Master's thesis

# Application of hydrocarbon degrading microorganism enumeration and catabolic genes detection for soil assessment

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Tiivistelmä/Referat – Abstract

Soil contamination with oily products poses great healthy and environmental risks to the polluted sites. The remediation difficulty mainly comes from the complexity of hydrocarbons. Different kinds of remediation technologies have been applied for hydrocarbon removal from soil. New technologies especially *in situ* bioremediation technologies are emerging constantly. Soil assessment is a key step in the remediation processes since it provides information about the contamination level and potential risks.

In the present study, hydrocarbon contaminated soil samples were collected from two sites (one site was contaminated by weathered oily sludge waste with some vegetated plots; the other was contaminated with fuel oil with short-chain hydrocarbons). The samples were analyzed for physicochemical properties and hydrocarbon degraders were enumerated. Four degrading strains were isolated from the samples and their 16S rRNA genes were sequenced. The samples and isolates were investigated to check the existence of three catabolic genes involved in petroleum degradation.

The objective was to reveal the intrinsic bioremediation potential of contaminated soils by investigating the key remediation "players" i.e. the degrader microorganisms and catabolic genes. The coexistence of abundant degraders and diverse catabolic genes give the soil a good potential for bioremediation. In addition, the relationships between degrader counts, genes detection and soil contamination levels can reveal how the contaminants affect the indigenous microbial community. The differences between vegetated and nonvegetated plots can also suggest if vegetation with legumes has good potential for hydrocarbon bioremediation.

According to the results, both sites were moderately contaminated with different hydrocarbon composition. In the landfarming site, the TPH depletion in vegetated fields was higher than the unvegetated bulk soil areas. However, the degrading microorganism counts had no significant differences between vegetated and nonvegetated plots. The hydrocarbon contamination level had no correlation with the degrader counts. In subsurface soils where aeration was quite limited, degrading microorganisms were much lower than those in surface soils. Catabolic genes were detected from the isolated strains but rarely from the contaminated soil samples. The contaminants co-extracted with soil DNA may inhibit the PCR-based gene detection. With more primer sets or primers targeting broader genetic diversity ranges, more detection results can be expected.



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Oil contaminated soil, bioremediation, MPN, *alkB, xylE, nah*

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# ABBREVIATIONS

| | |
|---|---|
| BTEX | benzene, toluene, ethylbenzene and xylenes |
| GC-FID | gas chromatograph-flame ionization detector |
| MNA | monitored natural attenuation |
| MPN | most probable number |
| NALP | non-aquatic liquid phase |
| PAH | polycyclic aromatic hydrocarbons |
| Q-PCR | quantitative polymerase chain reaction |
| SVOCs | semi volatile organic compounds |
| TCA | tricarboxylic acid |
| TPH | total petroleum hydrocarbons |
| TPHCWG | total petroleum hydrocarbons criteria working group |
| TSEM | total solvent extractable material |
| VOCs | volatile organic compounds |

# INTRODUCTION

Soil health is a key concern of academic researchers and the public with increasing awareness of health risk posed by contaminated sites. Oily products, as an extremely important energy source with broad application ranges, are a major soil contaminant. In the European Union, there are 3.5 million sites estimated to be potentially contaminated with 0.5 million sites being really contaminated and requiring remediation (Pereze, 2012). Every year, about 1.7 to 8.8 million metric tons of oil are released into the world's water, more than 90 % of which is directly related to accidents caused by human failures and activities (Megharaj et al., 2011).

As a complex mixture, different hydrocarbons have different physicochemical properties leading to diverse bioavailability and biodegradability. In general, hydrocarbons are classified into aliphatic (mainly *n*-alkanes), aromatics including monoaromatics such as BTEX (benzene, toluene, ethylbenzene and xylenes) and polycyclic aromatics (i.e. PAH i.e. polycyclic aromatic hydrocarbons), and asphaltics (Atlas, 1981). Previous researchers have found that there are different and diverse degradation pathways and responsible catabolic genes responsible for biodegradation of each hydrocarbon group. Many primer sets targeting diverse catabolic genes have been published in previous reports (Hendrickx et al., 2006; Nõvak et al., 2012). By amplification with these primers, the presence of catabolic genes in soil samples can be revealed. In this study, gene detection work was performed targeting three representative catabolic genes covering the metabolism pathways of *n*-alkanes, BTEX and PAH. However, gene detection is restricted with the current knowledge about known degradation pathways. Thus in the present study, hydrocarbon degrading microorganisms were also enumerated in order to get information without limits of known degradation pathways. The samples were also analyzed to for their physicochemical properties especially the residual oil contamination. The objectives were to reveal the degradation processes occurring in contaminated sites by studying the existence and relationships of hydrocarbon degrading microorganism and catabolic genes in contaminated sites. The results can suggest whether these two biological parameters can be informative in monitoring and assessment of bioremediation potential (existence of to-be-stimulated remediation "players") and soil health.

The samples were collected from two oil contaminated sites. One is a landfarming site used for oily refinery sludge waste disposal. The other site was used for fuel oil storage. The landfarming site has been investigated a lot previously by our group and the results have been published. Comparison with that information can also provide a time-scale perspective.

The oil storage site is a part of SOILI program which was a Finnish national oil contaminated sites remediation project launched by the oil industry since 1996.

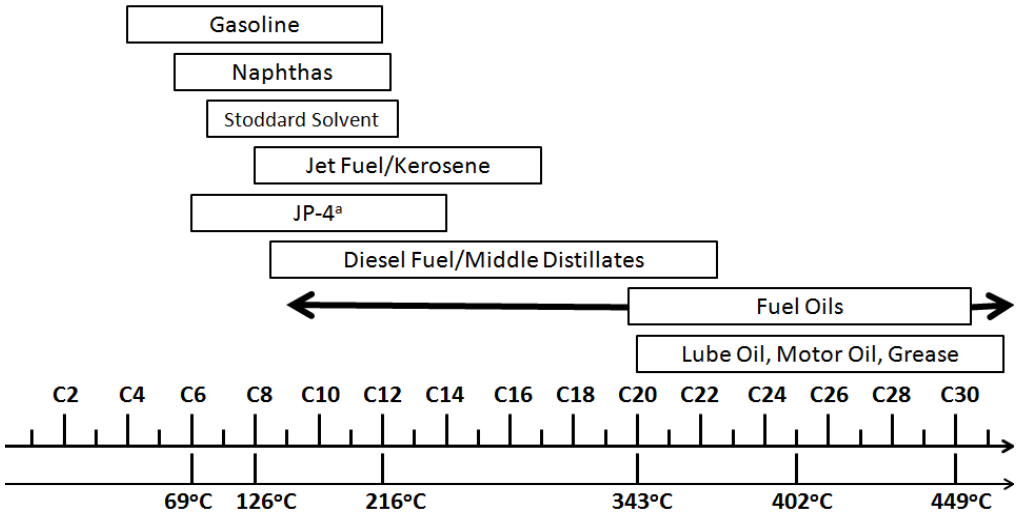
# LITERATURE REVIEW

## 1. Soil contamination

Soil, as the naturally occurring, unconsolidated minerals and organic material on the earth's surface that provides an environment for living organisms is where biosphere, interact with rocks and minerals (geosphere), water (hydrosphere), atmosphere and dead organic matter (detritosphere) (Paul, 2007). It provides shelter and supports agricultural production. Both are the most important activities of human beings. The maintenance of soil quality thus is a key concern of agronomists and soil scientists.

Nowadays, with the public awareness of the bioaccumulation effect of poorly degradable compounds through the food chain, soil quality especially at contaminated or potentially contaminated sites also attracts the attention from public and government. In the past decades, it is not a rare instance that a regional disease is associated with local soil or underground water contamination, such as the famous "itai-itai disease" as a result of long term cadmium intake from excessive cadmium accumulated rice (Nishi et al., 2012) and "cancer villages" located near industrial areas in developing countries (Tremblay, 2007). The soil contamination is easily underestimated because of its invisibility nature. However, the current soil contamination situation is quite severe. In the European Union, there are 3.5 million sites estimated to be potentially contaminated with 0.5 million sites being really contaminated and needing remediation (Pereze, 2012). In China, the soil contamination is widely believed to be extremely severe while the data about the contaminant types, distribution and extent are still unavailable.

Petroleum products with an extremely important role in nowadays industrial activities are a main source of soil contamination. The products from petroleum are widely used in many aspects of our life. The approximate carbon and boiling ranges of different petroleum products are shown in Figure 1. With diverse ranges of the number of carbon atoms, petroleum products have diverse physicochemical properties leading to different behavior in the environment (Table 1). With the industrial development and enlargement of human activities, increasing incidence of petroleum contamination draws more and more public concern. Human activities are the main source of significant hydrocarbon release to the environment (Mikkonen, 2012).




a. JP-4 "Jet Propellant" is a jet fuel consisting of 50-50 kerosene-gasoline blend

**Figure 1**.Summary of petroleum product types with respect to approximate carbon number and boiling point ranges. (Adopted from TPHCWG, 1998)

The hydrocarbons can enter the environment naturally by seepage, run-off and other means such as methane produced by methanogenic archaea, ethylene released by higher plants, bacteria and fungi, and isoprenoids e.g. carotenoids and terpenes of many plants, insects and microorganisms (Heider et al., 1999). However, there is about 1.7 to 8.8 million metric tons of oil released into the world's water every year (Megharaj et al., 2011). More than 90 % of them are directly related with accidents caused by human failures and activities including deliberate waste disposal (Megharaj et al., 2011).

The behavior of petroleum contaminants in the environment is described in quite detail in the Remediation technologies screening matrix and reference guide (second edition, 1994) released by the DOD Environmental Technology Transfer Committee of the United States (Marks et al., 1994). According to this guidance, petroleum contaminants in the unsaturated zone exist in four phases: vapor in the pore spaces; sorbed to subsurface solids; dissolved in water; or as NAPL (non-aquatic phase liquid). The nature and extent of transport are determined by the interactions among contaminant properties (e.g., density, vapor pressure, viscosity and hydrophobicity) and the subsurface environment (e.g., geology, aquifer mineralogy and groundwater hydrology).

It is more complicated to assess human health risks for petroleum and oil-contaminated sites than those polluted by a single compound due to the complex composition of petroleum (Park & Park, 2010). The regulatory agencies in the United States implement total petroleum hydrocarbon concentrations to establish target cleanup levels for soil as a common approach,

and they are followed by other countries (TPHCWG, 1998; Park & Park, 2010). In order to get more in-depth understanding of the health risks of TPH, several fractionation methods have been proposed. According to the silica gel chromatography, petroleum can be classified into a saturated or aliphatic fraction including *n*-alkanes, branched alkanes and cycloalkanes; an aromatic fraction including monoaromatic (BTEX, i.e. benzene, toluene, ethylbenzene, xylene) and polyaromatic hydrocarbon (PAH) compounds; as well as an asphaltic or polar fraction (Atlas, 1981). In the environment, aromatics are especially abundant because of applications such as fuels, industrial solvents (benzene, toluene), polymer synthesis (styrene) and starting materials for chemical synthesis (Sikkema et al., 1995) although non-polar mid-length alkanes ($C_{14}$-$C_{20}$) takes up to 90% of petrol and diesel (Stroud et al., 2007). However, this fractionation method is not straightforward for health risk analysis because each fraction is still a mixture of hydrocarbons.

The TPH Criteria Working Group (TPHCWG) proposed a commonly used fractionation methods. In this method, aliphatic and aromatic groups are classified into 13 TPH fractions based on their equivalent carbon number (Table 1). The different physicochemical properties of the fractions including octanol-water partition coefficient (Kow), air-water partition coefficient (Kaw) and octanol-air partition coefficient (Koa) indicate that the distribution of hydrocarbon mixtures may be dominated by certain fractions on different environmental conditions (Park & Park, 2010). This also allows each fraction to be used as a single compound in fate and transport models, risk assessments and compositional changes (e.g., weathering) (TPHCWG, 1998).

**Table 1**.TPHCWG petroleum hydrocarbon fractions and their physicochemical properties (Adopted from Park & Park, 2010)

| **Fraction** | **Solubility (mg/L)** | **Boiling point ($^o$C)** | **Melting point ($^o$C)** | **Molecular weight (g/mol)** | **log$K_{ow}$** | **log$K_{aw}$** | **log$K_{oa}$** |
|---|---|---|---|---|---|---|---|
| **Aliphatic compounds** | | | | | | | |
| EC5-6 | 36 | 51 | -100 | 81 | 3.07 | 1.37 | 1.70 |
| EC6-8 | 5.4 | 96 | -84 | 100 | 3.80 | 1.58 | 2.22 |
| EC8-10 | 0.43 | 150 | -63 | 130 | 4.79 | 1.88 | 2.91 |
| EC10-12 | 0.034 | 200 | -41 | 160 | 5.77 | 2.17 | 3.60 |
| EC12-16 | 0.0076 | 260 | -8 | 200 | 7.24 | 2.61 | 4.63 |
| EC16-21 | $3\times10^{-6}$ | 320 | 40 | 270 | 9.21 | 3.26 | 6,18 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Aromatic compounds** | | | | | | | |
| EC5-7 | 1800 | 80 | -78 | 78 | 2.13 | -0.66 | 2.77 |
| EC7-8 | 520 | 110 | -59 | 92 | 2.73 | -0.57 | 3.30 |
| EC8-10 | 650 | 150 | -25 | 120 | 3.70 | -0.48 | 4.19 |
| EC10-12 | 250 | 200 | -6 | 130 | 3.94 | -0.86 | 4.81 |
| EC12-16 | 5.8 | 260 | 22 | 150 | 4.28 | -1.43 | 5.72 |
| EC16-21 | 0.65 | 320 | 64 | 190 | 4.75 | -2.28 | 7.10 |
| EC21-35 | 0.0066 | 340 | 153 | 240 | 5.91 | -4.07 | 10.01 |

Short alkanes and monoaromatics are volatile. Volatile aromatics take up about 10% - 20% in diesel (Mikkonen, 2008). The solubility which depends on polarity and molecular size, is a key factor that determines the octanol-water partition coefficient (Kow). Furthermore, the octanol-water partition coefficient (log Kow) is a relative indicator of the tendency of an organic compound to absorb to soil (US EPA, 2009). Sorption to soil solids hinders the availability to degrader microorganisms (Mikkonen, 2008).

# 2. Site Investigation and Remediation Technologies

Concerning the high potential health risks of contaminated sites, a variety of regulations implemented by many counties restrict their use for other purpose before being remediated. The public and government concerns together with the gradual improvement of regulatory contexts throughout the world stimulate the development of contaminated sites investigation and remediation markets.

To determine the existence, the types and, the distribution of contaminants and to get information about to what extent the site was contaminated, the site should be characterized by investigation. The investigation also produces backup information for designing the remedial strategy. Site investigation should be conducted following certain standard procedures in order to get reliable and thoroughly understandable data. The major components of site characterization are (Guidance for conducting remedial investigations and feasibility studies under CERCLA, 1988):

• Conducting field investigations as appropriate;
• Analyzing field samples in the laboratory;
• Evaluating results of data analysis to characterize the site and develop a baseline risk assessment;

• Determining if data are sufficient for developing and evaluating potential remedial alternatives.

During these steps, re-scoping and additional sampling may occur if the results show that site conditions are significantly different than originally believed. Once the data has been collected and analyzed, it must be decided whether further sampling is needed to assess site risks and support the evaluation of potential remedial alternatives.

The information normally needed is categorized as surface features (including natural and artificial features), geology, soil, surface water hydrology, hydrogeology, meteorology, human populations, land use and ecology. Among the above mentioned properties, soil characteristics include soil type, holding capacity, temperature, solubility, ion speciation, absorption coefficients, leachability, cation exchange capacity and so on. The location and type of existing containment should be determined for all known sources. Besides, the nature and extent of contamination was defined and determined chemically.

The selection and use of innovative technologies to clean up hazardous waste sites is increasing rapidly, and new technologies are continuing to emerge. By analyzing the data from site investigation and considering the other factors such as operational costs, time frame, regulatory requirements, and especially clean/up goals, appropriate remedial technologies can be chosen (Ram et al., 1993).

Based on the location of remedial activities, remediation technologies can be divided into two classes: *in situ* technologies by which the pollution is treated on site and *ex situ* technologies which involve the removal of the pollution to be treated elsewhere (Megharaj et al., 2011).

When considering remedy for soil contaminants, it is important to verify whether the compounds are halogenated or nonhalogenated. This is important since the halogen bond and halogen itself can significantly affect performance of technology or require more extensive treatment than for nonhalogenated compounds (Marks et al., 1994). Petroleum, as an extreme mixture of hydrocarbons, is basically nonhalogenated. Some potentially applicable remediation technologies for sites with fuel or petroleum contaminants are presented in Table 2. It should be noted that a treatment technology may be applicable to treat a specific contaminant group, but may not be widely used because of factors such as cost, public acceptance or implementability.

For a specific contaminated site, the selection of appropriate technologies is often difficult but extremely crucial. Many important parameters such as site-specific conditions,

contaminant types and source, source control measures, and the potential impact of the possible remedial processes should be taken into account when making the decision (Khan et al., 2004).

