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THE BASICS OF RESEARCH IN MICROBIOLOGICAL SOIL REMEDIATION PRACTICES

DOCTORAL (PHD) THESIS

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADF	Aircraft de-icing fluid
ATP	Adenosine triphosphate
BGS	Background solution
bp	basis pair
cDNA	complementary DNA
CFU	Colony Forming Unit
DDBJ	DNA Database of Japan
DF	De-icing fluid
DEPC	Diethyl-pyrocarbonate
DIC	De-icing chemical
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
FDA	Fluorescein-diacetate analysis
FO	Formate
FSU	Friedrich-Schiller University
GPS	Global Positioning System
HAS	Hungarian Academy of Science
INT	Iodonitrotetrazolium chloride
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISM	Initial soil material
LOI	Lost of Ignition
LB	Lysogeny (Luria-Bertani) broth
MPN	Most Probable Number
OM	Organic matter
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PG	Propylene-glycol
pH	*pondus Hidrogenii*, which is a logarithmic measure of hydrogen ion concentration
PI	Plasticity index
RISSAC	Research Institute for Soil Science and Agricultural Chemistry
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
p-NP	*p*-nitrophenol
RDP	Ribosomal Database Project
TOC	Total Organic Carbon
TPF	Triphenyl-formazan
TPH	Total petroleum hydrocarbon
TE	Tris-EDTA
TES	2-[Tris(hydroxymethyl)-methylamino]-ethanesulfonic acid
TTC	Tetrazodium salt
SDW	Sterile distilled water
SRB	Sulphate-reducing bacteria
SOB	Super Optimal Broth
UV	Ultra violet
q.s.	*Quantum satis*/*Quantum suffitis* The amount which is needed or the quantity which is required.

1. ABSTRACTS

1.1. Abstract (English)

The basics of research in microbiological soil remediation practices

Current thesis involves the research of three European soils with three different types of contamination that have been analyzed with microbiological research methods with the objectives to monitor and collect biological data, gather information on the microbial connections to soil pollutions, and to isolate and identify microorganisms that can be used in the development of inoculums for soil bioremediation.

The tested sites involved contamination with propylene-glycol containing de-icing fluids (Gardermoen, Norway), crude-oil (Trecate, Italy) and Manganese-sludge (Őrbottyán, Hungary). Soil samples were gathered and tested for microbial abundance by using traditional Plate Count (PC) and MPN (Most Probable Number) assays, enzymatic activities were measured and microbial isolates were chosen based on abundancy. In the Gardermoen site the population composition was analyzed by molecular total DNA extraction and 16S rDNA sequencing. While on the Trecate and Őrbottyán site, abundant isolates were further tested for tolerance and antimicrobial resitance, and chosen isolates were identified by 16S rDNA sequencing. Furthermore, in the Manganese treated site phytoremediation was applied by planting *Elymus elongatus* Energy grass on the lysimeters combined with mycorrhizal fungi treatment.

In all research sites, the number of microbes and their activity were affected by the level of the contaminant, but the sensitivity of the biological parameters were different depending on the pollutant. When the contamination was organic propylene-glycol or crude-oil, the rate of groups which could degrade or tolerate the organic contaminant increased with the increased pollution concentrations. When the contaminant was an inorganic material, like Mn, the abundancy was less affected. Competition between microbial groups was detected in every site.

Enzymatic activities - as "early warning signals" - responded faster to soil pollution; reaching of the troxicity level was indicated by a drop of enzyme activity, independently from whether the contaminant was organic or inorganic; it was rather connected to toxicity. The response of the community was always selection toward microbial groups with either spore-forming ability or resistant groups and/or with biodegradable ability (in case of organic contamination). Abundant microbial isolates were gathered, and a few has been selected for identification.

In the Manganese treated site the results confirmed the successful application of the mycorrhiza treatment by having an increased plant biomass under higher concentration of Manganese and also more accumulated Mn in the root of the plants.

According to DNA based characterization and identification of abundant colonies, the microbial population selected toward degrader and stress tolerant bacteria in all three contaminated sites, depending on the contaminant. Among the identified isolates we found good candidates for further inoculum development, but other isolates should not be excluded either, they can be further assessed for bioremediation affinity.

By following the methods and tests described in the current thesis, we could realize a relatively fast and simple route to characterize the soil from a microbiological point of view and achieve possible isolates that are competitive, adapted on given contaminated areas, and are able to enhance bioremediation through degradation or collaboration with plants. These microorganisms can be used in the creation of complex inoculums in further researches, while the applied methods can be used to develop proper remediation, site rehabilitation practices and contamination monitoring systems.

1.2. Kivonat (Hungarian)

A talajjavítás mikrobiális eszközeinek kísérleti megalapozása

Jelen doktori értekezésben három különböző szennyezővel terhelt európai területet vizsgáltunk mikrobiológiai módszerekkel monitorozási és adatgyűjtési céllal, hogy képet alkothassunk a talajszennyezések és a mikroorganizmusok kapcsolatáról, tovább, hogy olyan mikroorganizmusokat izoláljunk és azonosítsunk, amelyek talajjavító oltóanyag-fejlesztéshez alkalmazhatók lehetnek.

A három vizsgált terület változatos szennyezettséggel rendelkezett, beleértve a propilén-glikol (PG) tartalmú jégmentesítővel (Gardermoen, Norvégia), a poliaromás szénhidogén kőolajjal (PAH) (Trecate, Olaszország) és az úrkúti Mangán-iszappal (Mn) szennyezett (Őrbottyán, Magyarország) területeket. Rendszeres talajmintázást követően meghatároztuk bizonyos, specifikus számolható mikroorganizmusok mennyiségét hagyományos lemezszámolással és MPN (legvalószínűbb szám) módszerrel. A Gardermoen területen molekuláris közösséganalízist végeztünk teljes DNS kinyeréssel és 16S rDNS szekvenálással, a Trecate és Őrbottyán területeken pedig az abundáns izolátumokat tovább teszteltük toleranciára és antimikrobiális ellenállásra, és a kiválasztott izolátumokat identifikáltuk 16S rDNS szekvenálással. A mangánnal kezelt területen továbbá fitoremediációs eljárást alkalmaztunk *Elymus elongatus* energiafű ültetésével a liziméterekre, és az eljárást mikorrhiza gomba kezeléssel kombináltuk.

Mindegyik kutatási területen kihatással volt a szennyeződés a mikroorganizmusok számára és a mikrobiális aktivitásra, de a szennyeződés típusától függően az adott biológia paraméterek érzékenysége eltérő volt. Szerves propilén-glikol és kőolaj szennyeződés esetén azoknak a mikrobiális csoportoknak az aránya növekedett a szennyeződéssel arányosan, amelyek képesek voltak lebontani vagy tolerálni a szennyezőket. A szervetlen Mn szennyezőnél a mikrobiális diverzitás megoszlása kevésbé volt érintett. Az egyes mikrobiális csoportok közötti kompetíció ennél megfigyelhető volt mindhárom területen.

Az enzimatikus aktivitás - "korai figyelmeztető jelként" - gyorsabban reagált a talajszennyezésekre, a toxicitási szint elérését csökkenéssel jelezte, függetlenül attól, hogy a szennyeződés szerves vagy szervetlen volt. Az aktivitás csökkenése inkább a toxikussághoz volt kapcsolható. A mikrobiális közösség a szennyeződésre adott válaszként szelektálódott vagy spóra-képző vagy rezisztens csoportok felé és/vagy amelyek képesek voltak a biológiai lebontásra (a szerves szennyezők esetén). Abundáns mikrobiális törzseket izoláltunk, amelyekből néhány beazonosításra került.

A mangánnal kezelt területen az eredmények alapján a mikorrhiza kezelés sikeresnek bizonyult, a növényi biomassza növekedett nagy koncentrációjú mangán jelenlétében is, továbbá, a kezelés hatására, a növény több Mn-t akkumulált a gyökerében.

A DNS alapú karakterizálás és abundáns törzsek izolálása alapján a populáció lebontó és stressz toleráns baktériumokra szelektált mindhárom vizsgált területen, a szennyeződés típusától függően.
Az azonosított törzsek között alkalmas törzseket találtunk későbbi oltóanyag fejlesztéshez, de a többi izolátumot sem kell kizárni, további vizsgálatok szükségesek annak megállapítására, hogy alkalmasak-e bioremediációhoz.

Az értekezésben szereplő módszereket és teszteket követve le tudtunk vezetni egy olyan útvonalat, amellyel versenyképes, adott szennyezőhöz adaptált és biodegradációra vagy együttműködésre képes, bioremediációt elősegítő mikroorganizmusokra tudtunk szert tenni. Ezeket a mikroorganizmusokat fel lehet használni egy további komplex oltóanyag készítési folyamatban, míg a felhasznált módszerek megfelelő remediációs, talajjavító és szennyezettség monitorozó rendszerek kifejlesztését teszik lehetővé.

1.3. Abstract (German)

Grundlagen der Forschung in mikrobiologischen Bodensanierungspraktiken

Die vorliegende Arbeit umfasst die Untersuchung von drei europäischen Böden mit drei verschiedenen Arten von Kontaminationen, die mit mikrobiologischen Untersuchungsmethoden analysiert wurden, um biologische Daten zu überwachen und zu sammeln, Informationen über die mikrobiellen Verbindungen zu Bodenverunreinigungen zu sammeln und Mikroorganismen zu isolieren und zu identifizieren. kann bei der Entwicklung von Inokulum für die biologische Bodensanierung verwendet werden.

Die untersuchten Standorte waren mit Propylenglykol enthaltenden Enteisungsflüssigkeiten (Gardermoen, Norwegen), Rohöl (Trecate, Italien) und Mangan-Schlamm (Őrbottyán, Ungarn) kontaminiert. Bodenproben wurden gesammelt und auf die mikrobielle Häufigkeit unter Verwendung herkömmlicher Plate Count (PC) und MPN (Most Probable Number) -Assays getestet, enzymatische Aktivitäten wurden gemessen und mikrobielle Isolate wurden basierend auf der Häufigkeit ausgewählt. In der Gardermoen-Fundstelle wurde die Populationszusammensetzung durch molekulare Gesamt-DNA-Extraktion und 16S-rDNA-Sequenzierung analysiert. An der Trecate- und der Őrbottyán-Stelle wurden reichlich vorhandene Isolate weiter auf Toleranz und Resistenz gegen antimikrobielle Substanzen getestet, und ausgewählte Isolate wurden durch 16S-rDNA-Sequenzierung identifiziert. Darüber hinaus wurde in der Mangan-behandelten Stelle Phytoremediation durch Einpflanzen von *Elymus elongatus* Energy Gras auf die Lysimeter in Kombination mit Mykorrhizapilzen Behandlung angewendet.

In allen Forschungsstandorten wurden die Anzahl der Mikroben und ihre Aktivität durch die Menge der Kontaminanten beeinflusst, aber die Empfindlichkeit der biologischen Parameter war je nach Schadstoff unterschiedlich. Wenn die Verunreinigung organisches Propylenglykol oder Rohöl war, nahm die Rate der Gruppen, die die organische Verunreinigung abbauen oder tolerieren könnten, mit den erhöhten Verunreinigungskonzentrationen zu. Wenn die Verunreinigung ein anorganisches Material war, wie Mn, war die Häufigkeit weniger beeinträchtigt. Die Konkurrenz zwischen mikrobiellen Gruppen wurde an jedem Ort nachgewiesen.

Enzymatische Aktivitäten - als "Frühwarnsignale" - reagierten schneller auf die Bodenverschmutzung; das Erreichen des Troxizitätsniveaus wurde durch einen Abfall der Enzymaktivität angezeigt, unabhängig davon, ob die Verunreinigung organisch oder anorganisch war; es war eher mit Toxizität verbunden. Die Reaktion der Gemeinschaft war immer die Selektion auf mikrobielle Gruppen mit entweder Sporen bildender Fähigkeit oder resistenten Gruppen und/oder mit biologisch abbaubarer Fähigkeit (im Falle einer organischen Kontamination). Es wurden zahlreiche mikrobielle Isolate gesammelt und einige wurden zur Identifizierung ausgewählt.

In der mit Mangan behandelten Stelle bestätigten die Ergebnisse die erfolgreiche Anwendung der Mykorrhiza-Behandlung, indem sie eine erhöhte Pflanzenbiomasse unter einer höheren Konzentration von Mangan und auch mehr akkumuliertes Mn in der Wurzel der Pflanzen aufwiesen.
Gemäß DNA-basierter Charakterisierung und Identifizierung von reichlich vorhandenen Kolonien wurde die mikrobielle Population in Abhängigkeit von der Verunreinigung in allen drei kontaminierten Stellen in Richtung abbauender und stresstoleranter Bakterien selektiert. Unter den identifizierten Isolaten fanden wir gute Kandidaten für eine weitere Inokulum-Entwicklung, aber auch andere Isolate sollten nicht ausgeschlossen werden, sie können weiter auf ihre biologische Sanierungsaffinität hin untersucht werden.

Indem wir die in der vorliegenden Arbeit beschriebenen Methoden und Tests befolgten, konnten wir einen relativ schnellen und einfachen Weg finden, um den Boden aus mikrobiologischer Sicht zu charakterisieren und mögliche isolierte, wettbewerbsfähige, an bestimmte kontaminierte Gebiete angepasste Isolate zu erzielen und zu verbessern. biologische Sanierung durch Abbau oder Zusammenarbeit mit Pflanzen. Diese Mikroorganismen können in weiteren Forschungen bei der Herstellung komplexer Inokulum verwendet werden, während die angewandten Methoden verwendet werden können, um geeignete Sanierungs-, Standort-Rehabilitationspraktiken und Kontaminationsüberwachungssysteme zu entwickeln.

2. INTRODUCTION AND IMPORTANCE OF THE TOPIC

2.1. Research background

The research study presented in this doctoral thesis was performed as part of two (2) projects: the SoilCAM ("*Soil contamination: Advanced integrated characterization and time-lapse monitoring*") project (*www.soilcam.eu*) and the Italian-Hungarian bilateral cooperation ("*Optimalization of soil-plant-microbe interactions in the bioremediation of soils polluted with toxic organic compounds and heavy metals" TET-10-1-2011-0173*).

The research sites of Norway (de-icing fluid contamination) and Italy (crude-oil contamination) were investigated under the SoilCAM project, while the Hungarian site (Manganese soil treatments) was studied during the Italian-Hungarian cooperation. Background data from the soil of the sites (for example chemical-physical and other measured soil parameters) was investigated by research partners from the projects.

The laboratory tests described in this thesis were performed in the Institute for Soil Sciences and Agricultural Chemistry, Centre for Agricultural Research, Hungarian Academy of Sciences (SoilCAM project and Ita-Hun cooperation) and in the Corvinus University of Budapest, Faculty of Horticultural Science, Department of Soil Science and Water Management (Ita-Hun cooperation) (belonging to the SZIE University since 2016)

2.2. Basis of the research

Soil degradation and/or soil pollution can be directly connected to the human life activities, in which case the natural physical, chemical and biological properties of the soil can change in an unfavorable way. Xenobiotics, by definition, are materials that are not supporting the survival of the living creatures, including human being, although they are considered as non-target organisms. The specificity of those agrochemicals is a crucial question [123].

These pesticides, xenobiotics and other dangerous pollutants are not easily recognized by degradation enzymes, and so they can be accumulating in soils and waters, and also in parallel in living organisms [1]. The ecological soil functions, like biomass production, filtering, buffering, transforming and storing, living habitat and genetical reserve function can be damaged [2]. Contaminants moreover can cause health hazard to both humans and animals. The pollution can be uptaken from the contaminated area, from either the water or soil, with direct contact, breathing in the evaporating contamination or with ingestion of those plant or animal products that contain the pollution in them [3].

In many cases, some materials are thought to be not harmful and used by many industries, but in higher doses or together with other materials they can become extreme polluters. For example, propylen-glycol (PG) is used in food and beauty industry as well, or as nutrient for animals, and is considered as easily degradable sugar-alcoholic compounds by the soil microorganisms. Still as a de-icing agent at northern airports it is used in such a big amount that it can pose a threat to the environment [4; 5]. Moreover, similar as many "heavy metal" type elements, they are essential "trace elements" for the growth of plants and microorganisms, their ability to denaturate protein molecules in supraoptimal high doses might result toxicity to living creatures [6].

Several important plant products like corn, peanut and sugar beet is very dependent on treatment against pests in the soil [7]. Pesticides can enter the soil and groundwater from the plants, which can once again cause extra burden on the environment. The degradation products of these pesticides can either cause negative or positive effect on microorganisms: they can either enhance or interfere with population, they can prevent or stimulate respiration, nitrogen transformation or substrate uptake, and moreover they can decrease growth for one or many microbial species [8].

The optimal operation of all the elements of an ecosystem highly depends on the healthy soil state, which needs to be revitalized after agricultural, mining and industrial usage, or after it was disturbed with traffic. It was proved that many waste treatment processes ignore the burden put on the soil, and because of this, they should not be used [9]. In most cases with sufficient organic and inorganic treatment – like increasing the water capacity of the soil, or restoring the microbial population etc. – the soil state can be repaired [3].

3. OBJECTIVES

1) Monitoring and collecting biological data from three contaminated sites (at Hungary and in Europe) under different environmental conditions:
   By gathering various physical-chemical and microbiological characteristics, the complexity of biological data might provide sufficient information for achieving tools of potential remediation practices of the contaminated soils.

2) Investigating the connection between pollutants and microorganisms and to receive information on the contamination's effect on soil quality:
   We assume that contamination might affect the orientation of the microbes, microbial activity and population composition might be based upon the distance of the pollutant concentrations and affecting periods.

3) Using additional molecular analysis of the soils, we might be able to gather information about population compositions and later to select microorganisms for further studies.
   We assume that population composition is highly affected by the characteristics of the pollutant (organic/inorganic, degradability, toxicity, concentrations, spreading etc.).

4) Selecting microorganims based on their abundancy and resistance to the given contamination avoiding antibiotic resistant isolates:
   We assume that by investigating the potential adaptation to certain environmental factors (i.e. the pollutants) we will be able to isolate and receive possible strains to create inoculants dedicated to the degradation of the contaminants at certain sites under the given area's environmental conditions.
   We also think that molecular methods combined with susceptibility tests are applicable to disqualify such potential plant-human pathogens, which cannot be applicable due to environmental reasons to soil remediation practices.

5) We assume that the above-mentioned methodology might lead us to a logical route to receive possible degrading bacteria for developing inoculums to increase the success and speed of the remediation of soils with similar contaminations.

4. LITERATURE REVIEW

4.1. Soil characteristics and soil pollution

Soil is a major component of the Earth's ecosystem. The soil is the second largest carbon reservoir on Earth, and it is potentially one of the most reactive to human disturbance and climate change. Soil acts as an engineering medium, a habitat for soil organisms, a recycling system for nutrients and organic wastes, a regulator of water quality, a modifier of atmospheric composition, and a medium for plant growth.

Soils offer plants physical support, air, water, temperature moderation, nutrients, and protection from toxins. Soils provide readily available nutrients to plants and animals by converting dead organic matter into various nutrient forms. For optimum plant growth, the soil components by volume should be roughly 50% solids (45% mineral and 5% organic matter), and 50% voids of which half is occupied by water and half by gas [10]. The percent soil mineral and organic content is typically treated as a constant, while the percent soil water and gas content is considered highly variable whereby a rise in one is simultaneously balanced by a reduction in the other. Pore space allows for the infiltration and movement of air and water, both of which are critical for life in soil. Compaction is a common problem with soils.

Most nutrients, with the exception of nitrogen, originate from minerals. Some nitrogen originates from rain (as dilute nitric acid), but most of the nitrogen is available in soils as a result of nitrogen fixation by bacteria. The action of microbes on organic matter and minerals may be to free nutrients for use, sequester them, or cause their loss from the soil by their volatilisation to gases or their leaching from the soil. The nutrients may be stored on soil colloids, or within live or dead organic matter.

The most influential factors in stabilizing soil fertility are the soil colloids, clay and humus [11]. Some nutrients are held in place by the clay and humus content of the soil otherwise they would be leached from the soil or released in response to changes of soil pH. The greatest influence on plant nutrient availability is the previously mentioned pH in the soil, which is a measure of the hydrogen ion (acid-forming) soil reactivity, and is in turn a function of the soil materials, precipitation level, and plant root behavior. The organic material of the soil also has a powerful effect on its development, fertility, and available moisture. Following water and soil colloids, organic material is next in importance to a soil's formation and fertility.
Since soil has a tremendous range of available niches and habitats, it contains most of the Earth's genetic diversity. A gram of soil can contain billions of organisms, belonging to thousands of species [12]. Soil has a mean prokaryotic density of roughly 10^{13} organisms per cubic meter.

Through agriculture, industry, and daily life, harmful chemicals have been released into the Earth’s soil. These pollutants found in soils present a variety of different human health risks. Soil contaminants are typically classified as organic and inorganic pollutants.

Industrialization resulted in an increased use of organic compounds that build up and persist in the environment. Main sources of organic pollutants are through anthropogenic activities, including the use of solvents, pesticides, and fuels affecting the runoff and indirectly the ground water [23]. Some of these organic compounds are highly toxic and they are associated with a variety of health issues around the world [13].

A common organic pollutant example is Polycyclic Aromatic Hydrocarbons (PAHs). Hydrocarbons are stored deep underground but are brought up to the surface to be transformed and utilized, primarily as an energy source known as fossil fuels. The majority of pollution currently comes from these byproducts in the form of PAHs, which are xenobiotic environmental pollutants that form when carbon materials are incompletely combusted. Some of the examples of PAHs include burning wood, fossil fuels and cigarette smoke [14].

Most inorganic pollutants that accumulate over time are due to human activities, for example pesticides, paints, wood preservers, mining and wastes, like batteries. There are also natural forms of contamination from normal biological processes, which include weathering of minerals over time, erosion, volcanic activities, biogenic sources and particles released by the vegetation.

Depending on their concentrations, these substances can have destructive consequences on ecosystems, and they can cause severe damage to humans and other organisms nearby. An apparently nontoxic chemical can be toxic at high doses, however highly toxic chemicals can be life saving when provided in appropriate doses [123].

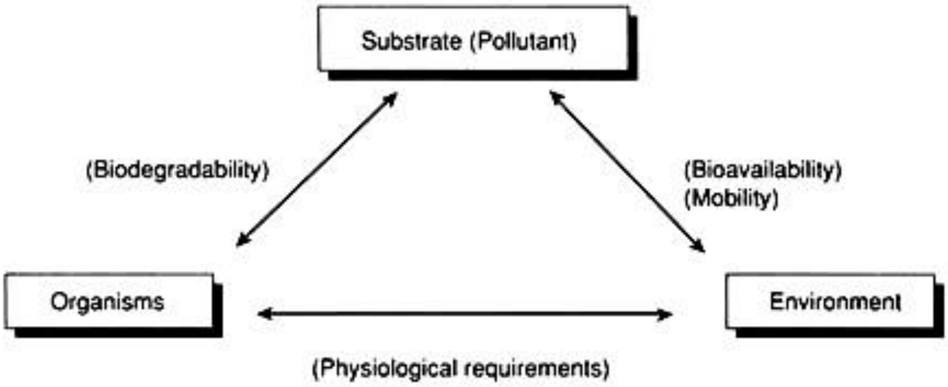
Soil pollution is a crucial factor, because of its combined impact on surface, groundwater and air, and connected to this phenomenon polluting materials can easily spread and accumulate in human organisms as well by direct and/or indirect consumption.

Toxic metals for example, as inorganic pollutants, can enter the environment at all life cycle stages of the metal compound. Metal leakage can occur from mining processes till the disposal of the metal wastes. However, in nature, the mobility of metals comes from the geological processes that can be released into the soil and aquatic environments. The environmental largest risk from metal contamination comes from the relationship between metals and compounds that are inherently incapable of being degraded by any natural procedures. The best solution to treat contamination is transporting the metals to locations where they cannot produce negative environmental effects [15].

4.2. Biodegradation of soil pollutants by microorganisms

Biodegradation is the biologically catalyzed modification of an organic chemical's structure. This modification can be through different metabolic pathways but does not necessarily mean a reduction in toxicity. Mineralization, one type of biodegradation, is defined as the conversion of an organic substance to its inorganic constituents, rendering the original compound harmless [16]. Transformation is defined as any metabolically-induced change in the chemical composition of a compound [17].

There is a close connection between the degrader biological organisms, the pollution and the environment, and changing any of these three factors can result in a change of the other two (**Figure 1**). Biodegradation from the part of the environment may be influenced by pH, temperature, moisture, carbon sources, soil texture, aerobic versus anaerobic conditions, the number of substituents, and then there is the concentration of the pollutant. It is impossible, however, to make a generalization about the best universal conditions for biodegradation. What’s toxic to some microbes is a nutrient to others, what might be a damaging pH to some is beneficial to others. A greater amount of substituents will cause slower degradation in aerobic environments, but faster degradation in anaerobic ones. High concentration of a pollutant generally results in faster rates of degradation. If the concentration drops below a threshold concentration, the enzymes that contribute to the degradation may not detect it [18]. On many polluted area the rate of degradation might be highly limited.



1. Figure: The connection of organisms, soils and contaminants, and their effect on each other (retrieved from [19]).

Microbial activity in the soil is very important regarding the agricultural and environmental protection function of the soils [20]. Microorganisms are widely diverse organisms that take part in biodegradation processes of the soil. They use the organic and inorganic materials of the environment (in full or partially) to build up their own biomolecules. Based on this, degradation can be whole or partial. The end product in the first one is carbon-dioxide and water, while the latter results in many different other compounds, which might be even more toxic than the starting material [21]. Specific microorganisms isolated from different water and soil environment can methabolize hydrocarbons [22]. Because of their variety, flexibility and versatility microorganisms are excellent choices to lead xenobiotic elements back to the natural biochemic cycle by using their catabolic pathways [1].

Bioremediation involves various microorganisms (but not only microorganisms) that are able to degrade and reduce toxicity of environmental pollutants [36]. Therefore, the interactions of microbes with the environment and pollutants are significant in determining effectiveness of bioremediation [28]. The involved microbes can be either naturally present in the site of bioremediation or isolated from other sites and inoculated artificially. Biodegradation often occurs as part of microbial metabolism and in some cases; microbes are able to directly harvest carbon and energy by breaking down pollutants [36]. Sections below go over bacteria and fungi, the commonly used organisms in bioremediation, and archaea, the more recently discovered group of organisms with unique potential in bioremediation. Degradation can be aerobe or anaerobe depending on the metabolic pathway used.

4.2.1. Bacteria in bioremediation

Bacteria are widely diverse organisms, and thus make excellent players in biodegradation and bioremediation. There are few universal toxins to bacteria, so there is likely an organism able to break down any given substrate, when provided with the right conditions (anaerobic versus aerobic environment, sufficient electron donors or acceptors, etc.).

Common degraders

Several specific bacteria species are known to participate in bioremediation.

Members of the gram-negative *Pseudomonas genus* are able to metabolize chemical pollutants in the environment, and as a result, can be used for bioremediation. Notable species demonstrated as suitable for use as bioremediation agents include:

- *P. alcaligenes*, which can degrade polycyclic aromatic hydrocarbons [48].
- *P. mendocina,* which is able to degrade toluene [49].
- *P. pseudoalcaligenes*, which is able to use cyanide as nitrogen source [50].
- *P. resinovorans*, which can degrade carbazole [51].
- *P. veronii*, which has been shown to degrade a variety of simple aromatic organic compounds [52; 65].
- *P. putida*, which has the ability to degrade organic solvents such as toluene [53]. At least one strain of this bacterium is able to convert morphine in aqueous solution into the stronger and somewhat expensive to manufacture drug hydromorphone (Dilaudid). It is also capable of degrading naphthalene, a product of petroleum refining, in contaminated soils [54].
- Strain KC of *P. stutzeri*, which is able to degrade carbon tetrachloride [55].

The genus *Rhodococcus* contains aerobic, non-sporulating, nonmotile Gram-positive bacteria closely related to *Mycobacterium* and *Corynebacterium*. They have the important ability of bioconversion, using biological systems to metabolize harmful environmental pollutants, including toluene, naphthalene, herbicides, and PCBs [56; 57].

Dechloromonas aromatica is a rod-shaped bacterium which can oxidize aromatics including benzoate, chlorobenzoate, and toluene, coupling the reaction with the reduction of oxygen, chlorate, or nitrate. It is the only organism able to oxidize benzene anaerobically. Due to the high propensity of benzene contamination, especially in ground and surface water, *D. aromatic* is especially useful for in situ bioremediation of this substance [58].

Alcanivorax borkumensis is a marine rod-shaped bacterium which consumes hydrocarbons, such as the ones found in fuel, and produces carbon dioxide. It grows rapidly in environments damaged by oil, and has been used to aid in cleaning the more than 830,000 gallons of oil from the Deepwater Horizon oil spill in the Gulf of Mexico [59].

Another example is *Deinococcus radiodurans*. It is a radiation-resistant extremophile bacterium that is genetically engineered for the bioremediation of solvents and heavy metals. An engineered strain of *Deinococcus radiodurans* has been shown to degrade ionic mercury and toluene in radioactive mixed waste environments [60].

CO_2 and H_2 autotrophs

Autotroph is an organism capable of biosynthesizing all cell material from carbon dioxide as the only carbon source. With respect to energy, autotrophs can obtain it from two sources: (1) photoautotrophs from radiation (sunlight) and (2) chemolithoautotrophs from the oxidation of reduced inorganic substrates. Autotrophs are capable of growth exclusively at the expense of inorganic nutrients, and they are of vital importance in the cycling of inorganic compounds on Earth including methanogens, which produce methane from H_2 and CO_2, and nitrifiers, which convert ammonia to nitrate. Autotrophs are the source of reduced carbon substrates for the heterotrophs. Autotrophs are key elements of the carbon cycle. For this reason, autotrophic organisms are also called primary producers [153].

An example of a CO_2 autotroph is *Alcaligenes eutrophus* [154], while *Methanobacterium wolfei*, *Methanobacterium formicicum*, *Methanococcus maripaludis* and *Methanosarcina barkeri* are H_2 autotrophs [155].

Nitrifiers and denitrifiers

The removal of nitrogen is a two stage process that involves nitrification and denitrification. During nitrification, ammonium is oxidized to nitrite by organisms like *Nitrosomonas europaea* [61]. Then, nitrite is further oxidized to nitrate by microbes like *Nitrobacter hamburgensis* [62]. Usually these two groups of microorganisms occur together in the environment. In anaerobic conditions nitrate produced during ammonium oxidation is used as a terminal electron acceptor by microbes like *Paracoccus denitrificans* and *Pseudomonas stutzeri* [63]. The result is N_2 gas.

Sulphate reducers

Sulphate-reducing bacteria (SRB) play an important part in the sulphur cycle in nature, both on land and in the sea. Their activities, chiefly because invariably accompanied by the production of hydrogen sulphide, frequently result in undesirable manifestations, sometimes of considerable economic importance, as in the corrosion of buried metal structures or the contamination of coal, gas and oil. They are said to contribute to the formation of petroleum and to its modification after formation. A recently discovered and potentially valuable property of some strains is their power of releasing oil from oil-bearing sediments [150].

Examples of sulphate-reducing bacteria are the genera of *Desulphococcus*, *Desulphomonile* and *Desulphobacterium* [151].

Sulphur oxidizers

Sulphur is one of the essential plant nutrients and it contributes to yield and quality of crops. The transfer of sulphur between the inorganic and organic pool is entirely caused by the activity of the soil biota, particularly the soil microbial biomass, which has the greatest potential for both mineralization and also for subsequent transformation of the oxidation state of sulphur. Sulphur oxidation is the most important step of sulphur cycle, which improves soil fertility [152].

Sulphur oxidizing microorganisms are primarily the gram negative bacteria currently classified as species of *Thiobacillus*, *Thiomicrospira* and *Thiosphaera*, but heterotrophs, such as some species of *Paracoccus*, *Xanthobacter*, *Alcaligens* and *Pseudomonas* can also exhibit chemolithotrophic growth on inorganic sulphur [152].

Fungi are also capable of oxidizing elemental sulphur, which include, *Alternaria tenius*, *Aureobasidium pullulans*, *Epicoccum nigrum*, a range of *Penicillium species*, *Scolecobasidium constrictum*, *Myrothecium cinctum* and *Aspergillus* [152].

Fe/Mn reducers

The oxidation of organic matter coupled to the reduction of Fe(III) or Mn(IV) is one of the most important biogeochemical reactions in aquatic sediments, soils, and groundwater. This process, which may have been the first globally significant mechanism for the oxidation of organic matter to carbon dioxide, plays an important role in the oxidation of natural and contaminant organic compounds in a variety of environments and contributes to other phenomena of widespread significance such as the release of metals and nutrients into water supplies, the magnetization of sediments, and the corrosion of metal [156].

Dissimilatory Fe(III) or Mn(IV) reduction can be defined as the use of Fe(III) or Mn(IV) as an external electron acceptor in metabolism, therefore a wide variety of fungi and bacteria reduce Fe(III) or Mn(IV) under various conditions while they metabolize fermentable sugars, amino acids, or oxidize sulphur, organic acids and aromatic compounds. Some examples are *Lactobacillus lactis*, *Clostridium sporogenes* and *Thiobacillus ferrooxidans* [156].

4.2.2. Fungal mycoremediation applications

Current bioremediation applications primarily utilize bacteria, with comparatively few attempts to use fungi. Fungi have fundamentally important roles because of their participation in the cycling of elements through decomposition and transformation of organic and inorganic materials. These characteristics can be translated into applications for bioremediation which could break down organic compounds and reduce the risks of metals. In some cases, fungi have an advantage over bacteria not just in metabolic versatility but also their environmental resilience. They are able to oxidize a diverse amount of chemicals and survive in harsh environmental conditions such as low moisture and high concentrations of pollutants – by producing spores.

Fungi are capable degrading PAH’s that are high in molecular weight (while bacteria in comparison are better at degrading smaller molecules) and they can function well in non-aqueous environments and low oxygen conditions, both are conditions where PAH’s can accumulate. Many fungi have evolved mechanisms that allow them to target specific PAHs. Fungi produce extracellular enzymes that degrade lignin, a process called mineralization the produces carbon dioxide as the end product [64].

Additionally, fungi have various ways of interacting with metals; some of the techniques are increasing or decreasing the mobility of metals, sorption, or even cellular uptake. After the metals have been absorbed by the fungus, they can be chemically altered to be stored or translocated through the hyphae and into various plants that participate in symbiosis [66]. Fungi therefore could also be a potentially powerful tool in soil bioremediation [67].

4.2.3. The aspects of choosing microorganisms for bioremediation

Although fungi demonstrate significant biochemical and ecological useful qualities, they are hardly utilized for biotechnological purposes. Instead, bacteria are most commonly used because they usually produce superior results in their numerous advantages ranging from their highly specific biochemical reactions to their capabilities of breaking down pollutants efficiently. Fungi are underused primarily because of the costs that come from providing oxygen to fungi in polluted environments. However, filamentous fungi could be highly valuable in situations where bacteria cannot perform. For example, fungi are useful in situations where contaminants are physically blockaded and bacteria cannot reach or in circumstances of environmental extremes such as high acidity or dryness prevent bacteria from functioning [66].

