



Bioremediation trial on PCB polluted soils – A bench study in Iceland

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Bioremediation trial on PCB polluted soils – A bench study in Iceland

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Magister Scientiarum degree in Environment and Natural Resources

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Abstract

Soils of today are under pressure of various pollutants, including polychlorinated biphenyls (PCBs) that are present in the soils at the old NATO facility in Keflavík, Iceland. Threats of PCBs to the environment are toxicity, ability to bioaccumulate, stability and low reactivity, low water solubility and high adsorption capacity to soil organic matter (Borja *et al.*, 2005).

The aim of this research was to propose a framework of protocols that can be adapted to bioremediate Icelandic soils that inhibit PCB contamination. To the author's best knowledge, no data has been reported, neither on PCB degradation rates nor PCB degrading genes in Icelandic soils, and very limited research exists on contamination issues in Icelandic soils. The study was outlined as a two-phase remediation bench study where different biostimulation methods at different temperatures were conducted. The study likewise included a microbiology investigation of the soils and bioavailability to earthworms (*Eisenia foetida*).

Pine needles biostimulation resulted in nearly 40 % degradation of total PCBs after two months incubation at 10°C. Successful amplification was obtained with aerobic PCB degrading gene *bphA*, and significantly different microbial communities were found in anaerobic soils compared to aerobic soils. Bioaccumulation factor (BAF) ranged from 0.82 to 0.89 in the earthworms, and both highly and less chlorinated congeners were accumulated. To conclude, a further small-scale field experiment with pine needles stimulation is recommended, with regular monitoring of the dynamic changes in the microbial communities in order to monitor early changes in other soil parameters.

Útdráttur

Jarðvegsmengun er útbreitt vandamál. Einn af mengunarvöldum í jarðvegi eru PCB efnasambönd líkt og finnast til dæmis á fyrrum umráðasvæði NATÓ á Reykjanesi. PCB eru þrávirk lífræn efni sem eru eitruð, geta safnast upp í lífkeðjunni, leysast illa í vatni og geta bundist í miklu magni við lífrænt efni í jarðvegi (Borja o.fl., 2005).

Markmið verkefnisins var að gera tillögu að verkferlum sem nota má til að brjóta niður PCB með lífrænum aðferðum í íslenskum jarðvegi. Eftir því sem höfundur best veit, hafa gögn hvorki verið birt um PCB niðurbrot né um tilvist PCB niðurbrotserfðavísa í íslenskum jarðvegi. Raunar eru mjög takmarkaðar rannsóknir til um jarðvegsmengun á Íslandi. Verkefnið var unnið í tveimur þrepum á rannsóknastofu þar sem jarðvegur var örvaður með völdum aðferðum við breytilegt hitastig. Ennfremur var örverumassi jarðvegsins og lífaðgengi PCB til ánamaðka (*Eisenia foetida*) kannað.

Örvun jarðvegsins með furunálum leiddi til nærri 40% niðurbrots á heildar PCB eftir tveggja mánaða tímabil við 10°C. Mögnun tókst á loftháða PCB niðurbrotserfðavísinum *bphA* í PCB menguðum jarðvegi. Loftfirrð meðferð jarðvegs olli breytingu á tegundasamsetningu örverumassans frá loftháðum jarðvegi. Lífmögnunarstuðull (BAF) var á bilinu 0.82 til 0.89 í ánamöðkum sem tóku upp lítið og mikið klórberandi PCB. Lagt er til að tilraun verði gerð við náttúrulegar aðstæður með furunálum með reglulegri vöktun örverusamfélags jarðvegsins sem getur gefið upplýsingar um snemmbúnaðar breytingar á öðrum þáttum jarðvegsins vegna örvunarmedhöndlunarinnar.

Dedication

This work is dedicated to Äite

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Abbreviations

BA = Brown Andosol

BAF = bioaccumulation factor

BESA = bromoethane sulfonic acid

BSA = bovine serum albumin

Bp = base pair

BP = biphenyl

CBA = chlorobenzoic acid

DNA = deoxyribonucleic acid

DI = deionized

EPA = US Environmental protection agency

GC = gas chromatograph

LH-PCR = length heterogeneity polymerase chain reaction

PCB = polychlorinated biphenyl

PCR = polymerase

SOM = soil organic matter

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1 Introduction

Soils are multi-component and complex systems that provide us with numerous ecosystem services; including nutrient cycling, regulation of biological populations in soils, maintenance of soil structure and carbon transformations (Kibblewhite *et al.*, 2008). In addition soils also function as an engineering medium and an interface between lithosphere, hydrosphere, atmosphere and biosphere (Brady and Weil, 2004).

At present, soil quality is under threat from anthropogenic stresses such as pollution. The European Union's Soil Thematic Strategy stresses the importance of soils for the ecosystems and society, and especially points out soil pollution as a concern for soil health (Van-Camp *et al.*, 2004). At the moment only nine EU Member States have a regulatory framework on soil protection, and Iceland is among the nations that do not have a comprehensive soil protection strategy.

A preliminary study on soil protection and an inventory of potential polluted sites in Iceland was carried out in 2005 by interviews with local environmental agencies (Meyles and Schmidt, 2005). Urban areas and infrastructure only cover approximately 1.4 % of Iceland, but the total minimum number of likely polluted areas in the country exceeds 200. It should be noted that this number does not include information from environmental agencies in the Westfjords and Western Iceland (Akranes and Borgarnes), or information about private properties. The most typical pollution sites were fuel filling stations, old landfills and shooting ranges (Meyles and Schmidt, 2005).

In this thesis, work initiated by Kadeco – Prónarfélag Keflavíkurflugvallar and Almenna Verkfræðistofan was continued, in order to find novel methods to biodegrade PCBs that would make incineration and complete destruction of the polluted soils redundant. At first

an introduction to soils is given, and thereafter the state of art about soil pollution and bioremediation of PCBs is discussed.

1.1 Soil formation

Soil types differ from each other in both the soil forming factors and soil forming processes. The soil forming factors include parent material, topography, climate, biota, anthropogenic effects and time (Chapin III, Matson and Mooney, 2002; Brady and Weil, 2004). Van Breemen and Buurman (2002) describe soil forming processes as set of physical, chemical and biological processes that affect the formation of a particular soil. Physical processes include movement of water and solutes within the soil profile, temperature effects as well as shrinkage and swelling of soil aggregates and clays. Chemical processes comprise chemical weathering and formation of secondary minerals, soil minerals and their physicochemical properties and redox processes. Biological processes take into account the complexity of decomposition of fresh organic and formation of soil organic matter. Soil fauna, e.g. earthworms, consume the soils continuously and cause bioturbation. That affects the soil formation greatly, for example by aerating the soil and sometimes counteracting with other soil forming processes (Van Breemen and Buurman, 2002; Chapin III, Matson and Mooney, 2002).

1.2 Andosols

Andosols are found in volcanic areas, such as Japan, Chile, New Zealand, Greece, Italy, Iceland and Azores (Chorover, 2002; Brady and Weil, 2004; Dahlgren *et al.*, 2004). They are young soils (5000-10000 years) and the main soil forming process is weathering of volcanic ash and other volcanic ejecta into amorphous minerals such as allophane ($\text{Al}_2\text{O}_3 \cdot (\text{SiO}_2)_{1.3-2} \cdot 2.5-3(\text{H}_2\text{O})$) and imogolite ($\text{Al}_2\text{SiO}_3(\text{OH})_4$) as well as ferrihydrite ($5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$) (Wada, 1985). Andosols have unique andic soil properties that include a high amount of poorly crystalline or amorphous minerals, high organic matter content and great water holding capacity (WHC). The minerals in Andosols have large surface areas, which results for example in great carbon sequestration potential. The characteristics of Andosols result in naturally highly fertile soils, although fertilizing might be needed due to strong phosphorous retention (Brady and Weil, 2004).

1.2.1 Andosols of Iceland

Iceland is situated on the Mid-Atlantic Ridge and experiences volcanic eruptions on average every 4 to 5 years (Thordarson and Larsen, 2007). The island, 103 000 km² in size, has been built up in the past 16 million years and is composed entirely of volcanic material. Therefore Icelandic soils receive great amounts of eolian volcanic material, basaltic glass particles and rhyolitic pumice grains, which get incorporated into the soil horizons (Arnalds, 2004). Soils are also formed by weathering of the parent material at the base of the soil profiles. The climate in Iceland ranges from Boreal to Sub-Arctic in the lowlands and is Arctic in the highlands (Einarsson, 1984). The mean annual temperature ranges from 2 to 5°C in the Icelandic lowlands and in the highlands from -2 to 2°C (Einarsson, 1984). Mean annual precipitation in the lowlands varies from 450 to > 2500 mm and from 300 to > 2500 mm in the highlands (Einarsson, 1984). Due to the several freeze-thaw cycles, cryoturbation occurs severely resulting in visible hummocks and solifluction features at the surface (Arnalds, 2004).

Iceland has six main soil types: Histosols, Histic Andosols, Gleyic Andosols, Brown Andosols, Vitrisols and Leptosols (Arnalds, 2004). Approximately 86 % of Icelandic soils are Andosols and more specifically 14 % of Icelandic soils are Brown Andosols (BA) (Appendix I), compared to Andosols only covering approximately 1 % of the world (Chrover, 2002; Arnalds, 2008). Brown Andosols are pedologically young soils that are basaltic in origin and receive high amounts of eolian and tephra material on the soil surface. They lack cohesion. These soils have distinct andic properties, which include the presence of amorphous clay minerals allophane and ferrihydrite, low bulk density (BD) (< 0.9 g cm⁻³), strong organic carbon binding capacity, high cation exchange capacity (CEC) and high P retention (Arnalds, 2004; Arnalds, 2008; Arnalds and Oskarsson, 2009). The mean summer (June to September) soil total biomass carbon (mic_c) in Icelandic Andosols has been estimated at 3823 mg kg⁻¹ and mean winter (October to December) mic_c at 2774 mg kg⁻¹ (Guicharnaud *et al.*, 2010). The same study measured mean summer dehydrogenase activity at 14 µg g⁻¹ h⁻¹ and mean winter dehydrogenase activity at 0.25 µg g⁻¹ h⁻¹.

2 State of the Art

2.1 Soil Pollution

Soil pollution is a result of an increased concentration of materials in soils that can have disadvantageous effects on living organisms (Ashman and Puri, 2002; Gobat *et al.*, 2004; Killham, 2004). Pollution can result from natural processes including forest fires and volcanic eruptions; however, they are caused primarily by various anthropogenic actions. Those include industrial waste materials and agricultural runoff, originating from both point sources and diffuse sources. The pollutants can enter the ecosystems both unintended, as in a nuclear accident, or intended, as in the case of waste dumps and usage of agricultural pesticides and herbicides in large quantities (Gobat *et al.*, 2004; Walker *et al.*, 2006). Soils can act as a natural sink and a resource for various substances and wastes in the environment. The high current magnitude of contaminants, especially close to point sources, in the soil environment prevents the soil organisms from recycling the pollutants and they accumulate in the soils. Soil pollutants are divided into two main groups: (1) organic pollutants and (2) inorganic pollutants (Ashman and Puri, 2002; Walker *et al.*, 2006).

Organic pollutants consist of many harmful substances such as hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), PCBs, detergents and pesticides (Ashman and Puri, 2002; Walker *et al.*, 2006). Organic pollutants are characterized by their high binding into or onto the soil organic matter (SOM) and clay particles of the soil. Microorganisms face great challenges in attempting to degrade these compounds, which leads to bioaccumulation of the pollutants in the food chain. Additional concerns with organic pollutants are their toxicity and persistence in the surrounding environment (Ashman and Puri, 2002; Walker *et al.*, 2006).

Inorganic pollutants are mainly heavy metals (Ashman and Puri, 2002; Walker *et al.*, 2006). Cadmium, arsenic, chromium and lead are examples of metals that originate from industrial processes, more specifically from sewage sludge, agrochemicals and burning of fossil fuels. They are toxic due to their ability to affect the energy-producing functions of the cell, which allows them to enter the food chain. The soil microorganisms are therefore not able to use these pollutants as nutrients and degrade them, but the pollutants will accumulate in the environment (Ashman and Puri, 2002; Walker *et al.*, 2006).

2.1.1 Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs), $C_{12}H_{10-n}Cl_n$, are organic hydrocarbons that have 1 to 10 chlorine atoms attached to biphenyl (Safe, 1994; Erickson, 1997; BEST, 2001; Abraham *et al.*, 2002; Ashman and Puri, 2002; Ohtsubo *et al.*, 2004; Vasilyeva and Strijakova, 2007; Figure 2.1). Between 1929 and late 1970s PCBs were produced and used in industrial applications such as transformers, capacitors, hydraulic liquids, lubricants, pesticide extenders, flame retardants and plastics. PCBs were mainly used in closed systems but due to accidents, leakage and poor storage practices they have entered into the natural environment. The use of PCBs in electrical equipment was considered as closed systems, whereas hydraulic systems were seen as nominally closed systems and plasticizers and carbonless copy paper were seen as open-ended applications. As much as 1.5 million metric tons of PCBs have been produced worldwide. There are 209 theoretically different PCB congeners (each have different amount of chlorine atoms attached to biphenyl), but 20-60 congeners are the most common ones in the commercial mixtures. Characteristics of these substances include thermal stability and low reactivity, low water solubility and high adsorption capacity to the soil organic matter. They are chemically very stable, soluble in organic solvents such as oil and fat, resistant to heat, have low flammability and high vaporization temperature (Erickson, 1997; Fagervold *et al.*, 2007; Jörundsdóttir, 2009).

PCBs are manmade pollutants that belong to Persistent Organic Pollutants (POPs) and should be phased out of use by 2025 according to the Stockholm POPs convention. Their use and production are already heavily restricted, having been banned since the 1970s in most countries, but existing machinery with PCBs got an exception in the convention (Axelrod *et al.*, 2005). Many commercial mixtures of PCBs existed worldwide, e.g.

Aroclor (USA), Chlophen (Germany), Kanechlor (Japan), Pheneclor and Pyralene (France) as well as Fenclor (Italy) (Safe, 1994; Jörundsdóttir, 2009) – of which Aroclor 1260 is the focus of this thesis.

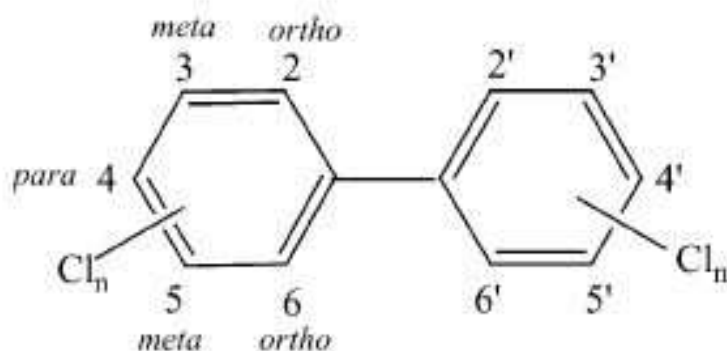


Figure 2.1 Demonstration of PCB structure. Source: Borja *et al.* (2005)

Commercial mixtures were produced by chlorination of biphenyls with chlorine gas under high heat (Erickson, 1997). Aroclor 1260 is one of the most common forms of PCBs used as dielectric fluid of liquid-filled transformers. Aroclor 1260 is a sticky resin that has 60 % of chlorine per weight and averages 6 chloride atoms per biphenyl molecule (Quensen III *et al.*, 1990), which makes it a mixture of highly chlorinated PCB congeners. It has been stated to be recalcitrant in the environment since its hydrophobicity makes it less bioavailable and it lacks less chlorinated congeners that are more easily available for the microorganisms in the soil environment (Fagervold *et al.*, 2007). The threats of PCBs to the surrounding environment are however not only determined by its chlorine content, but also by the individual congeners in the mixture and possible impurities, species that are affected and exposure route and duration (Safe, 1994).

Accumulation of PCBs in the environment was first documented by accident in the 1960s by Swedish scientist Sören Jensen, when he was studying DDT in environmental samples (Fagervold *et al.*, 2007). PCBs can bioaccumulate in organisms by uptake from various exposure routes as well as biomagnify when PCBs accumulate in higher concentrations at

higher levels in the food chain (Safe, 1994; BEST, 2001; Ashman and Puri, 2002; Borja *et al.*, 2005; Ohtsubo *et al.*, 2004; Pu *et al.*, 2006; Vasilyeva and Strijakova, 2007). This occurs when PCB is transferred from the tissue of the prey to the tissue of the predator repeatedly. PCBs have been demonstrated to be toxic in both laboratory and field studies. Humans can be exposed to PCBs for example at work (more historically than today), in accidents or through their living environment. In general PCBs can cause skin irritation, behavioral changes and liver damage in humans and are chronically toxic for fish, birds and mammals. PCBs have in addition been reported to have an effect on the primary food source of sea organisms, phytoplankton. There is also evidence of PCB being a probable carcinogen and therefore it is imperative to clean-up PCB pollution in the environment (Safe, 1994; BEST, 2001; Ashman and Puri, 2002; Borja *et al.*, 2005; Ohtsubo *et al.*, 2004; Pu *et al.*, 2006; Vasilyeva and Strijakova, 2007).

Critical values for PCB in soils vary between different countries and they might also be differently categorized in different sources, which are illustrated in Table 2.1. In 1996, a draft for limits for various pollutants in Icelandic soils was written, including PCBs, but it has not become legally binding as of now (UST, 1996). In Finland the threshold value (Sum of PCB 28, 52, 101, 118, 138, 153, 180) is 0.1 mg kg⁻¹ of the dry weight. Furthermore the lower critical value, for residential areas, is 0.5 mg kg⁻¹ of the dry weight and the higher critical value, industrial areas, is 5 mg kg⁻¹ of the dry weight (Reinikainen, 2007). In Sweden the Natural Protection Agency has divided the values into four different categories: less serious (< 0.02 mg kg⁻¹ of dry weight), rather serious (0.02-0.06 mg kg⁻¹ of dry weight), serious (0.06-0.2 mg kg⁻¹ of dry weight) and very serious (> 0.2 mg kg⁻¹ of dry weight) (Naturvårdsverket, 1999). The Netherlands has a target value (Sum of PCB 28, 52, 101, 138, 153, 180) of 0.02 mg kg⁻¹ and an intervention limit (Sum of PCB 28, 52, 101, 118, 138, 153, 180) of 1 mg kg⁻¹ (Pronk, 2000). In the EU's Soil Thematic Strategy the precautionary soil threshold values were set as follows: for soils with soil organic matter (SOM) content more than 8 % 0.1 mg kg⁻¹ of dry weight should not be exceeded and for soils with less than 8 % of SOM 0.05 mg kg⁻¹ should not be exceeded (Van-Camp *et al.*, 2004) USA has the highest target values, 1 mg kg⁻¹ for residential areas and as high as 10-25 mg kg⁻¹ for industrial areas (Erickson, 1997). A legally binding critical value for

Icelandic soils does not exist but may be evaluated and developed alongside criteria given for European soils taking into account Icelandic soil types, soil use and land management.

Table 2.1 Demonstration of critical values for PCB concentrations in soils in mg kg⁻¹ dry weight. SOM stands for soil organic matter. Source: UST, 1996, Erickson, 1997; Naturvårdsverket, 1999; Pronk, 2000; Van-Camp et al., 2004; Reinikainen, 2007.

	PCB values			
	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight
Iceland (Draft from 1996)		Lower limit 0.02 ¹	Upper limit 1 ²	
Finland		Threshold 0.1 ²	Lower critical 0.5 ²	Higher critical 5 ²
Sweden	Less serious 0.02 ³	Rather serious 0.02-0.06 ³	Serious 0.06-0.2 ³	Very serious > 0.2 ³
The Netherlands		Target 0.02 ¹	Intervention 1 ²	
EU (Draft from 2006)		< 8 % SOM 0.05 ¹	> 8 % SOM 0.1 ¹	
USA		Residential 1 ³	Industrial 10-25 ³	

¹Sum of PCB 28, 52, 101, 138, 153 and 180

²Sum of PCB 28, 52, 101, 118, 138, 153 and 180

³Total PCBs

2.2 PCBs in the soil environment

PCBs have been identified in major parts of the global ecosystems including air, water, soil, animals and even human tissues (Safe, 1994; MacDonald *et al.*, 2000). The biggest part of PCBs in the environment is located in soils and water sediments close to the previous localization of their production and use (Vasilyeva and Strijakova, 2007; Figure 2.2). Soils act both as a sink and a source for PCBs in the natural ecosystems. PCBs can enter the soil through wet and dry deposition from the atmosphere, through accumulation in vegetation as well as xenobiotic additions through leakage and accidents (MacDonald *et al.*, 2000). After various processes in soils (as will be explained in this section) PCBs may enter the aquatic environment, river and lakes, and subsequently to the oceans. Oceans can again act as a source through sea spray that transports the pollutants back to land and terrestrial waters (Wågman *et al.*, 2001; Öberg, 2002). In the soil environment PCBs fate and behaviour are governed by various factors, such as chemical properties, soil characteristics and environmental factors. Volatilization of less chlorinated PCBs may occur while the pollutant is new, but when the soil consolidates the rate becomes almost insignificant. Ageing of PCBs plays a major role in their cycle since only the more recalcitrant part of the compound might be left after time and the metabolites become natural compounds when animals produce them in their bodies. In many cases the environmental samples do not resemble the commercial mixtures of PCBs but are somewhat different due to e.g. natural attenuation (Safe, 1994; Semple *et al.*, 2001; Vasilyeva and Strijakova, 2007).

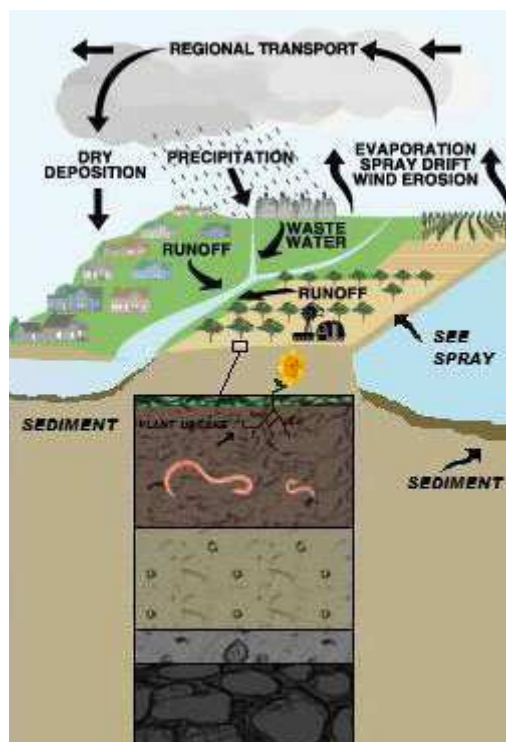


Figure 2.2 PCB cycle in the environment. Illustrated by Guðny Petursdóttir, as per transcript by author.

2.2.1 Binding of PCBs into the soil environment

The two major factors binding PCBs into the soil are SOM and the amount of soil clay. The time scale of the sorption to SOM varies a great deal between different congeners and chlorination levels, not to mention differences in commercial mixtures (Erickson, 1997; Reid *et al.*, 2000; Semple *et al.*, 2001; Mulligan & Yong, 2004; Ohtsubo *et al.*, 2004; Borja *et al.*, 2005; Vasilyeva and Strijakova, 2007). The clay and SOM concentrations also vary between different soil types. Volcanic ash soils, such as Icelandic Brown Andosol (BA), contain amorphous clay minerals, such as allophanes and ferrihydrites. Those result in positively charged soil colloids and strong bonding of soil organic matter (Joergensen and Castillo, 2001). This enables high amounts of pollutants to bind to soil particles, thereby decreasing their bioavailability. Positive charges provide sites for microbial growth on mineral surfaces. They can also stabilize organic residues, limiting the amount of carbon substrates used by microorganisms for their metabolism. This can result in a limited microbial growth and hence slowing down the degradation of organic pollutants like PCBs (Saggar *et al.*, 1994; Joergensen and Castillo, 2001; Kleber *et al.*, 2005). Even non-allophanic Andosols have shown the ability to resist against disaggregation of organo-

metallic complexes (Aran *et al.*, 2001). Erickson (1997), however, states that PCB is mainly bound to expandable, montmorillonite type clays and especially to their inter-crystalline water layers of the clay. Pu *et al.* (2006) showed the least mobilization of PCBs from soils with the high SOM concentrations compared to organic poor soils. Moreover, sorption of PCBs into soil colloids has been shown to increase significantly when the chlorine content of the PCB mixture increased (Pu *et al.*, 2006).

2.2.2 Bioavailability of PCBs

One of the most predominant factors affecting soil-PCB interactions and remediation processes is considered to be bioavailability (Wiegel and Wu, 2000; Semple *et al.*, 2001). Bioavailability is used to describe the available amount of a chemical to the microorganisms in a specific area under given time period. In the case of PCBs, this amount may not be equal to the total amount of PCBs in the soil, but describes the amount that is accessible to the microorganisms and is possible to be used in the soil environment (Semple *et al.*, 2007). Bioavailability is governed mainly by soil properties, such as clay content, organic matter content, and soil sorption and desorption processes. In addition, ageing, may make the compounds less bioavailable. In general terms, the more organic material or clay in the soil, the more contaminants will stay in the soil (Reid *et al.*, 2000; Wågman *et al.*, 2001; Pu *et al.*, 2006). Moreover, pollution concentration plays a role. If the concentration is very high, the soil can't hold onto all of it through sorption and other biogeochemical processes and therefore bioavailability increases (Reid *et al.*, 2000).

Both biological and chemical methods are used to give an indication of bioavailability. The amount of pollutant taken up by an organism, e.g. an earthworm, can be measured as a biological tool. The chemical extraction methods, such as soil washing, have often been criticised for presenting an overestimate or underestimate of the real situation, since they don't take into account the biological processes in the organisms. Furthermore bioavailability can be both species and organism dependent, which makes it difficult to get an overall estimate for bioavailability for a single chemical (Reid *et al.*, 2000; Paton *et al.*, 2005; Pu *et al.*, 2006). Earthworms, e.g. *Eisenia foetida*, are one example of a biological indicator used for assessment of bioavailability (Hallgren *et al.*, 2006). They act as a link in the transport of pollutants from the soil to consumers in the terrestrial food web, for

example as a major food source for birds. Contaminants enter the earthworms via passive diffusion from the soil solution through their outer membrane and via resorption of the compounds from soil material passing through their gut. This way they can give an estimate for the worst-case scenario of bioavailability (Ville *et al.*, 1995; Krauss *et al.*, 2000; Wågman *et al.*, 2001; Hallgren *et al.*, 2006).