**Table 2**. Treatment technologies for contaminated sites (adopted from Remediation Technologies Screening Matrix and Reference Guide 2nd edition, 1994)

| **Technology** | **Use Rating** | **Technology Function** | **Applicability** | **Cost** |
|---|---|---|---|---|
| **In situ biological treatment** | | | | |
| Biodegradation | Wide | Destruct | NA | NA |
| Phytoremediation | NA | NA | Heavy metals, radionuclides, chlorinated solvents, petroleum hydrocarbons, PCBs, PAHs, organophosphate insecticides, explosives, and surfactants | NA |
| Bioventing | Wide | Destruct | Any aerobic degradable contaminants | $30 US to $90 US/t of contaminated soil |
| **In situ physical/chemical treatment** | | | | |
| Soil Flushing | Limited | Extract | All types of soil contaminants | Rough estimates from $25 to $250 US per cubic yard |
| Soil Vapor Extraction (SVE) | Wide | Extract | Volatile organic compounds | $20 to $50 US/t of contaminated soil |
| In situ solidification/ stabilization | NA | Immob./ Dest. | Heavy metals and other inorganic compounds | $80 US per cubic meter for shallow applications to $330 US per cubic meter for deeper |

| | | | | |
|---|---|---|---|---|
| | | | | Applications |
| **Ex situ biological treatment (assuming excavation)** | | | | |
| Composting | Wide | Destruct | Most petroleum products, VOCs, SVOCs, and pesticides | $130 to $260 US per cubic yard |
| Bioslurry systems | Wide | Destruct | Non-halogenated SVOCs and VOCs | $130 to $200 US per cubic meter |
| Landfarming | Wide | Destruct | Petroleum hydrocarbons | $30 to $60 US/t and can take from 6 months to 2 years |
| **Ex situ physical/chemical treatment (assuming excavation)** | | | | |
| Soil washing | Limited | Extract | Semi-volatile organic compounds (SVOCs), petroleum and fuel residuals, heavy metals, PCBs, PAHs, and pesticides | $170 US/t of contaminated soil |
| **Ex situ thermal treatment (assuming excavation)** | | | | |
| Thermal Desorption | Limited | Extract | VOCs, PAHs, PCBs, and pesticides | $50 US to 330 US per metric ton |
| Incineration | Limited | Destruct | NA | NA |
| **Other treatment** | | | | |
| Natural Attenuation | Limited | Destruct | NA | NA |

NA means not available.

## 2.1 Physical and Chemical technologies

From Table 2 it can be concluded that besides biological treatments, both *in situ* and *ex situ*, SVE (soil vapor extraction), incineration and low temperature thermal treatment are also mature technologies considering the factors of development status, use rating and applicability.

Soil Vapor Extraction (SVE), also known as soil venting or vacuum extraction, is an *in situ* unsaturated soil (vadose-zone) remediation technology which applies a vacuum to the soil inducing a controlled air flow to remove volatile and some semi-volatile contaminants from the soil, and also extracted gas treatment unit before their release to the atmosphere (Marks et al., 1994; Khan et al., 2004). The treatment efficiency and remediation time of SVE are affected by several factors: operational conditions (such as soil temperature and airflow rate), contaminant properties (such as vapor pressure and solubility), and soil properties (such as water and organic matter content) (Albergaria et al., 2006; 2012). The airflow through subsurface provided by SVE also stimulates the biodegradation of contaminants, especially those that are less volatile (Khan et al., 2004). SVE is generally most successful for lighter, more volatile petroleum products such as gasoline.

Incineration uses high temperatures, 870 to 1200 $^{o}$C, to volatilize and combust organic constituents in hazardous wastes under aerobic conditions. The destruction and removal efficiency for properly operated incinerators exceeds the 99.99% requirement for hazardous waste and can be operated to meet the 99.9999% requirement for PCBs and dioxins. (Marks et al., 1994)

Low temperature thermal desorption (LTTD) systems are physical separation processes and are not designed to destroy organics which means that the treatment only volatilize water and organic contaminants. The volatilized gases are then carried to the gas treatment system. LTTD is a full-scale technology that has been proven successful for remediating petroleum hydrocarbon contamination in all types of soil. Decontaminated soil retains its physical properties and ability to support biological activity (Marks et al., 1994).

Soil flushing uses a solution to carry the contaminants to an area where they can be removed (Khan et al., 2004). It is accomplished by passing an extraction fluid through in-place soils using an injection or infiltration process. This treatment can be used for all kinds of soil contamination and is often used together with other remediation technologies such as activated carbon, biodegradation, and pump-and-treat. Soil permeability dramatically affects the efficiency of soil flushing. The target contaminants are the inorganics while it can also be

used to treat VOCs, SVOCs, fuels and pesticides. However, it may be less cost-effective than other alternative technologies.

## 2.2 Bioremediation technologies

Bioremediation refers to the use of living organisms to degrade environmental pollutants (Barea et al., 2005). According to the guideline of US EPA, bioremediation is feasible when there is about $10^3$ CFU/g soil of the microbial population (Lin et al., 2009). The excavation and transportation of a large quantity of polluted materials for *ex situ* treatments used by the physical and chemical technologies make them expensive. The increasing costs and limited efficiency of these conventional, engineering-based remediation technologies described above result in the application of alternative *in situ* technologies, especially those based on the biological remediation capabilities of plants and microorganisms (Chaudhry et al., 2005). The advantages of bioremediation are little waste production, minimal disturbance to the environment, lower costs, less equipment and labor needed, and little to no contact between operators and contaminants (Declercq et al., 2012). Because of the ability of microorganisms to degrade organic compounds, it is applicable to consider bioremediation and natural attenuation as a first option (Atlas, 1981).

### 2.2.1 Natural attenuation

Natural attenuation (NA), also known as intrinsic bioremediation is the simplest remediation method. It is referred to as bioremediation in this thesis, whilst it actually involves also physical and chemical remediation (Kauppi, 2011). Natural attenuation does not mean “no action”, although it is often perceived as such. According to the definition by USEPA (Environmental Protection Agency of the United States of America), natural attenuation refers to a variety of physical, chemical or biological processes that act, preferably without human interventions to reduce the mass, toxicity, mobility, volume or concentration of contaminants in soil and groundwater (Jussila, 2006; Declercq et al., 2012). The metabolic processes of the indigenous microbial community are the main degrading pathways of recalcitrant molecules or xenobiotics during which aerobic and anaerobic biodegradation, dispersion, dilution, sorption, volatilization, and other destruction of contaminants also take place (Rittmann, 2004). It is necessary to differentiate natural attenuation or the mere existence of NA processes on site from the monitored natural attenuation (MNA). The latter one refers to the utilization of NA as a remediation option for contaminated sites.

Ever since the 1990s, natural attenuation has gained interest in contaminated site management. For most countries, the first cases of MNA application took place at the end 1990s (Declercq et al., 2012). In the United States of America, natural attenuation has been selected for remediation at 45 USAF sites by AFCEE (Air Force Center for Environmental Excellence) (Marks et al., 1994). In Europe, the conducts of natural attenuation related research programs mainly started from the early 2000s. As for Finland, although MNA is now accepted by the experts and administrators as a remediation technology, it does not have an official legal status yet (Declercq et al., 2012). However, according to the report by Jørgensen (2006), with the obvious market potential, it is likely that the authorities would like to approve MNA in the future especially considering the new Finnish decree which makes risk based decisions possible.

In general, the application of MNA includes several steps. The steps differ in protocols of different countries, while there are several steps being of consensus. The common steps are shown in Fig. 2 (Declercq et al., 2012):

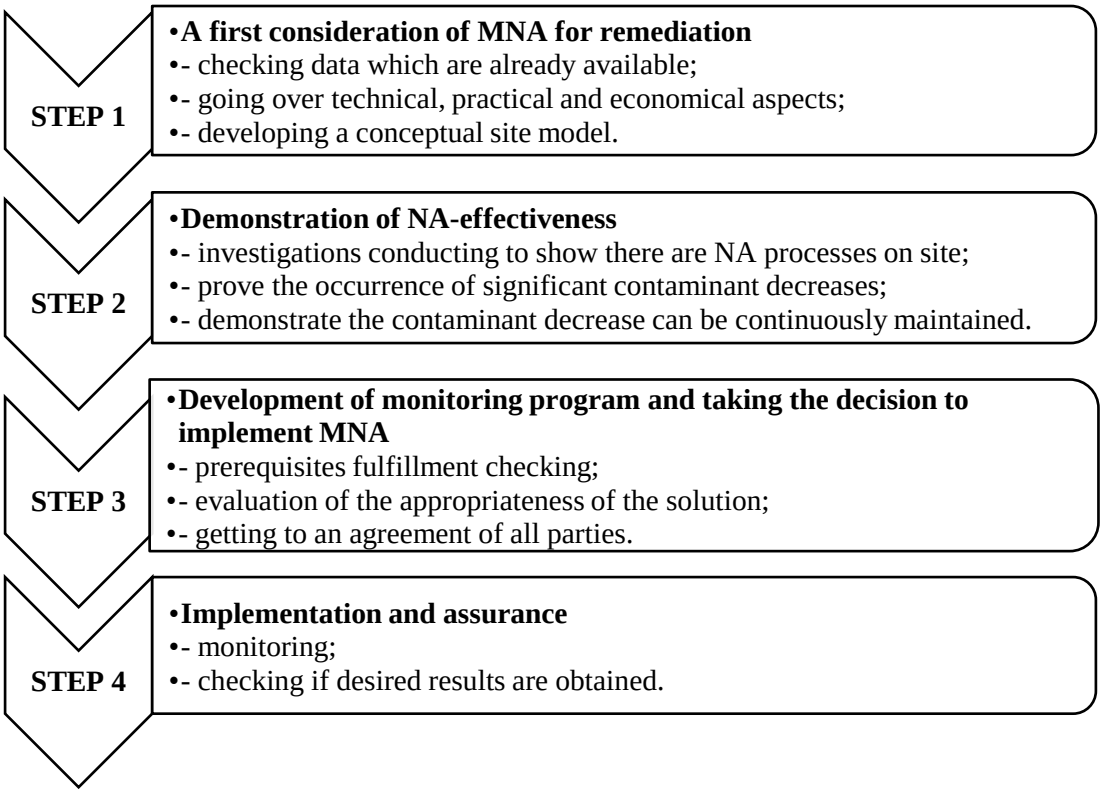



**Figure 2.** Common steps of conducting Monitored Natural Attenuation concluded from national protocols throughout Europe (Declercq et al., 2012).

Target degradation contaminants for natural attenuation include nonhalogenated VOCs, SVOCs and fuel hydrocarbons. The method of MNA as a remediation technology best suits the organic biodegradable contaminants such as petroleum hydrocarbons (Jørgensen, 2006). However, halogenated VOCs and SVOCs and pesticides may be less responsive to natural attenuation. MNA is even limited to be used for petroleum hydrocarbons/BTEX or chlorinated hydrocarbons contaminated sites in the national protocols of some countries concerning MNA conducting, such as The Netherlands, Spain and Sweden (Declercq et al., 2012). Despite of this, the MNA process was still used as a risk management strategy for risk reduction, for instance, at a site contaminated with chlorinated-hydrocarbons, where the main contaminants included trichloroethylene (TCE) and 1,1-dichloroethylene (1,1-DCE) (Tsai et al., 2012). Natural attenuation has also been selected as remediation method at sites where removal of contaminants has been determined to be technically impractical and where active remedial actions have been determined to be unable to significantly speed remediation processes (Marks et al., 1994). Often, MNA is used with other active methods, for example as a follow-up measurement after active remediation. However, it is possible to take even years to decades to clean up a site by MNA. The time needed for MNA remediation depends on many factors such as the type, amount and distribution of contaminants and type and conditions of soil (Declercq et al., 2012). Another limiting factor of MNA is that the suitable organism harboring catabolic functional genes might not be available at site (Jussila, 2006).

As can be seen from the common steps of the protocols' (Fig. 2), feasibility investigation and monitoring are the main human actions in MNA which are also the principal costs although it is insignificant compared with traditional clean-up cases (Jørgensen et al., 2006). Thus consideration of natural attenuation requires modeling and evaluation of contaminant degradation rates and pathways so as to make sure that the cleaning objectives can be reached in a reasonable timeframe (Marks et al., 1994). In another word, the MNA relies on the feasibility that NA processes are able to reach the site-specific remediation objective within a reasonable timeframe. In addition, sampling and analysis must be conducted throughout the process to confirm the degradation keeps at consistent rates with the objectives (Marks et al., 1994). It is especially important to monitor taking into account that the contaminants may immigrate before they are degraded (Marks et al., 1994). It is often said that MNA has two essential aspects: source control and long-term performance monitoring (Declercq et al., 2012).

The major tasks of MNA is to use chemical parameters such as the concentration of the xenobiotics, intermediate and end product formation, electron acceptor consumption and toxicity, and/or biological parameters such as microbial population structure and its degradation activity, to follow the natural degradation processes by microorganisms (Kuiper

et al., 2004). It is proposed that specific anaerobic intermediates for example (alkyl)benzylsuccinates, can be used as biomarkers for anaerobic biodegradation (Foght, 2008). According to van Hamme et al. (2003), many current analytical techniques require expensive equipment and extensive pretreatment of environmental samples. Besides, the classical analytical methods cannot distinguish between unavailable and bioavailable compounds. They believed that molecular and biochemical tools would help provide solutions to these problems. Jørgensen (2006) reported a project conducted during 2003 to 2006. The objective was to demonstrate the feasibility of MNA as a remediation technique for oil-contaminated sites in Finland. She also suggested the use of biological and ecological methods in addition to chemical analysis for monitoring and risk assessment. In case of petroleum hydrocarbon (PHCs), their fractions in the contaminated spots should be determined as well.

There are researches assessing biodegradation in contaminated sites by correlating an indirect biological activity measurement (e.g. $O_2$ consumption, biomass, enzyme activity etc.) to contaminant concentration decrease or mineralization (Franzmann et al., 1996; Bolliger et al., 1999). Gao et al. (2013) studied the qualities of oil-contaminated saline soils of different contamination levels. They used two composite indices (geometric mean of the assayed enzyme activities and integrated quality index derived from principal components analysis) as a soil quality evaluation method. Some researchers linked biodegradation potential with the enumeration of degrader microorganisms or the diversity of bacterial phylogenetic families based on molecular biological classification methods (Shi et al., 1999; Wilson et al., 1999; Nõvak et al., 2012; Suton et al., 2013). Genetic engineering technology is also developed to assess contaminated sites. Van Hamme et al. (2003) reviewed bacterial whole-cell biosensor technology which can measure the toxicity and bioavailability of contaminated environmental samples. The whole-cell biosensors are constructed by fusing a reporter gene to a promoter element induced by the target compound, offering the ability to characterize, identify, quantify, and determine the biodegradability of specific contaminants. Recently, some studies proposed the method of using contaminant catabolic functional genes as biomarker to monitor the degradation process (Baldwin et al., 2003; Hendrickx et al., 2006; Whyte et al., 2006).

However, in terms of an investigation and monitoring method which should be conducted throughout the whole MNA period of even up to years or decades, within acceptable accuracy and validity range, the simpler and cheaper to carry out the better. To reduce the workload and try to make the method to be more applicable, initial data about the contamination history and contamination types, and site risk assessment can be helpful in deciding which kinds of genes to be checked. For example, if the contamination source is

known as BTEX, the representative genes can be chosen from the gene pools responsible for BTEX degradation. Likewise, risk assessment can also draw information about the major kinds of contaminants posing greatest risks to the environment, thus gives clues to decide the representative genes for target contaminant. However, since the information about known biodegradation metabolic pathways and functional genes are limited, the genetic monitoring method may underestimate the *in situ* biodegradation capacity. Besides, the technical sensitivity of the methods may also be a hinder to provide accurate information. Hendrickx et al. (2006) checked the sensitivity of the gene detection method with designed primer sets. They found that the catabolic genes had a detection limit of ca. $10^3$-$10^4$ copies $g^{-1}$ soil, assuming one copy of the gene per cell. The detection limit was also found by other researchers (Kowalchuk et al., 1999).

In the present study, I investigated the contaminated soil samples by both the gene detection method and degrader microorganisms enumeration method, which is not restricted within the known catabolic pathways, to reveal whether these two parameters are correlated. And if so, how can results from these two methods used in assessment and even monitoring of bioremediation.

### 2.2.2 Biostimulation

The intentional stimulation of indigenous microorganisms by addition of electron acceptors and/or donors, water or fertilizer nutrient, in order to accelerate the biodegradation process, is referred to as biostimulation (Sarkar et al., 2004; Jussila, 2006). The biostimulation has traditionally focused on addition of N and P, either organically or inorganically. It is suggested by previous researches that nutrient supplementation stimulates bioremediation by increasing microbial biomass (Sarkar et al., 2004). The most adequate ratio to promote microbial growth is C:N:P of 100:10:1 (Dias et al., 2012). Oil contamination causes the significant increase of hydrocarbon amounts leading to an unbalanced C:N:P ratio. Thus the adjustment of the C:N:P ratio has been reported as an effective method in terms of the biodegradation. Various nutrient sources have been used in biostimulation such as inorganic fertilizer, urea, sawdust, compost, manure and biosolids (Namkoong et al., 2002; Sarkar et al., 2004).

Oil contaminants significantly decrease the air permeability of soil thus limiting the bacterial growth. Biostimulation also uses oxygen as a stimulating factor aiming at improving aeration conditions at contaminated sites which provides more oxygen for indigenous degrader microorganisms. Some researchers have indicated the significant effect of improving aeration condition to get more successful degradation (Embar et al., 2006). It is

also proposed by some report that the utilization of surfactants contribute to enhance microbial hydrocarbon degradation activity (Dias et al., 2012) because the surfactants improve the bioavailability of hydrocarbons.

However, no matter which kind of material was used as a stimulation addition, the source of nutrients, fertilization strategy and soil properties are reported to influence the performance of biostimulation (Dias et al., 2012).

### 2.2.3 Bioaugmentation

Bioaugmentation is a method to improve biodegradation by introducing into soil either wild-type or genetically modified indigenous microorganisms which obtain the degradation ability by gene transfer from a donor organism (Pepper et al., 2002; Kuiper et al., 2004; Jussila, 2006). The inoculants may be derived from the contaminated soil or obtained from a stock of microbes that have been previously proven of contaminants degradation property (Sarkar et al., 2005). The contact of microorganisms and contaminants is necessary for the success of microbial inocula in soils.

However, it has been discussed whether bioaugmentation really makes a significant difference in terms of enhancing biodegradation processes. Previous researches pointed out that bioaugmentation may fail to function when inoculated microbial biomass decline under biotic or abiotic stress; they may use other compounds in preference to the pollutants, they may be unable to move through the soil to the contaminated sites, or they may be less successfully compete with indigenous microflora (Margesin & Schinner, 1997; Pepper et al., 2002; Mrozik & Piotrowska-Seget, 2009).