4.3. Bioremediation practices

Bioremediation is a technology with a purpose to use the degradation abilities of organisms to remove pollutants from the soil in an environmental friendly way [21].

Bacteria that can degrade contaminants have a central role in the process [24]. The versatility of microbes to degrade a vast array of pollutants makes bioremediation a technology that can be applied in different soil conditions [25]. The natural treatment of soils can be significantly cheaper compared to other remediation methods and the execution is generally less disruptive to the polluted area. Both *in situ* (in place) and *ex situ* (removal and treatment in another place) remediation approaches are used [26].

A widely used approach to bioremediation involves stimulating naturally occurring microbial communities, providing them with nutrients and other needs, to break down a contaminant. This is termed biostimulation**.** Biostimulation can be achieved through changes in pH, moisture, aeration, or additions of electron donors, electron acceptors or nutrients.

Another bioremediation approach is termed bioaugmentation, where organisms selected for high degradation abilities are used to inoculate the contaminated site [25]. To intensify the degradation of the pollution not just amplified indigenous, but microbe species extraneous to the area can be used as well [27]. Biostimulation and bioaugmentation approaches are not mutually exclusive- they can be used simultaneously.

From an ecological point of view, bioremediation depends on the various interactions between three factors: substrate (pollutant), organisms, and environment, as shown in the figure at right [28]. The interactions of these factors affect biodegradability, bioavailability, and physiological requirements, which are important in assessing the feasibility of bioremediation.

Biodegradability, or whether a chemical can be degraded or not, is determined by the presence or absence of organisms that are able to degrade a chemical of interest and how widespread these organisms are in the site. The substrate (pollutant) can interact with its surrounding environment to change its bioavailability, or availability to organisms that are capable of degrading it; for example, substrate has low bioavailability if it is tightly bound to soil organic matter or trapped inside aggregates. Physiological requirements, or set of conditions required by organisms to carry out bioremediation in the environment, include nutrient availability, optimal pH, and availability of electron acceptors, such as oxygen and nitrate. Also, the environment needs to be habitable for organisms involved in bioremediation [19].

4.3.1. Environmental Monitoring

In order for bioremediation to be successful, it requires sufficient proof for the degradation of contaminants. Biological analysis of the soil (e.g. soil respiration, biomass, enzyme activity, microbial counts) can provide information about the presence of viable microorganisms in addition to the intensity, quality and time period of occurring environmental pollution and its effect on the soil [30; 27]. Further factors that need to be assessed are the concentration of the pollutant, the appearance of intermediate compounds, the amount of electron acceptors and dissolved oxygen and pH.

Current monitoring practices determine the disappearance of contaminants and their degradation products to regulatory levels that are monitored by toxicity testing, usually on single organisms or species to ensure there are no induced changes that may result in residual toxicity. The problem with these monitoring techniques is that the assessment of contaminants may result in an inaccurate indicator of residual toxicity [29].

To properly evaluate the biological detoxification results, it is not enough the measure the remaining hydrocarbon content after the degradation, but it is important to monitor the microbial processes as well [30]. The presence of natural degradation can be proved by the increase of the number and activity of microorganisms and the appearance of microbes known to be degraders of a given contaminant [26]. To degrade a pollutant the degrader microorganism usually needs to be resilient and selective to the pollution [31]. In practice we can count, isolate, identify, characterize degrader native microorganisms and evaluate them in the laboratory and on site also [24].

It is a fact however, that most of the bacteria found in the environment cannot be cultured in a laboratory environment. Currently about 5% is culturable; the rest is viable but cannot be cultured on solid or liquid media in laboratory condition [43]. This huge blind spot is hard to overcome at the moment, but we can utilize another monitoring method, which can still show us the non-culturable population and their specific reaction to environmental stresses, and this is the molecular analysis of the soil: microarray and methagenomic methods [24]. The most common molecular marker for microorganisms is the 16S rDNA is widely used to identify microorganisms. Molecular oecological information can provide infomation about the diversity and presence of contamination degrader microorganisms and can help us build strategies for a remediation plan [32].

In case we wish to use a microorganism not native in the area, in other words, bioaugmentation, we need to follow up on its survivability in the soil after inoculation; its presence therefore needs to be monitored frequently, not to mention its effect on the original population. Monitoring, for example, can be achieved with PCR and fingerprint methods, like temperature gradient gel electrophoresis [32].

Another important factor directly connected to the contamination is biological activity. The enzymes in the soil catalize methabolic degradation pathways for organic materials and they take part in the detoxification of xenobiotics [33; 34]. Soil activity is sensitive to environmental stresses, therefore soil enzymes can be good indicators of the different environmental effects – like pollution – and they can be monitored and compared in different soils with different analytical methods, like fluorescein-diacetate analysis [35]. Once sufficient evidence is provided, human intervention may be needed for a more effective cleanup process.

4.3.2. Bioremediation methods

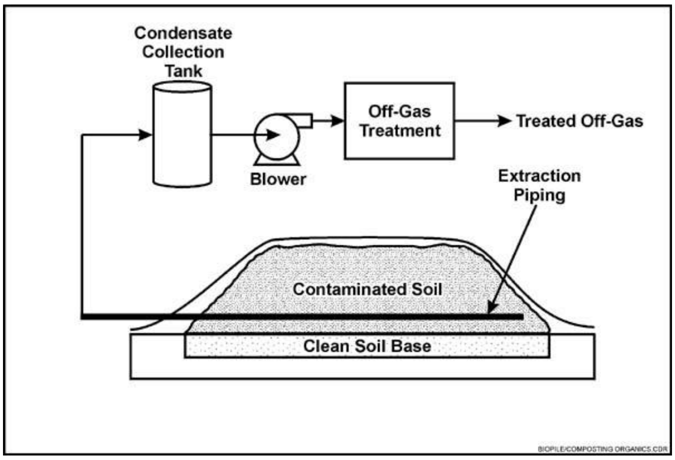
There are two types of remediation can be performed, "*ex situ*" which is done by removing the contaminated soil or water and treating it outside the source, and "*in situ*" which treatment happens in place within the contaminated area. There are some treatments methods that can be either ex situ or in situ. Some techniques may deal with the mobilization of pollutants, to move them out of an area, or immobilized to keep them out of an area such as a water table.

"Ex situ" techniques are those that are applied to soil and groundwater which has been removed from the site via excavation or pumping [36]. The methods used include **biopiling** and **composting**. *Ex situ* is used for smaller projects, primarily because larger excavation of the soil is not preferred. The movement of the soil can be more detrimental by destroying the preestablished horizons in the soil.

Biopiling

During biopiling excavated soils are mixed with soil amendments and placed on a treatment area. Biopiles are aerated with the use of perforated pipes and blowers in order to control the progression of biodegradation more efficiently by controlling the supply of oxygen, which in turn may affect other factors such as pH. A basic biopile design can be seen in **Figure 2** [37].

This system is primarily used to remediate systems with oil and hydrocarbon contamination. The remediated soil is placed in a liner to prevent further contamination of the soil and they may also be covered with plastic to control runoff, evaporation, and volatilization.



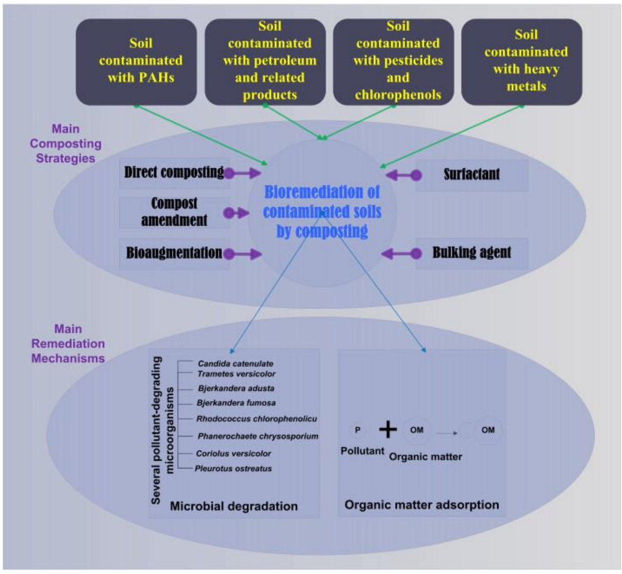
2. Figure: Design of a biopile: the contaminated soil mixed with amendments is palced on a treatment area, where oxygen is supplied with pipes (retrieved from [37])

Composting

During composting nutrients are added to soil that is mixed to increase aeration and activation of indigenous microorganisms. Composting is done in a separate container, then, when composting is complete, it is incorporated into the soil. Bioremediation by the utilization of compost relies on the adsorption capabilities of organic matter and the degradation capabilities of the microorganisms at present [38]. **Figure 3** can give us a short overview about the factors involved during this process.

Composting is recognized as one of the most cost-effective technologies used in soil bioremediation and it can be done on large and small scales. The use of composting is a very versatile technique for soil polluted by a wide range of organic pollutants and heavy metals, making it great for easier remediation involving various contaminants. The utilization of organic wastes for soil remediation is also helpful in decreasing the need for their storage and treatment. Organic matter that is generated from composting offers the benefit of improving soil quality and structure.

Composting is primarily used for remediation over a longer period of time, as the nutrients for the microbes are released gradually and require more time compared to quicker treatments such as biostimulation.



3. Figure: An overview of composting: contaminants, treating strategies and the mechanisms of remediation (retrieved from [38])

"*In situ*" techniques are applied to soil and groundwater at the site with minimal disturbance [36]. These methods include **biostimulation**, **bioleaching**, **biosorption**, and **bioventing**. "*In situ*" is preferred because it is often minimally invasive to the soil structure in comparison to ex situ, but it can be expensive due to specialized equipment.

Biostimulation

This method involves the addition of nutrients to a polluted site in order to encourage the growth of naturally occurring chemical-degrading microorganisms [39]. Biostimulation is primarily done by the addition of various nutrients that are limited in the soil as well as electron acceptors, such as phosphorus, nitrogen and oxygen, or increasing the amount of available carbon in order to increase the population or activity of naturally occurring microorganisms.

Other approaches are to optimize environmental conditions such as aeration, the addition of nutrients, altering pH and temperature control [40]. The primary advantage of biostimulation is that it is done by native microorganisms that are well-suited to the given environment, and are already well distributed spatially. The challenge is delivering additives so they are readily available to the subsurface microbes.

Co-metabolism strategies connected to biostimulation

Cometabolism is the biological transformation of a non-growth supportive substrate in the obligate presence of a growth supporting substrate or another transformable compound. This is based on the wider substrate specific property of some enzymes, meaning, that the enzyme accepts similar type and size of substrates and cuts them, but the resulting product is not able to move forward to the next reaction and through the metabolic pathway.

What makes it very important connecting to bioremediation is that certain PAHs and many xenobiotics are degraded by cometabolism [157; 158]. Therefore, if the energy supporting substrate is provided, cometabolism can be triggered, resulting in the degradation of the given contaminant in the soil.

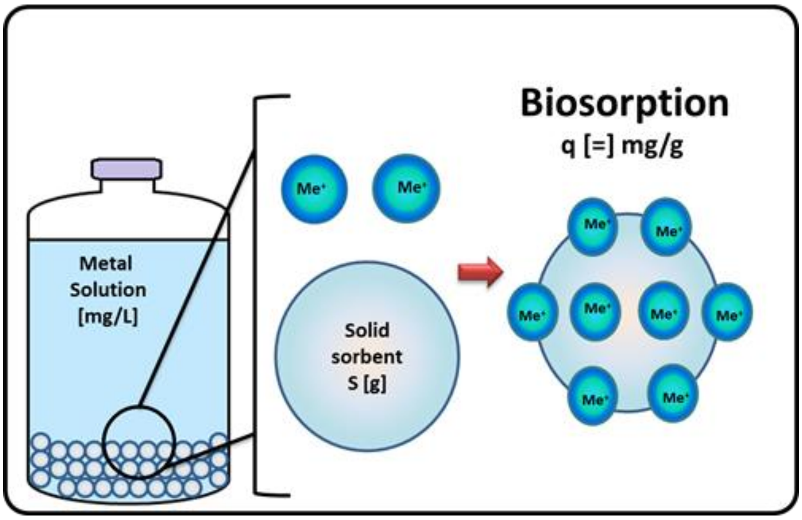
Cometabolism strategies stimulate only indigenous microbes with the ability to degrade the contaminant and cosubstrate, e.g., methane, propane, toluene, and others. This highly targeted stimulation insures that only those microbes that can degrade the contaminant are targeted, thus reducing amendment costs, well and formation plugging, etc. Cometabolic bioremediation has been used on some of the most recalcitrant contaminants, e.g., PCE, TCE, MTBE, TNT, dioxane, and atrazine. Methanotrophs have been demonstrated to produce methane monooxygenase, an oxidase that can degrade over 300 compounds. Cometabolic bioremediation also has the advantage of being able to degrade contaminants to trace concentrations, since the biodegrader is not dependent on the contaminant for carbon or energy [159].

Bioleaching

Metal bioleaching is the extraction of metals from soils utilizing a biological source such as microbes. This technique was first developed to extract minerals from ores. Specific microorganisms like *Thiobacillus ferrooxidans* and *T. thiooxidans* promote the solubilization of metals. Several species of fungi are also used for bioleaching. Two notable fungal strains, *Aspergillus niger* and *Penicillium simplicissimum* are capable of mobilizing metals such as copper, tin, aluminium, nickel, palladium, and zinc [41], which makes them much easier to remove from the soil.

Biosorption

Biosorption is the adsorption of metals and other ions of an aqueous solution by the use of microbes. The biosorption process involves a solid phase and a liquid phase containing a dissolved material to be sorbed [42]. **Figure 4** illustrates the biosorption process, which continues until equilibrium is established between the amount of solid-bound sorbent and its portion remaining in the solution. The degree of affinity for the sorbate determines its distribution between the solid and liquid phases.

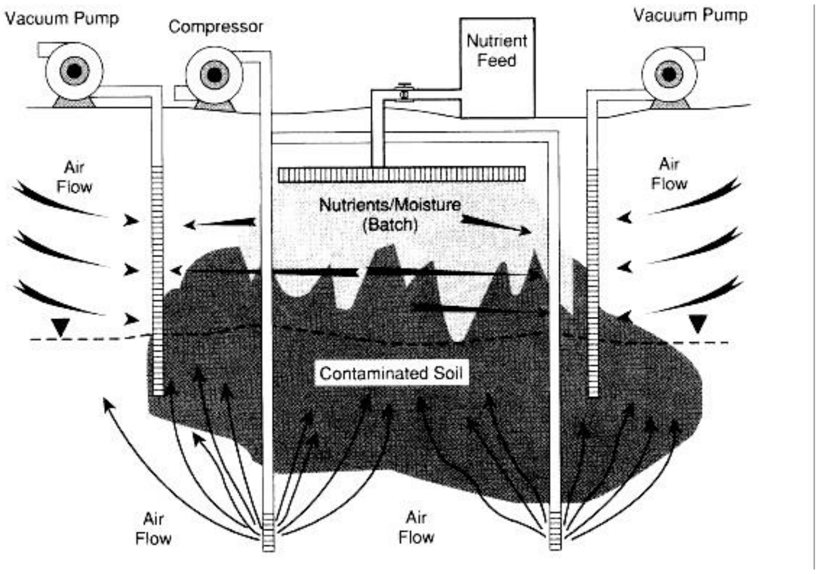


4. Figure: Removal of metals from aqueous solutions by biosorption
(retrieved from [111])

Bioventing

The availability of oxygen generally controls the rate at which aerobic bioremediation proceeds. Bioventing process involves the injection of air directly into the ground water below the contaminant plume. The air displaces the water in the ground providing the microorganisms with an electron acceptor needed for bioremediation. The air also helps to remove the volatile contaminants that can be captured by using a soil vapour recovery system. If nutrients or water are not present in sufficient quantities, they can be provided using an injection well. This system works because air movement helps to mix and distribute the nutrients to the microorganisms as shown in **Figure 5** [44].

This technology can be successfully applied to compounds ranging from gasoline or diesel, to heavier hydrocarbons. The addition of nutrients with the bioventing flow rates can achieve greater contaminant reductions than venting alone [43].



5. Figure: Bioventing by air and nutrient injection
(retrieved from [44])

Some methods can be either "*in situ*" or "*ex situ*" methods. The soil or water can be removed from the contamination source and treated, or treated at the source, the method chosen can be based on many factors such as how expensive the project may be or how much the contaminant needs to be treated. **Land farming** and **biofiltration** and **bioaugmentation** belong to this category.

Landfarming

During landfarming, contaminated soil is mixed with amendments - such as nutrients - and then they are tilled into the earth. Moisturation and pH buffering with agricultural lime or crushed limestone can also be applied. Another method is when the contaminated soil is applied into lined beds and periodically turned over or tilled to aerate the waste and enhance aerobic biological degradation. The topmost layer is the area of concentration for this method, so it is not ideal for deeper remediation. Land farming differs from composting because it actually incorporates contaminated soil into soil that is uncontaminated [46]. The higher zone of remediation will typically contain primarily lighter hydrocarbons that can be volatilized. The material is periodically tilled for aeration to hasten remediation of any nutrients and allow more oxygen to act as electron acceptors, as well as allowing volatilization to occur. Contaminants are degraded, transformed, and immobilized by microbiological processes and oxidation.

Factors that may limit the applicability and effectiveness of the process include [46]:

- A large amount of space is required.
- Conditions affecting biological degradation of contaminants (e.g., temperature, rain fall) are largely uncontrolled, which increases the length of time to complete remediation.
- Inorganic contaminants will not be biodegraded.
- Volatile contaminants, such as solvents, must be pretreated because they would volatilize into the atmosphere, causing air pollution.
- Dust control is an important consideration, especially during tilling and other material handling operations.
- Runoff collection facilities must be constructed and monitored.
- Topography, erosion, climate, soil stratigraphy, and permeability of the soil at the site must be evaluated to determine the optimum design of facility.
- Waste constitutes may be subject to "Land-ban" regulation and thus may not be applied to soil for treatment by landfarming (e.g., some petroleum sludges).

Biofilters

In the soil, biofilters are primarily used for the filtration of contaminated groundwater. Microorganisms fixed to a porous medium are used in the biofiltration process to break down pollutants present in the wastewater stream. With the progress of filtration process, microorganisms (aerobic, anaerobic, facultative, bacteria, fungi, algae, and protozoa) are gradually developed on the surface of the filter media and form a biological film or slim layer known as biofilm. The filter bed medium consists of relatively inert substances which ensure large surface attachment areas and additional nutrient supply. The overall effectiveness of a biofilter is largely governed by the properties and characteristics of the support medium, which include porosity, degree of compaction, water retention capabilities and the ability to host microbial populations. The crucial point for the successful operation of a biofilter is to control and maintain a healthy biomass on the surface of the filter. Critical biofilter operational and performance parameters include microbial inoculation, medium pH, temperature, medium moisture, and nutrient content [47].

Bioaugmentation

Bioaugmentation is the addition of native and/or non-native microorganisms proliferated in laboratory conditions that have the ability to degrade the contaminants that are harmful to the indigenous microbiota. The microbial strains can originate from the treated soil or from microbial banks [148].

Bioaugmentation is ideal for soils with low number of microbes that are capable of degrading targeted pollutants and in soils where contaminant compounds require multi stepped remediation. Augmentation techniques have a great potential for aromatic compound remediation. The most important step in successful bioaugmentation is selection of proper microbial strains. The success of bioaugmentation is also strongly depends on the ability of inoculants to survive in contaminated soil, which may vary due to predation and an environment that does not identically mimic the lab it was grown in.

The effectiveness of bioremediation can be increased "*in situ*" by the previously mentioned **biostimulation**, when we increase the living conditions of the inoculated microorganisms or the native microbial population by adding nutrient supplements, minerals to the soil. By doing so the degradation/transformation capacity of the microorganisms can increase, or their fitness can improve, but by changing the pH of the soil we can also achieve better bioavailability of the contaminant to be degraded [148].

Another approach of bioaugmentation involves the use of not just single strain cultures, but rather **microbial consortia** consisting of many hydrocarbon-degrading microorganisms (for example a consortium of *Rhodococcus sp*., *Acinetobacter sp*., *Pseudomonas sp*. is effective for PAH degradation). In many cases consortia were more effective than single strains by the fact that intermediates of a catabolic pathway of one strain may be further degraded by other strains possessing suitable catabolic pathway [149].

Although bioaugmentation has many advantages, it is still faces many disadvantages, environmental problems, such as the survival of strains introduced to soil [39]. The number of introduced microorganisms usually decreases shortly after soil inoculation, when the pollutant has been heavily removed. Laboratory strains may spread only on a small area and their number decreases if the bioavailability is not appropriate. Competition can occur between microbial populations [148]. The number of the inoculated microorganisms can also be reduced by predation by protozoa and bacteriophages [149]. Despite this the introduced species may linger for long periods of time, a wider use of non-natives runs the possible risk of creating a monoculture in the soil. Also, a horizontal gene transfer can occur between the newly added and indigenous microbial species where the effect of changes in the population cannot be predicted [45].

4.3.3. Advantages and disadvantages of bioremediation

Based on Vidali, M. (2001) [36] the following advantages and disadvantages can be concluded about bioremediation processes:

Advantages

- Bioremediation is a publicly accepted treatment of polluted soils because it is based upon natural processes. Microbes that metabolize contaminants increase in population when the contaminant is present. The inverse is true, degradation of the contaminant causes population decline of those microbes.
- Usually the products from treatment are harmless; such as carbon dioxide, water, and cellular biomass.
- Bioremediation is theoretically meant to completely degrade a wide range of pollutants into harmless products on site. This removes the risks involved with transportation for treatment and elimination of contaminated substances.
- Bioremediation is meant to completely eliminate specific pollutants without the risks of transferring contaminants from one environmental medium to another (land, air, water).
- Bioremediation can be a cheaper alternative to other technologies used for pollution mitigation.

Disadvantages

- Only biodegradable compounds are capable of undergoing bioremediation. Not every compound is capable to be fully degraded.
- The products of biodegradation may potentially be even more persistent or toxic than the original contaminant.
- Biological functions are usually extremely specific and require the presence of microbes that are capable of metabolizing the contaminants. In order for the correct microbes to be present, the appropriate environmental conditions, levels of nutrients, and contaminants need to be met.
- Scaling up the size of studies from small initial studies to commercial-scale field operations is difficult, and results might not match.
- The real environment contains contaminants that are mixed, unevenly distributed, and in different phases (solid, liquid, gas). More research needs to be completed to create technologies that can adapt.
- Compared to other treatment technologies, bioremediation often takes more time.
- Problems with ensuring adequate contact between the microbes and the contaminant.
- Preferential pathway and soil structure can leave uncertainty in remediation dispersal.

4.3.4. *Phytoremediation*

Phytoremediation is a type of bioremediation, where plants and associated soil microbes are used to reduce the concentrations or toxic effects of contaminants in the environments. It is a relatively recent technology and is perceived as cost-effective, efficient, novel, eco-friendly, and solar-driven technology with good public acceptance [92].

Substances that may be subjected to phytoremediation include metals (Pb, Zn, Cd, Cu, Ni, Hg), metalloids (As, Sb), inorganic compounds (NO^{3-} NH^{4+}, PO_4^{3-}), radioactive chemical elements (U, Cs, Sr), petroleum hydrocarbons, pesticides and herbicides (atrazine, bentazone, chlorinated and nitroaromatic compounds), explosives, chlorinated solvents and industrial organic wastes and others [146]. Phytoremediation techniques include 6 different modalities, depending on the chemical nature and properties of the contaminant (if it is inert, volatile or subject to degradation in the plant or in the soil) and the plant characteristics. These 6 strategies are presented in **Figure 6**.

Phytodegradation (Phytotransformation)

Organic contaminants are degraded (metabolized) or mineralized inside plant cells by specific enzymes that include nitroreductases (degradation of nitroaromatic compounds), dehalogenases (degradation of chlorinated solvents and pesticides) and laccases (degradation of anilines). *Populus* species and *Myriophyllium spicatum* are examples of plants that have these enzymatic systems.

Phytostabilization (Phytoimmobilization)

Contaminants, organic or inorganic, are incorporated into the lignin of the cell wall of roots cells or into humus. Metals are precipitated as insoluble forms by direct action of root exudates and subsequently trapped in the soil matrix. The main objective is to avoid mobilization of contaminants and limit their diffusion in the soil. Species of genera *Haumaniastrum*, *Eragrostis*, *Ascolepis*, *Gladiolus* and *Alyssum* are examples of plants cultivated for this purpose.

Phytovolatilization

This technique relies on the ability of some plants to absorb and volatilize certain metals/metalloids. Some element ions of the groups IIB, VA and VIA of the periodic table (specifically Hg, Se and As) are absorbed by the roots, converted into non-toxic forms, and then released into the atmosphere.
Examples are the species *Astragalus bisulcatus* and *Stanleya pinnata* for Se or transgenic plants (with bacterial genes), and *Arabidopsis thaliana*, *Nicotiana tabacum*, *Liriodendron tulipifera* or *Brassica napus* for Hg can be mentioned. This technique can also be used for organic compounds.

Phytoextraction (Phytoaccumulation, Phytoabsorption or Phytosequestration)

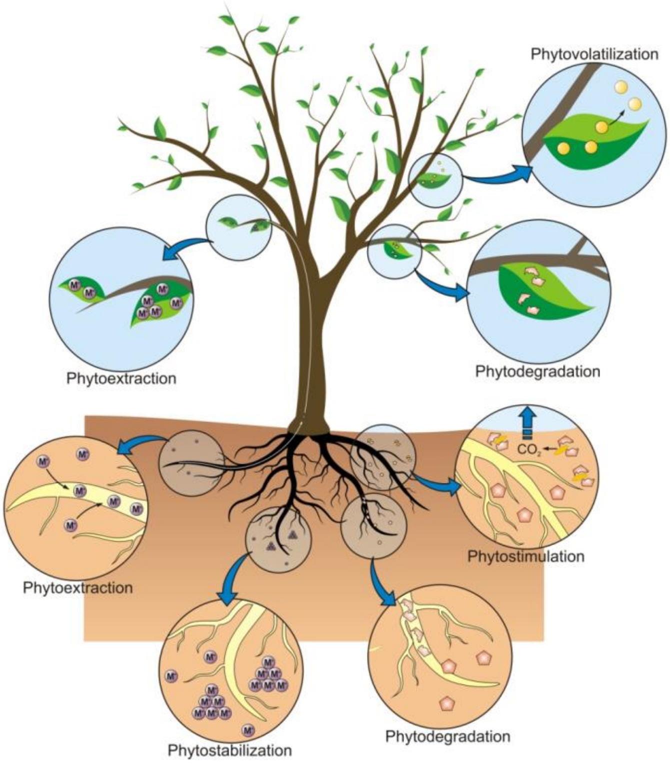
This involves the absorption of contaminants by roots followed by translocation and accumulation in the aerial parts. It is mainly applied to metals (Cd, Ni, Cu, Zn, Pb) but can also be used for other elements (Se, As) and organic compounds. This technique preferentially uses hyperaccumulator plants that have the ability to store high concentrations of specific metals in their aerial parts (0.01% to 1% dry weight, depending on the metal).
Elsholtzia splendens, *Alyssum bertolonii*, *Thlaspi caerulescens* and *Pteris vittata* are known examples of hyperaccumulator plants for Cu, Ni, Zn/Cd and As, respectively.

Phytofiltration

This uses plants to absorb, concentrate and/or precipitate contaminants, particularly heavy metals or radioactive elements, from an aqueous medium through their root system or other submerged organs. The plants are kept in a hydroponic system, whereby the effluents pass and are “filtered” by the roots (Rhizofiltration), or other organs that absorb and concentrate contaminants. Plants with high root biomass, or high absorption surface, with more accumulation capacity (aquatic hyperaccumulators) and tolerance to contaminants achieve the best results.
Promising examples include *Helianthus annus*, *Brassica juncea*, *Phragmites australis*, *Fontinalis antipyretica* and several species of *Salix*, *Populus*, *Lemna* and *Callitriche*.

Rhizodegradation (Phytostimulation)

Growing roots promote the proliferation of degrading rhizosphere microorganisms which utilize exudates and metabolites of plants as a source of carbon and energy. In addition, plants may exude biodegrading enzymes themselves. The application of phytostimulation is limited to organic contaminants. The microbial community in the rhizosphere is heterogeneous due to variable spatial distribution of nutrients, however species of the genus *Pseudomonas* are the predominant organisms associated with roots.



6. Figure: Phytoremediation strategies (derived from [146]).

4.3.5. Advantages and disadvantages of phytoremediation

Based on Paulo et. al. 2014 [146] and Mertens et. al. 2006 [147], the following advantages and disadvantages can be concluded about phytoremediation processes:

Advantages

- In situ and passive technique.
- Uses solar energy and is low cost.
- Has reduced environmental impact and contributes to the landscape improvement.
- High acceptance by the public.
- Provides habitat for animal life.
- Reduction in dispersal of dust and contaminants by wind.
- Reduction of surface runoff.
- Reduction of leaching and mobilization of contaminants in soil.
- Harvesting of the plants or organs that have accumulated metals is easy to accomplish with existing technology.
- The harvested biomass can be economically valuable.
- Plant process more easily controlled than those of microorganisms.

Disadvantages

- Limited to shallow soils or where contamination is localized to the surface (< 5 m).
- Still under development and therefore not accepted by many regulatory agencies.
- There is little knowledge of farming, genetics, reproduction and diseases of phytoremediating plants.
- Metal concentrations in the soil can be toxic and lethal to plants.
- Generally, plants are selective in metal remediation.
- Treatment slower than the traditional physico-chemical techniques.
- Contamination may spread through the food chain if accumulator plants are ingested by animals.
- Efficient phytoremediating plants may not adapt to climatic and environmental conditions at contaminated sites.
- Seasonality can affect phytoremediation processes.
- Aging of the plant can reduce the effectiveness of the processes.
- If the plants release compounds to increase the mobility of the metals, these can be leached into groundwater.
- The area to be decontaminated must be large enough to allow application of cultivation techniques.
- Toxicity and bioavailability of degradation products remain largely unknown.

5. METHODS AND MATERIALS

During the research period of 2010-2015 three research sites with three different types of contamination were analyzed, using similar and occasionally partly distinct research methods.

5.1. Research sites

5.1.1. Gardermoen, Norway (de-icing chemical contamination)

The Gardermoen aquifer, 40 km north of Oslo, Norway, is the largest rain fed unconfined aquifer in Norway. In 1998 the new main airport of Norway was opened here

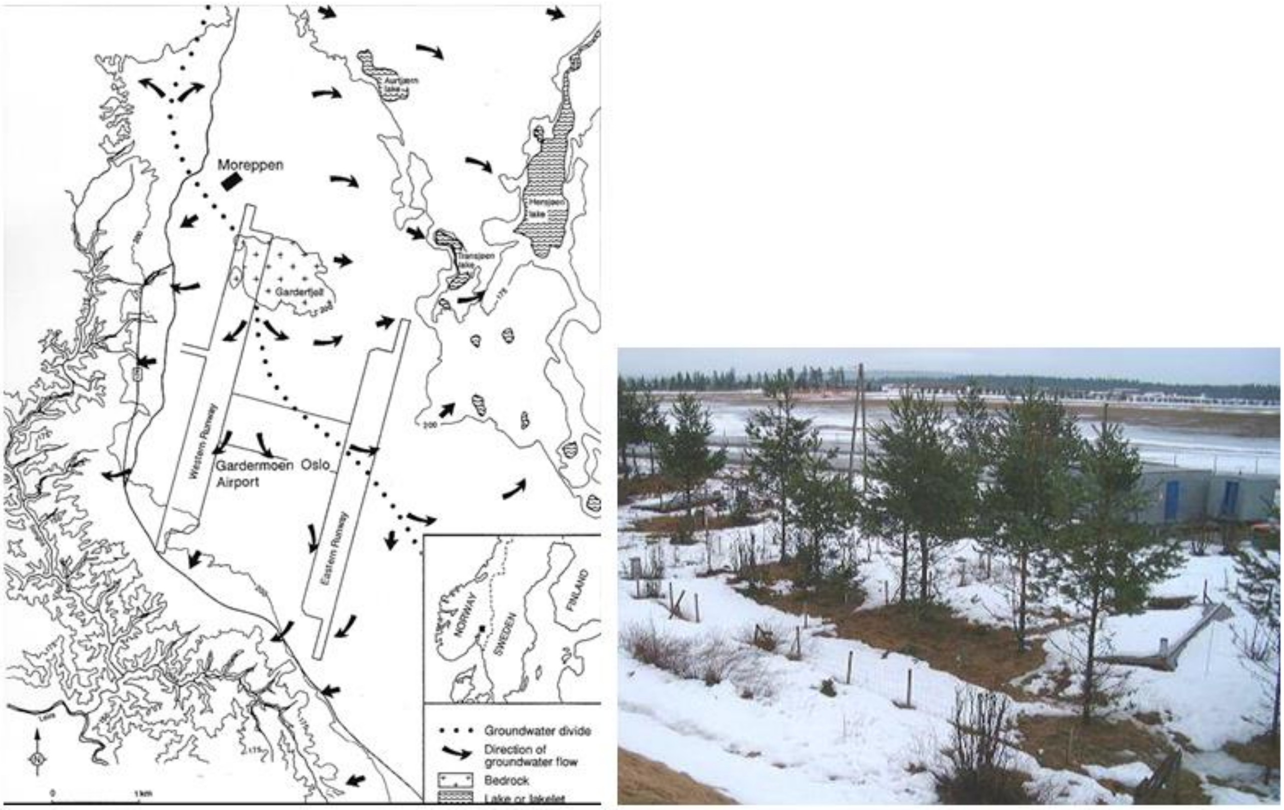
During the winter periods there is an intensive use of **propylene-glycol (PG) and formate (FO) based aircraft de-icing fluids** to prevent aircraft to freeze. Anti-icing of aircraft occurs in a remote platform [71]. During mechanical removal of snow from the runways and by the airborne drift of DF from the airplanes during take-off the DFs are mixed with snow (**Figure 7**). All de-icing chemicals used on the runways are spread diffusively along the runway.

7. Figure: Mechanical removal of snow from the runways in Oslo airport, Norway (SoilCAM project)

De-icing chemicals can infiltrate the sandy soil and groundwater during the snowmelt in the spring, causing pollution. Usually the groundwater in this area is 10 meters deep, but in higher grounds it can range between 2 and 20 m (SoilCAM Project, 2007).

There is an increased interest in monitoring the changes that these chemicals can cause in the microbe population found in these soils during the snowmelt. The de-icing chemicals are easily degradable, but may still threaten groundwater due to system overloading. The site is representative of contaminated sites where easily degradable and highly mobile contaminants are released to the soil.

There is a well equipped research station in the Oslo airport that was established in 1992 to be able to follow the spreading of de-icing fluids in the soil (**Figure 8**). It is necessary to increase the biological degradation of the chemicals in order to match the environmental regulations. So far several research projects have taken place in order to estimate the effects of aircraft de-icing fluids on the environment [93].



