonen et al. 2011a). For instance, Lindström et al. (2003) isolated several oil degrading bacterial species from galega rhizoshere and found that the ability to degrade *m*-toluate (3-methylbenzene) in the presence of the gene xylE, which is an indication of toluene degradation, was only detected within the genus *Pseudomonas*. Another research further indicated that *Pseudomonas* could increase the numbers and diversity of cultivable bacteria in *G. orientalis* rhizosphere in oil-contaminated soil (Kaksonen et al. 2006). A new greenhouse experiment showed that co-inoculation of fodder galega with *R. galega* HAMBI 540 and *P. trivialis* 3Re27 or with *R. galegae* HAMBI 540 and *P. extremorientalis* TSAU20 could increase yields, nodulation and N content of fodder galega, compared to plants inoculated with *R. galegae* HAMBI 540 alone (Egamberdieva et al. 2010).

However, it is unknown whether the results from greenhouse experiments, where conditions are controlled, can be transferred to the field. For example, the productivity of galega-grass swards is significantly affected by sowing time, climate, N fertilizers, and the frequency of cutting (Zolotarev 2010).

An extensive multi-year field experiment with crops (*Bromus inermis* and *Galega orientalis*) under PGPB and oil contamination of 7000 ppm are designed in our project in order to translate the results of greenhouse experiments into real applicability.

4.1 Objectives of the experiment

The overall objectives of the empirical study was to 1) determine if *Galega orientalis*, a legume, and *Bromus inermis*, a grass grown separately or in combination had a significant effect on phytoremediation of oil-contaminated soil, 2) investigate the effect of using plant growth promoting bacteria (PGPB) on plant growth and oil remediation, and 3) study the recovery ability of soil under moderate oil contamination by analyzing the differences in soil total DNA, soil bacterial diversity and soil bacterial community structure with time in Nordic field condition.

4.2 Hypotheses

The main hypotheses of the experiment are as follows:

1) Crops would enhance oil removal compared to bare soil.
2) Crop performance on oil removal: galega + bromus > bromus > galega.
3) PGPB would enhance oil removal and dry matter yields of galega.

5 Materials and methods

5.1 Experimental field design

The field was established in June 2009 in Viikki experimental farm, Helsinki (60°14‘N, 25°01‘E) by the ‘’Legume Futures’’ group. The general information about the field is listed in Table 4.

This field was originally planted with *Salix* species. The field experiment was a split-plot design, where plant species (galega, bromus, galega+bromus, unvegetated control) was the main factor and oil contamination (+/ -) and use of PGPB (+/-) were the subplots in factorial combination. The size of each plot was 2.5 x 1.5 m. There were four replicates in the experiment with a total of 64 plots as illustrated in Figure 4 and Figure 5.

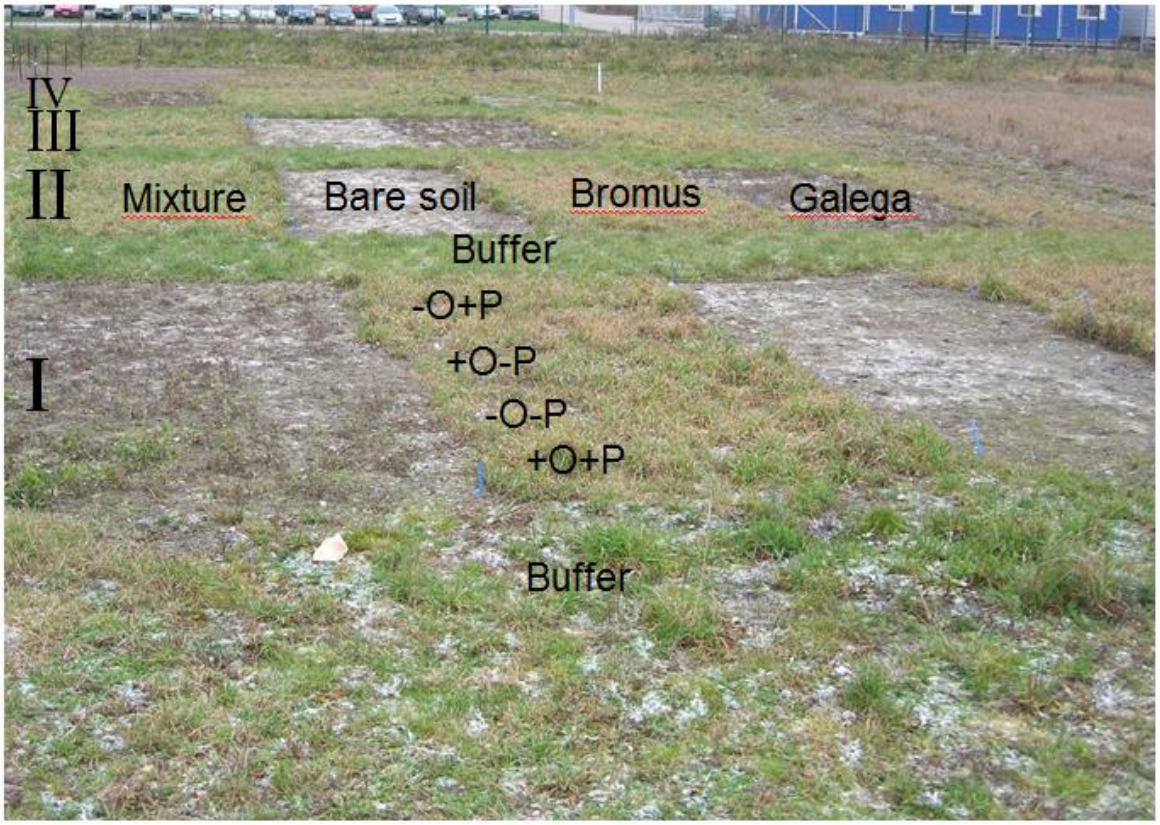

Figure 4. Layout of the experiment, photographed in November 2010. Four replicates I-IV of 4 main plots (Galega, Bromus, Mixture, Bare soil) each of which has 4 subplots of ±O (oil) and ± P (PGPB) in factorial combinations (Yan et al. 2012)

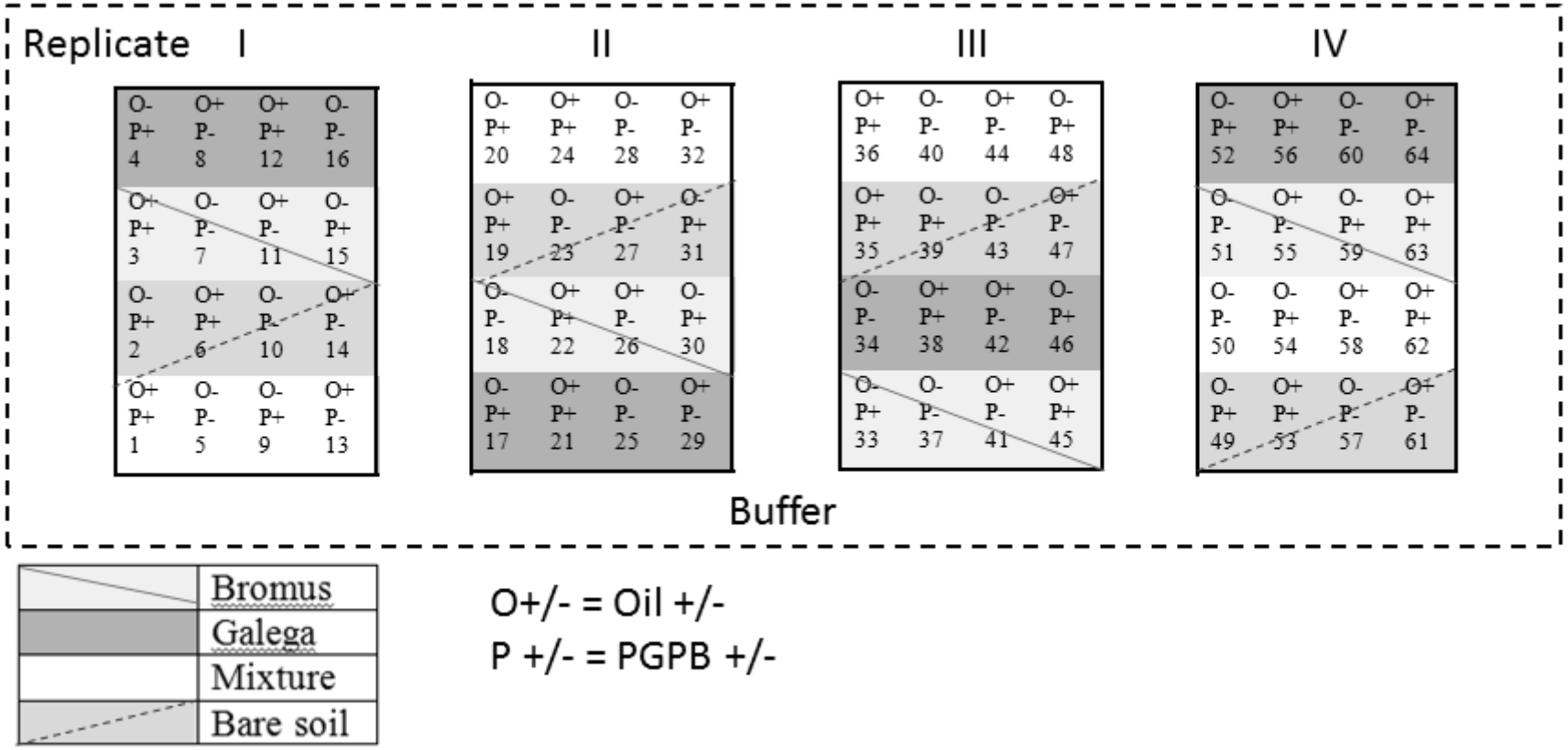

Figure 5. Layout of field experiment, showing species, oil and PGPB treatment. Numbers refer to plot number.

Table 4. General information about this field

Site size	About 420 m^2
Soil structure	Clay loam
Altitude	20 m
Vegetation zone	Boreal
Annual precipitation	650 mm
Annual mean temperature	4.9 ^{0}C
Farming systems	Organic / Intergrated
Oil+ (motor oil 'SAE 10W-30') treatment	7000 ppm or 7 g oil / kg dry soil

5.2 Selection of species

Two species, smooth brome (*Bromus inermis*) that is a cool-season perennial, sod-forming grass, fodder galega (*Galega orientalis*) that is a leguminous plant, two stains of *Pseudomonas* (*P. trivialis* 3Re27 and *P. extremorientalis* TSAU20) and *Rhizobium galegae* HAMBI 540 were selected in our experiment.

Figure 6. *Bromus inermis* (left) and *Galega orientalis* (right), photographed in June 2010.

