

**Pollutant remediation strategies in the soil: electrokinetic remediation,
biostimulation, and experimental design**

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Doctoral Programme in Interdisciplinary Environmental Sciences (DENVI)

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DOCTORAL DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in the Hule conference room, Niemenkatu 73, Lahti, on the 7th of May, 2021 at 12 o'clock.

Lahti, 2021

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ABSTRACT

Soil pollution is one of the world's biggest challenges. The most common soil pollutants include heavy metals and petroleum hydrocarbons. A great number of studies have developed strategies for pollutant remediation. For the removal of heavy metals including cadmium (Cd) in the soil, electrokinetic remediation has been rapidly developed in the recent decades as a both remediation and metal resource recovery method. However, field and full-scale applications of electrokinetic remediation in the removal of soil heavy metals are rare. As for organic pollutants including petroleum hydrocarbons, biostimulation with fertilization has been widely done and highly appreciated over physical and chemical methods ecologically and economically. Due to different soil physico-chemical properties and climates, fertilizers should be chosen carefully for the best outcome. Remediation strategies are often initially validated in the laboratory, which can still fail in the field tests or application usually due to soil heterogeneity that exists even within millimeters. A false conclusion may result from an improper experimental design in the laboratory, such as insufficient true replication (pseudoreplication) that fails to represent the sampling site, and composite sampling or randomized complete block design (RCBD) that only presents the average performance of the sampling site and can result in false positive or negative results for the component samples.

This doctoral thesis presents three strategies in soil remediation, i.e. electrokinetic remediation of soil heavy metal Cd (paper I), biodegradation of soil gasoline aromatics stimulated by fertilization (paper II), and optimization of experimental design (paper III). Electrokinetic remediation of Cd was monitored for 14 days, in a pilot (4 m²) test and a full-scale (200 m²) application in paddy agricultural soil, at soil layers 0-10 cm, 10-20 cm, and 40-50 cm. A voltage of 20 V was applied at both scales. In the biostimulation study, three nitrogen fertilizers, i.e. inorganic NPK, urea, and methylene urea were compared in the biostimulation of gasoline aromatics removal in the soil collected from the high Arctic permafrost active layer and experimented in the laboratory at 10 °C for 28 days. Finally, the experimental design study that was conducted in the laboratory at 4 °C for 28 days illustrated how pseudoreplication and sole use of plot averages, would affect the conclusions drawn in biodegradation studies. In the experimental design study, three ecologically independent plots of soil were collected and processed independently in parallel. The soil was spiked with gasoline and divided for natural attenuation and biostimulation with methylene urea fertilization. Biodegradation results were analyzed individually within each plot of soil (pseudoreplication) as well as in a between-plot comparison by using the mean values of the three plots (plot averages).

In 14 days, electrokinetic remediation showed a successful application at both scales, especially for the soil total Cd in the surface 0-10 cm layer, with a higher removal efficiency in the pilot test (87%)

than the full-scale application (74%). The final concentration of soil total Cd was below the hazard threshold set for paddy agricultural soils in China. The higher removal efficiency in the pilot test can be due to the higher voltage gradient between the electrodes. The removal efficiency of the plant available Cd was lower than the soil total Cd, which can be due to the enhanced desorption of Cd cations through cation exchange and/or dissolution by lactic acid during electrokinetic remediation.

In 28 days, biostimulation with urea fertilization managed to lower the concentration of the total gasoline aromatics below the initial level (by 47%). On day 7, the observed concentration of the total gasoline aromatics was higher than the initial level in the NPK and urea fertilized treatments, which indicated an enhanced desorption and extractability of soil gasoline aromatics. The enhanced desorption and thereafter bioaccessibility of gasoline aromatics in the NPK and urea fertilized treatments could be related to the enhanced biodegradation activity compared to natural attenuation and methylene urea fertilized treatment, mainly shown between day 7 and day 28.

In contrast to the successful application of electrokinetic remediation and biostimulation with urea fertilization, the effect of methylene urea in biostimulation showed positive, negative, and negligible results. The random results of natural attenuation and biostimulation with methylene urea fertilization indicated the patchiness of biodegraders even within one plot of soil. Additionally, the results based on the between-plot comparison showed a dilution effect and could pose false positive and negative results for the component soil.

Although electrokinetic remediation of heavy metal Cd and biodegradation of gasoline aromatics stimulated by urea fertilization showed success in the cases studied herein, the success of these remediation strategies cannot be generalized to other soil pollution cases due to soil heterogeneity at least, according to the third study. The results suggest that ecologically independent replication be adopted in the between-plot comparison and that parallel experiments and statistical analysis with within-plot replication for each replicate plot be performed to guide a more successful practical application of remediation strategies.

LIST OF ORIGINAL PAPERS

- I. Cai, Z., Sun, Y., Deng, Y., Sun, S., Zhen, X., Romantschuk, M., Sinkkonen, A. 2021. *In situ* electrokinetic (EK) remediation of the total and plant available cadmium (Cd) in paddy agricultural soil using low voltage gradients at pilot and full scales. Submitted manuscript.
- II. Sun, Y., Romantschuk, M., Bang-Andreasen, T., Rantalainen, A.L., Sinkkonen, A., 2020. Nitrogen fertilizers stimulate desorption and biodegradation of gasoline aromatics in the soil from high Arctic permafrost active layer: A laboratory study. *Int. Biodeterior. Biodegradation* 150, 104957. <https://doi.org/10.1016/j.ibiod.2020.104957>
- III. Sun, Y., Rantalainen, A., Romantschuk, M., Sinkkonen, A., 2018. Requirement of ecological replication with independent parallel analysis of each replicate plot to support soil remediation. *Int. Biodeterior. Biodegradation* 133, 133–141. <https://doi.org/10.1016/j.ibiod.2018.06.006>

THE AUTHOR'S CONTRIBUTION

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- II. Sun, Y. took part in the experimental design and implementation, did the chemical analysis and data analysis, wrote the paper with co-authors, and is the corresponding author.
- III. Sun, Y. did the chemical analysis and data analysis and wrote the paper with co-authors.

ABBREVIATIONS USED IN THE THESIS

BFR	Brominated flame retardant
BTEX	Benzene, toluene, ethylbenzene and xylene
DDT	Dichloro–diphenyl–trichloroethane
OCP	Organochlorine pesticide
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyls
PGPR	Plant growth promoting rhizobacteria
POP	Persistent organic pollutant
RCBD	Randomized complete block design

1. INTRODUCTION

Soil pollution is one of the biggest challenges that the world faces. Pollution occurs throughout the life cycle of products or services in all eco-societal sectors, e.g. industry, agriculture, household, and traffic (Tao et al., 2004; Ma et al., 2009; Omores et al., 2017; Lough et al., 2005; Zhang et al., 2008; Su et al., 2014; Shifaw, 2018). Some soils are severely polluted by heavy metals, pesticides, polycyclic aromatic hydrocarbons (PAHs), and solid wastes such as plastics and industrial sludge (Li et al., 2018). Ecosystems provide important services to the human society (Xu et al., 2016). When pollution is beyond the capability of nature in self-healing (natural attenuation), human interference needs to be done. The applied strategies can be physical, chemical, and biological. In general, biological methods are considered cheaper and more sustainable than physical and chemical methods, but biological methods take a longer time (Filler et al., 2008; Su et al., 2014). Physical methods are usually means of relocation of pollutants (sometimes followed by *ex situ* treatment) and chemical methods introduce compounds and metabolites that may turn out to be pollutants (Lee et al., 2001; Su et al., 2014; Koul and Taak, 2018; Sharma et al., 2018).

Environmental pollutants can be categorized into organic and inorganic pollutants. For organic pollutants, such as oil, herbicides, organic industrial sludge, biological treatments, i.e. bioremediation is highly appreciated, especially for *in situ* applications. Bioremediation includes various strategies, such as biodegradation, phytoremediation, and biosorption (Michalak et al., 2013; Azubuike et al., 2016). Biodegradation of soil oil pollutants has been vastly studied (Solano-Serena et al., 1999; Vidali, 2001; Moslemy et al., 2002; Gogoi et al., 2003; Prince et al., 2007; Filler et al., 2008; Österreicher-Cunha et al., 2009; Kauppi et al., 2011; Avanzi et al., 2015; Simpanen et al., 2016). Petroleum hydrocarbons are common organic pollutants. Big-scale oil pollution events usually take place during failed oil exploitation, storage and transport (Philp et al., 2005; Hilpert et al., 2015). In cities, soil oil pollution is often present in gas stations, e.g. tank leaks. Oil leaks also occur constantly in vehicles. Oil pollution occurs even in the remote Arctic areas due to e.g. Arctic oil exploitation (Filler et al., 2008). Biodegradation is especially preferred in remote areas where restoration is not so urgent, and excavation and transportation is inconvenient and expensive. Many studies have emphasized the ecological and economic feasibility and importance of biodegradation compared to physical and chemical remediation strategies in the Arctic areas (Aislabie et al., 2006; Aislabie and Foght, 2008; Filler et al., 2008; Naseri et al., 2014). However, due to many undesired environmental conditions, e.g. low temperature, low nutrient availability, and seasonal soil freezing and thawing, meaning short bioactive period, biodegradation in the arctic areas still faces big challenges (Filler et al., 2008).

Biodegradation of organic pollutants is the process that microbial organisms feed on the organic pollutants for carbon and energy, or degrade a pollutant while utilizing coexisting carbon and energy sources (Gu, 2016). The process can be enhanced by aeration, fertilization, surfactant addition, etc., which is referred to as biostimulation (Romantschuk et al., 2000; Adams et al., 2015). Biostimulation with fertilization is a very common strategy. Many studies have shown the improved removal of soil oil pollutants after fertilizer addition. Fertilization is also highly recommended to stimulate biodegradation in the Arctic areas (Braddock et al., 1997; Coulon et al., 2005; Walworth and Ferguson, 2008). The idea is to boost the growth of biodegraders with the addition of fertilizers that contain crucial life elements, i.e. C, N, and P.

As for inorganic pollutants, chemical immobilizers are often added to the polluted soils to reduce the bioaccessibility and consequently the accumulation of heavy metals in crops. The mechanisms include precipitation, adsorption, chelation, etc. (Basta and McGowen, 2004; Thompson and Goynes, 2012). Microbial consortia, as an alternative, have also been widely studied for the immobilization of soil heavy metals through biosorption (Unz and Shuttleworth, 1996; Michalak et al., 2013; Jin et al., 2018; Velkova et al., 2018). Phytoremediation is another biological method that has been studied worldwide for the immobilization and extraction of soil heavy metals; phytoremediation is both a remediation and recovery method (Newman and Reynolds, 2004; Ali et al., 2013; Cristaldi et al., 2017).