2.2.3 Bioaccumulation of PCBs

Bioaccumulation illustrates the process of how PCBs get into a single living organism, whereas biomagnification describes the process of PCB concentrating on higher trophic levels of the food chain (Botkin and Keller, 2005). Primary consumers and detritivores, organisms such as millipedes and terrestrial worms, play a significant role in bioaccumulation since they feed on dead organic matter in the soil (Figure 2.3). Bioaccumulation can be seen both as a passive and an active uptake process, which Streit (1992) described for example through the food chain approach. The main emphasis is put on the food chain, and a realistic picture of the food chain is used to stress the importance of bioaccumulation. Bioaccumulation may enhance the environmental threat of the compound by 1) storing the compound in the lipids of the organisms, 2) decreasing the degradation rates when the compound is not easily available for biological, chemical nor physical degradation, and 3) the compound affecting single individual's health. Furthermore, if a species is harmfully affected by the compound, it may have effects on the predator patterns and therefore on the whole ecosystem (Streit, 1992).

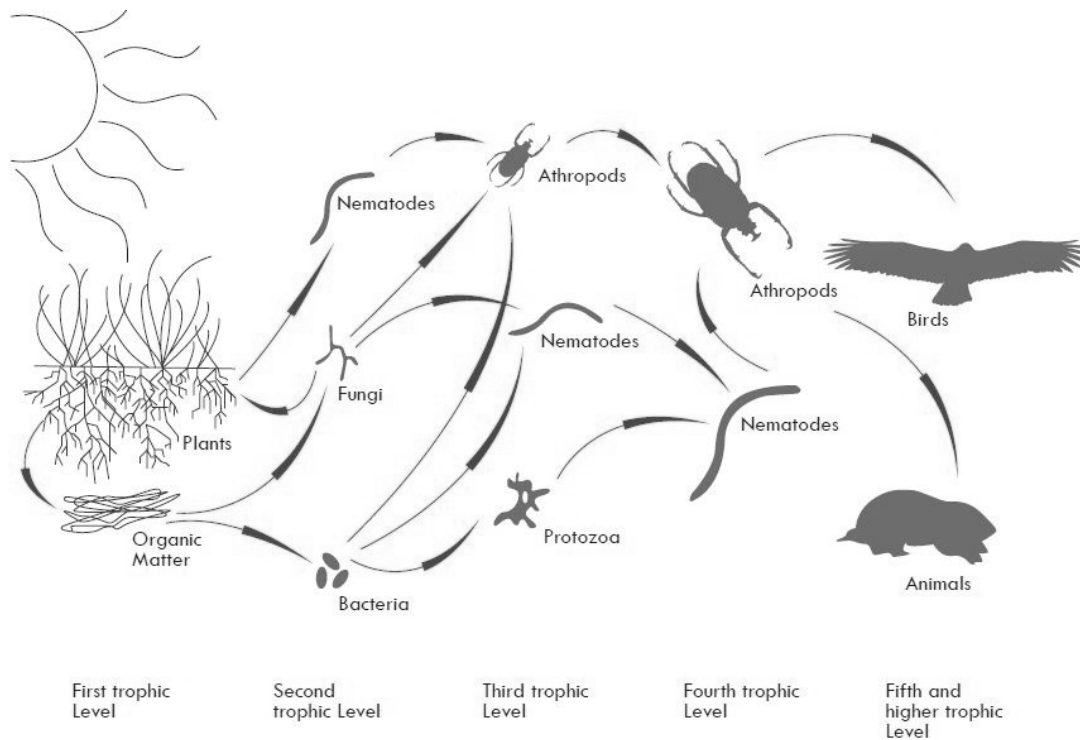


Figure 2.3 Demonstration of the soil food web. The first trophic level demonstrates the photosynthesizers whereas the second trophic level demonstrates the decomposers, mutualists, pathogens, parasites and root feeders. The third level includes shredders, predators and grazers whereas the fourth level includes higher-level predators followed by even higher-level predators on the fifth level. Adopted from US-NRCS (2010) and illustrated by Hildur Gunnlaugsdóttir.

2.2.4 Ageing of PCBs

After a considerable time in the environment PCBs undergo ageing, which may reduce the biodegradability of the compound significantly (Reid *et al.*, 2000; Semple *et al.*, 2001). The most recalcitrant parts of the compounds may only be left or the pollutant is moved to soil compartments that are more difficult to reach by the microorganisms (Semple *et al.*, 2001). Reid *et al.* (2000) and Semple *et al.* (2001) presented three possible pools together for contaminants as a result of ageing: firstly a fraction that can easily be loosened, secondly a fraction that desorbs more slowly, and thirdly a fraction that is seen as recalcitrant. In other words, the last fraction would be more or less impossible to reach or be desorbed. Main factors affecting ageing processes are the properties of the pollutant

itself as well as soil characteristics that have been discussed above (Semple *et al.*, 2001; Qi *et al.*, 2006).

2.3 Bioremediation of PCBs

High-temperature incineration or burial have been the most widely used methods of remediating PCB polluted soils, even though it is very expensive and can generate toxic dioxins (Gobat *et al.*, 2004; Borja *et al.*, 2005; Leigh *et al.*, 2006; Vasilyeva and Strijakova, 2007). Chemical and physical methods such as dispersion, sorption, soil washing, solvent extraction and abiotic transformations are also used. These chemical and physical methods are not in the scope of this thesis and will therefore not be discussed in more detail.

Bioremediation includes the biological processes that the soil has to degrade the pollutants, with the help of fungi, bacteria and plants (Gobat *et al.*, 2004; Welander, 2005). Bioremediation can save both economic and energy resources as well as being less disturbing for the natural environment at the site that is being cleaned (Luo *et al.*, 2008). The properties of PCBs in the soil environment explained in section 2.2 – including high hydrophobicity and ability to sorb tightly to soil organic matter – make bioremediation a challenge. Bioremediation can occur through mineralization and co-metabolism; and may be conducted as natural attenuation, biostimulation or bioaugmentation (Iwamoto and Nasu, 2001; Gobat *et al.*, 2004; Crawford and Crawford, 2005). In this chapter both anaerobic and aerobic biodegradation of PCBs will be outlined followed by a section on bioremediation of PCB polluted soils in cold regions where reaction rates are slower than in warmer climates.

2.3.1 Anaerobic bioremediation

Anaerobic bioremediation is carried out with the help of microorganisms that get energy from PCBs, which act as electron acceptors. In nature anaerobic conditions occur in flooded soils, river and pond sediments whereas in the laboratory anaerobic conditions are created in soil slurries and bioreactors (Vasilyeva and Strijakova, 2007). In unpolluted soils these bacteria are capable of anaerobic respiration with electron acceptors such as sulfates and nitrates and in the presence of PCBs they are able to switch to dehalorespiration. The

abundance of these bacteria is relatively low (about 10^2 cells/g) in natural environments, which explains the low rates of dehalorespiration without stimulation. This process is targeted for the highly chlorinated PCBs and the bacteria usually attack the chlorines in *para*- and *meta*-positions (see Figure 2.1) and replace them with hydrogen. Microbial dechlorination decreases the toxicity and the potential risk of the PCBs by transforming the higher chlorinated congeners to less chlorinated congeners. Therefore it makes them suitable for further aerobic biodegradation (Wiegel and Wu, 2000; Borja *et al.*, 2005; Vasilyeva and Strijakova, 2007; Field and Sierra-Alvarez, 2008). An example of the dechlorination process can be seen in Figure 2.4.

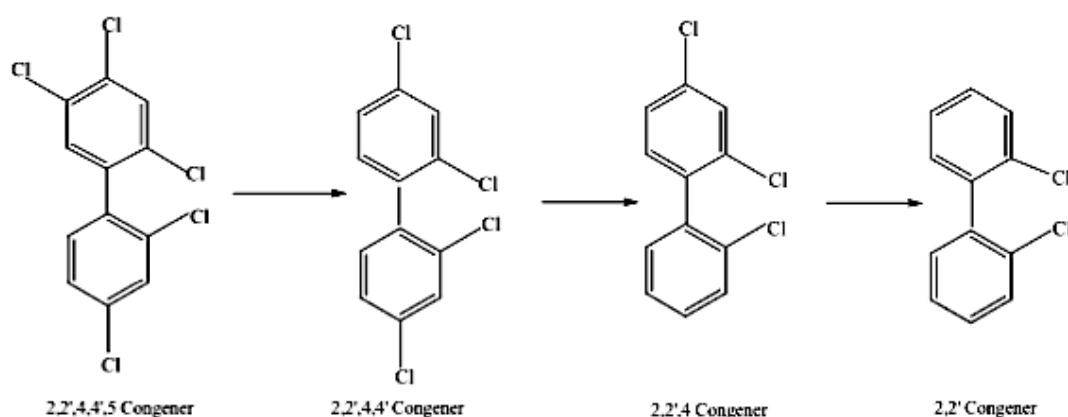


Figure 2.4 An example of anaerobic dechlorination. Source: Borja *et al.* (2005)

Biostimulation

Biostimulation targets the indigenous microorganisms in the soil and aims to give them the optimal living conditions. In order to activate the anaerobic microorganisms to biodegrade PCBs the soil studied has to be made anaerobic, e.g. by flooding the soil. In addition the process often has to be primed by individual PCB-congeners or other co-substrates (Tiedje *et al.*, 1993). It has been shown that keeping the soil pH close to neutral and temperature close to 25°C results in successful biodegradation (Vasilyeva and Strijakova, 2007). Tiedje *et al.* (1993) described a study in which Aroclor 1254 degradation was compared between soil that was made anaerobic by flooding and soil that was both made anaerobic and inoculated with anaerobic river sediment. The results were significantly higher with

inoculation, indicating that the indigenous anaerobic microorganism from the river sediment enhanced the process considerably (Tiedje *et al.*, 1993). Reductive dechlorination of Aroclor 1260 may be a more time demanding task than bioremediation of less chlorinated commercial mixtures. Quensen III *et al.* (1990) showed only 19% and 15% decrease in *meta*- and *para*-chlorines from Aroclor 1260 after 50 weeks when inoculated with two different anaerobic river sediments. In comparison the percentages for Aroclor 1242, using the same methodology, were 46 % and 85 % after 16 and 12 weeks, respectively. It should, however, be noted that one of the sediments inoculated was previously polluted with Aroclor 1242 and therefore a suitable community of microorganisms may have been present (Quensen III *et al.*, 1990). Alexander (1999) stated that anaerobic bioremediation without O₂ available may be the only way to degrade highly chlorinated PCBs.

Bioaugmentation

Bioaugmentation, i.e. adding microorganisms that are able to dechlorinate PCBs, has been the most successful approach of the anaerobic method but only under laboratory conditions (Vasilyeva and Strijakova, 2007). Therefore it was unclear whether it would work as successfully under field conditions. Table 2.2 presents the variety of microorganisms that are involved in reductive dechlorination of PCBs. The communities are very diverse, which explains why bioremediation methods may vary considerably from site to site due to the local PCB degrading community (Abraham *et al.*, 2002). The first complete dechlorination of 23456-CB to biphenyl was demonstrated by Natarajan *et al.* (1996), under laboratory conditions, where microbial granules and co-substrates were added in order to carry out the dechlorination.

Table 2.2 Summary of the genus of the microorganisms that have been used in experimental anaerobic dechlorination of PCBs.

Genus	Reference
<i>Clostridium</i>	Okeke et al., 2001
<i>Dehalococcoides</i>	Maymo-Gatell <i>et al.</i> , 1999; Smidt and de Vos, 2004; Borja <i>et al.</i> , 2005; Bedard, 2008; Field and Sierra-Alvarez, 2008; Adrian <i>et al.</i> , 2009
<i>Desulfitobacterium</i>	Sanford <i>et al.</i> , 1996; Wiegel <i>et al.</i> , 1999; Smidt and de Vos, 2004; Borja <i>et al.</i> , 2005
<i>Desulfomonile</i>	De Weerd and Suflita, 1990; Smidt and de Vos, 2004; Borja <i>et al.</i> , 2005
<i>Desulfuromonas</i>	Krumholz, 1997; Borja <i>et al.</i> , 2005
<i>Dehalospirillum</i>	Borja <i>et al.</i> , 2005
<i>Sulfospirillum</i>	Boyle <i>et al.</i> , 1999; Smidt and de Vos, 2004
<i>o</i> -17	Cutter <i>et al.</i> , 2001; Bedard, 2008
DF-1	Wu <i>et al.</i> , 2002
<i>Chloroflexi</i> (phylum)	Fagervold <i>et al.</i> , 2007; Bedard, 2008; Field and Sierra-Alvarez, 2008

2.3.2 Aerobic bioremediation

Aerobic conditions occur in surface soil and surface sediments in nature. They also exist in sewage sludge and can be created in bioreactors in the laboratory. Aerobic degradation relies on the oxidative destruction of PCBs, in which numerous genes are involved (Wiegel and Wu, 2000). In the most commonly described degradation pathway, namely the biphenyl pathway, PCBs are first transformed to chlorobenzoic acid (CBA) by bacteria that uses BP as a carbon and energy source. Further transformation occurs by CBA degrading bacteria. The end products of the process are many, but in general they are less toxic to the environment than the original PCBs. Aerobic degradation is, however, only capable of attacking lightly chlorinated congeners (Wiegel and Wu, 2000; Borja *et al.*, 2005; Pieper, 2005; Vasilyeva and Strijakova, 2007). The genes involved in the degradation process are *bph* gene clusters, as described in Figure 2.5 (Furukawa, 2000; Field and Sierra-Alvarez,

2008). First *bphA* gene activates BphA enzyme that converts biphenyl to dihydrodiol by dihydroxylation reaction, and thereafter BphB (dehydrogenase) is activated to convert dihydrodiol to 2,3-dihydroxybiphenyls. Next BphC (ring-cleavage dioxygenase), BphD (hydrolase), BphE (hydratase), BphF (aldolase) and BphG (acetaldehyde dehydrogenase) catalyse the degradation (Ohtsubo *et al.*, 2004). This reaction chain is, however, only an example and the exact route of it is dependent on e.g. the chlorination level of the PCB congener or mixture in question.

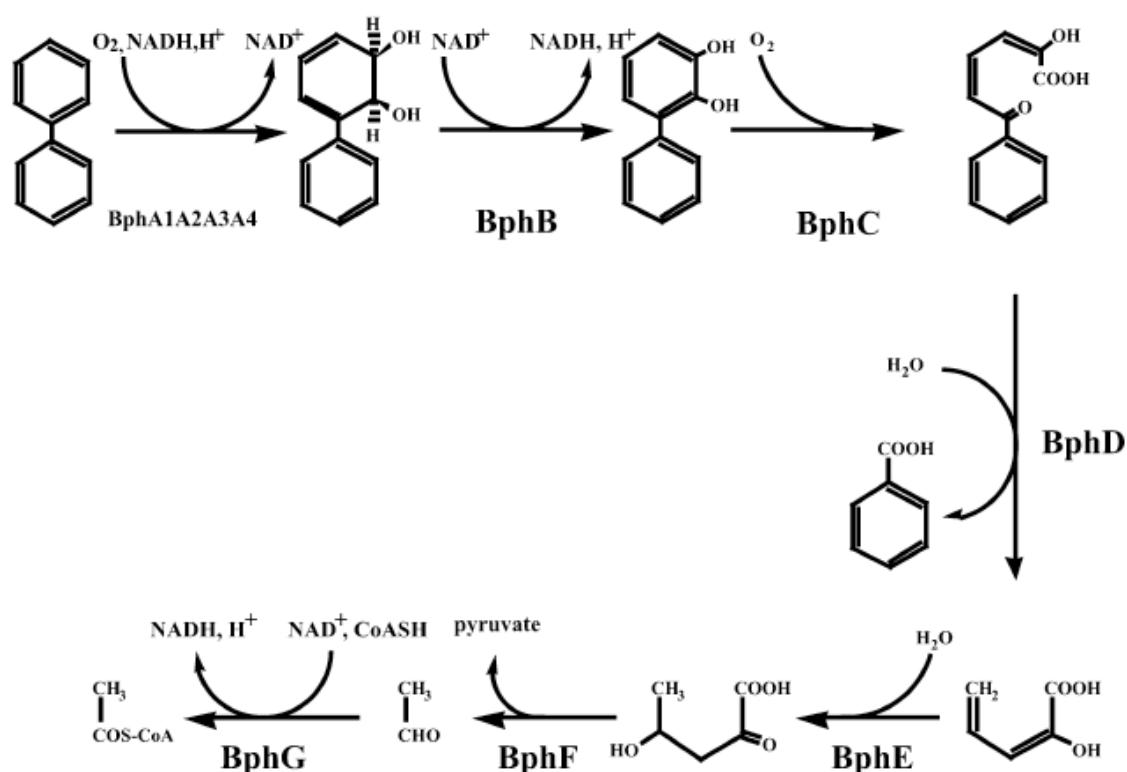


Figure 2.5 Demonstration of an example of aerobic degradation of PCBs through the upper BP-pathway. Source: Ohtsubo *et al.* (2004)

Biostimulation

Co-surfactants, oxygen and nutrients are commonly added to the polluted soils in order to stimulate the PCB degrading bacteria by enhancing their living conditions. PCBs may serve as a substrate for PCB degrading bacteria but it is very common that the degradation occurs through co-metabolisms, in other words there is a need for an additional substrate in order to keep the degradation process going (Pieper, 2005). Chemical surfactants may be toxic for biological systems, and therefore more expensive biosurfactants, such as carvone

or glucose, could be recommended (Abraham *et al.*, 2002). Landfarming is a bioremediation technique in which the soil is excavated and moved on top of an impermeable membrane. Thereafter the soil microorganisms can be stimulated by addition of nutrients, maintaining the optimal soil pH, moisture and aeration. In case of lack of space, windrows or biopiles can be created; these are high soil piles to which nutrients are commonly added and oxygen can be added by turning the piles occasionally (Adamsson, 1998).

Bioremediation with the help of fungi and plants

Flavonoids, terpenes and other plant-derived compounds may work as growth substrates for PCB degrading bacteria and therefore activate the PCB degrading process (Hernandez *et al.*, 1997; Pieper, 2005). Microorganisms have both chemical and physical means to interact with pollutants, which makes them quite powerful degradation activators in the soil environment (Semple *et al.*, 2001).

White rot fungi can be helpful in degrading PCBs since the low specificity of their enzymes, which are able to cleave for example lignin (Yadav *et al.*, 1995). Usually the products are polymerized or bound to the soil organic matter (Vasilyeva and Strijakova, 2007). However, the degradation potential is restricted to lower chlorinated compounds. Yadav *et al.* (1995) for example reported PCB degradation by 60.9, 30.5 and 17.6 % of Aroclor 1242, 1254 and 1260, respectively. Table 2.3 displays the variety of the genus of white rot fungi that are able to degrade PCBs.

Table 2.3 Listing of the genus of white rot fungi that have been demonstrated to be involved in aerobic degradation of PCBs.

Genus	Reference
<i>Phanerochaete</i>	Yadav <i>et al.</i> , 1995; De <i>et al.</i> , 2006; Vasilyeva and Strijakova, 2007
<i>Bjerkandera</i>	Vasilyeva and Strijakova, 2007
<i>Pleurotus</i>	Kubatova <i>et al.</i> , 2001; Vasilyeva and Strijakova, 2007
<i>Trametes</i>	Vasilyeva and Strijakova, 2007
<i>Rhizobium</i>	Vasilyeva and Strijakova, 2007

Composting employs microorganisms to degrade organic pollutants through four stages: mesophilic, thermophilic, cooling and maturation (Semple *et al.*, 2001). The compost, that is the result of composting, contains both inorganic and organic compounds. The power of composting lies on the diversity of microorganism populations. The downside may be that composting sometimes only binds the pollutants into the organic matter instead of degrading them. The pollutant can be stabilized for short time, but how stable it is, remains uncertain (Semple *et al.*, 2001). Michel Jr. *et al.* (2001) reported a successful treatment of PCBs when contaminated soil was composted with yard trimmings and up to 40 % decline in PCB concentrations was found. It was also estimated that less than 1 % of the PCBs in the soil were volatilized and therefore bioremediation would be the main cause for the reduction of PCBs (Michel *et al.*, 2001). Rhizoremediation has shown one of the best potentials for organic pollutants since the major part of soil microorganisms, and PCB degrading bacteria, are present in the plant rhizosphere. Plants such as alfalfa, black nightshade and tobacco have shown good potential for PCB degradation (Vasilyeva and Strijakova, 2007).

Bioaugmentation

Bioaugmentation has been used extensively in aerobic bioremediation, but it has been shown that bioaugmentation with a single species faces lots of challenges. The indigenous microorganisms are a complex community of species and the cometabolism of PCBs may generate toxic compounds for the single species added to the soil (Pieper, 2005). Table 2.4

demonstrates the variety of microorganisms that have been used in aerobic bioaugmentation.

Table 2.4 Genus of microorganisms that have been involved in experimental aerobic degradation of PCBs.

Genus	Reference
<i>Alcaligenes</i>	Clark <i>et al.</i> , 1979; Williams <i>et al.</i> , 1997; Furukawa, 2006
<i>Archromobacter</i>	Furukawa, 2006
<i>Pseudomonas</i>	Baxter <i>et al.</i> , 1975; Master and Mohn, 1998; Abraham <i>et al.</i> , 2002; Furukawa, 2006; Pieper and Seeger, 2008
<i>Burkholderia</i>	Master and Mohn, 1998; Nogales <i>et al.</i> , 2001; Abraham <i>et al.</i> , 2002; Furukawa, 2006; Pieper and Seeger, 2008; Field and Sierra-Alvarez, 2008
<i>Comamonas</i>	Furukawa, 2006; Pieper and Seeger, 2008
<i>Cupriavidus</i>	Pieper and Seeger, 2008
<i>Cytophagales</i>	Lloyd-Jones and Lau, 1998
<i>Sphingomonas</i>	Nogales <i>et al.</i> , 2001; Abraham <i>et al.</i> , 2002; Furukawa, 2006; Pieper and Seeger, 2008
<i>Acidivorax</i>	Pieper and Seeger, 2008
<i>Rhodococcus</i>	Williams <i>et al.</i> , 1997; Furukawa, 2006; Pieper and Seeger, 2008
<i>Bacillus</i>	Furukawa, 2006; Pieper and Seeger, 2008
<i>Variovorax</i>	Nogales <i>et al.</i> , 2001
<i>Nocardia</i>	Baxter <i>et al.</i> , 1975
<i>Ralstonia</i>	Furukawa, 2006
<i>Acinetobacter</i>	Furukawa, 2006

2.3.3 Sequential Anaerobic-Aerobic bioremediation

A combination of anaerobic and aerobic conditions theoretically enables complete degradation of PCBs. Ideally anaerobic treatment will first break down the PCBs to mono-, di- and triCBs and the aerobic treatment breaks down the remainder (BEST, 2001; Vasilyeva and Strijakova, 2007). Figure 2.6 shows examples of PCB degradation pathways and what reactions are most likely to occur under anaerobic, microaerophilic (biofilm in the figure) and aerobic conditions. Wiegel and Wu (2000) also stated that it is necessary to carry out both anaerobic and aerobic treatments to be able to degrade all the PCBs in the soil. This is due to the complex microbial communities that are involved in the process. Complete degradation of PCBs requires cooperation of various bacteria, and all of them may have somewhat different preferences on their living environment (Abraham *et al.*, 2002; Borja *et al.*, 2005).

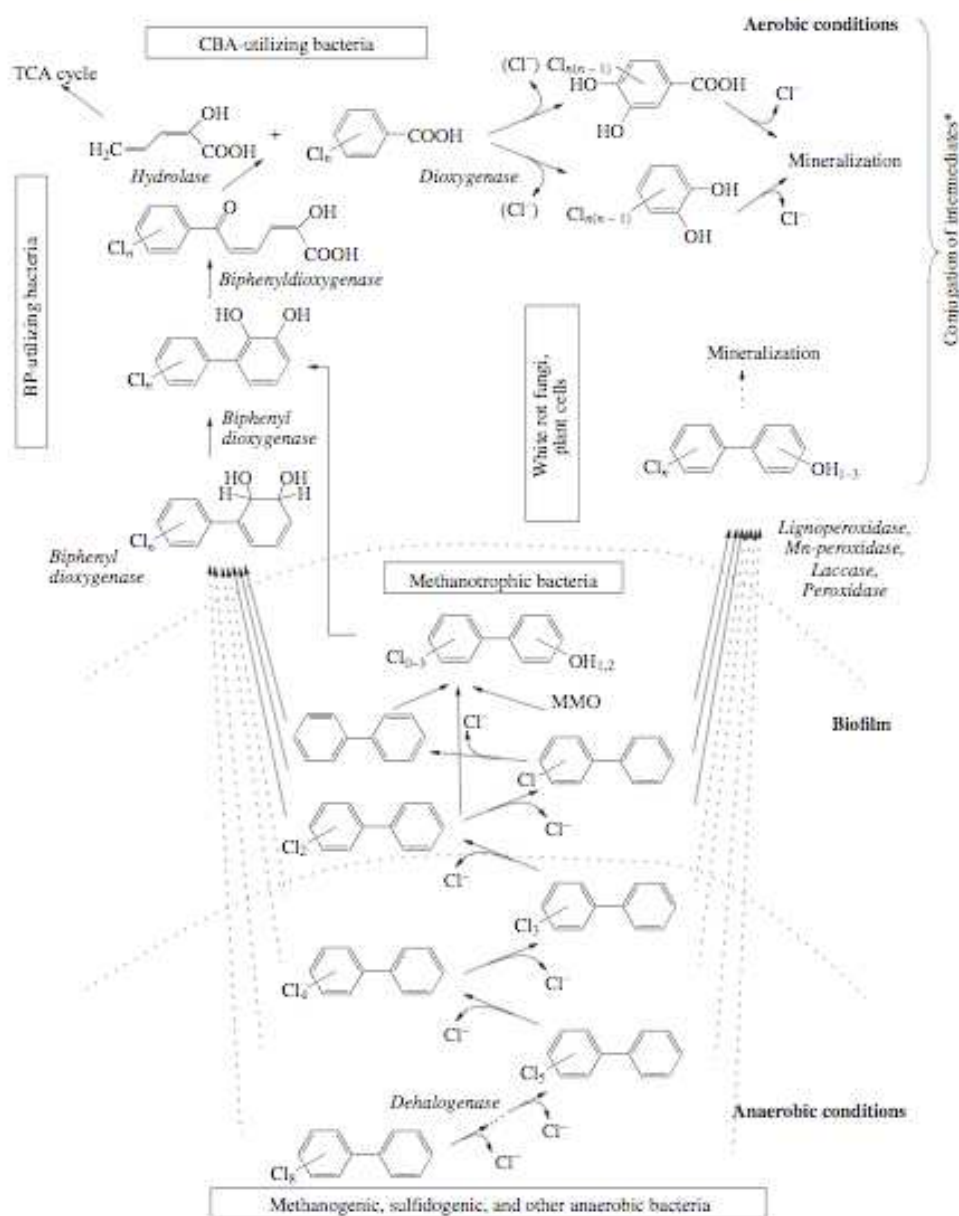


Figure 2.6 Examples of PCB degradation under anaerobic, microaerophilic (biofilm) and aerobic conditions. Source: Vasilyeva and Strijakova (2007)

The US EPA has attempted sequential anaerobic-aerobic bioremediation of PCBs by first adding anaerobic sludge and then aerobic PCB degraders, which resulted in high degradation in highly polluted soils and less degradation in moderately polluted soils (Tharakan *et al.*, 2006). Several studies have been carried out on Aroclor 1260. Zeeb *et al.* (2006) succeeded in 17-18 % degradation of aged Aroclor 1260 (20 ppm) by treating the soil three times with 3.5 months cultivation of marsh plants followed by one-month aerobic

period. Master *et al.* (2001) carried out a laboratory study on soil that had 59 ppm of aged Aroclor 1260. After 5 months of anaerobic treatment followed by bioaugmentation in aerobic conditions for 28 days, the PCB concentration was degraded to 20 ppm. However, Fava *et al.* (2003) only succeeded in 2 and 6 % degradation of Aroclor 1260 in solid and slurry bioreactors after 6 months of incubation. This might be due to the high initial concentrations, 890 and 8500 ppm, respectively.