5.3 Field preparation and management

5.3.1 Soil contamination and preparation

The oil used was a mixture of 'SAE 10W-30' motor engine oil, which was the waste oil from the tractors and cars belonging to the university at Viikki campus, Helsinki. Density of motor oil is about 0.89 kg/l. In an oil-contaminated plot, 7000 ppm or 7g kg^{-1} of motor oil was designed, assuming soil bulk density is 1.0 g/ml. For the 32 oil+ plots, a total of 6 kg (c.a. 6 L) oil was mixed with 10 kg white coarse sand (0.5 - 1.2 mm) in a concrete mixer. The same amount of pure sand was mixed without oil for the other 32 oil- plots. The oil/sand mix and pure sand was then spread out and

mixed into the top 20 cm layer in respective plots with a rotary tilling device on June 17, 2009.

5.3.2 Co-inoculation of PGPB and R. Galegae on Galega orientalis

Befores sowing, commercial galega seeds (Naturcom Oy, Ruukki, Finland) were first surface-sterilized and germinated on 1% water agar in the dark at 28 ^{0}C. The two strains of Pseudomonas, *P. trivialis* 3Re27 (Graz University of Technology, Graz, Austria) and *P. extremorientalis* TSAU20 (National University of Uzbekistan, Tashkent, Uzbekistan), were grown in King's B (KB) broth and *Rhizobium galegae* strain HAMBI 540 (University of Helsinki, Helsinki, Finland) in tryptone yeast (TY) broth overnight. One ml and each culture was pelleted by centrifugation, washed and suspended in PBS before diluted to an optical density of 0.1 at 620 nm. Cell suspensions with three strains were mixed in a ratio of 1:1:1 and vortexed to obtain a homogenous suspension. Germinated seeds were inoculated by placing them in the bacterial suspension with sterile forceps and shaken gently for a few seconds. The treatments were as follows: 1) seeds inoculated with *R. galegae* HAMBI 540 alone and 2) seeds inoculated with the combination of *R. galegae* HAMBI 540, *P. trivialis* 3Re27 and *P. extremorientalis* TSAU20. The inoculated seeds were mixed with the peat prior to sowing. Detailed procedures on co-inoculation can refer to Egamberdieva et al. (2010).

5.3.3 Sowing

The inoculated seeds were sown by hand on surface and lightly covered with soil by raking. First sowing was done on July 7, 2009. For pure plots, 25 kg/ha of galega and 35 kg/ha of bromus seeds were sown. For the mixture plots, 6 kg/ha of galega and 26 kg/ha of bromus seeds were sown. Due to a slow initial growth performance of galega, an extra sowing of galega seeds was done in May 2010.

5.3.4 Weeds control and fertilization

This field was treated with herbicide (Glyphosate) before the establishment of the field in June 2009 and Basagran® SG (165 gr/50L) in September 2009. Afterwards, weeds were controlled manually once every month. Weeds in the border area were not cleared as they can serve as buffer to prevent oil from surface spreading and disturbances from neighbouring fields. 60 kg/ha N-fertilizer was given to the grass plots in the summer of 2009 but none was given subsequently.

5.4 Data collection

5.4.1 Soil sampling

From July 2009 to May 2011, the soil was sampled four times (Table 5).

Table 5. Soil sampling time

Label	Year	Growing season	Date
A	2009	Beginning	July 16 - 17
B	2010	Beginning	May 17 - 25
C	2010	End	November 12
D	2011	Beginning	May 17 - 19

On each occasion, sixteen sub-samples were taken from the top soil (0-25 cm) in each plot (Figure 7) using an auger with the diameter of 2 cm. The sampling was designed so as not to disturb the plants. Since the germination of galega in the first year was not very successful, the samples were taken under galega canopy in the pure galega plots. In the mixture (galega+bromus) plots, 2 out of 16 sub-samples were taken under galega canopy. The 16 sub-samples were combined to one composite sample per plot, mixed, sieved through a 5 mm mesh, put in a plastic bag and stored at -20 °C before analysis. If the samples could not be pre-treated in one day, they were stored at -20 °C.

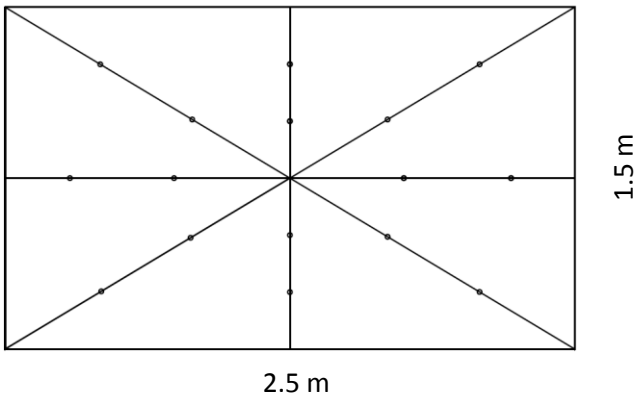

Figure 7. Soil sampling of a plot (the 16 dots represent the sampling points).

5.4.2 Plant harvest and chlorophyll content

The plants were harvested twice in a growing season in the year of 2010 and 2011. First cut was done when flowering began in late June and the second cut was done in late August. The total fresh biomass was weighed per plot by species in the case of the mixture (galega+bromus) plots. For each of the pure species plots, one kilogram

of fresh plant sample was taken for analyses. For the mixture plots, one kilogram of bromus and 200 grams of galega were taken for analyses.

Before each harvest, chlorophyll content was measured using a SPAD 502 Chlorophyll Meter (MINOLTA CAMERA, Japan) once. The leaves selected for measurement were healthy and unfolded ones located at the top of the plants. In the pure species plots, 20 leaves per plot were selected. In the mixture plots, 20 bromus leaves and at least 10 galega leaves were selected.

5.4.3 Meteorological data

According to the Finnish Meteorological Institute's statistics, there was a particularly warm summer in 2010 in Finland with several temperature broken records (Hutila 2011). Summer mean temperature in 2010 was 1.87 ^{0}C higher compared to that in 2009 (Table 6).

Table 6. Monthly average temperature (^{0}C) in summer 2009 and 2010 reported by Finnish Meteorological Institute in the observation in Kumpula campus

Month	Year 2009	Year 2010
May	11.4	11.9
June	14.4	14.9
July	17.3	22.2
August	16.6	18.2
Mean	14.9	16.8

5.5 Laboratory analyses

5.5.1 Soil chemical analysis

Several soil properties were measured from the 128 soil samples taken in July 2009 and November 2010, including: dry matter weight, apparent bulk density, electrical conductivity, pH in water, and total carbon and nitrogen. After thawing, the samples were air-dried at room temperature (1 week), ground by hand and sieved (2 mm) before analysis.

1) Dry matter content (DM)

The dry matter content (%) to an oven-dry basis was measured to convert bulk density and total C and N contents. Crucibles were put to oven overnight at 105 ºC

before weighing the dry weight. About 1 g of air dried soil sample was weighed before and after it was dried in a 105 ºC oven overnight together with crucibles. Dry matter content is calculated as the formula below:

$$\text{Dry matter content (d.m.)} = (W_{c+os} - W_c) / W_{as} * 100\%, \quad (1)$$

where W_c is the dry weight of the crucible, W_{c+os} is the total weight of the crucible and soil after oven drying and W_{as} is the weight of air dried soil.

2) Apparent bulk density

Apparent soil bulk density was determined using a special 10 ml measuring cup. The cup was weighed before filling soil in. Then the cup was gently knocked with a ruler for ten times to prevent compacting soil. The total weight of 10 ml pre-treated soil sample and the measuring cup was recorded and the apparent bulk density was calculated by the formula below:

$$\text{Apparent bulk density} = [(W_{m+s} - W_m) / \text{d.m.}] / V_m, \quad (2)$$

Where W_{m+s} is the total weight of the measuring cup and soil, W_m is the weight of the measuring cup and V_m is the volume of the measuring cup (10 ml).

3) Soil total C and N

Total carbon and nitrogen contents were determined using a Vario Max CN-analyzer. For each determination, about 500 mg of pre-treated soil sample was weighed in a special sample cup and then burned in an induction oven. The analyzer gives the results as per cent C and N by weight. The values were then converted into dry weight content by DM.

4) Electrical conductivity index and pH

Electrical conductivity (EC) and soil pH was measured in water suspension. EC was measured from the solution part of the water-soil suspension. About 2 g pre-treated soil sample was weighed into a 10 ml tube and then mixed with 5 ml deionized water, suspended and left overnight. Electrical conductivity was first measured from the upper solution of the water-soil suspension using a conductivity meter (MeterLab™ CDM210) the next morning. Soil pH was then measured by a pH-meter (SCHOTT CG842) in the suspension after mixing with a glass rod.

5.5.2 Total solvent extractable material (TSEM) of oil

The total solvent extractable materials (TSEM) analysis provides a gross measure of petroleum in the samples by gravimetric analysis (Mills et al. 1999). TSEM extraction was done by Anni-Mari Pulkkinen (Department of applied chemistry, University of Helsinki). The total solvent extractable materials (TSEM) were extracted from thawed samples according to Jørgensen et al. (2005) modified from the ISO 16703:2004 method. Soil samples (10 g) were extracted with 10 ml retention time window solution (HPLC-pure heptane + 30 ml decane + 30 mg tetracontane) and 20 ml acetone (HPLC-pure) by ultrasonication for 30 min. A blank sample (without soil) was prepared in every extraction day. Organic phase was removed by duplicate washing of the extract with deionized water at the ratio of 1:1, followed by centrifuge (2500 rmp / 5 min). Finally the organic phase was dried with water free Na_2SO_4 (c.a. 0.1 g).

TSEM was measured gravimetrically from the hydrocarbon extract (1 ml) evaporated under N_2 (+30 ^{0}C) until dry. TSEM of the same kind of motor oil as used in the oil treatment (Teboil Lubricants Classic Mineral Motor oil, moniaste SAE 10W-30, API SF/CD) was also extracted and determined.

5.5.3 Soil DNA analysis

1) Soil DNA extraction

Soil microbial DNA was directly extracted from 0.50 g fresh soil sample which had been stored in -20 °C freezer, with FastDNA SPIN kit for Soil (Qbiogene, USA), according to the manufacturer's instructions, except for the prolongation of the lysing matrix tube centrifugation time (5 min) and the final elution volume (75 µL – 150 µL).

Altogether there are 256 DNA extracts, 64 for each sampling time. The yield and quality of DNA extracts were checked on a 1 % agarose gel on 1 × TAE buffer.

2) Total DNA amount by fluorometric method

Double-stranded DNA can be bound to some fluorescent dyes, providing a quantitative assay for DNA (Invitrogen 2010). The quantity of extracted DNA was measured fluorometrically (PicoGreen dsDNA Quantification Reagent Kit;

Molecular Probes) according to Mikkonen at al. (2011b) with procedures shown in the appendix.