Another heavy metal remediation and recovery method is electrokinetic remediation. Electrokinetic remediation has been developed quickly during the past decades (Acar and Alshawabkeh, 1993; Acar et al., 1995; US Army Environmental Center, 2000; Kim et al., 2011; Ottosen, 2014). Electrokinetic remediation mobilizes soil heavy metals towards the relevant electrodes under forces of electroosmosis, electromigration, electrophoresis, and hydraulic potential gradient (Acar et al., 1995). The accumulated heavy metals at the electrodes are sequentially removed and recovered. Studies have also shown the potential of electrokinetic remediation in removing soil organic pollutants through migration, biostimulation by distributing nutrients, etc. (Suni et al., 2007; Lee and Kim, 2010; Cameselle and Gouveia, 2018; Koul and Taak, 2018).

Often, all the remediation strategies display high potential in tests especially at laboratory scales when experiment conditions and designs are ideal to acquire optimal results, which is unfortunately not practical in real contamination cases. For instance, in soil biodegradation experiments in the laboratory, it is common to use a single environment soil sample and mix the contaminated soil evenly before the remediation starts. This practice neglects the patchiness of biodegraders in natural soils. It means that a successful biodegradation in the laboratory experiment does not ensure one at a

contamination spot lacking biodegraders in real cases, since soil microbial patchiness occurs at scales as small as millimeters (Ghosh et al., 2000; Davis et al., 2003; Kauppi et al., 2011; Dechesne et al., 2014; Yu et al., 2015). It is a typical issue of pseudoreplication, that is, the sample size is too small for the results to be representative. Pseudoreplication has been pointed out already in the 1980s (Hurlbert, 1984). Varied biodegradation rates due to small-scale soil heterogeneity have also been illustrated (Davis et al., 2003). To gain more representative results, soil samples from several independent environments are taken and combined as a composite sample, or treated as ecological replicates in a randomized complete block design (RCBD) (Mills et al., 2004; Viñas et al., 2005; Jidere et al., 2012; Garg et al., 2016). Results gained from these experimental designs show an average performance of biodegradation in the sampled soils, meaning that these designs can pose false positive or negative results for individual sample components (Van Belle et al., 2001; Tan, 2005; Patil, 2011). This can further result in an oversight of valuable biodegraders and/or overestimate the performance of a biodegradation strategy in real cases.

2. LITERATURE REVIEW

2.1. Soil pollution

Soil pollution can take place through natural processes, such as precipitation of ash from volcanic eruption and/or forest fires; some natural pollution incidents can be induced by human disturbance, such as forest fires. However, anthropogenic soil pollution from e.g. industrial, domestic, and agricultural sectors contribute drastically.

Despite the natural existence of heavy metals in soils and rocks, the main pathways of exposure are anthropogenic. Mine exploitation, discharges from the metal industries, combustion of heavy metal containing materials, leaching, etc. release huge amounts of heavy metals into the environment (Lough et al., 2005; McConnell and Edwards, 2008; Zhang et al., 2008; Nzihou and Stanmore, 2013; Su et al., 2014; Shifaw, 2018; T. Li et al., 2019). Heavy metal pollution in agricultural soils near mining areas and smelting factories has particular concerns. These agricultural soils are usually polluted by heavy metal carrying water such as irrigation water, surface runoffs, and groundwater (Shifaw, 2018; C. Li et al., 2019; T. Li et al., 2019). One of the most famous and early alerting heavy metal pollution events is the Japan Itai-itai Disease (bone softening and kidney failure) that was caused by chromic Cd poisoning and was due to Cd pollution in water and rice fields in the downstream of the Kamioka mine (Kaji, 2012). The disease started in the early 20th century but was only identified as Cd poisoning after half a century and was still found in the 21st century.

In the developed countries, heavy metal pollution from mining and metallic industry is mainly historical (Arao et al., 2010; Tóth et al., 2016), while the less developed countries are still facing high and new pollution due to improper management and treatment (Bhuiyan et al., 2010; Huang et al., 2019). Heavy metal pollution in agricultural soils around mining areas in China has been a long-time issue (Wang et al., 2010; Liang et al., 2011). A great number of studies have been carried out concerning pollution level, crop contamination, toxicity, risk assessment, etc. of heavy metals from mines (Yu et al., 2017; Kong et al., 2018; Huang et al., 2020). The average concentrations of heavy metals Cd, Cr, Pb, Zn, Cu, Ag, Ni, and Hg in the soil around mining areas have all been found above the corresponding national standard values set for soil quality (Li et al., 2014). Especially for Cd, more than 85% of the collected samples did not meet the standard.

Many pollution control and remediation policies have taken place (T. Li et al., 2019). In the modern society, waste from industrial and/or domestic sectors are treated before being discharged to the environment or stored isolated from the environment. The discharged waste must have a low enough level of pollutants that should not form a threat to the environment. However, due to the increasing number of discharges and leaks, soil quality is facing challenges, especially in densely populated urban areas.

Urban green spaces are very important functional areas that provide citizens with places for leisure, recreation, exercise, and farming activities (Xu et al., 2016; WHO Regional Office for Europe, 2017). However, maintenance of urban green spaces, e.g. urban parks and gardens highly relies on the use of pesticides due to the vulnerability of urban ecosystems affected by pollution (WHO Regional Office for Europe, 2017; Li et al., 2018). Heavy traffic in the urban areas results in the soil pollution of petroleum hydrocarbons through leaks from vehicles and fuel stations. Emission and eventual deposition of PAHs happens through incomplete combustion from heating, vehicle engines, and factories (Li et al., 2018). Pollutants, including microplastics and heavy metals, also come from the wear of vehicle tires. Atmospheric deposition of combustion emissions from traffic, power plants, heating plants, etc. causes secondary pollution, both inorganic and organic in urban soils (Wright et al., 2018). Additionally, pollutants from building materials end up in the urban soils through rain-washes and/or atmospheric deposition (Al Bakri et al., 2008; Masoner et al., 2019). All these pollutants pose risks to urban citizens through direct skin contact, ingestion, and inhalation. Due to the rapid expansion of urban areas, alteration of land functions, e.g. former industrial and/or agricultural lands turning into residential lands, causes additional concerns (Fazal, 2000; Lester et al., 2013). With the popularity of urban green spaces and practice of urban garden farming, interactions

take place between urban soils and human beings, and hence more attention should be paid to urban soil pollution control and remediation.

In addition to agricultural and urban soils, soil pollution occurs in pristine soils. With a huge amount of oil stock and massive exploitation, the Arctic is also inevitably facing spills and consequently the environmental damage. Between 1986-1996, 19 oil spills occurred in the permafrost areas in the former Soviet Union (ESMAP, 2003). In 2020, a great spill from the storage tank build on the permafrost occurred in the northern Russia that released 20 000 tonnes of diesel oil to the environment (Rajendran et al., 2021), contaminating lands, rivers, groundwater, and eventually the Arctic Ocean. Due to the special climate in the Arctic areas, natural attenuation (e.g. evaporation and bioremediation) of these pollutants in the soil is challenging.

2.1.1. **Inorganic soil pollutants**

Heavy metals are the most frequently discussed and important inorganic soil pollutants, including mercury (Hg), lead (Pb), cadmium (Cd), chromium (Cr), nickel (Ni), copper (Cu), zinc (Zn), etc. Arsenic (As) is often treated as a heavy metal, even though it is recognized as a metalloid. Heavy metals As, Pb, Hg, and Cd are of particular concern due to their significant patho-physiological effects (Singh et al., 2017). In mining and metal smelting industries, multiple heavy metals usually co-exist. For example, Cu, Zn, Hg, Pb, As, Cd, etc. are commonly found co-present in gold mine tailings (Ferreira Da Silva et al., 2004; Abdul-Wahab and Marikar, 2011; Liang et al., 2011; Fashola et al., 2016).

Heavy metal pollution in agricultural soils and urban green spaces are additionally from the application of fertilizers and pesticides. Both inorganic and organic fertilizers can contain heavy metals, such as Cu, Zn, Cd, Ni, Mn, etc. that are present as trace metals in the raw materials, i.e. phosphate rocks and animal manures (Mortvedt, 1995). Heavy metal containing pesticides (insecticides, herbicides, fungicides, etc.), such as lead hydrogen arsenate and copper oxychloride are commonly used to kill insects or used as preservatives (Mortvedt, 1995; Singh et al., 2017). The excessive use of inorganic fertilizers and pesticides have resulted in the massive accumulation of heavy metals in agricultural soils and urban green spaces. In urban areas, soils are also commonly contaminated by heavy metals released from buildings and other concrete materials, exhaust from vehicles, abrasion of vehicle compartments, etc. through deposition and/or stormwater washing (Lough et al., 2005; Nzihou and Stanmore, 2013; Adamiec et al., 2016).

Extensive studies have indicated physiological impacts of heavy metals on microbes, plants, animals, and human beings (Domsch, 1984; Schützendübel and Polle, 2002; Järup, 2003; Singh et al., 2011,

2017; Jaishankar et al., 2014; Wang et al., 2016). Risks of soil heavy metals depend on the chemical forms of the metal elements. For instance, As has four oxidation states, i.e. -3, 0, +3, and +5, that can accordingly form inorganic and organic As compounds. In general, the toxicity of inorganic As compounds is remarkably higher than organic As compounds, and inorganic As³⁺ is more toxic and As⁵⁺ (WHO, 2001). The toxicity of heavy metals is also affected by soil types. For example, the effects of Cd on microbial activities were found smaller in soils with higher organic matter contents and in clays than sands (Vig et al., 2003), probably due to lower bioavailability (tighter adsorption to the soil).

Lee et al. (2002) found that the soil enzyme activities were repressed in the heavy metal (Pb, Cu, Zn, and Cd) polluted spots in a shooting range. The number of populations and/or composition of soil faunal and/or fungal communities were also found in relation to Pb pollution in a shooting range (Hui et al., 2009, 2011, 2012; Selonen et al., 2014). Reduced biological activities might further affect the microbial decomposition of soil organic matters and/or litters in heavy metal polluted soils (Domsch, 1984; Enya et al., 2020). What is worse is that the toxicity of some heavy metals can increase through microbial transformation. For instance, methylmercury (MeHg) that has high bioavailability and therefore has more toxicity concerns can be formed through microbial methylation in anoxic conditions (Xu et al., 2019). However, some microbial transformation can reduce the toxicity, e.g. microbial reduction of Cr⁶⁺ to the less toxic Cr³⁺ (Cheung and Gu, 2007)

The toxicity of combined uptake of some heavy metals and pesticides (inorganic and/or organic) has been found even more severe (Singh et al., 2017). For example, synergistic physiological effects, such as abnormal weight gain, relative organ weights, white cell counts, etc. have been illustrated by the many studies through simultaneous exposure to pesticide dimethoate and heavy metals Cd, As, and Hg respectively (Institóris et al., 2001, 2002; Graham et al., 2010; Rehman et al., 2017; Singh et al., 2017).