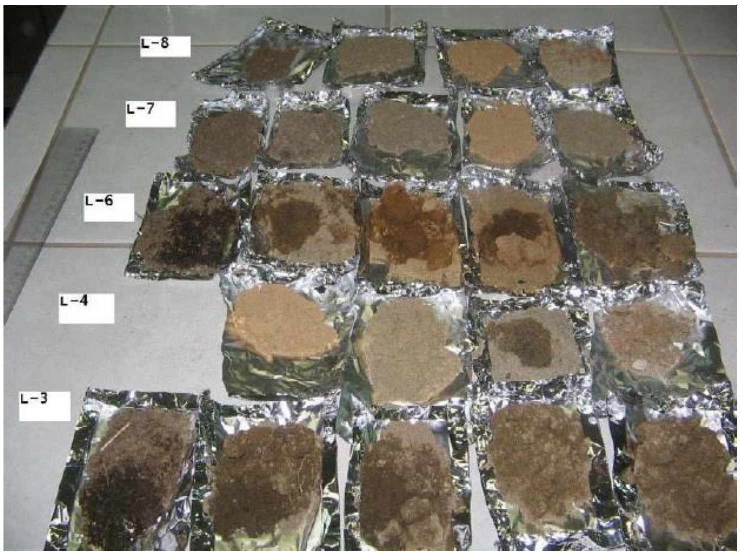
8. Figure: The airport covers much of the research study area. Moreppen is a field laboratory site for studying processes in the unsaturated zone

There are many types of de-icing chemicals which contain either propylene-glycol (PG) or ethylene-glycol with additional condensers, surfactants, corrosion inhibitors and UV sensitive coloring dyes. These de-icing fluids (DFs) are used to prevent aircraft from freezing during the cold seasons and discharged to the environment in large quantities, constituting a terrestrial and aquatic environmental risk.

Type 1 and type 2 aircraft DFs that are used at the Oslo airport are based on propylene-glycol [94]. Results from the 2005/2006 and 2006/2007 seasons indicate that it is mainly type 2 DF that is being dispersed to the area along the runways [71]. The primary concern regarding the environmental impacts of de-icing activities relates to oxygen consumed during the decomposition of de-icing fluids. The unsaturated zone acts as a natural purification system [93]. Although PG is easily degraded by soil bacterial communities, they still constitute a threat to the groundwater due to potential overloading of the system's degradative capacity if biodegradation rates are not sufficient compared to velocities in the unsaturated zone [95]. Aerobic microbial degradation is the most important environmental process affecting glycols in surface waters, whereas anaerobic metabolism of glycols is considerably slower. However, anaerobic degradation still needs to be taken account for because of the degradation activity's oxygen consumption that can turn the lower layer's environmental conditions anaerobic.

Contaminated soil samples used in our study were collected from the Gardermoen Airport site during two different campaigns: October 2008 and July 2009. The location of the site, where the soil samples are originating from can be found at the south-western part of the airport along the runway. The soil is being heavily contaminated with de-icing chemicals.

In October 2008, sampling for vertical profiling was performed overnight (when the airport was not operating) during lysimeter excavations near the western runway in a zone affected by DF pollution. The samples originated from 5 lysimeters and were collected in two replicates; one sample was used for aerobe microbial counts and the other for anaerobe microbial assessments. Anaerobic conditions were developed during transportation and storage by using Anaerocult sacks® (Darmstadt, Germany). Aerobic samples were applied for microbial counts of aerobic heterotrophs together with microscopic fungi, and also for enzymatic activity tests. After inspection of the soil profiles, one average sample per soil layer with the same soil properties was taken (as seen in **Figure 9**).



9. Figure: Vertical soil samples taken from the lysimeters of Gardermoen Airport research sites (photo taken by: Mónika Horváthné Domonkos, 2010)

Sample codes were consisting of the L and the number of the lysimeter, adding the number of the given soil layer, which corresponded to the depth of the layer, as seen in **Table 1**.

Horizontal samples were collected as 15 x 15 x 15 cm soil-plant blocks and transferred in this stage. Isolation boxes were used for the transportation of samples between the laboratories. The samples were stored at 5 °C prior until testing, preferably within one month.

During the studies the sampling points were not the same in 2008 and in 2009 due to the fact that we used the results of the first sampling campaign to adjust the distances of the second sampling campaign. In October 2008 samples were taken from about 15 cm deep at 10, 30, 60, and 150 m from the runway. The horizontal samples from 2008 were tested for fluorescein diacetate (FDA) enzymatic activity (no code-names were given). The second surface soil sampling campaign was done in July 2009, during which the sampling was done from the same depth at 0.2, 17, 27, 47, and 87 m from the runway (**Figure 10**). These samples were coded BB1-BB5 and were compared for total abundancy and amount of PG degraders.

Soil sample specimens were stored until use in plastic bags at 2-6^{o}C, for preferably no more than 2 weeks.

1. Table: Gardermoen Airport soil sample codes

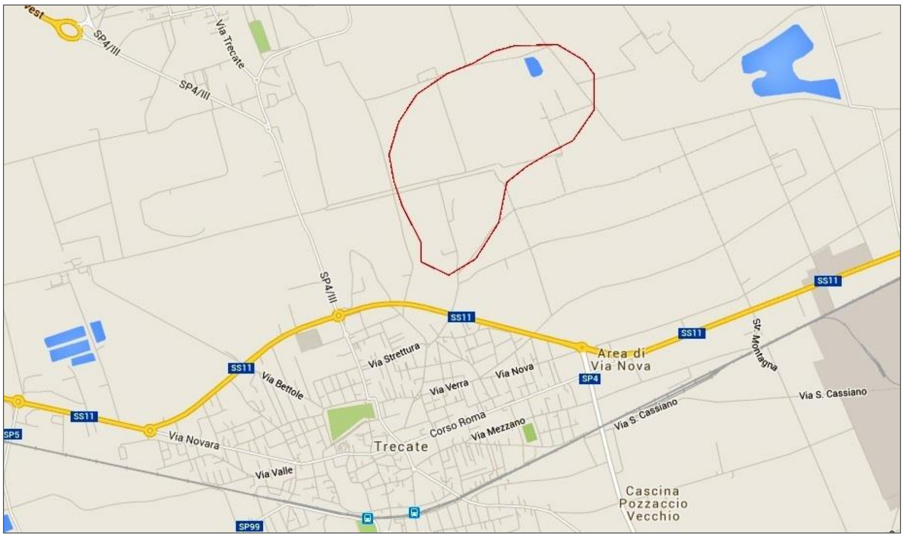
	Sample code	Depth of sample [cm]		Sample code	Depth of sample [cm]
Lysimeter 3	L3-1	0	**Lysimeter 7**	L7-1	0
	L3-2	32		L7-2	20
	L3-3	50		L7-3	40
	L3-4	80		L7-4	70
	L3-5	110		L7-5	80
Lysimeter 4	L4-1	30	**Lysimeter 8**	L8-1	0
	L4-2	57		L8-2	22
	L4-3	85		L8-3	60
	L4-4	110		L8-4	90
	L4-5	-		-	-
Lysimeter 6	L6-1	0			
	L6-2	20			
	L6-3	55			
	L6-4	82			
	L6-5	100			

10. Figure: Horizontal soil sampling locations of Gardermoen Airport research sites. Sample codes were BB1: 0.2 m, BB2: 17 m, BB3: 27 m, BB4: 47 m and BB5: 87 m (SoilCAM).

5.1.2. Trecate, Italy (crude-oil contamination)

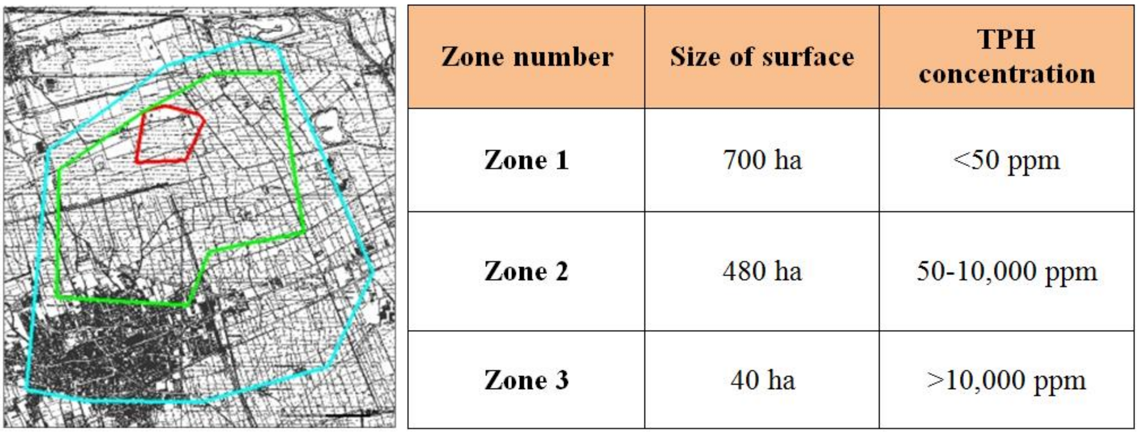
The test site of Trecate is part of the plain aquifer at Trecate in the Piemonte region of Italy (**Figure 11**). Groundwater levels at the site fluctuate by 6 m seasonally, with higher levels experienced during the summer period due to the surface recharge from agricultural irrigation practices. In 1994 the site was the scene of an inland crude oil spill following an oil well blow-out. The contamination resulted in approximately 15000 m^3 of middleweight **crude oil** being released overland contaminating both soil and groundwater.

The impacted area is devoted to agriculture and it has been used for producing high quality rice crops for more than a century. Following the preliminary clean-up bioremediation was selected as remediation approach. This was due to the fact that the Authorities and farmers' associations requested the impacted soil to be remediated without altering or changing its agricultural properties. The project was mainly based on in situ bioremediation. Preliminary identification of the soil's existing bacterial strains in the polluted area was performed as well as tractability tests at the laboratory scale, which confirmed that biodegradation is feasible through the soil's native bacterial communities.



11. Figure: Location of the Trecate test site where the oil blow-out occurred (retrieved using Google Maps)

The chemical composition of the crude oil contaminating the soil of the Trecate site is detailed in **Table 2**. During the SoilCAM project initially three different pollution zones were identified according to total petroleum hydrocarbon (TPH) values in the soil (**Figure 12**).



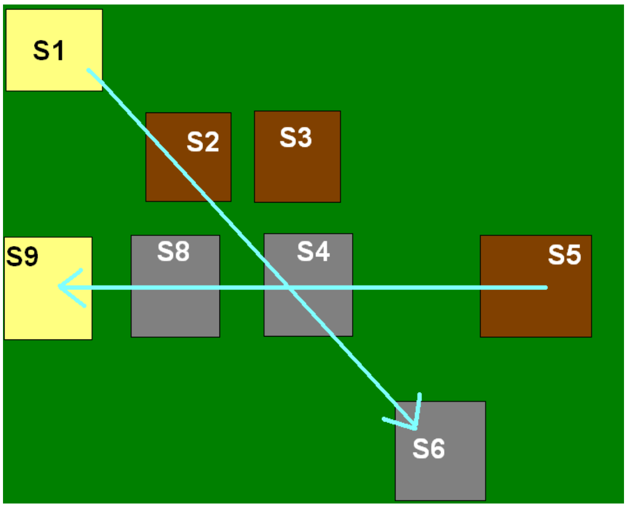
Zone number	Size of surface	TPH concentration
Zone 1	700 ha	<50 ppm
Zone 2	480 ha	50-10,000 ppm
Zone 3	40 ha	>10,000 ppm

12. Figure: Pollution zones of the crude-oil contaminated Trecate site. Zone 1 lies between the green and blue border, Zone 2 is between the red and green line, and Zone 3 is the red zone in the middle.

2. Table: Chemical composition of the crude-oil contamination of the Trecate site

Component	%
Water	0.10
H_2S	0.01
Hydrocarbons	88.0
Gaseous fraction, of which:	11.0
- Methane	4.0
- C2-C4 compounds	7.0
Liquid fraction	77.0
Polycyclic aromatic hydrocarbons (PAH)	8.0
Sulfur organic compounds	0.16
Light fuel	6.50
Total fuel	32.50
Kerosene	20.0
Diesel fuel	11.0
Lubricating oils	16.50
Residue	18.0
Loss	2.0

Samples were collected from 5 boreholes of the Trecate site (**Figure 13**), ranging from the soil surface up till the 12m deep soil layers. The excavation was executed in 1.2 m long soil-column specimens at each site. Sampling has been performed between 9-11th of February 2011. Among the sample points the borehole S1 is located on the northern part of the site, and this point is considered as uncontaminated with crude-oil. Those samples are used therefore as control throughout in the site-monitoring of Trecate, 2011 February. The borehole S4 was in the middle of the area, the borehole S6 could be found on the Northern-Southern line, and the borehole S8 was located on the Eastern-Western line of the area.



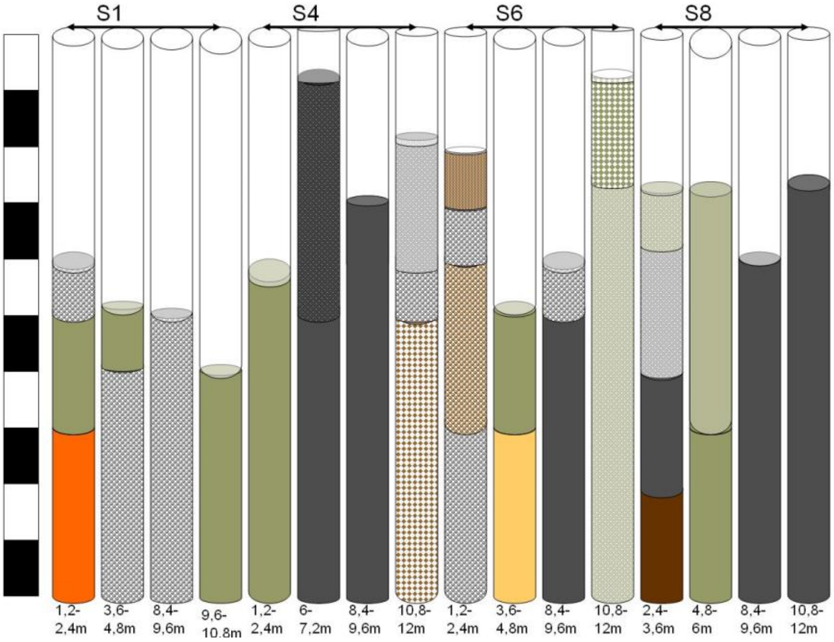
13. Figure: The location of the sampling boreholes in the Trecate field site. The blue arrows show the direction of the belowground water flow. The yellow sites are not polluted (control sites), the grey ones are crude-oil contaminated, and the brown sites have not been sampled for microbiological investigations.

Soil samples were collected from boreholes according to **Figure 13** from a 0-12 m depth from the soil surface. The water level of the subsoil is generally at about 10 meters deep, but later in the summer period it goes up to 5-6 meters. The samples therefore were divided to three parts on the basis of the water fluctuation and the pollution level of the subsoil:
1) the upper unsaturated zone ranging from 0 to 3.6 m deep layer
2) sub layer of 3.6-8.4 m, as the smear zone due to the water fluctuation and
3) the deepest layer of 8.4-12m deep, generally as saturated oil-polluted soils.

Soil-columns S1, S4, S6, S8 and S9 were sent to the RISSAC HAS from the Trecate site at the 9th of February 2011 to be tested for microbiological parameters.

Before the final storage the soil specimens in the refrigerator on 5°C, the columns were visually investigated on the basis of the quantity and quality of soils in the columns S1, S4, S6 and S8 (samples that were used for the microbiological research and one control sample). The S9 control column was not opened, as was stored right away as an extra control sample.

Results of the visual examinations are shown in **Figure 14**. The differences in colour and particle size are shown on the bases of visualization. Microbial investigations were performed from uniform, homogenized layers.



14. Figure: Visual separation of uniform layers by color and particle size of soil samples from the Trecate site (created by Mónika Horváthné Domonkos, 2011)

After the visual inspection of the soil columns, they were separated on the basis of differences in their particle sizes, in colours and also if contaminated or not. This was done in order to aid other research aspects.

As it is previously was stated, the boreholes of the S4, S6 and S8 sample points were highly contaminated with crude oil, and therefore they have been chosen for microbiological analysis, however along with the S1 control sample, only the S4 and S8 samples were processed at the start of the research. The S6 sample was stored, and was used later for isolation, identification and antimicrobial susceptibility tests, when the research progressed further. The following **Table 3** summarizes the samples, their properties and tests which they were used for:

3. Table: Trecate soil sample coding and performed tests

Sample borehole number	Crude oil contamination	Tested for
S1 (control)	no	Microbial abundance, FDA
S4	yes	Microbial abundance, FDA
S6	yes	Isolation, Microbial identification, Antimicrobial susceptibility testing
S8	yes	Microbial abundance, FDA
S9 (control)	no	Not tested, stored

5.1.3. Őrbottyán, Hungary (Manganese treatment)

The Mn-mine of Úrkút is producing tremendous annual amount of **Mn-sludge**. The utilization of sludge is potentially possible in agriculture; however, the effect of Mn-content need to be preliminary studied. Among field conditions, a so-called lysimeter experiment was set up with Mn-sludge treatments.

The experimental site of the Institute for Soil Sciences and Agricultural Chemistry is found between the Danube and Tisza River, the northern part of the sand ridge, close to the hillside of Gödöllő, in Őrbottyán, Hungary. The GPS location is shown on **Figure 15**.

15. Figure: Location of the Experimental site of the Institute for Soil Sciences and Agricultural Chemistry (retrieved from Google Maps)

The soil of the site is mostly mildly humous, with 0.8-1% humus and 1-5% $CaCO_3$ content. The soil is slightly alkaline, and poor in nutrients, as shown in **Table 4**.

4. Table: The physical-chemical properties of the sandy-soil of Őrbottyán, Hungary

pH (H_2O)	**pH (KCl)**	**PI**	**Salt %**	**$CaCO_3$ %**	**Humus %**	**Total N %**	**Total P $mgkg^{-1}$**
7.92	7.75	26.0	0.002	6.13	0.46	0.048	381.0
Al-K_2O $mgkg^{-1}$	**Al-P_2O_5 $mgkg^{-1}$**	**KCl-Mg $mgkg^{-1}$**	**NH_4-N $mgkg^{-1}$**	**NO_3-N $mgkg^{-1}$**	**EDTA Cu $mgkg^{-1}$**	**EDTA Zn $mgkg^{-1}$**	
31.50	74.0	37.80	3.05	1.75	0.855	0.699	

The soil of the research site was **originally not contaminated**. It has been **artificially polluted** by applying Mn-sludge originating from the Úrkút mine. As treatment in the separate lysimeters, increased doses of Manganese-containing sludge were used (except the control).

Manganese is essential to living organisms, and it is required to activate enzymes in plant photosynthesis [122], so only at higher levels can it be categorized as "contamination". In some Manganese rich soil layers even up to 3000 $mgkg^{-1}$ Mn can be found. Manganese in soil usually appears in different compounds. Some of these are soluble (Manganese-sulfate, Manganese-chloride), the other part is insoluble (Manganese-oxides, Manganese-carbonate) and often appear alongside ferric-oxides [124; 125].

Manganese appears in valence II, III and IV, silicates, carbonates and oxides [126]. Manganese-oxides can affect the accumulation of heavy metals, for example Co, Ni, Zn, Cd and Pb [127; 128]. During soil development Manganese migrates and washes out of the soil better than iron. Because of this acidic soils can be very poor in Mn [124]. Manganese levels can also drop because of agricultural usage. According to [129], drops of Mn levels were realized less than 20 years in sandy soils (lamellicarenosols) layer 'A'. The different Manganese valences can transform into each other in the soil and they can form organic complexes in the soil solution, from which other kations can remove them. Manganese affect reduction processes positively, so it can change microbial abundance depending on the doses. With several organic compounds Mn creates complexes that are insoluble and not usable by plants [125]. In these processes microorganisms also take part. In several occasions Mn-shortage can interfere with microorganisms degrading organic compounds, including contaminants, mostly in deeper, anaerobic soil layers. The amount of microbial populations can change according to Mn content of the soil, they can either help or prevent Manganese uptake for plants. The role of microorganisms in energy consumption of soils and soil quality is very important [20; 130; 131; 132; 133; 134; 135; 136].

There were 12 lysimeters dug in the ground on the experimental site in Őrbottyán (**Figure 16**). The radius of the lysimeters is 0.8 m and the surface is 0.5 m^2. The Mn-sludge was inserted and homogenized in the upper 20 cm of the carbonaceous sandy-soils according to the following treatments: 0: without Mn-sludge, 1: 500-, 2: 1000-, 3: 2500-, 4: 12500 $mgkg^{-1}$ Mn dose homogenized with the soil. The composition of the Mn-sludge can be seen in **Table 5**.

5. Table: Chemical composition (%) of Úrkút Mn-sludge

<table>
<tr><td>SiO_2</td><td colspan="2">29.0-33.0</td><td>CaO</td><td>3.0-7.0</td></tr>
<tr><td>TiO_2</td><td colspan="2">0.3-0.4</td><td>MgO</td><td>2.0-4.0</td></tr>
<tr><td>Al_2O_3</td><td colspan="2">6.0-10.0</td><td>K_2O</td><td>2.0-3.0</td></tr>
<tr><td>Fe_2O_3</td><td colspan="2">22.0-26.0</td><td>Na_2O</td><td>0.2-0.3</td></tr>
<tr><td>MnO_2</td><td colspan="2">13.0-19.0</td><td>P_2O_2</td><td>0.4-0.5</td></tr>
<tr><td>MnO</td><td colspan="2">2.0-3.0</td><td>Organic C</td><td>1</td></tr>
<tr><td>BaO</td><td colspan="2">0.05-0.1</td><td>LOI</td><td>8.5</td></tr>
<tr><td colspan="3">Major trace elements</td><td colspan="2">As, B, Cd, Co, Cr, Cu, V, Li, Ni, Pb, Zn, Sr, Sc</td></tr>
<tr><td colspan="5">Partial chemical analysis (%)</td></tr>
<tr><td>Mn</td><td>Fe</td><td>SiO_2</td><td>P</td><td>S</td></tr>
<tr><td>10.13</td><td>15.0-18.0</td><td>29.0-33.0</td><td>0.2</td><td>0.1</td></tr>
</table>

The first set of lysimeters were only treated with the increased doses of managanese sludge, while a second set of treatments increased additional mycorrhiza fungi.

Lysimeter treatment properties were according to the following (summarized in **Table 6**):

(a) “A” property: mycorrhiza
  1. mycorrhiza +
  2. mycorrhiza –

(b) „B” property: Mn-sludge concentration in the lysimeter ($mgkg^{-1}$)
  1. Control: 0 $mgkg^{-1}$
  2. Control: 0 $mgkg^{-1}$, with additional benomyl (this lysimeter was not tested; it was part of a different research).
  3. 500 $mgkg^{-1}$
  4. 1000 $mgkg^{-1}$
  5. 2500 $mgkg^{-1}$
  6. 12500 $mgkg^{-1}$

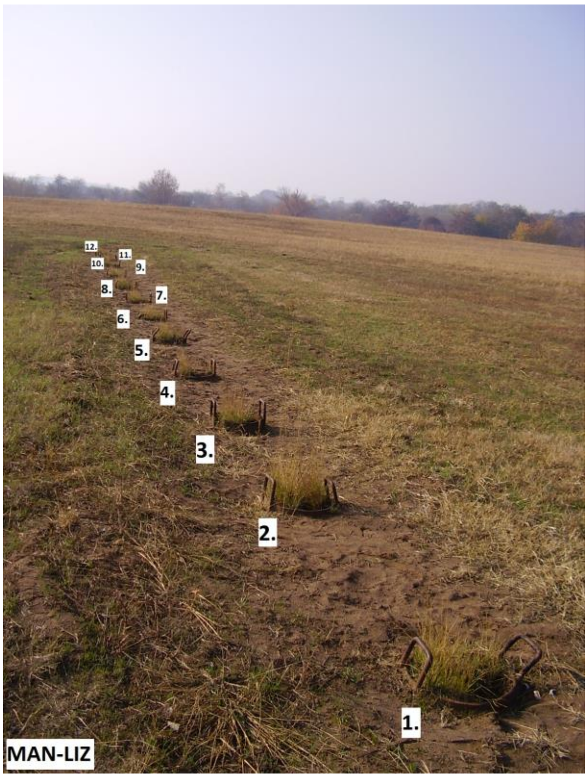
6. Table: Treatments and increasing Mn-doses in lysimeters at Őrbottyán, Hungary

Lysimeter number	**"A" (Myc)**	**"B" (Mn-sludge $mgkg^{-1}$)**	**Lysimeter number**	**"A" (Myc)**	**"B" (Mn-sludge $mgkg^{-1}$)**
1	+	0	7	-	0
2	+	0	8	-	0
3	+	500	9	-	500
4	+	1000	10	-	1000
5	+	2500	11	-	2500
6	+	12500	12	-	12500

Myc = inoculated (+) or not (-) with arbuscular mycorrhizal fungi

After the Mn-sludge and mycorrhiza treatments, Szarvasi 1 Energy grass (*Elymus elongatus L–Szarvas-1*) seeds were planted evenly on the lysimeters. They have been watered until sprouting.

Soil samples were gathered from lysimeters 1, 3, 4, 5, 6, 7, 9, 10, 11, 12 on the 19th of October 2011 and 23rd of August 2013. Each time 1 quarter 10 cm deep soil block was removed from the 10 open-pots, which contained the vegetation also. Soil samples have been stored on 2-6 °C until usage. Samples were not coded; they were either referred to on the basis of the number of the lysimeter, or according to the concentration of the manganese in the given lysimeter (see above). To differentiate from other site's samples ML or MAN-LIZ (short for Manganese-Lysimeter) was added before the sample numbers.



16. Figure: Open-pot experiment set-up in the Experimental site of the Institute for Soil Sciences and Agricultural Chemistry in Őrbottyán, Hungary (photo taken by: Nikoletta Horváth, 2012)

5.2. List of the performed analytical tests

The following table lists and summarizes all the tests performed on the soils of the three research sites:

Site	Test name	Method	Purpose of the test
Gardermoen, Norway	**Microbial abundance**	Traditional plating method on solid medium. Performed by the author and H. Wass-Matics. (Chapter 5.3.)	To estimate the number of different microbial communities, like heterotrophic bacteria, anaerobes and fungi in the contaminated soil, and connect their numbers to different contamination levels.
Trecate, Italy			
Őrbottyán, Hungary			
Gardermoen, Norway	**Total heterotrophic microbial abundance or specific degraders**	Most Probable Number method. Performed by the author and H. Wass-Matics. (Chapter 5.4.)	To statistically estimate the number of microorganisms capable to grow on specific liquid medium or sole carbon sources added to basal medium.
Őrbottyán, Hungary			
Gardermoen, Norway	**Total Organic Carbon content (TOC)**	Fumigation-extraction. Performed by a commissioned analytical laboratory. (Chapter 5.5.)	Organic carbon content can be used to measure microbial activity.
Gardermoen, Norway	**Microbial enzymatic activity**	FDA-β-glycosidase-hydrolase-dehydrogenase- analysis. FDA analysis was performed by the author and M. Horváthné Domonkos. Other enzymatic activity tests were carried out by project partners from the University of Naples Federico II. (Chapter 5.6.)	Enzymatic activities often show a significant positive correlation with numbers of soil microorganisms and amounts of organic carbon and together can be used as a measure of total microbial activity.
Trecate, Italy			
Őrbottyán, Hungary			
Gardermoen, Norway	**Column experiment**	Complex simulation tests, consisting of: Microbial abundance, tolerance tests and molecular community analysis. Performed by the author together with project partners from the FSU Jena. (Chapter 5.7.)	To receive an overview on how the microbial population changes and adapts to de-icing chemical (DIC) treatment.

Site	Test name	Method	Purpose of the test
Gardermoen, Norway	**Molecular population analysis**	Total microbial DNS extraction, preparation of clone libraries. Performed by the author with the help of project partners from the Friedrich-Schiller University (FSU) of Jena. (Chapter 5.8.1.)	The community structure of the soil before and after DIC treatment during our column experiments has been tested to achieve an overview on changes as the effect of de-icing chemical pollution.
Trecate, Italy Őrbottyán, Hungary	**Molecular identification of the most abundant colonies**	Total microbial DNA extraction was performed by the author and E. Farkas. Sequencing and identification have been carried out by a commissioned laboratory. (Chapter 5.8.2 and 5.8.3)	Identification is part of the description of the chosen isolates. Human pathogenic, objectionable microorganisms (with known antibiotic resistance) should be excluded from further research, because they are not to be used as inoculates.
Trecate, Italy	**Antibiotic susceptibility testing**	Kirby-Bauer / disc diffusion antibiotic testing. Performed by the author. (Chapter 5.9.)	To exclude isolates with antibiotic resistance. These strains should not be used in the soil because of a possible horizontal gene transfer.
Őrbottyán, Hungary	**Energy grass Manganese uptake**	Aqua regia digestion, carried out by a commissioned analytical laboratory [145].	To measure the amount of Manganese that was uptaken by the Energy grass plant used for phytoremediation of Manganese treated soils.
Őrbottyán, Hungary	**Plant biomass**	Traditional weighing by scale, performed by the author and H. Wass-Matics.	To estimate the growth of Energy grass plant on Manganese treated soil (treated with different doses of Manganese) we measured the weight of the plant biomass. This can show the effect on Mn on the energy grass growth.

5.3. Microbial counts using traditional Plate Count method

Microbial counts of the heterotrophic population can be used to estimate the overall microbiological activity of soil, which is useful to estimate both biodegradation capacity and toxicity effects. However, the assessment of the number and diversity of soil microbes is notoriously vitiated by the non-cultivability of a relevant fraction of microbial population in general purpose media [68] and the use of different culturing media and techniques or genomics is necessary to detect the microbial diversity [69].

Microbial abundance in the soil samples was measured by plating out 10-fold serial soil dilutions in 0.9% NaCl on standard nutrient for total heterotrophic bacteria and Rose-Bengal (SIGMA-Aldrich) for microscopic fungi agar plates. Anaerocult sacks (Merck) and anaerobe jars were used for the anaerobic bacterial counts. The 10^{-1} dilution was prepared by suspending 1g of soil in 9 ml 2mM tetrasodium-pyrophosphate and vortexed for two minutes to enhance the separation of bacterial cells and soil particles. Plates were incubated for 7 days at 22°C with at least two (but preferably three) parallel plates per soil sample. Colonies growing on the solid medium can be counted and the Colony Forming Unit (CFU) numbers of the microbial cells can be estimated (**Figure 17**). Strains isolated from the contaminated site showing highly abundant positive growth were isolated.

17. Figure: Example of microbial growth on solid medium in a Petri-dish (settle plate)
(photo taken by: Nikoletta Horváth, 2012)

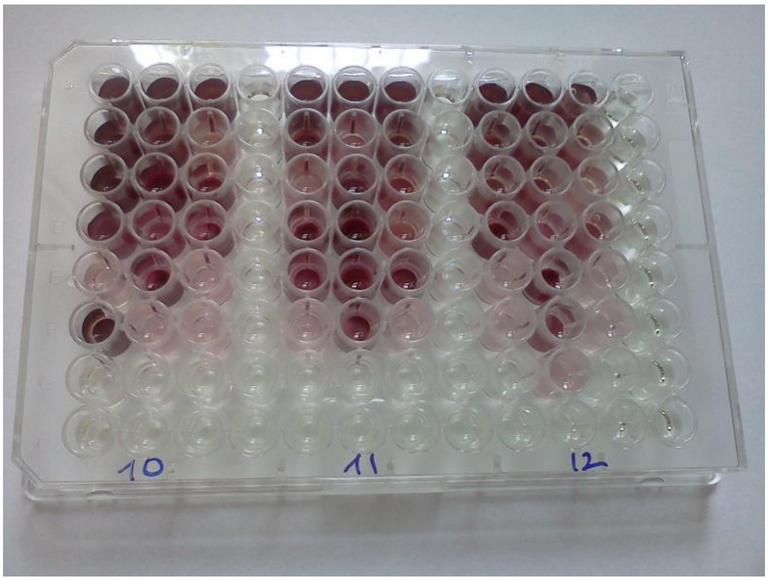
5.4. The MPN method

MPN (Most Probably Number) is a statistical method of distribution of microorganisms in solutions. Under preparation of a dilution series and observed growth, it is possible to calculate the probable number of cells. The dilution series can be prepared in 3, 5 or 10 fold replicates [70] using a 96 wells assay (ELISA) plates. For exact calculation of cell numbers all dilution steps should be included. The first dilution is the addition of 9 ml NaCl-solution to 1g of soil (dilution w/v).

All sort of cultivation media can be used, general standard nutrient, specific, selective culture media (not differentiating, because in a liquid solution the separation of cells cannot be seen) or a basal medium (consisting of materials only enough for bare survival of cells) capped with any specific carbon source to check degradability.

The presence of contaminants, that are toxic to micro-organisms, may hinder degradation. During soil bioremediation, using the contaminant as the sole carbon and energy source is considered favorable because complete oxidation to carbon dioxide and water can occur. These sole carbon source utilization patterns were originally developed for characterization of clinical bacterial isolates and not for the community analysis, but now this method can be used successfully to assess potential metabolic diversity of microbial communities in contaminated sites [83]. Every bioremediation process can benefit from the presence of microorganisms with the capability to tolerate and rapidly mineralize the target xenobiotic molecules [72].

After the defined incubation time of an MPN set-up, results can be evaluated by reading the 96 wells plates with either an ELISA-Reader, or cell growth can be visually measured with additional dying of the cells, for example by using iodonitro-tetrasolium, INT (**Figure 18**). The data can be exported to Excel.



18. Figure: Microbial growth (dyed with INT) as seen in a 96 wells microplate
(photo taken by: Nikoletta Horváth, 2015)

5.4.1. Monitoring of the rate of degraders in Gardermoen

Most probable number of soil bacteria capable of growing on 10000 ppm type 2 DF as a sole carbon source was determined by MPN method. 10-fold serial soil dilutions were made in basal medium (NaCl, 0.8 gl^{-1}; NH_4Cl, 1.0 gl^{-1}; KCl, 0.1 gl^{-1}; $MgSO_4(7\ H_2O)$, 0.02 gl^{-1}; KH_2PO_4, 1.35 gl^{-1}; K_2HPO_4, 1.75 gl^{-1}; $NaHCO_3$, 1.5 gl^{-1}; TES puffer, 4.6 gl^{-1}) from 10^{-1} to 10^{-8} dilutions as described by LIBISCH et al. 2010 [96] and then 10000 ppm DF was added. Five parallel 200µl aliquots from each dilution were transferred to sterile polystyrene 96-well microplates. Controls included inoculated dilutions with basal-medium only, without DF. After incubation at 22°C for 2 and 4 weeks the plates were tested for growth using the respiration indicator iodonitro-tetrazolium violet (INT) (Sigma-Aldrich), as described by JOHNSEN and HENRIKSEN, 2009 [97]. Approximately 50µl INT solution (3g/l INT dissolved in water) was added to each well, and the plates were incubated overnight at 22°C. Metabolically active bacteria reduce INT to the corresponding formazan forming a purple precipitate. The number of growth-positive wells at each dilution was determined by visual inspection of the plates. The statistical method of Cochran was applied to calculate MPN values using the MPN calculator [98].