3) 16S rRNA genes amplification

Based on the natural length variation of 16S ribosomal RNA genes, length heterogeneity analysis of polymerase chain reaction-amplified 16S rRNA genes (LH-PCR) has been widely used to study the pattern of microbial diversity and the response of a microbial community to environmental perturbation (e.g., release of pollutants) (Tiirola et al. 2003). The protocol for LH-PCR bacterial community fingerprinting was adopted from Tiirola et al. (2003) and Mikkonen (2008). The general bacterial primers fD1 (5'-AGAGTTTATCCTGGCT-CAG) (forward primer) and PRUN518r (5'-ATTACCGCGGCTGCTGG) (reverse primer) needed were ordered from Oligomer (Helsinki, Finland). Half of the reverse primer was labeled with FAM fluorophore at the 5' end, for running them in capillary electrophoresis. Humus is known to inhibit the amplification of DNA fragments and therefore the soil DNA extract was diluted 50 fold before amplification. Previous studies (Mikkonen et al. 2011) have shown that PCR reactions and capillary electrophosresis were highly reproducible with high profile similarity of replicate PCR products (> 99.8 %). Therefore, there was no need to do replicates of PCR amplification.

The master mix of PCR reagents was prepared by adding the regents of concentration and order shown in Table 7. The diluted soil DNA templates (1 µl each) were added separately after aliquoting the master mix with 49 µl in each reaction tube.

Table 7. Details on the composition of PCR reagents, modified from Mikkonen (2008)

Reagent	Product details	Final concentration	Amount (1x), µl	Master mix (20x), µl
water	Autoclaved Milli-Q water	-	33.00	660.00
10x Buffer	10x Taq reation buffer with $(NH_4)_2SO_4$	-	5.00	100.00
$MgCl_2$	25 mM	2 mM	4.00	80.00
BSA	Bovine serum albumin, acetylated 10 mg/ml, Promega	0.05%	2.50	50.00
dNTP	dNTP Mix, 10 mM each Finnzymes	0.2 mM each	1.00	20.00

fD1	10mM dilution in water	0.3 mM	1.50	30.00
½ PRUN518r	10mM dilution in 1/10 TE buffer	0.15 mM	0.75	15.00
½ PRUN518r (FAM)	10 mM dilution in 1/10 TE buffer	0.15 mM	0.75	15.00
Taq polymerase	Taq DNA polymerase 5 U/µl	2.5 U	0.50	10.00
Template	(1:50 diluted) soil DNA	variable	1.00	

DNA amplification was performed in a Peltier Thermal Cycler DNA Engine (PTC-200, MJ Research). Program settings for soil DNA amplification according to Tiirola et al. (2003) and Mikkonen (2008) are shown in Table 8.

Table 8. PCR thermal cycling program

Step	Program	Temperature (^{0}C)	Time (min)
1	Initial denaturation	95	5
2	Denaturation	94	45
3	Primer annealing	55	1
4	Extension	72	2
5	Repeat Steps 2-4 for 29 times		
6	Final	4	No limit

5 µl PCR products and 1 µl GeneRuler™ 100 bp DNA Ladder (Fermentas) were mixed gently with 6x DNA loading dye (1 µl) respectively, loaded on a 2 % agarose gel (with ethidium bromide) and then run at 100 V for 60 – 80 min. This process was used to check the quality and quantity of PCR products. The ready-to-use GeneRuler was performed as molecular size standards.

The sequence length of partial 16S rRNA genes amplified varied between 465 and 563 bp, a difference in fragment length large enough to allow separation of short 16S rRNA gene sequences (Tiirola et al. 2003). Because the longest marker of the commercial GeneScan-500 TAMRA Size Standard (Applied Biosystems, UK) is only 500 bp, self-made bp length standards (470 bp, 527 bp and 553 bp) were amplified using the same forward primer (Tiirola et al. 2003; Mikkonen 2008). The only difference was that the reverse primer was fully labeled by HEX for the

amplification of the standards, while in soil DNA amplification the reverse primer was half labeled by FAM. FAM and HEX are two fluorescence dyes and used to distinguish standards and soil PCR products in capillary electrophoresis. PCR products of these three size standards (10 µl each) were then mixed with 70 µl Hi-Di formamide and diluted 10 fold (0.1×standard mix).

4) Capillary electrophoresis (CE)

The amplicons were separated based on their length difference with polyacrylamide capillary electrophoresis by ABI PRISM 310 Genetic Analyzer with a 47 cm capillary and POP-6 Polymer (Applied Biosystems) (Mikkonen et al. 2011). The CE samples were prepared by thoroughly and carefully mixing Hi-Di formamide and HEX-labeled size standard (0.1×standard mix). The volumes of reagents for CE sample preparation (Table 9).

Table 9. Volumes of reagents for capillary electrophoresis

Reagents	Volume (µl)
PCR products	3.00
0.1× Size standard mix (HEX-labeled)	1.75 – 3.00
Hi-Di Formamide	9.00 – 10.25
Final total sample volume	15

The prepared samples were denatured with the same Peltier Thermal Cycler DNA Engine at 98 ^{0}C for 3-5 minutes and transferred immediately on ice before loaded on capillary electrophoresis.

The running condition for CE was defined as Mikkonen (2008) in the operating program (310 Data Collection Software) with injection seconds (10 s), injection voltage (15.0 kV), run temperature (60 ^{0}C) and run time (70 min) per sample. The raw data of the runs (curve-based) were collected, illustrated and exported from the program GeneScan version 3.7 for further analysis.

5.5.4 Plant dry matter content

Plant dry matter content is needed in the calculation of plant dry matter yield. Like the dry matter determination of the soil samples, plant samples were dried at 105 °C

overnight. Fresh plant sample (ca. 1 kg) was weighed before and after drying to calculate the dry matter content.

5.6 Statistical analysis

All soil chemical and plant data were analyzed using Microsoft Windows Excel 2007 and SPSS 15.0 for windows.

The oil carbon content (g C / kg oven-dry soil) in each sampling time was estimated in each contaminated plot as:

Total C content in contaminated plot (g C / kg oven-dry soil) – total C content in non-contaminated plot in the same crop and PGPB treatment within the same block (g C / kg oven-dry soil). (3)

The oil TSEM concentration (g TSEM / kg oven-dry soil) in each sampling time was estimated in each contaminated plot as:

TSEM concentration in the contaminated plot (g TSEM / kg oven-dry soil) – average TSEM concentration in the non-contaminated plots (g TSEM / kg oven-dry soil). (4)

The calculations (3) and (4) assumed that the C content and TSEM concentration of the contaminated plots prior to oil treatment were the same as the non-contaminated plots.

The loss of oil C content between July 2009 and November 2011 in each contaminated plot was then calculated as:

Total C content in July 2009 (g C / kg oven-dry soil) – total C content in November 2010 (g C / kg oven-dry soil). (5)

The loss of oil TSEM content between July 2009 and November 2011 in each contaminated plot was then calculated as:

TSEM concentration in July 2009 (g TSEM / kg oven-dry soil) – TSEM concentration in November 2010 (g TSEM / kg oven-dry soil), (6)

Calculations (5) and (6) assumed that the differences of total C content and TSEM in contaminated plots between July 2009 and November 2010 were all due to oil remediation.

The soil microbial fingerprint data were processed with BioNumerics v.6.6 software (Applied Maths, Sint-Martens-Latem, Belgium). The fingerprint electropherograms were first imported as 12-bit densitometric curves with the Curve Converter into the same set as an artificial gel. The sample (FAM-labeled) curves were aligned and normalized with the internal HEX-labeled standards. Each averaged fingerprint profile (in total 64 profiles) was calculated from 4 replicates using Create Averaged Fingerprint tool. The active area was set to the expected amplicon size of 460 - 565 base pairs (bp) which ranged between 18% - 65% in the profile. Multidimensional scaling (MDS) was performed using Pearson correlation as the similarity coefficient and Ward as the clustering method. Optimization was set to 0.45% (c.a. ±1 bp). The Shannon index (H') was calculated as:

$$H' = -\sum pi \ln pi, \quad (7)$$

where p_i is the relative height of the peak of the *i*th operational taxonomic unit (OTU).

Crop (bromus, galega, galega+bromus, bare control) was designated the main factor, oil (±) and PGPB (±) the subfactors in factorial combination. They were the fixed factors with four replicates as a ''random effect'' in the split-plot ANOVA model in SPSS below for the statistical analysis of pH, EC, total C, total N, bulk density, total microbial DNA and microbial diversity in soils under crop, oil (±) and PGPB (±) treatments.

Y_1 = residue (error) + crop + crop*replicate + oil + PGPB + oil*PGPB + oil*crop + crop*PGPB + oil*crop*PGPB. (8)

For TSEM and oil carbon analysis, the model tested was:

Y_2 = residue (error) + crop + crop*replicate + PGPB + crop*PGPB (9)

Crop was tested against crop * replicate to take out the effect of the main plot from the residual variance so it does not skew the error variance of the subplot stratum. Oil, PGPB and remaining interactions were tested against the subplot error mean square.

Pearson correlation was used to test the correlation between the soil physiochemical parameters. Two-way ANOVA was used to identify differences between treatments in terms of pH, EC, C stocks, soil total DNA, Shannon diversity index and TSEM content. All differences reported are significant at $p \leq 0.05$.

6 Results

6.1 Soil physicochemical properties

The physicochemical soil properties (apparent bulk density, pH in water, EC, total C and N and C/N ratio) values are shown in Table 10 in terms of crop and oil treatment for July 2009 and November 2010.

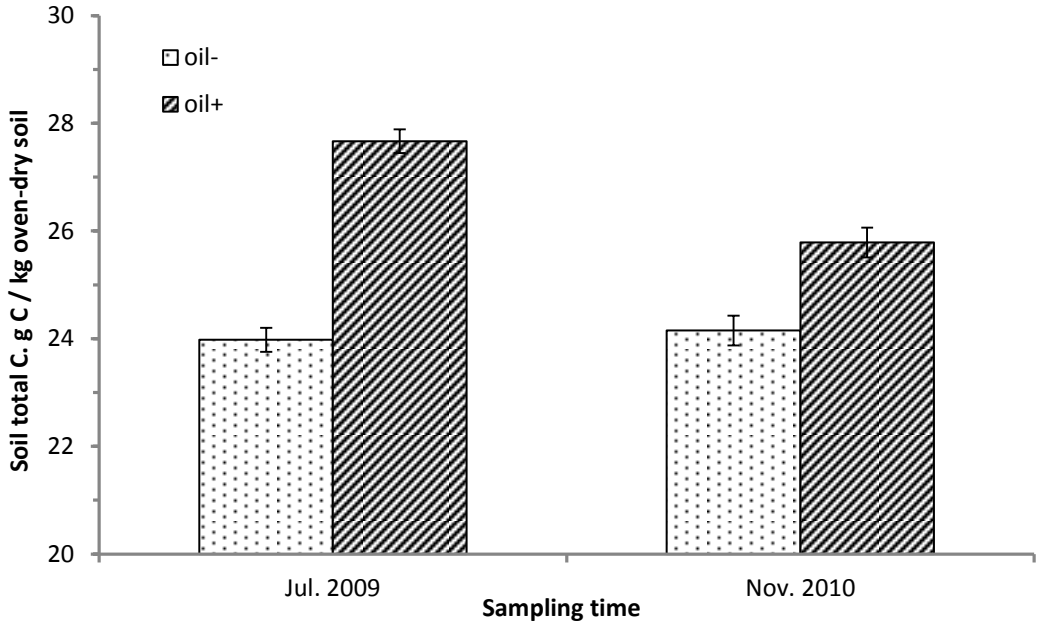

Figure 8. Soil total C content (g C / kg oven-dry soil) between years and oil treatments. Error bars represent SE of mean (n=32).