2.1.2. Organic soil pollutants

Organic pollutants are hydrocarbons mostly from petroleum and petroleum-derived products, among which the persistent organic pollutants (POPs) such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), halogenated aromatics, dioxins, and organochlorine pesticides (OCPs) including dichloro–diphenyl–trichloroethane (DDT), brominated flame retardants (BFRs), etc. are of the most concern (Li et al., 2018). Petroleum hydrocarbons are of especial concern since spills and leaks of petroleum hydrocarbons usually take place at big scales. Although the number of oil spills are decreasing worldwide, oil spill incidents continuously happen (ITOPF, 2019). For instance, one of the famous oil pipelines, Keystone pipeline has experienced more than 20 spills since

2010, affecting farmlands, wetlands, etc., with the latest great spill of 380 000 gallons in 2019 that polluted almost 20 000 m² lands (Hefflinger, 2019).

Petroleum hydrocarbons consist of a vast variety of compounds, including alkanes, alkenes, cycloalkanes, aromatics, resins, asphaltenes, etc. Generally, petroleum hydrocarbons with low carbon numbers (C1-C9) are relatively volatile and easy to be degraded, while long-chain hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) are very persistent in the environment (Greenwood et al., 2008). Among the soil organic pollutants, PAHs that are from both oil products and incomplete combustion of fossils and biomass, have been most frequently studied worldwide in urban and agricultural soils and along the urban and rural gradients (Tao et al., 2004; Ma et al., 2009; Vauramo et al., 2011; Omores et al., 2017; Roslund et al., 2018; Vari et al., 2020). Urban soils are facing more severe contamination of PAHs than rural soils due to far more industrial discharges, oil leaks, and combustion activities.

Soils are additionally contaminated by the organic pollutants in the building materials both indoors and outdoors, and other concrete materials (Liu et al., 2018; Wang, 2018). For instance, brominated flame retardants (BFRs) are widely used as additives in various products such as electronic, furniture, and vehicles to reduce the inflammability (Yu et al., 2016).

Persistent organic pollutants (POPs), including BTEX and PAHs especially pose risks to the ecosystems and human health. Many studies have shown the mutagenic, carcinogenic, and teratogenic effects (Varjani et al., 2017). Specific health effects include chronic effects, such as organ damage and growth of tumor, and acute effects such as vomiting and nausea (Varjani et al., 2017).

2.2. Bioavailability

Soil pollutants are a threat to ecosystems and human health only through interactions with living organisms, which can consist of two steps, namely desorption from the soil particles and partitioning into organisms; the two steps determine bioavailability (Committee on bioavailability of contaminants in soils and sediments, 2003; Cui et al., 2013). It means that even if there is a considerable amount of soil pollutants, the pollutants do not necessarily harm, e.g. the crops, for instance when the pollutants are tightly adsorbed to soil particles, in the forms of precipitates, or have low affinities to tissues (Cui et al., 2013). In these cases, the pollutants are not easily bioavailable, while free ions and chemicals in the soil water are very mobile and easily bioavailable.

Bioavailability of soil pollutants highly depends on the soil physiochemical properties, e.g. soil texture, organic matter, moisture content, and pH (Fine et al., 1997; Makovnikova and Barancikova, 2012). For instance, a relatively high pH contributes to the formation of heavy metal hydroxide

precipitates while a low pH results in the release of free heavy metal ions into soil water. Therefore, in addition to the removal of pollutants from the environments, a large number of soil remediation strategies are meant for the immobilization and consequently the reduced bioavailability of soil pollutants (Basta and McGowen, 2004; Velkova et al., 2018; Palansooriya et al., 2020). Soil texture, organic matter content, and moisture content affect the adsorption capability of pollutants (Fine et al., 1997; Vig et al., 2003; Makovnikova and Barancikova, 2012). For example, clays, higher organic matter contents, and lower moisture contents contribute to more adsorption of petroleum hydrocarbons to soil particles (Fine et al., 1997).

However, to actually remove pollutants from the soil, such as by biodegradation, one way to boost the process is to enhance the desorption of pollutants from soil particles. Surfactants are therefore widely applied in the biostimulation of hydrophobic pollutants, including petroleum hydrocarbons in soils, since surfactants can increase the bioavailability of hydrophobic pollutants by enhancing their desorption from the soil particles as well as increasing their solubility (Mulligan, 2005). In fact, microorganisms themselves can produce biosurfactants for accelerating biodegradation (Das et al., 2008; Mnif et al., 2011).

2.3. Biodegradation

Biodegradation is the mineralization of organic pollutants to carbon dioxide and other inorganic chemicals to the ultimate extent. Anthropogenic pollutants usually have structural similarities to chemicals in nature, meaning that these pollutants can go through similar biological processes for degradation. The efficiency of biodegradation in soils usually depends on the existence of biodegraders, temperature, oxygen content, nutrient levels, pH, salinity, and other physio-chemical conditions that affect bioavailability and bioactivity (Romantschuk et al., 2000; Filler et al., 2008; Adams et al., 2015). Many strategies have been applied to modify these factors to enhance the performance of biodegradation, i.e. biostimulation.

Various microorganisms have been found capable of degrading organic pollutants, including bacteria, fungi, Archaea, and microalgae (Haritash and Kaushik, 2009; Fuchs et al., 2011; Ghosal et al., 2016; Aydin et al., 2017). Proteobacteria and Actinobacteria are two major bacterial phyla that accommodate a great number of hydrocarbon-degrading species and many species, such as *Alkanivorax*, *Acinetobacter*, *Oleispira*, *Mycobacterium*, *Rhodococcus*, and *Nocardioides* spp. have been found to be able to use petroleum hydrocarbons as sole carbon and energy sources (Prince et al., 2010; Das and Chandran, 2011). Different organic pollutants are favored by different microorganisms in biodegradation. For instance, in oil contaminated environments, Alpha- and Gamma-proteobacteria are especially enriched (Prince et al., 2010; Hazen et al., 2016). Bioaugmentation that adds

biodegraders into the contaminated environment is one way to enhance biodegradation but not always successful simply because microbes have their own favorable living environments (Romantschuk et al., 2000; Kauppi et al., 2011; Baneshi et al., 2014; Adams et al., 2015).

Aerobic biodegradation is generally more efficient than anaerobic biodegradation; therefore a common method to stimulate biodegradation is aeration through tillage and/or addition of bulking agents (Kauppi et al., 2011). Instead of oxygen, SO_4^{2-} , NO_3^- , Fe^{3+} , Mn^{4+} , and S are the common electron acceptors in anaerobic biodegradation (Widdel et al., 2010).

Temperatures between 20 and 40 °C are usually optimal for biodegradation. In the Arctic areas, biodegradation of organic pollutants are usually limited due to low temperatures. However, biodegradation also occurs at temperatures below 10 °C and microbial activities has even been found at sub-zero conditions (Margesin, 2000; Aislabie and Foght, 2008; Rike et al., 2008; Margesin et al., 2013). In fact, biodegradation of petroleum hydrocarbons in cold areas is highly appreciated and many relevant studies have been carried out (Aislabie and Foght, 2008; Filler et al., 2008). Successful biodegradation cases in cold areas include the famous clean-up of *Exxon Valdez* oil spill in Alaska (Bragg et al., 1994).

Nitrogen and phosphorus are major limiting nutrients for microbial activity. Fertilizers that contain N and P are commonly added to enhance microbial activity and hence biodegradation. Many forms of fertilizers have been used, including ammonia, NPK, urea, methylene urea, biowaste, etc. (Coulon et al., 2005; Peltola et al., 2006; Walworth and Ferguson, 2008; Francioli et al., 2016). NPK is a mixture of nitrogen, phosphorous, and potassium (three of the essential elements for plant growth and constituting a microbial cell). Potassium is not really a critical element in bioremediation, but merely part of the ready-made NPK fertilizer. Nitrogen is often a limiting nutrient for microbial activity in soils and a suitable C/N ratio is decisive for a satisfactory bioremediation result. Phosphorous is another major limiting nutrient in bioremediation; the maximum enhancement in the degradation can be achieved when the addition of both nitrogen and phosphorous is applied. Ratio of C, N, and P after the addition of fertilizers in the soil is typically investigated for the best performance of biostimulation. The most recommended ratio of C, N, and P is 100:10:1 (Brown et al., 1983; Margesin and Schinner, 2001; Leys et al., 2005; da Silva et al., 2009; Kanissery and Sims, 2011; Kaouther Zaafouri, 2014; Tangahu et al., 2017). Urea and methylene urea are very common organic nitrogen fertilizers, the mineralization of which can result in a soil pH increase since nitrogen was released as ammonia (Peltola et al., 2006; Walworth and Ferguson, 2008; Ghaly and Ramakrishnan, 2013). Compared to urea, methylene urea is slow in nitrogen release; soil pH change is therefore minor and the fertilization effect is long lasting.

2.4. Electrokinetic remediation

Electrokinetic remediation, as both a pollution treatment and a resource recovery method, has been developed in the recent decades to remove pollutants, especially heavy metals in soils (Acar and Alshawabkeh, 1993; Acar et al., 1995; US Army Environmental Center, 2000; Kim et al., 2011; Ottosen, 2014). Electrokinetic remediation has shown outstanding performance in removing soil heavy metals, including Cd, As, Pb, Cu (Amrate et al., 2005; Alcántara et al., 2012; Kim et al., 2012, 2013; Ahmadipour et al., 2014).

In the electric field, hydroxyl and hydrogen ions are electrolyzed at the cathode and anode respectively, and move to the oppositely charged electrodes. Water migrates along with the hydrogen ions to the cathode, i.e. electroosmosis. Heavy metals migrate along with water, controlled by the forces of electromigration, electroosmosis, electrophoresis, and/or hydraulic gradient. As a result, positively charged heavy metals accumulate near the cathode through the formation of hydroxide precipitates and/or through electrochemical deposition onto the surface of the cathode. These heavy metal species can be removed physically easily thereafter. Soil pH is usually adjusted by weak acids, such as lactic acid and acetic acid to avoid metal precipitates far away from the electrodes and enhance soil metal desorption through cation exchange (Acar et al., 1995; Alshawabkeh et al., 2004). EDTA is another commonly added agent to enhance soil metal desorption through chelation (Amrate et al., 2005; Alcántara et al., 2012; Dong et al., 2013). In addition, electrokinetic remediation has been modified in many ways, such as with pulse current and approaching anodes to improve the removal efficiency (Chen et al., 2006; Shen et al., 2007; Yao et al., 2020).

The research of electrokinetic remediation so far has been mostly at bench scales. Laboratory studies (table 1) on the electrokinetic remediation of Cd have been widely conducted with soils from agricultural and industrial sites, commercially purchased, etc. (Kim and Kim, 2001; Kim et al., 2005; Chen et al., 2006; Chung and Lee, 2007; Lee et al., 2009; Ryu et al., 2009, 2010; Lu et al., 2012).