2.3.4 Bioremediation of PCBs in Cold Environments

Up to 85 % of the global biosphere is permanently exposed to temperatures below 5°C (Margesin, 2007). Degradation of pollutants, even natural attenuation, occurs in cold environments with the help of psychrotolerant bacteria but often with lower degradation rates than in warmer environments (Welander, 2005). Many still use the most common remediation methods (e.g. Kalinovich *et al.*, 2008), including incineration and burial, in cold environments instead of bioremediation. In a study by Lambo and Patel (2007) cold-adapted bacterium were isolated from a Canadian soil (Newfoundland) and exposed to Aroclor 1232. Degradation was similar at 5 and 30°C and the extent of removal were between 34 and 100% and between 18 and 100%, respectively (Lambo and Patel, 2007). The Russian town of Serpukhov encountered high PCB pollution levels due to industrial use of various PCB mixtures (Zharikov *et al.*, 2007). Thirty percent of the soils had up to 10 times more PCB than the regulatory 0.06 mg kg⁻¹, 30 % up to 10-50 times more, 5 % up to 100 times more, 14% up to 1000 times more and 3% more than 1000 times more PCBs than 0.06 mg kg⁻¹. Two months of bioremediation indicated that optimal temperature (20-30°C) and water content (60-70 %) were vital for the successful degradation, which was up to 90% (Zharikov *et al.*, 2007). Aislabie *et al.* (2006) listed temperature, available nutrients, and soil moisture and soil pH as the main variables for biodegradation in cold environments. Some studies have addressed the temperature problem by heating up the soil in order to increase degradation rates. Kuipers *et al.* (2003) treated aerobic Aroclor 1260 polluted soils from arctic Canada (Resolution Island and Saglek, Labrador) with anaerobic pond sediments in order to add anaerobic microorganisms and nutrients to the carbon poor soils. They compared weathered and nonweathered Aroclor 1260 pollution in soil and incubated at 21 and 30°C. At 30°C the average number of chlorine substituents per biphenyl molecule was decreased from 6.6 to 5.1 and from 6.2 to 4.5 for weathered and nonweathered Aroclor 1260, respectively. At 21°C the decrease for weathered Aroclor

1260 was from 6.7 to 5.1 and from 6.5 to 4.6 for two sites studied, respectively (Kuipers *et al.*, 2003). Master and Mohn (1998) compared Aroclor 1242 degradation at 7, 37 and 50°C by arctic soil bacteria from arctic Canada (Saglek, Cambridge Bay and Iqaluit) and by *Burkholderia*. Arctic soil bacteria were proven cold adapted, since the degradation of Aroclor 1242 was about the same at 37°C than at 7°C. The degradation at 50°C was up to 90 % lower than at 37°C. The study highlights the importance of using arctic, psychrotolerant microorganisms in bioremediation instead of mesophiles like *Burkholderia*, since the initial rates of degradation would be higher and the soil would not need to be heated up (Master and Mohn, 1998). Adamsson (1998) conducted an aerobic bioremediation study in Labrador (Canada), yielding a 20 % reduction of the initial 200 ppm of Aroclor 1260. This was accomplished by continuous oxygen addition and maintaining moisture at 40-60% of the water holding capacity (Adamsson, 1998). The degradation rates may be slower than at higher temperatures, but not as significant as would be expected based on the law of a doubling of enzymatic rates for each 10°C increase in temperature (Mohn *et al.*, 1997). Mohn *et al.* (1997) compared PCB degradation with added biphenyl at 7°C and 30°C in soils from arctic Canada (Saglek and Resolution Island). The study reported maximum Aroclor 1221 degradation of 14 to 40% removal at 7°C after 8 weeks and 71 to 76% removal at 30°C after 6 weeks. However, the degradation mainly occurred with the less chlorinated PCBs and therefore significant degradation might not occur with Aroclor mixtures having higher chlorine content, such as Aroclor 1260 (Mohn *et al.*, 1997).

2.4 Factors affecting bioremediation in polluted soils

Bioremediation relies on the microbial communities in the soil environment and their abilities to survive in a polluted soil (Wiegel and Wu, 2000; Vasileya and Strijakova, 2007). The theoretical, ideal PCB degrader would tolerate PCBs in the environment, produce surfactants that solubilise PCBs, does not accumulate toxic intermediates, has various genes that are involved in the biodegradation process and would survive throughout the whole process until end of the clean-up (Ohtsubo *et al.*, 2004). The natural environment is, however, very complex and to find or create a single microorganism that would exclusively be able to carry out bioremediation is rather unsustainable since it doesn't take into account the diversity or complexity of organisms living in polluted soil

environments. As Figure 2.7 illustrates, understanding contaminant degradation in soils requires knowledge of not only the contaminant but also the dynamic soil environment and the contaminant degraders. The composition of the microbial community and interactions among them seems to be the predominant factors in bioremediation (Wiegel and Wu, 2000; Borja *et al.*, 2005) and therefore different factors affecting the microbial communities are described in this chapter.

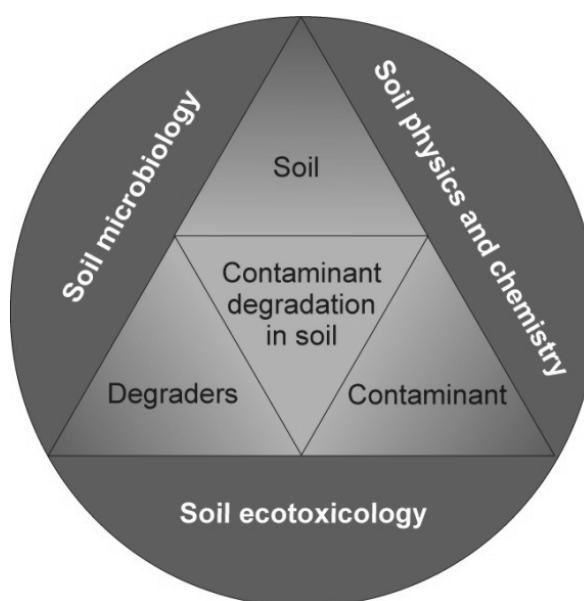


Figure 2.7 Illustration of factors affecting contaminant degradation in soils. Illustrated by Anu Mikkonen, University of Helsinki, Finland.

2.4.1 Effect of temperature on biodegradation

The optimal temperature for bioremediation of PCBs according to numerous sources is close to room temperature (25°C) (Tiedje *et al.*, 1993; Wu *et al.*, 1997; Alexander, 1999; Wiegel and Wu, 2000; Borja *et al.*, 2005). Many aerobic PCB degraders are mesophiles that have the optimal growth conditions between 22°C and 35°C (Alexander, 1999). Tiedje *et al.* (1993) studied anaerobic biodegradation at 12°C, 25°C, 37°C, 45°C and 60°C, finding out that optimal temperature was 25°C. Dechlorination occurred slower at 12°C and without success at the higher temperatures (Tiedje *et al.*, 1993). The temperature range in a study about anaerobic dechlorination of Aroclor 1260 was 8-34°C and 50-60°C (Wu *et al.*, 1997), and the optimal chlorine removal rate was found to be between 20°C and 27°C.

However, the highest variability in replicates was found at 18°C. This indicates that room temperature may not allow the full variety of dechlorination potentials to succeed, but favours certain degradation processes (Wu *et al.*, 1997).

Wiegel and Wu (2000) highlighted the importance of conducting studies at varying temperatures since temperature has an effect on the bioavailability of PCBs, growth of the microorganisms and the catalytic activity of enzymes. In nature, temperature changes seasonally, during weather events, and between day and night. This may activate different microorganism populations than those that have been studied in the laboratory under constant room temperature, as well as giving different picture of the degradation process (Wiegel and Wu, 2000).

2.4.2 Effect of soil pH on degradation

Soil pH has an important effect on the adsorption of PCBs into organic matter and therefore bioavailability and biodegradation as well (Jota and Hassett, 1991; Borja *et al.*, 2005). Wiegel and Wu (2000) reviewed studies on anaerobic dehalogenation of PCBs with affecting factors in mind, and found that dechlorination occurred between pH 5.0 and 8.0. The optimal pH for overall removal of chlorines was found to be 7.0-7.5 (Wiegel and Wu, 2000). Fava *et al.* (2003) also found close to neutral pH (6.0-7.5) to be optimal for the PCB degrading microbial community.

2.4.3 Carbon sources

One of the necessities for dechlorinating microorganisms is a sufficient amount of carbon substrates (Wiegel and Wu, 2000; Ohtsubo *et al.*, 2004; Borja *et al.*, 2005). The added carbon can affect the dechlorinating microorganisms directly or indirectly by improving the living conditions for other bacteria. Improvements in other microbial communities might supply the dechlorinators with more suitable electron donors or nutrients. In organic poor soils, carbon sources such as acetate, propionate, butyrate and hexanoic acid might be used whereas in organic rich soils the carbon added could be on the form of glucose, methanol, acetate or acetone (Tiedje *et al.*, 1993; Wiegel and Wu, 2000). Also terpenes have been shown to work as a successful carbon source (Vasilyeva and Strijakova, 2007). Commercial surfactants, both chemical and biosurfactants, are used to increase the

availability of PCBs to the microorganisms. PCBs that might be entrapped within the micelle are released when the surfactant is consumed by the microorganisms. However, surfactants remain a very costly carbon source and chemical surfactants may not be biodegradable in the environment themselves (Ohtsubo *et al.*, 2004).

2.4.4 Electron donors

Under anaerobic conditions PCBs can act as electron acceptors in the environment, which means they receive an electron during cellular respiration from an electron donor. Microorganisms such as bacteria gain energy from this process and PCBs are reduced. Other common electron acceptors are oxygen, nitrate (NO_3^-) and sulfate (SO_4) (Wiegel and Wu, 2000). The adequate amount of electron donors is crucial to the rate, extent and route of any anaerobic reductive dehalogenation process (Tiedje *et al.*, 1993; Wiegel and Wu, 2000; Abraham *et al.*, 2002; Borja *et al.*, 2005). Addition of H_2 as electron donor is one of the most common approaches; others include adding bromoethane sulfonic acid (BESA), ferric oxyhydroxide ($\text{FeO}(\text{OH})$), sodium sulfate (Na_2SO_4) or individual PCB congeners as electron acceptor. When electron acceptors are added, it is expected that sulfate will stimulate the growth of dechlorinating bacteria and they will then attack PCBs (Zwiernik *et al.*, 1998; Wiegel and Wu, 2000). This is especially important in deep anaerobic sediment layers, where limited addition of electron donors occur naturally (Tiedje *et al.*, 1993).

2.4.5 PCB concentration

A sufficient amount of PCB in the soil seems to be essential for biodegradation of the pollutant and to take off the activity of the microorganisms involved in the degradation process (Tiedje *et al.*, 1993; Bedard *et al.*, 1997; Kim and Rhee, 1997; Semple *et al.*, 2001; Borja *et al.*, 2005; Vasilyeva and Strijakova, 2007). Tiedje *et al.* (1993) even suggested that several hundreds to 1000 ppm of PCB are optimal concentrations for dechlorination to occur. If the concentrations were lower PCBs would be adsorbed to SOM and mineral compartments of the soil (Tiedje *et al.*, 1993). With very low concentrations of PCBs the biodegradation process only occurs close to immeasurable rates. Borja *et al.* (2005) stated that low concentrations of PCBs might not be sufficient enough to initiate the microbial activity or to sustain their activity in the degradation process. Another inhibitor could be a co-pollutant such as oil. A more easily available carbon source will be used first by the microorganisms, and as a result the PCB degradation rates decrease (Tiedje *et al.*, 1993).

Individual congeners have been used in several studies as primers for dechlorination. Bedard *et al.* (1997) showed that bioaugmentation alone was not successful, but needed addition of an individual PCB congener to prime the process. This was also stated in Wiegel and Wu (2000), in the context of highly polluted soils. However, concentrations that are too high may be toxic for the microorganisms and thereby prevent the biodegradation (Semple *et al.* (2001); Vasilyeva and Strijakova, 2007). The optimal PCB pollution range for successful aerobic and anaerobic biodegradation is 10-60 ppm and 500-1000 ppm, respectively, as suggested by Vasilyeva and Strijakova (2007).

2.5 The aims and research questions of the study

This research was undertaken to propose a framework of protocols that can be adapted to bioremediate Icelandic soils that inhibit PCB contamination in a sustainable manner. To the best knowledge of the author no data has been reported, neither on PCB degradation rates nor PCB degrading genes in Icelandic soils, and very limited research exists on contamination issues in general for soils of Iceland. Therefore the study was carried out as a two-phase remediation bench study where different biostimulation methods at different temperatures were conducted, the aim being to find the most suitable method that leads to decrease in PCB concentrations. The soil microbiology and degradation capacity of the soils in question, and bioavailability of PCBs were also studied.

The research questions of this bioremediation trial were:

1. Which PCB degradation methods are most suitable for polluted soils of the Keflavík area?
2. Do different PCB concentrations effect soil biological properties, and hence soil fertility?
3. Does the microbial community and degradation capacity differ between unpolluted soils and polluted soils, as well as between anaerobic and aerobic soils?
4. Are the PCBs bioavailable?
5. Can the laboratory methods be transferred to field scale application?

The first section of the thesis results focuses on describing soil biogeochemical properties, and the capacity of the soils to degrade the existing PCBs in the surrounding environment. In the second section of the thesis results, the suitability of different treatments to degrade PCBs and bioavailability are studied. Finally, the opportunities for future research and future bioremediation approaches and solutions will be covered. This thesis only focused on bioremediation and therefore the physical and chemical degradation methods of PCBs are not discussed. So far there are only a few studies on soil pollution in Iceland – there are even fewer studies on bioremediation – and therefore this study is of importance for fundamental research on soil pollution in Iceland.

2.6 Study Area

The former NATO facility and United States Naval Air Station Keflavík (NASKEF) was situated at Keflavík International Airport on the Reykjanes Peninsula from the WWII until the autumn of 2006 (Figure 2.8, Almenna Verkfræðistofan, 2008 and 2010). A number of various pollutants were documented in the surrounding environment and especially in soils when Icelandic authorities took over the airport. Despite the numerous pollutants in the study area, the focus of this research is only on the bioremediation of PCBs. PCBs were used at NASKEF in transformer oils in great quantities, but were mainly phased out of use during early 1990s (Almenna Verkfræðistofan, 2010).

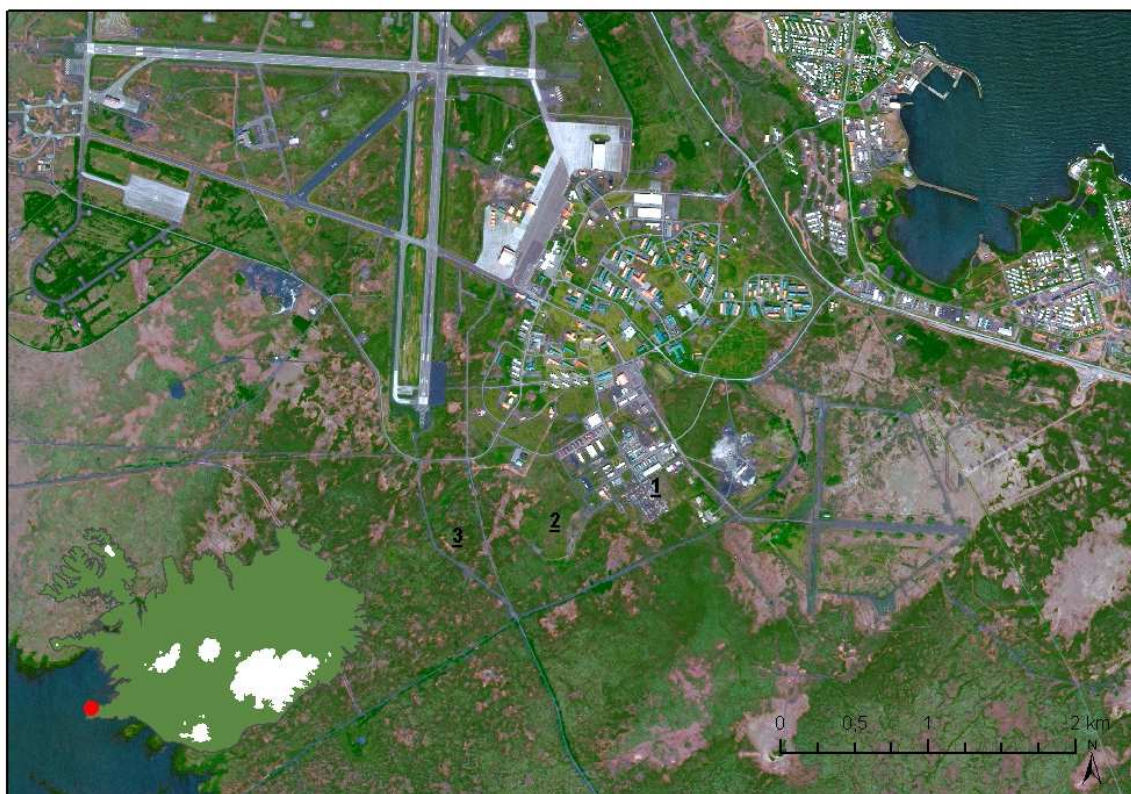


Figure 2.8 Aerial photograph of the study area. 1 denotes the former site of Sala Varnarliðseigna (army sales), 2 denotes the current location of the soil that has 1-50 ppm of PCBs and 3 denotes the current location of the soil that has < 1 ppm of PCBs. Map composed by Sigmundur Helgi Brink, Agricultural University of Iceland.

The soil in the area is classified as Brown Andosol (BA) (Arnalds, 2004; Arnalds *et al.*, 2009). Almenna Verkfræðistofan has estimated the extent and scale of PCB contaminated soils on the former site of Sala Varnarliðseigna (army sales) (see Appendix A and Figure 2.8) on behalf of Kadeco - Próunarfélag Keflavíkurflugvallar (Almenna Verkfræðistofan, 2008). Based on these estimates soils have been divided into three categories: more than 50 ppm PCB, 1-50 ppm PCB and less than 1 ppm PCB. The groundwater around the study site has also been investigated, but no higher values than the blank were found in the investigation (ÍSOR, 2008).

The most contaminated soils were excavated into High Density Polyethylene (HDPE) barrels and sent for incineration to an approved waste disposal facility in Germany

(Almenna Verkfræðistofan, 2010). Soils with 1-50 ppm (320 m³), which are used in this study, have been excavated and disposed in an area near the site of interest (see Figure 2.8, point 2; and Figure 3.1). Soils with < 1ppm of PCBs have been placed in an old landfill site near the original site and the landfill will be closed so that minimum disturbance to the surrounding ecosystems will occur (see Figure 2.8, point 3; and Figure 3.1). The Icelandic Environment Agency classifies the NASKEF site as one of the 6 largest polluted sites in Iceland and stresses the importance of future studies in the area as well as finding methodologies to treat the pollution (Meyles and Schmidt, 2005).

The climate is relatively mild, cold temperate oceanic in the study area. According to the Icelandic Meteorological Office database (2010) – between 1961 and 1990 – the mean annual precipitation in the area was 1100 mm and mean annual temperature was 4.4°C. The average minimum temperature, -0.1°C, was in January and highest, 10.2°C, in July. The average wind velocity was 12.5 m s⁻¹; the predominant wind directions were from southeast and northeast.

3 Materials and Methods

3.1 Soil samples

Eight surface soils samples for the experiments were collected from eight points of the 1-50 ppm polluted soil pile (Figure 3.1) in May 2009 according to the sampling guidelines in NORDTEST Technical Report No. 329 (Karstensen et al., 1997), with the exception that a 2 mm steel sieve was used instead of an 8 mm sieve. They were stored in glass jars at 4°C in the dark until ready for the experiment commencement. Before the secondary laboratory experiment, in October 2009, more soil was sampled from the < “1 ppm” field area in order to mix a less polluted soil to work with (final concentration 27 ppm). A control soil sample for laboratory analysis was sampled from the vicinity of the polluted soil, and confirmed not to contain any detectable PCBs.



Figure 3.1 The field site where the 8 samples were taken in May 2009 and < 1 ppm soil in October 2009. On the left, the 1-50 ppm polluted soil pile with 8 sampling bags (closest is sample nr 8). On the right, the 1-50 ppm polluted soil pile in front, then blue High Density Polyethylene (HDPE) barrels with > 50 ppm polluted soil and in the back the area < 1 ppm polluted soil. Photographed by Taru Lehtinen

A clarification of PCBs discussed in this thesis is given in Table 3.1. The PCB congeners, their structure, chemical name and molecular formula are presented. After this, when sum of six PCBs is mentioned, it reflects PCB 28, 52, 101, 138, 153 and 180. Sum of seven PCBs reflects PCB 28, 52, 101, 118, 138, 153 and 180; and sum of nine PCBs reflects PCB 28, 52, 101, 118, 138, 153, 170, 180 and 187.

Table 3.1 Summary of PCB congeners discussed here, and a clarification of the structure, name and molecular formula of each PCB congener. Source: Fiedler (2010)

PCB congener	Structure	Chemical name	Molecular formula
28	2,4,4'	Trichlorobiphenyls	C ₁₂ H ₇ Cl ₃
52	2,2',5,6'	Tetrachlorobiphenyl	C ₁₂ H ₆ Cl ₄
101	2,2',4,5,5'	pentachlorobiphenyl	C ₁₂ H ₅ Cl ₅
118	2,3',4,4',5	Pentachlorobiphenyls	C ₁₂ H ₅ Cl ₅
138	2,2',3,4,4',5	Hexachlorobiphenyls	C ₁₂ H ₄ Cl ₆
153	2,2',4,4',5,5'	Hexachlorobiphenyl	C ₁₂ H ₄ Cl ₆
170	2,2',3,3',4,4',5	Heptachlorobiphenyl	C ₁₂ H ₃ Cl ₇
180	2,2',3,4,4',5,5'	Heptachlorobiphenyl	C ₁₂ H ₃ Cl ₇
187	2,2',3,4',5,5',6	Heptachlorobiphenyl	C ₁₂ H ₃ Cl ₇

A complete description of the PCB amounts in the soil samples from eight samples taken in May 2009 is presented in Table 3.2, as well as the 27 ppm soil mixture used in experiment 2.

Table 3.2 Summary of mean PCB concentration results ($n = 2$) from 8 soil samples taken in May 2009 (\pm one standard error). Sample nr 8 denotes samples closest on the left in Figure 3.1, nr 7 the next soil sample in the picture and so forth. Columns Nr4 and Nr 5 indicate the soils used for experiment 1 and column Mixture indicates the soil used for experiment 2.