5.4.2. Manganese tolerance tests in the soil of Őrbottyán

The overall tolerance of the microbial population in non-treated and the soil treated with 12500 $mgkg^{-1}$Mn dose have been measured by adding increased doses of Manganese (0, 1250, 2500, 5000, 7500 and 10000 $mgkg^{-1}$) to liquid bacterial medium (standard nutrient). After incubation, the Most Probable Number (MPN) of these microbes was checked in 96-well microplates.

5.5. Total Organic Carbon measurements

The fumigation-extraction method was used to estimate biomass C with extractable C converted to microbial C using standard factors, according to [102]. TOC measurements were performed by means of a TOC analyzer (TOC-VCSH with Solid Sample Module SSM-5000A by Shimadzu Co., Kyoto, Japan).

5.6. Enzymatic activity

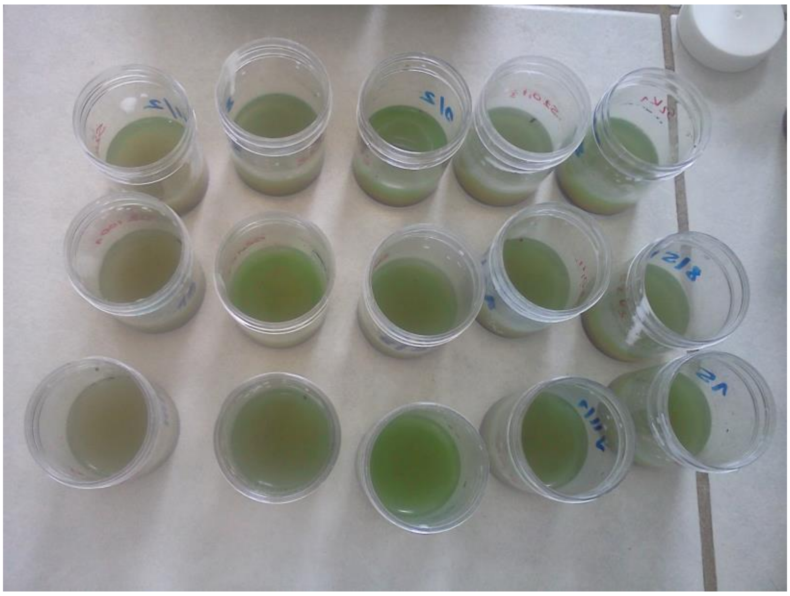
Soil enzyme activities are the direct response of the soil community to metabolic requirements and available nutrients [73]. However, since enzyme activities are generally substrate-specific, the measurement of a single enzyme activity cannot be related alone to the overall microbiological activity of soil, which includes a broad range of enzyme reactions [74].

The measurement of hydrolase activities required for carbon assimilation, such as β-glycosidase (involved in cellulose degradation) may also be affected by extracellular enzymes immobilized by soil colloids and not pertaining to viable microbes in soil.

Dehydrogenase activity is expressed by intracellular enzymes that occur in every viable microbial cell and is often measured to determine overall microbiological activity of soil. The problem with this measurement is that the electron acceptors (2, 3, 5-triphenyltetrazolium chloride (TTC) or 2-p-Iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT)) used in the assays are not very effective, and thus the measurements may often underestimate the true dehydrogenase activity [75].

Fluorescein diacetate (FDA) hydrolysis is another widely accepted method for measuring total catabolic microbial activity in soils and soil-plant systems [76]. The amount of fluorescein produced by the hydrolysis of FDA was proven to be directly proportional to the growing microbial population in several studies [77; 78]. **Figure 19** demonstrates this reaction. And indeed, non-specific esterases, proteases and lipases, which have been shown to hydrolyse FDA, are widespread among the major decomposers (bacteria and fungi) in soil. Therefore, FDA hydrolase assays are widely employed to monitor the effect of organic and inorganic contaminants on soil microbial activity [79; 80; 81].

In summary, enzymatic activities often show a significant positive correlation with numbers of soil microorganisms and amounts of organic carbon [82] and together can be used as a measure of total microbial activity. Correlation analysis can be used to evaluate the interrelation between the measurements.



19. Figure: The amount of fluorescein produced by the hydrolysis of FDA can be directly proportional to the size of the microbial population (photo taken by: Nikoletta Horváth, 2011)

5.7. Column experiments

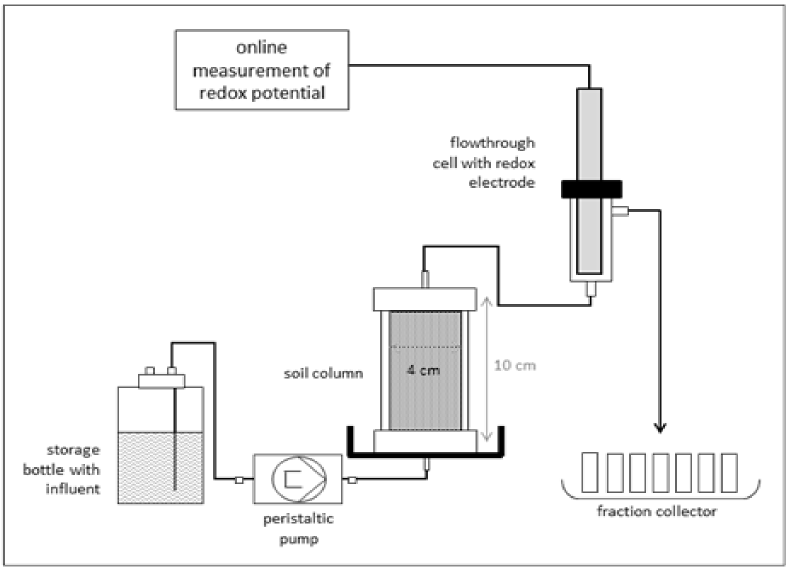
This experiment has been executed as a joined project of the Institute for Soil Sciences and Agricultural Chemistry of Hungary and the Friedrich-Schiller University of Jena (Institute of Geosciences and Institute of Microbiology - microbial Phytopathology) still within the framework of the SoilCAM Eu FP project.

The column experiments were conducted with homogenised soil obtained by ram drilling nearby the western run of Gardermoen. The subsoil samples had a silty sand texture and derived from a depth of 30 to 70 cm.

The experiments were run using six stainless steel columns (volume 170 ml, length 10 cm) (**Figure 20 and 21**). A peristaltic pump was used to pump the influent to the columns. The soil columns were saturated from bottom to top using a flow rate of 0.5 pore volumes (PV) per day (0.7 mlh^{-1}) to prevent air entrapment. After saturation the flow rate was increased to 1.5 PV (2.1 mlh^{-1}). To account the effect of low temperatures during snowmelt on degradation, the experiment was conducted at 4°C. The background solution (BGS) contained 6.9 mgl^{-1} $NaNO_3$, 27.0 mgl^{-1} $CaSO_4$ x 2 H_2O, and 1.6 mgl^{-1} $KHCO_3$. Bromide (1 mmoll^{-1} LiBr) was used as a tracer to determine column flow characteristics. Bromide and the de-icing chemicals (DIC) Propylene-glycol (PG) and Formate (FO) were added to the background solution of the six soil columns (**Table 7**).

The columns were treated as followed: 50 mgl^{-1} PG and 500 mgl^{-1} FO (S1 and S2), 50 mgl^{-1} PG without formate addition (S4 and S5), control without treatment (S3). During the second pulse column S6 served as control as well, but switched to an increased 1000 mgl^{-1} concentration of PG during pulse 3. Effluent fractions were collected in 3-5 times/PV using a fraction collector and they were sent for microbial and chemical analysis to check the changes in total heterotrophic cell numbers compared to the initial soil material. The effluent fractions were also analyzed for pH, total organic carbon, bromide, formate and PG content.

MPN method was used for the estimation of the total microbial abundance. The set-up of such a plate is demonstrated on **Figure 22**. MPN based analysis has been made also in order to see the tolerance level of microbes against PG and formate found in these soils by adding 500 – 20000 mgl^{-1} PG and FO as sole carbon source to the culture medium.

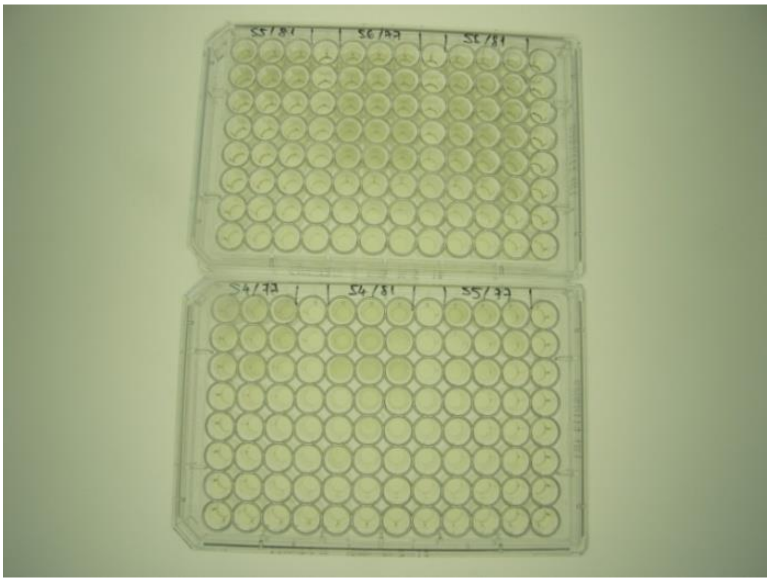


20. Figure: Sketch of a column set-up for the column experiment with propylen-glycol contaminated soil

21. Figure: Photo of column set-up in a 4°C tempered fridge during the column experiment with propylen-glycol contaminated soil (photo taken by: Heidi Lissner, 2012)

7. Table: Bromide and DICs (PG, FO) addition during 3 pulse sequences of the column experiment.

	Pulse duration	**S1 & S2**	**S4 & S5**	**S3**	**S6**
Pulse 1	17.02.2012-21.02.2012	1 $mmoll^{-1}$ Br	1 $mmoll^{-1}$ Br	1 $mmoll^{-1}$ Br	1 $mmoll^{-1}$ Br
Pulse 2	27.02.2012-03.03.2012	50 mgl^{-1} PG, 400 mgl^{-1} FO	50 mgl^{-1} PG	control	control
Pulse 3	11.03.2012-20.03.2012	1 $mmoll^{-1}$ Br, 50 mgl^{-1} PG, 400 mgl^{-1} FO	1 $mmoll^{-1}$ Br, 50 mgl^{-1} PG	control 1 $mmoll^{-1}$ Br	1 $mmoll^{-1}$ Br, 1000 mgl^{-1} PG



22. Figure: Microtiter plates used for the MPN method during the column experiment with propylen-glycol contaminated soil (photo taken by: Nikoletta Horváth, 2012)

5.8. Molecular methods

Microbial diversity describes complexity and variability at different levels of biological organization. It encompasses genetic variability within taxons (species), and the number (richness) and relative abundance (evenness) of taxons and functional groups (guilds) in communities [83; 84].

Until recently, methods to analyze soil microbial ecosystems were mainly focused on measuring biomass, processes such as respiration or analysing total or culturable numbers of bacteria. However these methods only provide data on processes or bacterial numbers, and are not suitable for the analysis of the community composition or diversity [85]. In order to find out which bacteria are active during the degradation phase, the diversity of the active microbiota in a degrading soil remediation system can be investigated by molecular analysis [86].

Nucleic acid based tools are powerful for relating biodegradation processes to specific microbial populations. These methods offer insight into bioremediation by providing evidence of biodegradation, by implicating specific organisms or populations in biotransformation events, and by quantifying environmentally relevant organisms [87].

Thus, direct amplification of rRNA genes from environmental samples, 16S rDNA cloning, sequencing and phylogenetic analysis appears to be a direct and powerful method to explore the soil bacterial communities [83]. The 16S ribosomal RNA are useful for these studies, because it’s present in all bacteria, and this gene contains both conserved regions, which can be used for primer design for polymerase chain reaction (PCR) amplifications, and variable regions which can be used to distinguish sequences from each other [88; 89]. These ribosomal RNA genes are obtained by cloning DNA isolated directly from the environment. However, nucleic acid extraction is arguably the most important stage in a molecular microbial investigation.

The problem with such analyses is how best to evaluate the efficiency of lysis and DNA recovery and how to assess whether the DNA extract obtained is representative of the indigenous microbiota. Quantitative nucleic acid recovery also extracts large amounts of humified organic materials. These humic substances are soluble and contaminate the DNA inhibiting subsequent molecular analysis such as the polymerase chain reaction (PCR) [90]. PCR amplification of 16S rRNA genes is using consensus bacterial primers and separation of the resultant PCR amplicons by cloning. Clones or bands on gradient gels can be sequenced, and the resultant information used to infer something about the diversity of the original sample.

Sequences are generally submitted to and can be retrieved from the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany; Genbank (NCBI), Bethesda, MD, USA; The DNA Database of Japan (DDBJ) Mishima, Japan, and the Ribosomal Database Project (RDP), University of Illinois, Illinois, USA. The RDP is a free to use site, and provides a wide and excellent range of analytical services including both phenetic and phylogenetic analyses of query sequences online [91].

5.8.1. Population analysis in propylene-glycol contaminated soil

The community structure of the soil before and after DIC treatment during our column experiments has been tested by molecular methods. Soil DNA extraction was done using PowerSoil DNA Isolation Kit.

For PCR **(Polymerase chain reaction) the following reaction mix has been used:**

- 2 µl forward primer (10 pmol/µl) - 10x dilution,
- 2 µl reverse primer (10 pmol/µl) - 10x dilution,
- 4 µl PCR-buffer (5x),
- 2 µl dNTPs 2 µl per nucleotide stock solution ad 100 µl Aqua dest,
- 0,2 µl Taq-DNA-polymerase (5 U/µl),
- 1 µl Template DNA (dilution depending on concentration)
- 8,8 µl DEPC treated water

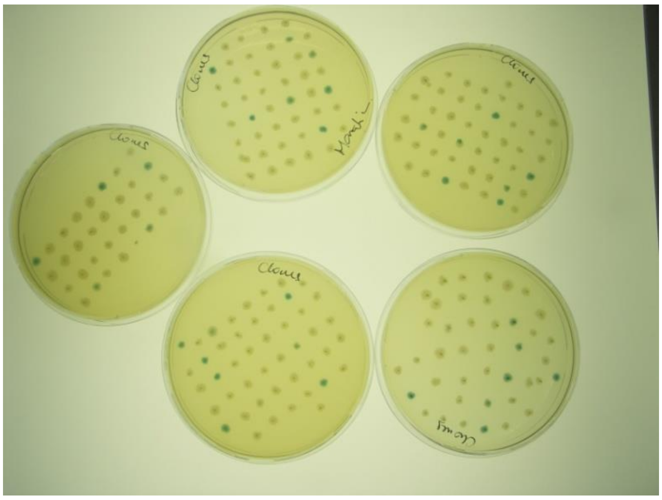
The following universal 16S rDNA primers were used to amplify 99% of known 16S rDNA species [107]:
16SfD1 5'-AGA GTT TGA TCC TGG CTC AG-3'
16SrP2 5'-ACG GCT ACC TTG TTA CGA CTT-3'

The following temperature program was set for amplification:

Temperature	94^{o}C	94^{o}C	52-56^{o}C	72 ^{o}C	72 ^{o}C	8-12^{o}C
Length (time)	3 min	1 min	1 min (30 cycles)	1 min	10 min	N/A (storage)

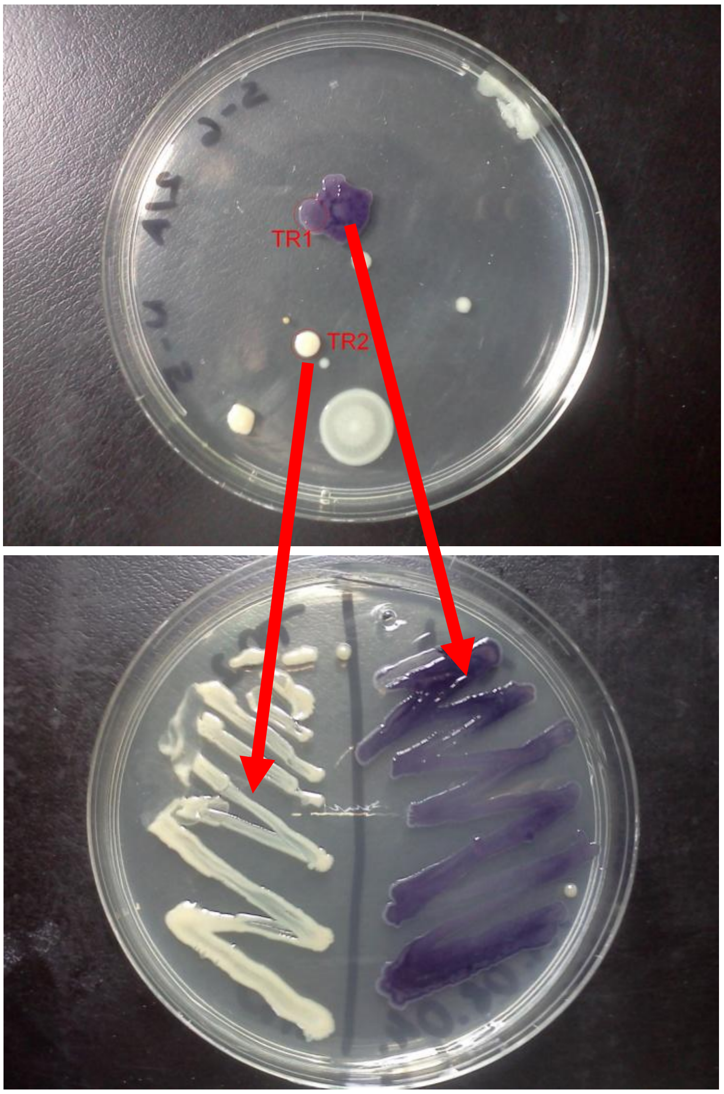
Control of the PCR product was done by Agarose-gel electrophoresis (1.8 %) on 80V, staining with ethidium-bromide. Following this QIAquick® Gel Extraction Kit was used for DNA extraction from the agarose gel. cDNA library (100 clones from each treatment, homogenizing the parallels and the initial soil material) was prepared has for sequencing using QIAGEN PCR Cloning Kit. Sequencing has been carried out by a commissioned laboratory, and the prepared sequences has been submitted and retrieved from the RDB.

Colonies showing growth in the agar plates were visually separated by size, form and colours. Typical, reoccurring and abundant colonies were isolated and cleaned (in replicates from different plates, under different code names) as shown on **Figure 23**.



23. Figure: *E. coli* clones during blue-white selection on solid Std 1 agar plates containing Ampicillin and X-gal (photo taken by: Nikoletta Horváth, 2012)

5.8.2. Isolation and identification of abundant microorganisms from crude-oil contaminated soil



24. Figure: Isolation of selected bacterial colonies from the crude oil contaminated soil (borehole S-6, Trecate). TR1 and TR2 are codenames for isolates (photo taken by: Nikoletta Horváth, 2011)

25. Figure: Isolation of selected bacterial colonies from the crude oil contaminated soil (borehole S-6, Trecate). TR23 and TR22 are codenames for isolates (photo taken by: Nikoletta Horváth, 2011)

Two typically reoccurring colony (TR2 from **Figure 24** and TR22 from **Figure 25**) have been sent for identification after isolation to a commissioned laboratory (Biomi Kft., biomi.hu, Hungay) using partial 16s rDNA sequencing.

The identification method was as follows:

The DNA has been isolated using ABI Prepman Ultra Kit. The 16s rRNA gene has been amplified by PCR method. The components of the PCR reaction mix have been measured together according to **Table 8** (except the DNA template). 2 µl DNA templates have been measured to each vial containing the 23 µl PCR reaction mix. Negative control contained 2 µl sterile MiliQ instead of the template DNA.

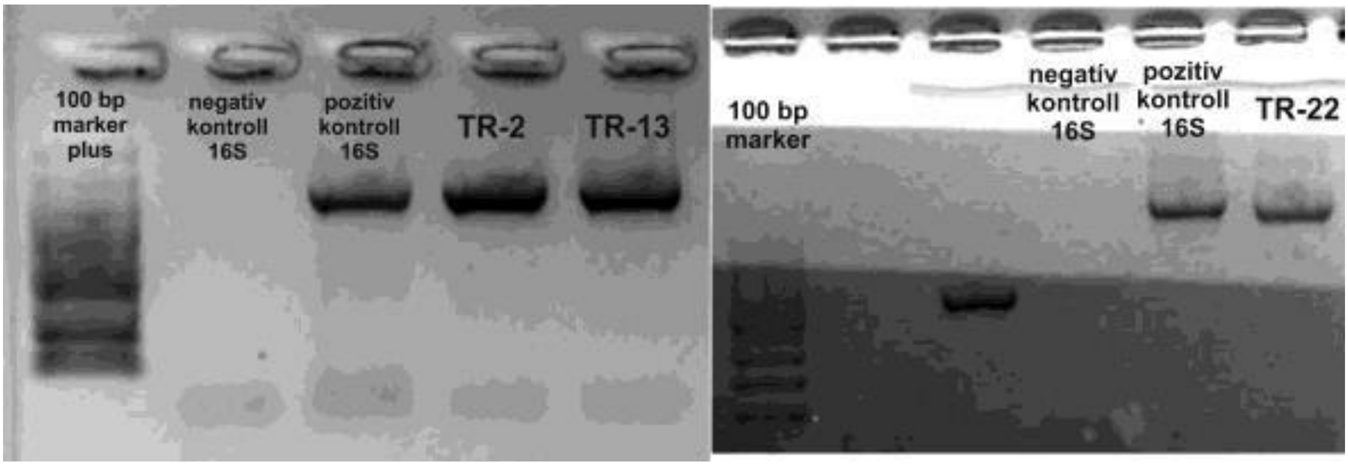
8. Table: PCR reaction mix components (Trecate samples)

Component	Amount/sample
2x PCR SigmaJumpStart Master mix	12,5 µl
dH_2O	7,5 µl
27F(10µM)	1,5 µl
1492R (10µM)	1,5 µl
DNS templát	2,0 µl
End volume	**25 µl**

The PCR temperature profile was the following:

Name of the step	Temperature	Length (time)	Number of cycles
-	94 °C	2 min	-
Denaturation	94 °C	30 sec	30
Annealing	52 °C	30 sec	
Extension	72 °C	1 min	
Terminal extension	72 °C	7 min	-
Cooling down	4 °C	q.s.	-

Detection of the PCR product happened with agarose-gel electroforesis (in 1% agarose gel, 1xTBE buffer on 100V for 20 minutes); the result is shown on **Figure 26**. DNA dyeing was performed with GelGreen dye detected by UV screening.



26. Figure: Result of the agarose-gel electroforesis in case of isolates from the Trecate site. From left to right: 100 bp marker plus, negative control, positive control, TR-2 sample, TR-13 sample, 100 bp marker, negative control, positive control, TR-22 sample.

Sequencing reaction mix has been prepared with BigDye™ Terminator v. 3.1 CycleSequencing Kit according to the protocol. The marker PCR reaction mix per each sample is described in **Table 9**. The PCR product has been cleaned by ethanol precipitation, and afterward it has been suspended in 10 µl Hi-Di formamide solution and denaturated on 96°C for 3 minutes. Separation of the sequences happened by using ABI 3130 x l Genetical Analysator (POP-7 polymer, 60°C).

9. Table: Marker PCR reaction mix components (Trecate samples)

Component	Amount/sample
BigDyeTerminator v3.1 Cyclesequencing Ready Reaction mix and BigDye puffer 1:1	1.4 µl
dH_2O	6.4 µl
519R / 27F (10µM)	0.7 µl
DNA template	1.5 µl
End volume	**10 µl**

Sequencing PCR temperature profile:

Name of the step	Temperature	Length (time)	Number of cycles
-	96 °C	5 min	-
Denaturation	96 °C	30 sec	50
Annealing	55 °C	20 sec	
Extension	60 °C	4 min	
Cooling down	4 °C	q.s.	-

From the sequences prepared with a 519R primer, the 27F primer sequence has been cut and the received partial 16S rDNA fragment has been compared to 2 databases:

1. **NCBI** (*National Center for Biotechnology Information*)
2. **EzTaxon-e type stain database** [113]

5.8.3. Isolation and identification of Manganese tolerant strains from Őrbottyán

From the growth positive wells of microplates from the Mn tolerance tests (Chapter 6.3.4.) 100 µl solution have been transferred to liquid medium (standard nutrient: 5 %-os peptone, 0,3%-os bovine extract, 0.5% NaCl in 1 l distilled water) spiked with 10.000 mg Manganese.

The liquid medium has been incubated on 37 °C for 1 day, then 100 µl of this stock solution have been spread on solid nutrient medium on Petri dishes (5 %-os peptone, 0.3%-os bovine extract, 0.5% NaCl, 1 distilled water, agar). The plates were incubated for 3-7 days on room temperature. When colonies were visually separable and isolation could be performed, clean isolates have been prepared on solid nutrient plates.

After another growth period on room temperature, out of several candidates we selected four isolates, one originating from the control sample, where there were very few tolerant isolates, and three originating from the Manganese treated soil. These isolates were capable to grow in the liquid media containing 10000 mgkg^{-1} Mn. The chosen 4 isolates have been identified by 16s rDNA sequencing.

DNA isolation, the 16s rDNA sequence amplification, the preparation of clone library and the cleaning of the plasmid have been performed on the house according to the followings:

DNA isolation

From the cleaned isolates DNA has been extracted by phenol-chloroform method:

I. Cell disruption

We added 100 µl Solution I:

- 0.990 g glukose (Sigma)
- 2.5 ml Tris 1M pH=8 (Reanal)
- 2 ml EDTA 500 mM (Sigma)
- filled up to 100 ml with sterile distilled water (SDW)

to the cells, used a strong vortex, while the pellett dissolved.

II. Added 200 µl Solution II to the compound:

- 0.08 g NaOH (Sigma)
- 1 ml 10%-os SDS (Sigma)
- filled up to 10 ml with sterile distilled water
- and mixed it carefuy 6-8 times, until it became mucosal, then we stored Eppendorf tubes on ice for 5 minutes.

III. Pipetted 150 µl Solution III to the samples:

- 29.44 g K-acetate (Fulka)
- 60 ml SDW
- 11.5 ml acetic acid (Reanal)
- filling it up to 100 ml with SDW

then we carefully mixed it by turning upside-down, until the white precipitate appeared, then we stored it for 5 minutes on ice.

Samples afterwards have been centrifuged for 10 minutes on 4 ^{o}C, and then the supernatant has been pipetted to a clean Eppendorf tube.

IV. Extraction with phenol

400 µl phenol-cloroform-(isoamilalcohol) has been measured to the samples in 1:1 rate, then we centrifuged it for 5 minutes.

From the supernatant 300 µl has been pipetted to a clean Eppendorf tube.

We added 750 µl absolute ethanol to the previously gathered supernatants, and then mixed the samples by turning the tube upside-down. After a few minutes of waiting we centrifuged the samples for 10 minutes.

V. Alcoholic washing

After emptying and drying the alcohol we washed the samples with 1 ml 70% alcohol.

After adding the alcohol we centrifuged the samples for 3-4 minutes, then we emptied the alchol and dryed the samples for another 10 minutes under vacuum.

VI. RNase digestion

The samples have been recovered in RNase containing 1xTE solution. Per each sample 50 µl 1xTE and 1 µl RNase was added, and then they were put to 37 ^{o}C waterbath for 30 minutes.

The samples have been tested with agarose gel electrophoresis. 1.8% agarose gel has been prepared (200 ml 1xTBE buffer + 3.6 g agarose). To the 5-5 µl samples and the marker DNA (Affymetrix) 0.5 µl MaestroSafe dye (Maestrogen) has been added and filled to the pockes of the gel with the negative control. Running was 30 minutes on 80 V in 1x TBE buffer.

Amplification of 16S coding sequencing

I. Amplification

Amplification of the extracted 16S rDNA sequence was performed by usin PCR. The used primers were: 16SfD1 (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 16SrP2 (5’-ACG GCT ACC TTG TTA CGA CTT-3’) [137].

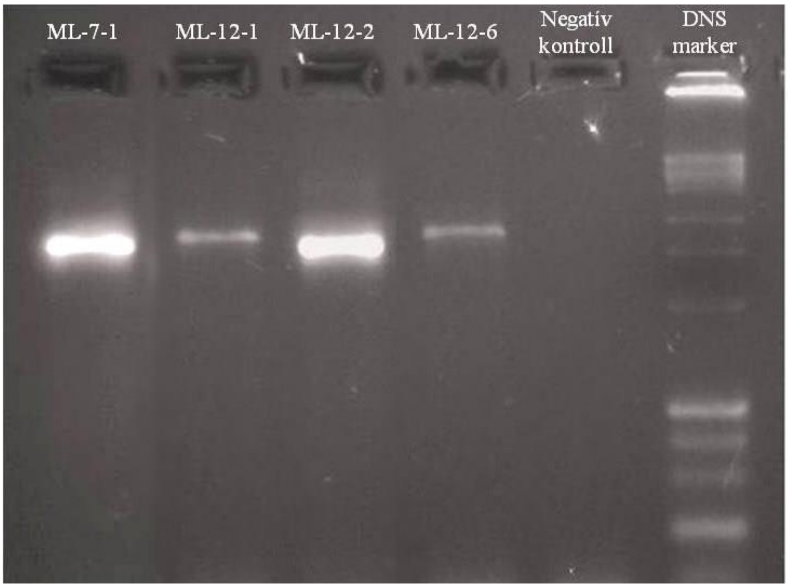
For the PCR premix the followings were measured together for an end volume of 50 µl per samples:

- Taq buffer (Thermo Scientific): 5 µl
- MgCl2 (Thermo Scientific): 3 µl
- dNTP (Thermo Scientific): 1 µl
- Forward primer: 2.5 µl
- Reverse primer: 2.5 µl
- Taq polimerase (Thermo Scientific): 1,25 µl
- SDW: 31.5 µl
- Template DNS: 3 µl

The temperature profile of the PCR was the following:

	Temperature (^{o}C)	**Time (minute)**
Denaturation	94	5
Annelation (35 cycles)	94	30
	52-56	30
	72	1.5
Extension	72	7
Storage	4	∞

PCR products were run in 1.8% agarose-gel as presented in **Figure 27**.



27. Figure: PCR products of Mn resistant isolates from calcareous sandy soil, Őrbottyán, Gödöllő. Sample ML-7 is from a non Mn treated soil, while ML-12-1,2,6 are from Mn treated soils. The 5th column contained the negative control, and the last column was the DNA marker.

II. Cleaning of the PCR product

For the cleaning of the PCR product, the samples were recovered in RNase containing 1xTE solution, and they were stored for 30 minutes in a 37 °C thermostate.

Afterwards we centrifuged them and added 50 µl 20% PEG (Polyethylene glycol), vortexed for a short time and let them stand in ice for 1 hour.

After 1 hour the samples were centrifuged for 10 minutes in 4 °C then we emptied the PEG from the Eppendorf tube with a fast movement and tapped the tube carefully to remove the residual.

Following the removal of the PEG we added 1 ml 70% alcohol, centrifuged on 13.000 rpm for 10 minutes and then we emptied the supernatant. We repeated this last step once more (from the addition of the alcohol), and we vauum centrifuged the samples for 10 minutes.

We added SDW to the cleaned PCR product.

Preparation of clone library

I. Ligation was performed by QIAGEN PCR Cloning Kit using the provided protocol.

II. Preparation of the supercompetent cell

The supercompetent cell was *E.coli strain* DH5α (prepared by the Department of Viticulture, Faculty of Horticultural Science, Szent István University, Budapest, Hungary), until usage it was stored on -70 °C. Taking out of the freezer we let it “warm up” on ice. After the suspension melt we mixed the cells with a pipette and we measured 100 µl cell suspensions to sterile test tubes. Afterwards 5 µl plasmids have been added to the cell cultures, which were then put into 16 °C waterbath for 1 hour.

III. Inserting the plasmids into the bacterial cells

Heatshock had been used for 30 seconds to insert the plasmids. The test tubes then were put into 42 °C waterbath for 40 seconds. After the heat treatment we put the tubes into ice again for 2 minutes.

IV. Propagation of bacterial cells in growth medium

We added 400 µl SOB medium to the cells [138]:

- 2% tryptone: 2 g
- 0.5% yeast extract: 0.5 g
- 10 mM NaCl: 0.05844 g
- 2.5 mM KCl (MKCl = 74.56 g/mol): 0.01864 g
- 10 mM MgCl2 (MMgCl2 = 203.31 g/mol): 0.20331g
- 10 mM MgSO4 (MMgSO4 = 120,37 g/mol): 0.120 g

Cell suspensions were put to a 200 rpm shaker thermostate on 37 °C for 1 hour.

Afterwards 40 µl X-gal, 8 µl IPTG and 100 µl cell suspensions were spread on solid LB agar media [139] containing additional 100 mg/l ampicillin (pH=8):

- 1% tryptone
- 0.5% yeast extract
- 1% NaCl
- distilled water

Plates were incubated on 37 °C until colonies were visually separable. White colonies were moved to new solid LB agar media containing 100 mg/l ampicillin with a sterile pipette tip and the same colonies were also transferred to liquid LB media containing 100 mg/l ampicillin. They were put to a shaker thermostate for 1 hour on 37 °C.

Plasmid extraction and cleaning

I. DNA extraction

DNA were extracted from the liquid cell cultures with the previously described phenol-chloroform method, and cleaned with PEG (Page 99).

II. Plasmid precipitation

Follwing the RN-ase digestion the samples have been centrifuged, then 30 µl
20 %-os PEG (6000) has been added to each of the sample with an additional 2.5 M NaCl.
We shortly vortexed the samples and stored them on ice for 1 hour. After 1 hour the samples have been centrifuged for 10 minutes on 4 ^{o}C.

III. PEG removal

With a fast movement we empty the PEG from the Eppendorf tube, carefully tapping the tube to remove the residuals.
We added 1 ml 70 % alcohol, centrifuged for 3 minutes then we emptied the alcohol and dried it. This step was repeated 2 times.
Drying took place for 10 minutes under vacuum.

IV. Sample recovery

Samples have been recovered in 50 µl SDW and vortexed. Afterwards we enzymatic digested the cleaned plasmids in the following mixture:

- Fast Digest buffer (Fermentas): 1 µl
- *Eco*RI enzime: 0.3 µl
- Plasmid DNA: 0.5 µl
- SDW: 8.2 µl

The plasmid concentration of the samples has been measured by a photometer with 100x dilution on 260 nm (WPA UV1101 Biotech Photometer).

V. Sequencing and data process

An appropriate volume of sample has been prepared with the sequencing priers according to the cleaned plasmid concentration (end volume of 0.75 µg plasmid).
Primers: M13F (5'-GTAAAACGACGGCCAGT-3') (Thermo Scientific) and M13R (5' CAGGAAACAGCTATGAC-3') (Thermo Scientific).
The final volume of 20 µl was reached by adding SDW according to **Table 10**.

The prepared tubes have been sent to Sanger sequencing for a commissioned laboratory (Biocenter Laboratóriumi Szolgáltató Kft., www.biocenter.hu).

The received chromatograms have been edited with Bioedit program and the sequences have been aligned in the Ribosomal Database Project.