Soil total carbon content in the contaminated plots differed between years ($p \leq 0.05$, mean 27.67 g/kg vs. 25.79 g/kg), but did not differ in the non-oil treated plots (Figure 8). Crops and PGPB treatments did not result in statistically significant differences in soil carbon content between the two sampling occasions.

No difference was found in soil total nitrogen concentration and pH either between treatments or years. The C/N ratio was higher in the oil-contaminated soils than in the non-contaminated soils ($p \leq 0.05$), especially in the first year samples. However, because of the differences in carbon content, soil carbon content is a simple and good indicator for oil contamination and remediation in soils.

Table 10. Physicochemical soil properties of bioremediation field experiment in Viikki. Values are mean and standard deviation (n=128).

Crop treatment	Oil treatment	Sampling time	Apparent BD (g/ml)	pH in water	EC (µs/cm, 22^0 C)	Total N (g/kg)	Total C (g/kg)	C/N ratio
Bare soil	oil-	Jul. 2009	1.18±0.02	6.44±0.16	77.5±12.6	2.23±0.20	23.77±1.22	11:1
		Nov. 2010	1.12±0.05	6.29±0.15	77.3±18.3	2.19±0.07	22.91±0.73	10:1
	oil+	Jul. 2009	1.18±0.04	6.51±0.22	55.4±7.4	2.16±0.14	27.32±2.31	13:1
		Nov. 2010	1.12±0.06	6.38±0.18	59.7±12.8	2.17±0.12	24.88±1.65	11:1
Bromus	oil-	Jul. 2009	1.17±0.02	6.51±0.15	83.7±9.9	2.16±0.14	23.18±1.59	11:1
		Nov. 2010	1.13±0.04	6.36±0.10	62.8±10.9	2.21±0.21	23.82±3.14	11:1
	oil+	Jul. 2009	1.17±0.03	6.52±0.19	49.6±5.7	2.15±0.25	26.55±1.87	12:1
		Nov. 2010	1.13±0.03	6.34±0.10	50.9±5.3	2.24±0.12	25.60±1.14	11:1
Galega	oil-	Jul. 2009	1.17±0.05	6.52±0.27	86.0±9.0	2.27±0.20	24.02±0.82	11:1
		Nov. 2010	1.13±0.03	6.44±0.11	53.7±8.7	2.29±0.16	24.14±1.74	11:1
	oil+	Jul. 2009	1.17±0.03	6.55±0.13	58.3±5.9	2.25±0.20	28.33±1.77	13:1
		Nov. 2010	1.12±0.04	6.40±0.10	49.7±4.8	2.20±0.23	25.93±1.23	12:1
Mixture	oil-	Jul. 2009	1.15±0.02	6.52±0.30	88.1±20.4	2.19±0.20	24.95±1.39	11:1
		Nov. 2010	1.12±0.05	6.38±0.10	54.9±7.9	2.36±0.12	25.74±2.01	11:1
	oil+	Jul. 2009	1.17±0.02	6.53±0.17	58.3±12.1	2.27±0.21	28.46±2.28	13:1
		Nov. 2010	1.13±0.03	6.38±0.13	56.9±6.5	2.33±0.09	26.73±1.81	11:1

Soil electrical conductivity was correlated with soil total C content in July 2009 (2-tailed Pearson correlation, -0.474, *p*= 0.00). The EC values remained stable in oil treated soils between two occasions, whereas in November 2010 the values decreased ($p \leq 0.05$) in non-contaminated plots (Figure 9). The EC values were not different between crop treatments in the first year (data not shown). In November 2010, EC values were highest ($p \leq 0.05$) in the non-contaminated bare plots and at the same level in all the rest (Figure 10).

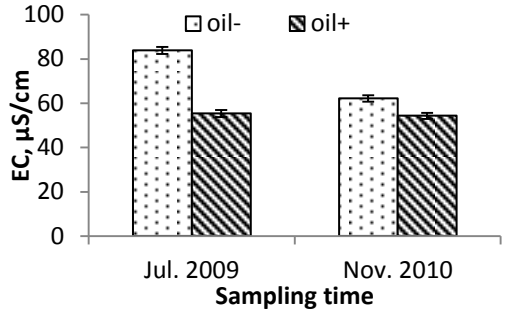

Figure 9. Soil Electrical Conductivity (µS/cm) between oil treatments in Jul. 2009 and Nov. 2010. Error bars represent SE of mean (n=32).

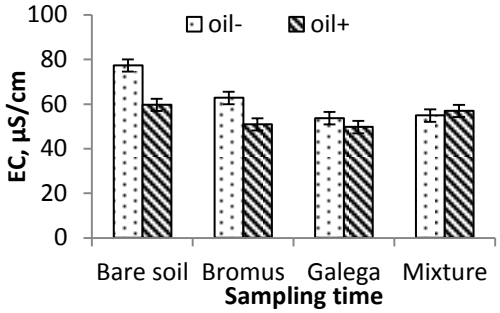

Figure 10. Soil electrical conductivity (µs/cm) between oil and crop treatments in Nov. 2010. Error bars represent SE of mean (n=8).

6.2 Oil content

The oil content of the soil was determined as the difference of 1) total solvent extractable material (TSEM) and 2) carbon content between oil treated plots and the corresponding non-oil treated plots. To test the TSEM method, TSEM of motor oil (Teboil Lubricants Classic Mineral Motor oil, moniaste SAE 10W-30, API SF/CD) was extracted and yielded 100 %.

The differences of TSEM concentration between oil-contaminated and non-contaminated plots are due to the oil treatment (Figure 11). TSEM concentration in non-contaminated plots remained stable (around 1.00 g TSEM / kg of oven-dry basis). The average TSEM in oil-contaminated plots (n=32) decreased with time, e.g., by 47 % from July 2009 to May 2011. There was a high decrease rate (a steep descent) in TSEM content in oil-contaminated plots in the growing season from May 2010 to November 2010. However, in May 2011 the oil was still not completely remediated.. Crops (Galega, Bromus, and Mixture) did not influence TSEM values. To our surprise, TSEM concentrations in bare soil were lower than in the vegetated

plots in May 2011 ($p \leq 0.05$) and the decrease of TSEM from July 2009 to May 2011 in bare plots was also greater than in vegetated ones ($p \leq 0.05$). PGPB treatment did not affect the TSEM values.

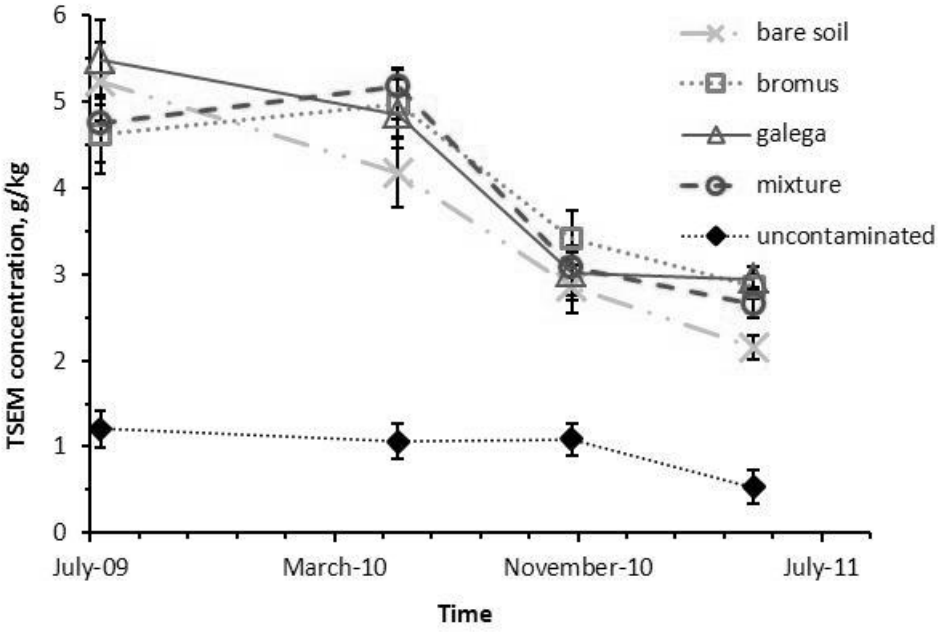

Figure 11. TSEM concentration (g TSEM / kg oven-dry soil) between crop treatments and years in the oil treated plots. Error bars represent SE of mean. The non-contaminated value was averaged by four non-contaminated soil samples in four sampling occasions.

The relationship between soil total carbon and TSEM in oil-contaminated plots is illustrated in Figure 12. Soil TSEM concentration had a positive correlation (n=32, 2-tailed Pearson correlation coefficient 0.405, $p \leq 0.05$) with the total carbon content in contaminated plots in Jul. 2009, whereas no such correlation was detected in Nov. 2010.

The relationship between estimated oil carbon content and oil TSEM is illustrated in Figure 13. Oil TSEM concentration had no correlation (n=32, 2-tailed Pearson correlation) with the estimated oil carbon content in oil treated plots neither in Jul. 2009 nor in Nov. 2010.

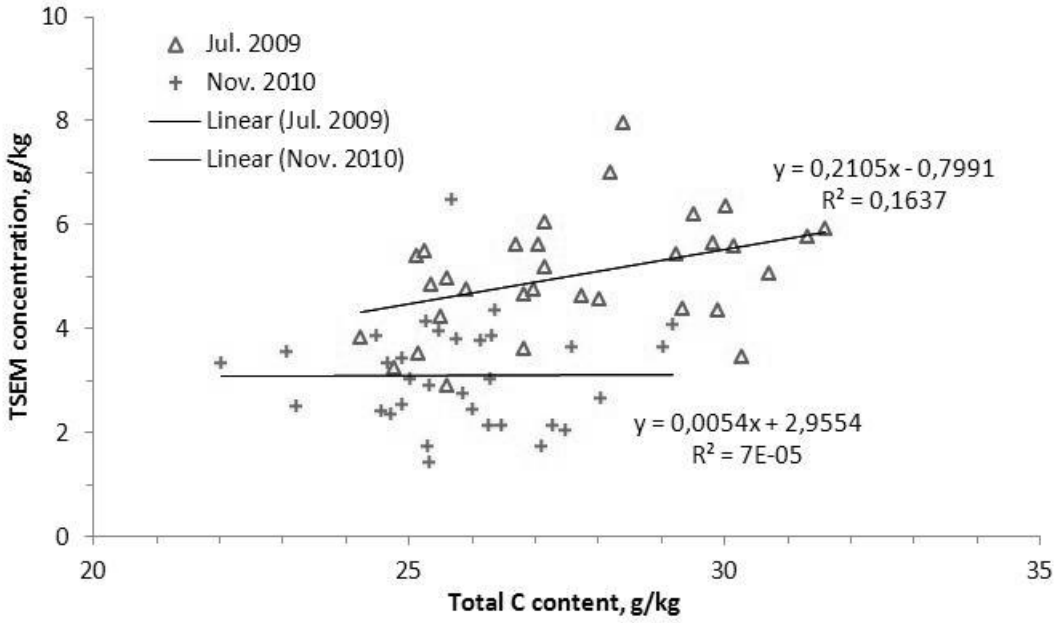

Figure 12. Relationship between total carbon (g C / m^2 oven-dry soil) and TSEM concentration (g TSEM / kg oven-dry soil) in Jul. 2009 and Nov. 2010 in the 32 oil treated plots.