Several technologies are being developed to solve these problems. These approaches involve the use of genetically engineered microorganisms and gene bioaugmentation. Various carriers with microorganisms immobilized on them and activated soils can be settled to get microorganisms to the contaminants (Mrozik & Piotrowska-Seget, 2009).

### 2.2.4 *Ex-situ* bioremediation technologies

All the bioremediation technologies mentioned above are *in situ* treatment. However, the first developed bioremediation technologies were *ex situ* technologies, which treat excavated soil in contrast to *in situ* technologies. The most commonly used *ex situ* technologies include slurry-phase remediation, treatment-bed remediation, biopile or composting (Khan et al., 2004). It is basically possible to add microbial inocula, i.e. bioaugmentation, to all these types of technologies (Jøgensen et al., 2000). Among these *ex-*

*situ* technologies, biopile technology has been demonstrated to function in field pilot or full scale especially for petroleum hydrocarbons (Jøgensen et al., 2000).

Slurry-phase remediation makes use of an added water phase to improve the physical mixing. Treatment-bed remediation is usually accomplished with nutrient supply, i.e. biostimulation. Biopiles have aeration piping devices and may be amended with bulking agents. The technology is termed as composting if organic material is added (Jøgensen et al., 2000).

# 3. Hydrocarbon-utilizing microorganisms

Despite only taking up less than 5% of the soil space, living microbes including bacteria, archaea and fungi are responsible for more than 80-90% of all the soil processes such as nutrient cycling, organic matter transformation, and maintenance of soil structure, with more than 90% of the energy flow in soil passing through microbial decomposers (Nannipieri et al. 2003; Ulrich & Becher, 2006). Soil microorganisms also control ecosystem functioning through decomposition and nutrient cycling. As soil microorganisms are responsible for the vast majority of the biogeochemical processes in soil, soil functioning is largely dependent on their activities. Moreover, it is widely accepted that the microbial community structure and dynamics can provide vital information about the soil quality, land use change and ecosystem health since they respond to the environmental changes more rapidly than soil physiochemical characters (Doran & Zeiss, 2000; Mikkonen, 2008). It is clear from a large number of studies that the distribution changes and sizable population increase of hydrocarbon-utilizing microorganism can occur when environmental samples are exposed to petroleum hydrocarbons (Atlas, 1981). Thus, it is important to identify the key players in contaminant biodegradation in order to understand, evaluate and develop *in situ* bioremediation strategies (Harayama et al., 2004).

The degradation of petroleum hydrocarbon is an ability shared by not only a few microbial genera but a diverse group of bacteria and fungi, even some cyanobacteria and algae. They are widely distributed in marine, freshwater and soil habitats. It is not surprising that microorganisms have obtained hydrocarbon utilizing ability considering that these compounds are naturally occurring (Atlas, 1981). A number of bacterial species are also known to degrade PAHs and most of them are isolated from contaminated soil or sediments (Haritash & Kaushik, 2009). It has also been demonstrated by both laboratory and field studies that a considerable subsurface microbial community is able to metabolize pollutants (Margesin & Schinner, 1997). Generally, the hydrocarbon-utilizing microorganisms in unpolluted ecosystems consists of only less than 0.1% of the microbial community, but this

number can elevate up to 100% in oil-polluted ecosystems (Atlas, 1981) while overall microbial diversity declines (Aislabie et al., 2004). Atlas (1981) believes that the degree of elevation reflects the degree or extent of exposure of that ecosystem to hydrocarbon contaminants.

There are some important hydrocarbon utilizing genera such as *Pseudomonas*, *Achromobacter*, *Arthrobacter*, *Micrococcus*, *Nocardia*, *Vibrio*, *Acinetobacter*, *Brevibacterium*, *Corynebacterium*, *Flavobacterium*, *Candida*, *Rhodotorula* and *Sporobolomyces* (Atlas, 1981). Previous studies have even found the evolution of some obligate hydrocarbon degraders (also named as obligate hydrocarbonoclastic bacteria) of indigenous marine bacterial genera *Oleispira*, *Marinobacter*, *Thalassolituus*, *Alcanivorax* and *Cycloclasticus*–which are present at low or undetectable levels before pollution occurrence but were found to dominate in oil polluted microcosms (Yakimov et al., 2007; Brooijmans et al., 2009). For example, *Alcanivorax* strains grow on *n*-alkanes and branched alkanes while they cannot grow on any sugars or amino acids as carbon sources. *Cycloclasticus* strains grow on aromatic hydrocarbons, naphthalene, phenanthrene and anthracene, whereas *Oleispira* strains grow on the aliphatic hydrocarbons, alkanoles and alkanoates (Harayama et al., 2004). In general, there are only small numbers of these organisms but they can grow and multiply rapidly provided with hydrocarbons as carbon and energy source (Head et al., 2006). There are some researches indicating that the *Alcanivorax* spp. responds to the oiling event within days and its population size can decline significantly within weeks correlating with the removal of saturated hydrocarbons (Head et al., 2006). *Alcanivorax* spp. is a global player of hydrocarbon degradation since it has been detected across the world such as United States, Singapore, China, Germany, Japan and so on (Yakimov et al., 2007). Their selective advantage is suggested come from their more effectiveness on using branched-chain alkanes (Head et al., 2006).

Soil contaminants change the chemical and physical properties of soil structure such as the soil pH value, total organic matter and electricity conductivity. As a result of the physicochemical properties changes, microbial communities show response to the contaminant exposure. Sutton et al. (2013) found a highly significant reduction of microbial diversity correlated with TPH contamination. Bundy *et al*. (2002) found that the microbial community in different soil types showed no convergence whilst more dissimilarities after diesel contamination and bioremediation treatment. It indicated that different soils have different indigenous microorganisms with hydrocarbon degradation potential which should be taken into consideration for impact and risk assessments. Some researchers also tried to figure out the competition between indigenous and exogenous degraders and their conclusion

confirms that the degrading ability determines the success of predominant strain (Harayama et al., 2004).

The development and recovery of the microbial community can make an important parameter for impact monitoring and oil-contaminated soil recovery. Nowadays, some soil monitoring programs already included microbial ecology as an indicating parameter. Mikkonen (2012) suggested it should also be considered when conducting contaminated soil risk assessment and management which primarily aims are protection of human health and prevention of contaminant spreading. Nõvak et al. (2012) revealed the bacterial community profiling of contaminated soil samples by DGGE (denaturing gradient gel electrophoresis) method. Suton et al. (2013) identified OTUs (operational taxonomy units) which are similar to microbes involved in biodegradation and correlated these results with the degradation potential. They claimed that with the noticed reduction of microbial diversity, a large portion of the microbial community present in contaminated sites could be targeted with relatively few molecular assays. All these works mentioned above provide a perspective to estimate potential common patterns of community structure associated with biodegradation. This, in turn helps develop new tools to monitor and assess bioremediation processes (Head et al., 2006).

Hydrocarbon catabolism has long been considered as a strictly aerobic process. However, particular microorganisms were demonstrated with anaerobic degradation capacity (Coates et al., 1997; Heider et al., 1999). All the anaerobic hydrocarbon degrading strains are denitrifying, ferric iron-reducing, sulfate-reducing bacteria or bacteria capable of reducing proton to hydrogen (Heider et al., 1999). Some of the denitrifying bacteria were formerly classified as *Pseudomonas* sp. and now as *Thauera* and *Azoarcus* genera within the *β-Proteobacteria* (Heider et al., 1999). *Geobacter metallireducens*, a ferric iron-reducing strain was reported to degrade toluene under anaerobic condition and several sulfate-reducing strains were reported with capability of utilizing alkanes and alkenes (Heider et al., 1999). Chang et al. (2002) measured the biodegradation rates of PAH under three anaerobic conditions. Their results showed that degradation rates decreases from sulfate reducing conditions to methanogenic conditions, and to nitrate-reducing conditions. Their results also indicated that sulfate-reducing bacteria, methanogen and eubacteria were involved in PAH biodegradation, and sulfate-reducing bacteria was a major component in the PAH-adapted consortia. A latest study by Jaekel et al. (2013) reported the anaerobic degradation of propane and butane by sulfate-reducing bacteria enriched from marine hydrocarbon cold seeps. They found the enriched cultures formed a distinct phylogenetic cluster affiliated with the *Desulfosarcina-Desulfococcus* cluster within the δ-*Proteobacteria*.

# 4. Biodegradation mechanisms of hydrocarbons

During last decades, many studies have been conducted about the metabolic pathways of hydrocarbon compounds degradation. The substrate compounds must enter the cell prior to their degradation. However, direct contact between hydrocarbons, usually lipophilic compounds or in other word hydrophobic, and the cell membrane is prevented by the presence of the cell wall and/or the hydrophilic parts of the outer membrane (Sikkema et al., 1995). The lipophilicity of a compound depends on various physical and chemical characteristics such as molecular surface area, molecular volume and polarity. The bioavailability of a compound can be critically measured by its dissolution rate (Sikkema et al., 1995).

Alkanes, as nonpolar molecules are chemically very inert. Their lower water solubility (Table 1), tendency to accumulate in cell membrane and energy consumption for activation hinder their metabolism (Rojo, 2009). Aromatics are more soluble and volatile indicating that a large proportion of them may be volatilized before causing harm to soil biota (Mikkonen, 2008). Generally, the biodegradability of hydrocarbons can be ranked as: linear alkanes > branched alkanes > low-molecular-weight alkyl aromatics> monoaromatics > cyclic alkanes> polyaromatics> > asphaltenes (van Hamme et al., 2003; Brooijmans et al., 2009). The increase in the relative abundance of the polar fractions, as well as the loss of saturated and aromatic hydrocarbons, is a characteristic of biodegradation (Head et al., 2006).

## 4.1 Bioavailability of hydrocarbons in soil

The degradation of hydrocarbon by microbes is greatly influenced by the bioavailability of hydrocarbons. The chemical properties of hydrocarbons determine its bioavailability. To be degraded, the hydrocarbons have to be firstly absorbed by degrading microbes which means the process of transmembrane transportation. The absorption contains two stages: the exposure of hydrocarbons to microbes and the transmembrane transportation. It has been shown that the hydrocarbons can cause an increase of microbial membrane permeability (Li & Liu, 2002). Different mechanisms have evolved by the degrader species to increase absorbance of the hydrocarbons for example, the secretion of biosurfactants or emulsifiers (Whang et al., 2008).

Biosurfactants are surface active compounds which can be used as environmentally friendly dispersion and remediation agents in remediation processes such as bioremediation, soil washing and soil flushing (Thavasi et al., 2011). Various types of biosurfactants such as

glycolipids, lipopolysaccharides, oligosaccharides, and lipopeptides have been reported to be produced by diverse bacterial genera (Thavasi et al., 2011). Biosurfactants can form spherical or lamellar micelles which have hydrophobic cores where hydrophobic compounds become solubilized leading to a transfer of hydrocarbons from solid, liquid, or sorbed PAH-pools into the water phase (Johnsen et al., 2005).

## 4.2 Aerobic degradation

For alkanes, the most common pathway is monoterminal oxidation. The first step is the oxidization of a terminal methyl to form primary alcohol. The primary alcohol is then oxidized to appropriate aldehyde and fatty acid. After the formation of the fatty acid, the β-oxidation takes place resulting in the formation and removal of acetyl coenzyme A, by which the fatty acid is shortened by a two-carbon atom (Li & Liu, 2002). There are also several other oxidation pathways, such as diterminal oxidation and subterminal oxidation (Li & Liu, 2002). In the cases of diterminal pathway, oxidation of both ends of the alkane molecule takes place through ω-hydroxylation (ω position i.e. the terminal methyl group) of fatty acids, and then further converted into a dicarboxylic acid and processed by β-oxidation (Rojo, 2009). As for subterminal oxidation, alkanes are oxidized to secondary alcohol and then to the corresponding ketone and ester. The ester then is hydrolysed generating an alcohol and a fatty acid (Rojo, 2009).

The aromatic hydrocarbons are important components of crude oil and the priority pollutants of soil remediation. Diverse microorganisms have evolved to be capable of utilizing aromatic hydrocarbons since they are also naturally occurring organic compounds. The key steps in the degradation of aromatic hydrocarbons are the initial oxidative attack and the cleavage of the benzene ring (Hendrickx et al., 2006). The most common way of initial oxidation is through forming of *cis*-dihydrodiols by incorporation of both oxygen atoms of an oxygen molecule and then to catechols (Johnsen et al., 2005). Microorganisms can cleave the benzene ring in different ways with the catalysis of appropriate enzymes (Li & Liu, 2002): the *ortho*- or *meta*- cleavage pathways leading to the formation of central intermediates such as protocatechuates and catechols and then are further converted to tricarboxylic acid (TCA) cycle intermediates (Johnsen et al., 2005; Peng et al., 2008). The *ortho*- and *meta*- cleavage pathways differ in the cleavage site. *Ortho* cleavage catalyzed by intradiol dioxygenases (either homomultimers or composed of two different subunits containing ferric iron) cuts between the two hydrolated carbons, while *meta* cleavage catalyzed by extradiol dioxygenases (multimers of a single subunit containing ferrous iron) cuts between one hydrolated carbon and an adjacent nonhydrolated carbon (Harayama & Rekik, 1989; van der Meer et al., 1992). Normally, *ortho*-genes are located on chromosome

and *meta*-genes on plasmids, however, genetically modified *ortho*-genes can also locate on catabolic plasmids (van der Meer et al., 1992).

## 4.3 Anaerobic degradation

It was for a long time believed that oxygen was required by the microbes when degrading petroleum hydrocarbons. The aerobic biodegradation of hydrocarbons have been known and studied for many years. However, the study of anaerobic degradation is relatively recent and new sights are constantly generating. It is reported that, with nitrate, ferrous iron, manganese or sulfate as electron acceptor or under conditions of methanogenesis, the anaerobic degradation of several classes of petroleum hydrocarbons such as alkanes, mono- and polycyclic aromatic compounds can also happen (Yakimov et al., 2007; Foght, 2008). It was firstly recognized in 1980s that some bacteria are capable to metabolize hydrocarbons without the presence of molecular oxygen (Heider et al., 1999). In the beginning, anaerobic degradation activity is only observed in enrichment cultures and sediments or ground waters, but now some bacterial strains capable of anaerobic petroleum hydrocarbons degradation have been isolated (Foght, 2008). As currently known, several alkylbenzenes such as toluene, ethylbenzene as well as benzene and naphthalene (low molecular mass soluble compounds), as well as alkanes or alkenes can be anaerobically utilized as substrates by some bacterial species (Harwood et al., 1998; Heider et al., 1999).

It is proposed that the initial activation of alkanes have two biochemical mechanisms: addition of fumarate and carboxylation (Mbadinga et al., 2011). The fumarate addition mechanism is shared by more diverse microorganisms than the other mechanism (Mbadinga et al., 2011). For degradation of toluene, alkylbenzene and ethylbenzene, they are oxidized to benzoyl-CoA, a common intermediate in anaerobic catabolism of many aromatic compounds (Heider et al., 1999). However, the degradation of non-substituted aromatic hydrocarbons (such as benzene, naphthalene and phenanthrene) is much less understood. But it is evidenced by more and more studies that initial activation reactions of both benzene and naphthalene are direct carboxylation, although the detailed mechanism remains unclear (Foght, 2008). The further degradation follows the benzoyl-CoA or naphthalene-CoA pathways (Foght, 2008). It is necessary to mention that the central benzoyl-CoA pathways are different as for many aspects in different denitrifying, phototrophic and fermenting bacteria (Harwood et al., 1998).

Generally, anaerobic degrading microbes are less diverse than aerobic degraders and the degrading process needs a longer period of time (Coates & Woodward, 1997).

## 4.4 Influential factors

Microbial degradation in soil is not just determined by the degrader microorganisms (Head et al., 2006) but also influenced by the direct and indirect interaction with other non-degrading community members and the environment especially physicochemical properties of both the contaminants and soil conditions (Towell et al., 2011).

Oil concentration has a significant effect on the hydrocarbon mineralization. Towell et al. (2011) studied the effect of oil concentration on cable oil biodegradation. With increased oil concentration, lag phases decreased, whilst maximum degrading rates and cumulative extents of mineralization significantly increased. The most effective degradation happened with 10 ppm and 100 ppm oil concentration. Another factor affecting biodegradation studied by Towell et al. (2011) was the inoculum amounts. According to their results, $10^6$ CFU $g^{-1}$ soil is the optimum. With increasing inoculated degraders (commercial petroleum hydrocarbon catabolic inocula mixtures), the lag phases increased. The lag phase with $10^7$ CFU $g^{-1}$ soil inoculums was about 170 hours at 50 ppm oil concentration.

Mohn & Stewart (2000) studied limiting factors for hydrocarbon biodegradation at low temperature. The soil samples used by them were from previously contaminated sites and their soil microcosms were incubated at 7 $^0$C. They found that both N and P limited biological mineralization of dodecane in Arctic tundra soils. Mineralization rates can hardly be detected when neither N nor P was added. The competition for nutrients between degrader microorganisms and other microbial community members can be a stress for biodegradation. However, there is no clear signal to relate bacterial community composition to nutrient concentrations (Head et al., 2006).

# 5. Functional genes involved in degradation

Soil biological activity parameters such as the soil respiration or even degrader community structure do not specifically reflect biodegradation processes. The catabolism of petroleum hydrocarbons is not restricted within some certain genera especially considering most bacterial phylogenetic families are still unknown. Also, many catabolic pathways are carried on plasmids and most of the degradation plasmids can be conjugated i.e. self-transmissible (Jussila, 2006). Some research results indicated the occurrence of horizontal transfer of catabolic genes as well (Hendrickx et al., 2006).

Recently, detection of functional genes involved in contaminant degradation has been used as a more direct and straightforward method to monitor biodegradation of petroleum

hydrocarbons (Hendrickx et al., 2006; Nõvak et al., 2012). Moreover, any specific gene on plasmids is maintained in the population by only a few cells which requires minimal energy from the population as a whole, but ready to spread upon selective pressure such as oil contamination (Llosa & de la Cruz, 2005). Thus, the abundance kinetics of specific catabolic genes in contaminated sites can provide a perspective to intercept remediation processes. Some researchers have used quantification molecular biological tool to enumerate the copy numbers of functional genes as an evaluation of the feasibility of MNA and biodegradation (Hosoda et al., 2005; Baldwin et al., 2007). Also, Monard et al. (2012) monitored the degradation functional gene expression to assess *in situ* microbial abilities of soils to degrade contaminants.