10. Table: Content of the sequencing mix prepared with the cleaned plasmids and sent in for identification for the comissioned laboratory (Manganese resistant strains)

Sample ID*	Plasmid concentration (ng/µl)	Volume of the plasmid (µl)	Sequencing primer (µl)	Volume of the SDW (µl)
ML-7-1 c	200	3.75	2.5	13.75
ML-7-1 d	125	6	2.5	11.5
ML-12-1 f	80	10	2.5	7.5
ML-12-1 j	130	6	2.5	11.5
ML-12-2 b	100	7.5	2.5	10
ML-12-2 c	80	10	2.5	7.5
ML-12-2 d	80	10	2.5	7.5
ML-12-6 c	100	7.5	2.5	10
ML-12-6 f	180	4	2.5	13.5

*Sample ID was created as: ML (short for Manganese-Lysimeter) - the number of the lysimeter it was sampled from - alphabet for the paralells

5.9. Antibiotic susceptibility testing

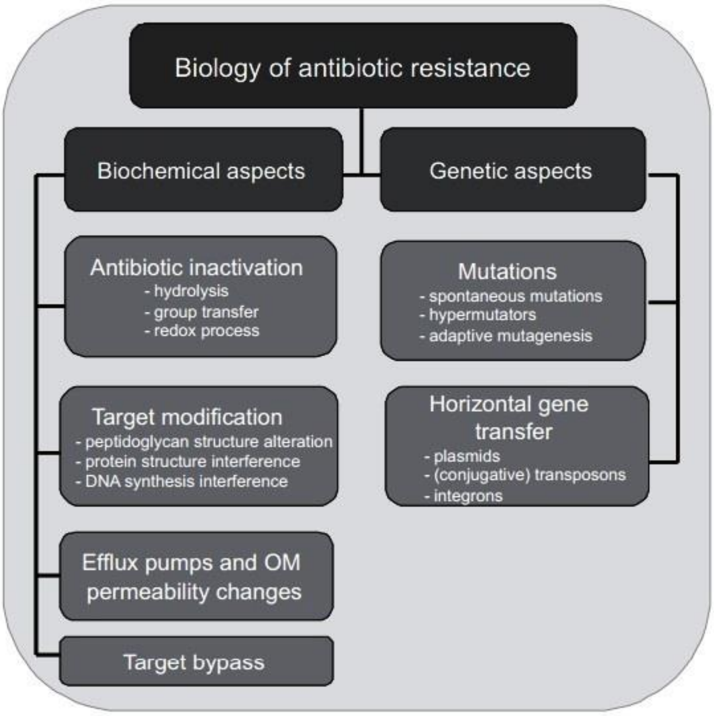
Bacteria may be intrinsically resistant to more than one class of antimicrobial agents, or may acquire resistance by de novo mutation or via the acquisition of resistance genes from other microorganisms [120]. There several mechanisms through which bacteria can exert antibiotic resistance (**Figure 28**).

Biochemical antibiotic resistance contains [121]:
a) Antibiotic inactivation - direct inactivation of the active antibiotic molecule,
b) Target modification – alteration of the sensitivity to the antibiotic by modification of the target,
c) Efflux pumps and outer membrane (OM) permeability changes – reduction of the concentration of drug without modification of the compound itself,
d) Target bypass – some bacteria become refractory to specific antibiotics by bypassing the inactivation of a given enzyme.

Single bacterial strain may possess several types of resistance mechanisms. Which of these mechanisms prevails depends on the nature of the antibiotic, its target site, the bacterial species and whether it is mediated by a resistance plasmid or by a chromosomal mutation.

Antibiotic susceptibility testing (AST) is usually carried out to determine if an antibiotic will be successful in inhibiting a bacterium from infection/growth. When a bacterial isolate is planned to be used as inoculate for bioremediation or any other biotechnological purpose, one must consider the unfavorable effects of placing a non-native species into the soil. As time passes by, mutations can occur, horizontal gene transfers can take place, and if our inoculums had antibiotic resistance genes, it can cause problems when we want to eliminate them, or it can be transferred or native species in the soil, even disrupting the current colonies [120].

Testing for antibiotic sensitivity was done by the Kirby-Bauer method [144]. Small wafers containing antibiotics are placed onto a plate upon which bacteria are growing. If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition, is seen around the wafer indicating poor growth. If the ring appears, it means the microorganisms was not capable to grow around the antimicrobial agent, therefore, it is not resistant against it.



28. Figure: The way of acquiring antibiotic resistance in microorganisms (retrieved from [121])

6. RESULTS AND DISCUSSION

6.1. Results of the propylen-glycol contaminated soils of Gardermoen

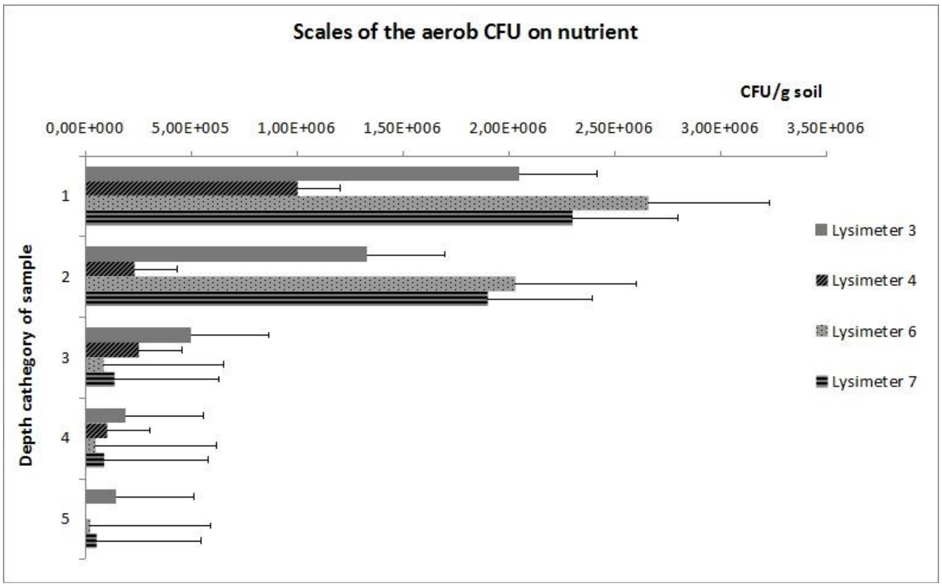
In this study the objective has been to map the degradation potential for de-icing chemicals in the soil along an actively used runway. This was done by quantifying the microbial abundance of microbes at increasing distances and depths from the source of PG in soil next to western runway of Oslo airport, Gardermoen. The hypothesis is that areas closest to the runway that has received repeated supply of easily degradable de-icing chemicals would show modified microbial abundance distributions.

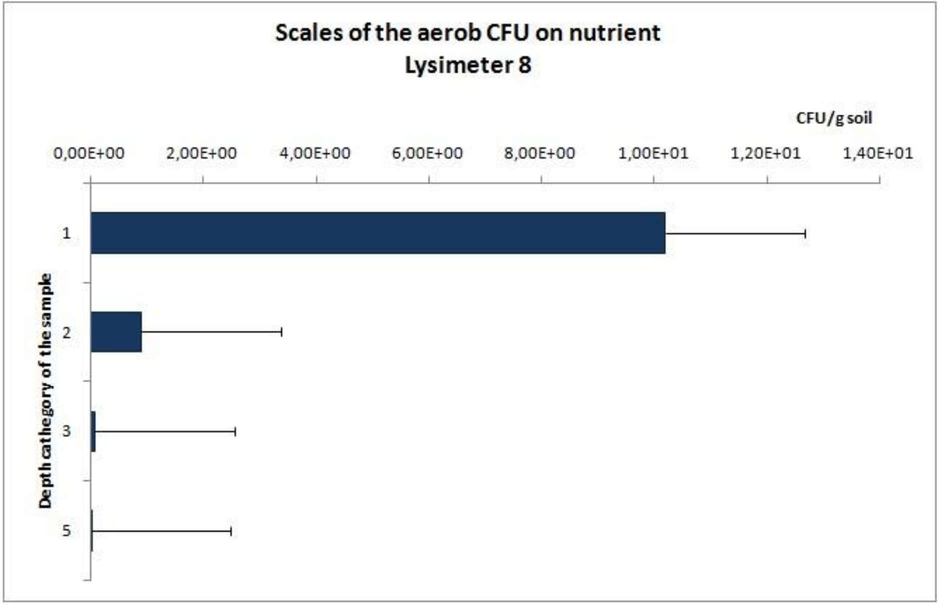
The vertical and horizontal soil distributions in the contaminated area were characterized by several bioindicators widely employed to characterize microbial abundance and activity of soils, namely: i) microbial counts (CFU of aerobic and anaerobic heterotrophic bacteria, CFU of microscopic fungi, most probable numbers (MPN) of PG-degrading aerobes), ii) enzymatic activities (β-glucosidase, FDA hydrolase, dehydrogenase) and iii) organic carbon content (biomass carbon and total organic carbon (TOC)).

6.1.1. Vertical and horizontal microbial abundance in PG contaminated soils

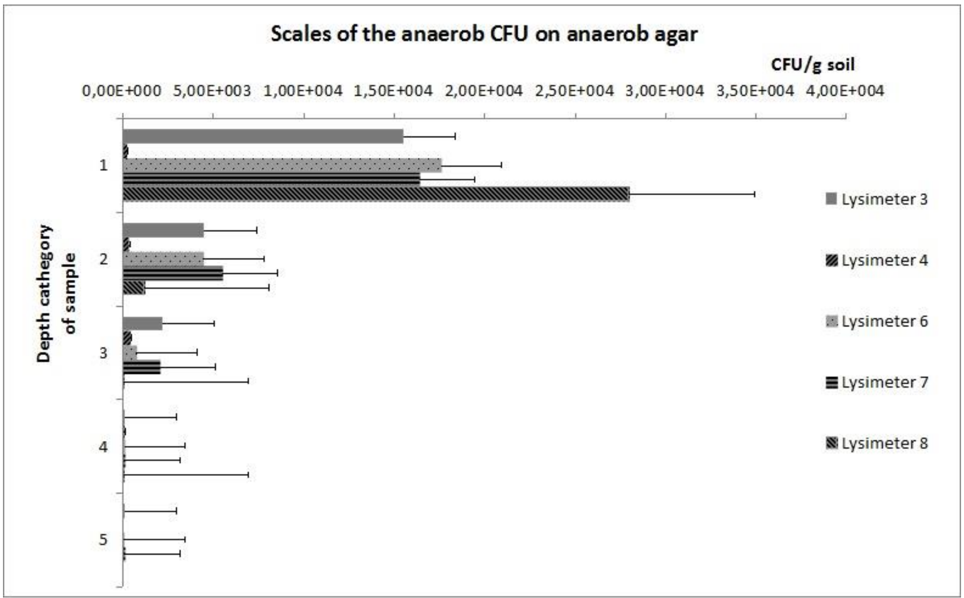
Lysimeter sample locations (vertical samples) were all from contaminated areas of the Oslo airport representing the different polluted sites of the airport; their depth level ranging from the surface to 110cm deep (not exactly the same for each lysimeter).

During the evaluation of the results of the vertical samples the highest microbe colony forming unit number (CFU g^{-1}) was observed in the top 20-30cm of the soil. Heterotrophic cell numbers counted on the nutrient agar (**Figure 29**) were 2 orders of magnitude higher than anaerobic (**Figure 30**) and fungal (**Figure 31**) cell numbers measured from the same samples. In all three groups the CFU g^{-1} decreases toward the deeper soil layers implying a drop in oxygen and nutrient levels. Even though the samples were taken from the same area, the five lysimeters show significant difference in microbial cell numbers.

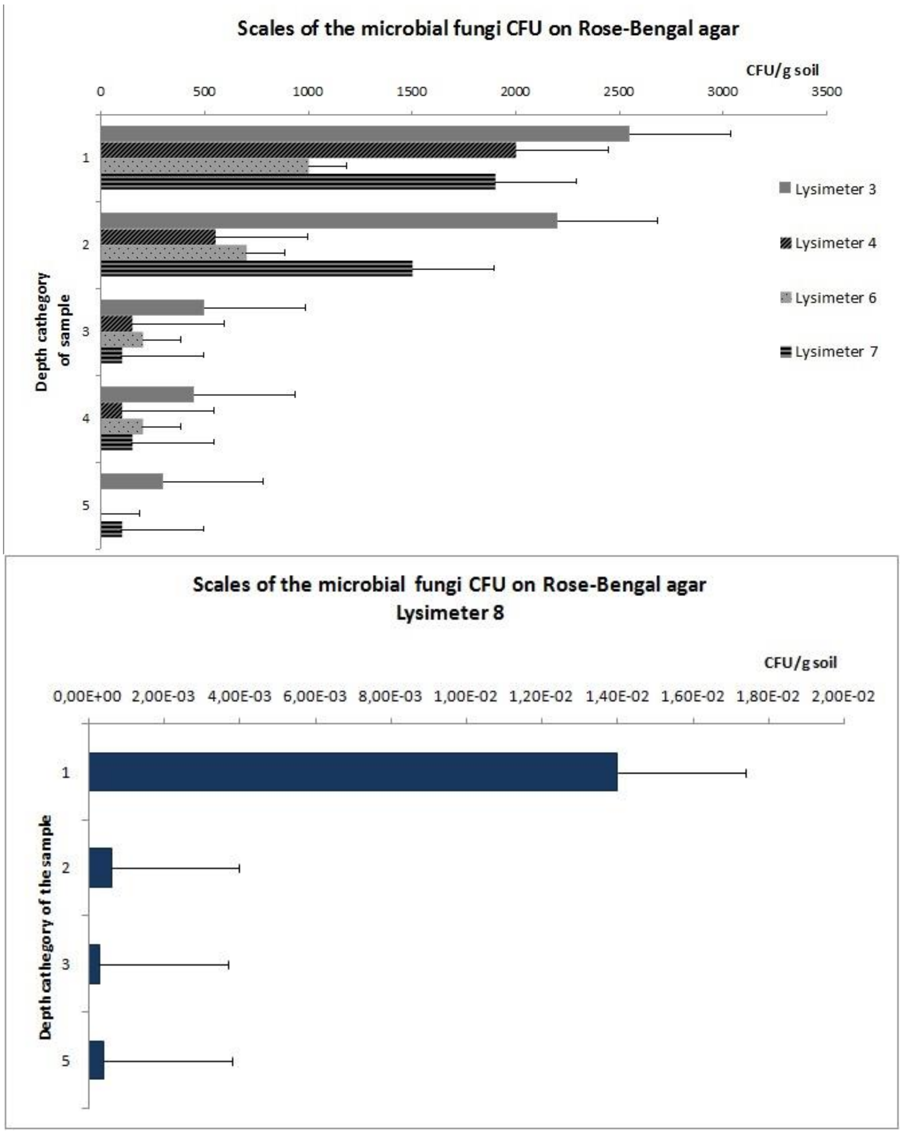




29. Figure: Rate of the total aerobic microbial CFU according to depth in lysimeters from Oslo airport. Lysimeter 8 is presented separately because of the magnitude difference.



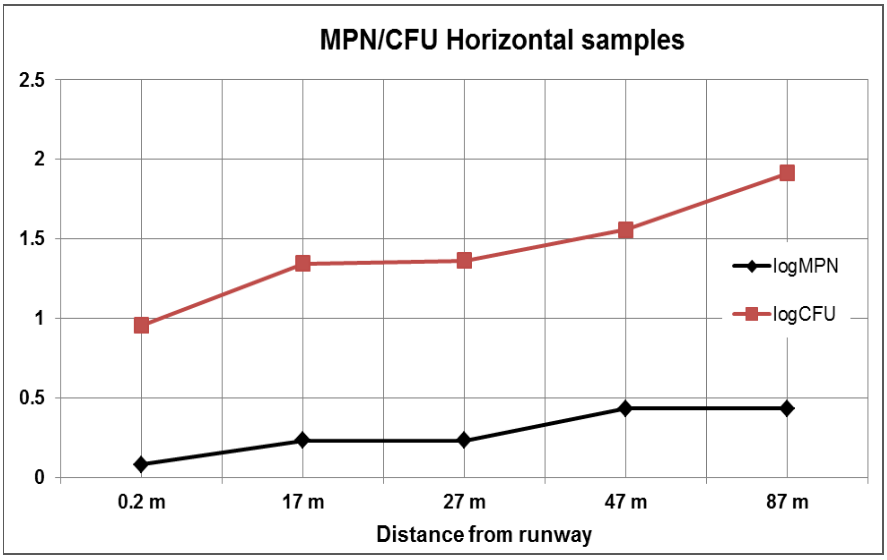
30. Figure: Rate of the anaerobic microbial CFU g^{-1} according to depth in lysimeters from Oslo airport



31. Figure: Rate of the microbial fungi CFU g^{-1}according to depth in each lysimeter from Oslo airport. Lysimeter 8 is presented separately because of the magnitude difference.

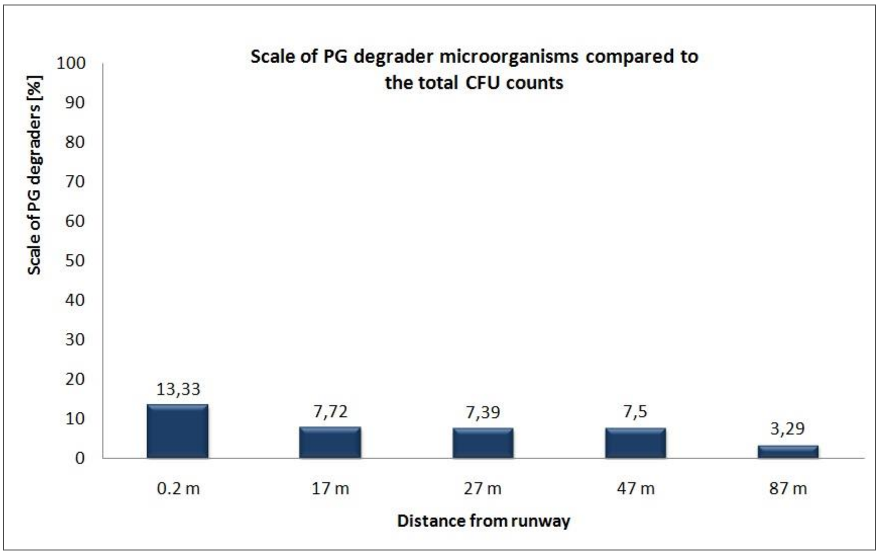
<u>Horizontal samples</u> were taken at an increasing distance from the runway. Normally high concentrations of pollutants inhibit microbial growth and reproduction. Results show that the number of microorganisms increases proportionately with distance, and thus with the decreasing concentration of chemicals (**Figure 32**). Approximately 85m from the runway CFU g^{-1} numbers were around 10 times higher than in the close vicinity of centre of the pollution.

To estimate the number of degrader microorganisms we used MPN tests where PG were added as sole carbon source to the medium, therefore, only bacteria capable to degrade PG as sole carbon source were able to proliferate. MPN g^{-1} results from horizontal samples showed a similar trend with the CFU g^{-1} numbers: from the runway the most probable number of bacterial cells grows in accordance with the contamination decrease (**Figure 33**). Comparing the number of PG degraders to CFU cell count results from nutrient agar - which show the total number of heterotrophic bacteria (**Figure 32**) - we can see a significant difference in magnitudes.



32. Figure: Total aerob CFU and Most Probable Number of bacteria capable to degrade PG at an increased at an increased distance from the runway in Oslo airport.

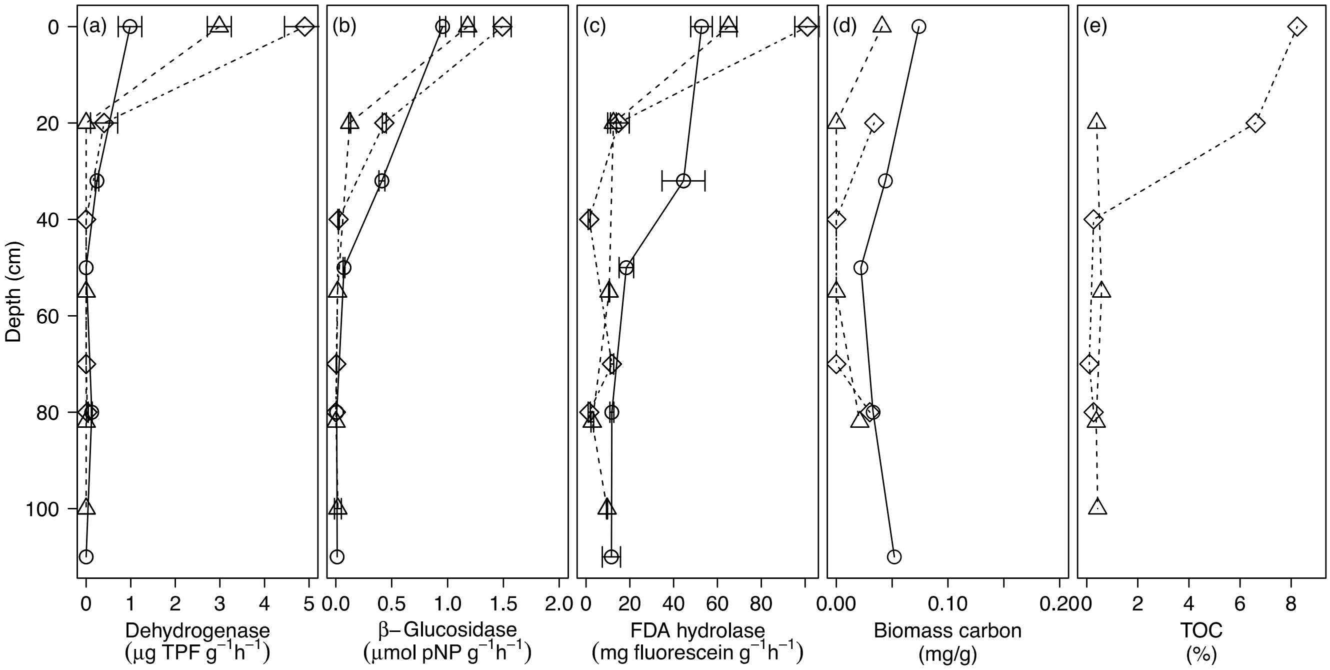
The amount of degraders was much higher; closer to the runway 13.33% of the total CFU g^{-1} (**Figure 33**), but further from the runway the percentage of PG degraders decrease to 3.29%.



33. Figure: Scale of PG derader microbes (logMPN) compared to the total aerob heterotroph numbers (logCFU) at an increased distance from the runway in Oslo airport.

6.1.2. *Microbial enzymatic activity in propylene-glycol contaminated soils*

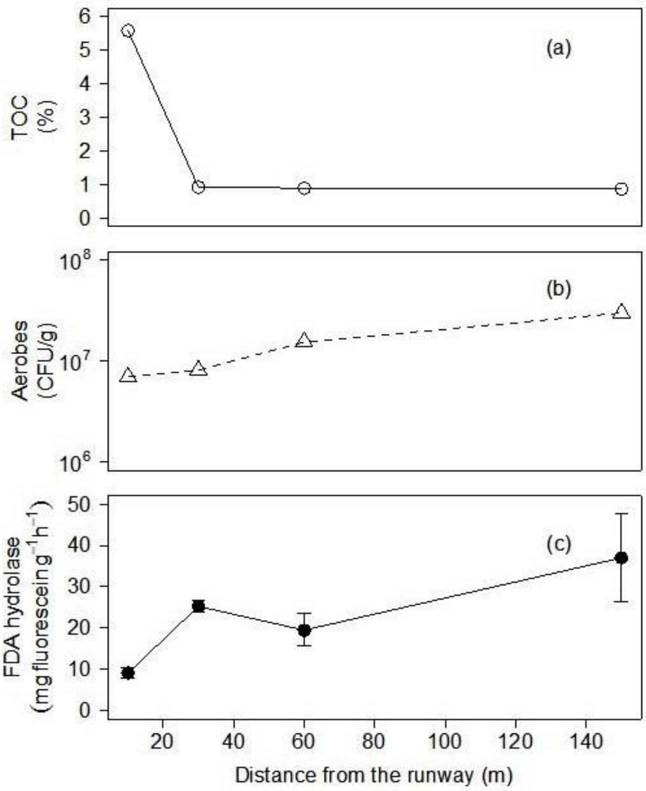
In the vertical samples dehydrogenase, FDA hydrolase and β-glucosidase enzyme activities decreased with increasing depth (**Figure 34 a-c**) following trends that closely mirror those found for CFU counts (**Figure 29-31**).



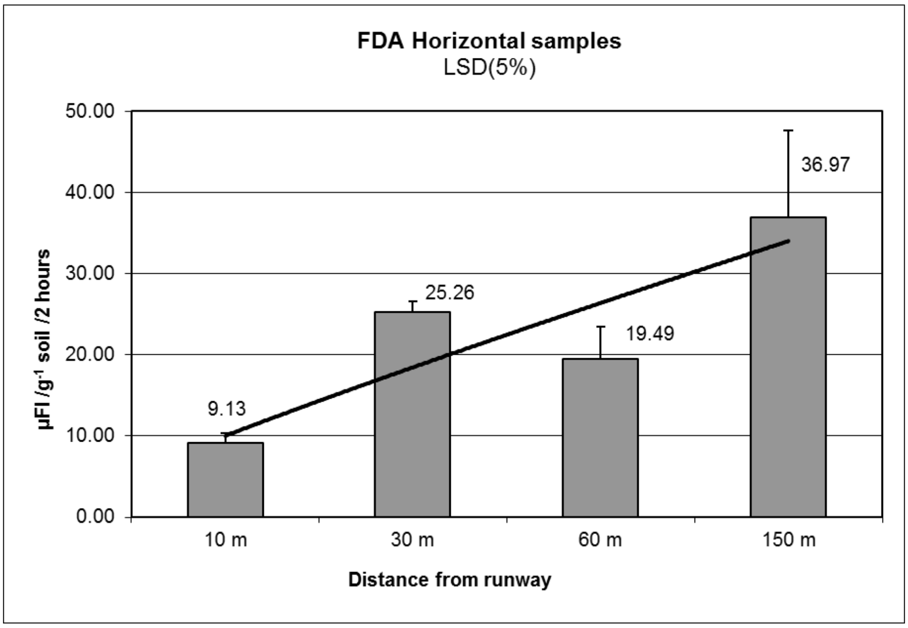
34. Figure: Comparison of enzymatic activities (a-c), biomass (d) and total organic carbon (e) levels according to soil depth in the Oslo airport (retrieved from [101])

Unexpectedly and in contrast with vertical profiles, aerobic heterotrophic CFU and FDA hydrolase activity are inversely correlated with TOC and with ADF load in the horizontal samples. Indeed, aerobic heterotrophic CFUs on Nutrient agar and FDA hydrolase activity show that the number of microorganisms increases with the distance from the runway (**Figure 35 b-c**). Approximately 150 m from the runway, CFU/g numbers were around 5 times higher than in close vicinity to the runway.

Along with CFU and MPN counts measured from horizontal samples taken at an increasing distance from the runway (Chapter 6.1.1.) the FDA enzymatic activity was also measured to estimate microbial activity and compare it to microbial abundance. Normally high concentrations of pollutants inhibit microbial growth and reproduction, as mentioned before. CFU results already proved that the number of microorganisms increases proportionately with distance, and thus with the decreasing concentration of chemicals. This statement was further strengthened by the microbial activity results, which correlated very well with CFU counts. According to **Figure 36**, the enzymatic activity levels also increase as we go further from the runway.



35. Figure: Soil organic carbon (*a*), CFU of heterotrophic aerobes (*b*) FDA hydrolase activity (*c*) at increasing distances from the runway, October 2008 survey, Oslo airport (retrieved from [101])



36. Figure: FDA enzymatic activity measured at an increased distance from the runway in Oslo airport

6.1.3. Organic carbon content in propylene-glycol contaminated soil

Only a few samples were tested for microbial biomass C and TOC measurements in the vertical soil samples. Biomass carbon measurements (**Figure 34 d-e**) were probably not reliable due to the very low amounts of biomass in soil and showed low correlation with both activities and CFU counts. The few TOC values (**Figure 34 e**) show high correlation with total aerobic, anaerobic and microfungi CFUs.

In the horizontal samples from the 2008 sampling campaign soil organic carbon was relatively much higher (5.58 %) near the runway (**Figure 35 a**) in comparison with the C-content at the 30, 60, and 150 m distances from the runway (0.93, 0.91, 0.88 C% respectively). The greater amount could be probably the result of ADF infiltration, more particularly at close vicinity to the runway.

6.1.4. Simulation of de-icing chemical contamination and potential microbial adaptation

Columns are scientific tools that are used to more accurately simulate the soil's seep, layered conditions in the laboratory [104]. Although the column experiments better at stimulating the hydraulic and geometric properties of subsurface porous media, they are time and labor intensive (5). However, previous column experiments proved successful combined with molecular methods [105; 104; 106].

The main objective of our column experiments was to receive an overview on how the microbial population changes and adapts to de-icing chemical (DIC) treatment. Indicators of soil respiration, organic matter degradation, and microbial biomass are classic methods for describing soil environments, but at a minimum a community profiling method also required for better understanding the effect of these compounds on the microbial population structure [103].

In columns treated with PG and formate (S1 & S2), PG reached faster its initial concentration of 50 mg/L during the first pulse of application than formate. A different observation was made during the second feeding of the columns with both chemicals. The maximum concentration of PG was 80 % and therefore remained below that of formate with 100 %. Recovered PG concentrations in the columns without formate addition (S4 & S5) reached maximum values of 65 % and 70 % during the two applications, indicating the accelerated degradation compared to S1 and S2, particularly during first application. After addition of 1000 mgl^{-1} PG to column 6 the effluent normalized PG concentrations almost increased concurrently with normalized bromide concentrations, implying that relative to the initial concentration PG was hardly degraded. The increase of residence time during the first flow interruption enhanced significantly the degradation efficiency of PG in all columns. The minimum effluent concentrations of PG thereby were 15 % in S1 and 0 % in S4 and S5 of the initial concentration. Impact of flow interruption on degradation was less during the second application of the chemicals, including also the concentration step of 1000 mgl^{-1} PG in S6 (Appendix 4).

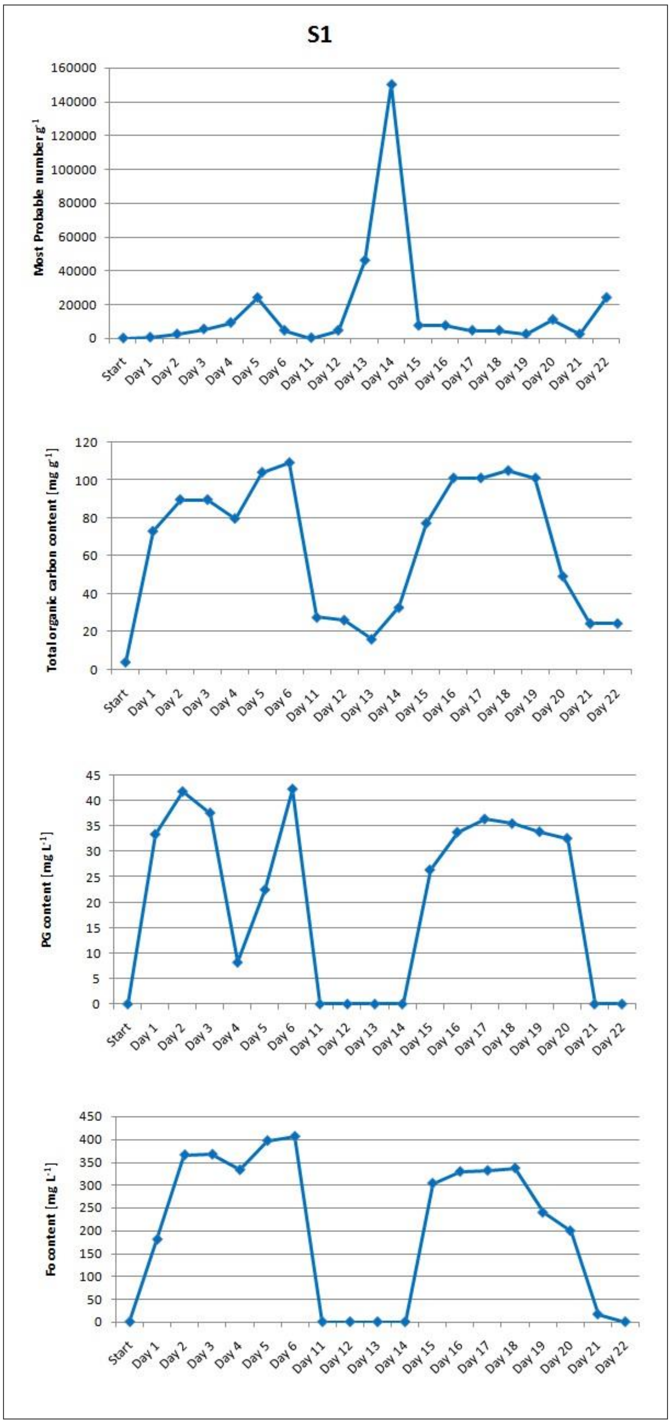
It can be inferred that the joint application of PG and formate inhibits PG degradation. When added alone PG was degraded up to 40 % compared of the initial concentration. The extension of the retention time showed a very positive effect on PG degradation, particularly during the first treatment of DIC application. This may be due to the better availability of potential electron acceptors, such as Mn and Fe oxides, while with proceeding of the treatment more of those electron acceptors are depleted. Further, PG degradation increased during the second application of both DIC, indicating the adaption of soil organisms to consume both carbon sources equally.

Microbial abundance shows a high correlation with the amount of the different organic compounds as we can see on **Figures 37-41**. Since PG and FO are degradable and usable as carbon sources, a trend can be followed. S1 and S2 were parallels and treated the same way. When the level of the pollutant was higher (Day 1-11), the number of microbes increased in the following days (after Day 11). This is due to the higher amount of available carbon source. However, since there was a flow interruption at the end of the first week, and the microorganisms used up most of the carbon sources found in the column, the level of pollutants dropped. This decreased the microbial numbers right after. After Day 21 when the organic carbon level normalized we could see a slight increase in the microbial numbers right before the end of the experiment (**Figure 37-38**).