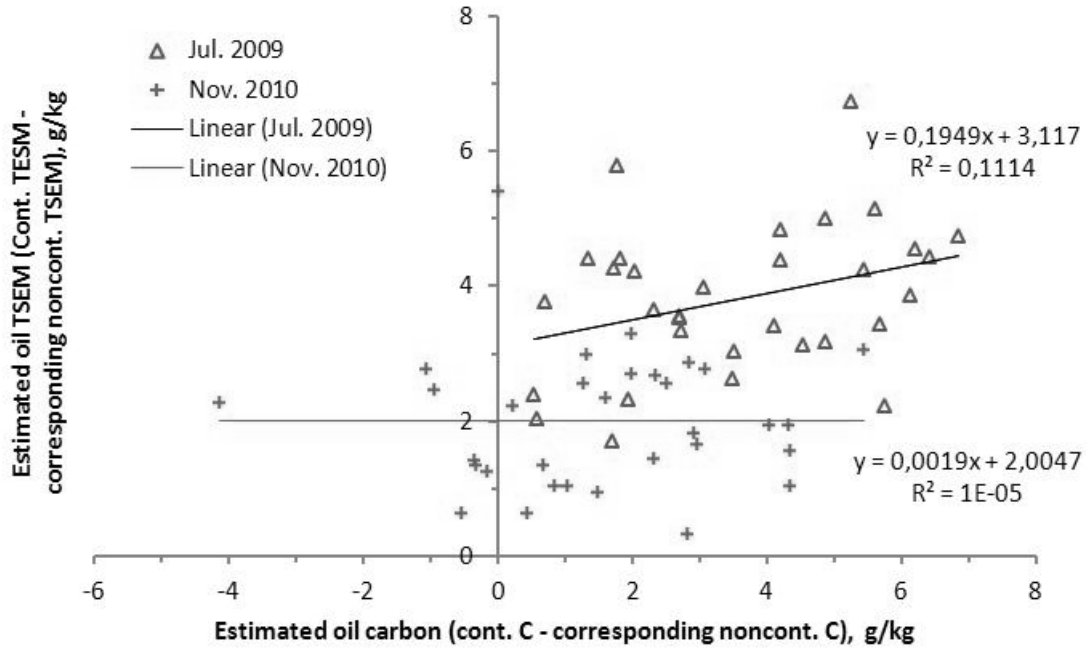

Figure 13. Relationship between estimated oil carbon content (g C / kg oven-dry soil) and oil TSEM concentration (g TSEM / kg oven-dry soil) in Jul. 2009 (n=32) and Nov. 2010 (n=32) in the oil treated plots.

Soil carbon loss and soil TSEM loss are used to indicate oil remediation in the oil-contaminated plots between years. TSEM loss had a positive correlation (n=32, 2-tailed Pearson correlation coefficient 0.442, $p \leq 0.05$) with the estimated oil carbon loss (Figure 14) between July 2009 and November 2010.

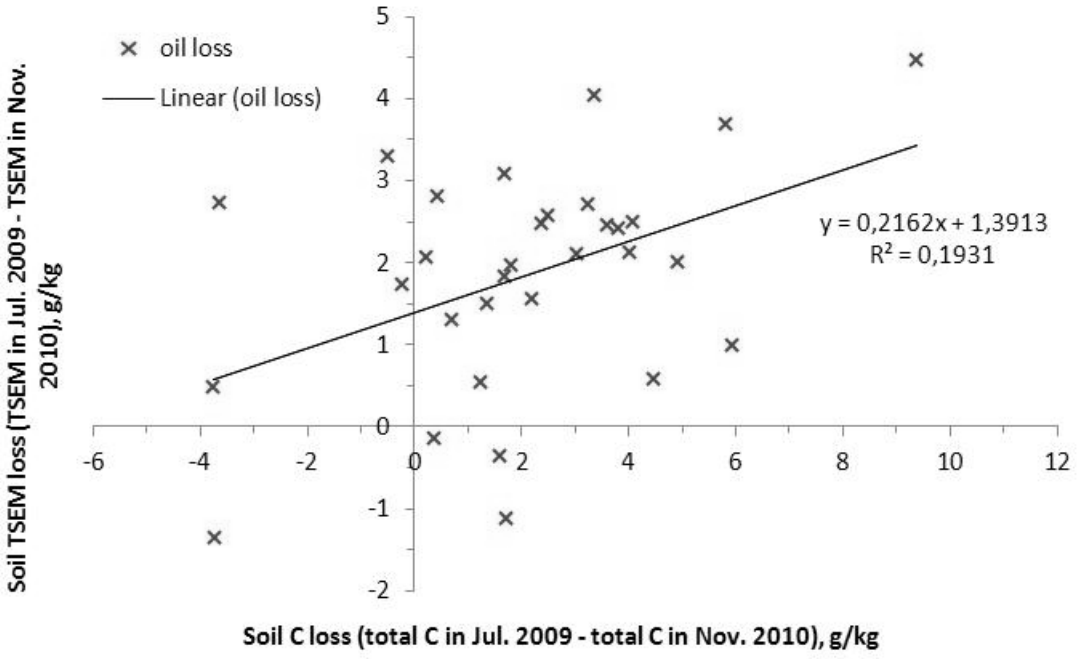

Figure 14. Relationship between estimated soil carbon loss (g C / kg oven-dry soil) and soil TSEM loss (g TSEM / kg oven-dry soil) between Jul. 2009 (n=32) and Nov. 2010 (n=32) in the oil treated plots.

6.3 Plant chlorophyll content and dry matter yields

Plant chlorophyll content was not affected by oil contamination (data not shown). The annual DM yields for the crops were the sum of two cuts. The DM yield in the first cut in June was always much higher than the later one in August. Mixture plots had larger yields in both 2010 and 2011 compared to the pure crop plots.

Surprisingly, the presence of oil slightly increased the dry matter yields of the two crops by 13% in 2010 ($p \leq 0.05$) (Figure 15). However, the enhancement of oil on crop yield was gone in 2011 when there was less oil. Galega yields doubled to 9.53 t/ha in the second year, whereas the bromus yields almost remained unchanged (7.48 t/ha) (Figure 16).

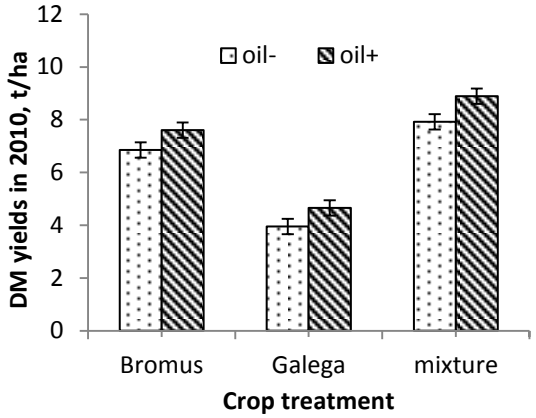

Figure 15. Comparison of the dry matter yields of different crops and their combinations between contaminated and non-contaminated plots in 2010. Error bars represent SE of the mean.

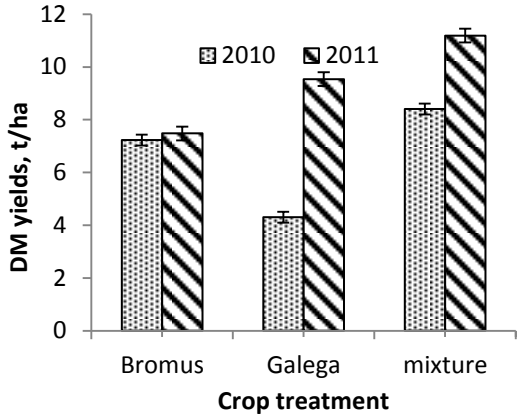

Figure 16. Dry matter yields of crops between years. Oil contamination was not separated from the annual mean values of different crops and their combinations. Error bars represent SE of the mean.

Bromus gained much higher dry matter yields in mixture than in pure plots by 43 % in 2011 ($p \leq 0.05$), although less bromus seeds were sown in the mixture.

6.4 Soil microbial dynamics

6.4.1 Soil total microbial DNA

The total microbial DNA amount was used as an indicator of the total microbial biomass. None of the treatments (crops, oil and PGPB) made a significant difference on soil total microbial DNA amounts. Below, the y-axes do not start from 0.

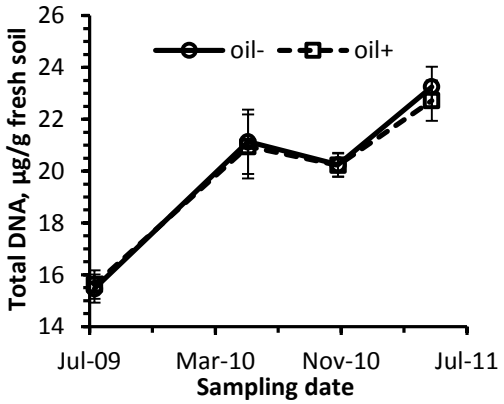

Figure 17. Development of the microbial total DNA in contaminated soil (n=32) and non-contaminated soil (n=32). Error bars represent SE of mean.

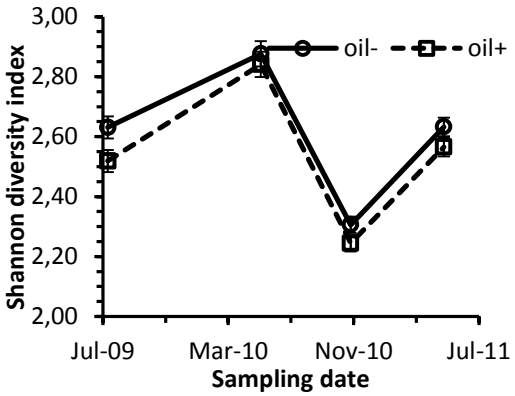

Figure 18. Development of the bacterial community diversity in contaminated soil (n=32) and non-contaminated soil (n=32). Error bars represent SE of mean.

In general, soil total microbial biomass increased over time except for a slight decrease in the autumn of 2010 both in oil-contaminated plots and non-contaminated plots (Figure 17). Soil total microbial DNA increased almost 50% from July 2009 to May 2011 ($p \leq 0.05$).