These studied soils were artificially contaminated (spiked) or originally contaminated at the sampling sites. In most cases, the removal efficiency in the spiked soils ($\geq 80\%$) were much higher than those in the originally contaminated soils ($< 50\%$). Although the spiked soils were allowed for settlement up to 3 months in those cases, Cd adsorption to the soil particles could still be loose which resulted in a high removal efficiency. High removal efficiencies were also found in the originally contaminated soils with advanced electrokinetic remediation methods. Chen et al. (2006) employed electrokinetic remediation with an approaching anode towards the cathode leading to a removal efficiency of 73% in an originally contaminated soil near a plating factory. Lu et al. (2012) adopted

polarity exchange in the electrokinetic remediation of an industrial site soil and the removal efficiency of Cd was up to 94% when the interval of polarity exchange was 48 h.

Additionally, a voltage gradient of 100 V m^{-1} was commonly used in these studies. However, the recommended maximum voltage for outdoor use in wet environments in China is 35 V (GB/T 3805-2008), which makes *in situ* electrokinetic remediation with a similar voltage gradient impossible in China. Therefore, more relevant voltage gradients should be studied according to local regulations.

Table 1. Previous laboratory studies on electrokinetic remediation of Cd in the soil. The highest removal efficiency and the corresponding treatment in each study are listed in this table.

Origin of Cd/soil, depth of soil	Laboratory scale, cm, L×W×D	Voltage gradient, V m ⁻¹	Electrolytes/additives	Time	Highest removal efficiency, total Cd or otherwise clarified	EK enhancement	Authors
Spiked soil near an airport, depth not mentioned (settlement for 48 h)	10x10x11, rectangular	200	Detonized water	200 h	90 %	Permeable reactive barriers	Chung and Lee, 2007
Spiked commercial soil (settlement for 1 week)	34(Ø)x5(H), vertical cylindrical	10-20 A fixed current (0.5 A)	0.01 M KNO ₃ (catholyte and anolyte)	100 h	92 %, HCl acid soluble	Ion exchange membrane	Kim et al., 2005
a. Spiked agricultural soil, top 0-15 cm (settlement for 3 months)	21x5.5x11, rectangular	100	Not used	60 h	a. 80 %, spiked b. 73 %, site contamination	Approaching anode	Chen et al., 2006
b. Soil near a plating factory, top 0-15 cm							
Industrial site soil, top 5-20 cm	15(L)×9.3(Ø), horizontal cylindrical	100	Tap water (catholyte and anolyte)	192 h	94 %	Polarity exchange, interval 48 h	Lu et al., 2012
Mine tailing soil, depth not mentioned	15x9x9, rectangular	80-200 A fixed current (0.1 A)	0.5 N H ₂ SO ₄ (catholyte), 0.005 N H ₂ SO ₄ (anolyte)	120 h	95 %, exchangeable 13 %, total	Conventional	Kim and Kim, 2001
Mine area soil, top 0-30 cm	20x4x5, rectangular	100	0.5 M H ₂ SO ₄ (catholyte), tap water (anolyte)	10 d	47 %	Microbial leaching	Lee et al., 2009
Agricultural soil near a mining area, depth 0-30 cm	20x4x4, rectangular	100	Tap water (catholyte), 0.1 M MgSO ₄ (anolyte)	14 d	32 %, HCl acid soluble	Conventional	Ryu et al., 2009
Agricultural soil near a mining area, depth not mentioned	20x4x4, rectangular	100-200	Tap water (catholyte), 0.1 M MgSO ₄ (anolyte)	14 d	24 %	Pulsed EK remediation	Ryu et al., 2010
Soil near a lead and zinc factory, top 0-15 cm	22(L)×6.2(Ø), horizontal cylindrical	266	EDTA (catholyte and anolyte)	5 d	43 %	Conventional	Beyrami, in press

2.5. Joint applications of biodegradation, electrokinetic remediation, and other remediation strategies

In many studies, joint applications of different remediation methods have shown enhanced performance compared to the sole use of one method. For instance, due to the special interaction between microbial community and plants at the rhizosphere, phytoremediation can be coupled with bioaugmentation to enhance phytoextraction of heavy metals and degradation of organic pollutants (Płociniczak et al., 2013, 2017; Baneshi et al., 2014; Saravanan et al., 2020). Plant Growth Promoting Rhizobacteria (PGPR) have especially been adopted to enhance pollutant removal efficiency (Khan et al., 2013; Gkorezis et al., 2016; Saravanan et al., 2020).

The desorption and migration characteristics make electrokinetic remediation very compatible with other remediation methods. To enhance the desorption, acids, surfactants, and chelating agents are added in the soil in electrokinetic remediation (Zhang et al., 2019). Electrokinetic remediation can also be coupled with chemical oxidation, bioremediation, soil vapor extraction (Suni et al., 2007; Lee and Kim, 2010; Cameselle and Gouveia, 2018; Koul and Taak, 2018; Simpanen et al., 2018). Electrokinetic remediation can distribute species, such as iron ions and persulfate that induce chemical oxidation, and nutrients and biodegraders that boost biodegradation. Products from chemical oxidation and biodegradation that are more water soluble can then be migrated through electrokinetic remediation. When electrokinetic remediation is coupled with phytoremediation, plant growth can be boosted through nutrient delivery (Cameselle and Gouveia, 2018).

Additionally, chemical oxidation often produces byproducts that are more water soluble and therefore more bioavailable, which makes biodegradation more feasible. Hence, chemical oxidation followed by bioremediation has shown improved removal efficiency for organic pollutants such as PAHs (Nam et al., 2001; Liao et al., 2018; Talvenmäki, 2020).

2.6. Experimental design

Remediation strategies, including biodegradation, are often investigated at bench scales, followed by pilot tests and finally full-scale applications in real cases. Real-case applications can be *in situ* treatments or *ex situ* treatments after excavation. In *ex situ* treatments, polluted soils were usually mixed properly for better results while it is more challenging in *in situ* cases due to heterogeneous distribution of pollutants and degradation strains in soils. Basically, the test or application scale goes up when results continuously show promise, while aborted if failure happens. In biodegradation, not all microbes in the studied environment function as biodegraders of organic pollutants or all kinds of organic pollutants. Therefore, the existence of the relevant biodegraders is essential for the success of biodegradation in the tests or practical applications. Unfortunately, the environmental microbial

community is highly heterogeneous (Hurlbert, 1984; Davis et al., 2003; Prosser, 2010), meaning that success or failure in the laboratory experiments does not ensure the same results in the field pilot tests or real cases. Therefore, it is especially important to have a well-designed experiment in the early phases to have repeatable successful results in the scaled-up biodegradation practices or to be convincing that the failure of the biodegradation strategy in the early experiments is not due to inferior experimental designs.

2.6.1. Pseudoreplication

Pseudoreplication refers to situations in which a single environmental sample was studied or samples are mistreated as true ecological replicates in experimental designs and data analysis to make general conclusions (Hurlbert, 1984). Pseudoreplication in ecological studies has been pointed out since the 1980s (Hurlbert, 1984), and has been continuously criticized over the years (Davis et al., 2003; Prosser, 2010). However, not all studies including those that are published in the high-level international journals recognize the importance of having ecologically independent replicates to make general conclusions (Prosser 2010).

The use of pseudoreplication happens also in soil biodegradation studies (Davis et al., 2003). In the laboratory, the soil to be studied is often taken from a single environment, mixed and divided into independent units as replicates in the experiment and statistical data analysis. Both sampling a single unit from the environment and mixing the soil sample before replicate division ignore the heterogeneity of environmental soil. Statistical results gained from this kind of experimental design does not assist in conclusion making for the sampled environment, and not even for the sampled soil due to the mixing action before replicate division. Since soil microbial community is naturally heterogeneous, mixing can distribute the biodegraders all over the soil and the divided replicates after mixing are surely not actual representatives for the sampled soil in biodegradation characteristics. As a matter of fact, it is only normal that biodegradation in the environment is patchy (Ghosh et al., 2000; Davis et al., 2003; Kauppi et al., 2011; Dechesne et al., 2014; Yu et al., 2015).

2.6.2. Composite sampling

It is practically impossible to involve the whole microbial community of the sampled area in biodegradation studies. Therefore, ecological replicates are usually taken. In principle, the representability of analyzed sample replicates for the sampled area increases as the number of replicates increases. However, it also creates more burden of labor and cost. As a compromise, composite sampling is often applied in soil pollutant biodegradation studies (Walton and Anderson, 1990; Viñas et al., 2005; Jensen et al., 2009; Garg et al., 2016). Composite sampling is a practice of pooling individual samples (Van Belle et al., 2001; Tan, 2005; Patil, 2011). It is commonly done in

medical disease screening (Dorfman, 1943; Ciampa et al., 2010; Bilder and Tebbs, 2012). A composite sample represents the component samples at an average level and it is well-known that false positive and negative results can happen (Van Belle et al., 2001; Patil, 2011). A negative result of a disease for a composite sample can at times simply mean that the measured index is below detection limit after being pooled, i.e. averaged or diluted (dilution effect); some component samples can still carry the disease. Similarly, a positive result for a composite sample can simply mean that at least one component but not necessarily all carries the disease. The same can occur to composite sampling in biodegradation studies.

In biodegradation studies, a composite sample can be a pool of replicate samples from one environment or from several different environments (Walton and Anderson, 1990; Viñas et al., 2005; Jensen et al., 2009; Garg et al., 2016). The composite sample is often well mixed and then divided into independent replicates in the laboratory experiments and for statistical data analysis. Again, environmental heterogeneity is neglected. A composite sample that contains degradation strains does not ensure that all the component samples contain the strains and a promising statistical result gained from replicates derived from a well-mixed composite sample is not equivalent to what is gained from actual ecologically independent replicates. Additionally, an effective degradation strain in certain sample components can be overlooked in composite sampling due to a dilution effect.

2.6.3. Randomized complete block design (RCBD)

RCBD is a very common design in soil studies (Mills et al., 2004; Fageria, 2007; Jidere et al., 2012; Tavares et al., 2016). It is a design that applies ecologically independent replicates, i.e. blocks. All treatments have one sample in each soil block so that all treatments have ecologically independent replicates with similar variance between treatments, assuming that the soil is as homogeneous as possible in each soil block. Compared to pseudoreplication and composite sampling, RCBD takes into account environmental heterogeneity, but treatment effects can still be biased due to the fact that soil is heterogeneous even within one soil block (Davis et al., 2003). In biodegradation, degradation strains are the key factor and yet soil microbes are as patchy as at millimeter scales in addition to patchy pollutants (Ghosh et al., 2000; Davis et al., 2003; Kauppi et al., 2011; Dechesne et al., 2014; Yu et al., 2015).