In this chapter, I review some well investigated hydrocarbon catabolic genes including *alkB* gene for alkane monooxygenase, *xylE* gene for catechol dioxygenase, and *nahAc* gene for naphthalene dioxygenase (**Chapter 5.1, 5.2 & 5.3**). The degradability of petroleum mixture depends on the hydrocarbon content (Atlas, 1981). As an extreme complicated mixture, petroleum hydrocarbons can be classified into many fractions and there are different functional genes responsible for biodegradation of certain hydrocarbon classes. Table 3 lists some functional genes involved in hydrocarbon biodegradation pathways described in previous literature.

**Table 3.** Fuel hydrocarbon degradation genes

| Gene | Protein | Primers (5'-3') | Reference strains | Amplified Fragment size (bp) | Reference |
|---|---|---|---|---|---|
| ***n*-alkanes** | | | | | |
| ***alma (C32+)*** | Flavin-binding monooxygenase | f GGNGGNACNTGGGAYCTNTT<br>r ATRTCNGCYTTNAGNGTCC | *Acinetobacter and Alcanivorax genera* | 1100 | Wang& Shao, 2012 |
| ***alkB*1** | alkane monooxygenase | f ATCTGGGCGCGTTGGGATTTGAGCG<br>r CGCATGGTGATCGCTGTGCCGCTGC | *Rhodococcus* spp. | 629 | Whyte et al., 2002 |
| ***alkB*2** | alkane monooxygenase | f ACTCTGGCGCAGTCGTTTTACGGCC<br>r CCCACTGGGCAGGTTGGGCGCACCG | *Rhodococcus* spp. | 552 | Whyte et al., 2002 |
| ***alkB*** | alkane monooxygenase | f TGGCCGGCTACTCCGATGATCGGAATCTGG<br>r CGCGTGGTGATCCGAGTGCCGCTGAAGGTG | *Pseudomonas oleovorans* | 870 | Kok et al., 1989; Whyte et al.,1996; Whyte et al., 2002 |
| ***alkM*** | alkane monooxygenase | f CCTGTCTCATTTGGCGCTCGTTCCTACAGG<br>r GTGATGATCTGAATGTCGTTGTAACTGG | *Acinetobacter* spp. | NA | Whyte et al., 2002 |
| ***cyp153 (C5-C16)*** | cytochrome P450 enzymes | f ATGTTYATYGCNATGGAYCCN<br>r GCGRTTVCCCATRCARCGRTG | *Alcanivoraxdieselolei* | 820 | Wang et al., 2011 |
| **Aromatic hydrocarbons** | | | | | |
| ***Cdo*** | Subfamily I.2.C of catechol extradioldioxygenases | f CATGTCAACATGCGCGTAATG<br>r CATGTCTGTGTTGAAGCCGTA | -- | 255 | Hendrickx et al., 2006 |

| | | | | | |
|---|---|---|---|---|---|
| ***tbmD*** | Subfamily 1 of α-subunits of hydroxylase component of multicomponent | f GCCTGACCATGGATGC(C/G)TACTGG<br>r CGCCAGAACCACTTGTC(A/G)(A/G)TCCA | *Pseudomonas* sp. strain JS 150 | 640 | Hendrickx et al., 2006; Baldwin et al., 2003 |
| ***tmoA*** | Subfamily 2 of α-subunits of hydroxylase component of multicomponent | f CGAAACCGGCTT(C/T)ACCAA(C/T)ATG<br>r ACCGGGATATTT(C/T)TCTTC(C/G)AGCCA | -- | 505 | Hendrickx et al., 2006 |
| ***xylM*** | Toluene monooxygenase | f TGAGGCTGAAACTTTACGTAGA<br>r CTCACCTGGAGTTGCGTAC | *Pseudomonas putida* | 475 | Baldwin et al., 2003 |
| ***xylA*** | Electron transfer component of two-component side chain mono-oxygenases | f CCAGGTGGAATTTTCAGTGGTTGG<br>r AATTAACTCGAAGCGCCCACCCCA | -- | 291 | Hendrickx et al., 2006 |
| ***todC1*** | Subfamilies D.1.B+D.1.C+ D.2.A+D.2.B+D .2.C of a-subunits of Type D iron–sulfur multi-component aromatic dioxygenases | f CAGTGCCGCCA(C/T)CGTGG(C/T)ATG<br>r GCCACTTCCATG(C/T)CC(A/G)CCCCA | -- | 510 | Hendrickx et al., 2006 |
| ***xylE1*** | Subfamily I.2.A of catechol extradioldioxygenases | f CCGCCGACCTGATC(A/T)(C/G)CATG<br>r TCAGGTCA(G/T)CACGGTCA(G/T)GA | *Pseudomonas putida* | 242 | Hendrickx et al., 2006 |
| ***xylE2*** | Subfamily I.2.B of catechol extradioldioxygenases | f GTAATTCGCCCTGGCTA(C/T)GTICA<br>r GGTGTTCACCGTCATGAAGCG(C/G/T)TC | *Pseudomonas putida* | 906 | Hendrickx et al., 2006 |
| **Polycyclic aromatic hydrocarbons** | | | | | |
| ***nid*** | Pyrenedioxygenase large subunit | f ATCTTCGGGCGCGCCTGGGTGTTTCTCGG<br>r AATTGTCGGCGGCTGTCTTCCAGTTCGC | *Mycobacterium* sp. strain PRY-1 | 323 | Margesin et al., 2003 |
| ***nahAc*** | Naphthalene 1,2-dioxygenase Fe-S protein large subunit | f CAAAA(A/G)CACCTGATT(C/T)ATGG<br>r A(C/T)(A/G)CG(A/G)G(C/G)GACTTCTTTCA A | *Pseudomonas putida* | 377 | Baldwin et al., 2003 |

## 5.1 Alkane monooxygenase (*alkB*)

AlkB alkane hydroxylase systems, as presently the best-characterized alkane hydroxylase, were firstly described in *Pseudomonas oleovorans* GPo1 (latter classified as a *Pseudomonas putida* strain) where it is located on the OCT plasmid and organized in two operons: *alkBFGHJKL* and *alkST* (Kok et al., 1989; Heiss-Blanquet et al., 2005; Liu et al., 2011). It catalyses the first step of alkane degradation by oxidizing alkanes to alkanols with help of two electron transfer proteins, rubredoxin (AlkG) and rubredoxinreductase (AlkT) (van Beilen et al., 1994). They are widely spread in nature with more than 250 AlkB

homologues identified in at least 45 bacterial species (Paisse et al., 2011). Meanwhile, the *alkB*-like hydroxylase genes have also been detected in a wide range alkane-degrading bacteria, including α-, β-, and γ- proteobacteria; as well as in some high G + C content Gram-positive bacteria (Smits et al., 2002).

There are four *alkB* gene groups described by previous researches. They consists of '*Pseudomonas* 1' group which includes *alkB* sequences from *Pseudomonas putida* strains and other Gram-negative bacteria, '*Pseudomonas* 2' group which also comprises *alkB* gene sequences from *Pseudomonas* species, especially *Pseudomonas aeruginosa* strains. In addition to these two groups, *Rhodococcus* group which includes sequences of species belonging to *Rhodococcus, Burkholderia, Nocardioides, Prauserella* and *Mycobacteria*, as well *Acinetobacter* group of only *Acinetobacter* strains, are also clustered (Heiss-Blanquet et al., 2005). Smits et al. (2002) indicated that the alkane hydroxylases of *Pseudomonas* are as distantly related to each other as to the other studied strains which belong to species of *Alcanivorax borkumensis*, *Mycobacterium tuberculosis* and *Prauserell arugosa*. The sequence comparison results of van Beiden (2003) also pointed out the high gene sequence diversity, even among *alkB* genes within the same species. It may be therefore difficult to analyze alkane degrading populations or to assess the alkane biodegradation potential with only a single primer set and even lead to false negative results (Whyte et al., 1996). However, using group-specific primers or primer sets can to some extent avoid this problem (Whyte et al., 2002; Margesin et al., 2003).

With broad distribution range, diverse genotypes and adequate previous research results, *alkB* gene has been considered to be a promising functional biomarker for monitoring the bioremediation capacities of oil contamination. Many studies tried to relate the degradation processes or contaminants mineralization with the kinetic changes of *alkB* gene diversity, abundance or expression *in situ* (Paisse et al., 2011). However, their results suggested that there is no clear relationship between the contamination level and *alkB* gene expression. Another factor makes it more complex to correlate mineralization and the gene expression is that alkanes derived from plant litter highly influences the abundance, activity and community structure of *alkB* harbouring bacteria, as postulated by Schulz et al. (2012). Recently, there is also consideration of using other alkane monooxygenase genes as indicator. The most promising ones include *almA* and *cyp153* genes. Flavin-binding monooxygenase (*alm*A) gene was involved in long chain alkane degradation by *Acinetobacter* and *Alcanivorax* genera. The first experimentally confirmed enzyme involved in long chain alkane metabolism of $C_{32}$ or longer was encoded by *almA* gene (Wang & Shao, 2012a). Besides, Cytochrome P450 of the CYP153 family was found to be involved in the oxidation of C5 to C16 n-alkanes in *Acinetobacter* (Wang & Shao, 2012b).

## 5.2 Catechol -2,3- dioxygenase (*xylE)*

BTEX degradation functional proteins can be found in several subfamilies for example subfamily I.2.A within family I.2 and in subfamily I.3.B within family of I.3 of the phylogenetic tree of catechol 2, 3-dioxygenases (C23O) amino acid sequences (Eltis & Bolin, 1996). Subfamily I.2.A mainly contains the C23O sequences of fluorescent *Pseudomonas* bacteria (Hendrickx et al., 2006). The degradation of BTEX initiates from the oxidative attack by direct oxidation of the aromatic ring via a mono-oxygenase or a dioxygenase or oxidation of the alkyl side chain via mono-oxygenases through upper pathway (Ramos et al., 1997; Hendrickx et al., 2006). Afterwards, aromatic ring cleavage happens by the catalysis of catechol 2, 3-dioxygenases (C23O) through *meta*-cleavage pathway and then be degraded into Krebs cycle (TCA cycle) intermediates (Ramos et al., 1997; Johnsen et al., 2005).

The TOL plasmid (archetypal pWW0), one of the best understood catabolic plasmids, is self-transmissible and is a member of incompatibility group P-9. Out of its 117 kilo base pairs, approximately 40 kilo base pair is needed for the catabolic pathway and the regulatory genes (Burlage et al., 1989). The mechanism of *xyl* genes on TOL plasmid is now well understood. There are four transcriptional units in *xyl* genes of *Pseudomonas putida* TOL plasmid which catabolize toluene and xylenes: the upper-operon responsible for conversion toluene/xylene to benzoate/alkylbenzoates; the *meta*-operon encoding enzymes further converse these compounds into Krebs cycle (TCA cycle) intermediates; and *xylS* and *xylR*, which are involved in transcriptional control (Ramos et al., 1997). As for the upper-operon *xylUWCAMBN*, the *xylU*, *xylW* and *xylN* gene products are not required for toluene/xylenes degradation, while *xylM*, *xylA*, *xylB* and *xylC* are needed for upper pathway (Ramos et al., 1997). The *meta*-operon with over 11 kb, as one of the largest operons in prokaryotes, comprises 13 genes, *xylXYZLTEGFJQKIH* (Ramos et al., 1997). In the earlier stage of *meta*-cleavage pathway, *xylXYZ* and *xylL* further oxidize the benzyle alcohol to corresponding catechol and *xylE* functions at the first step of ring cleavage (Ramos et al., 1997). Early studies also found that catechol can also be degraded through *ortho*-cleavage pathway encoded by chromosomal genes (Burlage et al., 1989). Expression of the *xyl* gene clusters is submitted to interplay between plasmid-encoded regulators, encoded by *xylS* and *xylR*, and host factors (Ramos et al., 1997). The complex XylR/*m*-xylene, consisting of binding-enhancer protein XylR and pathway substrate compounds (i.e. *m*-xylene), activates the promoter (*Pu*) for upper operon transcription and increases expression of the activator of the lower operon (XylS) (Velázquez et al., 2005). Other global regulation mechanisms also control the expression of *xyl* genes such as catabolite repression mediated by intermediates,

growth phase control caused by IHF (integration host factor) binding to *Pu* and sigma factor competition in stationary phase (Velázquez et al., 2005).

Catechol 2,3-dioxygenase is the product of *xylE*. The enzyme has four identical subunits each with a molecular mass of 35 kDa and molecular iron is required for its activity (Burlage et al., 1989). The gene has been purified, characterized, sequenced and incorporated into convenient transcriptional fusion vectors (Burlage et al., 1989). The *xylE* gene is mainly found in Gram-negative bacteria, however also reported in some Gram-positive bacteria such as *Rhodococcus* and *Arthrobacter* (Hendrickx et al., 2006).

## 5.3 Naphthalene dioxygenase (*nah*)

The NAH7 plasmid with 83 kilo base pairs is well studied genetically in *Pseudomonas putida* G7. It has three operons: *nah* for the upper-pathway enzymes catalyzing naphthalene to salicylate (i.e. monoaromatics), *sal* encoding the lower-pathway enzymes for *meta*-ring cleavage through which the salicylate are catalyzed to TCA cycle intermediates (i.e. pyruvate and acetaldehyde), and one encoding regulator (NahR) (Harayama & Rekik, 1989; van der Meer et al., 1992; Habe & Omori, 2003). The *sal* operon genes are similar to those on plasmid pWW0 and the sequences of both operons are homologous (van der Meer et al., 1992). The *nahR* located between the *nah* and *sal* operons on NAH7 and it encodes the enzyme which is a positive control regulator required for high-level expression of *nah* genes (Habe & Omori, 2003).

The upper pathway genes *nahAaAbAcAdBFCQED* are similar to those of the catabolic genes from the plasmids of several other studied *Pseudomonas* strains in terms of organization and sequences (Habe & Omori, 2003). In these genes, the *nahAa* encodes NDO (naphthalene dioxygenase) ferrodoxin reductase, *nahAb* for NDO ferrodoxin, *nahAc* for the a subunit of NDO, *nahAd* for the b subunit of NDO, *nahB* for naphthalene *cis*-dihydrodioldehydrogenase, *nahF* for salicylaldehyde dehydrogenase, *nahC* for 1,2-dihydroxynaphthalene dioxygenase, *nahQ*, *nahE* for *trans*-*o*-hydroxybenzylidenepyruvate hydratase-aldolase, and *nahD* for 2-hydroxychromene-2-carboxylate isomerase (Habe & Omori, 2003). The *sal* operon for salicylate oxidation includes the genes of *nahG*, *nahH*, *nahI*, *nahN*, and *nahL*. The *nahH* encodes enzyme of catechol 2,3-dioxygenase, *nahI* for hydroxymuconic semialdehyde dehydrogenase, *nahN* for hydroxymuconic semialdehyde hydrolase, and *nahL* for 2-oxopent-4-enoate hydratase (Harayama et al., 1987).

The catechol 2,3-dioxygenases encoded by TOL and NAH plasmids with similar sequences are also similar to other extradiol ring cleavage enzymes (van der Meer et al.,

1992). There were 20% nucleotide sequence difference and 16% amino acid sequence difference between *nahH* (i.e. the gene for catechol 2,3-dioxygenase from plasmid NAH7) and *xylE* (i.e. the gene for catechol 2,3-dioxygenase from plasmid pWW0) (Harayama et al., 1987). It is suggested that the catechol oxidation genes from pWW0 and NAH derived from a common ancestor (Harayama et al., 1987). The *nah*-like genes from *Pseudomonas* species are highly conserved and have been well investigated for the structure-function relationships and evolutionary trails of the catabolic enzymes (Habe & Omori, 2003). Recently, evolutionarily different PAH-catabolic genes have now been characterized from more diverse bacterial species including other Gram-negative species and Gram-positive species (Habe & Omori, 2003).

# 6. Theories of the methods

## 6.1 MPN (most probable number) method

To enumerate the microorganism numbers, the MPN (most-probable-number) method was used. The MPN method has been suggested for enumerating hydrocarbon-utilizing microorganisms as early as in 1980s, since the procedures make it possible to direct assess the ability to actually utilize hydrocarbons and eliminate the need for a solidifying agent which might overestimate the number of microbial degraders. The liquid media for MPN procedures removes the trace organic contaminants being of a medium with hydrocarbon as a sole carbon source (Atlas, 1981).

There are several principles behind the MPN method. The basic concept of this method is that the pattern of growth vs. non-growth can provide information. Highly selective media or incubation conditions targeting one or small groups of microorganisms are often used to estimate cell numbers of microbial species with certain property. In this study, by the Bushnell-Hass broth which is carbon free and supplemented with petroleum as sole carbon sources, the microorganisms capable of petroleum degradation can be selectively incubated (Madigan et al., 2012). The selective broths support the growth of organisms and turn turbid. For example, if one replicate tube with inoculum of the sample contains a bacterial cell, it will show growth whilst its replicated tubes may not receive any bacteria because of pipetting or sampling and would not turn turbid. The accuracy can be greatly increased by diluting the inoculum and using replicates (Sutton, 2010). For example, with 10-fold serial dilutions of soil suspension, the first dilutions should show positive growth and the last dilution with growth should have originated from ten or fewer cells. To get more accuracy, the MPN method can be performed with replicated treatments (Madigan et al., 2012). The

raw data derived from MPN procedures were analyzed by computer program to get the soil degrader enumeration results.