6.4.2 Soil bacterial community diversity and structure

1) Shannon diversity index

Shannon diversity is calculated from the number of operational taxonomic units (OTUs) (peaks) and the relative abundance (height) of the OTUs in the community data so as to identify the bacteria diversity in soils.

Crop and PGPB treatments had no effect on the bacterial diversity. The bacterial diversity in non-contaminated (oil-) plots was significantly higher ($p \leq 0.05$) than in contaminated (oil+) plots one month after oil treatment (Figure 18). Afterwards, no difference in bacterial diversity between contaminated and non-contaminated plots was observed. The Shannon diversity values in both the contaminated and non-contaminated plots peaked in spring 2010, and the lowest values occurred in autumn 2010.

2) Grouping pattern of the bacterial community profiles in MDS ordination

The Multi-Dimensional Scaling (MDS) ordination (Figure 19) visualizes the similarities of the 32 averaged LH-PCR profiles of the non-PGPB treated plots over time according to curve-based Pearson correlation.

Soil bacterial communities of the oil-contaminated soils were dramatically different from those of non-contaminated soils one month after oil spreading. At the second sampling, this difference was seen but not as clearly. At the third (Nov. 2010) and fourth (May 2011) samplings, profiles of bacterial community between contaminated plots and uncontaminated plots had no big difference any more. The effect of crop was minor and cannot be detected in the community profile. The profiles of autumn samples were clustered whereas those of spring samples were more dispersed. PGPB did not show significant effect on these profiles.

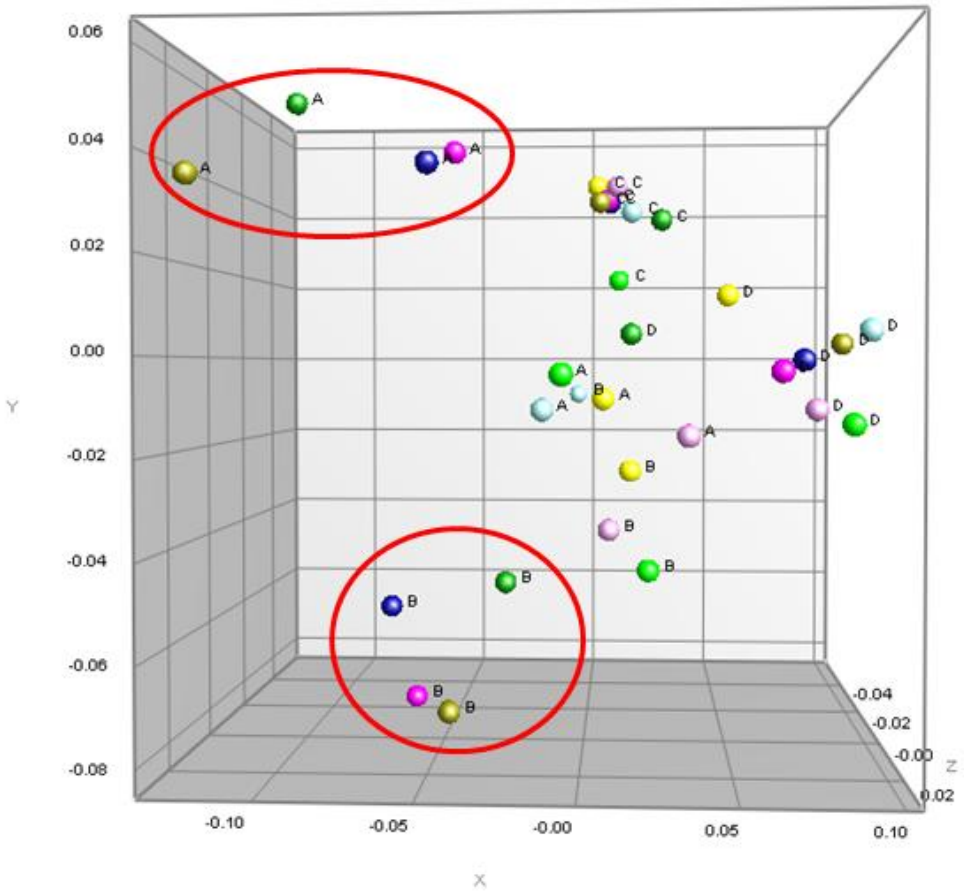

Figure 19. Multi-dimensional scaling ordination for 32 averaged LH-PCR profiles under plant treatment of Galega (blue), Bromus (green), Galega+Bromus (pink) and bare soil (yellow) in non-PGPB treatment. The profiles under oil+ treatment are shown with darker colors and those under oil- with lighter colors. ''A'', ''B'', ''C'' and ''D'' next to the symbols refer to the sampling times of July 2009, May 2010, November 2010 and May, 2011 respectively. The circulated groups are bacterial community profiles in oil treated plots taken one month after the oil treatment in July 2009 and one year after oil spill in May 2010, respectively.

3) Bacterial community structure in curve-based analysis

In the curve-based analysis (Figure 20), changes in bacterial community structure following oil contamination were dramatic. Profiles were normalized by the total fluorescence intensity. The height of the relative fluorescence units reflects the quantity of the operational taxonomic unit (OTU) at a certain fragment length. It was clear that the 16S rRNA fragment length of 497 bp responded rapidly and grew to dominate the bacterial community as soon as oil was spread on the the plots in July 2009. At the same time, the abundance of the fragment (521 bp) was higher in

contaminated soils than in non-contaminated soils both in pure bromus and mixture plots.

The mixture plot (Figure 21) is presented to represent the dynamic change of the bacterial community structure over time after oil treatment. The alterations due to oil treatment reduced with time. The fragment at 497 bp decreased as the oil was degraded and the dominance was not significant anymore 10 months later in May 2010. In the third and last soil samplings, the soil bacterial community profiles in contaminated plots were almost the same as those in non-contaminated plots.

Fragments with the lengths of 470 bp and 534 bp (Figure 20 and Figure 21) reduced slightly after oil treatment. However, they recovered in the later sampling times and they remained as two of the dominate fragments all the time.

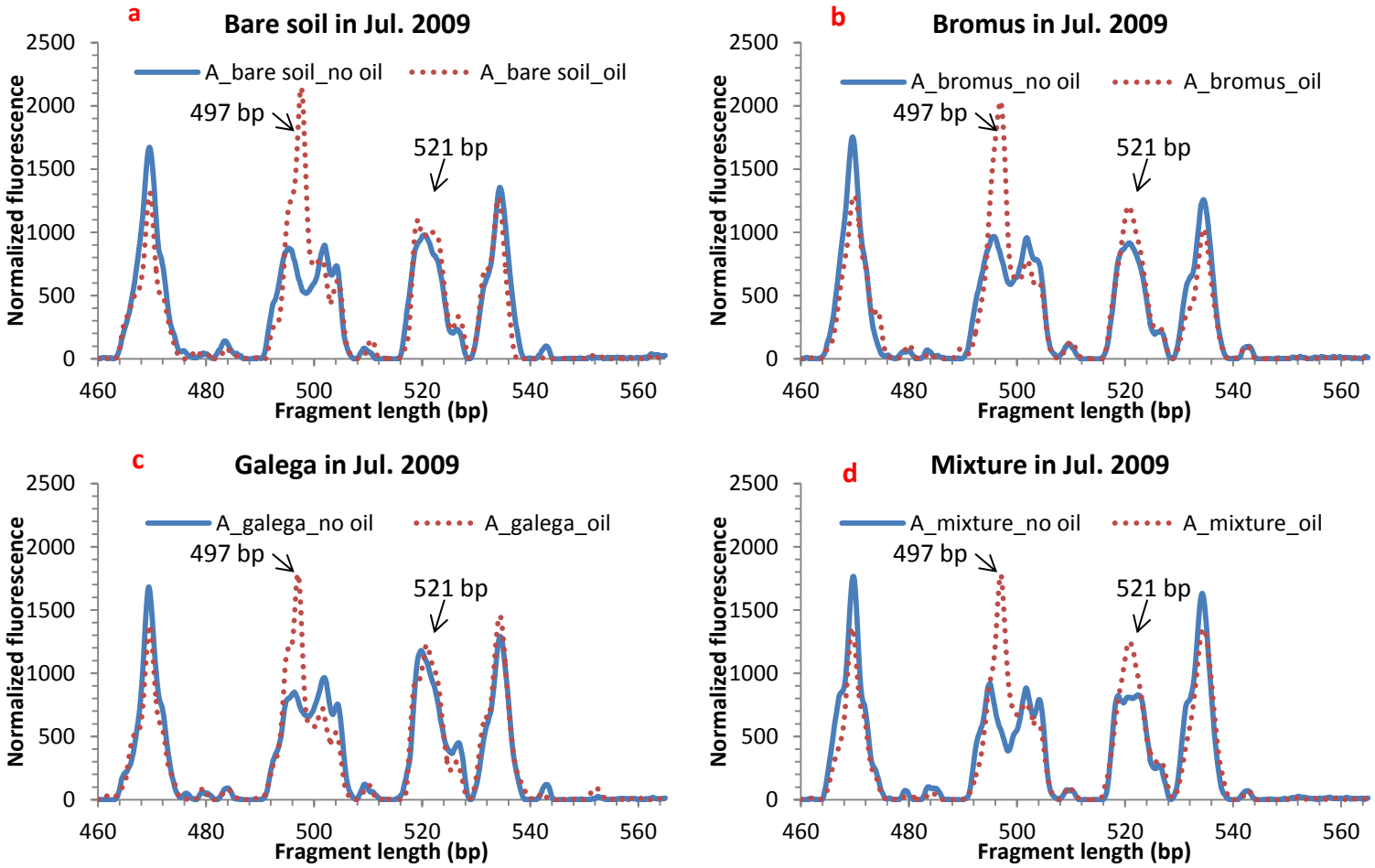

Figure 20. Curve- based bacterial community profiles from the crop treatments (a: bare soil, b: bromus, c: galega, and d: mixture) in July 2009, one month after oil spill. Each profile is the arithmetic average of 4 replicate fingerprints, normalized by total fluorescence intensity. The regions (fragment lengths) corresponding to the major bacterial community changes are indicated.