3. AIMS OF THE STUDY

This doctoral thesis aimed for developing soil remediation strategies. One of the major aims was to test electrokinetic remediation in the removal of soil heavy metal Cd in field conditions (Paper I). Specifically, the study meant to investigate the effect of voltage gradient on the removal efficiency

of Cd by comparing pilot and scaled-up (full-scale application) tests. Additionally, the study aimed to compare the removal efficiency of the total and plant available Cd as well as in different soil layers. The hypotheses were 1) the removal efficiency of soil Cd is higher in the pilot test than in the full-scale application; 2) the removal efficiency is higher for the plant available Cd than total Cd; and 3) the removal efficiency is higher in the surface soil layer.

Another major aim was to optimize biodegradation of gasoline aromatic pollutants by fertilization (biostimulation) in the polluted Arctic soil (Paper II). The study was mainly to compare three nitrogen fertilizers: inorganic NPK, urea, and methylene urea in biostimulation and to illustrate the effects of fertilizer type, temperature, and contamination age on soil pH alteration in association to biostimulation. The hypotheses were 1) NPK is the most efficient fertilizer in biostimulation due to more various nutrient supply; 2) urea changes soil pH more than methylene urea and NPK; 3) soil pH is altered more by fertilizers when the temperature is higher; and 4) soil pH alteration by fertilizers depends on the contamination age of the soil.

Finally, the doctoral thesis was to illustrate how different results and conclusions could be obtained from biodegradation studies using a single source of soil and using ecologically independent soil replication, and thereafter to optimize experimental design and the consequent data analysis for guiding a more successful application of *in situ* biodegradation strategies in real cases (Paper III). The hypotheses were 1) results and conclusions vary among parallel experiments using within-plot soil replication and using ecologically independent replication; and 2) dilution effect happens when using ecologically independent replication.

4. MATERIALS AND METHODS

4.1. Experimental design

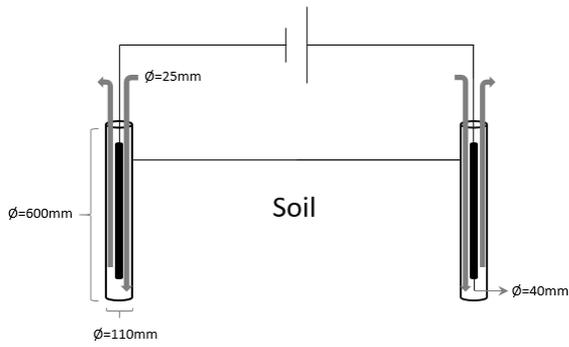
4.1.1. Electrokinetic remediation

The electrokinetic remediation study consisted of a pilot (4 m²) test and a full-scale (200 m²) application of electrokinetic remediation in a Cd contaminated farmland (paddy agricultural soil) near the Fankou lead-zinc mining area in Gaolian village (25°04' N, 113°40' E), Dongtang town, Renhua district, Shaoguan city, Guangdong province, China. The depth of the treated soil was 50 cm. The electrode assembly is illustrated in the schematic diagram (Fig.1). PVC pipes (60 × 6.3 cm², length × diameter) were first inserted into the soil, and the electrodes were then placed into the PVC pipes. Two additional thin pipes (d=2.5 cm) were also inserted into the PVC pipes for pumping electrolyte.

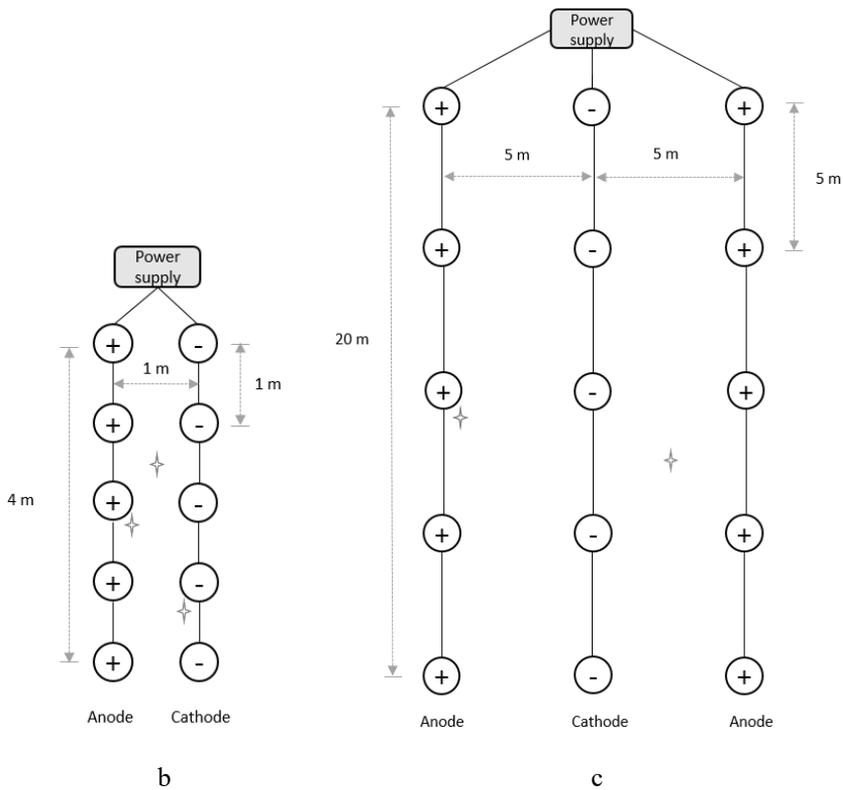
In the pilot test, there were five pairs of series-wound electrodes. The distance between the opposite and adjacent electrodes was 1 m. In the full-scale application, five series-wound anodes with a 5 m

interval were placed on each side of the farmland edges. The distance between the two edges was 10 m. Five series-wound cathodes with 5 m interval were placed in the center line and shared by anodes. 20 V was applied for both the pilot test and full-scale application. During electrokinetic remediation, 0.5 mol·L⁻¹ lactic acid electrolyte was added to all the electrode PVC pipes every 24 hours for the first 72 hours, followed by a 68 h break. Later, lactic acid was added every 48 hours. The electrolyte was added to all the PVC pipes to keep the liquid close to the soil surface level. Sampling spots are shown in the schematic diagram (Fig.1). Soil Cd was measured as the total Cd and plant available Cd respectively, at soil layers 0-10 cm, 10-20 cm, and 40-50 cm before and after electrokinetic remediation.

The detailed experimental procedure can be found in paper I.



a



b

c

Fig. 1. Schematic diagram of the experimental setup: a) electrode installation in the soil; b) electrode assembly in the pilot test; c) electrode assembly in the full-scale application. Star marks show the sampling spots.

4.1.2. **Biostimulation with fertilization**

This study was conducted in the laboratory. Gasoline (unleaded gasoline octane 95) contaminated soil was sampled from a high Arctic permafrost active layer near the Zackenberg research station (74°28' N, 20°34' W) in Northeast Greenland. The contamination happened 24 hours before sampling. Some clean soil was also collected in the surrounding area as control. The treatments were designed as non-contaminated control and four contaminated treatments. The four contaminated treatments were NPK (inorganic, 14-3-23, HORNUM®) fertilized, urea fertilized, methylene urea fertilized, and non-fertilized. The fertilizers were added to the soil at a nitrogen concentration of 305 mg (g dry wt soil)⁻¹. Three replicates (10 g soil each) were prepared for each treatment and each destructive sampling day, i.e. days 0, 7, 21, 28. The experiment temperature was 10 °C that was relevant to the sampled area where the mean air and soil temperature has been around 5 °C -10 °C in summer in the last decades.

A separate experiment was done with the same fertilization design to monitor the soil pH effects of the fertilizers to two differently aged soils at 4 °C, 10 °C, and room temperature (RT) respectively. The soils were sampled from Finland and had similar phyio-chemical properties to those of the soil from Greenland. One soil was contaminated by gasoline and diesel 24 h ago and the other had been contaminated between the early 1990s and 2015. Soil pH was followed on days 0, 7, 14, 21, and 28.

The detailed experimental procedure can be found in paper II.

4.1.3. **Effects of experimental design on biodegradation results**

This study was conducted in the laboratory. Three ecologically independent plots of soil (about 10 L per plot) was sampled from the soil surface layer (3-15 cm) in a pristine boreal pine forest in Hollola (67°67'N 34°18'E), Finland. The three plots were at least 5 m apart in the sampling area, samples were not pooled and were processed independently. The experiment design is shown in the schematic diagram (Fig.2). Each of the three plots of soil was designed with three treatments: non-contaminated control, methylene urea fertilized and gasoline contaminated, and non-fertilized and gasoline contaminated. Gasoline (unleaded gasoline octane 98) contamination was by spiking. The gasoline concentration was about 0.39 mL (g dry wt soil)⁻¹, 0.44 mL (g dry wt soil)⁻¹, and 0.52 mL (g dry wt soil)⁻¹ in the soil from plots 1, 2, and 3 respectively. Methylene urea was added at a concentration of about 2.5 mg (g dry wt soil)⁻¹, 2.9 mg (g dry wt soil)⁻¹, and 3.4 mg (g dry wt soil)⁻¹ in the soil from plots 1, 2, and 3 respectively. Additionally, each treatment of each plot of soil was designed with three technically independent replicates (10 g per replicate) treated as pseudoreplicates, i.e. within-plot comparison. Hence, there were three sets of within-plot comparisons from the three plots of soil. The means of pseudoreplicates of each plot of soil then constructed the true ecological replicates, i.e.

between-plot comparison. The temperature of the experiment was set at 4 °C, which was relevant in Finnish winter. The sampling is destructive and the sampling days are 0, 7, and 28.

The detailed experimental procedure can be found in paper III.

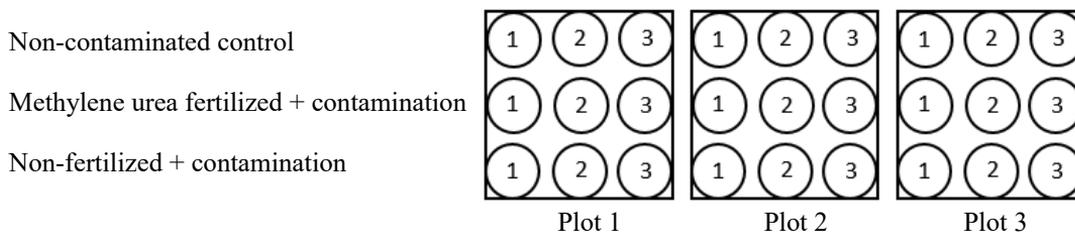


Fig.2. Experimental design. Circles with numbers in the squares are technically independent replicates for each treatment within each plot of soil.

4.2. Soil analysis

The soil analysis methods for Cd, gasoline aromatics, and pH are summarized in table 2. Details can be found in the relevant papers.