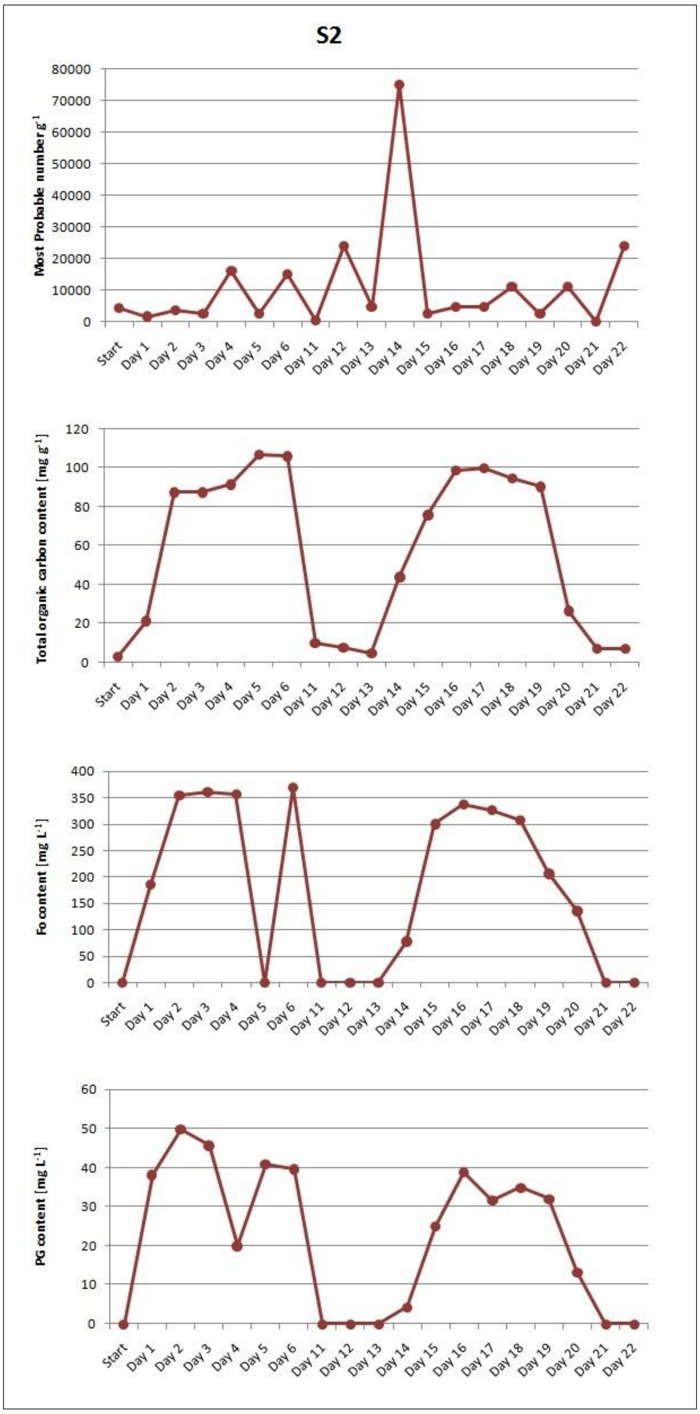
3 column being a control did not present us with such trends, since the total organic carbon levels were comparably low, as well as the number of microbes. However, S4 and S5 once again shows the trend that the increasing amount of degradable organic contaminants provides higher cell numbers in the following days (**Figure 39-40**).

S6 was a control parallel for S3 for the first pulse. The initial microbial numbers were significantly higher compared to the other columns, which can be due to the heterogeneous composition of the soil. The cell numbers dropped drastically during the first pulse, where no addition of carbon source occurred. The CFU numbers drop from $9x10^4$ - $2x10^5$ g^{-1} to $2x10^3$ - $4x10^4$ g^{-1}. After the second pulse however addition of higher concentration PG increased the amount of microorganisms back to around $2x10^5$ g^{-1}, representing the trend previously mentioned (**Figure 41**). In some columns the redox potential dropped significantly, which can be due to the present of anaerobic degradation.

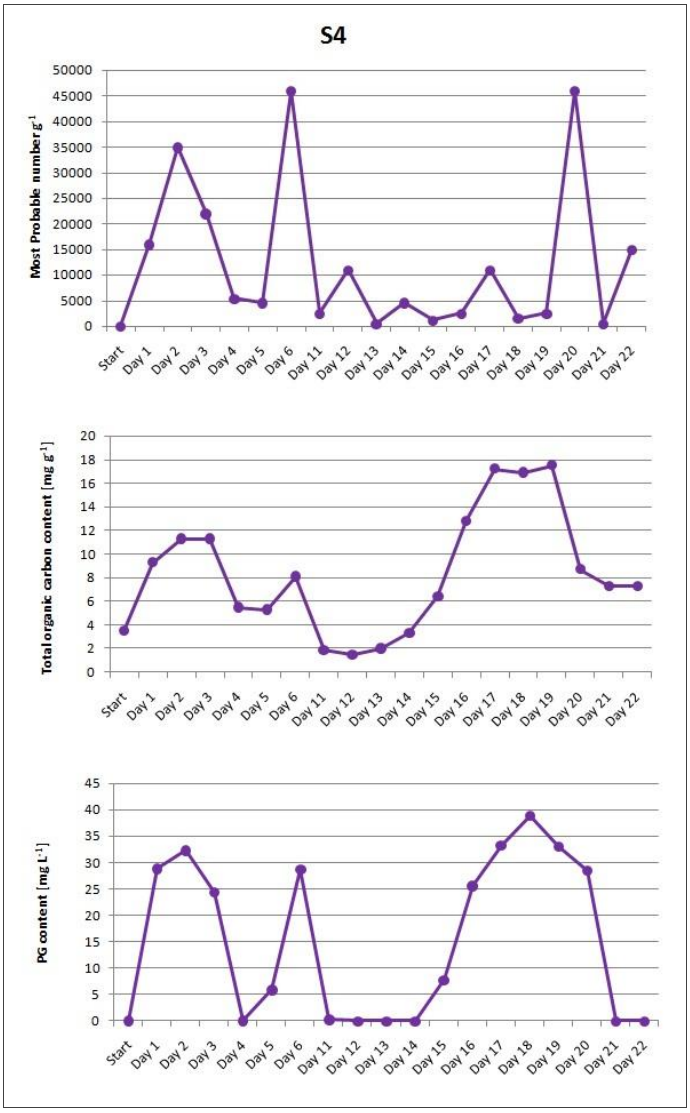
The soil profile showed little changes in pH values ranging from 6.4 to 7.4.



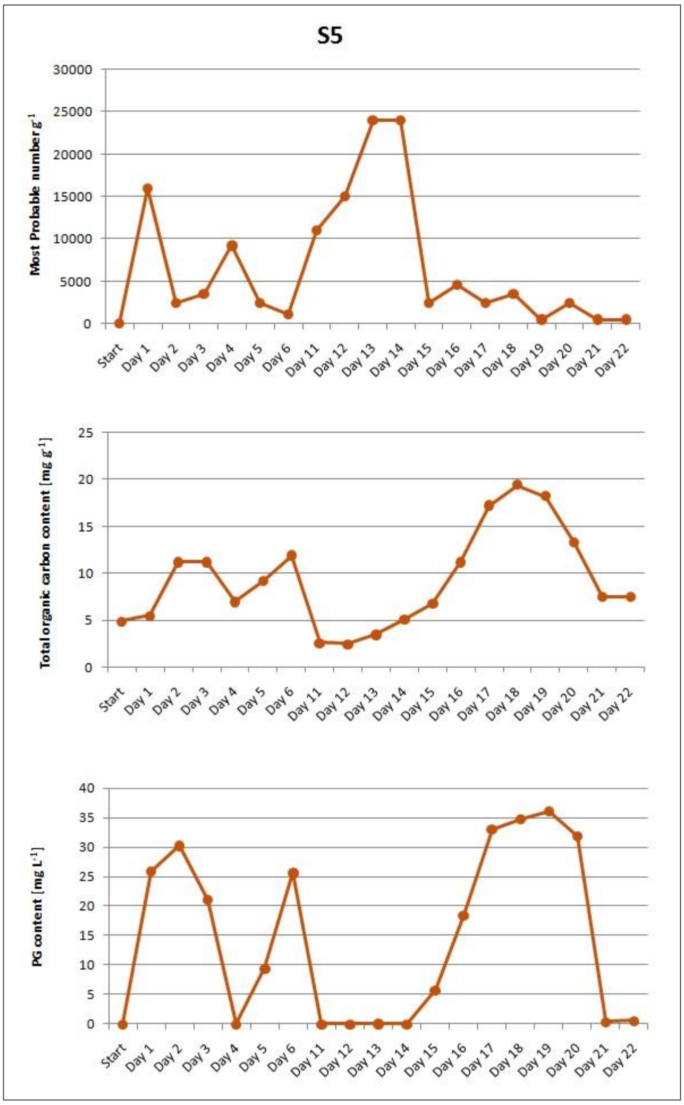
37. Figure: Comparison of the estimated number of microorganisms and the TOC, PG and FO content measured in Column S1 effluent samples.



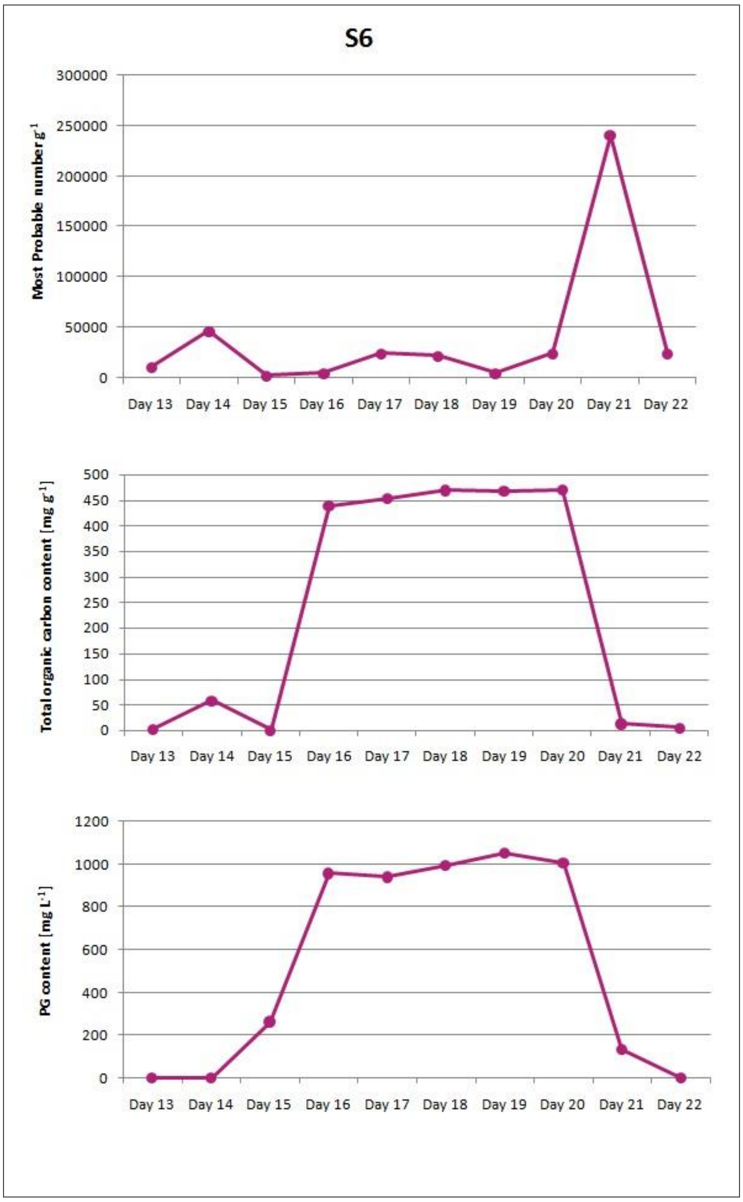
38. Figure: Comparison of the estimated number of microorganisms and the TOC, PG and FO content measured in Column S2 effluent samples.



39. Figure: Comparison of the estimated number of microorganisms and the TOC and PG content measured in Column S4 effluent samples

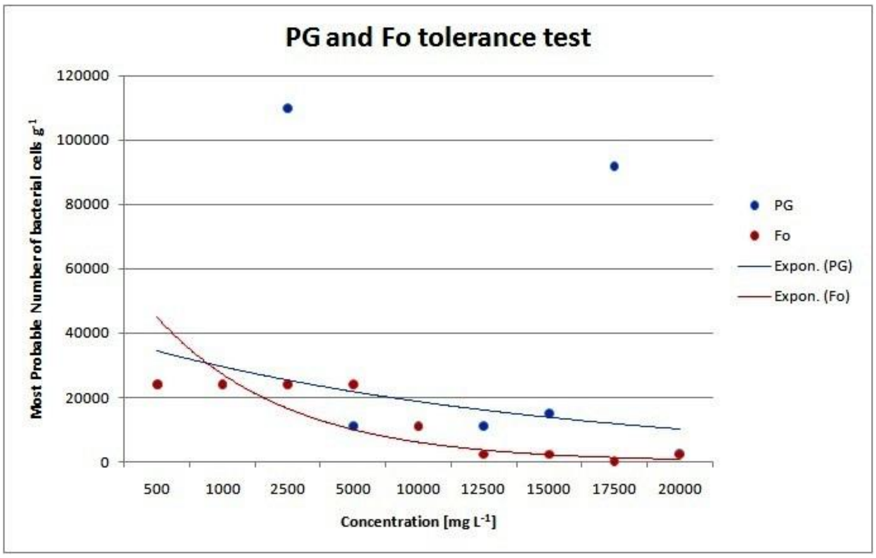


40. Figure: Comparison of the estimated number of microorganisms and the TOC and PG content measured in Column S5 effluent samples



41. Figure: Comparison of the estimated number of microorganisms and the TOC and PG content measured in Column S6 effluent samples.

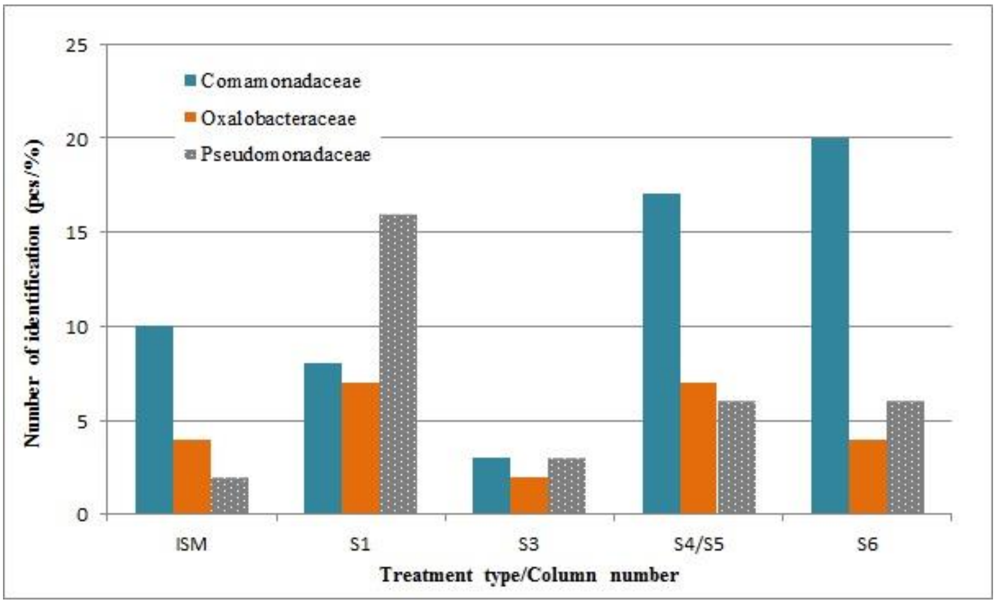
While the tolerance test used a fairly large-step scale on evaluating the toxicity level of the contaminants, it is clear, that microbes capable to grown on higher DIC concentration levels were still present between 15000 and 20000 mgl^{-1} DIC, although in lower numbers (**Figure 42**). Microbes found in these soils seem to be able to endure even 10 times more DIC concentration that can be found in the soil during snowmelt. Bacteria may respond to environmental stresses not only changing their number but also by biochemical activity, and they can adapt after the first pollution stress [88].



42. Figure: Microbial cell numbers during treatment with increased concentration of DICs

Pseudomonas are known to be r-strategist bacteria, ubiquitary in soil and easily cultured. As r-strategists, through their rapid growth rate, *Pseudomonas* can take over and dominate environments where nutrients are abundant [86]. The presence of PG degrading *Pseudomonas* strains has also been demonstrated in previous studies [108; 96; 109; 110]. Their ability to make efficient use of readily available PG determined the prevalence of this genus within the mixed population of enrichment cultures. However, a single bacterium usually has only a relatively small degradation range, and thus not all fractions of hydrocarbons can be degraded by a single species [86]. This community structure is representative only to low temperature, the bacterial population dynamics may change with the seasons [85]. However, the main reason for community analysis is not just to characterize the soil and monitor the effect of xenobitotic contamination, but to detect microorganisms that can be used for bioaugumentation.

In our studies, among the 100 clones from each treatment, three dominant bacterial families could be detected: *Pseudomonadaceae*, *Oxalobacteraceae* and *Comamonadaceae*. They occurred in comparably more times and reacted significantly to the different organic compound treatments.



43. Figure: Changes in the number of the 3 dominant bacterium families in the Initial Soil Material (ISM) and in the columns after DIC treatment (S1-S6)

The number of representing *Pseudomonas species* in the columns treated with the pollutants were found to be increasing, especially in S1/S2 column. The chemical data suggest, that both PG and formate degradation is intensive, although when both present formate inhibits PG degradation. Still the *Pseudomonadacea* family represented a higher part in the column treated with both compounds (S1/S2), while the *Comamonadaceae* was found to be more abundant in columns treated with only PG (S4/S5 and S6) (**Figure 43**). The community profiling therefore suggest selection toward microbial groups capable to degrade PG and FO in soils contaminated with DF.

6.1.5. Summary of the research in the deicing-fluid contaminated Gardermoen site

Summarizing the results of microbiological investigations at Gardermoen site, we can state the followings:

There were 2 order of magnitude (i.e. 100 times) less total anaerobic microbiological counts found both at the surface (horizontal) samples (L3-1 sample) and also at the below-ground (vertical) samples (i.e. sample L3-3 at the 50 cm layer) in comparison with the total aerobic heterotrophic counts.

Regarding the surface samples, the anaerobic counts and the microbes capable of growing in DF as sole carbon source in BM (basal medium) was found to be about 1.5-2.0 order of magnitude less (~10^4) than the total anaerobic counts (close to 10^6). Dominant route of PG degradation is assumed to be aerobic, due to the fact, that the aerobic counts and the MPN of PG degraders were always greater in the surface horizontal samples. In the surface soil samples the abundance of PG degrading bacteria is near the same as the total aerobic heterotrophic counts. The degradation of PG therefore is not a specific behavior, cannot be fixed to a certain subpopulation of the whole microbial community. In comparison with this finding the abundance of microbes capable of DF degradation is about 1 order of magnitude (1.e. 10 times) less in the soil samples. This fact shows that some of the components of DF other than the PG can result in a reduction capacity of microbial growth. The increased abundance of total heterotrophic and PG degrader microorganisms at an increasing distance from the runway however shows similarity even with the magnitude difference. This correlation was also verified by the enzymatic activity data, which correlated well with CFU counts.

Additional column experiments were used to combine two bioremediation techniques to simulate direct injection in contaminated soils. During our experiments it was proved that the impact of the tested pollutants is indeed represented in either cell numbers or community structure. We could detect the most abundant populations represented in a community profile with 16s rDNA analysis technique (Pseudomonacea, Oxalobacteraceae, Comamondaceae). In our samples they represented themselves in comparably higher numbers and reacted well to DIC treatments, providing proof of selection toward groups capable to degrade PG and FO.

Bacteria that can tolerate high level of contamination, and can survive in the harsh conditions like low temperature are favorable for bioaugmentation processes. Isolating, identifying and testing these abundant bacterial strains are needed in order to move forward to bioremediation experiments. Further combined microcosm and biostimulation experiments can lead to possible inoculants that can be injected to soils contaminated with de-icing chemicals.

6.2. Results of the crude-oil contaminated Trecate site

6.2.1. Microbial abundance in crude-oil contaminated soils

The Trecate site was sampled as it was described in Chapter 5.1.2. There was a non-contaminated area selected for comparison purposes (labelled as S1) and 2 other contaminated sampling areas (S4 and S8) were considered as being crude oil-polluted in the deep soil layers and therefore tested for microbial abundance. The countable microbial components, established as the total aerobe heterotrophs in nutrient agar plates are shown in the following figures (**Figure 44 a, b, c**).

Regarding the aerobic microbes, the abundance was found to be rather variable as a function of different soil layers. In case of the control (S1) soil the highest amount of bacteria 2.3 x 10^6 was found in the sample originating from the 4.3 m deep layer. Approximately the same quantity 2.75 x 10^6 was estimated in the borehole S4 at the 6.1 m deep soil layer. A much higher, at least 10 times more amount of microbes could be found in the most contaminated borehole of S8, where the highest amount of 21.5 x 10^6 bacteria could be measured.

The abundance of the anaerobic microbial community was also estimated. Anaerobe results for the 3 boreholes are shown in **Figures 45 a, b and c**. In the S-1 control soil a much higher amount of anaerobic microbes (7.5 x 10^3) could be found near the soil-surfaces at 1.6 m. In deeper soil layers there were not much differences found in the microbial counts, with a number of 5-20 x 10^3 in each g of soil-samples. Regarding the borehole S8, there were 2 highest peak recorded at the layers 6.5 and 11.3 m. At those layers the abundance of microbes was 150.5 x 10^3 and 181.5 x 10^3, respectively. These numbers are about 10-times greater than in case of the control borehole, S-1. The highest microbial abundance of anaerobes could be realized at borehole S8. At the layer 5.1 - 5.5 m, the abundance of anaerobe microbes was 284.5 x 10^3 - and 260 x 10^3 in each g of soil on a dry soil base. Microbial counts were rather low in other soil samples from the S-8.

6.2.2. Microbial enzymatic activity in crude-oil contaminated soils

The activity of microbes in the soil could be assessed by enzymatic assays. The best methods are which are showing the metabolically active part of the total microbes, present in the soils. The fluorescein diacetate analysis (FDA) is a generally accepted method, which could be correlated with microbial abundance.
Result of the fluorescein diacetate (FDA) analysis is shown in the **Figure 36.** The soil-samples were originating from the non-contaminated borehole S1 (control) of Trecate, Italy and the boreholes of S4 and S8, considered as crude-oil contaminated areas.

Regarding the control area presented on **Figure 46a**, the highest activity was recorded at the soil layer 1.7m, where the activity was found to be about 6-times more than in other layers investigated.

A greater amount of fluorescein dye was measured in the samples which were originating from the boreholes of S4 and S8. **Figures 46b and c** are showing, that the highest value of FDA activity can be about 10-times more in case of the borehole S8, in comparison with the S4. However, there is not much activity difference at the layers of 6.1-11m deep from the borehole of S4 (**Figure 46b**), which is considered fully saturated by oil-pollutants. The same balanced activity however was not found at the borehole S8 where the oil-saturation was unequal from 5m below. From those 49 samples of the borehole S8, the FDA activity was the highest only at the smear zone, at the layers of 5.1-5.5 meters (see **Figure 45c**).

Figure 44a

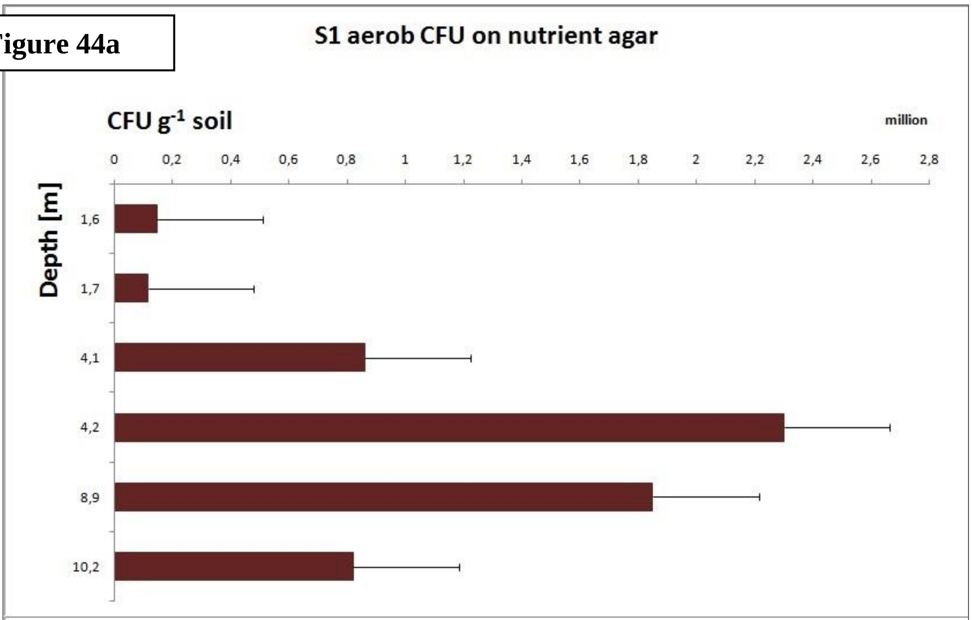


Figure 44b

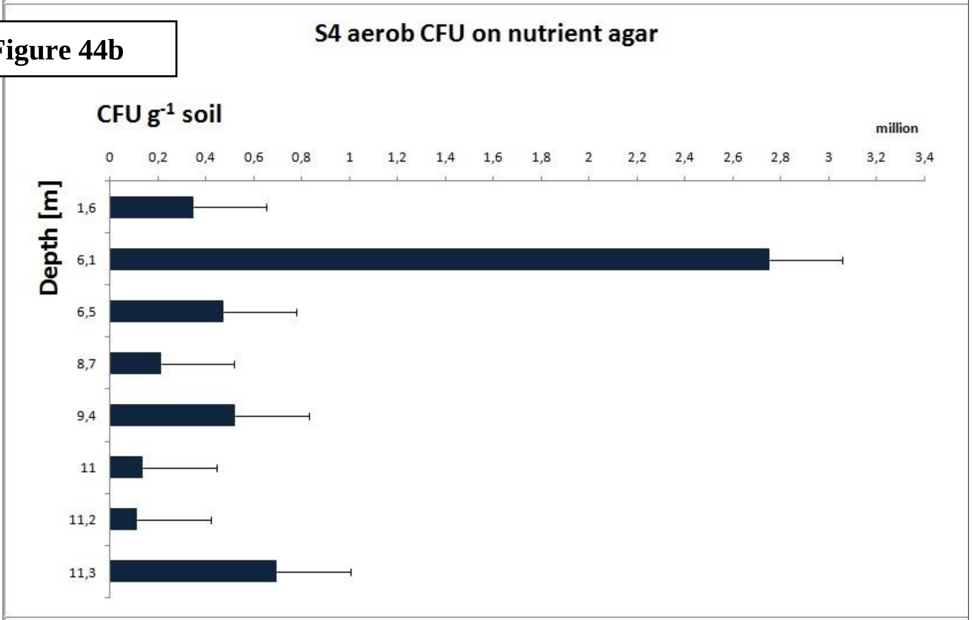
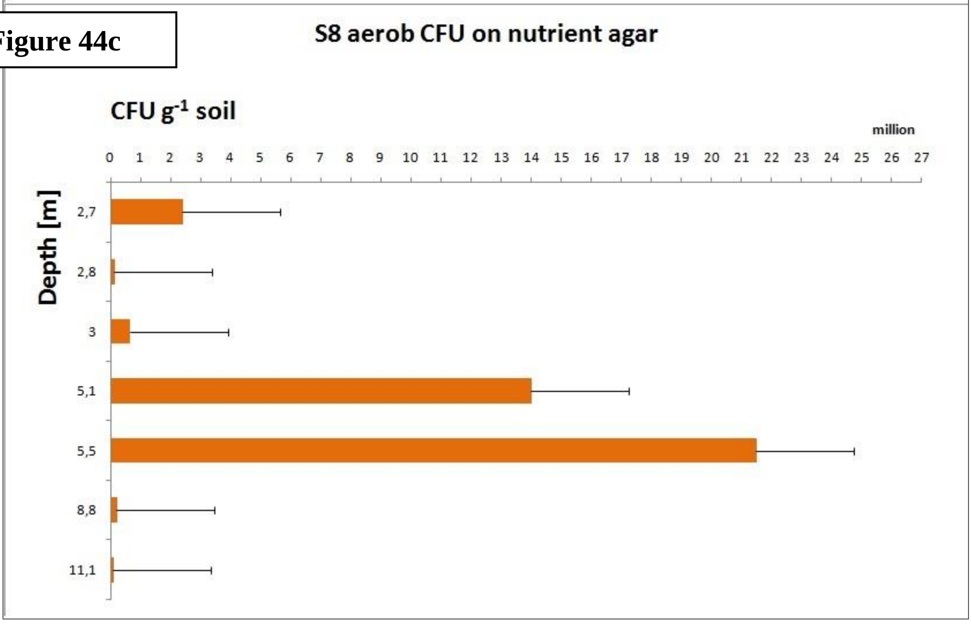


Figure 44c



44. Figure: Colony forming units (CFU) of total aerob microbes, grown on Nutrient agar plates from boreholes a.) S-1 (control), b.) S-4 and c.) S-8 (crude-oil contaminated) of the Tecate site.

Figure 45a

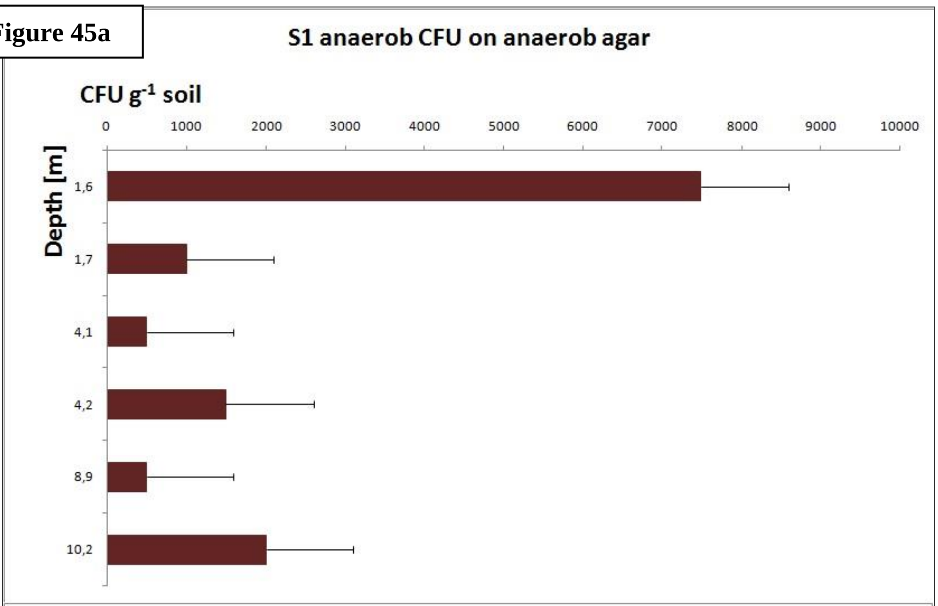


Figure 45b

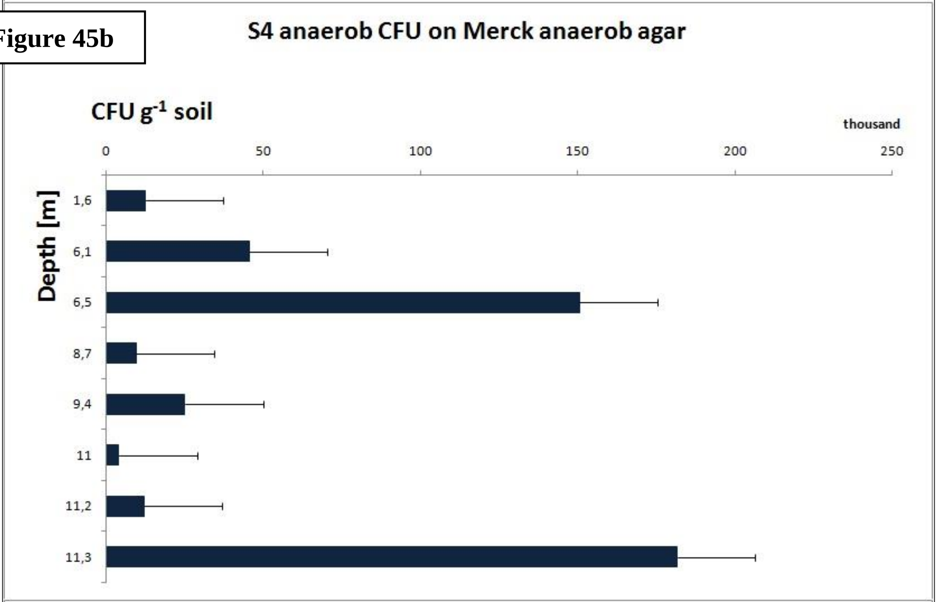
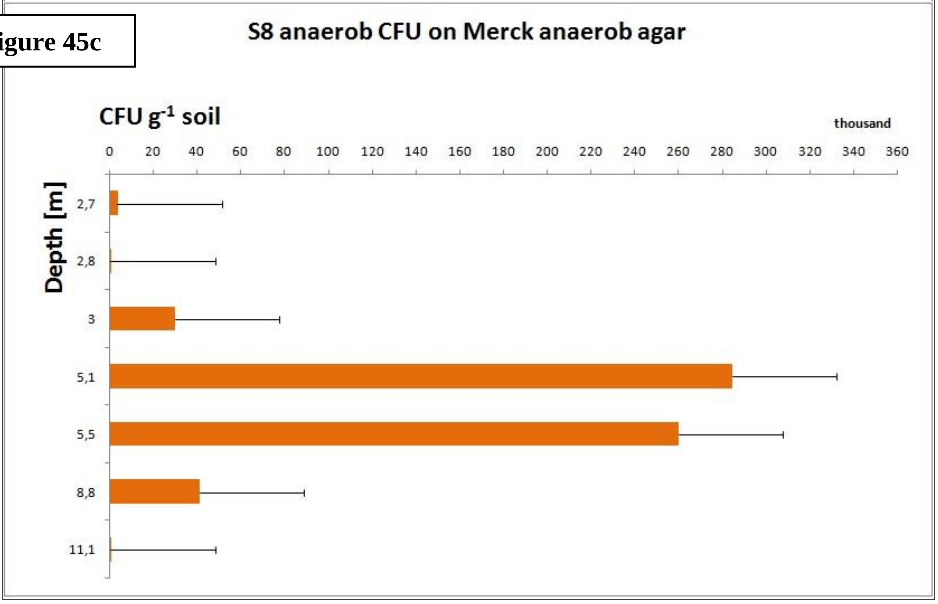


Figure 45c



45. Figure: Colony forming units (CFU) of anaerobic microbes in the soils of Trecate site. Borehole a.) S-1 is the control, non-contaminated, the other two borehole, b.) S-4 and c.) S-8 is oil-contaminated.