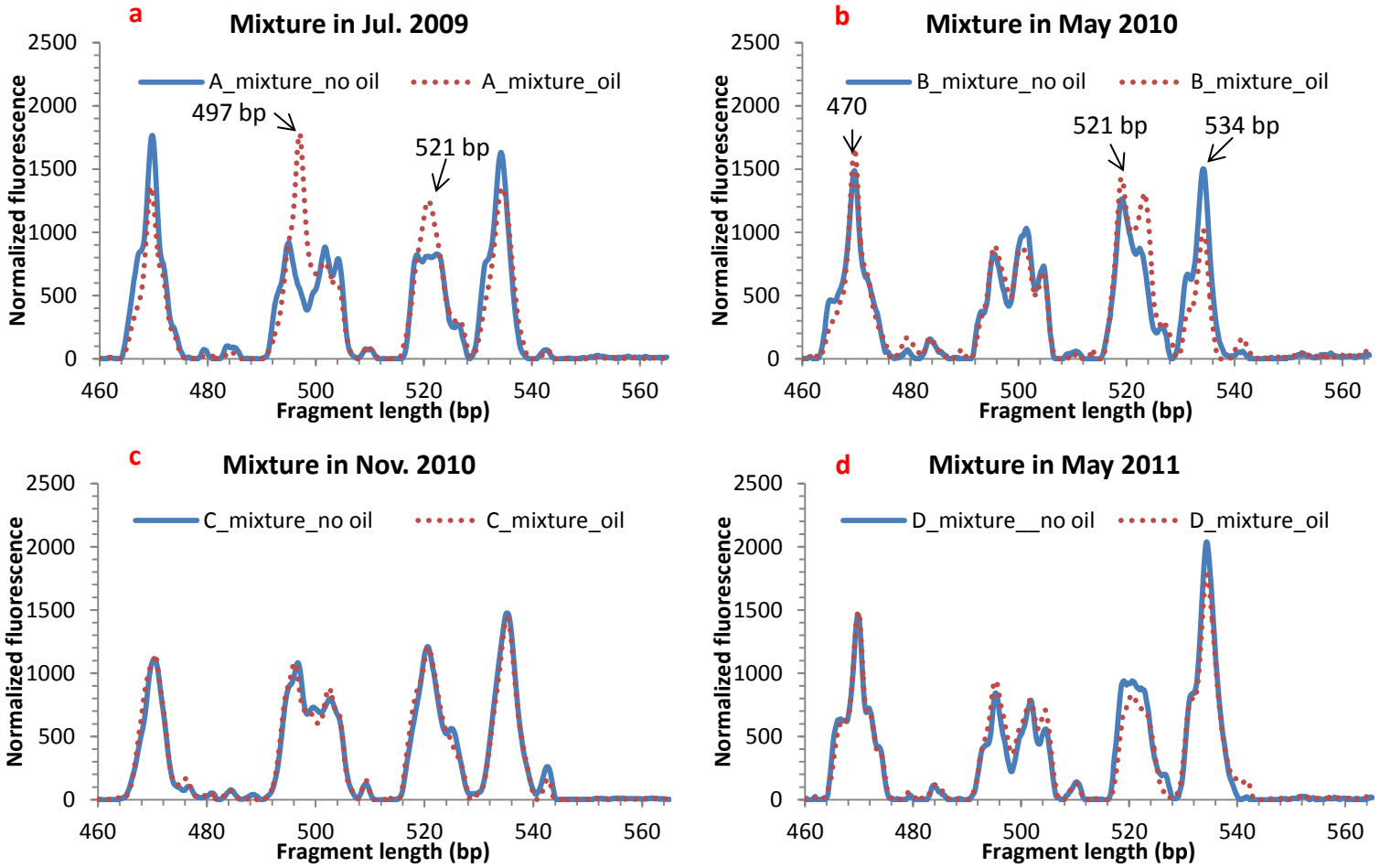

Figure 21. Curve- based bacterial community profiles from mixture (*Galega+Bromus*) treatment over time (a: in Jul. 2009, b: in May 2010, c: in Nov. 2010 and d: May 2011. Each profile is the arithmetic average of 4 replicate fingerprints, normalized by total fluorescence intensity. The regions (fragment lengths) corresponding to the major bacterial community changes are indicated.

7 Discussion

Soil is not only a nonrenewable natural resource, but also a living dynamic ecosystem (FAO 2005). It takes extremely slow physicochemical and biological processes for soil to be formed from rocks into binding particles and then aggregates with the help of soil organisms (Banwart 2011). The health of a soil not only affects the metabolic activities of plants and soil microorganisms, but also has a significant impact on the human social and economic activities. Soil quality is highly dependent on the interaction among soil physical, chemical, and biological properties (Dexter 2004). Soil properties can be affected by petroleum hydrocarbon contamination (PHC) contamination and they in turn affect PHC remediation.

The total solvent extractable material (TSEM) yield in pure motor oil (Teboil Lubricants Classic Mineral Motor oil, moniaste SAE 10W-30, API SF/CD) was around 100 %, meaning that the method for TSEM extraction and determination was working very well for this kind of motor oil. Soil samples are considered "clean", when the oil concentration is below 1 g/kg.

7.1 Hydrocarbon contamination and soil physicochemical properties

The degree of hydrocarbon degradation is mainly affected by the characteristics of the hydrocarbon in the contamination and only to a lesser extent by soil properties (Aichberger et al. 2005). However, several soil properties (e.g. the soil texture, content of soil organic matter, C/N, and pH) have been shown to be greatly involved in hydrocarbon degradation in non-vegetated soils (Chiapusio et al. 2007).

7.1.1 Soil organic matter

Soil organic matter (SOM) is a reservoir of plant nutrients and carbon. Usually, organic matter in soil originates from natural materials (e.g. plant or animal litters that return to soil and microbial biomass. Soil may also contain man-made chemicals (e.g. pesticides, hydrocarbons, plastics and industrial by-products) (Ward 2008; FAO 2005). SOM influences the bioavailability of nutrients as well as soil enzymes produced by bacteria, fungi and plant roots (Chaudhary 2012).

Bioavailability is well explained as one of the most limiting factors in bioremediation of persistent organic pollutants in soil (Wenzel 2009). Compared to the other soil characteristics, soil organic matter is the major factor which affects the distribution

and bioavailability of petroleum hydrocarbons (Chaudhary 2012). Many studies proved that there is a close relationship between soil organic matter and PHC bioavailability to plants. SOM increased significantly after an oil contamination (Liu et al. 2007). Bioavailability of PHCs in soil decreases as the soil organic matter content increases; that is to say, when SOM accumulated for a certain amount after an oil spill, phytoremediation of PHCs can be limited (Weissenfels et al. 1992).

The TSEM concentration is a direct indicator of oil content in oil-contaminated soil. There is no doubt that TSEM loss had a significant positive correlation with the estimated oil carbon loss between July 2009 and November 2010. One can easily get the conclusion that the loss of TSEM and carbon were brought by the remediation of PHCs in the oil treated soils. PHCs might have either physically left the soil matrix or been converted into soil organic matter by heterotrophic microorganisms.

We observed a significantly positive correlation between TSEM concentration and total carbon content one month after oil treatment. This correlation disappeared in later sampling times, indicating that as oil content was reduced, the impact of oil carbon on soil total carbon was smaller. However, the estimated oil TSEM did not correlate with the estimated oil carbon content, indicating that it was not appropriate to estimate oil carbon content by the difference of total carbon content between the oil treated plots and the corresponding non-oil treated plots. Soil organic carbon (without oil carbon), which constitutes the largest part of carbon content in the soil, might have a different dynamics in oil treated and non-oil treated plots. Thus soil organic carbon differed in content between oil treated and non-oil treated plots. The negative values of estimated oil TSEM and oil carbon content in the samples from Nov. 2010 could also well demonstrate this explanation.

7.1.2 Soil texture and structure

Chiapusio et al. (2007) investigated the importance of soil texture on the remediation of PAHs and found that clay soil is the best for remediation of PAH contamination because in clay soil PAHs and nutrients are more bioavailable and bioaccessible to microorganisms. The clay loam soil in our field can therefore be expected to have provided an optimal environment for microorganisms to degrade the oil contamination.

7.1.3 Soil pH and electrical conductivity (EC)

Soil pH influences on plant growth, metal ion solubility, microbial activity and clay dispersion (Haynes & Naidu 1998). The ideal pH range to promote biodegradation of soil contamination is between 6 and 8, depending on the species of the degraders (ATSDR 1999). In our study, soil pH was optimal (6.5) throughout the first two years, providing a suitable environment for microbial PHC degradation. Oil treatment made no difference to soil pH.

In our study, both oil and crop treatment significantly reduced soil electrical conductivity. The EC values of the oil-contaminated plots remained stable between July 2009 and November 2010, whereas the EC values reduced significantly in November 2010, indicating that crops played a greater role in the change of soil electrical conductivity than oil contamination in the second year.

7.1.4 Soil total N and C/N ratio

Since soil is a well-buffered system, soil total N, soluble K and effective P are not largely influenced by PHCs (Liu et al. 2007). There was no significant difference in soil total N concentration between oil-contaminated and non-contaminated plots in our field experiment.

The addition of oil increased the C content and ratio of C/N of the soil. C/N ratios less than 25:1 lead to mineralization and ratios greater than 30:1 lead to immobilization (Xu & Johnson 1997). In order to stimulate and enhance the biodegradation, 60 kg/ha N-fertilizer was given to the pure bromus field in 2009. The C/N ratio in all plots was found to be lower than 25:1, increasing the availability of N for microorganisms in the soil. The chlorophyll contents of the crops in all plots (particularly in pure bromus plots) indicated that there was no N-deficiency in the first two experimental years.

7.1.5 Climatic factors affecting soil moisture and temperature

The soil moisture content affects biodegradation of oils by the dissolution and dispersive actions of the residual compounds and byproducts (Frankenberger 1992). The volatilization of benzene, toluene, ethylbenzene and xylene (BTEX) from gasoline-contaminated soils is enhanced by the decreasing soil moisture content (Frankenberger 1992). Soil moisture also plays an important role in metabolic

activities of oil degrading microorganisms by affecting the availability of oxygen in the soil (Adams et al. 2011).

All biological transformations are influenced by temperature (ATSDR 1999). Biodegradation is reduced in winter when soil is frozen (Rike et al. 2003), but microbial activity increases with temperature until the temperature rises to a level at which the enzyme denaturation occurs (ATSDR 1999). The optimal temperature for biodegradation ranges from 18 ºC to 30 ºC and the minimum rates are at 5 ºC or lower (Frankenberger 1992). For oil degrading bacteria (e.g. *Acinetobacter* sp. LT4, *Pseudomonas* sp. B2, BJ8, CY11, and *Achromobacter* sp.BJ5), degrading activities are found optimal at 27 ºC (Huang et al. 2007). Temperature also affects the volatility of lighter hydrocarbons (< C_{18}). High summer temperatures tend to enhance volatilization, especially when the soil begins to dry out (ATSDR 1999).

There was a steeper decrease in TSEM content from May to November in 2010. Low moisture content and high temperature in the field are likely the crucial factors responsible for the decrease. Soil sampling was difficult in 2010 as a result of hardening of the soil at low moisture content. The high temperature in summer 2010 possibly not only accelerated the physical removal of oil through volatilization but also enhanced soil microbial activity.

SOM is responsible for the brown and black color of soil. The mixture of soil and organic compounds (e.g. oil) with darker color is most likely to raise soil temperature, especially at the surface layer due to the higher capacity of adsorbing radiation by the dark color (ATSDR 1999). As a result, bare soil adsorbed more radiation than vegetated soil. The higher TSEM loss in bare contaminated soil than vegetated contaminated soil from July 2009 to May 2011 may be explained by the greater volatilization of the oil hydrocarbons in bare plots.