4.3. Statistical analysis

The statistical analysis methods for the fate of Cd and gasoline aromatics in the soil and fertilizer-induced soil pH change, and the study scale are summarized in table 3.

Table 2. Soil analysis methods.

Study object	Measurement	Study source
Total Cd	0.3 g, complete soil digestion (HCl-HNO ₃ - HF-HClO ₄), graphite furnace atomic absorption spectrophotometer (Hitachi Z-2000)	Paper I
Plant available Cd	10 g, leaching (DTPA-CaCl ₂ -TEA), graphite furnace atomic absorption spectrophotometer (Hitachi Z-2000)	Paper I
Gasoline aromatics	1 g, ISO 14507, GC-MS	Paper II & III
Soil pH	10 g, HJ 962-2018, Chinese standard	Paper I
Soil pH	0.5 g, ISO 10390	Paper II

Table 3. Statistical analysis methods.

Study object	Statistical analysis	Study source	Scale
Cd concentration over time	Repeated Measures ANOVA (Natural logarithmic values of concentration)	Paper I	Field
Gasoline aromatic concentration on each sampling day between different treatments and in comparison to day 0	One-Way ANOVA with Dunnett's post hoc test	Paper II	Field
Effects of fertilizer type, temperature, contamination age on soil pH alteration	Repeated Measures ANOVA; time as the within-subject factor, and fertilizer type, temperature, and contamination age as the between-subject factors	Paper II	Laboratory
Gasoline aromatic concentration over time in within-plot comparisons (three technically independent replicates, i.e. pseudoreplicates within each plot of soil)	Repeated Measures ANOVA; time as the within-subject factor, and treatment and plot as the between-subject factors	Paper III	Laboratory
Gasoline aromatic concentration over time in between-plot comparison (three means of pseudoreplicates from the three plots of soil)	Repeated Measures ANOVA; time as the within-subject factor and treatment as the between-subject factor	Paper III	Laboratory

5. RESULTS AND DISCUSSION

5.1. Electrokinetic remediation

Electrokinetic remediation of soil heavy metal Cd in the field showed success in the electrokinetic remediation study. After 14 days' electrokinetic remediation, the concentration of the total Cd dropped ($F=32.304$, $df=1$, $p<0.0005$) from 0.19-2.26 to 0.08-0.31 mg (kg dry wt soil)⁻¹ and the concentration of the plant available Cd dropped ($F=9.731$, $df=1$, $p=0.012$) from 0.08-0.92 mg to 0.03-0.11 mg (kg dry wt soil)⁻¹. The final concentration of the total Cd was below the hazard threshold, i.e. 0.4 mg (kg dry wt soil)⁻¹ set for paddy agricultural soils in China (Li et al., 2019).

Removal of the total and plant available Cd varied at different soil layers (Fig.3&4). Basically, the removal efficiency was higher in the soil surface layer, i.e. 0-10 cm. For the total Cd, significant removal was found at all soil layers, i.e. 0-10 cm, 10-20 cm, and 40-50 cm in the pilot test, with the removal efficiency decreasing from 87% to 54% from the surface to the lower layers, while significant removal (74%) was only found in the surface layer in the full-scale application (Fig.3). As for the plant available Cd, significant removal was only found in the pilot test which happened in the surface layer by 64% (Fig.4). The lower removal efficiency in the lower soil layers could be due to the gravity-induced migration of Cd from the upper to the lower soil layers (Acar et al., 1995).

The removal efficiency of Cd was generally higher in the pilot test than full-scale application, which was not surprising since the voltage gradient in the pilot test was much higher. While scaling up the application, a higher voltage should be applied to enhance the removal efficiency, although safety should be concerned while exposing high voltage electricity outdoors.

Comparing the total and plant available Cd, the removal efficiency was generally higher for the total Cd, although the plant available Cd is supposed to be more water soluble and have higher mobility. However, in electrokinetic remediation, the plant available Cd is basically produced through cation exchange (Acar et al., 1995) and/or dissolution by the added lactic acid, which offsets the removal and could result in a lowered removal efficiency than the total Cd. However, this should only be a matter of time.

No earlier research that investigated *in situ* electrokinetic remediation of Cd in agricultural soils has been found. However, there are a number of laboratory studies on electrokinetic remediation of Cd in different soils, some of which are listed in table 1 (in section 2.4). Considering the much lower voltage gradients (20 V m^{-1} in the pilot test and 4 V m^{-1} in the full-scale application) used in the study herein, the removal of the total Cd was rather successful in the top 0-10 cm layer, i.e. 87% in the pilot test and 74% in the full-scale application. However, a much higher voltage gradient of 100 V m^{-1} was frequently used in the earlier studies and the removal efficiencies for real contamination cases were mostly below 50% (table 1).

This study was the first to show a significant removal of heavy metal Cd in the soil by *in situ* electrokinetic remediation as a full-scale application, which shows a high potential of *in situ* electrokinetic remediation of soil heavy metals with a low voltage demand. A low voltage demand is especially important for *in situ* electrokinetic remediation in China due to the restriction of outdoor voltage use.

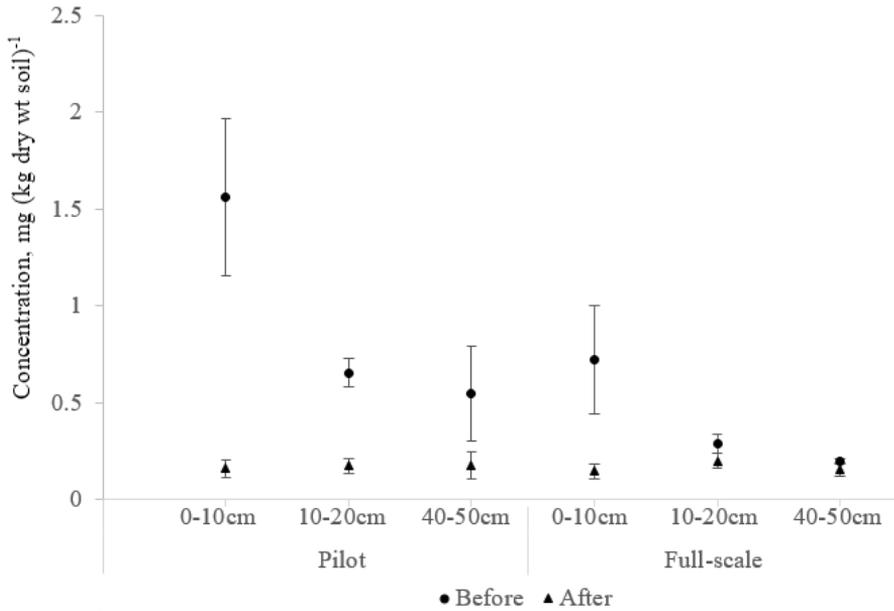


Fig. 3. Concentration of the total Cd in the soil layers 0-10 cm, 10-20 cm, and 40-50 cm before and after electrokinetic remediation in the pilot test and full-scale application. Mean \pm SE.

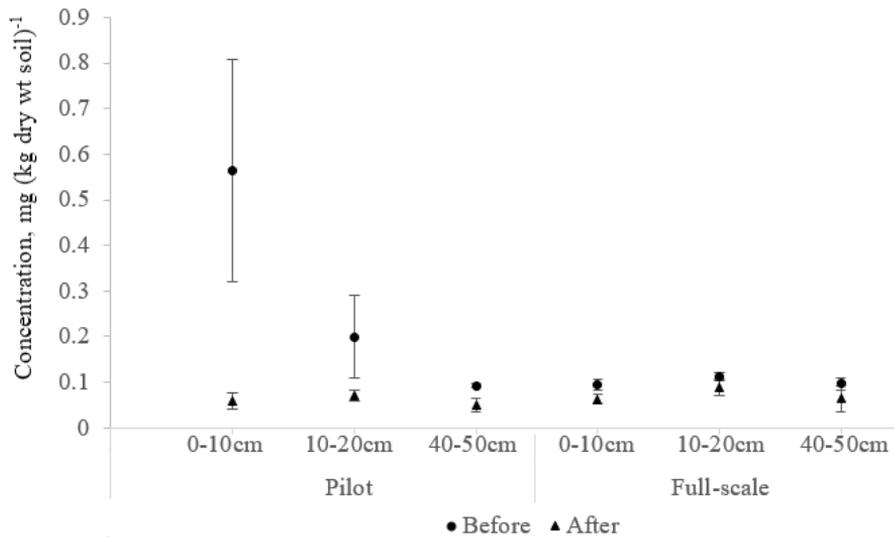


Fig. 4. Concentration of the plant available Cd in the soil layers 0-10 cm, 10-20 cm, and 40-50 cm before and after electrokinetic remediation in the pilot test and full-scale application. Mean \pm SE.

5.2. Biostimulation by fertilization

Unlike any remediation means of heavy metals (Ukonmaanaho et al., 1998; Tulonen et al., 2006; Fu et al., 2014; Zwolak et al., 2019), removal of organic pollutants through biodegradation actually destroys the chemicals. Biostimulation by fertilization is a very common way to enhance biodegradation (Braddock et al., 1997; Romantschuk et al., 2000; Coulon et al., 2005; Walworth and Ferguson, 2008; Kauppi et al., 2011; Adams et al., 2015).

Gasoline aromatics were not detected in the clean soil samples throughout the experiment. Hence, the soil mentioned below refers to the contaminated soil. Fertilizers including NPK, urea, and methylene urea have all shown enhancement in biodegradation of organic pollutants. In the biostimulation study of this doctoral thesis, it was found that some nitrogen fertilizers stimulated the desorption of gasoline aromatics from soil particles which could have contributed to the enhanced biodegradation of gasoline aromatics in the soil in addition to functioning as a nutrient supplement for the biodegraders.

The fate of the total gasoline aromatics over time varied in different treatments (Fig.5). During the 28-day experiment, the concentration of the total gasoline aromatics in the soil was found higher in the NPK and urea fertilized treatments on day 7 compared to day 0 ($F=21.18$, $df=4$, $p<0.0005$), followed by a decreasing trend until the end of the experiment ($F=8.26$, $df=4$, $p=0.002$). The concentration of the total gasoline aromatics in the urea fertilized treatment was 47% lower ($p=0.009$, Dunnett's test) in the end compared to the initial level while the decreasing trend in the NPK fertilized treatment was not as significant and the concentration stayed around the initial level ($p=0.102$, Dunnett's test). The concentration of the total gasoline aromatics was stable in the non-fertilized treatment (natural attenuation) and it did not fluctuate significantly in the methylene urea fertilized treatment.