Figure 46a

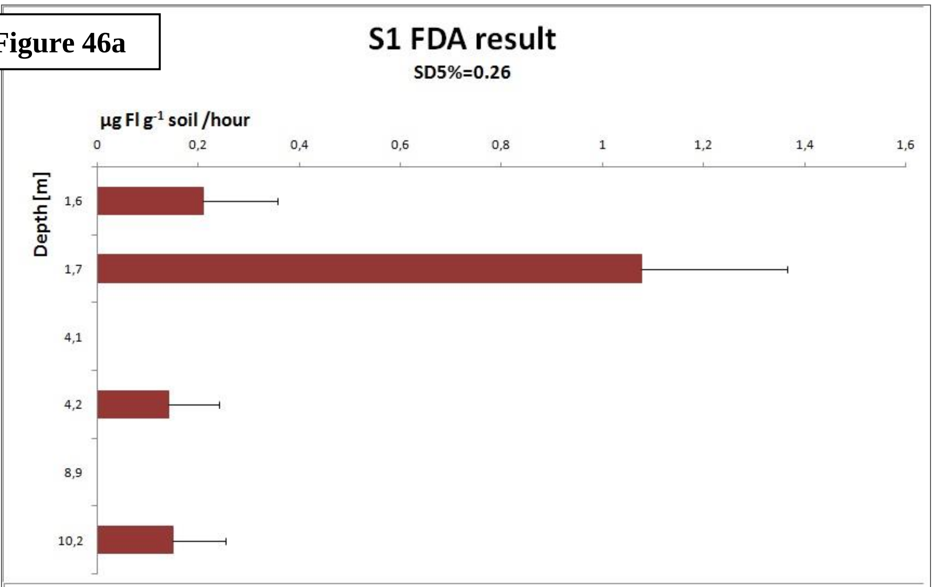


Figure 46b

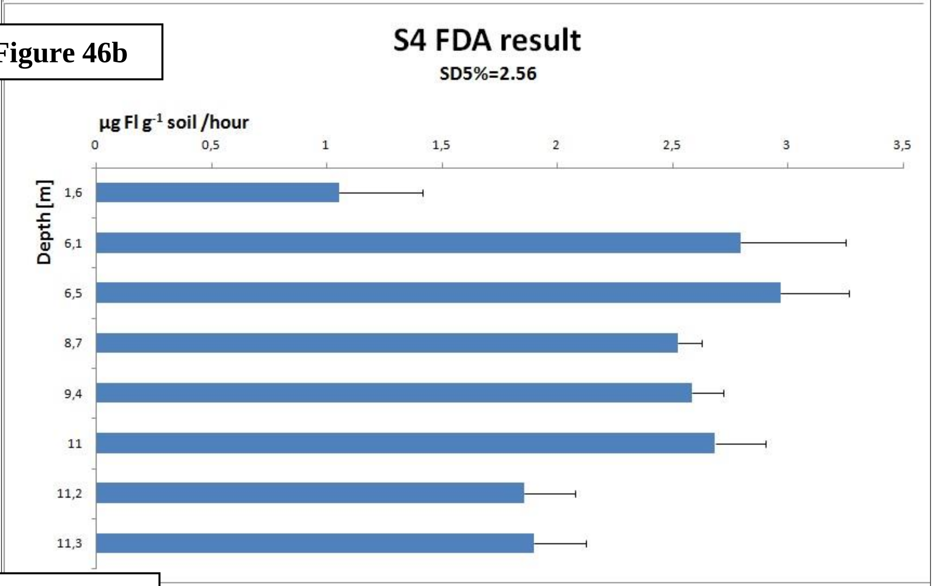
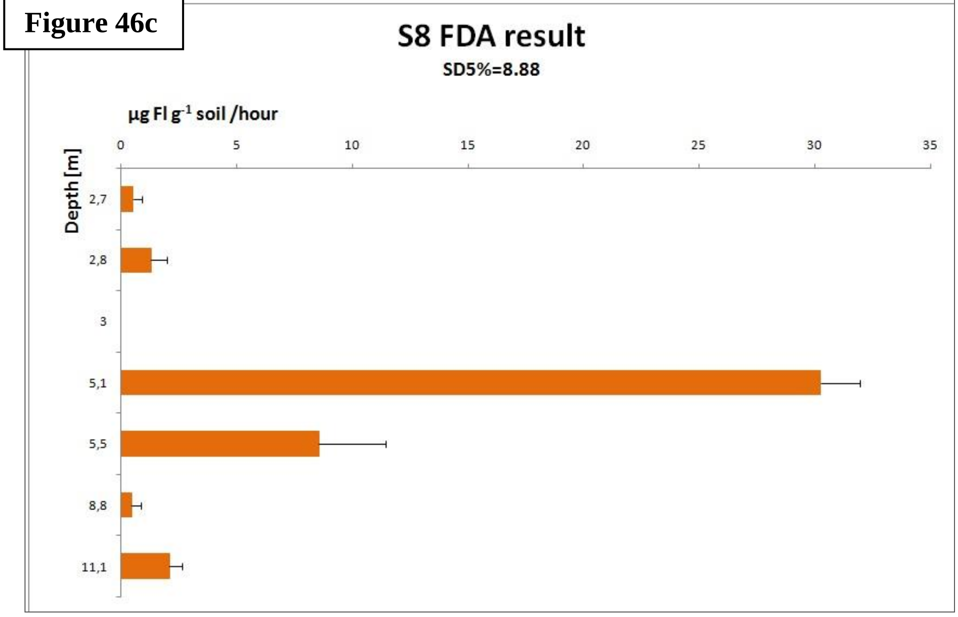


Figure 46c



46. Figure: Enzyme activity, measured by fluorescent diacetat analysis (FDA) in the soils, sampled from the borehole a.) S-1 (control sample), b.) S-4 and c.) S-8, considered as oil-contaminated areas of the Trecate site.

6.2.3. Identification of isolates from the crude-oil contaminated soil

Alignment results from the identification are presented in **Table 11 and 12**.

According to results Isolate TR-2 is part of the *Pseudomonas genus* at 100% identicality. Species cannot be a 100% determined, because it shows more than 98% similarity to several other *Pseudomonas species*. The partial 16S rDNA gene fragment (443 nucleotides long) did not contain polimorf nucleotides. Comparing the results from 2 databases could narrow the possibility down to 2 species, *Pseudomonas extremaustralis* and *Pseudomonas veronii. P. extremaustralis* was first isolated from a temporary pond in Antarctica [114], so the likelyhood that our strain belongs to this species are low, although genetically they are very closely related. *P. veronii* was originally isolated from natural springs in France; therefore our isolate is more likely this species originating from a neighboring country.

Pseudomonas veronii is a Gram-negative, rod-shaped, fluorescent, motile bacterium, and it may be used for bioremediation of contaminated soils, as it has been shown to degrade a variety of organic compounds. *P. veronii* strains have been implicated in human pathogenicity, heavy metal biosorption, and dibenzo-p-dioxin, chloroaromatic and alkyl methyl ketone degradation. *P. veronii* strains were also pointed out as key catabolic players in a BTEX contaminated area where benzene had been the major contaminant [115]. This candidate therefore can be considered for future inoculum development.

Isolate TR-22 is 100% part of the *Bacillus genus*. Species cannot be a 100% determined, because it shows more than 98% similarity to several other *Bacillus species*. The partial 16S rDNA gene fragment (447 nucleotide long) contained 1 polimorf nucleotide (Y = C or T).

The "*Bacillus cereus* group" of Gram-positive, spore-forming bacteria forms a homogeneous independent branch within the *Bacillus genus* and comprises eleven closely related species, for example *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* etc. [118].

Comparing the 2 databases (**Table 11 and 12**) the possible species were narrowed down to *Bacillus thuringiensis* and *Bacillus toyonensis.*

Bacillus thuringiensis is a Gram-positive, rod shaped, spore-forming, soil-dwelling bacterium. During sporulation many *B. thuringiensis* strains produce crystal proteins (proteinaceous inclusions) called δ-endotoxins that have insecticidal action [116]. This has led to their use as insecticides, and more recently to genetically modified crops using their genes [117]. The species were tested in bioremediation processes as well and were found to be able to bioaccumulate Zinc and Lead [163] and also to volatilize Mercury [164]. Since this species does not show potential toward oil remediation, it is not a very good candidate for future inoculum development in case of the Trecate site.

Bacillus toyonensis is Gram-positive, motile, facultative anaerobic rods forming ellipsoidal central to subterminal spores in non-swollen sporangia. The length of the rods range between 3 and 4 m, and cells occur singly, in pairs and occasionally in short chains or filaments. Colonies are generally large, flat with entire to undulated edges, and often form swarming rings around the colony extending through the plate. In general, colonies are matt to granulated, with a whitish to cream color. Growth occurs between 10 and 45 ◦C, and optimum growth occurs at 35 ◦C [119]. *Bacillus toyonensis* has not been described as a common bioremediating bacterium, but it has been demonstrated with the ability to produce bioflucculant, which makes it a very good candidate for wastewater treatment [165; 28], but it needs to be further tested for oil contamination in the soil.

11. Table: NCBI Blast alignment results of TR-2 and TR-22 isolates' identification from the Trecate site

Species	**Accession#**	**Identicality (%)**	**Nucleotid difference***
TR-2			
Pseudomonas orientalis strain IHB B 10273	KR233760	100	0/443
Pseudomonas simiae strain IHBB 9152	KR085825	100	0/443
Pseudomonas trivialis strain IHBB745	CP011507	100	0/443
Pseudomonas veronii strain PAZ1	KP858504	100	0/443
Pseudomonas fluorescens bv. I t strain A-Tom2	KP126778	100	0/443
Pseudomonas denitrificans isolate 05CF15-5C	LC007966	100	0/443
Pseudomonas extremaustralis strain N-16	KM386366	100	0/443
Pseudomonas fluorescens strain ATCC 17397	JN630889	100	0/443
Pseudomonas fluorescens strain ATCC 17397	JN630889	100	0/443
TR-22			
Bacillus thuringiensis strain HD12	CP014847	99	1/447
Bacillus toyonensis strain AIMST. Acse2	KT861456	99	1/447
Bacillus cereus strain FSL K6-0069	KU198625	99	1/447
Bacillus cereus strain FSL K6-0069	KU198625	99	1/447

*database considers polimorf nucleotides as difference

12. Table: EzTaxon-e type stain database alignment results of TR-2 and TR-22 isolates' identification from the Trecate site

Species	**Type stain**	**Accession#**	**Identicality (%)**	**Nucleotid difference***
TR-2				
Pseudomonas extremaustralis	14-3(T)	AHIP01000073	100.00	0/443
Pseudomonas veronii	CIP 104663(T)	AF064460	100.00	0/443
Pseudomonas antarctica	CMS 35(T)	AJ537601	99.77	1/443
Pseudomonas trivialis	DSM 14937(T)	AJ492831	99.77	1/443
Pseudomonas extremorientalis	KMM 3447(T)	AF405328	99.77	1/443
Pseudomonas poae	DSM 14936(T)	AJ492829	99.77	1/443
Pseudomonas meridiana	CMS 38(T)	AJ537602	99.77	1/443
Pseudomonas simiae	OLi(T)	AJ936933	99.74	1/391
Pseudomonas orientalis	CFML 96-170(T)	AF064457	99.55	2/443

Species	**Type stain**	**Accession#**	**Identicality (%)**	**Nucleotid difference***
TR-2				
Pseudomonas costantinii	CFBP 5705(T)	AF374472	99.32	3/443
Pseudomonas grimontii	CFML 97-514(T)	AF268029	99.31	3/434
Pseudomonas lurida	DSM 15835(T)	AJ581999	99.10	4/443
Pseudomonas chlororaphis subsp. aureofaciens	NBRC 3521(T)	BBQB01000031	99.10	4/443
Pseudomonas arsenicoxydans	VC-1(T)	FN645213	99.10	4/443
Pseudomonas viridiflava	LMG 2352(T)	Z76671	99.10	4/443
Pseudomonas palleroniana	CFBP 4389(T)	AY091527	99.03	4/411
Pseudomonas prosekii	AN/28/1(T)	JN814372	99.00	4/400
Pseudomonas chlororaphis subsp. aurantiaca	NCIB 10068(T)	DQ682655	98.87	5/443
Pseudomonas asturiensis	LPPA 221(T)	FM865870	98.87	5/443
Pseudomonas rhodesiae	CIP 104664(T)	AF064459	98.87	5/443
Pseudomonas chlororaphis subsp. piscium	JF3835(T)	FJ168539	98.81	4/336
Pseudomonas marginalis	ATCC 10844(T)	AJ308309	98.81	4/336
Pseudomonas tolaasii	ATCC 33618(T)	D84028	98.60	6/428
Pseudomonas lini	CFBP 5737(T)	AY035996	98.42	7/442
Pseudomonas psychrophila	E-3(T)	AB041885	98.42	7/442
Pseudomonas fluorescens	DSM 50090(T)	Z76662	98.40	7/437
Pseudomonas yamanorum	8H1(T)	EU557337	98.19	8/443
Pseudomonas mediterranea	CFBP 5447(T)	AUPB01000004	98.19	8/443
Pseudomonas fragi	ATCC 4973(T)	AF094733	98.19	8/443
Pseudomonas panacis	CG20106(T)	AY787208	98.19	8/443
Pseudomonas migulae	CIP 105470(T)	AF074383	98.19	8/443
Pseudomonas brenneri	CFML 97-391(T)	AF268968	98.16	8/435
TR-22				
Bacillus thuringiensis	ATCC 10792(T)	ACNF01000156	100.00	0/446
Bacillus toyonensis	BCT-7112(T)	CP006863	100.00	0/446
Bacillus anthracis	ATCC 14578(T)	AB190217	99.50	2/402
Bacillus cereus	ATCC 14579(T)	AE016877	99.33	3/446
Bacillus anthracis	Ames	AE016879	99.10	4/446
Bacillus pseudomycoides	DSM 12442(T)	ACMX01000133	98.65	6/446
Bacillus weihenstephanensis	NBRC 101238(T)	BAUY01000093	98.43	7/446
Bacillus mycoides	DSM 2048(T)	ACMU01000002	98.43	7/446
Bacillus bingmayongensis	FJAT-13831(T)	JN885201	98.10	8/420
Bacillus gaemokensis	BL3-6 KCTC 13318(T)	FJ416489	97.86	8/373

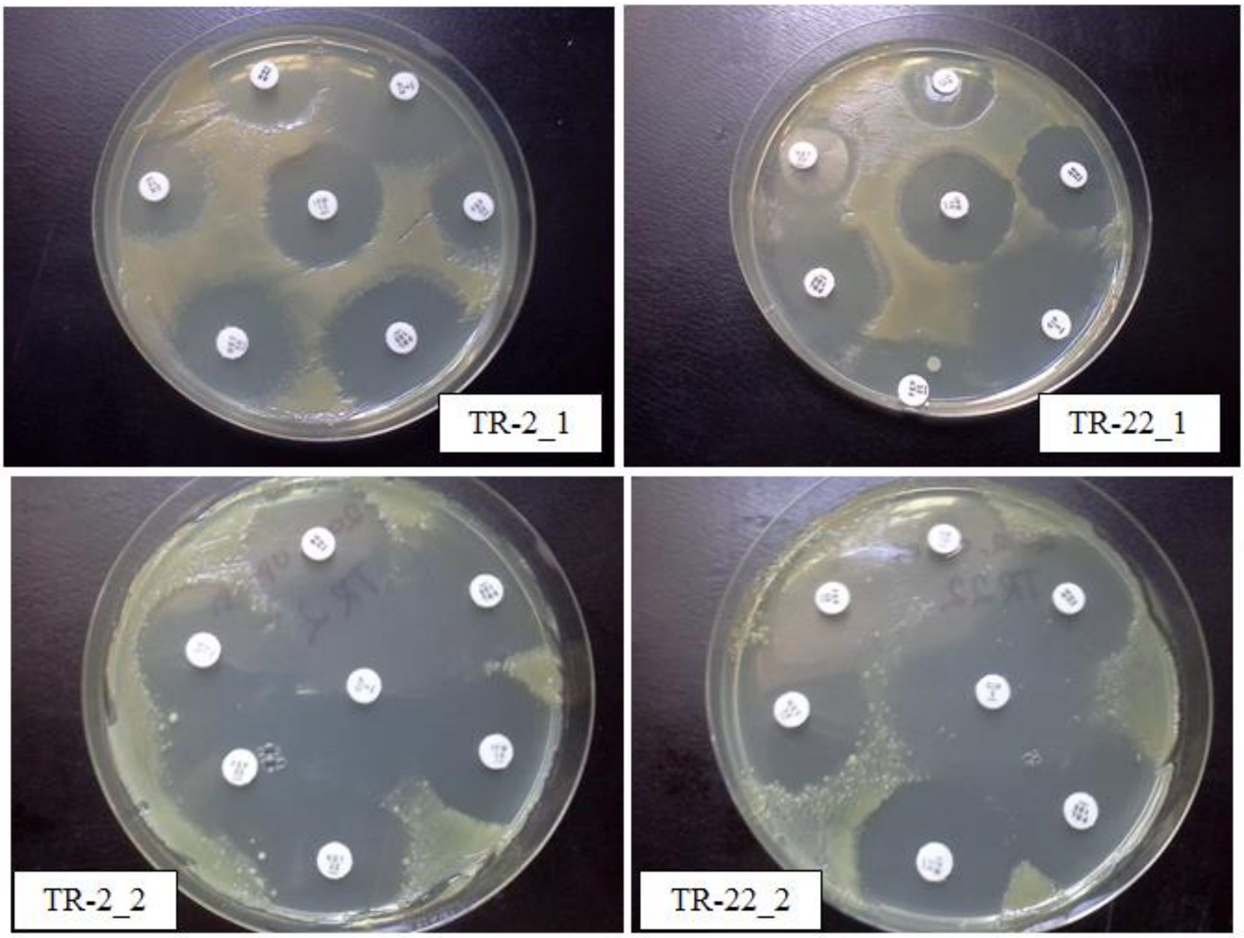
**database ignores polimorf nucleotides*

The two identified bacterial isolates (TR-2 and TR-22) were subjected to antibiotic susceptibility testing against 7 anitbiotics (in 2 paralells) listed in **Table 13**. The listed antibiotics have been choosen to cover different types of antibiotic agents based on [166].

13. Table: Antibiotics used for susceptibility testing of the strains isolated from crude-oil contaminated Trecate site

Antibiotic	Concetration (μg)	Disk
Netilmicin	30	NET30
Gentamicin	10	CN10
Amikacin	30	AK30
Cefepime	30	FEP30
Ciprofloxacin	5	CIP5
Piperacillin	100	PRL100
Imipenem	10	IPM10

Results from the susceptibility test indicate both of our chosen isolates were sensitive against all 7 antibiotics (see **Figure 47**) therefore, bioremediation usage is possible for each strain.



47. Figure: Susceptibility test on TR-2 and TR-22 isolates isolated from crude-oil contaminated Trecate site (photo taken by: Nikoletta Horváth, 2012)

6.2.4. Summary of the research in the crude-oil contaminated Trecate site

The Trecate site of Italy was contaminated by crude-oil in 1994. Sampling of belowground soil-layers was performed in February 2011, when specimens from 5 boreholes have been transferred to the RISSAC Budapest, Hungary. During the current research 1 control are (S-1) and 2 crude-oil contaminated (S-4 and S-8) have been tested on.

Classic microbiological plating method was used to measure the colony forming units (CFU). Selective plates were utilized for aerobic and anaerobic microbial content separation in the soil samples. Regarding the monitoring of microbial status in the soils, the activity of microbial community was also studied by catabolic enzymatic analysis (FDA). The final goal was to isolate and identify bacteria capable to enhance the biodegradation and bioremediation of oil contaminants in the soils.

The below-ground microbiological parameters were variable in the soils, independently from the origin of the sample. At the control, non-contaminated borehole, the abundance was rather dependent on the soil-physical, -chemical conditions. Among the potential parameters affecting the microbial abundance, the organic matter (OM) content and the soil-pore-distribution are known mainly from the literature.

There were differences found in the abundance (presence) of aerobic and anaerobic microbial components in the different layers of the boreholes. This finding could show the necessity of measuring the oxygen tension in those soil samples beside the other more general soil-physical, chemical parameters.

At boreholes, which were contaminated with oil-pollutants, a greater microbiological abundance and activity was found in generally at all measured parameters in the smear zone, at those layers, that occasionally are affected by the water-fluctuation. In those soils the available nutrient content are generally higher, which might affect positively the microbiological parameters, including both the abundance (CFU) and the enzymatic, catabolic activity (FDA).

Beside abundancy and enzymatic activity, microbial characterization can provide an appropriate tool for after isolating microbes, to define potential candidates for later remediation technologies. Therefore chosen isolates (based on frequency of appearance and growth speed) were sent for 16S rDNA identification by a commissioned laboratory. The 2 isolates chosen as candidates for further bioremediation application were identified as *Pseudomonas veronii* (TR-2) and the other was a Bacillus species, closely related, either *B. thuringiensis* or *B. toyonensis* (TR-22). Both of the strains were tested against 7 antimicrobial agents to make sure antimicrobial resistance genes were not present that could exclude them as bioremediator candidates. The susceptibility test was favorable.

Pseudomonas veronii is a known bioremediatior bacteria, therefore, it is advisable to use this strain for inoculum development. The other strain also has biotechnological uses, but it needs to be further assessed, because *B. thuringiensis* and *B. toyonensis* had not been studied in oil bioremediation before.

6.3. Results of the Manganese treated soil of Őrbottyán

6.3.1. Biomass of the Energy grass on Manganese treated soil

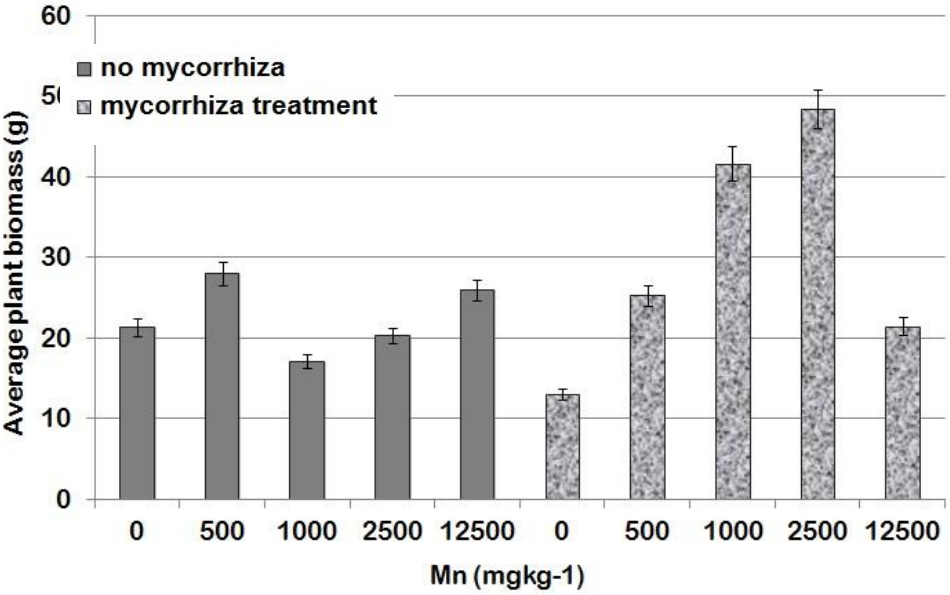
After the vegetation period of the Energy grass biomass data from the root and shoot growth have been gathered by traditional weighing method.

When comparing the growth of the Energy grass with and without mycorrhizal treatment after 1 year on the Mn treated soil, the average plant biomass reacted positively to mycorrhizal treatment, and showed increase up to 12500 mg Manganese, where it drops significally, indicating that the Manganese reached to toxic levels, and the plant reacted to it with decreased biomass production, as shown in **Figure 48**.

After measurements of the Mn content of the plants treated with mycorrhiza (tested by a commissioned analytical laboratory of the RISSAC, HAS) we realized that extreme high doses of Manganese could be found in the root, which is the first protecting barrier of the plant (**Table 14**).

Energy grass has been studied before in connection to several other heavy metals. The plant was shown to be sensitive to high external concentrations of Cu and Cd and fairly tolerant to Ni and Pb. Cu and Cd toxicity leading to the inhibition of growth, transpiration and Chl biosynthesis was attributed to the uptake and translocation of these metals. It was also described as a Zn accumulator [160]. In this study the plant did not show any change in root and shoot biomass during metal treatment, which confirmed our results without mycorrhiza treatment.

Therefore, we can state, that mycorrhizal treatment was very successful regarding both Manganese uptake and plant biomass increment. Moreover, when treated with mycorrhiza - therefore enhancing its stress tolerance - the plant could be used as an indicator to Manganese concentration. This proves that rhizospheric processes involving microorganisms can indeed help Manganese uptake for plants from the soil. An optimal Manganese level of the plant-fungi symbiosis might be defined, as described by [144].



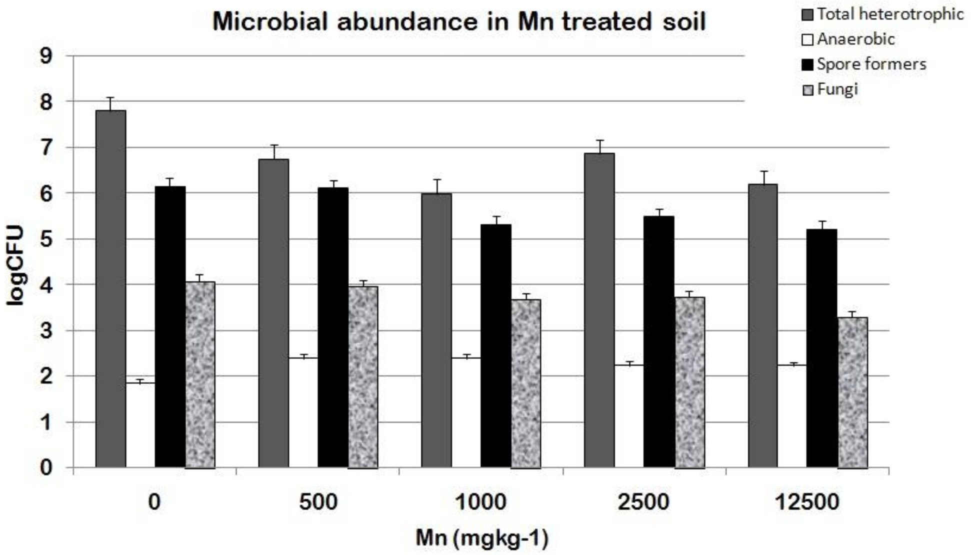
48. Figure: Measured plant biomass of Energy grass Szarvasi-1 after 1 year of different doses of Mn-treatment in calcareous sandy soil, Örbottyán, Hungary

14. Table: Manganese content in the soil, and in the shoot and root of energy grass at increasing Mn-doses with mycorrhiza inoculation.

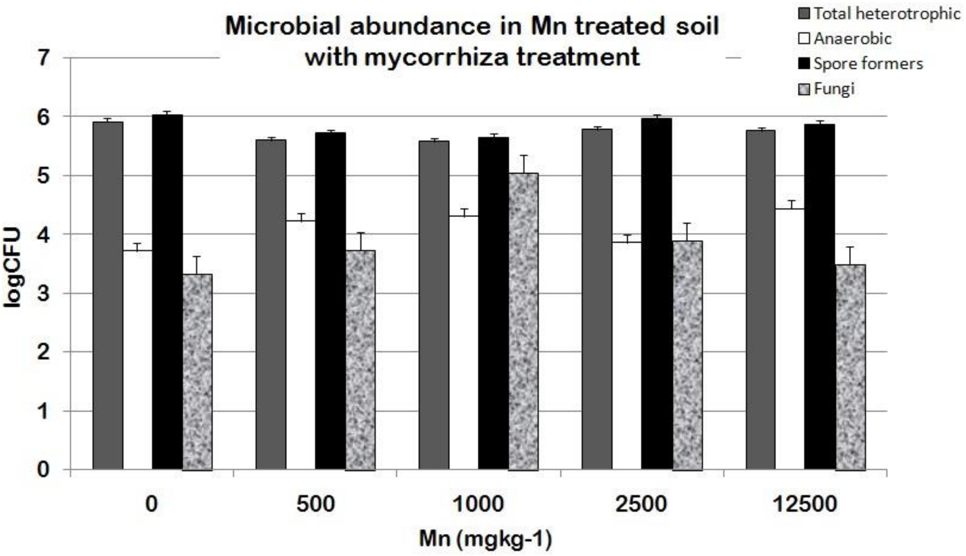
Mn doses (mgkg^{-1})	**Mn content (mgkg^{-1})**		
	soil	**shoot**	**root**
0	231	619	1065
500	1132	590	2305
1000	1216	2302	5215
2500	1592	1878	5521
12500	7310	1714	13967

6.3.2. The effect of Manganese on microbial abundance

Figure 49 and 50 shows the abundance of some culturable physiological groups of microorganisms from the experimental site. We can determine that the tested microbial groups with or without additional mycorrhiza react differently to these Mn treatments. Anaerobic microorganisms, that require oxygen-free environment, showed a definite increase in numbers with mycorrhizal treatment. The culturable numbers of this group at the lowest Manganese doses (500, 1000 mgkg^{-1}) increased with two magnitudes, but we found ten times higher numbers at 2500, 12500 mgkg^{-1} doses too. Total heterotrophic bacterial numbers decreased significally with mycorrhizal treatment at the highest Mn doses. Sensitivity of spore formers and fungi with these treatments is not very definite. Increased anaerobic numbers and decreased heterotrophic aerobic numbers indicate that additional mycorrhizal treatment might be competitive for heterotrophic bacteria, and anaerobic bacteria can get a more significant role in these soils.



49. Figure: Abundance of different microbial groups at different Mn-doses without mycorrhizal treatment in calcareous sandy soil, Örbottyán, Hungary

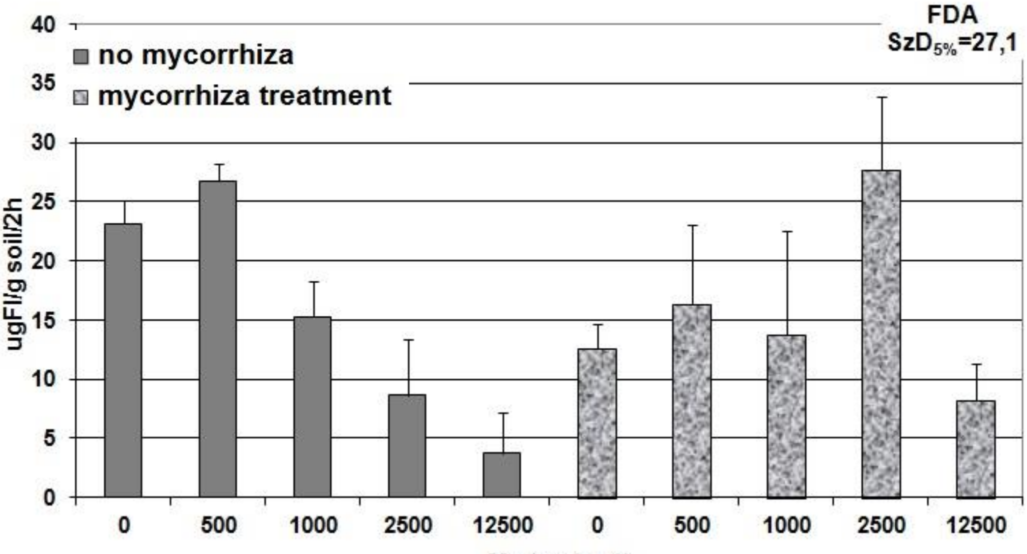


50. Figure: Abundance of different microbial groups at different Mn-doses with mycorrhizal treatment in calcareous sandy soil, Örbottyán, Hungary

6.3.3. Microbial enzymatic activity after Manganese treatment

The enzymatic activity in the soil after 1 year treatment was proved to be affected by the increased doses of Manganese. Without additional micorrhiza, higher concentration of Mn reduced activity starting from 500 mgkg^{-1}. Compared to the previous statement, with mycorrhizal treatment, enzymatic activity increased up to 2500 mgkg^{-1} Manganese, however, according to results, 12500 mgkg^{-1} of Mn content was highly toxic, and activity decreased significantly at this point (**Figure 51**).

We can therefore conclude that even with mycorrhizal treatment, toxic levels of Mn contamination will not be tolerated; however, the concentration at which point Mn starts to have a negative effect on microbial activity can be postponed.



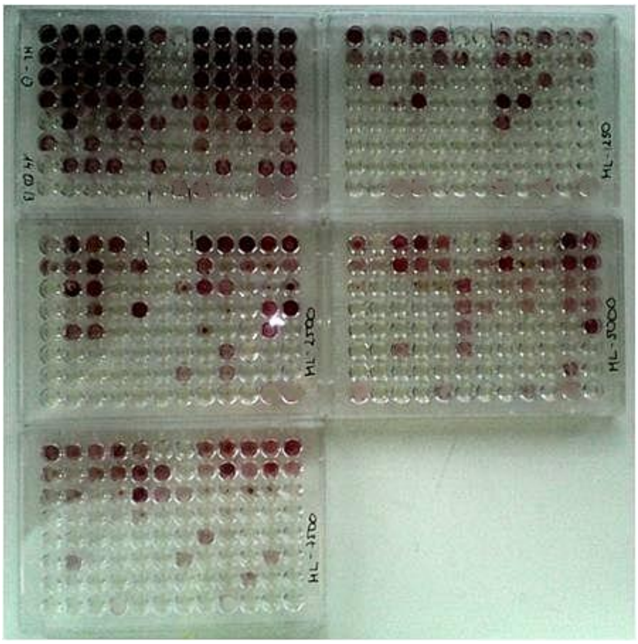
51. Figure: Measured FDA enzymatic activity at different Mn-doses in calcareous sandy soil, Örbottyán, Hungary

6.3.4. The appearance of Manganese tolerance

After 3 years of Manganese treatment (sampling from 2013), we wished to estimate how the population adapted to the different levels of Mn. During Manganese tolerance tests our goal was to see how the several years of Manganese treatment affected the tolerance of microbial populations, and in the end to select specific bacteria that can endure high levels of Mn doses.

The overall tolerance of the microbial population in the non-treated soil (ML7) and the soil treated with 12500 mgkg^{-1}Mn dose (ML12) have been tested by MPN method (the test is presented on **Figure 52**).

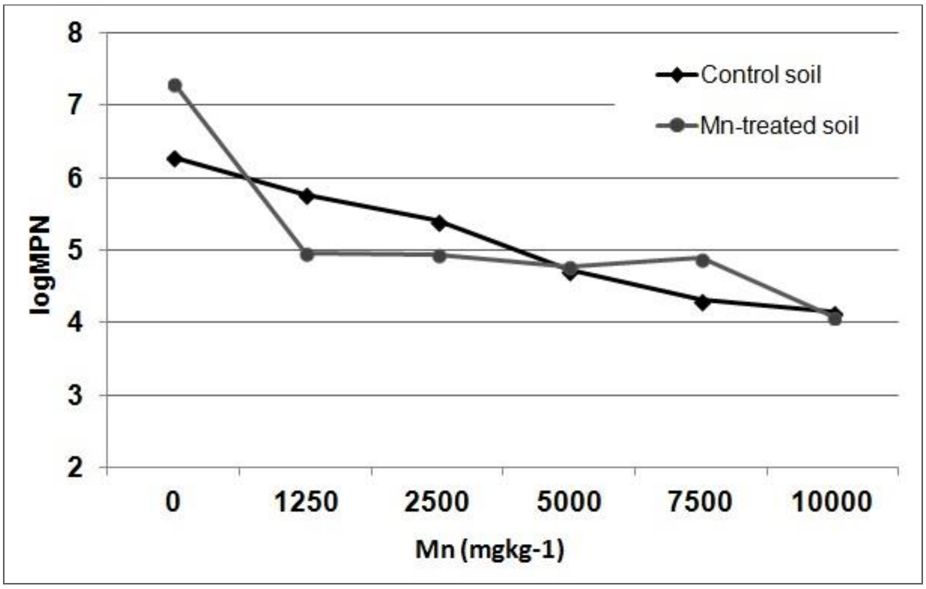
Enzymatic activity measurements (**Figure 51**) showed us in 2011 that 12500 mgkg^{-1} proved to be toxic even with mycorrhiza treatment. As such we tested two soils from the experimental site, one without any treatment during the 3 years - this acted as control sample (ML7) - and one, which was treated with the highest doses of Manganese, 12500 mgkg^{-1} (ML12). Soils from both samples have been incubated in media containing 0, 1250, 2500, 5000, 7500 and 10000 mgkg^{-1} Mn.