7.2 Hydrocarbon contamination and crop growth

Interactions between plants, soil and contaminants are rather complex. Plants are able to remediate oil-contaminated soils by several mechanisms, including hydraulic control, volatilization, stabilization, transformation, degradation and rhizodegradation (Kamath et al. 2007).

Galega orientalis only reaches its highest stable annual dry matter yield and seed yield in the second or third year after cultivation, due to a slow growth pattern (e.g. low germination and growth rate) in the seedling year (Adamovich 2002 & Zolotarev 2010). Our results well proved this point. In the second year (2012), galega dry matter yield doubled to 9.54 t/ha.

The roots of *Bromus inermis* develop within five days of germination (Global Invasive Species Database 2010). DM yields in pure bromus plots did not change between years and N-deficiency was not observed in these plots. Galega and rhizobia symbiosis enhanced the growth of bromus, resulting in higher bromus DM yields in the mixture plots (without N-fertilizer added) than in the pure bromus plots (with N-fertilizer added) in 2011. This positive effect may most likely be contributed by the nitrogen fixation of legumes and rhizobia symbiosis. In addition, rhizobia have been proved to be associated with plant hormone (IAA) synthesis gene (*nthAB*) (Amadou et al. 2008), which might benefit the growth of bromus in mixture plots. The yields were highest in the mixture plots, which greatly reflected the benefits brought by the legume-cropping system.

Galega orientalis and *Bromus inermis* were both tolerant to oil-contaminated soils at the concentration of 7000 ppm. Surprisingly, oil contamination slightly increased the dry matter yield of both crop species in the first year. The effect of oil on crop yields was gone in the second year when the oil concentration was reduced.

The cultivation of the crops (galega and bromus) was hypothesized to have positive impact on soil properties and microbial dynamics (e.g. soiltotal DNA, bacterial diversity and bacterial community profiles) and therefore to enhance the remediation on oil-contaminated soil. Nevertheless, oil loss was greater in bare soils than vegetated soils. There are two possible causes for this result. The competition for resources (e.g. nutrients and oxygen) between crops and soil microorganisms limited the role crops played in oil remediation or the most easy-degradable PHCs might have been removed from the soil surface by bioremediation and physical removal before galega and bromus established their roots in the soil. Plant growth promoting bacteria (PGPB) had no effect on plant growth probably due to the resource competition with other organisms in soil or to climatic factors regulating plant growth. There was no significant difference in oil (TSEM) concentration and total C

content between the two crop species and their combination, indicating that oil reduction in our study was most likely caused by volatilization, leaching and bioremediation, but not phytoremediation. The total solvent extractable materials (TSEM) analysis does not distinguish between petroleum hydrocarbons and naturally occurring compounds such as plant waxes and chlorophyll in the sample (Mills et al. 1999). It may partly explain why the TSEM concentration was always the lowest in the bare plots, where there were no plant waxes and chlorophyll.

7.3 Hydrocarbon contamination and soil microorganisms

There are three categories of soil biota: soil flora (plants), soil fauna (animals) and soil microorganisms (microbes) (McCauley 2005). Soil fauna act as a conditioner in decomposing organic matter (e.g., plant debris) and promoting nutrient cycling in soil and therefore soil fauna can be expected to help bioremediation in the soil (Yin et al. 2010). Sinha et al. (2008) showed that earthworms are highly tolerant of many chemical contaminants including PAHs and can purify the pollutants by bio-accumulating them in their tissues. Earthworms were commonly observed in our field.

Soil microorganisms are the main decomposers of organic matter in ecosystems. Bacteria, molds, algae and fungi are common microorganisms in soil (Sullivan 1999). The smallest and most numerous of the free-living microorganisms in the soil are bacteria, which decompose plant debris and enhance plant nutrient up-take. Some bacteria release nitrogen, sulfur, phosphorus and trace elements from organic matter and some can even break down soil minerals and release potassium, phosphorus, magnesium, calcium and iron (Sullivan 1999). Soil bacteria play a great role in the bioremediation of contaminated soils. The bacterial diversity and community structure highly are highly sensitive to the change of soil condition, especially to pertubations such as soil contamination. Thus it is used as a tool to detect oil contamination in our field experiment.

Bacteria in our field were highly resilient to oil contamination. The bacterial diversity in the contaminated plots gradually returned to the original non-contaminated state after two years from oil spreading. In curve-based analysis, bacterial community structure in the contaminated soil developed in the same direction as in the non-contaminated soil. Soil microbial biomass kept increasing

after oil treatment, indicating a good adaptation of soil microbes to PHC contamination with exception to a seasonal slight decrease in the autumn 2010. A larger microbial population indicates a faster remediation of organic contaminants in soils. Oil treatment has been found to increase soil total microbial DNA content (Mikkonen 2008). In our experiment, there was no statistical difference in soil total microbial DNA between contaminated plots and non-contaminated plots.

Oil contamination was observed to decrease the bacterial diversity significantly in the first few weeks after oil treatment. A 16S rRNA gene fragment at 497 bp was very abundant at the beginning in contaminated soils, indicating a prompt growth of a bacterium that is likely a PHC degrader. However, the quantity of this fragment had decreased before the second sampling, when oil (TSEM) concentration in contaminated soils was still high. This phenomenon might be brought by two reasons. The easily degraded oil components had been degraded, and this bacterium with fragment of 497 bp was not as efficient in degrading the rest oil components with longer chains or oil contaminants might have been converted to some form of SOM that was also extracted by our method in the second sampling. Bromus seemed to enhance oil degrading activities of a bacterium with 16S rRNA gene fragment length of 521 bp, resulting in a higher abundance in bromus plots. Besides the 497 and 521 bacteria described above, other bacteria with fragments of 470 bp and 534 bp also appeared to endure oil contamination and to remain dominant in whole bacterial community over time. They may therefore be promising for use in remediation of oil-contaminated soils.

The clustering characteristics of bacterial community in the MDS analysis and the overlap of bacterial community structure in the curve-based analysis indicated that there was a clear seasonal change in total microbial DNA content and bacterial diversity. Climatic factors (soil temperature and moisture) and soil C/N ratio are prominent factors which can result in seasonal variation of soil microbial community (Habekost et al. 2008). Change in soil temperature is probably the most important factor to explain the seasonal change of microbial DNA content and bacterial diversity in our experiment. Lower temperature limited the activities of enzymes and bacteria in the autumn season, leading to a reduction in microbial DNA content. Soil moisture affects the availability of oxygen in the soil, limiting the metabolic activities of oil degrading microorganisms (Adams et al. 2011). Soil moisture was

higher in autumn in our field because of lower evapotranspiration, resulting in an anaerobic soil environment. Activities of aerobic soil microorganisms were probably restricted or inhibited in this season, resulting in the sharp decrease both in soil total DNA and bacterial diversity. Most oil-degrading bacteria prefer aerobic conditions (ATSDR 1999). Anaerobic soil conditions might also be responsible for the clustering pattern of bacterial community structures between the oil-contaminated plots and non-contaminated plots in the autumn 2010.

Generally, plant roots have less impact on bulk soil than rhizosphere soil in terms of microbial growth and soil properties. The above analyses were all done on the basis of bulk soil, instead of rhizosphere soil. It might explain why crops (galega and bromus) did not make any difference on soil microbial population in our experiment.

8 General conclusions

It appears that bioremediation by soil microorganisms and physical removal including volatilization and leaching were the main processes of oil reduction in our experiment. Climate factors (e.g. temperature and precipitation) had an overriding influence on the removal of oil and soil microbial activities in our study. Soil condition in our field was optimal for biodegradation of hydrocarbons, having a neutral pH and an optimal C/N ratio. Soil physiochemical properties were very well buffered under oil treatment, expect for the sensitive parameters of electrical conductivity and the total carbon content in the first year.

Compared to soil parameters, the changes in soil microbial population and community are a more sensitive indicator of petroleum hydrocarbon contamination and recovery. The soil bacterial communities showed a vast potential to recover from oil contamination, even though the recovery was slower under field conditions as compared to the greenhouse condition (Mikkonen et al. 2010). The seasonal change of soil microorganisms was remarkable, which needs to be taken into account before planning soil sampling.

Surprisingly, crops (*Galega orientalis* and *Bromus inermis*) and PGPB treatment had no significant effect neither on soil physiochemical and microbiological properties (microbial DNA content, bacterial diversity and bacterial community structure) nor on the loss of oil in our experiment, which largely differed from our hypothesis. Except for the climatic factors, resource competition between crops and microorganisms might have resulted in the better oil remediation in bare soils than in vegetated soils. Nevertheless, crops were found to have a high tolerance to oil contamination and surprisingly, the oil contamination seemed to increase the growth of both crop species. Bromus in mixture plots (without commercial nitrogen fertilization) had better yield than in pure plots (with commercial nitrogen fertilization) as a result of biological nitrogen fixation of *Galega orientalis* and *Rhizobium galegae*. Therefore the mixture of galega and bromus can be suggested to apply to future phytoremediation projects.

However, the oil contamination was not completely cleaned up till the last sampling in May 2011. Long-term stimulation and monitoring on soil parameters, oil content, crop growth and microbial community are still needed. In the future, sequencing will

be applied to identify the microbes and genes that were associated in PHC remediation in our experiment.

As nitrogen is the most frequently deficient nutrient in most contaminated soils, legume-cropping system is a good candidate in future phytoremediation projects with considerable economic values. In the tropics, woody legumes which are abundant there are going to benefit the removing of petroleum hydrocarbon contamination in soils *(*Vitosek et al. 2002). Azotobacter, azospirillum, rhizobium, actinomycete, frankia, blue-green algae and anabaena are the commonly used microorganisms which can economically fix nitrogen in soils (Havlin et al. 2010). Provided with proper management strategies for irrigation, fertilization, weed control (mowing, mulching, or spraying) and pest control (ITRC 2009), N-fixing bacteria assisted crop system is therefore ideal for the successful remediation of contaminated soils.

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APPENDIX

Protocol description for total DNA quantification with Fluorometric method

Protocol name ..... Qubit
Protocol number ..... N/A
Name of the plate type ..... Generic 8x12 size plate
Number of repeats 1
Delay between repeats ..... 0 s
Measurement height ..... Default
Protocol notes .....

Shaking duration ..... 10.0 s
Shaking speed ..... Slow
Shaking diameter ..... 0.10 mm
Shaking type ..... Orbital
Repeated operation ..... Yes

Delay duration ..... 120.0 s
Repeated operation ..... Yes

Shaking duration ..... 10.0 s
Shaking speed ..... Slow
Shaking diameter ..... 0.10 mm
Shaking type ..... Orbital
Repeated operation ..... Yes

Name of the label ..... Fluorescein (1.0s)
Label technology ..... Prompt fluorometry
CW-lamp filter name F485
CW-lamp filter slot A5
Emission filter name F535
Emission filter slot A5
Measurement time ..... 1.0 s
Emission aperture ..... Small
CW-lamp energy 18944
Second measurement CW-lamp energy . 0
Emission side ..... Above
CW-Lamp Control ..... Stabilized Energy
Excitation Aperture ..... N/A