## 6.2 Oil analysis: TSEM and TPH

The oil content analysis techniques used in this study, TSEM (total solvent extractable material) and TPH (total petroleum hydrocarbon), are both nonspectroscopic. The method was based on the European Standard for the quantitative determination of total petroleum hydrocarbon (TPH) in solid waste by gas chromatography (ISO 16703: 2004), which was released by European Committee for Standardization in April 2004. It is an easily performed laboratory method since it extracts oil from samples by ultrasonic mixture and shaking at room temperature (ISO 16703: 2004). The extracted oil measured gravimetrically after nitrogen drying of solvent is the resultant TSEM, which stands for the amount of solvent extractable materials in samples. TSEM content is a rough estimation of oil content. The purified oil extraction by Florisil measured on GC-FID determines the TPH content. By this method, the measured TPH covers the non-polar compounds within a boiling range of 175 $^{o}$C (*n*-decane $C_{10}H_{22}$) to 525 $^{o}$C (*n*-tetracontane $C_{40}H_{82}$) that do not absorb on Florisil including isoalkanes, cycloalkanes, alkylbenzenes, alkylnaphthalenes and polycyclic aromatic compounds. It excludes the interference of polar components in soils originating from animal, vegetable fats and oils. However, some reports have indicated certain lipids, pigments and plant resins may exist after purification (Saari et al., 2007).

# 7. Objectives of the study

The success of bioremediation depends on the behavior of *in situ* bacterial degrading microorganisms. Their ability of metabolizing contaminants relies on the existence of degradation functional genes. Before performing *in situ* bioremediation technologies for an oil-contaminated site, the existence of indigenous degrader microorganisms should be confirmed. As for bioaugmentation, the fate of augmented degraders should also be investigated. However, traditional enumeration methods of the bacterial population such as plate counting or even MPN is rather time consuming and often an underestimate because of the existence of unculturable bacteria. Whilst, the genetic monitoring method (i.e. functional genes detection) may also underestimate the *in situ* biodegradation capacity since it is limited to known biodegradation metabolic pathways. Therefore, a culture-independent molecular method, i.e. PCR amplification with primer sets targeted functional degradation genes designed based on sequences from diverse degrading bacteria, together with a culture-dependent method, i.e. MPN method to enumerate degrader microorganisms, may provide an

useful solution for detecting, monitoring and characterizing intrinsic hydrocarbon degradation capacity of soil.

In addition, the bioremediation of oil-contaminated soils is a clean solution which gets more and more attention and application. However, there is no commonly accepted or utilized protocol which can provide information about bioremediation potential in the contaminated sites. It is often empirically decided whether a contaminated site is applicable for bioremediation and which kind of bioremediation technologies should be used while no indicators of biological activities are monitored. Generally speaking, this study targets at proposing an simple but reliable method for contaminated site investigation and to monitor the bioremediation processes. The method provides information about bioremediation potential by investigating key remediation "players" i.e. the degrader microorganisms and catabolic genes. To achieve this applicable objective, the protocol should be easily performed and can provide clear, strong and general conclusions for remedial decision making.

The present study consists mainly of three parts: 1) analysis of physicochemical properties of the soil samples, especially the residual oil contamination level, 2) enumeration of contaminant bacterial degraders, and 3) the existence of functional genes. By this methodology, the abundance of degrading microorganisms and the distribution of hydrocarbon degradation genes in contaminated soil samples can be revealed. Correlated with the residual contaminated level, the results of degrader enumeration and gene detection can suggest if these two biological parameters can be informative in assessment of bioremediation potential (existence of to-be-stimulated remediation "players") and soil health and, monitoring of bioremediation processes. In addition, the comparisons between the vegetated soil samples and un-vegetated samples can also indicate whether rhizoremediation makes difference in the sampling field. The present study can also give a suggestion on whether these genes are appropriate to be used as biomarkers. The results also indicate possible direction for future researches in this field.

# 8. Materials and Methods

## 8.1 Sampling

The samples were collected from two oil contaminated sites in Porvoo, Finland. The first sampling site is a landfarming field used for disposal and treatment of oily refinery

sludges. There were disposal of oily refinery sludge between 1980 and 2005. The field was fine sand soil and had received about 30,000 tons of moderately oil-contaminated sludges per 3.5 ha (Wallenius et al., 2012). During and after that period, the field have been fertilized with urea and ploughed twice per year down to 20 cm. The site was investigated by a previous study in our group (Mikkonen et al. 2012) which found the horizontal and vertical contamination gradient. A series of six adjacent 3 × 3 m plots with 1 m gaps were setup covering the horizontal gradient. These plots were planted with legumes and fenced while outside of the plots area was bare soil. Samples were collected from the vegetated area and also the outside bare soil area. The vegetated samples were taken from three plots along the horizontal gradient while the unvegetated samples were taken from the horizontally parallel plots in unvegetated areas along the gradient. They were labeled as "ve1-3" for vegetated samples and "unve1-3" for unvegetated samples. The number 1, 2, 3 indicate the increasing in initial contamination level. So there were three paired samples (ve1 & unve1; ve2 & unve2; ve3 & unve3) and each paired samples were considered to have same initial contamination level.

The samples were collected from both surface soil and subsurface soil. Sampling of surface soils was conducted compositely by drilling 8 or 12 subsamples at a depth of 0-20 cm radically 0.5 and 1 m from each plot center with a manual gouge auger (Ø 20 mm). Subsurface samples (down to 80 cm below surface) with heavy contaminantion were taken by digging pits between the surface plots. For each composite subsurface sample, ten cores of subsamples (Ø 2 cm, height 20 cm) were collected. In total, six surface samples (three vegetated samples and three unvegetated samples) and two subsurface (one vegetated and one unvegetated) samples were taken.

The other sampling site was another oil contaminated site in Porvoo which was included in the Soili Project. Soili was a national oil contaminated sites remediation project launched by the oil industry in Finland in 1996, based on an agreement between the Federation, the Ministry of the Environment and the Association of Finnish Local and Regional Authorities. The site was used for fuel oil storage and supply in past decades. There was a remediation operation in 2001 during which 1,835 tons of contaminated masses were removed. In late September 2012, *in situ* remediation started and should last for 18 months. The sampling was done before the in-situ remediation started. The samples were collected from depths of 20 cm, 50 cm, 1 m and 2 m. For each depth, two composite samples were collected in order to cover the high spatial heterogeneity of the digging site. Ten subsamples from each depth were mixed and taken as a composite sample.

All the samples were transported in ice boxes, sieved by Ø 4 mm mesh and stored at -80$^{o}$C until further molecular analysis and -4$^{o}$C for microbiological analysis.

## 8.2 Analysis of soil chemical properties

Soil samples were mixed and sieved through a 5 mm mesh and stored at -20$^{o}$C before analysis. The soil samples from both sampling sites were characterized with several chemical properties: pH value, dry matter content, organic matter content and total petroleum hydrocarbon (TPH) content.

1) Soil pH

Twenty grams of fresh soil was weighed and put into two bottles as replicates, adding 50 ml 0.01 $CaCl_2$ solutions for each bottle. The suspended soil suspension pH was then measured by a pH meter (SCHOTT CG842).

2) Dry matter content

For dry matter content measurement, crucibles was filled with fresh soil to 1/3 full and weighed to nearest 0.01 g. The soils in crucibles were dried at 105 $^{o}$C in the oven overnight. After cooling at room temperature in a desiccator for about one hour, the crucibles were weighed again. The dry matter content was calculated following this formula:

Dry matter content (%) = (dry soil weight/wet soil weight) × 100

3) Organic matter content

To measure the amount of organic matter, the dried soil was burned at 500 $^{o}$C for 4 hours in a muffle furnace after which the temperature was lowered to 100 $^{o}$C. After cooling in a desiccator at room temperature, the crucibles were weighed again. The amount of organic matter in terms of percentage was calculated following the formula:

Organic matter content (%) = [(dry soil - ash)/dry soil] × 100

4) Oil extraction

Oil was extracted from air dried soil samples with two replicates according to the ISO 16703:2004. The soil samples were air dried three days at room temperature and grinded in a 2 mm mortar before analysis. Ten grams (0.001 g accuracy) of each soil sample were weighed in a centrifuge tube and 10 ml RTW-solution (30 mg tetracontane + 30 µldecane in

1 l heptane) and 20 ml acetone (HPLC pure) was added into the tube. After gentle shaking, the tubes were ultrasonicated for 30 min with lightly closed tubes. The ultrasonicator was cooled with about 500 ml ice.

After ultralsonication, 30 ml of deionized water was added into the centrifuge tube and shaken for 1 min. The tubes were centrifuged without lids at 2000 ×g for 5 min. The upper organic phase was removed to a new 25 ml glass tube using a glass pasteur-pipette. Ten ml of dioinized water was added to the glass tube and shaken gently for 1 min. The tubes were centrifuged again at 2000 ×g for 5 min. After removing the lower water phase, one spoon of $Na_2SO_4$ (approx. 5 ml) was added to remove the residual water. The water-dried extract was then removed to a new 10 ml storage glass tube and stored at -20 $^{o}$C.

5) Total Solvent Extractable Materials (TSEM) measurement

One ml of extracted oil was added to a preweighed glass tube. The oil was carefully evaporated by nitrogen at +30 $^{o}$C. After cooling to room temperature, the tube was weighed again. The amount of TSEM was calculated by the formula:

TSEM = ((glass tube with evaporated oil) - (empty glass tube) × 10)/dry weigh of 10 g soil samples

6) Total Petroleum Hydrocarbon (TPH) content

The Total Petroleum Hydrocarbon (TPH) content was measured by GC-FID (Agilent Technologies 6890N Network). The method is used for quantification of the mineral hydrocarbon content between 100 mg/kg and 10000 mg/kg dry weight soil in field-moist soil samples by gas chromatography. The targeted hydrocarbon of this method covers a boiling range of 175 $^{o}$C to 525 $^{o}$C, of *n*-alkanes from C10 to C40, of isoalkanes, cycloalkanes, alkylbenzenes, alkylnaphthalenes and polycyclic aromatic compounds which are not absorbed on the specific column during the purification procedure, here refered to as Florisil (ISO 16703:2004). Before measured in GC, the oil extracts were firstly purified. Polar substances were trapped to Florisil (Mikkonen et al., 2011) and non-polar hydrocarbons C10-C40 were measured according to ISO 16703:2004. The oil extracts were mixed with RTW-solution (Retention Time Window solution: heptane with decane and tetracontane as tracers) at the ratio of 1:4. Oil extracts were purified by the purification columns (washed glass wool was put into the bottom of a glass column plus two grams of activated Florisil and two grams of dried $Na_2SO_4$) and the eluted solutions were collected in a 10 ml storage tubes with a lid and stored at -20 $^{o}$C. The eluted solution was the loading sample of GC measurement.

TPH were quantified by loading one microliter of purified oil extract onto GC-FID (Agilent Technologies 6890N Network) with polar methylpolysiloxane (5% fenyl) capillary column (30 m, Ø 0.32 mm, film thickness 0.25 mm, pre-column 5 m; Restek, USA). Two replicates of each sample were run with 1 ml splitless injection with the temperature program 60 $^{o}$C 2 min-10 $^{o}$C min$^{-1}$-320 $^{o}$C 25 min-50 $^{o}$C min$^{-1}$-340 $^{o}$C 7 min. The GC validity checks were performed according to ISO 16703:2004. The chromatograms were integrated manually with Chemstation A.09.03. The GC results were calculated by the formula derived from the standard curve.

The GC standard curve was drawn with the GC results of fuel oil at the concentration of 0.2, 0.4, 0.6, 0.8, 1.0, 1.4 and 2.0 g/l. The formula for GC result and TPH content is y=7628.7x-615.84 ($r^2$=0.998) is shown in Figure 3.

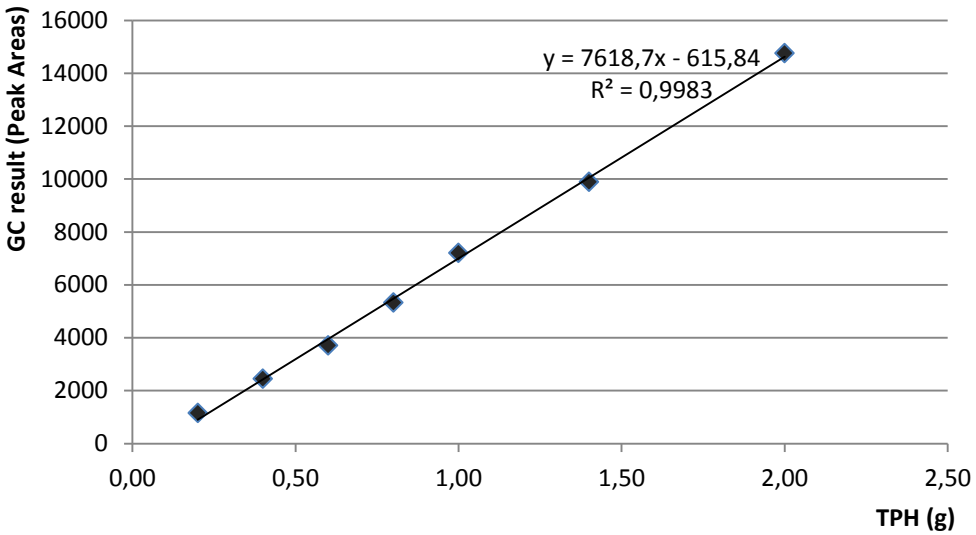



**Figure 3**. Gas Chromatograph-Flame Ionizing Detector Standard curve of oil concentrations

## 8.3 Enumeration of hydrocarbon degraders using MPN method

After sieving, five grams of fresh soil were suspended in 45 ml phosphate buffer solution and shaken at 400 rpm for 10 minutes. In order to let soil particles sediment, the soil suspension was left sitting on the bench for 20 minutes. After sedimentation, 10-fold serial dilutions of soil suspension with Bushnell-Haas media were made from $10^{-1}$ to $10^{-8}$ dilution. Out of each dilution, 200 µl solutions were added to 12 replicated wells of a 96-well microplate (Nunc$^{TM}$, Denmark). For each well, 2µl of 98 E5 petroleum oil was added as sole

carbon source. The oil was filter sterilized before use. An identical plate without oil was also prepared as negative control. The plates were incubated in the dark at room temperature for 4 weeks.

After incubation, the plates were detected for growth using an absorbance reader (LabsystemsiEMS Reader MF) at the wavelength 620 nm (Mikkonen et al. 2011). The threshold of positive growth was determined according to the background absorbance from negative plates. The absorbance values of positive growth wells were obviously separated from those of negative wells. The MPN results were calculated with an Excel version Calculator from an online source (Curiale, 2000). The Calculator followed the format:

$$\sum_{i=1}^{k}\left(\frac{x_i d_i w_i}{1-\exp(-d_i w_i \hat{\mu})} - n_i d_i w_i\right) = 0$$

where

exp(x) means $e^x$, and

k denotes the number of dilutions,

$x_i$ denotes the number of positive tubes in *i*th dilution,

$d_i w_i$ denotes the amount of the original sample put in each tube in the *i*th dilution,

$n_i$ denotes the number of tubes in the *i*th dilution.

The 95% confidence limits were calculated using this format:

Lower $\hat{\mu} \cdot \exp(-2\hat{\sigma}_{\ln \hat{\mu}})$ Upper $\hat{\mu} \cdot \exp(2\hat{\sigma}_{\ln \hat{\mu}})$

The samples from the landfarming site were also enumerated for naphthalene degraders by MPN method. The setup of the 96-well microplates was the same as described for the hydrocarbon degrader enumeration but the carbon source was naphthalene instead of fuel petroleum. The 96-well microplates without lids were set in plastic boxes (which were originally used for food) with naphthalene particles spread evenly around it to give a gaseous naphthalene source.

## 8.4 Reference strains and isolation of hydrocarbon-degrading strains

The genes extracted from reference strains were used as positive controls in PCR amplification. All the reference strains were obtained from HAMBI culture collection at the Division of Microbiology and Biotechnology, Department of Food and Environmental Sciences at the University of Helsinki. The reference strains were cultured in TY (Tryptone - Yeast extract) broth at +28 °C with shaking at 230 rpm for 2 to 4 days until obvious growth

was observed. HAMBI 2359 (*Pseudomonas oleovorans*) was used as reference strain for *alkB* gene detection - HAMBI 8 (*Pseudomonas putida*) for *xylE1* gene detection, and HAMBI 9 (*Pseudomonas putida PpG7*) for *nahAc* gene detection.

A mixture aliquot of 40 µl from the wells with growth of oil- or naphthalene degraders in the 96-well plates were transferred to Bushnell-Haas agar plates supplemented with 80 µl filter fertilized oil as sole carbon source. The plates were incubated at +28˚C for 3 days. Four dominant colonies with distinct characteristics on selective media plates were then transferred to TGY agar plates and incubated at +28˚C for 7 days. Pure colonies from the TGY agar plates were inoculated into TY broth and incubated at +28˚C shaking at 230 rpm for three days or until clear growth showed up. The isolates were transferred again from TY broth to TGY agar plates and Bushnell-Haas oil plates to check for purity and oil degradation ability. The isolates were observed under microscopes after Gram staining.

## 8.5 DNA extraction from soil, reference strains and isolates

DNA was extracted from soil samples by a commercial soil DNA extraction kit (UltraClean™ Soil DNA Isolation Kit, MoBio 12800-50). The extraction procedures followed the kit instructions. Soil samples were added to a bead beating tube containing beads, lysis solution, bead solution and Inhibitor Removal Solution. The cellular components were lysed and the released DNA was bound to a silica spin filter. The bound DNA was then washed and eluted for further analysis.

The DNA of reference strains and pure cultures of isolated degraders were extracted utilizing commercial microbialDNA isolation kits (Ultraclean™ microbial DNA isolation kit 12224-50, MoBio, USA). The extraction procedures followed the kit instruction. Microbial cells were resuspended in bead solution and were added to a provided bead beating tube. The cells were lysed by a combination of heat, detergent, and mechanical force. Released DNA from the lysed cells were bounded to a silica spin filter and recovered in buffer after washing (Ultraclean™ microbial DNA isolation kit 12224-50 Protocol).

## 8.6 PCR amplification of functional genes

The DNA extracts from soil samples and pure cultures of the isolates were amplified by PCR reactions with properly designed primer sets to detect targeted functional genes. The amplification regions of the functional genes by the primer sets were checked by alignment of the primer and known gene sequences.