PCB	Nr 1	Nr 2	Nr 3	Nr 4	Nr 5	Nr 6	Nr 7	Nr 8	Mixture
	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight	mg kg ⁻¹ dry weight
28	0.41 (± 0.04)	0.18 (± 0.02)	0.10 (± 0.00)	0.11 (± 0.00)	0.05 (± 0.00)	0.27 (± 0.00)	2.60 (± 0.08)	0.19 (± 0.02)	0.45 (± 0.00)
52	3.59 (± 0.46)	0.89 (± 0.06)	0.55 (± 0.03)	0.20 (± 0.01)	0.09 (± 0.01)	0.40 (± 0.00)	3.08 (± 0.13)	0.97 (± 0.11)	0.23 (± 0.01)
101	49.7 (± 4.35)	10.5 (± 0.84)	6.85 (± 0.42)	1.72 (± 0.19)	0.66 (± 0.07)	2.46 (± 0.00)	12.4 (± 0.40)	12.2 (± 1.42)	1.32 (± 0.10)
118	6.64 (± 0.57)	1.61 (± 0.09)	1.04 (± 0.05)	0.28 (± 0.02)	0.12 (± 0.01)	0.41 (± 0.00)	2.18 (± 0.06)	1.83 (± 0.20)	0.57 (± 0.02)
138	112 (± 13.2)	26.0 (± 1.55)	16.6 (± 1.25)	4.11 (± 0.50)	0.53 (± 0.19)	6.01 (± 0.01)	30.91 (± 1.90)	28.14 (± 3.20)	2.21 (± 0.19)
153	189 (± 17.5)	40.9 (± 4.35)	25.9 (± 1.70)	7.01 (± 0.73)	2.54 (± 0.32)	11.6 (± 0.06)	58.2 (± 3.05)	44.9 (± 5.95)	3.24 (± 0.28)
170	90.4 (± 6.25)	20.1 (± 1.70)	7.71 (± 4.01)	3.53 (± 0.33)	1.40 (± 0.18)	5.92 (± 0.09)	31.8 (± 1.60)	22.0 (± 2.60)	1.87 (± 0.17)
180	205 (± 18.0)	46.3 (± 3.40)	17.8 (± 8.83)	8.11 (± 0.85)	3.36 (± 0.33)	15.5 (± 0.25)	85.8 (± 5.55)	50.1 (± 6.05)	3.48 (± 0.29)
187	98.6 (± 10.4)	22.5 (± 1.60)	17.2 (± 0.45)	4.06 (± 0.33)	1.74 (± 0.18)	8.15 (± 0.01)	49.2 (± 3.20)	23.8 (± 2.65)	2.05 (± 0.21)
$\Sigma 6^1$	560 (± 53.5)	125 (± 10.2)	67.7 (± 12.2)	21.3 (± 2.28)	7.23 (± 0.91)	36.2 (± 0.18)	193 (± 11.1)	136 (± 16.7)	10.9 (± 0.89)
$\Sigma 7^2$	567 (± 54.1)	126 (± 10.3)	68.7 (± 12.3)	21.5 (± 2.30)	7.35 (± 0.92)	36.6 (± 0.18)	195 (± 11.2)	138 (± 16.9)	11.5 (± 0.87)
$\Sigma 9^3$	756 (± 70.8)	169 (± 13.6)	93.7 (± 16.7)	29.1 (± 2.95)	10.5 (± 1.27)	50.7 (± 0.25)	276 (± 16.0)	184 (± 22.2)	15.4 (± 1.27)
Total	1310 (± 126)	218 (± 26.7)	148 (± 2.09)	54.4 (± 4.46)	24.5 (± 2.38)	67.1 (± 1.05)	383 (± 30.9)	211 (± 37.2)	27.0 (± 3.54)

¹PCB 28, 52, 101, 138, 153 and 180

²PCB 28, 52, 101, 118, 138, 153 and 180

³PCB 28, 52, 101, 118, 138, 153, 170, 180 and 187

A comparison between a random sample taken in May 2009 and standard Aroclor 1260 100 ppm is provided in Figure 3.1. The GC peaks are relatively similar and therefore the PCB pollution at the field site was identified as Aroclor 1260.

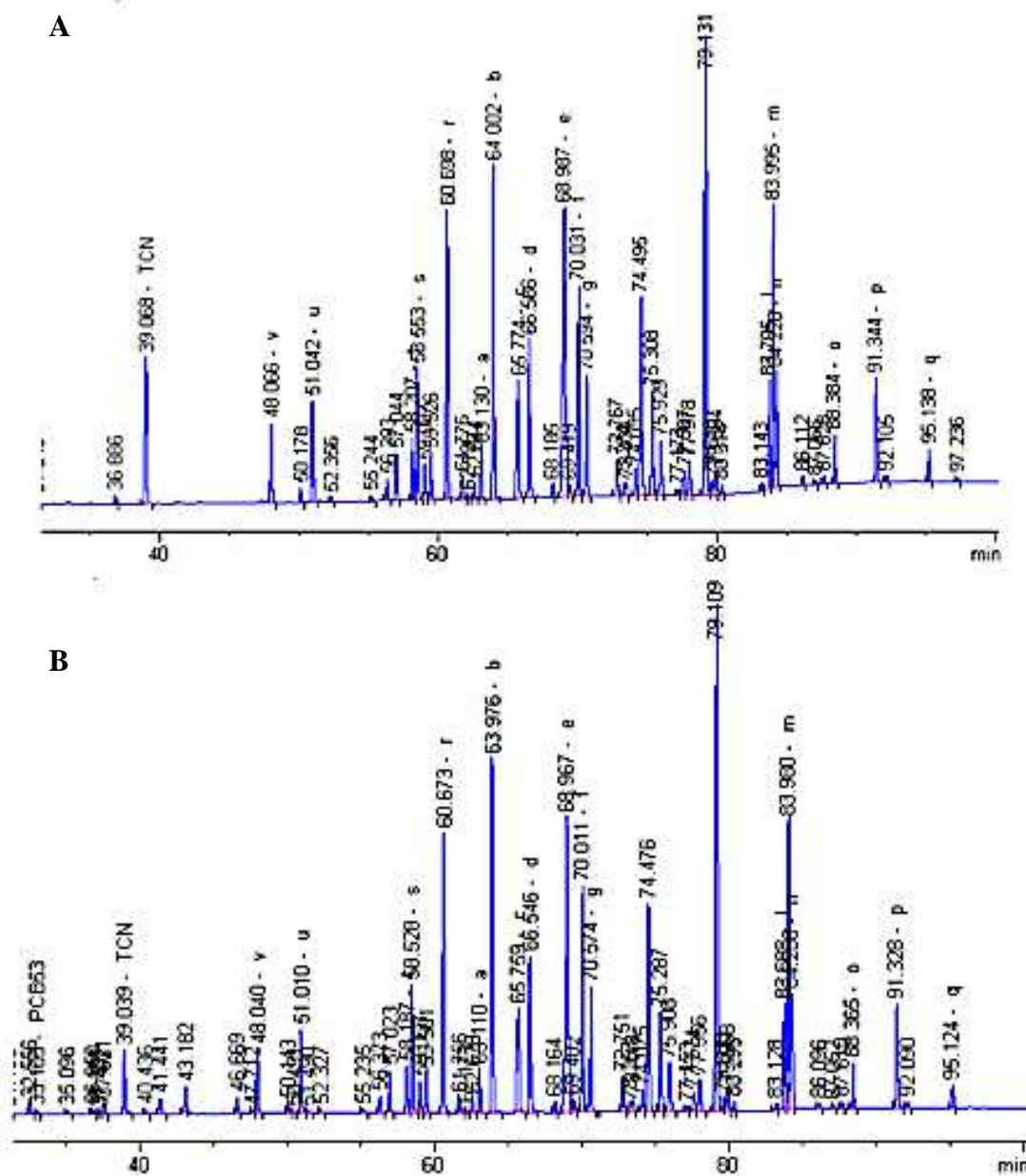


Figure 3.1 Comparison between Aroclor 1260 100 ppm standard (A) and a random sample (B) selected from eight surface samples taken in May 2009, showing the GC peaks of both.

The majority of the research was conducted in the soil laboratory facilities at the Agricultural University of Iceland in Keldnaholt. The PCB analyses were carried out at the Department of Pharmacology and Toxicology at the University of Iceland, soil respiration (CO₂) measurements at Reykjavik Energy and the detailed soil microbiology investigations at the Department of Food and Environmental Sciences at the University of Helsinki, Finland.

3.2 Soil characteristics

3.2.1 Soil physics and chemistry

Soil moisture Content

Soil moisture content was measured in order to see if it correlates with PCB concentration. Field moist soil was dried at 105°C for 24 hours. Both wet and dry samples were weighed and recorded. Thereafter moisture content (MC %) was calculated with the following formula (Page *et al.*, 1982).

$$\text{MC \%} = (\text{water weight})/(\text{wet soil weight}) \times 100.$$

Water Holding Capacity (WHC)

WHC was measured so that the soils could be adjusted to 60 % of it for the treatments and that correlation between PCB concentration and WHC could be studied. It was determined by the “filter paper method” (Whatman Nr 42) according to Smith and Mullins (2001). At the initial stage of the WHC analysis the filter papers weights were recorded filled up to $\frac{3}{4}$ with moist soil. Soils were thereafter wetted thoroughly with deionised water. (DI) After two hours drainage the funnels with wet filter paper and wet soil were weighed and weights recorded. The samples were dried at 105°C overnight and reweighed. The water holding capacity was calculated by the following formula (Smith and Mullins, 2001).

$$\text{WHC} = (\text{water weight})/(\text{dry soil weight}) \times 100$$

Soil pH

Soil pH was measured in contemplation of testing correlation with PCB concentration. Soil pH was determined according to Blakemore *et al.* (1987). Five g of oven-dried soil (< 2 mm) in 25 ml of deionised water were shaken for 2 hours; thereafter the soil pH was

measured with a glass calomel electrode (Oakton pH/mV/°C Meter pH 1000 Series, Illinois, USA).

Soil total organic carbon and nitrogen content (C_{tot} and N_{tot})

C_{tot} and N_{tot} were determined according to Blakemore *et al.* (1987), in order to characterise and classify the soils used in the experiments and to see if these characteristics correlated with the PCB concentration of the soils. Homogenised (< 2 mm, dried at 105°C for 24 hours) samples were measured by thermal combustion (Elementar macro Elemental Analyzer vario MAX CN, Analysensysteme GmbH, Germany).

Cation Exchange Capacity (CEC)

CEC was measured so that correlation between CEC and PCB concentration could be studied. The determination was carried out according to Blakemore *et al.* (1987) using Sampletek Vacuum Extractor (Marco Industries, INC). One g of macerated filter pads, approximately 1.5 g of silica sand, one g of homogenized sample (< 2 mm) and a second 1.5 g of silica sand were inserted into the leaching tubes. Tubes were placed in the vacuum extractor and receiving syringes fitted. Fifteen ml of 1 M ammonium acetate, NH_4OAc , was added into leaching tubes and let to stand for 15-20 minutes. Reservoirs were fitted to leaching tubes and samples were extracted rapidly until 1 cm of NH_4OAc remained above the sample. Then 25 ml of 1 M NH_4OAc was added to reservoirs and the extractor was run to full extraction for 12 hours. Leachate was transferred to 50 ml plastic centrifuge bottles (Sarstedt, Germany) and made up to 50 ml with deionised water for exchangeable bases (Na^+ , Mg^{2+} , K^+ , Ca^{2+}) determination by gas diffusion with FIALAB 3500B (Fialab Instruments, USA) at the Innovation Center Iceland. Leaching tubes were then cleaned with ethanol by first rinsing with 10 ml of wash ethanol, and then extracted with 50 ml wash ethanol for 3 hours. 10 ml of 1 M sodium chloride, NaCl , was then added to the leaching tubes and extracted until the liquid was 1 cm deep. 25 ml of NaCl was added to the fitted reservoirs and the extraction run to complete extraction for 12 hours. Leachates were handled as previously described and analysed for $\text{NH}_4\text{-H}$ in a flow injection FIAstar 5010 analyzer (Tecator, Hoganas, Sweden) for CEC determination (Schollenberg and Simon, 1945; Rhoades, 1982; Blakemore *et al.*, 1987).

Allophane and Ferrihydrite content

Allophane and ferrihydrite have large surface areas that can attract pollutants in the soil, and therefore allophane and ferrihydrite contents were estimated by oxalate extraction according to the modified method of Blakemore *et al.* (1987). 0.15 g of homogenized oven dried (105°C for 24 hours) soil was weighed into 50 ml plastic centrifuge bottles (Sarstedt, Germany) together with 30 ml of 0.2 M ammonium oxalate solution that was adjusted to pH 3 with oxalic acid. Samples were shaken for 4 hours in the dark to inhibit chemical reactions within samples. After centrifuging samples for 20 minutes at 3500 rpm the solution was transferred with plastic pipette to a 12 ml plastic tube. The samples were kept refrigerated until the chemical analysis at the Innovation Center Iceland. The content (%) of Al, Si, Fe and Mn were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (Spectro, Germany). Allophane content was estimated by multiplying Si % with 6 (Parfitt, 1990) and ferrihydrite content by multiplying Fe % with 1.7 (Parfitt and Childs, 1988).

3.2.2 Soil biological analyses

Soil total microbial biomass carbon (mic_c)

Soil mic_c represents the size of the complete microbial community in the soil and is used as an indicator of soil health (Caravaca and Roldán, 2003). The determination was carried out by a chloroform fumigation-extraction method based on Vance *et al.* (1987). Five g of field moist soil (< 2mm) was weighed and added to glass Universal bottles. Fumigated samples were placed in a dessicator (~25°C) for 24 hours with moist paper towels and a 100 ml glass beaker containing 80 ml chloroform (CHCl_3) and anti-bumping granules. The desiccator was evacuated using a vacuum pump until CHCl_3 was boiling, then the valve was closed and pump stopped. Non-fumigated samples were extracted (Whatman No 42) during the fumigation with 25 ml of 0.5 M K_2SO_4 for 30 minutes. Fumigated samples were extracted as described above and all samples were stored at 4°C until the analyses. The extracts were diluted with deionised water (1:10) prior to the total organic carbon (TOC) analysis in an aqueous carbon analyzer (LABTOC Polluting and Process Monitoring) with UV digestion and infrared detector. The total soil microbial biomass C was calculated with k_{EC} -factor 0.35 for mineral soils (Sparling and West, 1988). Soil total microbial biomass carbon was measured before the treatments, and after both preliminary and secondary

bioremediation experiments. After the laboratory experiments three soil sample replicates were bulked together to represent a mean for each treatment.

Soil dehydrogenase activity

It was of importance to measure dehydrogenase activity, since it is an enzyme connected to aerobic PCB degradation, and is involved in the carbon cycle in soils. Soil dehydrogenase activity was determined according to the modified method of Trevors (1984). One g of soil was weighed and added to sterilized and foil covered plastic 50 ml centrifuge bottles (Sarstedt, Germany). 10 ml of sterile substrate solution (0.1 % p-iodonitrotetrazolium chloride (INT) and 0.5 M TES buffer, adjusted to pH 7.8 with 0.5 M NaOH) was added and samples were placed on an end-over shaker for 18 hours (~25°C). After shaking, 10 ml of ethanol was added to inhibit all microbial activity. Samples were centrifuged (Universal 320R, Hettich, Germany) for 20 minutes at 4°C and 2700g. Thereafter samples were transferred into luminometer cuvettes and absorbance was measured at 490 nm on a linear spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Sweden). Standard curves were determined using 1, 2, 3 and 5 ppm iodonitotetrazolium formazan (INTF) and the enzyme activity was expressed as $\mu\text{g g}^{-1} \text{h}^{-1}$ of dry soil (Trevors, 1984). Soil dehydrogenase activity was measured before the treatments, and after both preliminary and secondary bioremediation experiments. After the laboratory experiments three soil sample replicates were bulked together to represent a mean for each treatment.

Soil DNA extraction and quantification

Soil DNA analyses were necessary in order to see how the microbial community in the polluted soils differs from the control soils and whether indigenous PCB degrading genes are present in the polluted soils. Soil DNA was extracted from control soil, 25 ppm and 50 ppm polluted soils by direct soil DNA extraction using the commercial kit PowerSoilTM DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, USA). Polluted soils were extracted before and after anaerobic treatment in order to be analysed for anaerobic genes. Extraction was performed according to the kit protocol with 0.25 g sample size, except for step 5 where FastPrep[®] Instrument (Qbiogene, USA) instead of vortex was used for mechanical cell lysis (30 seconds, speed 5.5 m sec⁻¹). The DNA extracts were stored at -20°C.

The yield and quality of DNA extracts were checked by gel electrophoresis on a 1.5 % agarose gel at 0.5 * Sodium Borate buffer. Bands were visualized with ethidium bromide using Bio-Rad gel documentation system with Quantity One 4.6.7 program. PicoGreen dsDNA Quantitation Reagent kit (Molecular Probes, USA) with Qubit® Fluorometer (Invitrogen™, Finland) and NanoDrop® ND1000 UV-Vis spectrophotometer (NanoDrop Technologies, USA) were used for an accurate quantification of soil DNA, according to manufacturer's instructions.

Bacterial community analysis with Length Heterogeneity Polymerase Chain Reaction (LH-PCR)

The primers used for the general analysis of bacterial community structure were adopted from Tiirola *et al.* (2003), as described in Mikkonen (2008), and were fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and PRUN518r (5'-ATTACCGCGGCTGCTGG-3'). Primers were ordered from Oligomer (Helsinki, Finland). The composition of the PCR reaction (final volume 50 µl) was according to Mikkonen (2008) and original references therein. The seven first reagents (see Table 3.3) were mixed together first, and the template was added after the master mix had been aliquoted into Multiply-µStrip 0.2 ml (Sarstedt, Germany) 8-tube strips.

Table 3.3 PCR reagents and their concentrations in a final volume of 50 μ l.

Reagent	Product details	Final conc	Amount/reaction
Water	Autoclaved Milli-Q water	N/A	36.5 μ l
10x Buffer	10x Biotools reaction buffer	2 mM MgCl ₂	5 μ l
BSA	BSA Acetylated 10 mg/ml, Promega	0.05%	2.5 μ l
dNTP	dNTP Mix, 10 mM each, Finnzymes	0.2 mM each	1 μ l
fD1	10 mM dilution in water	0.3 μ M	1.5 μ l
(1/2 FAM) PRUN518r	10 mM dilution in 1/10 TE- buffer	0.3 μ M	1.5 μ l
Biotools polymerase	Biotools DNA polymerase 1 U / μ l	1 U	1 μ l
Template	(diluted) soil DNA extract	variable	1 μ l

The programme used for the PCR reaction was modified from Tirola *et al.* (2003) and described in detail in Mikkonen (2008). The program comprised initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation for 45 s at 94°C, primer annealing for 1 min at 55°C, and finally elongation for 2 min at 72°C. Amplification was done in a Peltier Thermal Cycler DNA Engine (PTC-200, MJ Research). The PCR products were separated by agarose gel electrophoresis (5 μ l of product mixed with Blue/Orange Loading Dye 6x (Promega), loaded on a 1.5 % agarose gel at 0.5 * Sodium Borate buffer, ran for 15 min at 250V) and visualized with ethidium bromide. pGEM DNA Markers (Promega) functioned as a molecular size standards.

PCR products were prepared for capillary electrophoresis according to Mikkonen (2008). 13.5 µl of Hi-Di Formamide and 0.5 µl of HEX-labeled LH-PCR size standard were first aliquoted into Multiply-µStrip 0.2 ml (Sarstedt, Germany) 8-tube strips, and thereafter 1 µl of PCR product was added to each tube. The final volume in each tube was 15 µl. Peltier Thermal Cycler DNA Engine at 98°C for 3-5 minutes was used for sample denaturation and thereafter samples were transferred onto ice immediately. The size separation by capillary electrophoresis was performed with an ABI PRISM® 310 Genetic Analyzer, which was equipped with a 47 cm long capillary (sequencing capillary) and POP-6™ Polymer (Applied Biosystems, UK), as described in Mikkonen (2008). Bacterial community profiles on PCR product size range 460-560 bp were analyzed with BioNumerics v. 6.1 according to Mikkonen (2008). Arithmetic average profiles were constructed from analytical replicates, normalised with molecular size standards. Total profile signal intensities were normalised and the fingerprint profiles visualised in Excel 2007. Profile similarities were calculated with Pearson correlation (optimization 0.44 %, approximately 1 bp) and dendrograms drawn with Ward's clustering algorithm.

PCR amplification of PCB degrading genes

The primers used for PCR amplification of PCB degrading genes are described in Table 3.4. All primers were ordered from Oligomer (Helsinki, Finland). The gene *bphA* has been associated with aerobic PCB degradation (Witzig *et al.*, 2006). Genes *pcbA*, *pcbB* and *ohb* have been associated with degradation of PCB dechlorination products (Rodrigues *et al.*, 2001 and 2006) and *cbrA* to dechlorination by *Dehalococcoides* (Watts *et al.*, 2005). The last primer set targets 16S rDNA of known dechlorinating members of the *Chloroflexi* genus (Wagner *et al.*, 2009).

Table 3.4 Summary of primers used in this study for PCR amplification of PCB degrading genes.

Gene	Primer	Sequence	Reference
<i>bphA</i>	bphAf668-3	5'GT TCC GTG TAA CTG GAA RTW YGC 3'	Witzig <i>et al.</i> (2006)
	bphAr1153-2	5'CCA GTT CTC GCC RTC RTC YTG HTC 3'	
<i>fcba</i>	forward	5'AACTGATCCGCCGAGACAACATCC 3'	Rodrigues <i>et al.</i> (2001)
	reverse	5'AGGCATTTTTTCGAGACGCTTCA 3'	
<i>fcbb</i>	forward	5'GGTCCAGCGCGAAATCCAGTC 3'	
	reverse	5'CCCCCGCACACCGCATCAAG 3'	
<i>ohb</i>	F580 <i>ohb</i>	5'GCGGACAAGCGTTTCGATACAGGA 3'	Rodrigues <i>et al.</i> (2006)
	R580 <i>ohb</i>	5'GCTTGCAGTTGCGCTTGATGAT 3'	
<i>cbrA</i>	cbdbA84_f	5'CTTATATCCTCAAAGCCTGA 3'	Wagner <i>et al.</i> (2009)
	cbdbA84_r	5'TGTTGTTGGCAACTGCTTC 3'	
<i>cbrA</i>	cluster 2a_f	5'GTYTTCMAKGAYHTKGACGA 3'	
	cluster 2a-1_r	5'TCRATTTMTYAGGYAKCAC 3'	
<i>Chloroflexi</i> (<i>phylum</i>)	fD1	5'AGAGTTTGATCCTGGCTCAG 3'	Tirola <i>et al.</i> (2003)
	Dehal1265R	5'GCTATTCCTACCTGCTGTACC 3'	Watts <i>et al.</i> (2005)

The compositions of the PCR reaction were modified from the original references and Mikkonen (2008). The reactions were carried out in a final volume of 50 µl, and the reagents are listed in Table 3.5. The Master mix was prepared by mixing all reagents except the template first. Thereafter the template was added individually into each reaction tube (Multiply-µStrip 0.2 ml 8-tube strips, Sarstedt, Germany). For *fcba* and *fcbb*, a PCR reaction with 10 µl of both primers was also attempted.

Table 3.5 PCR reagents and their concentrations in the final 50 μ l reaction.

Reagent	Product details	Final conc	Amount/reaction
Water	Autoclaved Milli-Q water	N/A	34.5 μ l
10x Buffer	10x Biotools reaction buffer	2 mM MgCl ₂	5 μ l
BSA	BSA Acetylated 10 mg/ml, Promega	0.05%	2.5 μ l
dNTP	dNTP Mix, 10 mM each, Finnzymes	0.2 mM each	1 μ l
Forward primer	10 mM dilution in water	0.5 μ M	2.5 μ l
Reverse primer	10 mM dilution in 1/10 TE- buffer	0.5 μ M	2.5 μ l
Biotools polymerase	Biotools DNA polymerase 1 U / μ l	1 U	1 μ l
Template	(diluted) soil DNA extract	Variable	1 μ l

The programmes used for the PCR reactions were modified from Mikkonen (2008). It comprised initial denaturation for 5 min at 95°C followed by N cycles of denaturation for 45 s at 94°C, primer annealing for 1 min at various temperatures, and finally elongation for 2 min at 72°C. The exact number (N) of cycles and annealing temperatures used for each individual gene can be seen below in Table 3.6.

Table 3.6 Listing of primer annealing temperatures and number (N) of cycles used in the PCR amplification reactions for PCB degrading genes.

Gene	Primer annealing °C	N cycles
<i>bphA</i>	58°C	35
<i>fcba</i>	60°C and 58°C	30 and 35
<i>fcba</i>	55°C	30 and 35
<i>ohb</i>	58°C	35
<i>cbrA</i> short	55°C	40
<i>cbrA</i> long	48°C	40
<i>Chloroflexi</i> (phylum)	62°C and 57°C	30 and 35

Amplification and checking of the PCR products were carried out as described above in the section Bacterial community analysis with Length Heterogeneity Polymerase Chain Reaction (LH-PCR).

3.2.3 Soil PCB analyses

Analysis of PCB in soil samples was undertaken according to the Nordic Guidelines for chemical analysis of contaminated soil samples (Karstensen *et al.*, 1997). One g of soil (exact weights were recorded) was placed into a 12 ml borosilicate glass tube together with 100 µl of 0.5 ng µl⁻¹ PCB 53 recovery standard, and for the control sample 10 µl of 500 ppm Aroclor 1260 in transformer oil was also added. One ml of pyrophosphate solution, Na₄P₂O₇, and 6 ml of acetone, OC(CH₃)₂, were added to the samples, after which they were sonicated for 5 minutes, rotated for an hour and centrifuged (10 minutes 3000 rpm). The organic phase was transferred with a Pasteur pipette to a 30 ml glass bottle that contained 10 ml of 0.2 M NaCl/0.1M H₃PO₄. Thereafter 2 ml of acetone and 6 ml of n-hexane, C₆H₁₄, was added to the samples and the same procedure was carried out as previously in order to transfer the organic phase into the 30 ml glass bottles with a Pasteur pipette. The 30 ml bottles were shaken carefully and phases were allowed to separate. The

organic phase was transferred to a 12 ml glass bottle with a Pasteur pipette. The water phase was further washed and shaken with 5 ml of hexane:diethylether ($\text{C}_6\text{H}_{14}:\text{CH}_3\text{-CH}_2)_2\text{O}$; 9:1) and the organic phase transferred into 12 ml glass bottles with a Pasteur pipette. The samples were then evaporated with nitrogen and dissolved in 0.7 g of 1,2,3,4-tetrachloronaphthalene (TCN), the internal standard. Five ml of concentrated sulfuric acid, H_2SO_4 , was used to clean the samples and then samples were centrifuged (10 minutes, 3000 rpm). The organic phase was transferred into gas chromatograph (GC) bottles with a Pasteur pipette (Karstensen *et al.*, 1997).

The determination of total PCBs and PCB congeners were carried out with Agilent 6890N GC with DB1701 column (60 m, 0.25 mm i.d., 0.25 μm film) equipped with an Electron capture detector (ECD) according to NORDTEST Technical Report No. 329 (Karstensen *et al.*, 1997). PCBs were analyzed first from the eight samples taken in May 2009, before the treatment, as well as from three replicates from each treatment after both primary and secondary treatments.