52. Figure: MPN microplates of Managnese treated soil samples incubated in media spiked with 0, 1250, 2500, 5000 and 7500 mgkg^{-1} Mn (photo taken by: Nikoletta Horváth, 2014)

According to the MPN results presented on **Figure 53**, bacterial growth from both the control and Mn-treated sample shows decrease toward higher doses of Manganese, however the trend shows difference. While the control group was very linear in decrease, the Mn-treated bacteria, that should have already been adapted to higher doses of Manganese, acted more sensitively at the first dose of Mn, their numbers dropped from 10^7 to 10^5. After that, however, we could not detect significant change in their number up until we reached 10000 mgkg^{-1}, which is very close to the toxicity border we previously measured. This made us realize that a more precise toxicity level would be between 7.500 and 10000 mgkg^{-1}, where the magnitude of cell numbers counted only around 10^4 compared to 10^6-10^7.

The difference in trend can be explained by previous experiments with adapted bacteria, where long term exposure to high level toxic compounds could cause an overall decrease of fitness in the population compared to those that were never in contact with the contaminating metal before.



53. Figure: Comparison of the most probable number of bacteria from non-treated control soil and Mn-treated soil (additional doses of Manganese in the media) after 3 years of treatment, Örbottyán, Hungary

6.3.5. Identification of Manganese tolerant strains

The isolates from the control sample (ML-7) has been identified as ***Bacillus sp.*** on the genus level with a similarity ratio of 92.3% and 94.1% (**Table 15 and 16**) to *Bacillus pumilus*. From the Manganese treated soils (ML-12) the two isolates were neither clearly admittable to any identified *Arthrobacter species*, only to the ***Arthrobacter genus*** with highest scoring similarity ratios of 90.4% nor 93.9 % (**Table 17**).

Bacillus species are rod-shaped, endospore-forming aerobic or facultative anaerobic, Gram-positive bacteria; in some species cultures may turn Gram-negative with age [141]. They are well known for their spore forming abilities. These spores allow them to survive harsh conditions, and environmental stresses. Since this soil was not affected by Manganese before, only spore formers were found to be able to survive the short time Mn stress during the Mn tolerance tests (Chapter 6.3.4.).

On the other hand, the found *Arthrobacter species* has been subjected to high doses of Mn for years. The *Arthrobacter genus* is a genus of bacteria that is commonly found in soil. All species in this genus are Gram-positive obligate aerobes that are rods during exponential growth and cocci in their stationary phase. They are chemoorganotrophs and have an oxidative metabolism. *Arthrobacter species* have been particularly useful in the bioremediation of groundwater contaminated with pesticides and herbicides; this is made possible because of their adaptable genomes, which can handle stressful conditions and environments [161]. Because of their metabolic diversity, *Arthrobacter species* have been taken advantage of for their ability to biodegrade various types of pollutants in our environment. Species of the genus, like *Arthrobacter sp.* FB24, are quite resistant to certain heavy metals that are toxic. *Arthrobacter aurescens* strain TC1 can metabolize more than 23 kinds of s-triazine compounds, an important fact to consider since these compounds are found in pesticides, resins, dyes, and explosives [161]. *Arthrobacter crystallopoietes* can reduce hexavalent chromium levels in soil, which may mean that there are potential future applications for it in bioremediation as well [162]. They have species capable to oxidize Manganese, and some of them are known PGPR bacteria, that can help reducing heavy metal stress on plants [140].

While we did not identify many candidates for further inoculation development, the isolates we chose were abundant, and not many types of colonies were present during plate cultivation. In case of non-treated soils, when subjected to higher doses of Manganese, the reaction was selection toward tolerant strains with endospores, while soils under Managanese treatment for years selected toward possible Mn oxidizers, these are very good candidates for bioremediation inoculums. As it was described in the Trecate site before (Chapter 6.2.3.), some *Bacillus species* can also be used in heavy metal bioremediation, therefore the selected isolates need to be further identified to the species level and assessed whether they have an affinity toward Mn bioaccumulation.

15. Table: Identification sequencing results from the RDP, Mn-treated soil samples, ML-7-1

Identicality (%)	***Species* and Accession**	**Identicality (%)**	***Species* and Accession**
ML-7-1			
91.7	*Bacillus sp.* 19499; LMG 19499; AJ315067	92.1	*Bacillus sp.* 19499; LMG 19499; AJ315067
91.7	*Bacillus pumilus*; OS-52.a; AM237360	92.1	*Bacillus pumilus*; OS-52.a; AM237360
91.7	*Bacillus sp.* R-31208; AM910309	92.1	*Bacillus sp.* R-31208; AM910309
91.7	*Bacillus pumilus*; DT83; EU780103	92.1	*Bacillus pumilus*; DT83; EU780103
91.7	*Bacillus pumilus*; XJSL4-8; GQ903417	92.1	*Bacillus pumilus*; XJSL4-8; GQ903417
91.7	*Bacillus pumilus*; XJSL4-9; GQ903418	92.1	*Bacillus pumilus*; XJSL4-9; GQ903418
91.7	*Bacillus pumilus*; SB 3182; GU191909	92.1	*Bacillus pumilus*; SB 3182; GU191909
91.7	*Bacillus pumilus*; SB 3002; GU191914	92.1	*Bacillus pumilus*; SB 3002; GU191914
91.7	uncultured *Bacillus sp.*; Filt.138; HM152725	92.1	uncultured *Bacillus sp.*; Filt.138; HM152725
91.7	uncultured *Bacillus sp.*; Filt.149; HM152736	92.1	uncultured *Bacillus sp.*; Filt.149; HM152736
91.7	*Bacillus pumilus*; cp42; JN082266	92.1	*Bacillus pumilus*; cp42; JN082266
91.7	*Bacillus pumilus*; PR16; JQ435676	92.1	*Bacillus pumilus*; PR16; JQ435676
91.7	*Bacillus pumilus*; CE4; JQ435701	92.1	*Bacillus pumilus*; CE4; JQ435701
91.7	*Bacillus pumilus*; IARI-ABSL-40; JX428992	92.1	*Bacillus pumilus*; IARI-ABSL-40; JX428992
91.7	*Bacillus pumilus*; LErs14; JX485760	92.1	*Bacillus pumilus*; LErs14; JX485760
91.7	*Bacillus pumilus*; LEr04; JX897981	92.1	*Bacillus pumilus*; LEr04; JX897981
91.7	*Bacillus pumilus*; ToIr-MA; KC806242	92.1	*Bacillus pumilus*; ToIr-MA; KC806242
91.7	*Bacillus pumilus*; MCCC 1A00439; JX680074	92.1	*Bacillus pumilus*; MCCC 1A00439; JX680074
91.7	*Bacillus pumilus*; MCCC 1A06991; JX680106	92.1	*Bacillus pumilus*; MCCC 1A06991; JX680106
91.7	*Bacillus pumilus*; MCCC 1A06996; JX680107	92.1	*Bacillus pumilus*; MCCC 1A06996; JX680107
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus*; BPT-18; EF523475
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
92.3	*Bacillus pumilus*; TCCC11014; EU231626	89.6	*Bacillus pumilus* SAFR-032; CP000813
92.3	*Bacillus pumilus*; TCCC11015; EU231627	89.6	*Bacillus pumilus*; TCCC11014; EU231626
92.3	*Bacillus sp.* FIA07; FIA07_1; EU308307	89.6	*Bacillus pumilus*; TCCC11015; EU231627
92.3	*Bacillus pumilus*; SB 3002; GU191914	89.6	*Bacillus sp.* B14(2008b); EU851052
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus sp.* LD82; AM913908
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus sp.* FIA07; FIA07_1; EU308307
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus*; SB 3002; GU191914
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.7	*Bacillus pumilus*; AUCAB17; GU297609
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus*; BHK6; AB298784
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus*; BF6-1; GQ861531
92.3	*Bacillus pumilus* SAFR-032; CP000813	89.6	uncultured *Bacillus sp.*; Filt.147; HM152734
92.3	*Bacillus pumilus*; PR13; JQ435673	89.9	uncultured bacterium; 12TCLN172; AB637098
92.3	*Bacillus pumilus*; CE4; JQ435701	89.9	*Bacillus sp.* 3021; JX566586

16. Table: Identification sequencing results from the RDP, Mn-treated soil samples, ML-7-l and 2

Identi-cality (%)	***Species* and Accession**	**Identi-cality (%)**	***Species* and Accession**
ML-7-2			
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus*; BPT-18; EF523475
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus* SAFR-032; CP000813
94.1	*Bacillus pumilus*; TCCC11014; EU231626	89.6	*Bacillus pumilus* SAFR-032; CP000813
94.1	*Bacillus pumilus*; TCCC11015; EU231627	89.6	*Bacillus pumilus*; TCCC11014; EU231626
94.1	*Bacillus sp*. FIA07; FIA07_1; EU308307	89.6	*Bacillus pumilus*; TCCC11015; EU231627
94.1	*Bacillus pumilus*; SB 3002; GU191914	89.6	*Bacillus sp*. B14(2008b); EU851052
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus sp*. LD82; AM913908
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus sp*. FIA07; FIA07_1; EU308307
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus*; SB 3002; GU191914
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.7	*Bacillus pumilus*; AUCAB17; GU297609
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus*; BHK6; AB298784
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	*Bacillus pumilus*; BF6-1; GQ861531
94.1	*Bacillus pumilus* SAFR-032; CP000813	89.6	uncultured *Bacillus sp*.; Filt.147; HM152734
94.1	*Bacillus pumilus*; PR13; JQ435673	89.9	uncultured bacterium; 12TCLN172; AB637098
94.1	*Bacillus pumilus*; IARI-BC-5; JX312578	89.9	*Bacillus sp*. 3021; JX566586

17. Table: Identification sequencing results from the RDP, Mn-treated soil samples, ML-12-1 and 2

Identicality (%)	***Species* and Accession**	**Identicality (%)**	***Species* and Accession**
ML-12-1		ML-12-2	
90.1	uncultured bacterium; 300I-E04; AY661985	92.4	*Arthrobacter sp*. CPA2; AY310305
90.1	*Arthrobacter sp*. R33S; AY572475	92.4	*Arthrobacter sp*. J3.36; DQ157996
90.1	*Arthrobacter sp*. CPA2; AY310305	92.4	*Arthrobacter sp*. J3.41; DQ157999
90.1	*Arthrobacter sp*. J3.36; DQ157996	92.4	*Arthrobacter sp*. J3.45; DQ158000
90.1	*Arthrobacter sp*. J3.37; DQ157997	92.4	*Arthrobacter sp*. J3.51; DQ158003
90.1	*Arthrobacter sp*. J3.41; DQ157999	93.1	*Arthrobacter sp*. AK-1; EF110913
90.1	*Arthrobacter sp*. J3.45; DQ158000	92.6	*Arthrobacter oxydans*; Z1369; EU086823
90.1	*Arthrobacter sp*. J3.51; DQ158003	93.0	*Arthrobacter oxydans*; Z1656; EU086824
90.1	*Arthrobacter sp*. J3.53; DQ158004	93.1	*Arthrobacter phenanthrenivorans* Sphe3; CP002379
90.1	*Arthrobacter sp*. J3.73; DQ158006	93.1	*Arthrobacter phenanthrenivorans* Sphe3; CP002379
90.4	*Arthrobacter sp*. AK-1; EF110913	93.1	*Arthrobacter phenanthrenivorans* Sphe3; CP002379
90.1	*Arthrobacter oxydans*; Z1656; EU086824	93.1	*Arthrobacter phenanthrenivorans* Sphe3; CP002379
90.1	*Arthrobacter sp*. IWF3; GU120650	93.1	uncultured bacterium; 12TCLN380; AB637306
90.4	*Arthrobacter phenanthrenivorans* Sphe3; CP002379	93.1	uncultured bacterium; 12TCLN384; AB637310
90.4	*Arthrobacter phenanthrenivorans* Sphe3; CP002379	92.8	*Arthrobacter sp*. PL20g1b_S1; JF274911
90.4	*Arthrobacter phenanthrenivorans* Sphe3; CP002379	93.1	*Arthrobacter polychromogenes*; HWG-A27; JQ684250
90.4	*Arthrobacter phenanthrenivorans* Sphe3; CP002379	93.1	uncultured bacterium; 4-1048; KC554204
90.4	uncultured bacterium; CT0C2AB07; JQ427459	92.6	*Arthrobacter phenanthrenivorans*; L6; KC934752
91.1	uncultured bacterium; AC0C2BB07; JQ427644	93.9	*Arthrobacter phenanthrenivorans*; L16; KC934760
90.4	uncultured bacterium; 4-1048; KC554204	93.9	*Arthrobacter phenanthrenivorans*; L50; KC934786
		91.8	*Arthrobacter sp*. CPA2; AY310305
		91.8	*Arthrobacter sp*. J3.36; DQ157996
		91.8	*Arthrobacter sp*. J3.41; DQ157999
		91.8	*Arthrobacter sp*. J3.45; DQ158000
		91.8	*Arthrobacter sp*. J3.51; DQ158003
		92.5	*Arthrobacter sp*. AK-1; EF110913
		91.9	*Arthrobacter oxydans*; Z1369; EU086823
		92.3	*Arthrobacter oxydans*; Z1656; EU086824
		92.5	*Arthrobacter phenanthrenivorans* Sphe3; CP002379
		92.5	*Arthrobacter phenanthrenivorans* Sphe3; CP002379
		92.5	*Arthrobacter phenanthrenivorans* Sphe3; CP002379
		92.5	*Arthrobacter phenanthrenivorans* Sphe3; CP002379
		92.5	uncultured bacterium; 12TCLN380; AB637306
		92.5	uncultured bacterium; 12TCLN384; AB637310
		92.1	*Arthrobacter sp*. PL20g1b_S1; JF274911
		92.5	*Arthrobacter polychromogenes*; HWG-A27; JQ684250
		92.5	uncultured bacterium; 4-1048; KC554204
		91.9	*Arthrobacter phenanthrenivorans*; L6; KC934752
		93.2	*Arthrobacter phenanthrenivorans*; L16; KC934760
		93.2	*Arthrobacter phenanthrenivorans*; L50; KC934786

6.3.6. Summary of the research in the Manganese treated soil of Őrbottyán

Mn-sludge originating from the Úrkut Mn-ore (Hungary) was used in increasing amount (0, 500, 1000, 2500 and 12500 $mgkg^{-1}$ soil) in a lysimeter open-pot experiment, in order to test the sludge as a potential fertilizer product. Energy grass (*Elymus elongatus* L–Szarvas-1) was planted and the upper layer (0-20cm) of the calcareous sandy soil (Őrbottyán, Hungary) was treated with the sludge and additional mycorrhizal fungi, before the sowing of Energy grass.

After the first year, Energy grass plant biomass increased and could tolerate phytotoxic levels of manganese with mycorrhiza treatment, indicating that the *Elymus elongatus* L-Szarvas-1 can be considered a Mn-stress tolerant plant, useful for recultivation in manganese contaminated soils. The mycorrhizal treatment also helped the plants to uptake high concentration of Manganese to their root according to the Mn measurements in the root.

The abundance of countable microorganisms was investigated after the first year with the estimation of several physiological groups of aerobic, anaerobic bacteria and microbial fungi along with overall enzymatic activity. The bacterial abundance in the soil was usually balanced, and not affected by the increased doses of Mn. Previous researches proved that Manganese can serve as a potential source of energy for bacteria when organic nutrient requirements are low, therefore low concentration of Mn can have stimulatory effects [142]. Because the microbial community is adaptive and still active at lower concentrations of Manganese treatment, plants that have them involved in their rhizospheric processes are also tolerant to some levels of Mn. Mycorrhizal presence however affected the ratio of the different microbial groups, as the treatment might have provided competition for heterotrophic bacteria making anaerobes appear in higher numbers.

The enzymatic activity results of the first year showed that microorganisms lose their activity at higher doses of Mn, which can result in loss of functions.

After 3 years of treatment the biomass of the Energy grass was measured, and soil were tested for Manganese tolerance. Mycorrhizal treatment was very successful regarding both Manganese uptake and plant biomass increment, and by enhancing stress tolerance, the plant could be used as an indicator to Manganese concentration. In presence of mycorrhizal fungi even toxic levels of Mn were tolerated.

Mn tolerant isolates have been selected and identified with 16s rDNA sequencing. The structure of the microbial population changed as a result of several years of Manganese treatment. Long term exposure to Mn induced a selection toward *Arthrobacter species* that can tolerate Mn, and are possible PGPR species, helping the uptake for the plants. These *Arthrobacter* strains are good candidates for future inoculum development. The isolated *Bacillus species* from non-treated soils need to be further assessed to check affinity toward metal accumulation.

7. CONCLUSIONS AND FUTURE ASPECTS

Between 2010 and 2014 three contaminated research sites of propylene-glycol containing de-icing agent, crude-oil and Manganese have been studied, mainly focusing on the microbiological aspects of the contamination in connection with the soil parameters and vegetation.

By gathering various physical-chemical and microbiological characteristics, we found similarities in microbial abundance, enzymatic activities correlated to the given pollutants in the contaminated areas.

In all research sites the number of microbes (quantity) and their activities (quality) were affected by the increasing level of the contamination. The abundance of groups which could degrade or tolerate the pollutant increased with higher concentrations in general, whereas the total number of heterotrophic microorganisms decreased. We assume that it is a trend which is highly dependent on the type of the pollutant and also on the exposition time. The pollution is considered to be an environmental stress factor that can result in direct and indirect effects on the microbial community.

Enzymatic activities were also affected negatively by the toxic level of contaminations on all three sites. Increased amount of pollutant can change the environmental conditions in ways that are not favorable for the presence, the surviving ability and as a consequence the activity of microorganisms. In some cases the appearance of the pollution in low concentration caused a slight activity increase, the reason because below toxic levels organic pollutants might be used as carbon or nitrogen sources by various microorganisms. The enzymatic reaction to the appearance of the pollution and concentration levels was fast.

The response of the community was always dependent on the contamination with selection toward microbial groups based on their capability to degrade the given pollutant and to survive in the conditions which might have been resulted from the contamination on site (eg. lack of oxygen, available nutrient, water reservoirs etc.).

Along the similarities, we identified some differences as well regarding the previously mentioned biological parameters on the three sites, caused by the character of the pollutant.

When the contaminant was not a degradable organic, but inorganic Manganese, the abundancy was less affected and responsive to the change of contamination concentration while below the toxicity levels.

Competition was also apparent in case of mycorrhiza fungi used as inoculum treatment. Their presence could be determinative in any soil-plant-microbe-systems, resulting in a change regarding the abundance of given microbial groups compared to the soils not treated with arbuscular mycorrhiza fungi. Competition and changes in the abundance of several specific microbial groups were also detectable in case of the crude-oil contaminated site. Differences among the environmental conditions and the available nutrients or oxygen might select toward those groups that tolerate or favor stressful conditions. Because of crude-oil contamination, the oxygen levels dropped in certain soil layers, which can also be connected to enhanced microbial activities where easily degradable nutrients are present. In this case after time passes, and anaerobe conditions take place, the rate of anaerobes increase, because the conditions are now not favorable for their competitive partners.

Based on the previous statements regarding similarities and differences in the results of our study, we could conclude that both measured biological soil parameters: abundancy and enzymatic activity could be very usable indicators of environmental pollution levels and toxicity, and analysis of them can prove to be useful when estimating the degradation rate of soils. However, abundancy is not reliable by itself for several reasons: if abundancy is measured by using traditional Plate Count or any other cultivating method using culture media (like MPN), only a small portion of the community will produce detectable colony growth, the majority of viable cells cannot be counted with these methods.

Another problem is the sensitivity of microorganisms to the given contamination, and its toxicity. In case of not degradable pollutants the community might not react with abundancy changes in a short time, possibly because the given xenobiotic material does not contribute to growth directly. Eventually the level of the contaminant will increase to a point where it starts to affect soil functions and might inhibit normal enzymatic activities, but up until that point, it will not affect greatly the microbial population. Additionally, if the given contamination produces a secondary effect on the environment, like a change in the soil pH or anaerobic conditions that will also directly affect the microbial population. At that point it is hard to make a direct correlation between the contaminant concentration and the microbial abundancy of all or specific microbial groups.

In contrast to abundancy, enzymatic activities seem to be more sensitive to any change regarding soil-environmental conditions and they could produce a faster reaction - a so called “early warning signal” - to indicate the negative sub-optimal consequences of the contaminants. Those measured biological parameters, therefore, which are closely related to biological functions, are suggested to be highly applicable indicators in the environmental studies. This biological parameter is more effecting in indicating than microbial abundancy only.

Although the change in population composition was mentioned as a similarity in the three studied sites, there were some differences as well. While organic, degradable propylene-glycol and crude-oil sites resulted with a selection toward degrading microbial populations, like *Pseudomonas*, the soil treated with inorganic Manganese resulted in either spore-former *Bacillus* species or Mn oxidizers. While this outcome was not a new scientific result, it was very important for future inoculum preparation if we want to progress further in our bioremediation treatment research.

In this study, seasonality is also a notable factor, which affects abundancy, microbial community composition and enzymatic activity biological parameters. As we could see on the Gardermoen site, cold weather could inhibit microbial growth and activity, slowing down the degradation processes. Therefore, it is highly advisable to count with seasonality in areas where humidity, temperature or snowmelt can affect soil parameters greatly.

Although similarities and differences could be realized regarding methods used on all three sites (abundancy, enzymatic activities, isolation – identification), in the Gardermoen site we also used DNA based microbial total population analysis as an additional test.

Based on our results DNA based microbial population analysis was also a very good indicator of any change in the soil environment. While we cannot make a direct correlation to the contamination concentration, and the population composition is not as fast as enzymatic activity in reacting to the pollution, we can identify the presence of the contaminant by a shift in the population. Total microbial DNA extraction, PCR and sequencing is better when it comes to soil characterization, because, as mentioned before, cultivation and isolation is limited to only a few % of the total population. The identified strains were only limited to those which could be cultivated. This fact can provide some obstacles in applicability and also in improving remediation practices in general.

On the other hand, DNA extraction from soil also has disadvantages. The extraction process can be limiting, for example, chemical DNA extraction methods might not work on spores, and part of the population DNA will not be recovered. Low concentration of DNA may not yield result, or species with low number will not show up. Moreover, recovered DNA can originate from either living or dead microorganisms, which can give a false positive result.

Therefore, cultivation is still being highly dependent on. When it comes to the end goal of the research, namely a production of inoculums that can enhance bioremediation processes, species that are not cultivable can only be used as pollution indicators. While it is common to perform biostimulation of native microorganisms by adding nutrients or oxygen, it is hard to test the outcome in laboratory conditions. Mentioned nutrients are not specifically for one or two microbial groups, the whole community can use it. It is hard to target only those populations that are involved in the degradation of the given contaminant. While this is true, it is also problematic, that after creating single strain or consortium inoculums in laboratory conditions, they will not stay alive or perform well in the "to be treated" area. As such, it seems logical to perform large scale field tests after inoculums are selected in the laboratory, with the knowledge of the original population composition and a way to be able to follow the inoculums after they have been placed out in the field.

As laboratory tests were mentioned, it needs to be noted that gathering and identifying microorganisms and testing their degradation capabilities are not enough when it comes to strain selection. The selected isolates need to be tested for other characteristics relevant to the contamination treatment as well, and only a few might be selected. One objective is to exclude human pathogens and microorganisms with antimicrobial resistance, as they should not be used as inoculums for remediation. But there could be other possible approaches. Slow growing microorganisms might not be useful as inoculums either, or ones requiring special environmental conditions, like pH or temperature, that would be problematic to provide in the field. While our research mainly focused on gathering and selecting native degrader microorganisms, bioremediation practices can involve the use of microorganisms from microbial banks or other sites with similar contamination, that were already characterized.

During our research we managed to select and isolate native microorganisms that were not just adapted and tolerant to the given contamination, but were able to perform biodegradation with organic contamination. These isolates were recovered from the more polluted parts in contrast to the not contaminated, control soils.

The Manganese treated Őrbottyán site was in this regard different than the other two. Since the pollutant is inorganic, we were not searching for microorganisms with degradation capability only, but also with tolerance abilities. What the objective was for these types of soils to identify organisms which enhance phytoremediation with the test plant Energy grass, or to stabilize the contaminant Mn. Additional mycorrhiza inoculums were used and it has been proved that their presence could enhance the remediation process by providing collaboration between plants and microorganisms, to assist the plant in uptaking these elements. Although without mycorrhizal treatment the plant did not provide any special indication of the contaminant, with mycorrhizal treatment the plant showed increased growth until toxic levels of Mn, where the plant biomass decreased. This means that Energy grass and the involved phytoremediation practice can also contribute to contamination characterization, and not just degradation or contamination control. More sensitive plants might indicate the presence and increase of given metals and xenobiotics faster, so these could be planted on sites where contamination monitoring is required. Since the plant accumulated metals in the root - performed phytoremediation - it is also useful for removal and retrieval of the metals.

While our studies involved only some microbiological tools from the palette, there are many other notable techniques that could provide good indicator results connected to the contamination: ATP measurements, substrate induced respiration or other molecular approaches, like fluorescent *in situ* hybridization or DNA fingerprint techniques. When designing the monitoring methodology for different contaminated soils, these methods can also be used and correlated to the concentration of the pollution.

So why is continuous monitoring useful? Where can we use this knowledge? While creating treatment inoculums or biostimulating packages are good corrective actions for soil contamination that are already in place, it would be even better if bioremediation knowledge and soil characterization could result in creating a method or device that can prevent contaminated sites to reach toxic levels of pollution by continuously monitoring soil parameters. And what if the continuous monitoring provides signs of contamination appearance or overloading?

Two future approaches could be realized: treatment, resting of the area, or the combination of the two. One could start treating the area with appropriate bioremediation methods, or, simply move to another area where the processes causing the contamination were not yet used. For example, if the Gardermoen Airport uses an area where the airplanes receive the anti-freezing treatment, and the monitoring results indicate that the area is getting overload, they could move the de-freezing platform to another area while treatments are in place and the contamination concentration drops. Biostimulation or inoculums could be used to enhance natural remediation on the burdened area.

To summarize our experiences: by using the methods and tests described in the current thesis, we could realize a relatively fast and simple route to characterize contaminated soil by correlating the biological results to the pollutant concentration, and to achieve possible isolates that are competitive, adapted on given contaminated areas, and are able to enhance bioremediation through degradation or collaboration with plants. Applied tests and methodology during our study could be used as a basis to develop general monitoring tools at the contaminated sites. The tested microbiological parameters can indicate soil contamination and toxicity and can be used for developing proper remediation, site rehabilitation practices and contamination monitoring systems.

8. NEW SCIENTIFIC RESULTS

1) By gathering various physical-chemical and microbiological characteristics in three differently contaminated soils from Europe, positive interrelation was found between the studied physical-chemical and the biological parameters. Some of the biological parameters, depending on the type of the pollutant and the soil, could be defined as indicators of soil status and functioning. We suggest therefore involving specific biological parameters to site characterization and to risk assessment of pollutants.

2) Sensitivity and abundance of biological indicators are being highly dependent on the type and severity of the contaminants. Among the various parameters studied, the enzymatic activities proved to react faster and more accurately compared to the used microbial counting methods. Since some of the applied tests have limitations, more biological parameters, including enzymatic activity assays and molecular methods are suggested be used.

3) Contamination affects not only the presence and abundancy of microbes, but also their microbiological activities. Various abundancy reactions were found depending on the studied microbial physiological groups. Pollution and other environmental stress might act as a selecting factor, resulting in an increased number of tolerant microbial genera and species. Among various physiological groups, spore-forming bacteria can survive more efficiently in polluted ecosystems.

4) Both DNA based total population analysis and single isolate identification produced results that verified changes in certain soil microbial communities. For characterization, total microbial DNA extraction and population analysis fits better, however, for future applications, isolates need to be prepared. At this step of the process species level identification of the selected strains is necessary.

5) Abundant and degrader/tolerant microorganisms were identified, which can survive among certain environmental stress conditions. Isolated and selected microorganisms should be tested in order to avoid using human pathogenic species and strains with antimicrobial resistant genes considering human and environmental risks.

6) Among the possible candidates, we found microorganisms with multi-degradative abilities and some with specific characteristics. *Pseudomonas* strains are suggested to be used for inoculum development in case of both the propylen-glycol and crude-oil contaminated site, while the *Arthrobacter* strains should be selected for application against Manganese sludge.

7) Energy grass has been described in several studies with the ability to accumulate different heavy metals, but we managed to enhance this accumulation property, the biomass of the plant along with stress tolerance by applying additional mycorrhizal fungi treatment.

8) Applied tests and methodology during our study could be used as a basis to develop general monitoring tools at the contaminated sites. The tested microbiological parameters can indicate soil contamination and toxicity and can be used for developing proper remediation, site rehabilitation practices and contamination monitoring systems.

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10.PUBLICATIONS

10.1. Scientific articles (English)

1) B. Biró, **N. Horváth,** M. Domonkos, A. Füzy, T. Szili-Kovács, B. Libisch and A. Anton (2012): *Report on soil environmental condition, status and development*
Published in: Deliverable 2.3 of EU-Fp7 Soil-CAM project, http://www.soilcam.eu.

2) B. Biró, G. Toscano, **N. Horváth**, H. Matics, M. Domonkos, R. Scotti, A. M. Rao, H. K. French (2014): *Vertical and horizontal distributions of microbial abundances and enzymatic activities in propylene glycol affected soils*
Published in: Environ. Sci. Pollut. Res. (2014) 21: 9095. doi:10.1007/s11356-014-2686-1
IF (2014): 2.828

3) H. Lissner, M. Wehrer, M. Reinicke, **N. Horváth**, K.U. Totsche (2015): *Constraints of propylene glycol degradation at low temperatures and saturated flow conditions*
Published in: Environ. Sci. Pollut. Res. (2015) 22: 3158. doi:10.1007/s11356-014-3506-3
IF (2015): 2.760

10.2. Presentations published in full length (English)

1) **N. Horváth**, H. Matics, M. Domonkos, B. Biró (2012): *Monitoring of microbial abundance and the selection of propylene-glycol degrading bacteria from contaminated soil*
Presented and published in: International Scientific Conference for PhD Students, the University of West Hungary Győr, Hungary, 19-20 March, 2013. p. 174-179., Talentum Project, TÁMOP 4. 2. 2. B – 10/1 – 2010 – 0018

2) B. Biró, **N. Horváth**, H. Matics, M. Domonkos, X. Malov (2013): *Enhanced degradation of de-icing fluids in soils and soil-plant systems by improving soil nutrient status and quality*
Presented in**:** 12th Alps-Adria Scientific Workshop Opatija, Doberdó, Venezia – Croatia – Italy 2013. Published in: Növénytermelés 2013 supplement, 62: 393-397. DOI: 10.12666/Novenyterm. 62. 2013. suppl.

3) **N. Horváth**, H. Matics, M. Domonkos, B. Biró (2014): *Microbial abudance in Mn treated calcareous sandy soil cultivated with energy grass*
Presented and published in: XVII. Apáczai-napok Nemzetközi Tudományos Konferencia, Győr, Hungary, 2013.10.25: Apaczai Csere Janos Faculty, University of West Hungary, pp. 24-25

10.3. Presentations published as abstracts (English)

1) B. Biró, **N. Horváth**, M. Domonkos, B. Libisch (2011): *Horizontal microbiological characterization of forest soils targeted by deicing fluids*
Presented in: 16th International Congress of the Hungarian Society for Microbiology, 12-15 October 2010, Keszthely, Hungary. Published in: Acta Microbiologica et Immunologica Hungarica Vol. 58, P. 128, 2011

2) B. Biró, **N. Horváth**, H. Matics, K. Czakó-Vér, T. Takács, M. P. Polgári (2012): *Microbiological responses and Mn-stress alleviation in the mycorrhizosphere of Elymus elongatus energie grass*
Presented in: A Magyar Mikrobiológiai Társaság Nagygyűlése, Keszthely, Hungary, 25-26 October 2012. Published in: Acta Microbiologica et Immunologica Hungarica, Vol. 60: (Suppl) p. 10. (2013)

3) **N. Horváth,** H. Lissner, M. Reinicke, B. Biró (2012): *Monitoring the microbial abundance in propylene-glycol and formate contaminated soils in a column experiment*
Presented in: A Magyar Mikrobiológiai Társaság Nagygyűlése, Keszthely, Hungary, 25-26 October 2012. Published in: Acta Microbiologica et Immunologica Hungarica, Vol. 60: (Suppl) p. 24.

4) B. Biró, **N. Horváth**, M. Domonkos, H. K. French (2012): *Microbial monitoring and most-probable number of microbes in soils capable of degrading aircraft deicing fluids*
Published in: Geophysical Research Abstracts Vol. 14, EGU2012-12812, 2012

10.4. Contributions to other publications not connected to the topic (Hungarian)

1) B. Biró, I. Angerer, M. Domonkos, H. Matics, **N. Horvath**, E. Kiss (2013): *Nehézfémek és néhány szerves szennyezőanyag mennyisége és a mikrobiológiai tulajdonságok alakulása dunaújvárosi talajmintákban*
Publication in: Tájékoztató Dunaújváros m.j.v. környezeti állapotáról. editor: Tóth. T., Text Press, Dunaújváros, pp. 153-162., ISSN 1786-7592 (2013)

2) B. Biró, H. Matics, **N. Horváth**, K. Czakó-Vér (2012): *Talajok tápanyagkiegészítésének hatása néhány mezőgazdasági és környezetvédelmi funkcióra*
Presentation in: Talajtani, Vizgazdálkodási és Növénytermesztési Tudományos Nap, Debrecen, Hungary, 25. November 2012. Publication In: Proc. of 1. TVN. MTA ATK TAKI Kiadó, Budapest

3) H. Matics, H. Tiricz, **N. Horváth**, B. Biró (2013): *Érzékeny és toleráns rhizobiumok szaporodása növekvő toxikus elemdózisok függvényében*
Publication In: Fiatal kutatók az egészséges élelmiszerért. DE tudományos képzési műhelyeinek támogatása. TÁMOP-4.2.2/B-10/1-2010-0024. ISBN 978-963-473-601-1 Editor: Bódi Éva, Fekete István, Kovács Béla, Debrecen, 141-146 p. 2013.02.19.

4) H. Matics, A. Ferenczy, M. Domonkos, **N. Horváth**, B. Biró (2013): *Cink és nikkel hatásának vizsgálata két szerveződési szinten bio-tesztekkel*
Publication in: Újabb kutatási eredmények a növénytudományokban. DE tudományos képzési műhelyeinek támogatása. TÁMOP-4.2.2/B-10/1-2010-0024. ISBN 978-615-5183-40-9 Szerk: Sándor Zs., Szabó A., Debrecen, 35-40 p. 2013.04.05

5) H. Matics, B. Kaltenecker, **N. Horváth**, B. Biró (2013): *Az adaptáció hatása nitrogén-kötő baktériumok Cr és Zn érzékenységére*
Presentation and publication in: XVII. Apáczai-napok Nemzetközi Tudományos Konferencia, Győr, Hungary, 2013.10.25: Apaczai Csere Janos Faculty, University of West Hungary, p. 24

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