1) *alkB* gene primer set

The *alkB* gene primer in this study followed the primer set used by Kok et al., (1989); Whyte et al.,1996 and Whyte et al., 2002. The primer was designed according to the *alkB* gene sequence of *Pesudomonas* AJ233397 (Figure 4). The targeted amplification region is 870 base pair. DNA extraction from strain HAMBI 2359 (*Pseudomonas oleovorans*) was used as positive control for *alkB* gene detection.

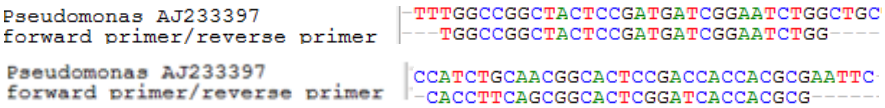



**Figure 4**. Nucleotide sequence alignment of *alkB* gene from *Pseudomonas* AJ233397 and *alkB* primer set.

2) *xylE1* gene primer set

The degenerate primer sequences for *xylE1* amplification followed those used by Hendrickx et al. (2006). The target regions on *xylE* gene sequences of the primer were checked by alignment the primer sequences with gene sequences from strains with *xylE* gene (Figure 5). The alignment results showed that the primer set targeted the conserved region of the *xylE* gene and should successfully amplify the targeted gene from *Pseudomonas putida* and *Achromobacter xylosoxidans* at least. The amplified product is at length of 242 base pair. DNA extract from strain HAMBI 8 (*Pseudomonas putida*) was used as positive control for *xylE1* gene detection.

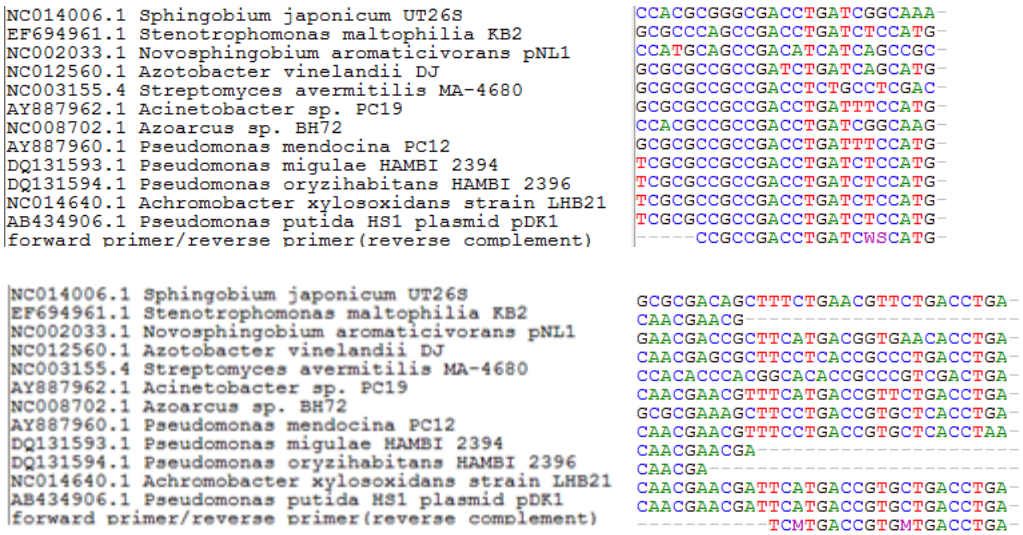



**Figure 5**. Nucleotide sequences alignment of *xylE* genes from previously investigated strains and the *xylE1* primer set.

3) *nahAc* gene primer set

The degenerate primer for *nah* gene amplification was designed and used by Baldwin et al., (2003). The length of amplified products was 377 base pairs. DNA from strain HAMBI 9 (*Pseudomonas putida PpG7*) was used as positive control for *nahAc* gene detection. Naphthalene dioxygenase specific primers (NAH primers) target the N.2.A subfamily of naphthalene dioxygenases with high sequence identity to *nahAc* from *Pseudomonas putida* G7 (Baldwin et al., 2003). The primers were designed based on a review of diverse group of *nahAc* genes. It would amplify some but perhaps not all naphthalene dioxygenase genes from N.2.A subfamily (Baldwin et al., 2003).

Soil DNA extracts were diluted before PCR reactions. The PCR reaction mixtures followed the recipes of **Table 4**. The PCR programs for the functional genes used in this project are shown in Table **5**.

**Table 4**. PCR solutions for functional genes amplification

| | *alkB* | *xylE1* | *nahC* |
|---|---|---|---|
| Buffer | 5 µl (10× KCl buffer) | 2.5 µl (10×$(NH_4)_2SO_4$ buffer) | 2.5 µl (10× KCl buffer) |
| $MgCl_2$ | 2.5 mM | 2.5 mM | 2.5 mM |
| dNTP | 200 µM each | 200 µMeach | 200 µMeach |
| forward primer | 0.2 µM | 0.4 µM | 0.2 µM |
| reverse primer | 0.2 µM | 0.4 µM | 0.2 µM |
| DNA polymerase | 1.0 U (*Taq*) | 1.0 U (*Taq*) | 1.0 U (*Taq*) |
| Template | 1 µl | 1 µl | 1 µl |
| BSA | 10 mg/ml | 10 mg/ml | 10 mg/ml |
| Water | 35.6 µl | 12.8 µl | 14.8 µl |
| Total | 50 µl | 25 µl | 25 µl |

**Table 5**. PCR conditions for functional genes amplification

| Times step was performed | Temperature | | | Duration of step | | |
|---|---|---|---|---|---|---|
| | *alkB* | *xylE1* | *nahC* | *alkB* | *xylE1* | *nahC* |
| 1× | 94˚C | 95˚C | 95˚C | 5 min | 5 min | 10 min |
| 30× (35× for *xylE1*and 16s rDNA) | 94˚C | 94˚C | 95˚C | 1 min | 1 min | 1 min |
| | 60˚C | 61.5˚C | 49˚C | 1 min | 1 min | 1 min |
| | 72˚C | 72˚C | 72˚C | 1 min | 2 min | 2 min |
| 1× | 72˚C | 72˚C | 72˚C | 3 min | 10 min | 10 min |
| 1× | 4˚C | 4˚C | 4˚C | Hold | hold | Hold |

The PCR products were checked in 1.2% agarose gel electrophoresis and stained by ethidium bromide under trans-ultraviolet light (Bio-Rad Laboratories Molecular Imager® Gel Doc™ XR system and QUANTITY ONE® 1-D V 4.6.7). The bands were compared with standard ladder (Fermentas MassRuler ladder mix).

## 8.7 Sequencing of 16S rRNA genes of the isolated degrader strains

The 16S rDNA of isolates were amplified with fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rD1 (5'-AAG GAG GTG ATC CAG CC-3') primer set. The PCR solution for 16S RNA gene amplification contained 5 µl 10× buffer, 0.25 µl fD1 and rD1 primer, 1 µl dNTP mixture, 1 µl template DNA, 42.25 µl water and 0.25 µl DreamTaq Enzyme (Thermo Scientific). The program for 16S rDNA amplification was the initial denaturation at 98˚C for 30 s, 35 cycles of 98˚C for 10 s, annealing temperature for 30 s and 72˚C for 45 s and final extension at 72˚C for 5 min. The annealing temperatures for four isolates were 61˚C, 61˚C, 62˚C and 61˚C respectively. The PCR conditions for each isolate were optimized until optimum products showed up on gel electrophoresis picture.

All the PCR amplification products were loaded on to 1 % agarose gel. The bands with a length of 1.5 kilobase pair of nucleotide were excised and the DNA contained in the gels was purified by gel DNA purification kits following kits instruction (Thermo Scientific GeneJET Gel Extraction Kit). The DNA fragment of interest excised from the gel was put into a microcentrifuge tube. The binding buffer dissolves agarose and the DNA was bound to the silica membrane in the column. The bound DNA was washed to get rid of impurities and the purified DNA was eluted from the column with elution buffer. Before sending for sequencing, the concentration of eluted DNA was measured by Nanodrop® ND-1000 spectrophotometer (Thermo Scientific) to ensure there was sufficient amount of DNA for sequencing. Purified PCR products were sequenced by DNA Sequencing and Genomics Laboratory, Institute of Biotechnology at University of Helsinki. The sequencing results were aligned by BioEdit and sequences with noise were deleted. The edited sequences were checked online by Blast and the sequences of the isolates together with several sequences from Blast results were shown in Table 8.

## 8.8 Statistical analysis

The soil samples physicochemical properties and the degrader microorganism enumeration results were analyzed statistically to reveal the correlation relationships between each pair of these parameters. The degrader microorganism enumeration results

from vegetated and unvegetated samples were analyzed to check whether there was a significant difference between them.

The statistical analyses were performed by using Pearson Correlation, one-way and two-way ANOVA in Microsoft-Excel (2010) and SPSS (13.0).

# 9. Results

## 9.1 Physicochemical properties of soil samples

The results of the measurements of the physicochemical properties of the soil samples are shown in Table 6. For the oil storage field, the pH value had a decreasing tendency towards deeper samples as well as the dry matter content. The organic matter content reached its highest value between 50 cm depth and 1 m depth with quite limited organic matter in surface soil. The TSEM (total solvent extractable materials) contents were very low compared to those of the samples from the landfarming site; however the results still showed an increasing trend towards deeper soil. The TPH (total petroleum hydrocarbon) contents were only detected with significant amounts in three subsurface samples (one from 1 m, and two from 2 m) with an increasing trend. Surprisingly, the TPH values of both 2 m depth samples were even higher than their TSEM values. For landfarming field samples, the pH values of vegetated surface samples were lower than those of unvegetated surface samples. The dry matter content was heterogeneous and there was a decreasing tendency along the samples (from sample 1 to sample 3), however, the organic matter content, TSEM content and TPH content increased.

**Table 6**. Physicochemical properties of soil samples from two contaminated sites

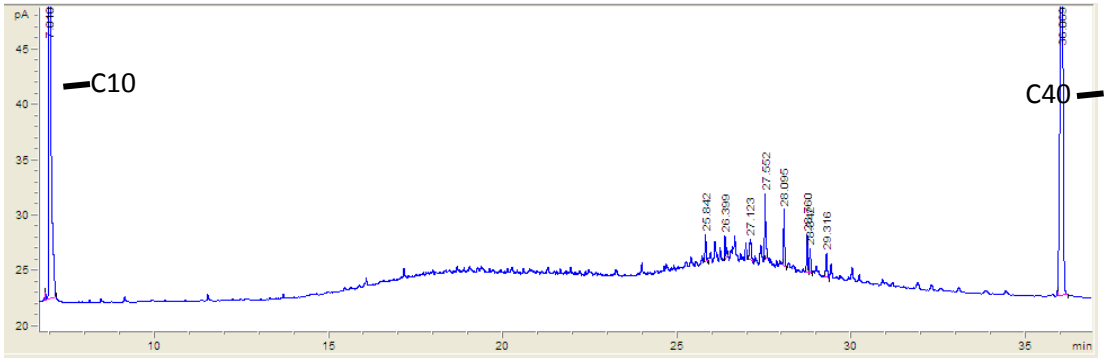
| Sample Code | pH[a] | Dry matter content (%) | Organic matter content (%) | TSEM (g/kg $dw^{-1}$soil) | TPH (g/kg $dw^{-1}$ soil) |
|---|---|---|---|---|---|
| Oil storage field | | | | | |
| 20I (20 cm) | 6.85 | 94.71 | 0.56 | 0.1 | NA |
| 20II (20 cm) | 7.35 | 91.50 | 1.07 | 0 | NA |
| 50I (50 cm) | 6.17 | 87.04 | 1.75 | 0.198 | NA |
| 50II (50 cm) | 6.28 | 81.64 | 3.39 | 0.096 | NA |
| 1I (1 m) | 6.28 | 79.88 | 3.70 | 0.298 | NA |
| 1II (1 m) | 6.49 | 81.96 | 2.98 | 1.208 | 1.15 |
| 2I (2 m) | 5.78 | 80.15 | 2.42 | 2.072 | 4.68 |
| 2II (2 m) | 5.95 | 82.93 | 1.83 | 2.343 | 5.61 |
| Landfarming field | | | | | |
| vegetated 1 | 5.29 | 72.10 | 18.70 | 32.60 | 4.29 |
| vegetated 2 | 5.05 | 71.30 | 19.91 | 35.54 | 5.20 |
| vegetated 3 | 5.05 | 68.76 | 21.90 | 40.52 | 6.17 |
| unvegetated 1 | 6.34 | 75.44 | 18.66 | 36.52 | 5.41 |
| unvegetated 2 | 6.47 | 69.35 | 20.60 | 39.75 | 5.78 |
| unvegetated 3 | 6.42 | 67.12 | 21.30 | 44.32 | 6.51 |
| A (vegetated subsurface) | 5.58 | 80.73 | 2.01 | 1.51 | NA |
| B (unvegetated subsurface) | 6.26 | 73.69 | 4.17 | 1.03 | NA |

a. Measured in 0.01 M $CaCl_2$ solution
b. TSEM means total solvent extractable materials; TPH means total petroleum hydrocarbons; NA means not detectable.

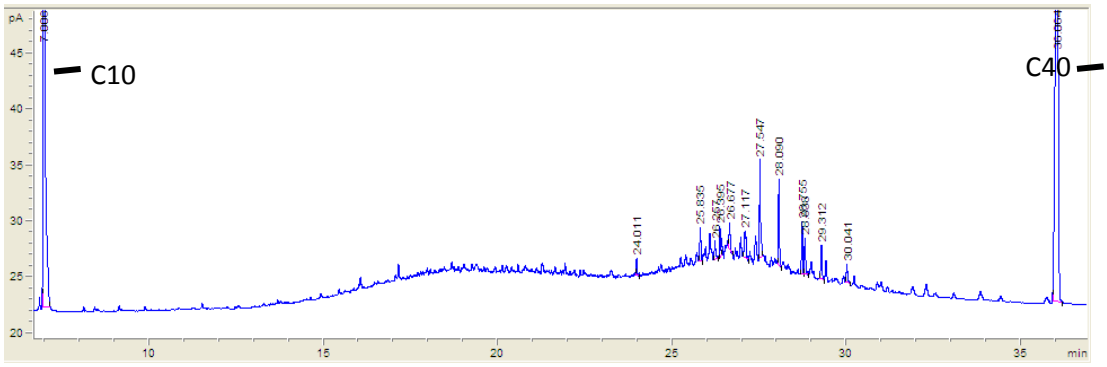
To reveal whether there were significant differences between vegetated and unvegetated surface samples in terms of these parameters, two-way ANOVA was used as the statistical analysis method. There were significant differences between vegetated surface samples and unvegetated surface samples in terms of pH value ($P<0.001$), TSEM ($P<0.01$) and TPH ($P<0.01$). However, the dry matter content and organic matter content of vegetated and unvegetated samples had no significant difference.

The correlation relationships between these parameters were analyzed by Pearson Correlation method. For the oil storage site, the TSEM and TPH had a weak positive correlation ($P<0.1$). There were positive correlation relationship between the pH with dry matter content ($P<0.05$), and negative with TSEM ($P<0.1$). The dry matter content had a negative correlation with organic matter content ($P<0.01$). For the landfarming site, the TPH correlated with organic matter content ($P<0.05$) and TSEM ($P<0.001$) positively. The TSEM content positively correlated with dry matter content ($P<0.05$) and organic matter content ($P<0.001$). The dry matter content and organic matter content had a negative correlation ($P<0.02$). These will be discussed in Section 9.1.

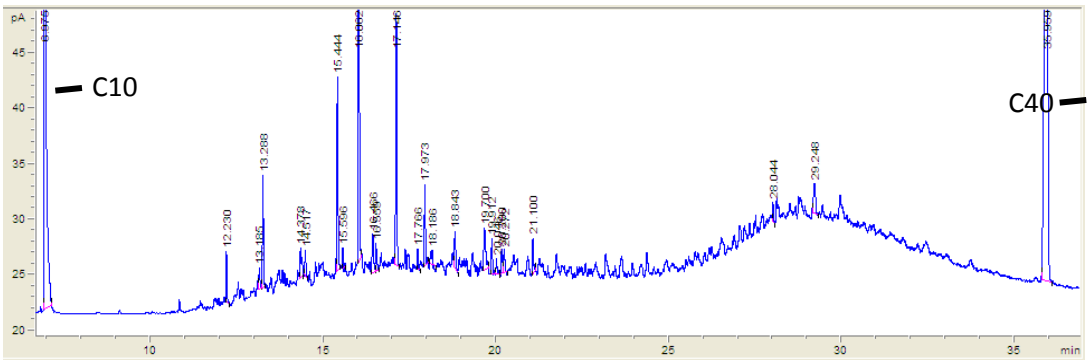
The GC results also revealed the compositions of residual TPH in the examined samples (Figure 6). As shown in the GC-FID chromatograms and in comparison with chromatogram of the alkane standard, the residual hydrocarbons in samples from the landfarming site were mainly long-chain hydrocarbons (C30-C40 with peaks shown up with retention time after 25 min). However, as for the oil storage samples, there were mainly short-chain hydrocarbons (C10-C20 with peaks shown up with retention time before 20 min). The compositions of hydrocarbons of vegetated samples (Figure 6a.) and unvegetated samples (Figure 6b.) from the landfarming site were quite similar while the amounts of vegetated samples were higher.