3.3 Experiment 1 – Biostimulation and PCB concentration effect on PCB degradation and soil biological properties

Experiment 1 was initiated in June 2009 at room temperature ($\sim 25^\circ\text{C}$) for 4 months on 25 ppm and 50 ppm polluted soil from the field area. As seen in Table 3.7 there were six anaerobic and six aerobic treatments. Soil treatments were: sterile control, active control, 100, 200 and 300 kg N/ha and 60 % (field moist weight) plant detritus and roots. After treating the polluted soil, ten g of field moist soil mixture was transferred to 20 ml amber vials (Agilent technologies, Germany) that served as microcosms. Microcosms were covered with ultraclean screw cap with septa (Agilent technologies, Germany); the aerobic samples were only closed loosely in order to let air in continuously to the samples. Anaerobic conditions were yielded by addition of 10 ml deionized water into the microcosms. The aerobic soil microcosms were adjusted to 60% WCH for optimal microbial activity (Alexander, 1999) and the weight of the aerobic samples were corrected with addition of deionized (DI) water on a weekly basis. Total treatments were twelve and each was replicated five times. Therefore 60 anaerobic and 60 aerobic 20 ml vials were set up.

Table 3.7 Experimental set up for the preliminary laboratory PCB degrading experiment. All treatments were carried out at room temperature (~25°C).

<i>Treatment</i>	<i>Anaerobic</i>	<i>Aerobic</i>
1	Sterile control	Sterile control
2	Active control	Active control
3	Fertilizer (100 kg N ha ⁻¹)	Fertilizer (100 kg N ha ⁻¹)
4	Fertilizer (200 kg N ha ⁻¹)	Fertilizer (200 kg N ha ⁻¹)
5	Fertilizer (300 kg N ha ⁻¹)	Fertilizer (300 kg N ha ⁻¹)
6	Plant detritus and roots (60% by field moist weight)	Plant detritus and roots (60% by field moist weight)

3.3.1 Soil respiration (CO₂)

Analysis of soil respiration (CO₂) in the microcosms to monitor metabolic activity was measured by gas chromatography (Agilent 7890A with CTC Combipal Autosampler). Ten µl were injected through a 125°C inlet with 10:1 split onto a J&W HP-PLOT 19095P-QO4 column (30 m, 30µm wall thickness and 0.53 mm inner diameter) with He 5.0 carrier gas at 10 psi and detected by a TCD sensor.

3.3.2 Bioavailability of PCBs in soil to earthworms

The uptake of PCBs by earthworms (*Eisenia foetida*) was determined according to Hallgren *et al.* (2006) with minor modifications. Earthworms were obtained from Guðmundur Óskar Sigurðsson from Vogar, Iceland, who breeds and sells earthworms to owners of household compost-containers. They were delivered in a humus-rich compost soil derived from degraded organic waste material from households. Earthworms and compost soil were analysed and confirmed to contain no PCBs. The earthworms were 3.0-9.5 cm long and weighed 0.11-0.67 g.

Microcosms were prepared by carefully mixing 20 g field moist polluted soil and 20 g of fresh compost-soil in a glass jar. Deionised water (5 g) was added to achieve a suitably

moist environment. Nine identical microcosms were prepared, three with unpolluted control soil, three with soil mixture containing 12.5 ppm of PCBs and three with soil mixture containing 25 ppm of PCBs (see Table 3.8 for exact concentrations). Ten earthworms were weighed and added to each jar. The jars were covered with parafilm and placed in desiccators where the drying-stones had been replaced by water to create stable humidity and no evaporation. The desiccators were stored in darkness at room temperature (~25°C) for a period of 10 days. After incubation, the earthworms were removed, rinsed and placed into the freezer (-20°C) until analysis (Hallgren *et al.*, 2006).

Table 3.8 Summary of mean PCB concentrations (n = 2) in the soil mixtures used in the bioavailability experiment.

PCB congener	12.5 ppm PCB soil mixture	25 ppm PCB soil mixture
PCB 28	0.03	0.06
PCB 52	0.05	0.10
PCB 101	0.33	0.86
PCB 118	0.06	0.14
PCB 138	0.27	2.06
PCB 153	1.27	3.51
PCB 170	0.7	1.77
PCB 180	1.68	4.06
PCB 187	0.87	2.03
Σ 6 PCB	3.62	10.6
Σ 7 PCB	3.68	10.8
Σ 9 PCB	5.25	14.6
Total PCBs	12.3	27.2

Extraction of PCBs in the samples was carried out by the Jensen extraction method (Jensen *et al.*, 1983), as described in Ólafsdóttir *et al.* (1995), Jensen *et al.* (2003) and Ólafsdóttir *et*

al. (2005). The melted earthworms were homogenised to fine-grained material with a mortar and pestle. The glass apparatus used consisted of two cylindrical 100 ml separating funnels, one placed above the other. The upper funnel was equipped with a glass filter at the bottom. A 1-2 g sample was transferred to the upper funnel and 100 µl of recovery standard (PCB-53) was added. The sample was then homogenised by vigorous shaking with 25 ml of acetone and 10 ml of n-hexane and let sit for 30 min. Then 25 ml of n-hexane:diethylether ($C_6H_{14}:CH_3-CH_2)_2O$; 9:1) was added to the sample, the solution was mixed well and let sit again for 30 minutes and then transferred to the lower funnel as described above. Finally, the sample was shaken with 25 ml of n-hexane:diethylether ($C_6H_{14}:CH_3-CH_2)_2O$; 9:1) and transferred to the lower funnel. All non-dissolved particles remained on the glass filter of the upper funnel. The lower funnel contained 25 ml of 0.2 M NaCl 0.9 % in 0.1 M H_3PO_4 . To avoid the formation of an emulsion, the lower funnel was not shaken but just sealed and turned upside down 20 times. After phase separation, the lower aqueous phase was transferred to a 100 ml beaker. To avoid water in the organic extracts, it was necessary to effectively rotate the lower funnel and transfer any additional water into the beaker. The organic phase was decanted into a pre-weighed 30 ml glass bottle and evaporated with nitrogen until dry. The aqueous phase was returned to the upper funnel and reextracted with 10 ml of n-hexane and evaporated again. The sample was dissolved in 0.7 g of TCN. Five ml of sulfuric acid, H_2SO_4 , was used to clean the samples and then samples were centrifuged (10 minutes, 3000 rpm). The organic phase was transferred into GC bottles with a Pasteur pipette. The total PCBs and individual PCB congeners were determined by gas chromatography as described above in section 3.2.3 Soil PCB analyses.

3.4 Experiment 2 – Biostimulation and temperature effect on PCB degradation and soil biological properties

Experiment 2 was carried out in a similar manner as the preliminary experiment, with slight changes. Instead of two pollution levels, only 27 ppm soil was used and the samples were incubated at 10°C and 30°C in refrigerated incubators. The duration of the experiment was two months. All aerobic treatments were carried out at 60 % WHC. Table 3.9 shows the treatments that the soil was subjected to. 200 kg N ha⁻¹ fertilizer treatment was omitted and pulverized white clover (C_{tot} 13.74 %, N_{tot} 3.11, C:N 42.80) and pine

needles were (C_{tot} 51.87 %, N_{tot} 0.75 %, C:N 70.13) added as plant treatments due to practicality issues in the laboratory measurements (Hernandez *et al.*, 1997). The aerobic treatments were carried out in 40 ml amber vials with thermo seal liners and screw caps (all from SUPELCO, USA), in order to guarantee continuous airflow to the samples and to make the handling of the samples more convenient.

Table 3.9 Experimental set up for the secondary laboratory PCB biodegradation experiment. All treatments were carried out at 10°C and 30°C.

<i>Treatment</i>	<i>Anaerobic</i>	<i>Aerobic</i>
1	Sterile control	Sterile control
2	Active control	Active control
3	Fertilizer (50 kg N/ha)	Fertilizer (50 kg N/ha)
4	Fertilizer (100 kg N/ha)	Fertilizer (100 kg N/ha)
5	Pulverized White clover (<i>Trifolium repens</i>) (0.5g dry mass in 10g field moist soil)	Pulverized White clover (<i>Trifolium repens</i>) (0.5g dry mass in 10g field moist soil)
6	Pulverized Lodgepole pine needles (<i>Pinus contorta</i>) (0.5g dry mass in 10g field moist soil)	Pulverized Lodgepole pine needles (<i>Pinus contorta</i>) (0.5g dry mass in 10g field moist soil)

3.5 Statistical and data analysis

Statistical analyses of the results were performed with SAS 9.1 for Windows and Excel Analysis ToolPak for Mac. Pearson's product moment correlations were carried out to inspect if there were significant linear correlations between two different variables. One-way analysis of variance (ANOVA) was undertaken to test for significant differences between measured values in microcosms with different biostimulation treatments. Anu Mikkonen at the University of Helsinki, Finland performed statistical analyses for LH-PCR data, as described under section "Bacterial community analysis with Length Heterogeneity PCR (LH-PCR)".

4 Results

4.1 Soil Characteristics

Soil characteristics (soil moisture content, WHC, soil pH, C_{tot} , N_{tot} , C:N, CEC, allophane, ferrihydrite) for the control soil, and soils from experiment 1 (25 ppm and 50 ppm) and experiment 2 (27 ppm) are described in the sections below.

4.1.1 Soil Physics and Chemistry

Soil moisture content

The soils used in experiment 1 had the lowest moisture contents, followed by the soils in experiment 2 and finally the control soils. Soil moisture contents were 24.6 %, 11.6 %, 11.8 % and 19.9 % for control, 25 ppm, 50 ppm and 27 ppm soil; respectively (Table 4.1). PCB concentration was significantly negatively correlated with moisture content ($r = 0.62$, $p < 0.001$).

Water Holding Capacity (WHC)

Measured WHC values are presented in Table 4.1 and were greatest for control soils and lowest for soils used in experiment 1. Soil WHC was $95.6 \text{ g } 100 \text{ g}^{-1}$, $32.7 \text{ g } 100 \text{ g}^{-1}$, $33.1 \text{ g } 100 \text{ g}^{-1}$ and $56.8 \text{ g } 100 \text{ g}^{-1}$ for controls, 25 ppm soil, 50 ppm soil and 27 ppm soil, respectively. Soil PCB concentration and WCH had a significant negative correlation ($r = 0.67$, $p < 0.001$).

Soil pH

Mean values for soil pH ranged from 6.0 to 6.9. Control soils had the lowest pH, 6.0, and soils from experiment 2 the highest values (soil pH 6.9). The soil pH for 25 ppm soils was 6.3 and 6.6 for 50 ppm soils (Table 4.1). Soil pH had a significant positive correlation with PCB concentrations ($r = 0.48$, $p < 0.05$).

Soil total organic carbon and nitrogen content (C_{tot} and N_{tot})

Soil C_{tot} ranged from 1.0 % in the most polluted soils to 2.1 % in the control soils. Soil N_{tot} ranged from 0.02 % in the most polluted soils (50 ppm) to 0.16 % in the control soil. The ratio between C_{tot} and N_{tot} ranged from 13 in the control soil to 58 in the 25 ppm soils

(Table 4.1). PCB concentration had a negative significant correlation with C_{tot} ($r = 0.79$, $p < 0.001$) and N_{tot} ($r = 0.56$, $p < 0.001$).

Cation Exchange Capacity

Measured soil CEC is presented in Table 4.1. Control soils had the highest CEC and 25 ppm soil the lowest, 13 meq 100 g⁻¹ and 4.8 meq 100 g⁻¹, respectively. PCB concentrations had significant negative relationships with CEC ($r = 0.61$, $p < 0.001$).

Allophane and Ferrihydrite content

The allophane and ferrihydrite contents for the control soils were 8.8 % and 5.0 %, respectively. In experiment 1 soils, the allophane content was 8.2 % and 9.2 % for 25 ppm and 50 ppm soils, respectively, and the ferrihydrite content was 4.1 % and 4.5 %, respectively (Table 4.1).

Table 4.1 Soil physiochemical characteristics presented as mean values of three replicates (± 1 standard error, if not given results is based on one replicate). MC indicates soil moisture content, WHC water holding capacity, CEC cation exchange capacity, A allophane, F ferrihydrite.

Soil	MC %	WHC g 100 g ⁻¹	pH H ₂ O	C _{tot} %	N _{tot} %	C:N	CEC meq 100 g ⁻¹	A %	F %	A+F %
Control	24.6	95.6 (± 6.93)	6.0 (± 0.01)	2.1 (± 0.0)	0.16 (± 0.00)	13 (± 0.31)	13 (± 0.57)	8.8 (± 0.07)	5.0 (± 0.06)	13.8 (± 0.12)
25 ppm	11.6	32.7	6.3 (± 0.02)	1.7 (± 0.1)	0.03 (± 0.00)	58 (± 6.33)	4.8 (± 0.18)	8.2 (± 0.42)	4.1 (± 0.20)	12.4 (± 0.61)
50 ppm	11.8	33.1	6.6 (± 0.01)	1.0 (± 0.0)	0.02 (± 0.00)	45 (± 2.28)	5.4 (± 0.20)	9.2 (± 0.16)	4.5 (± 0.05)	13.7 (± 0.21)
27 ppm	19.9	56.8 (± 0.62)	6.9 (± 0.01)	2.0 (± 0.1)	0.14 (± 0.00)	15 (± 0.35)	7.9 (± 0.70)	12.5 (± 0.39)	6.7 (± 0.20)	19.2 (± 0.59)

4.1.2 Soil biological analyses

Soil total microbial biomass carbon (mic_c)

Soil mic_c was highest for the soils used in experiment 2 and lowest in the control soil (under detection limits). The mean values for mic_c 9.31 mg kg^{-1} , 7.41 mg kg^{-1} and 57.23 mg kg^{-1} for 25 ppm soils, 50 ppm soils and 27 ppm soils, respectively (Table 4.2).

Soil dehydrogenase activity

Soil dehydrogenase activity was highest in control soils and lowest in the soils used for experiment 1. The activity for the control soil was 5.65 $\mu\text{g g}^{-1} \text{h}^{-1}$ compared to 1.76 $\mu\text{g g}^{-1} \text{h}^{-1}$, 1.87 $\mu\text{g g}^{-1} \text{h}^{-1}$ and 2.71 $\mu\text{g g}^{-1} \text{h}^{-1}$ for 25 ppm, 50 ppm and 27 ppm soils, respectively (Table 4.2). PCB concentration had a significant negative correlation dehydrogenase activity ($r = 0.67$, $p < 0.001$).

Table 4.2 Summary of soil total microbial biomass carbon (mic_c) and dehydrogenase activity before the laboratory experiments, presented as mean values of three replicates (\pm one standard error, SE).

Soil	mic_c mg kg^{-1}	Dehydrogenase activity $\mu\text{g g}^{-1} \text{h}^{-1}$
Control	u.d.l. ¹	5.65 (± 0.54)
25 ppm	9.31 (± 5.82)	1.76 (± 0.20)
50 ppm	7.41 (± 0.10)	1.87 (± 0.15)
27 ppm	57.2 (± 12.5)	2.71 (± 0.08)

¹under detection limits

Soil DNA extraction and quantification

Soil DNA extractions checked by gel electrophoresis are presented in Figure 4.1 and 4.2, and the DNA yields are presented with two different techniques in Figure 4.3.

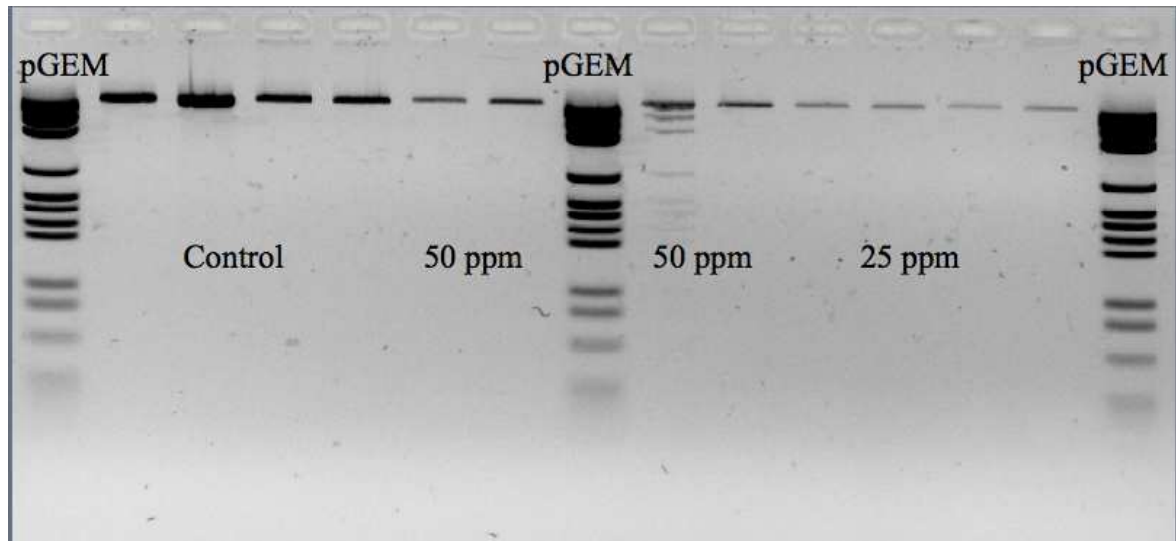


Figure 4.1 DNA extractions on a 1.5 % agarose gel from the soil samples. Four first bands from the left illustrate control soils, four next ones 50 ppm soil and four last ones 25 ppm soil

Soil DNA was obtained from all samples, however the yields were quite low for the polluted soils (Figure 4.3). The detection of soil DNA was strongest for control soils (Figure 4.1) and lowest for the anaerobic polluted soil samples, based on the gel electrophoresis pictures (Figure 4.2).

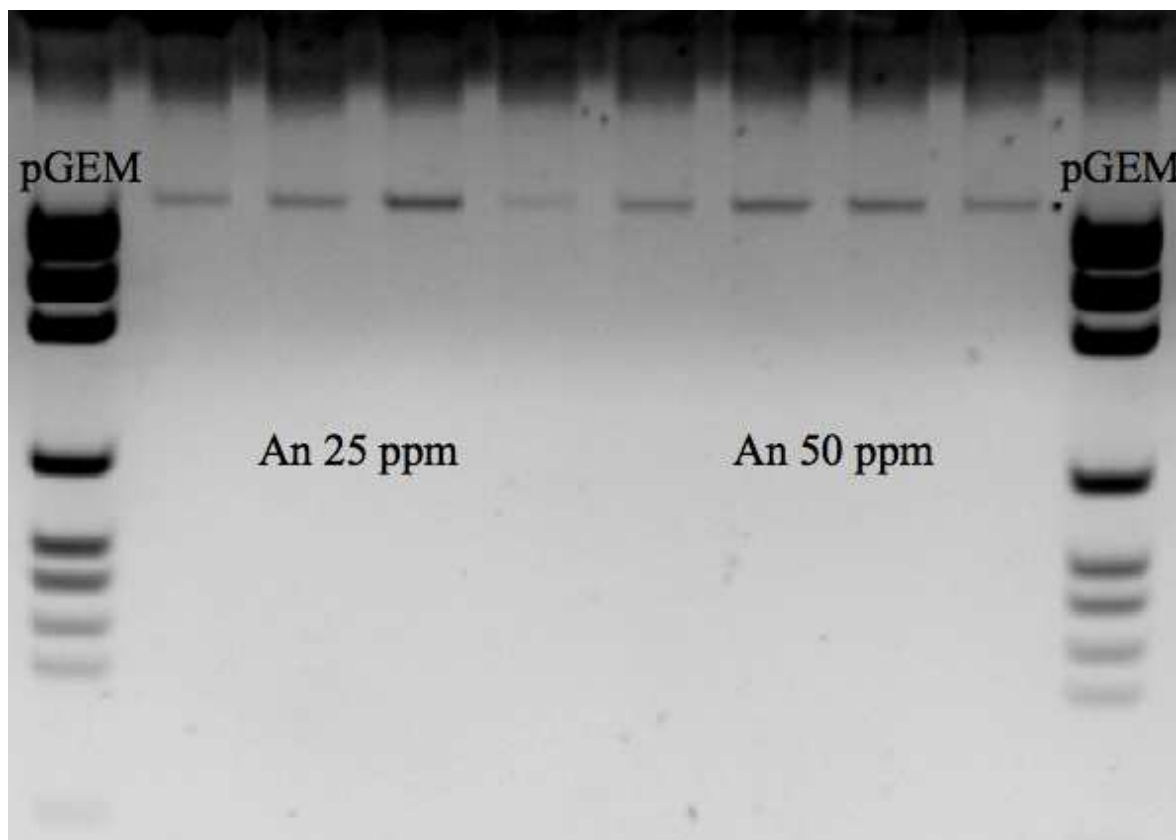


Figure 4.2 DNA extractions on a 1.5 % agarose gel from the anaerobic soil samples. Four first bands from the left illustrate anaerobic 25 ppm soils and the four next ones anaerobic 50 ppm soils. pGEM denotes size standard for DNA bands.

In general, the soil DNA yields were significantly higher ($p < 0.05$) with the NanoDrop® method than the PicoGreen® (Figure 4.3). When analysed separately there was no significant difference between the methods for control soils and 50 ppm soils. In contrast, the difference between the two chosen methods was significant for 25 ppm soils ($p < 0.05$), anaerobic 25 ppm soils ($p < 0.001$) and anaerobic 50 ppm soils ($p < 0.001$). In all cases except the control soils, PicoGreen® method gave a lower DNA yield than NanoDrop® method. DNA yields did not correlate with PCB concentration of the soils.

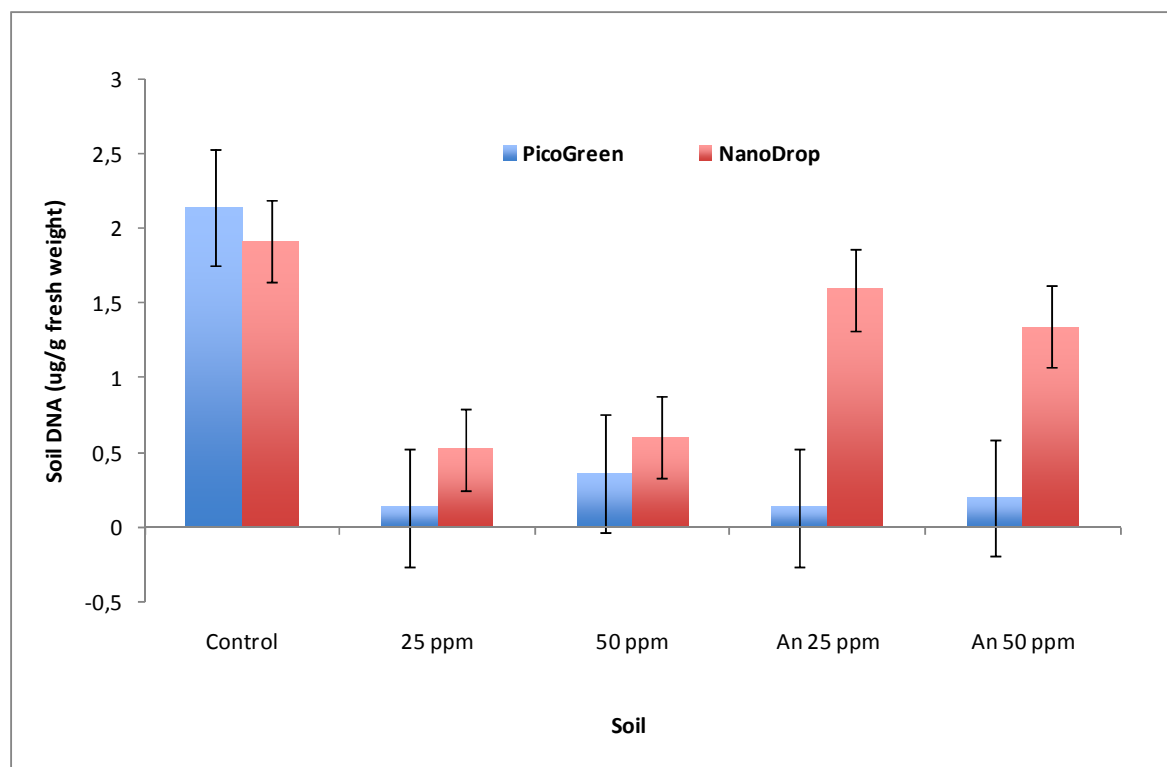


Figure 4.3 Soil DNA yields presented with two different methods, PicoGreen® and NanoDrop®. An indicates anaerobic soils. Columns represent mean \pm SE ($n = 4$).

Bacterial community analysis with length heterogeneity PCR (LH-PCR)

Good PCR products were obtained with primers used for the general analysis of bacterial community structure, as shown in Figure 4.4 and 4.5).

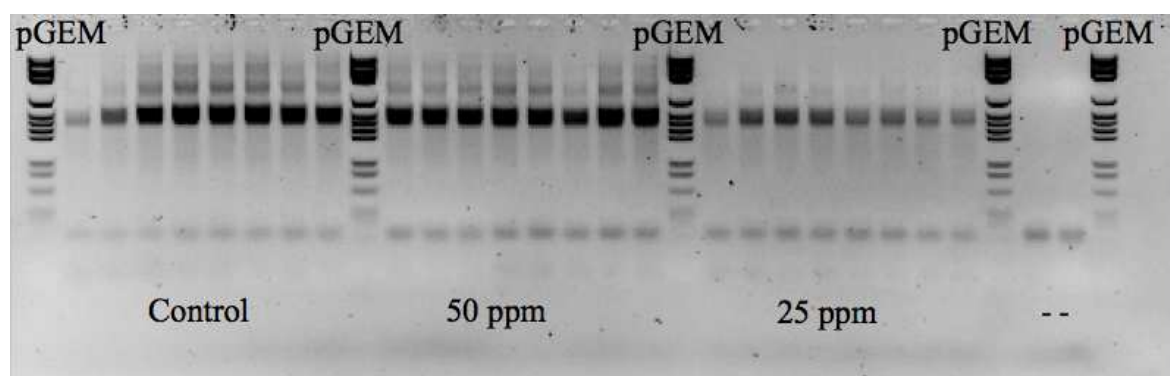


Figure 4.4 Presentaion of PCR products obtained with primers used for the general analysis of bacterial community structure on a 1.5 % agarose gel. – denotes negative control.