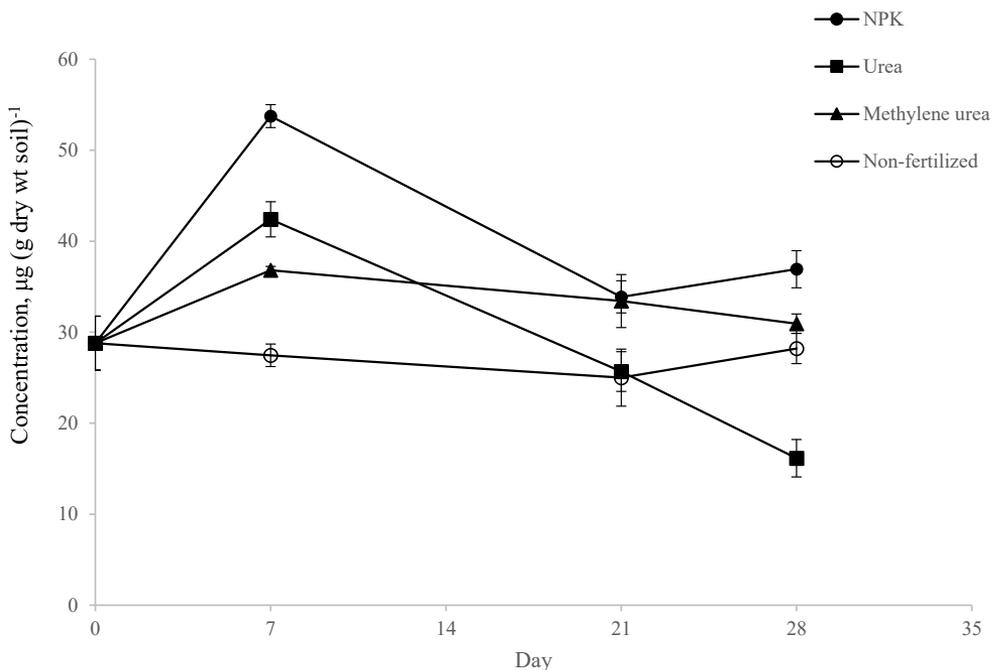


Fig.5. Measured concentration of the total gasoline aromatics in all the treatments over time. Mean \pm SE.

Temperature and nutrient availability are some of the key factors for microbial activity. This experiment was conducted at 10 °C while the optimal temperatures for biodegradation are between 20-40 °C (Vidali, 2001). Although biodegradation was also found at temperatures below 10 °C, the soil was basically mineral barren soil, which together with the relatively low temperature could explain why natural attenuation did not take place.

Gasoline aromatics were not removed in all the fertilized treatments, especially in the methylene urea fertilized treatment. However, methylene urea showed better performance than urea in an earlier study by Peltola et al. (2006). Organic nitrogen fertilizers including urea and methylene urea usually result in an increase in soil pH while hydrolyzed to release ammonia. Soil pH alteration by organic fertilizers is especially concerned. A soil pH between 6 and 8 is optimal for biodegradation (Vidali, 2001; International Centre for Soil and Contaminated Sites (ICCS), 2006; Pawar, 2015). Compared to urea, methylene urea is slower in nitrogen releasing, meaning the effect of methylene urea on soil pH alteration is also minor, which was credited to for the better performance of methylene urea in biostimulation by Peltola et al. (2006). In that study, the temperature was above 20 °C, while the

temperature was 10 °C in this study. Like all chemical reactions, hydrolysis is faster at higher temperatures.

In this study, the soil (of both contamination ages) pH was originally below 5.5 and was altered depending on the fertilizer, temperature and contamination age (Fig.1 in paper II). Soil pH was elevated over time in the urea and methylene urea fertilized treatments at all temperatures with more increase in the urea fertilized treatment, while it was stable in the non-fertilized and NPK fertilized treatments ($F = 1969.18$, $df = 24$, $p < 0.0005$). With urea fertilization, the soil pH increased more in the aged contaminated soil than in the newly contaminated soil at any temperature ($F = 66.42$, $df = 12$, $p < 0.0005$). Additionally, soil pH was increased more at higher temperatures in the urea and methylene urea fertilized treatments ($F = 112.53$, $df = 24$, $p < 0.0005$). At 10 °C, the soil pH was under 6 almost the whole period in the methylene urea fertilized treatment, while it was between 6-7.5 after day 14 until the end of the experiment. Therefore, in this study urea likely contributed to biostimulation by providing nitrogen more efficiently without increasing the soil pH in an undesired way.

The observed significant concentration increase of the total gasoline aromatics on day 7 in the NPK and urea fertilized treatments apparently reflected an increased desorption and improved extractability of gasoline aromatics (Fig.5). Biodegradation prerequisites access of organic pollutants by the biodegraders. Some portion of organic pollutants can be tightly adsorbed to soil particles and become long-lasting in the environment. Desorption is part of biodegradation processes in the soil, which is also referred to as bioaccessibility and can be measured standardly by partial extraction using a mild solvent, and only the free or weakly adsorbed hydrocarbons are targeted (Cui et al., 2013). In this study, the ISO standard extraction method used a strong solvent, n-hexane that is supposed to extract the hydrocarbons with the help of acetone to the utmost extent, including the tightly adsorbed ones in the soil. Even so, an increased amount of desorbed/extracted gasoline aromatics was observed in the urea and NPK fertilized treatments compared to day 0; no or insignificant increase was found in the non-fertilized and methylene urea fertilized treatments. It means that urea and NPK had enhanced the desorption of gasoline aromatics in the soil.

It is common to add surfactants to enhance the desorption of soil organic pollutants by increasing the solubility in soil water. An apparent rise in the concentration of soil organic pollutants has also been observed earlier (Simpanen et al. 2016), and was in that case concluded to be related to an increased extractability of the substances during the early biostimulation process by the surfactant. In fact, urea has been found having a surfactant feature, increasing hydrocarbon solubility (Alonso and Dill, 1991; Peruffo et al., 1994; Mulligan, 2005; Hua et al., 2008; Zangi et al., 2009; Bianco et al., 2011). As a

matter of fact, many microbes produce biosurfactants to enhance the desorption and degradation of organic pollutants (Das et al., 2008; Mnif et al., 2011; Joo and Kim, 2013). Therefore, biostimulation of organic pollutants related to biosurfactant production and thereafter the enhanced desorption can be further studied.

Additionally, metal-chelating chemicals have also been used to enhance the desorption of soil organic pollutants by breaking the organo-metallic complexes (Yang et al., 2001; Chen et al., 2017). Chelates contain high amounts of elements, such as nitrogen, oxygen, and phosphorus that have free-paired electrons to complex metals. Urea ($\text{CH}_4\text{N}_2\text{O}$) and NPK (nitrate and ammonium, phosphate, citrate and/or water soluble phosphorus, potassium, magnesium, and sulfur) indeed contain a high content of these functional elements, and phosphate (the main component in NPK in this study) is actually a competitive adsorbate on metal oxides in the mineral soil (Borggaard et al., 1990; Johnson and Scow, 1999; Dong et al., 2013; Moyo et al., 2014). Although there was also a trend of concentration increase of methylene urea, it was not statistically verified (Fig.5; $p=0.08$, Dunnett's test). The enhanced desorption and therefore the bioaccessibility of soil gasoline aromatics by urea and NPK can therefore partially explain the biostimulation effect after the dominant desorption phase between day 7 and day 28.

Although biostimulation with urea fertilization showed a high success compared to NPK and methylene urea in the above study, the study was done using a single source of soil in the laboratory at specific conditions, e.g. at 10 °C. The performance of urea in biostimulation cannot yet be generalized in all or most cases. One earlier study already showed the disadvantage of urea over methylene urea by increasing soil pH unfavorably at a much higher temperature (Peltola et al., 2006), not to speak of the high patchiness of microbes in the environment.

5.3. Effects of experimental design on biodegradation results

No gasoline aromatics were detected in the clean control soil throughout the experiment. Therefore, the soil below refers to the contaminated soil. In the experimental design study of this doctoral thesis, it was apparent that biodegradation performance was different in the soil from different plots. Biodegradation results of the total gasoline aromatics in the soil were treatment and plot dependent (Fig.6). For example, significant removal of the total gasoline aromatics was found only in the methylene urea fertilized treatment in the soil from plot 2 ($F=12.052$, $df=2$, $p=0.002$) while it was found only in the non-fertilized treatment in the soil from plot 3 ($F=12.925$, $df=2$, $p=0.001$). Based on the results gained from the plot 2 soil, it could be concluded that natural attenuation of soil gasoline aromatics was not promising and biostimulation with methylene urea fertilization was a successful intervention. On the contrary, based on the results gained from the plot 3 soil, it seemed

that natural attenuation did a better job than biostimulation. Similarly, biodegradation performance varied among the plots of soil when individual gasoline aromatics were compared. For example, the removal of naphthalene was outstanding in either methylene urea fertilized ($F=73.657$, $df=2$, $p<0.001$) or non-fertilized treatment ($F=40.085$, $df=2$, $p<0.001$) in the plot 1 soil, while significant removal was only in the methylene urea fertilized treatment in the plot 2 ($F=11.583$, $df=2$, $p=0.002$) soil and in the non-fertilized treatment in the plot 3 ($F=26.165$, $df=2$, $p<0.001$) soil respectively (Fig.7).

When the three plots were treated as ecologically independent replicates, which was referred to as between-plot comparison, no statistically significant removal of the total gasoline aromatics was observed in either methylene urea fertilized or non-fertilized treatment (Fig.6). It is not surprising that the variation was big among the plots of soil and the dilution effect happened to the between-plot comparison (Davis et al., 2003). For instance, in the non-fertilized treatment, even though there was biodegradation in the plot 3 soil, the average biodegradation performance of the three plots was negligible due to the negative biodegradation performance in the plot 1 or 2 soil. While looking at the results of naphthalene, biodegradation was still observed in the between-plot comparison in either treatment ($F=8.703$, $df=2$, $p=0.010$) after being averaged or diluted, e.g. by the negative biodegradation performance in the plot 2 soil in the non-fertilized treatment (Fig.7). Methylene urea did not make a difference in biodegradation of naphthalene in the between-plot comparison ($F=3.038$, $df=1$, $p=0.109$).

Due to the difference in biodegradation results among the three plots of soil and between-plot comparison, it is impossible to conclude whether methylene urea successfully stimulated biodegradation, i.e. biostimulation. The effect of methylene urea fertilization seemed to be positive, negative, and negligible in different cases. It is most likely that the positive biodegradation results in certain soil samples are simply due to the random existence of degradation strains. After all, microbial patchiness exists within millimeters in the soil (Ghosh et al., 2000; Davis et al., 2003; Kauppi et al., 2011; Dechesne et al., 2014; Yu et al., 2015).