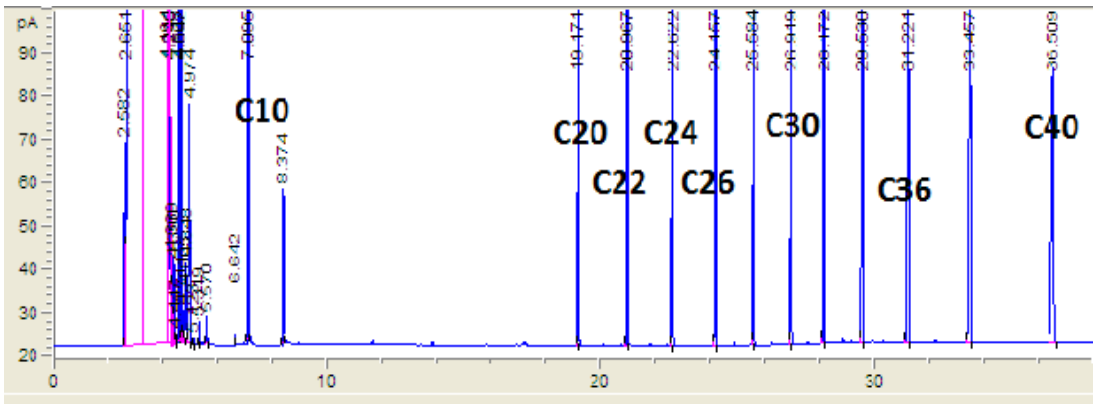
a.




b.




c.




d.

**Figure 6**. TPH (total petroleum hydrocarbon) content results from GC-FID. C10=decane, C40=tetracontane. a. TPH content in the sample from vegetated surface soils of landfarming site, b. TPH content in the sample from un-vegetated surface soils of landfarming site, c. TPH content in the sample from oil storage site, d. TPH chromatogram of alkane standard.

## 9.2 MPN enumeration of degrader microorganisms

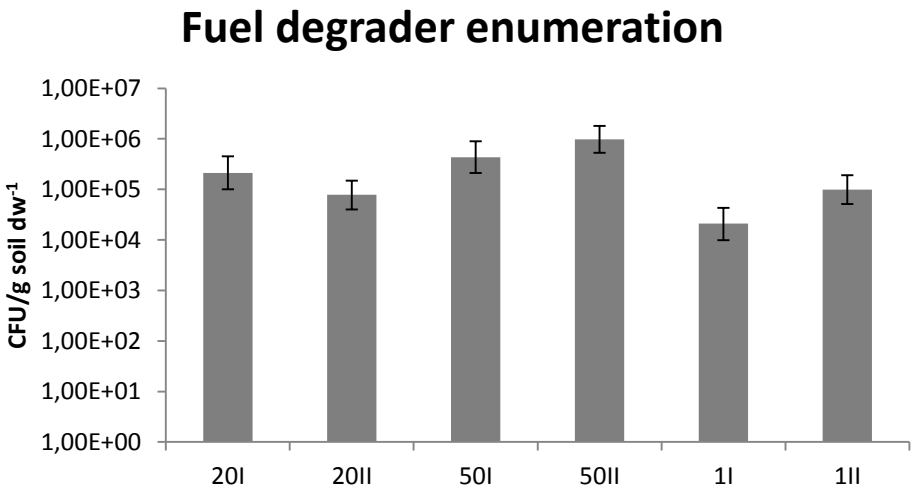
The results from the enumeration of the petroleum hydrocarbon degrader and naphthalene degrader microorganisms are shown in Table 7. As for the oil storage site, the petroleum degraders were most abundant at 50 cm depth and were almost undetectable at 2

m depth with quite limited aeration conditions (Figure 7a). The degrading microorganism counts had no correlation with any soil physicochemical property.

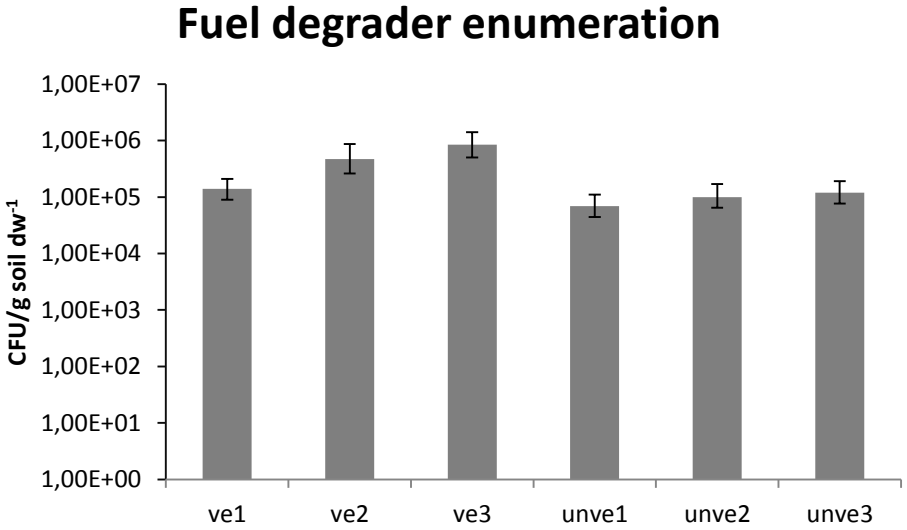
For the landfarming site, there were more petroleum degraders in vegetated samples than in unvegetated samples. However, the difference was not significant (Figure 7b). The naphthalene degraders in the surface samples of the landfarming site were at about same quantitative levels in the vegetated and unvegetated samples (Figure 7c). Both petroleum degraders and naphthalene degraders were much less numerous in subsurface samples than in surface samples (Table 7). In the landfarming site, there was no trend or correlation between the degrader counts with the soil physicochemical properties.

**Table 7**. MPN results of soil samples

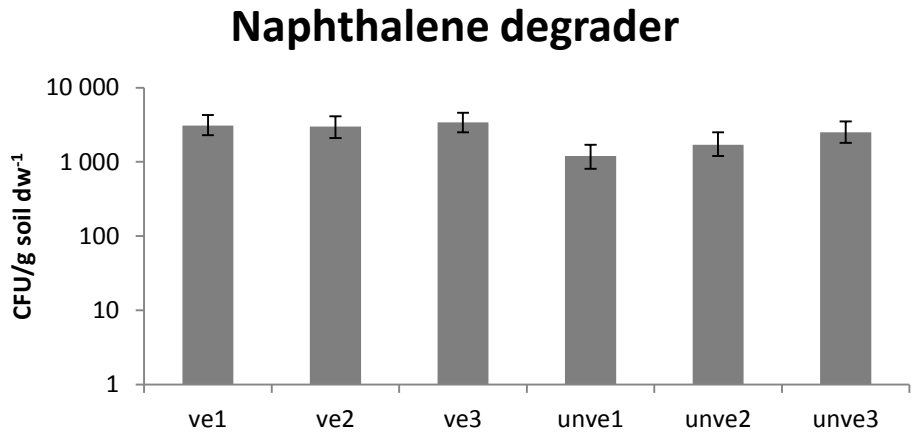
| **Depth of sample** | **No. of sample** | **MPN ($\times 10^4$ CFU/g dw soil)** | **95% Confidence Limits ($\times 10^4$ CFU/g dw soil)** | |
|---|---|---|---|---|
| **Numbers of petroleum hydrocarbon degraders** | | | | |
| **Oil storage site** | | | | |
| **20 cm** | I | 210 | 100 | 450 |
| **20 cm** | II | 78 | 40 | 150 |
| **50 cm** | I | 430 | 210 | 890 |
| **50 cm** | II | 980 | 530 | 1800 |
| **1 m** | I | 21 | 9.9 | 43 |
| **1 m** | II | 99 | 51 | 190 |
| **2 m** | I | 0.16 | 0.071 | 0.35 |
| **2 m** | II | 0.22 | 0.1 | 0.46 |
| | | | | |
| **Landfarming site** | | | | |
| **Vegetated surface** | 1 | 14 | 8.9 | 21 |
| | 2 | 37 | 19 | 69 |
| | 3 | 24 | 12 | 49 |
| **Unvegetated surface** | 1 | 6.9 | 4.4 | 11 |
| | 2 | 10 | 6.5 | 17 |
| | 3 | 12 | 7.7 | 19 |
| **Vegetated subsurface (80cm)** | - | 0.22 | 0.15 | 0.31 |
| **Unvegetated subsurface (80cm)** | - | 0.2 | 0.14 | 0.28 |
| | | | | |
| **Naphthalene degrader** | | | | |
| **Vegetated surface** | 1 | 0.31 | 0,23 | 0,43 |
| | 2 | 0.30 | 0,21 | 0,41 |
| | 3 | 0.34 | 0,25 | 0,46 |
| **Unvegetated surface** | 1 | 0.22 | 0,131 | 0,27 |
| | 2 | 0.37 | 0,28 | 0,45 |
| | 3 | 0.25 | 0,18 | 0,35 |




a.




b.

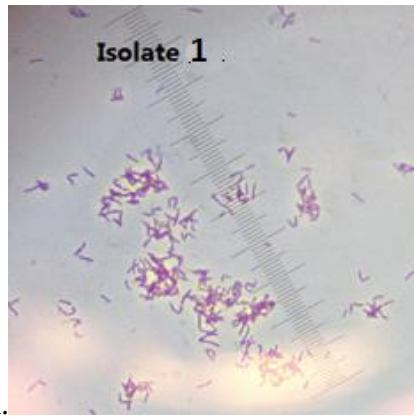


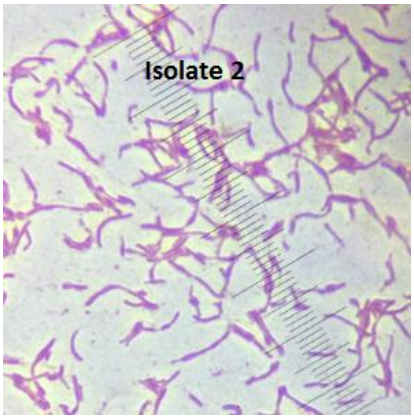

c.

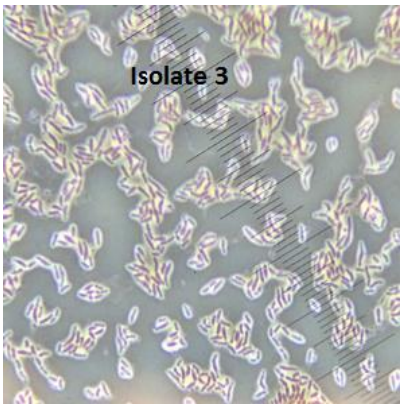
**Figure 7**. Hydrocarbon degrading microorganism counts for the soil samples, a. petroleum degrading microorganism counts in oil storage site; b. petroleum degrading microorganism counts in landfarming site; c. naphthalene degrading microorganism counts in landfarming site.

## 9.3 Isolated petroleum degrader strains

The Gram stained isolates were observed and pictures of the isolates are shown in Figure 8. Isolate 1, 2 and 3 were all Gram positive rod shaped bacteria. Isolate 4 was Gram negative rod bacteria. Cells of Isolate 1 were sized 2 by 6 µm. Cells of Isolate 2 measured 1 by 9 µm and cells of Isolate 3 were sized 1 by 3 µm whereas cells of Isolate 4 were 2 by 5 µm.

a. 

b. 

c. 

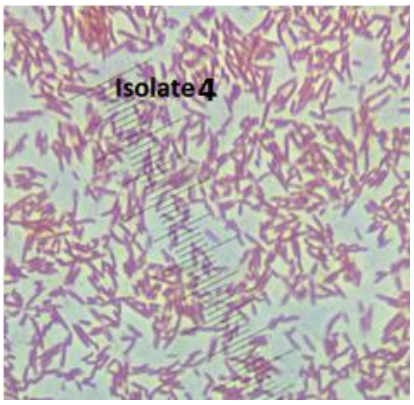
d. 


**Figure 8**. Gram stained cells of the petroleum degrader isolates. a. Isolate 1, b. Isolate 2, c. Isolate 3, d. Isolate 4.

The sequences of the isolates were checked and edited using BioEdit and the edited sequences were analyzed in BLAST online. The Blast results are shown in Table 8.

**Table 8**. The BLAST results of the 16S rRNA gene sequences of the isolates

| Bacterial strain | Identity | Accession number |
|---|---|---|
| Isolate 1 (939 bp) | | |
| *Arthrobacter nitroguajacolicus* strain: W-1 | 100 % | AB363932.1 |
| *Arthrobacter boritolerans* strain: KCTC 19791 | 99 % | AB288060.1 |
| Isolate 2 (739 bp) | | |
| *Arthrobacter oryzae* S32114 | 99 % | AB648957.1 |
| *Arthrobacter pascens* strain N25 | 99 % | JN903380.1 |
| Isolate 3 (898 bp) | | |
| *Arthrobacter sulfonivorans* strain Asd M3-2 | 100 % | FM955860.1 |
| *Arthrobacter defluvii* strain NB-8 | 99 % | JX869972.1 |
| Isolate 4 (962 bp) | | |
| *Pseudomonas migulae* strain R-20812 | 100 % | AJ786780.1 |
| *Pseudomonas fluorescens* strain P69 | 99 % | AY973265.1 |
| *Pseudomonas putida* NBRC 12653 | 99 % | AB680304.1 |

As can be seen from the table, the isolates were grouped into two clusters: *Pseudomonas* which is a Gram-negative genus belonging to γ-*Proteobacteria* and *Arthrobacter* which is a Gram-positive genus belonging to *Actinobacteria*. The isolates 1, 2 and 3 had the highest similarity with *Arthrobacter*. Isolate 1 had 100 % sequence identity with *Arthrobacter nitroguajacolicus* (AB363932.1). Isolate 2 was 99 % similar to *Arthrobacter oryzae* (AB648957.1) and *Arthrobacter pascens* (JN903380.1) in terms of 16S rRNA gene sequences. Isolate 3 had 100 % sequence identity with *Arthrobacter sulfonivorans* (FM955860.1). Isolate 4 had 100 % sequence identity with *Pseudomonas migulae* (AJ786780.1) and 99 % sequence identity with *Pseudomonas fluorescens* (AY973265.1) and *Pseudomonas putida* (AB680304.1).

## 9.4 Functional genes detection

The PCR amplification products with primer sets targeted at the functional genes were checked together with products of reference strains in DNA amplification. The positive bands of certain length (870 bp for *alkB*, 242 bp for *xylE1*, 377 bp for *nahAc*) were considered to demonstrate the existence of the corresponding gene (Figure 9).

The yields of the DNA extracted from soil samples were relatively low (i.e. ~20 ng/µl) and the A260/A230 was less than 1.0 indicating humic acid contamination. The DNA extraction from bacterial isolates had good yield and purity.

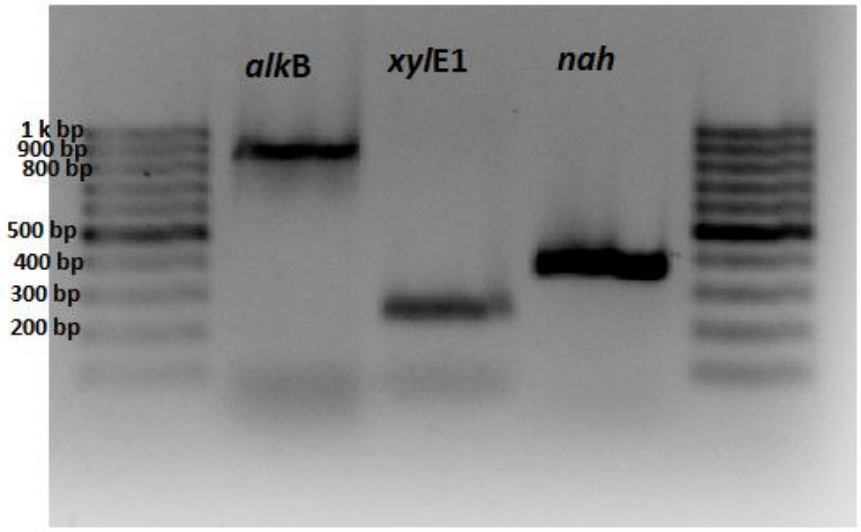



**Figure 9**. The agarose gel picture of *alkB, xylE1* and *nahAc* gene amplification products from reference strains DNA.

For the soil samples, the *xyl*E1 gene was detected only in one vegetated surface sample and the vegetated subsurface sample although the degrader microorganisms there were quite low in abundance compared to those of the surface samples. However there was no positive detection in any of the unvegetated samples or the samples from oil storage site. The *alkB* and *nahAc* genes were not detected from any of the soil samples. For the isolated strains, isolate 1 was found to carry the *xylE1* gene while isolate 2 and 3 showed a weak amplification with secondary PCR products for *xylE1* gene detection. Isolate 4 was found to carry with the *nahAc* gene. *AlkB* gene was not detected in any of the isolated strains.

# 10. Discussion

According to the results, both sites were moderately contaminated with different hydrocarbon composition. Some of the soil physicochemical properties in these sites showed correlation relationships with the contamination. In the landfarming site, the TPH depletion in vegetated fields was higher than the unvegetated bulk soil areas. However, the degrading microorganism counts had no significant differences between vegetated and nonvegetated plots. The hydrocarbon contamination level had no correlation with the degrader counts. In subsurface soils where aeration was quite limited, degrading microorganisms were much lower than those in surface soils. Catabolic genes were detected from the isolated strains but rarely from the contaminated soil samples.

One of the original objectives of the project was to explore whether catabolic gene detection can be an applicable indicator for soil oil-contamination assessment. However, with limited results from gene detection work, here I will discuss more about the degrader enumeration and the isolated degrader strains.

## 10.1 Soil physicochemical properties

Soil environments are very variable in terms of their physicochemical properties which are important for microbial growth and bioavailability of contaminants. The dry matter content reflects the moisture condition which is mainly determined by the soil texture, weather, and geological conditions. Some studies performed in artificial microcosms have shown that moisture have a significant effect on bioremediation of hydrocarbon (Gray et al., 2000; Yerushalmi et al., 2003; Ramírez et al., 2009). It is reported that the optimal moisture content for aerobic hydrocarbon biodegradation is 50-80 % of soil water holding capacity. However, the hydrocarbon biodegradation in clayed soils experiences more difficulties because the pollutants are sequestrated by the organic compounds. Ramírez et al. (2009) studied the best experimental condition for hydrocarbon degradation in clayed and weathered polluted soil. According to their results, the highest degradation rate was found in the treatments with water content corresponding to 350% water holding capacity.