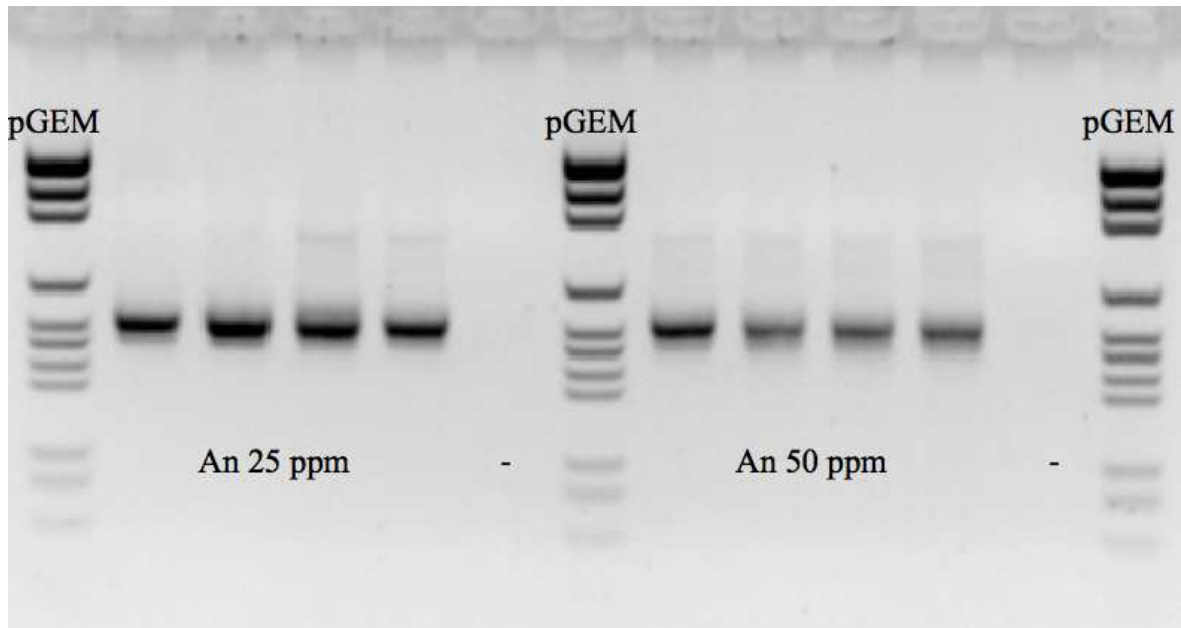


Figure 4.5 Presentation of PCR products obtained with primers used for the general analysis of beacterial community structure on a 1.5 % agarose gel. – denotes negative control.

Figure 4.6 presents the average LH-PCR profiles obtained from the soil samples. The aerobic samples form a rather similar picture (control, 25 ppm and 50 ppm soils), whereas the anaerobic samples formed a less diverse picture of the bacterial community. Especially one peak (approximately 520 bs) became much more common in the anaerobic samples and the peaks with shorter fragment lengths become nearly absent.

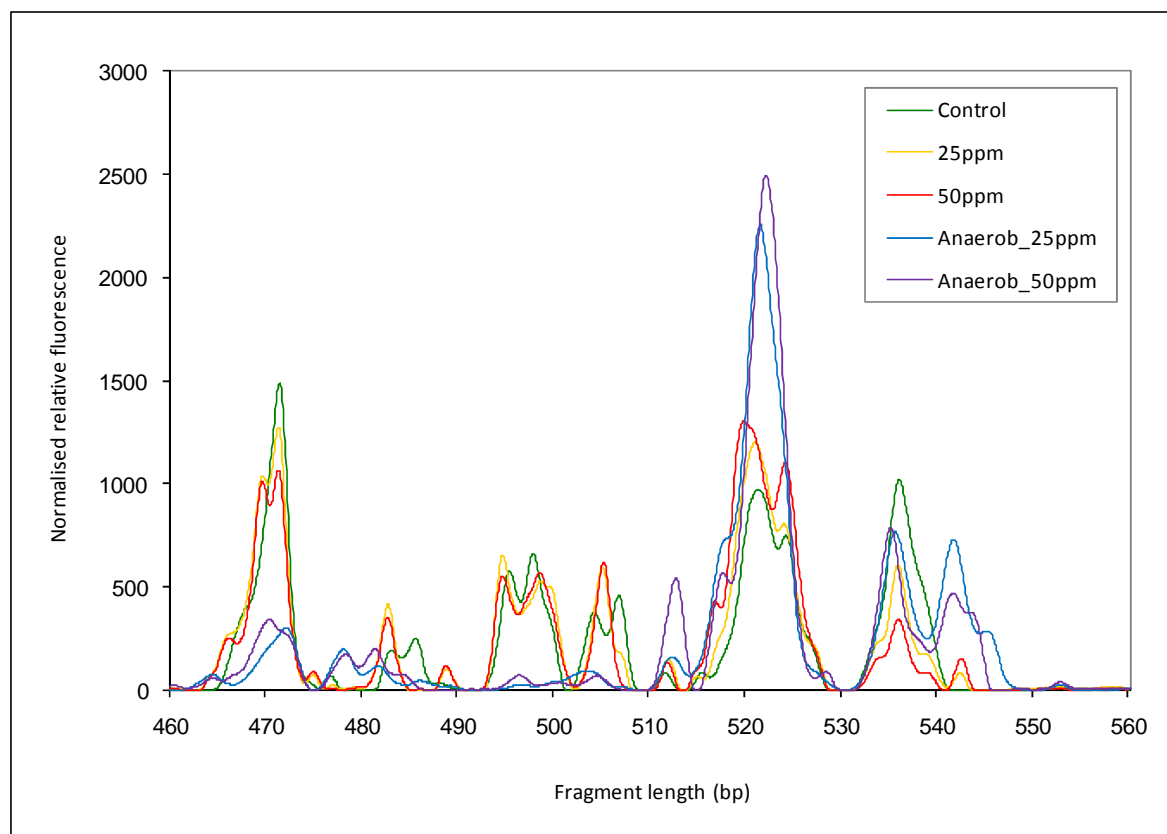


Figure 4.6 Averaged LH-PCR profiles of bacterial communities on the different soils (control, 25 ppm, 50 ppm, anaerobic 25 ppm and anaerobic 50 ppm). Figure from Anu Mikkonen, University of Helsinki, Finland.

Table 4.3 shows the similarities of the bacterial communities in the average profiles. The control soil samples were closest to the aerobic 25 ppm and 50 ppm soils (similarity 91.0 and 82.2, respectively), whereas the anaerobic 25 ppm and 50 ppm soils differed much more from the control (similarity 56.9 and 58.9, respectively). The aerobic 25 ppm and 50 ppm were fairly similar (96.2), as well as the bacterial communities from anaerobic 25 ppm and 50 ppm soils (similarity 95.8).

Table 4.3 Similarities (% of Pearson correlation) of bacterial communities in the average profiles.

	50 ppm	25 ppm	Control	Anaerobic 25 ppm	Anaerobic 50 ppm
50 ppm	100				
25 ppm	96.2	100			
Control	82.2	91.0	100		
Anaerobic 25 ppm	68.6	63.0	56.9	100	
Aerobic 50 ppm	70.6	66.7	58.9	95.8	100

Figure 4.7 further shows the clustering of the average LH-PCR profiles. Ward dendrograms on the left hand side of the figure shows that control soils grouped on their own, aerobic 25 ppm and 50 ppm soils as one group and anaerobic 25 ppm and 50 ppm soils represent another group of soil microorganisms.

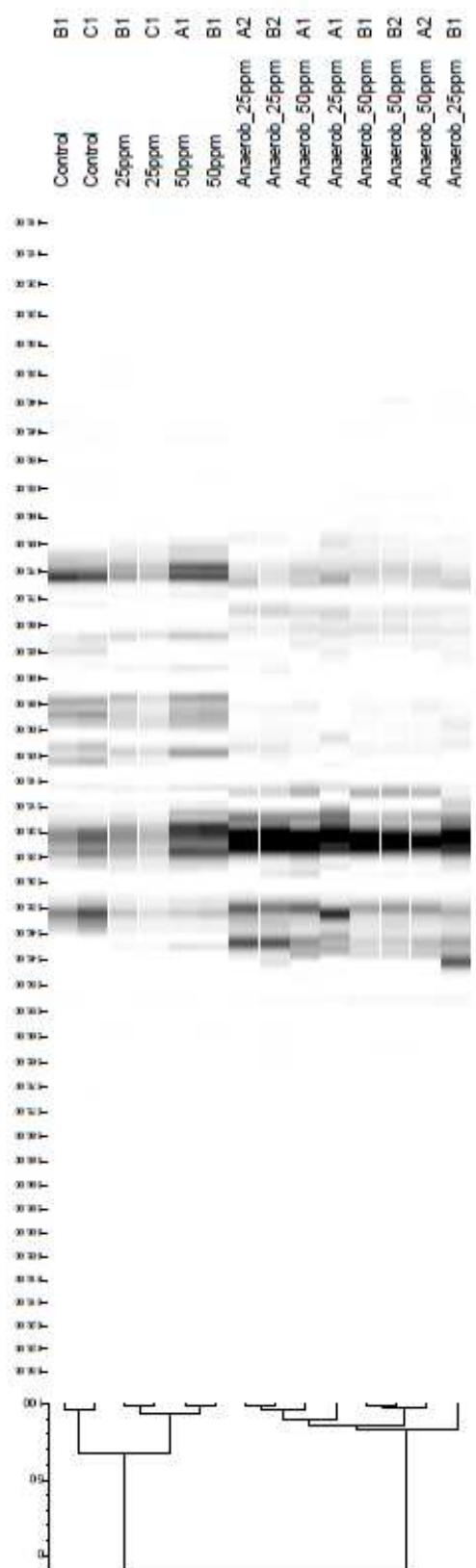


Figure 4.7 Similarity and clustering of average LH-PCR profiles. The fingerprint area included in the analysis was 460-565 bases and clustering was based on Pearson correlation, similarities illustrated in Ward dendrogram.

PCR amplification of PCB degrading genes

The PCR amplification of aerobic *bphA* was successful whereas the other genes were not successfully amplified. However, *bphA* was only detected in the aerobically stored soils and not from the anaerobic soils. Figure 4.8 below presents that lesser quantities of the *bphA* gene were detected in the control soils than in the polluted soils. The amplification was the strongest with the highest pollution level, i.e. 50 ppm. No other genes tested in this study were detected by gel electrophoresis.

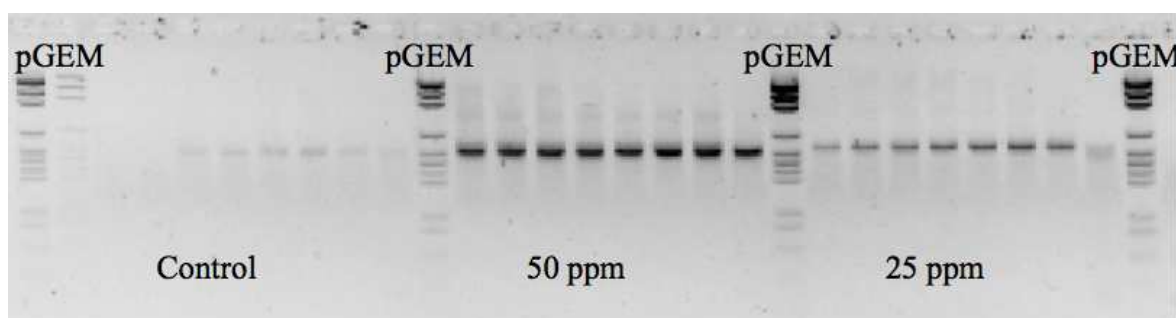


Figure 4.8 Amplification of PCR products from the *bphA* gene specific primers on 1.5 % agarose gel. pGEM denotes the DNA Markers that functioned as molecular size standards.

4.2 Experiment 1 – Biostimulation effect on PCB degradation and soil biological properties

In general, no detected degradation of total PCBs was observed with biostimulation in experiment 1, which consisted of different fertiliser treatments and a treatment with plant detritus and roots. A change in congener distribution was however detected at the end of the biostimulation experiment (Figures 4.9 and 4.10).

Figure 4.9 presents the 25 pm soils and the relative abundance of nine different PCB congeners; PCB 28, 52, 101, 118, 138, 153, 170, 180 and 187. The relative abundance of the less chlorinated congeners is higher in the anaerobic samples than in the aerobic samples. One treatment, i.e. treatment with plant detritus and roots, changed the congener distribution the most. It resulted in a decrease in the higher chlorinated PCB 180 and

increase in the less chlorinated congeners. Changes were, however, statistically insignificant.

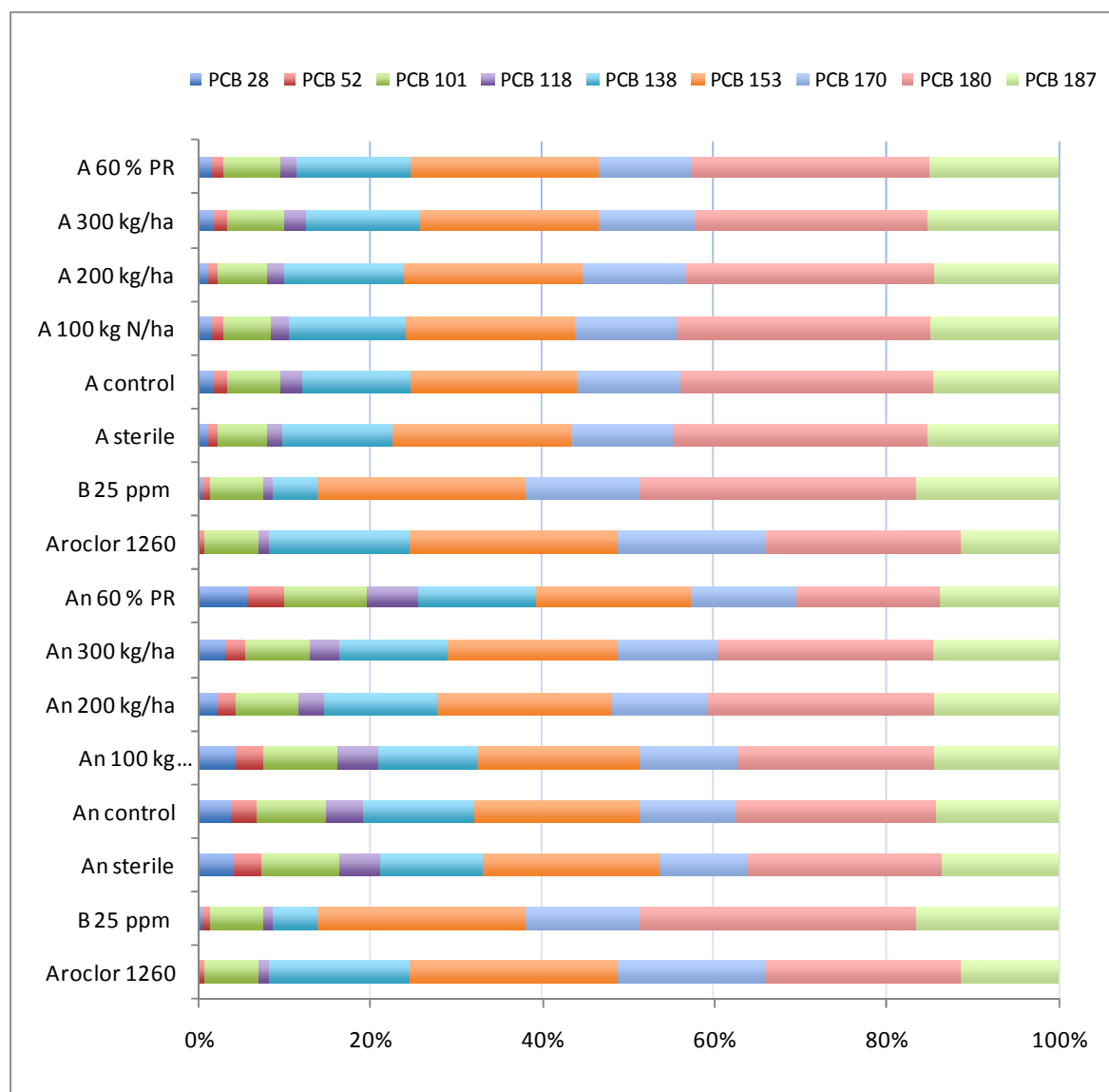


Figure 4.9 PCB congener distribution of the 25 ppm soils after experiment 1. A denotes aerobic treatment, An anaerobic treatment and B represent values before the treatments.

Figure 4.10 revealed a similar pattern for the 50 ppm soils after experiment 1, but no significant difference were observed either. Again, the plant detritus and roots treatment yielded higher relative abundance of less chlorinated congeners and less relative

abundance of especially PCB 180. Aerobic treatment with 200 kg N ha⁻¹ showed an increase in abundance of PCB 153 and decrease in PCB 170.

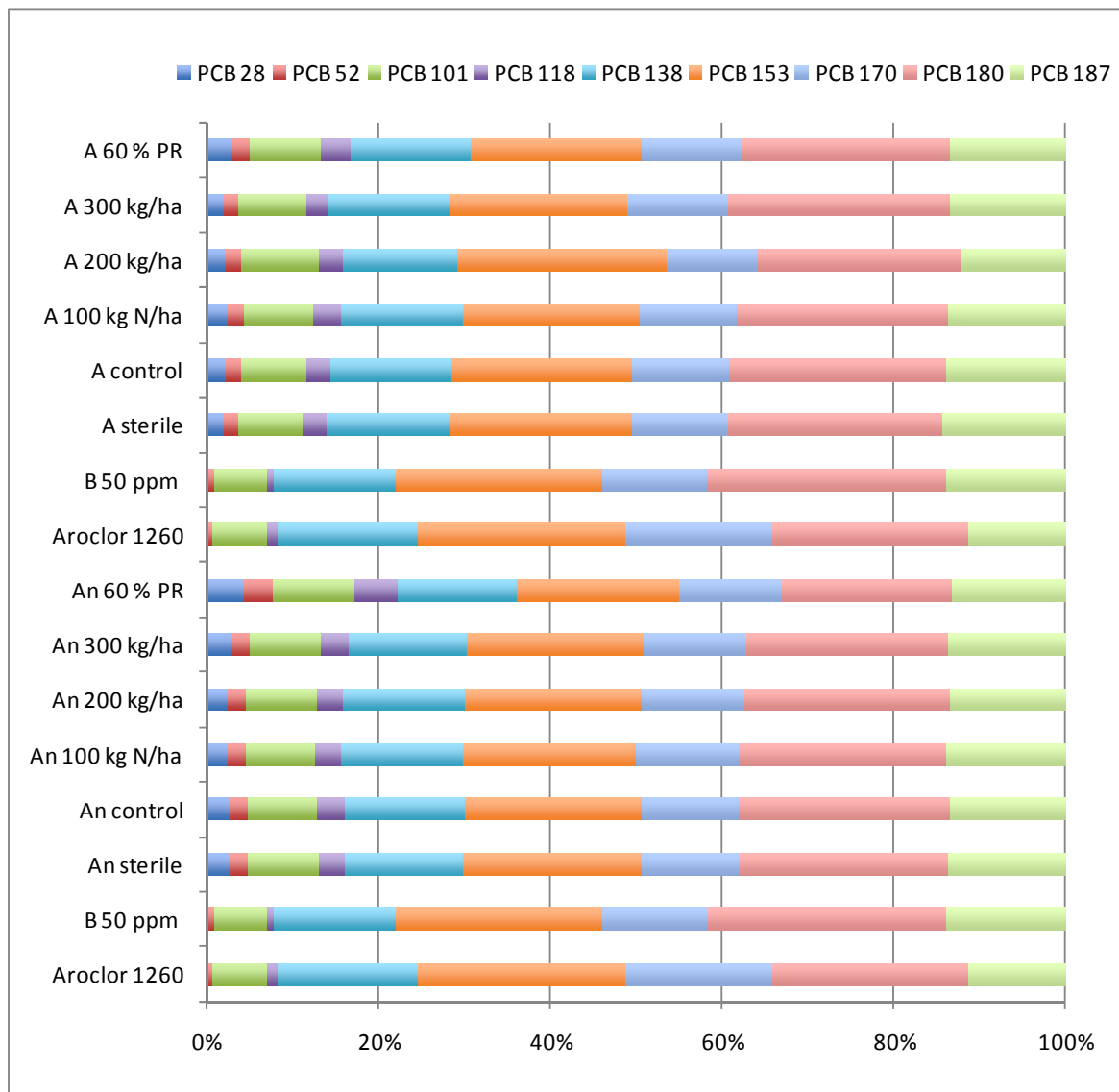


Figure 4.10 PCB congener distribution for the 50 ppm soils after experiment 1. A indicates aerobic treatment, An indicates anaerobic treatment and B values before treatments.

In general there were no correlations between mic_c , dehydrogenase activity and PCB concentration in the studied soils during experiment 1. When all 25 ppm soils and mic_c were analysed separately there was a significant positive correlation ($r = 0.85$, $p < 0.001$) between PCB concentration and mic_c . Figure 4.11 represents the mic_c for all the treatments and both 25 ppm and 50 ppm soils, before and after the preliminary laboratory experiment.

The highest values for mic_c were generally measured for plant detritus and roots treatments (10900 mg kg^{-1} , 1180 mg kg^{-1} and 97.3 mg kg^{-1} for anaerobic 25 ppm, aerobic 25 ppm and aerobic 50 ppm soils, respectively). In general, lowest values were measured for anaerobic treatments in 50 ppm soils.

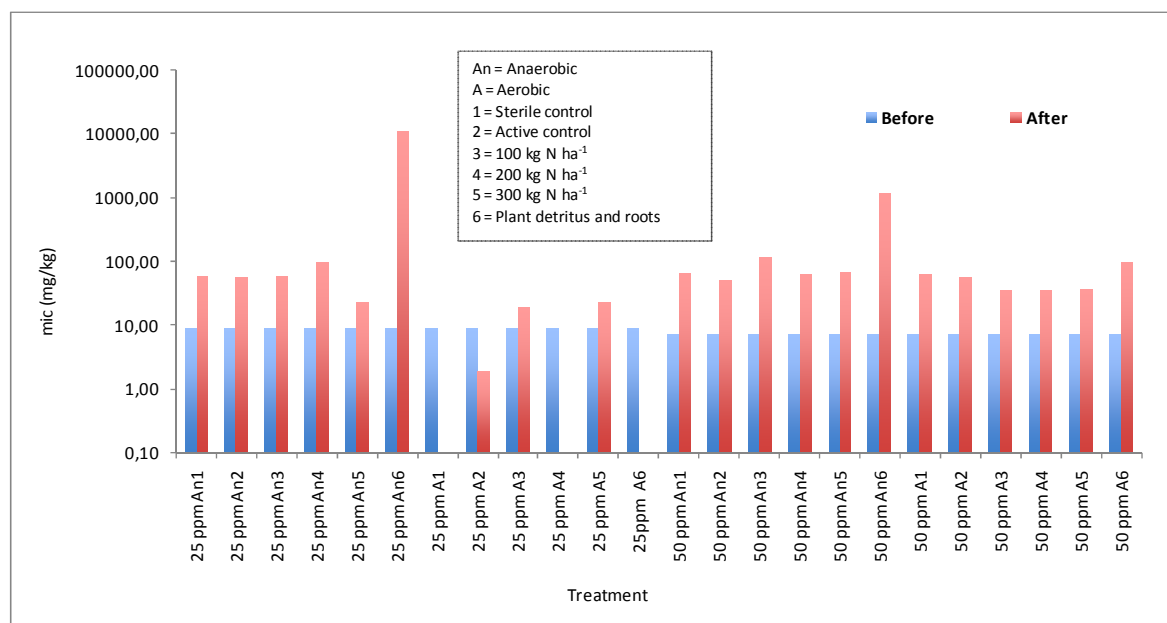


Figure 4.11 Presentation of mic_c before and after experiment 1. Columns present mean values of three replicates for before and one measurement from a sample bulked from three samples after the treatments.

Figure 4.12 illustrates the changes in the soil dehydrogenase activity during experiment 1. The soil dehydrogenase activity increased in the majority of the treatments for the 25 ppm soils, but not for the 50 ppm soils. Clearly the most increase was observed in the treatments with plant detritus and roots in all soils. Treatment with 100 kg N ha^{-1} also resulted in an increase in the soil dehydrogenase activity in all soils. A clear decrease in dehydrogenase activity was observed in 25 ppm soils with aerobic 300 kg N ha^{-1} treatment, and in the same aerobic treatment for 50 ppm soils. In addition, the anaerobic treatment with 200 kg N ha^{-1} decreased the dehydrogenase activity.

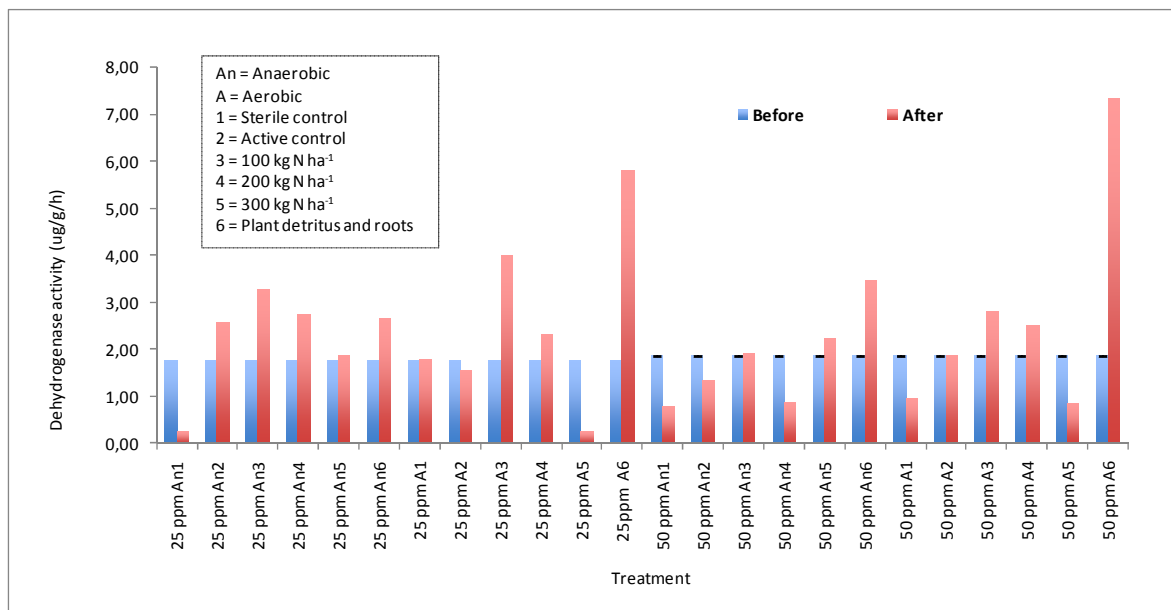


Figure 4.12 Presentation of dehydrogenase activity before and after experiment 1. Columns represent mean values of three replicates for before and one measurement from a sample bulked from three samples after the treatments.