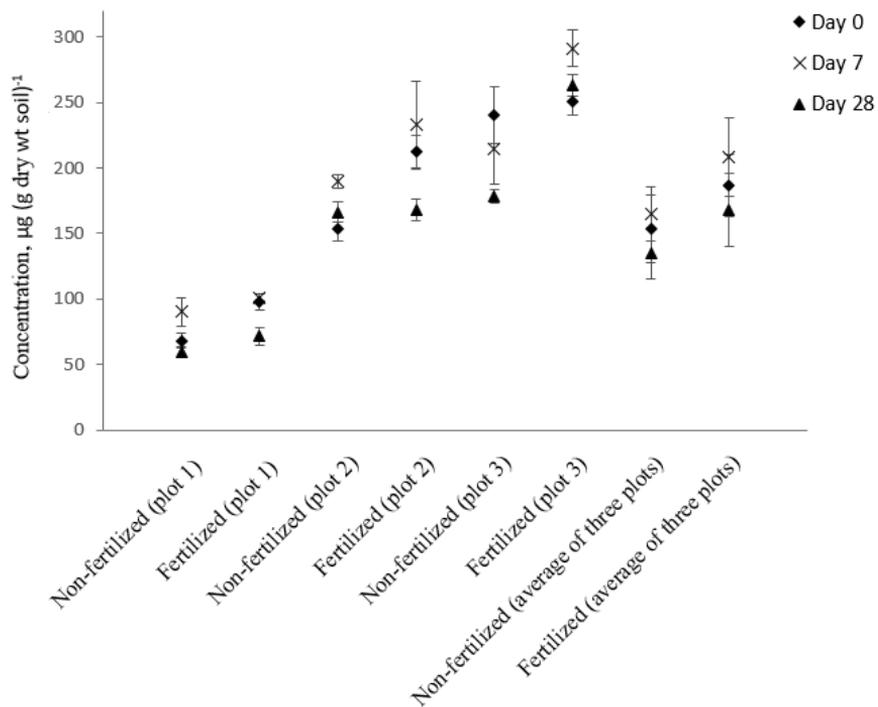


Fig. 6. Total gasoline concentration throughout the experiment in all the within-plot and between-plot comparisons. Mean \pm SE.

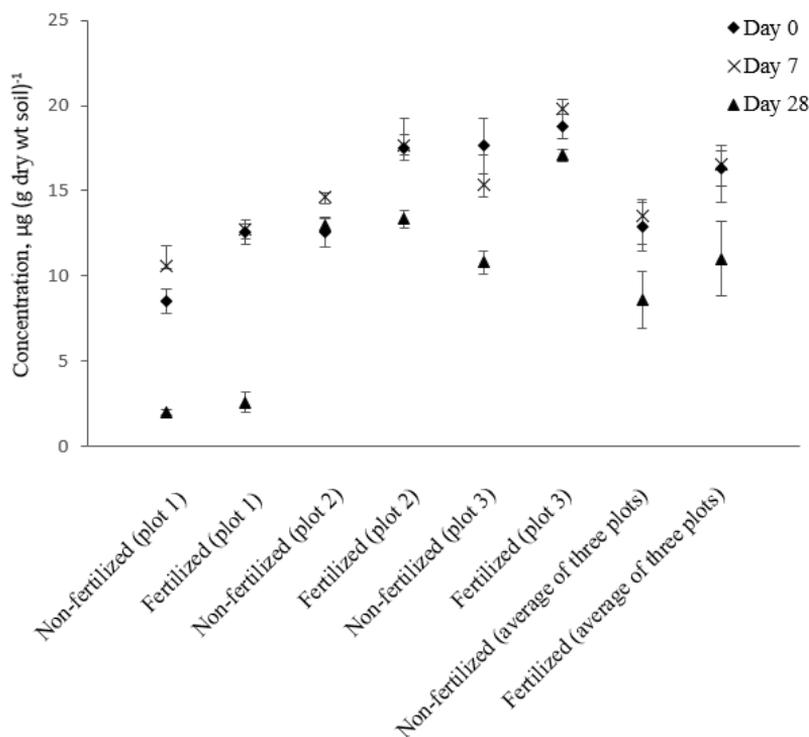


Fig.7. Naphthalene concentration throughout the experiment in all the within-plot and between-plot comparisons. Mean \pm SE.

With the all-inclusive case analysis, it was clear that whether positive or negative biodegradation result was gained from the between-plot comparison, it was not consistent in all the individual plots of soil (within-plot comparisons). It means that whether the results were gained from pseudoreplication or ecologically relevant replication, the conclusions cannot be easily generalized. Soil heterogeneity can be simply the reason why promising laboratory studies on bioremediation strategies do not succeed in *in situ* full-scale applications. It is rational that biodegradation results vary even at small scales and small-scale biodegradation results fail to represent field cases (Davis et al. 2003). To reduce the risk of drawing false conclusions or increase the chance of success of practical bioremediation strategies, e.g. phytoremediation, rhizoremediation, and bioaugmentation in real contamination cases, it is suggested to have ecologically relevant replicate plots with parallel independent analysis to each replicate plot before rushing into field application.

Similar methods and data analysis can be coupled with RCBD that is a very common design in agriculture and in bioremediation studies (Mills et al., 2004; Fageria, 2007; Jidere et al., 2012;

Tavares et al., 2016). In RCBD, ecologically independent blocks are taken as replicates like the between-plot comparison here. The difference is that only one replicate per treatment per block is applied, which neglects the soil variation between the treatments within each block. Therefore, the suggestion here is to have at least three replicates for each treatment in each block and have parallel data analysis to each block to minimize the interference of soil heterogeneity even within the same block on the treatment effects.

5.4. Outlooks

Although the results of the current *in situ* electrokinetic remediation of soil Cd (paper I) and urea fertilized biostimulation of soil gasoline aromatics in the laboratory (paper II) were quite promising, improvements and considerations can still be done. In electrokinetic remediation, a higher voltage gradient can be used in scaled-up applications for a better result. A vertical electric field can be applied for enhancing the removal efficiency of heavy metals in the lower soil layers by migrating the heavy metals upwards (Zhang et al., 2010). While applying biodegradation with urea fertilization in the field, the field temperature should be taken into consideration since a relatively high temperature can elevate the soil pH undesirably for microbial degradation. According to the laboratory results (paper II), 10 °C was proper for urea application in a mineral soil. Therefore, urea can be potentially a good fit for mineral soils in cold areas in the summer period in terms of biostimulation with fertilization.

In future studies, joint applications of different remediation strategies should be further investigated. For instance, for soils contaminated by both organic and heavy metal pollutants, biodegradation and electrokinetic remediation can be coupled to incorporate the features e.g. enhanced desorption, migration, nutrient distribution, biodegradation, biosorption for enhancing the remediation efficiency. Additionally, field tests should be more encouraged. However, before rushing into field applications, for any application of *in situ* remediation strategies in soils, environmental patchiness should be taken into consideration and soil heterogeneity is not limited to microbial patchiness. For instance, electrokinetic remediation of heavy metals highly depends on the form or state of the metal species, e.g. whether the metal species are charged and/or tightly desorbed into the soil. However, artificial contamination and mixing in laboratory experiments especially neglect pollution characteristics in the field. Laboratory and small-scale field tests should be done with ecological replication with parallel experiments and analysis to each component soil for a guiding a more successful *in situ* remediation.

6. CONCLUSIONS

In this doctoral thesis, several remediation strategies were presented, including remediation technologies and research methodology.

Electrokinetic remediation showed high potential in removing Cd in paddy agricultural soil especially in the surface 0-10 cm layer, with a removal efficiency of 87% in the pilot test and 74% in the full-scale application in 14 days. The removal efficiency was generally higher in the pilot test and for the total Cd. The lower removal efficiency for the plant available Cd might be due to the enhanced desorption of Cd cations from soil particles through cation exchange and/or dissolution by lactic acid in electrokinetic remediation.

As for the removal of soil gasoline aromatics, biostimulation with urea fertilization successfully lowered the concentration of soil total gasoline aromatics below the initial level by 47% in 28 days at 10 °C. The increased concentration of the total gasoline aromatics observed in the NPK and urea fertilized treatments on day 7 showed an enhanced desorption (bioaccessibility) and extractability of soil gasoline aromatics, which might be related to the biodegradation activity between day 7 and day 28.

With the advantages of electrokinetic remediation, such as desorbing and mobilizing pollutants, and distributing additives or nutrients, joint application of electrokinetic remediation and biodegradation can be further studied in the future.

Despite the success of electrokinetic remediation and biostimulation with urea fertilization in this doctoral thesis, it is not ensured that the same strategies would succeed in other field cases, as shown in the third study. Biodegradation activity, i.e. natural attenuation and biostimulation with methylene urea fertilization showed random results among the within-plot and between-plot comparisons, which could be simply due to soil heterogeneity even within the same plot of soil. To illustrate a more thorough remediation potential in practical applications, it is encouraged to apply ecologically independent plot replication with parallel experiment and analysis with within-plot replication to each component plot of soil.

ACKNOWLEDGEMENTS

The doctoral thesis studies were mainly carried out in the Ecosystems and Environment Research Programme at the University of Helsinki, Finland and Guangdong Polytechnic of Environmental Protection Engineering, China, supported by funds from Maa-ja vesitekniikan tuki ry, Finland (37316), Finnish Cultural Foundation (Päijät-Häme Regional Fund, 70171883), Academy of Finland (139847), EU MSC Action (project MicroArctic, 675546), and Science and Technology Planning Project of Guangdong Province, China (NO.2018A050506046).

I would like to express my sincerest gratitude to my main supervisor Professor Martin Romantschuk for giving inspiring and precious advice, modifying my research ideas and manuscripts, helping me apply for grants, and providing essential financial support, etc. Thanks to Professor Martin Romantschuk, I am able to finish my doctoral project and thesis with the least trouble. I would also like to show my great appreciation to my other supervisor Docent Aki Sinkkonen for giving assistance in statistical analysis, modifying my manuscripts, especially for guiding me in writing my first manuscript (paper III) in my doctoral studies piece by piece, and providing help with analyzing some of my samples. I would also like to thank Docent Anna-Lea Rantalainen for her help with chemical analysis, giving advice, and modifying some of my manuscripts. Docent Anna-Lea Rantalainen has been always helpful when I come to her with problems. Thanks also go to Toke Bang-Andreasen and Carsten Suhr Jacobsen, Aarhus University, for carrying out the experiment of paper II.

In addition, I would like to thank my doctoral thesis committee members, Professor Kristina Lindström and Doctor Kristian Spilling for their time, help, and advice throughout my doctoral studies. Special thanks go to Professor Shuiyu Sun and Associate Professor Zongping Cai at Guangdong Polytechnic of Environmental Protection Engineering, China, for the great research collaboration. I am also grateful to the AlmaLab staff for their help when I practiced experiments and analysis in the laboratory.

In the end, I would like to show my deepest gratitude to my beloved ones, family and friends for the help and support, especially to Santeri Vehma for the most precious, patient, unconditional, and endless help and support throughout my doctoral studies.

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