The samples of the present study were taken in the autumn before heavy snow started. For samples from landfarming site, soils were drier in surface samples and dry matter content increased along with increasing horizontal contamination gradient but there was no strong correlation between them. It is reasonable considering that the hydrophobia of hydrocarbons in soils can also increase soil hydrophobicity, however is not likely to affect its moisture content. Balks et al. (2002) examined the effects of hydrocarbon spills on moisture

conditions by comparing contaminated soils and nearby pristine soils. Their results showed that soil hydrophobia was higher, however, water holding capacity showed no noteworthy difference between contaminated and non-contaminated soils. A similar scenario of minor increased soil hydrophobia with non-altered moisture penetration into soil by oil spills was also found by Aislabie et al. (2004).

Organic matter content comprises of all the organic matter in the soil. The oil storage site had very low organic matter content compared with the landfarming samples, which reflects its quite sandy soil texture. For the landfarming site, the subsurface samples had quite low organic matter compared to the surface samples where plants residues contributed to soil organic matter. Microbial biomass is generally considered to be positively related to soil organic matter content in most ecosystems. However, both of the investigated contaminated sites showed no correlation between degrading microorganism counts and soil organic matter content.

As well known, bioremediation requires an optimal pH range to be successful. In return, the metabolites accumulated during degradation and released $CO_2$ by microbial respiration can change the soil pH value. Oxidation of oil during biodegradation leads to an increase of acidity (Head et al., 2003). Roberts (1998) believed that a pH range of 6.5 to 8.5 is optimum for hydrocarbon degradation. Besides, Dibble and Bartha (1979) proposed an optimal pH range of 5.0 to 7.8 for oily sludge mineralization in soils. In this study, all the samples were in such a pH range which indicates that both contaminated sites had a good acidity-alkalinity condition for bioremediation. It was also indicated that landfarming with urea fertilization, as a treatment technology for oily sludge waste, had no significant alteration of soil pH in terms of hydrocarbon biodegradation. Kästner et al. (1998) studied the effect of soil pH on survival of introduced PAH-degrading bacteria. They found the neutralization increased the CFU by 10-fold after 7 days. Other researchers also proposed neutralization is favorable for bacterial oil degradation (Leahy & Colwell, 1990).

In the present study, vegetated samples were more acidic compared to unvegetated samples ($P<0.001$). The plant roots secretion, soil humic acids and biological nitrogen fixation by legumes may be responsible for the acidifying effect. The protons secreted by legume nodules during the nitrogen fixation processes may contribute to lower soil pH (Mikkonen et al., 2011). Mikkonen et al. (2011) detected a change in the soil pH during fuel oil rhizoremediation. Their results showed that the rhizosphere soils showed a minor acidifying effect while the pH of pot soil increased slightly. This acidifying effect was encountered in both of their vegetated treatments during the last weeks of the experiment

when the biodegradation processes were almost completed and the legume root systems were well developed.

## 10.2 Residual contaminants in sampling sites

The landfarming site was previously investigated by Mikkonen et al. (2012), and their results revealed the hydrocarbon fractions and contamination gradients. Their samples were taken from the vegetated plots whereas the unvegetated plots in this work have not been investigated. In this section, their results and the results from this study will be compared and discussed.

The TSEM and especially TPH contents are normally used as a contamination indicator for oil contaminated soils. TSEM (total solvent extractable materials) measures all the materials which can be extracted by the solvent. According to the results, it correlated with soil dry matter content and organic matter content since most organic matter was extractable by the solvent used in this study. Thus TSEM is a rough estimation of oil contamination level. TPH directly reflects the real contamination posed by external contaminants because it excludes the polar hydrocarbons which can be accumulated during bioremediation processes (Head et al., 2006) and the finally mineralized hydrocarbons. Thus, the change of TPH on sites is used as a direct indicator measuring the removal of hydrocarbon contamination. Nevertheless, the accumulated polar fractions which are more toxic and resistant to biodegradation, could pose longer environmental effect (Head et al., 2003). In this study, TPH (total petroleum hydrocarbon) took up about 13-15 % of TSEM. The results of the previous study for the same site found a similar percentage composition of TSEM by TPH: 11-17 %. Their samples were taken in 2008 which was four and a half years before sampling of this study. The TPH seemed to vary at a stable percentage range of TSEM during the period between these two sampling times, The TPH, which measures the non-polar hydrocarbons between C10 and C40 was only the least abundant group of the hydrocarbons present in the contaminated sites according to the previous study. However, it directly reflects the real contamination posed by external contaminants because it excludes the polar hydrocarbons which can be accumulated during bioremediation processes (Head et al., 2006) and the finally mineralized hydrocarbons. Thus, the change of TPH on sites is used as a direct indicator measuring the removal of hydrocarbon contamination. Nevertheless, the accumulated polar fractions which are more toxic and resistant to biodegradation, could pose longer environmental effects (Head et al., 2003).

In the oil storage site, the TPH contents in samples from oil storage site were even higher than their corresponding TSEM contents, whilst TPH as the non-polar composition

group of TSEM can only be part of TSEM. The sum of three separate hydrocarbon groups (aliphatic, aromatic and polar) comprised up to 116 % of TSEM in the previous study because of technical noise. However, such big differences up to almost 200 % as recorded here are still unacceptable as reliable results. The possible explanation is that the TPH measurements by GC-FID were performed several days later than the gravimetrical TSEM measurement and during that time the volatized solvent (mainly comprised by heptane) increased the hydrocarbon concentration in the storage glass tubes. This suggested the TPH measurements must be performed soon after extraction from soil samples or at least stored in GC vials which are better sealed. In spite of these augmented TPH contents, the contamination levels of these samples can still be estimated by TSEM. Also, the site investigation was also performed by the SOILI program and according to their results this site had a ~3.7 g/kg soil contamination of short carbon chain hydrocarbons (C5-C10) (SOILI site investigation report, 2012).

In the landfarming site, the TSEM (~32 to 44 g/kg soil) and TPH (~4.5 to 6.5 g/ kg soil) remained at similar quantity levels as those of the previous study: TSEM (~35 to 41 g/kg soil) and TPH (~4.3 to 6.8 g/ kg soil) (Mikkonen et al., 2012). This indicates that the biodegradation in terms of TPH removal was quite slow for years, although the initial contamination examined in the previous study of ~3 to 7 g/ kg soil was only moderate. In a greenhouse experiment with fuel contamination of 3 g/kg dw soil performed by Mikkonen et al. (2012), up to about 90 % hydrocarbons were removed from soils after 21- weeks. Apparently, this efficiency of hydrocarbon bioremediation in optimal conditions was confronted with a huge obstacle when applying to the landfarming site as described in the beginning of this paragraph. Except that the biodegradation preferred the well-controlled conditions in greenhouse experiment than the harsh environmental conditions, the different compositions of contaminant source is also an explanation for the variable bioremediation rates. The oily refinery sludge waste in the landfarming site were mainly severely weathered long-chain hydrocarbons: C30-C40 (as shown in Figure 6), while the fuel oil used in greenhouse experiment was mainly easily degradable short-chain hydrocarbons (<C20). The long-chain hydrocarbons especially C30-C40 were reported to be more recalcitrant to biodegradation than short chain hydrocarbons (Balba et al., 2007). The weathered nature of oily sludge can also decrease its biodegradability (Head et al., 2003). The GC chromatograms of samples from the oil storage site showed the main composition of short chain hydrocarbons (C10-C20). It is thus reasonable to suggest that the oil storage site was mainly contaminated with easily biodegradable hydrocarbons. Considering the existence of hydrocarbon degrading microorganisms up to 1 m depth and the probably limited aeration conditions at the subsurface soils, biostimulation with aeration combined with

bioaugmentation into subsurface soils can make a practical remediation solution for the oil storage site.

It was indicated from the results that the vegetation surface samples had significant less TSEM ($P<0.01$) and TPH ($P<0.01$) compared with unvegetated surface samples. In some cases, the plants themselves can absorb minute amounts of hydrocarbons from the soil and translocate them into their different parts: sequestered in root tissues, transported into shoots and leaves, and then stored in vacuole or volatile (Khan et al., 2013). However, it needs more study at this landfarming site to confirm the stimulated effect of bioremediation by legume plantation. Samples from more plots and more sampling frequency can provide more data supporting or debating this assumption.

## 10.3 Degrading microorganisms enumeration and the isolated strains

In this study, petroleum degrading microorganisms were found to be present in the landfarming site with ~$10^4$-$10^5$ CFU/g soil and in oil storage site with ~$10^5$-$10^7$ CFU/g soil. The previous study by Mikkonen et al. (2012) also got similar counts, 1.3-4.4 × $10^5$ $g^{-1}$ soil from the landfarming site. They found no trend or correlation with oil concentration, either. The naphthalene degraders were about $10^3$-$10^4$ CFU/g soil which is at the same level as previously enumerated (2.2 × $10^3$-1.5 × $10^4$ $g^{-1}$) for the same site by Wallenius et al. (2012). Thus, it is indicated that the degrading microorganisms remained at a stable quantity range in this old weathered landfarming site.

The petroleum degrader microorganisms are commonly found in oil contaminated soils and sediments. Ali et al. (2012) even isolated hydrocarbon degrading bacteria from leave surfaces of two legumes (peas and beans) growing in oily soils. Their results suggested the phytoremediation through phyllosphere bacterial degradation can be a useful technology in remediating atmospheric hydrocarbon pollutants (Ali et al., 2012). However, the enumeration of degrading microorganisms in different sites varies a lot. It is affected by diverse *in-situ* factors including contamination level, contaminant properties and different soil physicochemical properties. Aislabie et al. (2001) enumerated the hydrocarbon degraders from three contaminated sites and found that the MPN results varied from non-detectable to more than $10^8$ $g^{-1}$ soil dry weigh. I in this study, the counts of petroleum degraders in the oil storage site were higher than those of the landfarming site since the former site was less weathered and mainly consisted of more biodegradable hydrocarbons. The different time scale under contamination exposure of the two soil ecosystems may be another explanation. It has been reported in some studies that after the initial increase of

degraders by oil contamination, the microbial community experienced a decrease of degraders (Bachoon et al., 2001; Mikkonen et al., 2011). Aislabie et al. (2001) also pointed out that the degraders had higher a proportion of microbial community in the recently contaminated site than the site with more than 30 years contamination.

As well known, the oil contamination stimulates indigenous hydrocarbon degrading microorganisms (Atlas, 1981; Aislabie et al., 2004). Atlas (1981) even believed that the extent of stimulation reflects the degree or extent of contaminant exposure. The hydrocarbon degraders are often low or below detection level from pristine soils (Balks et al., 2002; Aislabie et al., 2004).

The subsurface samples of both sites had a much lower degrading microorganism enumeration than their corresponding surface samples, although the contamination levels were higher in deeper soil. The limited aeration conditions in subsurface soils may be responsible for this. Subsurface soils are normally more anaerobic compared to surface soils due to compaction and higher oil contamination level. The aerobic degradation of oil consumes oxygen leading to more anaerobic conditions; especially considering the NAPL hydrocarbons in soil pores makes the soil texture less permeable.

The isolated strains in the present study were grouped with *Arthrobacter* and *Pseudomonas* strains which were reported with hydrocarbon degrading capacity (Foght & Westlake, 1996; Ionata et al., 2005; Margesin et al., 2013). As early as in the 1970s, Jensen (1975) reported that the most important species of oil degraders belonged to the genera *Arthrobacter* and *Pseudomonas* in his studies on soil bacterial flora after oily waste application. It has been reported that the *Arthrobacter* sp. strains are capable of alkane degradation (Ionata et al., 2005) and aromatic hydrocarbons degradation (Margesin et al., 2013). A large number of *Pseudomonas* species with oil degradation capacity have been isolated (Atlas, 1988). Moorthy et al. (2010) isolated three dominant bacterial strains from oil contaminated site and they found out that the *Pseudomonas* showed higher degrading ability than the other two isolates. Zhang et al. (2011) isolated a *Pseudomonas* strain of great practical significance in oil-contaminant bioremediation. The isolate could oxidize *n*-alkanes and PAH with three of four aromatic rings. The capacity of biosurfactant production by some *Pseudomonas* strains has also been reported and studied which improve the bioavailability of the hydrophobic oil compounds (Atlas, 1988; Obayori et al., 2009).

In this study, the *Arthrobacter* isolates were detected only to carry the *xylE1* gene while the *Pseudomonas* isolate was only detected to carry the *nahAc* gene. The detection of *nahAc* in the *Pseudomonas* isolate is not a surprise because the primer was designed with high

sequence identity to *nahAc* from *Pseudomonas putida* G7 (Baldwin et al., 2003). Previous studies reported that the *xylE1* gene was detected in Gram-positive species by Hendrix et al. (2006), although it is normally detected in Gram-negative species. Hendrix et al. (2006) suggested it might indicate the occurrence of horizontal transfer of catabolic genes located on plasmids. One difficulty for bioaugmentation technology is that the introduced degrading microorganism consortia are usually less competitive than indigenous microbial population leading to their short existence period in situ. Thus it is helpful to confirm the occurrence of horizontal transfer of the catabolic gene which indicates the transfer of degradation capacity.

Gene detection results of the isolates showed that each isolated degrading strain only possess one catabolic gene indicating that the degrader stains may only be capable of some certain degradation pathways. It is known that the TOL plasmid carrying *xyl* genes and NAH7 plasmid where *nah* genes locate belong to the IncP-9 incompatibility group and these two plasmids were found to be incompatible (Austen & Dunn, 1977). Little is known about the incompatibility of OCT plasmid carrying *alk* genes. However, there is not much available research about the incompatibility of catabolic genes because these genes were often examined only for characteristics related with degradative pathways other than the regions necessary for plasmid replication, conjugation and incompatibility (Burlage et al., 1990).

## 10.4 Catabolic gene detection from oil-contaminated soils

From the point of view of the objective of this study, it is difficult to get any insight in how the gene detection can be used as a soil assessment method for bioremediation potential. However, some suggestions can be proposed for researches in the future.

Two technical difficulties should be solved in order to get more positive results. Firstly, DNA extraction from oil contaminated soil samples usually had a problem because of the presence of organic matter like humic acids and the contaminants. The presence of these compounds can cause further challenges to gene amplification (Mahmoudi et al., 2011). For the landfarming site, previously applied urea may also interfere with DNA detection and measurements because urea and humic impurities exhibit similar solubility to properties of DNA (Lakay et al., 2007). Mahmoudi et al. (2011) compared the effectiveness of four commonly used commercial kits to extract pure, ready-for downstream use DNA from PAH contaminated soils. According to their results, the Ultraclean kits which were used in this study gave the lowest DNA yield and poor quality DNA extraction leading to variable quality of PCR reactions. In order to get more reliable results, essential purification method should be performed in similar studies in the future.

The other problem is that with diverse sequences of catabolic gene in different species, one single primer set may not successfully reveal its distribution in whole microbial population. It has been suggested to use group specific and more primer sets in order to get better amplification results (Whyte et al., 2002; Margesin et al., 2003). New primer design targeting a broader range of gene lineages are emerging, which also improves the gene detection technology (Passe et al., 2011). Besides, the sensitivity of primer sets is another influential factor. Hendrickx et al. (2006) reported that the catabolic genes had a detection limit of ca. $10^3$-$10^4$ copies $g^{-1}$ soil, assuming one copy of the gene per cell. The detection limit was also found by Kowalchuk et al. (1999). The maintenance of such catabolic genes in pristine soil microbial community is only by minority level. So it can still be pointed out that the primer set requires highly spiked abundance of genes in microbial population, although not much other reports has been published from this perspective.

It can be expected to get more catabolic genes detection results following these two suggestions. However, even in other studies, the attitude towards using gene detection as an assessment method is not consistent. Paisse et al. (2011) suggested that the detected *alkB* gene diversity and expression is not directly correlated with oil contamination and thus not an ideal method to monitor bacterial communities in contaminated sites. Similar results were also reported in other studies (Palmroth et al., 2007; Wang et al., 2012a). Despite the previous studies, the relationships between gene existence or expression and oil contamination are still not well understood. Much more work should still be done in the future to figure out the relationship and to apply the knowledge in environmental monitoring and assessment.

# 11. Conclusions

Oil contamination affects the environment and in return, the environmental conditions impact its bioremediation processes. As a complex mixture, different hydrocarbon groups have different bioavailability and biodegradability leading to different intrinsic bioremediation applicability. Thus the hydrocarbon composition depending on contamination source should be figured out by site investigation before taking remedial actions. To give fundamental information for designing remedy strategy, site investigation in this study provides several general conclusions.

Hydrocarbon degrading microorganisms exist in contaminated sites at a relatively high abundance level compared to non-contaminated soil. However, the degrader counts vary in different sites and probably vary in response to contaminant exposure time. Plantation of legumes had no significant effect in terms of stimulating the abundance of degrading microorganisms in the landfarming site contaminated with oily refinery sludge waste. However, the residual TPH and TSEM content in the vegetated fields were significantly lower than the bulk soil. *Arthrobacter* and *Pseudomonas* species were the dominant strains isolated from the contaminated sites in this study and were also reported by other similar studies to be important degrading species. The bioremediation of oily sludge wastes which consist mainly of long-chain hydrocarbons is quite slow from the perspective of TPH removal.

Each of the isolated degrading strains had only one catabolic gene responsible for degradation of certain hydrocarbon groups. It suggested that the degrader microorganism may survive through one specific degradation pathway rather than targeting the whole range of hydrocarbon mixtures.

This study got limited results from catabolic genes detection of contaminated soil samples. Contaminated soil samples may experience some inhibitions in DNA extraction especially in humic acid rich soils. Oil-contaminated soils have contaminants that can be co-extracted with soil DNA. The contaminants can interfere with further application of PCR-based methods. To reveal the presence of catabolic genes in microbial populations of contaminated sites, more degenerate primers targeting broader species range should be used.

This study is a good pioneering work attempting to assess oil contaminated sites from the perspectives of bioremediation potential. It also connected the scientific investigation with practical affairs by giving reliable suggestions about remedial strategy. To get more understanding and knowledge about the application in soil assessment, more work should be

conducted in the future such as optimizing the techniques of amplifying indicator genes and figuring out the threshold value of soil properties for promising bioremediation.

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