4.2.1 Soil respiration (CO₂)

Soil respiration was measured after two and four months of the four-month experiment, as shown in Table 4.4. Generally, soil respiration did not differ significantly between the two measurements, however being greatest after the initial two months but decreasing thereafter. The difference for 25 ppm soils was however significant between measurements after two months and four months ($p < 0.05$). Highest CO₂ concentrations were measured in the plant detritus and root microcosm treatments, for all soils in general.

Table 4.4 Summary of mean soil respiration (\pm standard error) after two and four months of experiment 1 ($n = 5$). 25 represents soil with 25 ppm of PCB and 50 represent soil with 50 ppm of PCB. An represents anaerobic treatment and A aerobic treatment.

Treatment	2 months	4 months
	$\mu\text{g CO}_2 \text{ g}^{-1} \text{ day}^{-1}$	$\mu\text{g CO}_2 \text{ g}^{-1} \text{ day}^{-1}$
25An Sterile control	0.19 (± 0.01)	0.01 (± 0.02)
25An Active control	2.19 (± 0.06)	0.06 (± 0.05)
25An Fertilizer (100 kg N ha ⁻¹)	3.64 (± 0.10)	0.53 (± 0.39)
25An Fertilizer (200 kg N ha ⁻¹)	3.96 (± 0.10)	0.18 (± 0.21)
25An Fertilizer (300 kg N ha ⁻¹)	4.76 (± 0.07)	0.08 (± 0.08)
25An Plant detritus and roots	11.8 (± 1.02)	3.50 (± 2.09)
25A Sterile control	7.01 (± 1.21)	0.04 (± 0.05)
25A Active control	4.55 (± 1.32)	0.11 (± 0.09)
25A Fertilizer (100 kg N ha ⁻¹)	14.7 (± 7.02)	0.81 (± 0.16)
25A Fertilizer (200 kg N ha ⁻¹)	1.28 (± 0.80)	1.08 (± 0.54)
25A Fertilizer (300 kg N ha ⁻¹)	0.05 (± 0.05)	1.00 (± 0.29)
25A Plant detritus and roots	9.08 (± 3.14)	10.6 (± 7.52)
50An Sterile control	0.16 (± 0.03)	0.00 (± 0.02)
50An Active control	2.61 (± 0.10)	0.98 (± 0.41)
50An Fertilizer (100 kg N ha ⁻¹)	3.06 (± 0.06)	0.76 (± 0.25)
50An Fertilizer (200 kg N ha ⁻¹)	3.70 (± 0.04)	1.18 (± 0.11)
50An Fertilizer (300 kg N ha ⁻¹)	3.82 (± 0.09)	1.18 (± 0.19)
50An Plant detritus and roots	20.7 (± 5.22)	4.76 (± 3.34)
50A Sterile control	4.09 (± 1.58)	0.14 (± 0.06)
50A Active control	2.60 (± 0.43)	0.17 (± 0.06)
50A Fertilizer (100 kg N ha ⁻¹)	5.23 (± 4.52)	0.27 (± 0.13)
50A Fertilizer (200 kg N ha ⁻¹)	0.89 (± 0.60)	1.65 (± 1.45)
50A Fertilizer (300 kg N ha ⁻¹)	3.77 (± 1.50)	0.86 (± 0.62)
50A Plant detritus and roots	103 (± 13.2)	5.56 (± 0.79)

4.2.2 Bioavailability of PCBs in soil to earthworms

The earthworms in the control soil appeared to be in good condition throughout the study whereas the earthworms in the polluted soils faced more mortality. Of the 30 earthworms added in the beginning of the experiment only 5 remained alive in the less polluted soil mixture (12.5 ppm) and 9 in the more polluted soil mixture (25 ppm). In addition, 10 earthworms in the more polluted soil mixture were found in very poor condition and almost entirely incorporated into the soil mixture. The PCB accumulation in earthworms, in 12.5 ppm PCB soil mixture and 25 ppm PCB soil mixture, is presented in Table 4.7 and Figures 4.12, 4.13 and 4.14.

Bioaccumulation factor (BAF, PCB concentration in earthworms (mg g^{-1} fresh weight) divided by PCB concentration in the soil mixture (mg g^{-1} dry weight)) for earthworms was 0.89 and 0.82 for earthworms in 12.5 ppm PCB soil mixture and in 25 ppm PCB soil mixture, respectively.

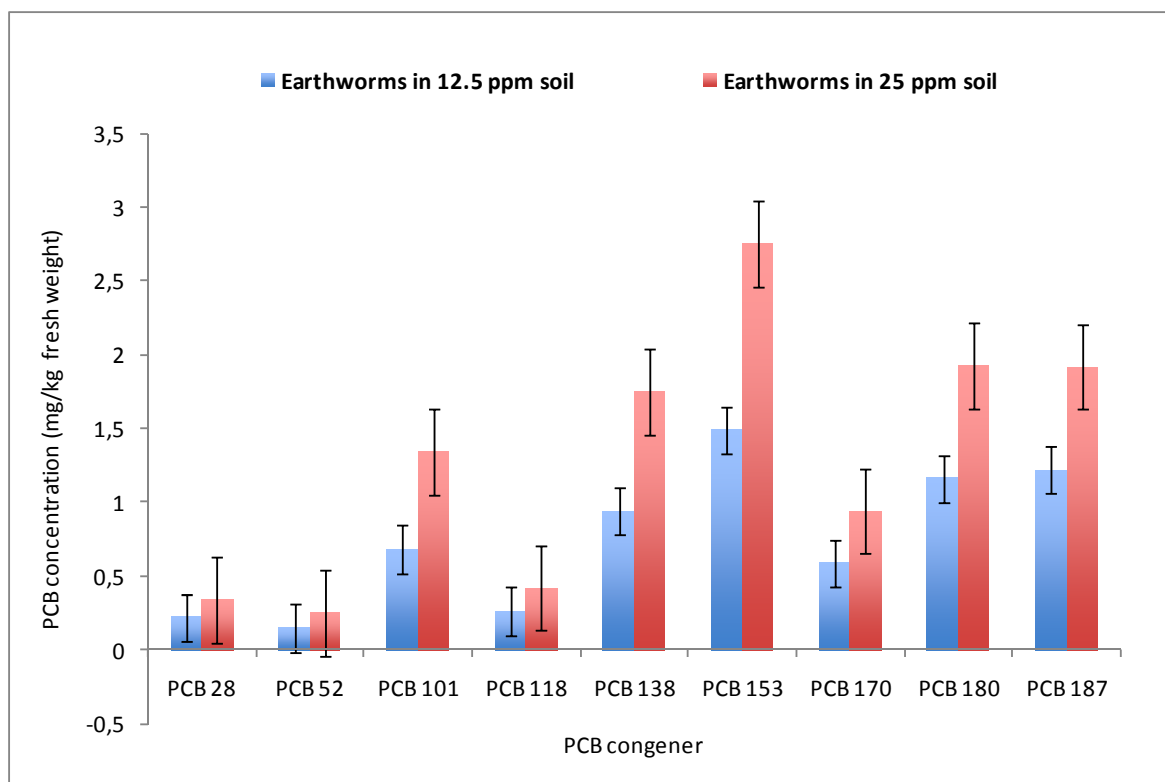


Figure 4.13 PCB concentrations in earthworms (*Eisenia foetida*) in mg kg^{-1} fresh weight determined by Jensen method. Columns represent means for three and four replicates for 12.5 ppm PCB soil mixture and 25 ppm PCB soil mixture, respectively.

Figures 4.14 and 4.15 represent PCB concentration for individual congeners and total PCBs, in earthworms from 12.5 ppm soil mixture and 25 ppm soil mixture. The earthworms accumulated both higher chlorinated and less chlorinated PCBs and the total PCBs were significantly higher than even sum of 9 individual congeners. Earthworms accumulated PCB 153 in greatest quantity in both soil mixtures. The highly chlorinated PCB 180 and 187 were accumulated in great quantity, as well as PCB 138 and 101. This pattern of accumulation represents the distribution of PCB congeners in the Aroclor 1260 mixture (Figure 4.16).

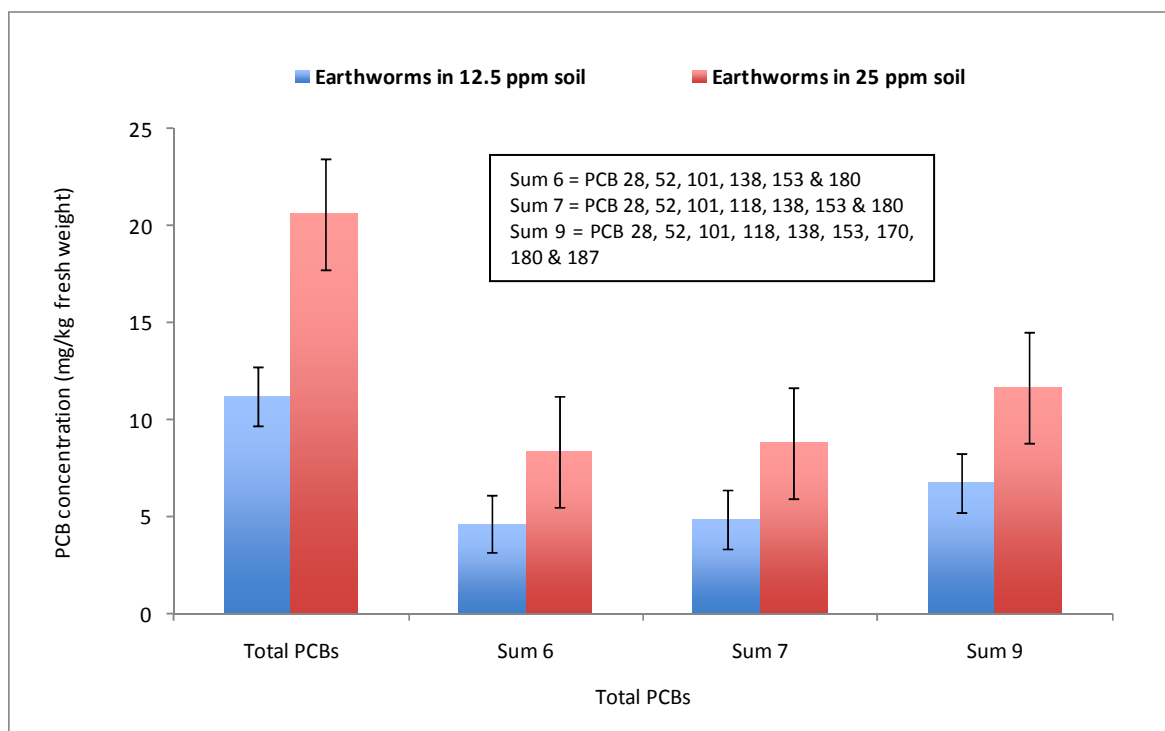


Figure 4.14 Total PCBs in earthworms (*Eisenia foetida*). Columns represent means for three and four replicates for earthworms in 12.5 ppm soil and earthworms in 25 ppm soil, respectively.

Furthermore Figure 4.16 shows the relative abundance of individual PCB congeners in the earthworm samples, compared to the relative abundance of individual congeners in the soils mixture and Aroclor 1260. All the nine PCB congeners studied were accumulated in the samples, of which PCB 153 in greatest quantity.

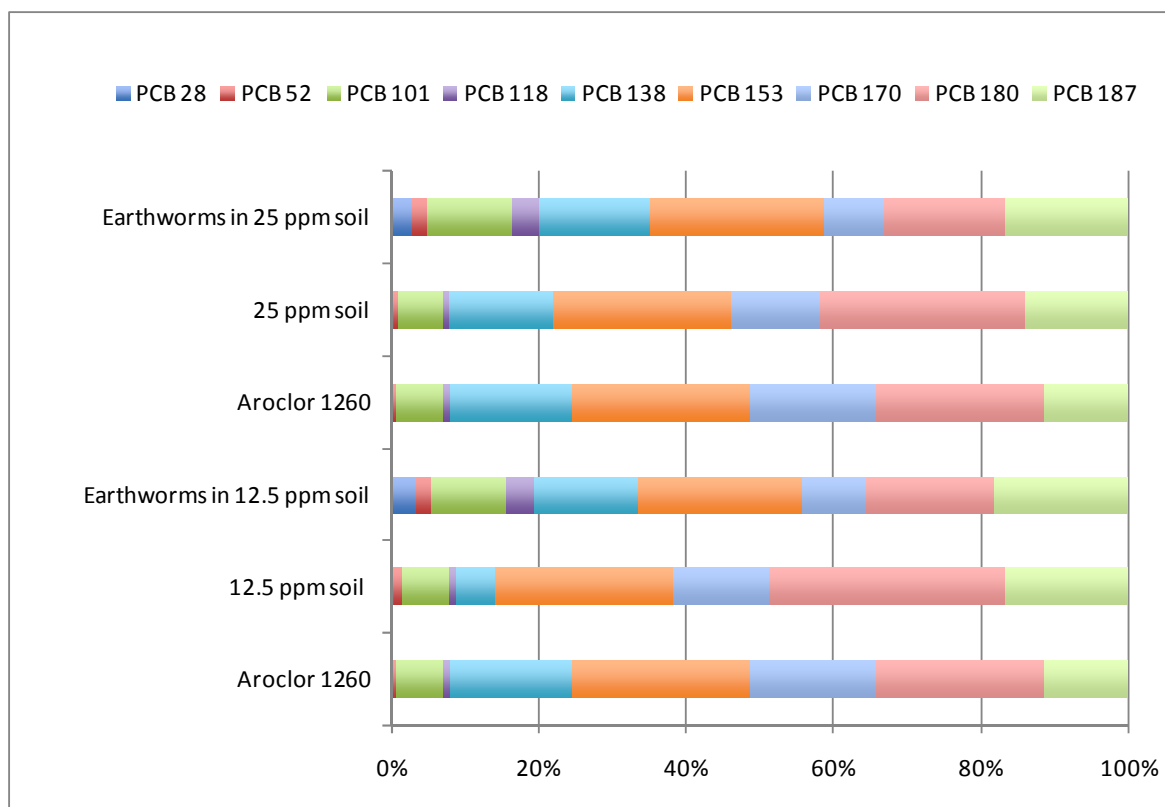


Figure 4.15 Distribution of accumulated PCB congeners in earthworms in 10 days.

4.3 Experiment 2 – Biostimulation and temperature effect on PCB degradation and soil biological properties

Contrary to experiment 1, experiment 2 was carried out at two temperatures, i.e 10°C and 30°C, but at one pollution level, i.e. 27 ppm. For the most part of the treatments, no detectable decrease in total PCBs were observed. Degradation of total PCBs was obtained in the aerobic pine needles treatment at 10°C, but not at 30°C. The pine needles treatment yielded a 37.7 % reduction in the total PCBs after two months, final concentration being 16.9 ppm compared to 27.0 ppm at the beginning of the experiment.

Figure 4.17 presents the congener distribution in the different treatments from the secondary experiment carried out at 10°C. No statistically significant changes were observed after experiment 2.

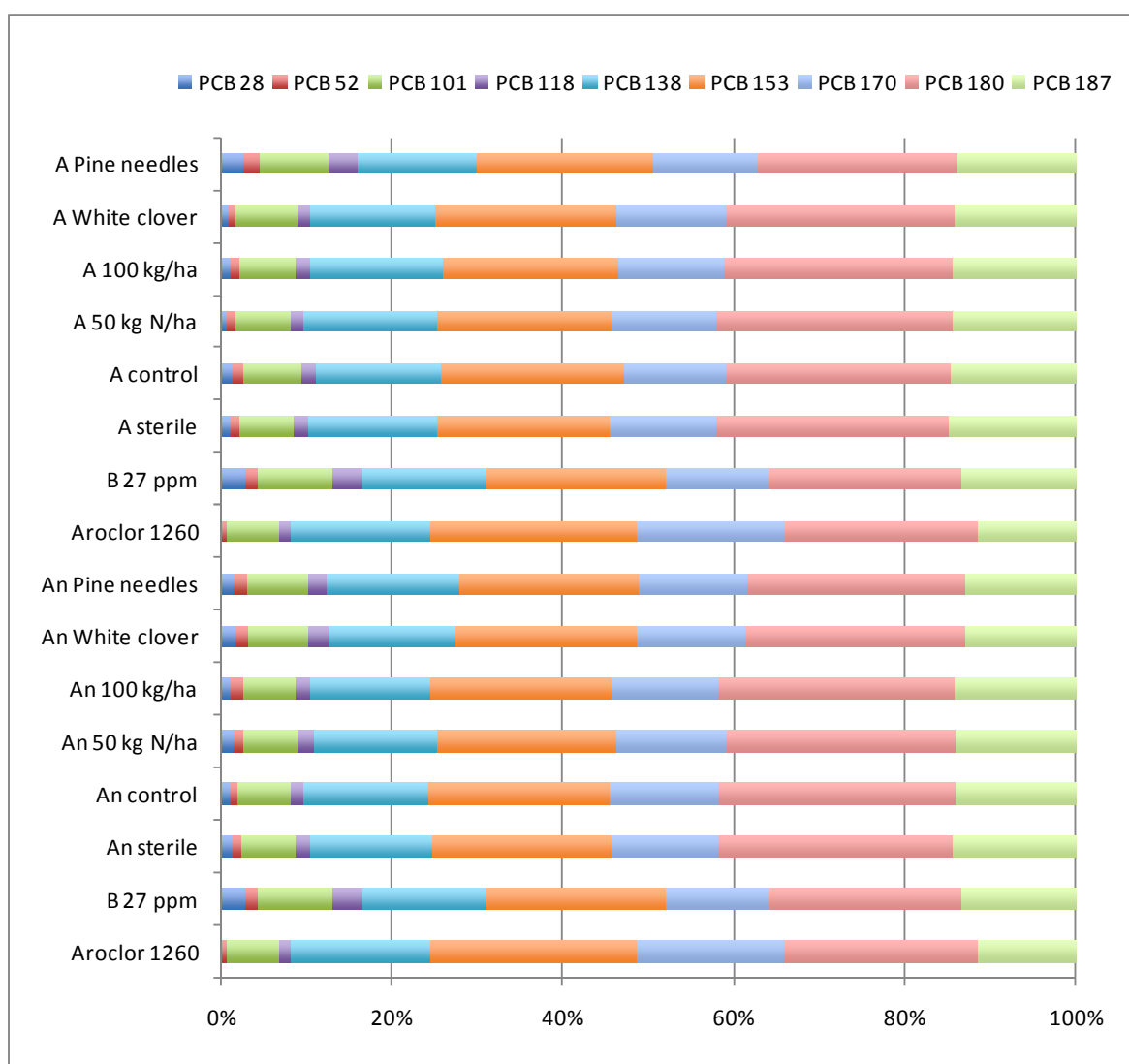


Figure 4.16 PCB congener distribution after experiment 2 which was carried out at 10°C. A indicates aerobic treatment, An indicates anaerobic treatment and B values before treatments.

Congener distribution in the treatments at 30°C is presented in Figure 4.18. The relative abundance of different congeners remained rather similar in all treatments throughout the experiment, and no changes were statistically significant.

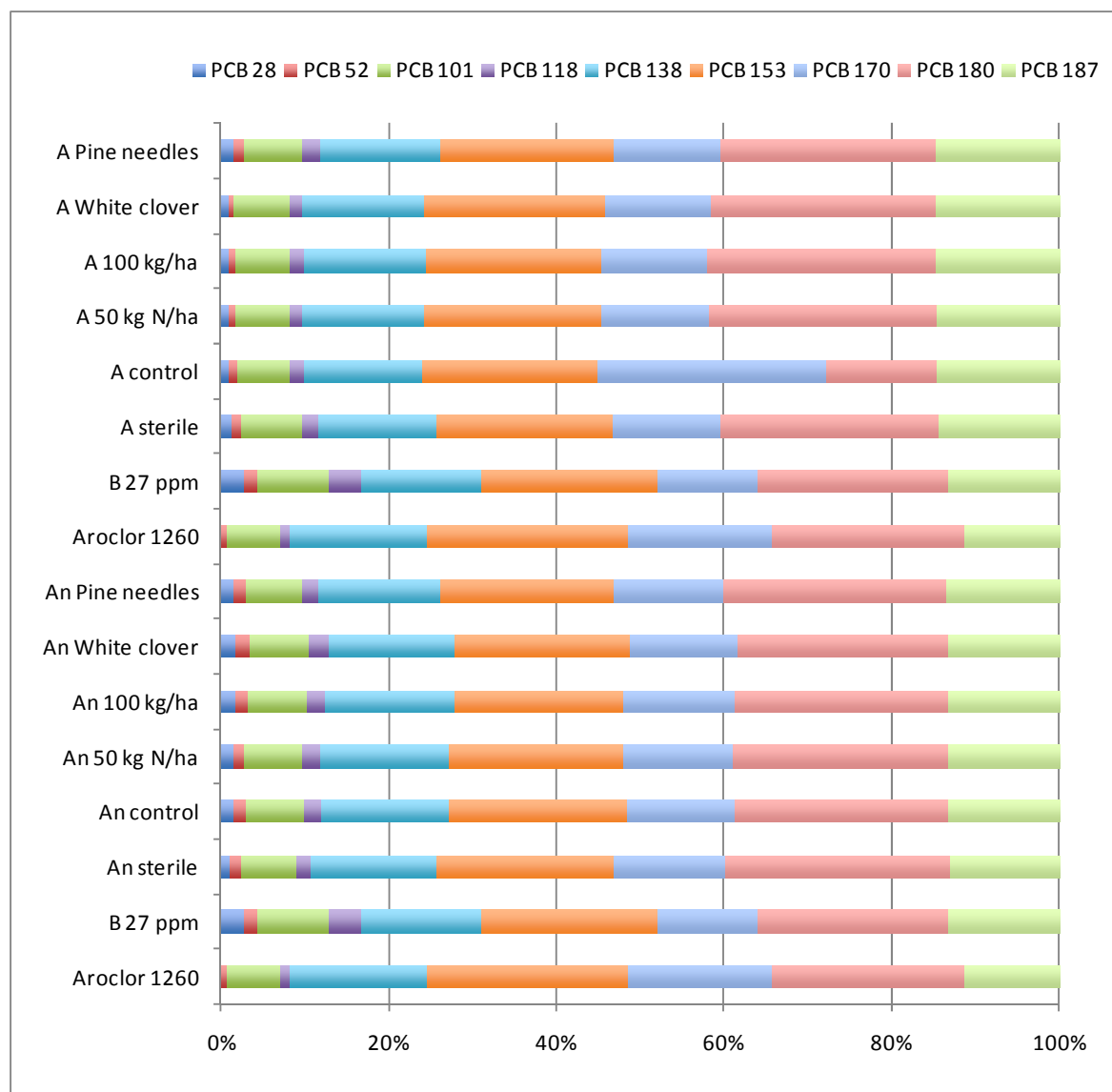


Figure 4.17 PCB congener distribution after experiment 2 which was carried out at 30°C. A indicates aerobic treatment, An indicates anaerobic treatment and B values before treatments.

Large variation was obtained in mic_c values between the treatments, and mic_c was above detection limits only in one treatment, i.e. anaerobic pine needles treatment, at 10°C (Figure 4.19). At 30°C, the aerobic and anaerobic white clover treatments had the greatest mic_c . Mic_c was also above detection limits in anaerobic 100 kg N ha⁻¹ treatment and pine needles treatment, but not in the any other treatment microcosm.

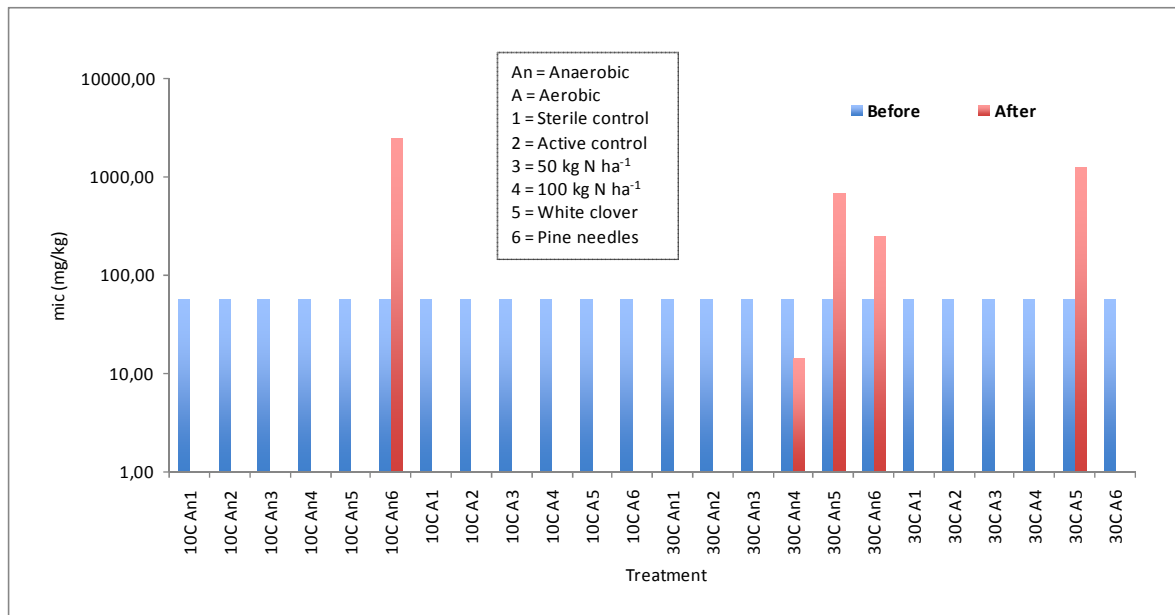


Figure 4.18 Presentation of mic_c in the soil before and after experiment 2. Columns represent mean values of three replicates for before and one measurement from a sample bulked from three samples after the treatments. 10C denotes treatments at 10°C and 30C denotes treatments at 30°C. If no red column is presented for a treatment, mic_c was under detection limits.

The highest values for dehydrogenase activity were measured for white clover treatments and second highest for the pine needles treatment (Figure 4.20). In general, the dehydrogenase activity was lower after the treatments, except for white clover and pine needles treatments. The white clover treatments yielded the highest dehydrogenase activities. All treatments with nitrogen addition (50 and 100 kg N ha⁻¹) resulted in decrease of dehydrogenase activity.

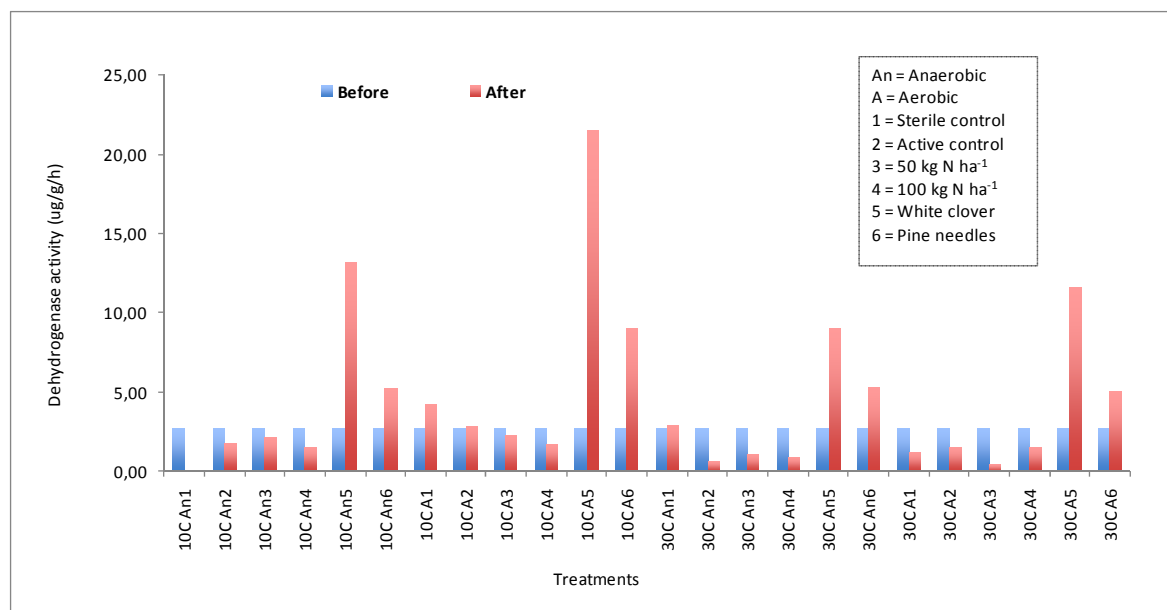


Figure 4.19 Demonstration of dehydrogenase activity before and after experiment 2. Columns represent mean values of three replicates for before and one measurement from a sample bulked from three samples after the treatments. 10C denotes treatments at 10°C and 30C denotes treatments at 30°C

5 Discussion and Conclusions

5.1 Effect of soil PCB concentration on soil biogeochemical properties

The positive relationship between soil PCB concentration and soil pH may be a reflection on the lower C_{tot} in the polluted soils compared to the unpolluted soils (Brady and Weil, 2004). The existence of soil organic matter, and especially humus, has been linked to lower soil pH (Killham, 1994), and this study agrees with these findings. The variation in soil pH in all soils was well within the reported range for Icelandic Andosols (e.g. Guicharnaud, 2009), and for Brown Andosols in particular (e.g. Arnalds, 2004; Sigurgeirsson *et al.*, 2005). The moisture content was lower when PCB concentration was higher, which could indicate differences in the sampling points and the overall wetness of the sampling area. The lower C_{tot} and N_{tot} values in the polluted soils compared to unpolluted soils could also explain the low moisture content, (Brady and Weil, 2004). The negative relationship between PCB concentration and WHC could be explained by pollution forming oily and sticky (Erickson, 1997) coating on the surface of soil particles and therefore the soil becomes more hydrophobic and WHC decreases (Aislabie *et al.*, 2006). The low C_{tot} , in which carbon for the most part comes from the PCBs, may be another reason for low WHC (Rey *et al.*, 2005). C_{tot} , N_{tot} and dehydrogenase activity decreased with higher pollution level, signalling less healthy soils, i.e. the soils would not be able to sustain plant productivity with higher degree of pollution (Doran and Zeiss, 2000). Kizilkaya *et al.* (2004) showed positive relationship between C_{tot} and dehydrogenase activity, which is in accordance to the current study. Furthermore the negative relationships between PCB concentration and other biogeochemical properties, including WHC, indicate less fertile soils when pollution increases, of which Andosols are known of (Dahlgren *et al.*, 2004). CEC was relatively low compared to Icelandic soils in general (Sigurgeirsson *et al.*, 2005) and lower for polluted soils than the control soils. This could be explained by the lower C_{tot} values in polluted soils, since organic matter is one of the most important controlling factors of CEC (Brady and Weil, 2004; Dahlgren *et al.*, 2004). Another controlling factor is the total amount of colloidal material in soils, i.e. allophane, ferrihydrite and organic matter content in Icelandic soils (Dahlgren *et al.*, 2004; Sigurgeirsson *et al.*, 2005).

Allophane and ferrihydrite contents represented typical values for Icelandic Brown Andosols (Sigurgeirsson *et al.*, 2005) although lower than reported for Icelandic Andosols (Guicharnaud and Paton, 2006), which further indicates the low C_{tot} values as a primary determinant for low CEC.

The snapshot of the soil microbial community that was created by LH-PCR gave an interesting insight into the differences in the microbial community in aerobic versus anaerobic soils as well as unpolluted versus polluted soils. This is in accordance with Killham (1994), in which the differences in soil microbial communities in extreme conditions are highlighted. It may also reflect the different microbial communities being active in anaerobic biodegradation of PCBs (e.g. Wiegel and Wu, 2000; Vasileya and Strijakova, 2007) than in aerobic biodegradation (Wiegel and Wu, 2000; Pieper, 2005). In the current study the microbial community in the anaerobic soils was less diverse (Figure 4.5) than in the aerobic soils, and therefore there may not be as many potential degradation candidates as in aerobic soils. It could also indicate the response of the microbial community to the great shift in oxygen levels, when the soils were flooded (Mikkonen, 2008). It has been previously shown that flooding may cause the aerobic bacteria to decline in numbers (Killham, 1994), which was anticipated when flooding was carried out in the current study in order to activate the anaerobic bacteria in the soils. Other factors indicating the great difference between anaerobic versus aerobic soils was the absence of the aerobic PCB degrading gene, *bphA*, after the flooding which had been anticipated (Wiegel and Wu, 2000). The greater abundance of microorganisms in aerobic topsoil compared to subsoil was also shown by Or *et al.* (2007), however the microbial communities in these different conditions may have different characteristics and preferences. Therefore it is not just the number of microorganisms that matter for a community to be active but also that the correct species are present, since the microbial metabolisms under anaerobic conditions compared to aerobic is of a different type (Wiegel and Wu, 2000; Or *et al.*, 2007). The differences between different pollution levels in the profiles generated by LH-PCR were not as great as between the oxygen conditions, but the communities were still grouped in polluted and unpolluted soils as illustrated in a Ward dendrogram (Figure 4.7). Similar results have previously been presented concurrently with different land management practices (e.g. Wu *et al.*, 2008).

5.2 PCB degradation and lack thereof

Limited PCB degradation was achieved in the experiments presented in this study, and only biostimulation with pine needles resulted in reduction of total PCBs. Reduction with the successful aerobic treatment at 10°C was 37.7 % in two months, in contrast to previous findings that Aroclor 1260 would not be susceptible for aerobic biodegradation (Crawford and Crawford, 2005). The degradation success with pine needles could be explained by the terpenes in the pine needles acting like a natural substrate for biphenyl-degrading bacteria, as described in Hernandez *et al.* (1997) and Park *et al.* (1999). It should be noted that the current successful biostimulation was carried out at 10°C compared to room temperature in the original reference. In the study by Hernandez *et al.* (1997) the same kind of pine needles stimulation was carried out as in the current study, except on Aroclor 1242, that resulted in total degradation of the 100 ppm of spiked Aroclor 1242 after six months. In the current study the same experimental setup was used with pulverized white clover as well, but without any degradation. This may be due to lack of terpenes and instead existence of flavonoids, which are phenolic compounds commonly produced by vascular plants (Donnelly *et al.*, 1994), in their structure. However, in other studies flavonoids have been identified as potential growth substrates for PCB degrading bacteria (Donnelly *et al.*, 1994; Pieper, 2005).

The treatments with plant detritus and roots (experiment 1) did not result in degradation of the total PCBs in the samples, but changed the PCB congener distribution (Figure 4.9 and 4.10). The experiment was carried out according to suggestions given by Michael Jr *et al.* (2001), except that it was carried out at the microcosm scale as opposed to the field scale approach. Michael Jr *et al.* (2001) showed no degradation of highly chlorinated PCB congeners, but 40 % decrease of less chlorinated congeners with the same 60 % addition of plant detritus and roots as in the current study. Differences in degradation results may come from differences in PCB mixtures; the soil mixture in Michael Jr *et al.* (2001) had 4 chlorines per biphenyl compared to 6 chlorines per biphenyl in this study. The initial PCB concentration of the soil mixtures also differed: being 8.9 ppm in the original reference (Michael Jr *et al.*, 2001) as opposed to 25 ppm and 50 ppm in this study. The time frame was longer in Michael Jr *et al.* (2001) compared to this study, 370 days and four months,

respectively. The biogeochemical characteristics of the soils should also be borne in mind. In this study the C:N ratio was 58.0 (25 ppm soils) and 45.1 (50 ppm soils), compared to 39.8 in the study of Michael Jr *et al.* (2001). The lack of degradation could therefore indicate lack of easily available nitrogen for the microorganisms and a need for more plant detritus and roots in this study (Michael Jr *et al.*, 2001; Brady and Weil, 2004). Other studies have also shown low degradation rates for Aroclor 1260 (Quensen III *et al.*, 1990), explained by higher degree of chlorination compared to Aroclor 1242, 1248 and 1254 mixtures. Fertilizer treatments did not decrease total PCBs neither in experiment 1 nor experiment 2, even though it has been described as one of the most cost-effective approaches to bioremediation of PCBs (Crawford and Crawford, 2005). The general lack of degradation opposes studies that have resulted in high degradation percentages such as 90 % in only two months (Zharikov *et al.*, 2007). Andosols are, however, known for retaining pollutants by colloidal material (Sigurgeirsson *et al.*, 2005) and high phosphorous retention due to soil colloids (Shoji *et al.*, 1993). This may be a likely reason for lack of degradation in this study, and fertilizer may have been sorbed to the allophane and ferrihydrite surfaces, which would make the nutrients unavailable for active soil microorganisms (Shoji *et al.*, 1993). One of the differences in experimental setup in this study compared to many successful laboratory approaches (e.g. Fagervold *et al.*, 2007; Pieper and Seeger, 2008) was that the indigenous microorganisms were biostimulated, but no bioaugmentation was induced. The addition of specific microorganisms has been described as the most successful method to stimulate degradation (Vasileya and Strijakova, 2007), however, it has mainly worked under laboratory conditions. The complexity of microbial communities *in situ* has been found surprisingly diverse (Abraham *et al.*, 2002), and therefore addition of a single microbial strain may not result in high degradation rates in the natural environment. The time frame of the current study should also be noted; experiment 1 was carried out for four months and experiment 2 for two months. Longer time frame, e.g. 50 weeks (Quensen III *et al.*, 1990) or an augmentation of a factor of three in length, could have yielded different results than in the current study.

The change in relative abundance of the individual congeners towards increase in less chlorinated congeners may indicate dechlorination of PCBs (Borja *et al.*, 2005). This was observed in experiment 1 in the anaerobic plant detritus and root treatments at both PCB

concentrations (Figures 4.9 and 4.10), and to a lesser degree in experiment 2 in the aerobic pine needles treatment at 10°C. Degradation of total PCBs was only observed in the pine needles treatment.

The PCB degradation was not temperature dependent in general or affected by PCB concentration in the microcosms. This was demonstrated in experiment 1, in which two different PCB concentrations at room temperature were biostimulated and in experiment 2 where two temperatures were studied for one PCB concentration. However, the most successful biostimulation was an aerobic pine needles treatment at 10°C. PCB degradation by cold-adapted bacteria has been reported widely (e.g. Welandar, 2005; Lambo and Patel, 2007), and biodegradation in cold environments, including frozen soils (Aislabie *et al.*, 2006), is far from impossible. Factors enhancing biodegradation at 10°C pine needles treatment were 60 % WHC (Aislabie *et al.*, 2006), close to neutral soil pH (Wiegel and Wu, 2000; Fava *et al.*, 2003), and possibly also the temperature (Wu *et al.*, 1997; Guicharnaud *et al.*, 2010). This result gives an indication that bioremediation at average Icelandic summer field temperatures, with appropriate biostimulation, could be feasible. This is supported by reported findings of the study by Guicharnaud *et al.* (2010), which showed that the biological properties of the Icelandic soils are adopted to work at low temperatures, and governed by substrate availability to microorganisms rather than temperature. However, the main part of the literature gives an indication that bioremediation occurs slower at lower temperatures (e.g. Mohn *et al.*, 1997; Master and Mohn, 1998), and an optimal temperature would be close to room temperature (Tiedje *et al.*, 1993; Vasileya and Strijakova), which is not in agreement with the results of this study. Wu *et al.* (1997), however, suggested that room temperature might enhance only certain PCB degradation processes, which doesn't mean it is the only favorable temperature for biodegradation to take place. Furthermore it has been reported that higher temperature enhances pollutant degradation at higher extent at high pollution levels, but not necessary at lower pollution levels where nutrients play a more significant role (Walworth *et al.*, 2001). Icelandic soils are experiencing numerous freeze and thaw cycles throughout the year (Arnalds, 2004), and therefore the microorganisms may be more likely to adjust to low temperatures (Guicharnaud *et al.*, 2010).

The difference between degrading freshly spiked PCBs and aged PCBs should also be noted. In the soils of this study, the PCBs have been in the soils possibly from as long as since the late 1940s, and after late 1990s there hasn't been as much addition since the use of PCBs was phased out at the NATO base. Kuipers *et al.* (2003) studied the difference of Aroclor 1260 degradation with the help of inoculated anaerobic bacterial culture in freshly spiked and aged soils, finding that the degradation was far more effective in soils that had freshly added Aroclor 1260. According to Kuipers *et al.* (2003), the addition of anaerobic sediment to the soils was essential for dechlorination to be initiated. In this study it was shown study that flooding the soils made the microbial community less diverse than it was in the aerobic soils, which could indicate different microorganisms being active in anaerobic conditions (Wiegel and Wu, 2000). Pollutants may become nearly recalcitrant after a considerable time in the soil environment by for example binding and stabilizing to the positive surfaces of the minerals (Joergensen and Castillo, 2001; Semple *et al.*, 2001), such as allophane and ferrihydrite in the soils studied in this experiment. Moreover, the highly chlorinated Aroclor 1260 present in these soils is more likely to bind to soil colloids than less chlorinated PCB mixtures (Pu *et al.*, 2006), and therefore inhibit the degradation. Binding to SOM may also inhibit PCB degradation (Reid *et al.*, 2000; Semple *et al.*, 2001), but is most likely not the inhibiting factor in this case since there is very little organic carbon and nitrogen in the soils (Table 4.1).

Even though this study did not show PCB concentration dependence, there may not have been enough PCBs for dechlorination to take place, which could explain the lack of degradation. Anan'eva *et al.* (2005) and Vasilyeva and Strijakova (2007) have suggested 140-700 ppm and 500-1000 ppm, respectively, for an optimal PCB concentration for successful dechlorination to take place. Optimal PCB concentration range for aerobic biodegradation is lower, 10-60 ppm, according to Vasileya and Strijakova (2007). The optimal ranges for anaerobic biodegradation are considerably higher than the pollution levels in the soils used in the current study, which could explain the lack of degradation. Tiedje *et al.* (1993) suggested that with lower pollution levels the PCB might bind into the SOM and mineral particles. In the case of the current study, it is likely that the pollutant have been adsorbed to the allophane and ferrihydrite in the soils.

The existence of PCBs in soils has been accompanied by reduction in microbial biomass (Anan'eva *et al.*, 2005), which was not entirely the case in the current study. The control soil had less mic_c than the polluted soils, however, the amounts in the polluted soils were also low compared to Icelandic Andosols (Guicharnaud, 2009). The low mic_c could be explained by the low C_{tot} and N_{tot} , as well as very high carbon to nitrogen ratio (C:N), since low C:N ratio has been shown to favour microbial growth (Poll *et al.*, 2003). When C:N is higher than 20, microbes are not able to get sufficient amount of nitrogen to synthesize proteins, enzyme activity is also inhibited (Gianfreda *et al.*, 2005) and organic matter will be immobilized within the soil microbial biomass (Scott and Brinkley, 1997). Soil mic_c has been recognized as a parameter that reacts rapidly to environmental changes and enables reliable results (Insam, 2001). In this study, soil mic_c generally increased in experiment 1 but decreased in experiment 2. In the most successful degradation part of this study, the aerobic pine needles treatment at 10°C, mic_c was however under detection limits and that does not agree with the results of Anan'eva *et al.* (2005). The poor response of mic_c to the biostimulation could be explained by the overall goal to biostimulate only PCB degrading organisms in the soil. Insam (2001) suggested that fluxes from the soil or closer look into composition of microbial communities in soils should be studied in addition, if activation of specific functions of the soils were only undertaken. Biostimulation should also enhance the soil respiration (Walworth *et al.*, 2001). In this study (experiment 1) the highest respiration was measured after two months whereafter it decreased to almost none in many of the soil microcosms. The reduction in respiration after four months (experiment 1) may indicate the need for biostimulation more regularly, and not only at the beginning of the experiment (Walworth *et al.*, 2001).

Soil enzymes are considered as a relatively easy tool to analyze changes in the soil environment, since they respond quickly to any ecological disturbance (Gianfreda *et al.*, 2005). For PCB polluted soils dehydrogenase is an important enzyme to monitor since it is involved in the aerobic degradation of PCBs (Ohtsubo *et al.*, 2004), through the biphenyl pathway, and in general it is involved in the carbon cycle in the soils (Gianfreda *et al.*, 2005). The increase in dehydrogenase activity that was obtained in the experiments is in agreement with Wilke and Bräutingam (1992). High application of nitrogen (fertilizer treatments) alone was not favourable for dehydrogenase activity. The highest activities

were obtained from soils that were treated with plant detritus and roots in experiment 1. In experiment 2 dehydrogenase activity increased the most in the white clover and pine needles treatments. The dehydrogenase activity did not increase in as many treatments as in experiment 1, which may relate to the experimental length or unfavorable temperatures (10°C and 30°C instead of room temperature). If more time would have been given, different results could have been obtained. The importance of analyzing and monitoring changes in enzymatic activity instead of just mic_c is pointed out in this case, since an increase in mic_c did not necessarily mean an increase in any enzyme activities (Insam, 2001). In this study a monitoring of only one enzyme activity was chosen, but in the future it would be advisable to focus on at least two more enzymes (e.g. urease and phosphatase) to find those to monitor that react fastest to the changes in the environment. Insam (2001) argues that enzymes alone cannot give a reliable picture of the functionality of the soil biota, but should be taken together with analyzing of microbial pools or fluxes, as was undertaken in this study.

5.3 Bioavailability of PCBs

Even though a great deal of degradation did not occur in other treatments than with pine needles, the pollutant in the studied soils was bioavailable to earthworms (*Eisenia foetida*) and therefore there is a risk of the pollutant to the surrounding environment. *E. foetida* is widely used earthworm in bioavailability studies and results obtained may be considered as worst case scenarios (Hallgren et al., 2006). Earthworms process a large quantity of soil on a daily basis and in addition they may absorb pollutants through their thin external barrier, which may increase the bioaccumulation of PCBs in the food web when higher organisms, such as birds, consume earthworms (Ville et al., 1995). An explanation of the high bioavailability in this experiment could be the low organic matter content of the soils, since the pollutant is not bound to SOM (e.g. Wågman et al., 2001). According to Hickman and Reid (2008) the high bioavailability could be explained by the normal biological, chemical and physical actions of the earthworms. Ideally, earthworms will be able to release some of the most recalcitrant parts of the pollutants and at the same time enhance the soil properties including porosity and aeration of the soil. It seems, that in this study, the earthworms were able to consume the pollutant and they accumulated both highly and less chlorinated PCB congeners (Figure 4.12). This is in accordance with other studies done on Aroclor mixtures (e.g. Tharakan et al., 2006). It should be borne in mind that the bioavailability test was

undertaken at room temperature, which may have increased the bioaccumulation. The species, *E. foetida*, used in the study originates from southern Mediterranean (Walker et al., 2006), and may not be as active in Icelandic field conditions.

5.4 Indigenous PCB degrading capacity

Extensive literature exists on aerobic PCB degradation and genes involved in the degradation process (e.g. Ohtsubo *et al.*, 2004; Pieper, 2005; Pieper and Seeger, 2008), but the coverage of anaerobic degradation pathways and genes are not as extensive. In this study, strong amplification of aerobic *bphA* gene was obtained (Figure 4.6), which indicates the capability of the soils to aerobically degrade PCBs. In aerobic oxidative PCB degradation, *bphA* encodes the first fundamental step of the biphenyl upper pathway, in which biphenyl is converted to dihydrodiol and further to chlorobenzoic acid (CBA). In order to state whether aerobic PCB degrading bacteria, such as *Pseudomona* and *Bulkholderia*, are present in the polluted soils, further research is needed.

Degradation of products from anaerobic dechlorination of PCBs has been reported with genes *fcba* and *fcbb* (Rodrigues *et al.*, 2001 and 2006), as well as *ohb* (Rodrigues *et al.*, 2006). These genes, searched for with same primers as in the literature mentioned, were not present in the soils in the current study, and neither were *cbrA* and *Chloroflexi* phylum. *Chloroflexi* phylum and *cbrA* have been associated with PCB dechlorination, and would be important players in the first steps of degrading highly chlorinated PCB mixtures in soils, such as Aroclor 1260. It is likely that the bioremediation approach with bioaugmentation would be beneficial for these soils, since the indigenous degraders needed for the highest chlorinated PCB congeners are not present. It is also likely that the degraders might be in such a low quantities that they were not detected, or alternatively that more aggressive biostimulation, e.g. priming with individual congeners (Tiedje *et al.*, 1993), would activate the PCB degrading microorganisms. It should also be noted that significantly less literature and successful studies are available on anaerobic PCB degradation and degraders than aerobic, and many of the dynamic processes are still unknown in the scientific community worldwide.

5.5 Concluding remarks

This study demonstrated a nearly 40 % aerobic biodegradation of PCBs with pine needles biostimulation at 10°C in two months. In the soils from experiment 1 (both 25 and 50 ppm of PCBs soils) indigenous aerobic PCB degrading gene, *bphA*, was successfully amplified. When soils were flooded, the gene could not be amplified as was undertaken successfully before the flooding. The average profile generated by LH-PCR also proved the anaerobic soils to have less diverse microbial community than the aerobic soils. Earthworms accumulated both less and higher chlorinated PCBs effectively, which indicates potential for biomagnification of PCBs in the food chain. Overall the soils, Brown Andosols, of this study retained PCB effectively in the soil and only a small degree of PCB degradation occurred. However, since the PCBs were bioavailable to earthworms, a possible risk for the surrounding ecosystems exists (e.g. birds), especially taking into account the high mortality of the earthworms in the polluted soils.

The success with pine needles treatment is of great significance since Aroclor 1260 has been considered as recalcitrant for aerobic degradation and for any microbial degradation previously. Moreover, the treatment working best at 10°C, gives good indications for remediation being successful at Icelandic field summer temperatures. Terpenes, which are present in pine needles, may be seen as beneficial stimulation method since they are natural compounds, easily available in Iceland and they can promote bioavailability of PCBs. This is also of importance for cold environment remediation research in general.

Regarding remediation opportunities for Icelandic Andosols, and primarily for the field area of this study, a summer field scale pine needles stimulation trial is recommended to optimize the method under field conditions. If positive results are obtained at summer temperatures, a further field scale application is recommended.

5.6 Future Prospects

The results of this study suggest several interesting research opportunities. An almost 40 % decrease in total PCBs in two months at 10°C after application of pine needles gives a strong indication that a field scale application could be carried out successfully. Laboratory analysis with larger soil mass, in mesocosms, or preferably a small-scale field experiment would give fundamental knowledge on how pine needles treatment would work under less controlled conditions than microcosms. Terpenes from other plants, including orange peel, ivy leaves or eucalyptus leaves may work in a similar way for natural biostimulation for PCB degradation as pine needles (Hernandez *et al.*, 1997). Pine needles are, however, the easiest to find in Iceland. It would be interesting to study the *bphA* gene in future bioremediation projects with pine needles, in order to investigate whether stronger amplification could be obtained from biostimulation soils than from controls. In the current study *bphA* was amplified from soils without any treatments, and amplification of genes was not studied throughout the experimental length. A positive sign from this research is that the best results were obtained under aerobic conditions at 10°C, which indicates that a field scale approach in Icelandic temperatures could be successful. Bioremediation research in cold environments has mainly been focusing on oil pollution, and more research on PCB bioremediation is recommended.

Further research on anaerobic bioremediation of PCBs is needed, since the scientific community has not yet found all the processes and organisms involved in this process. It would be interesting to undertake an experiment with soils that have higher pollution levels than the ones tried in this experiment, to see whether the pollution level is a critical parameter for dechlorination. Sequential anaerobic-aerobic bioremediation trial would also be interesting, since it has been described as a possible way to fully degrade PCBs (Wiegel and Wu, 2000; Borja *et al.*, 2005). Promising results were obtained in Tharakan *et al.* (2006), with and without earthworms involved in the remediation process. Research on sequential degradation of Aroclor 1260 has been carried out (e.g. Master *et al.*, 2001), that could be used as a reference material for future experimental setups.

Based on the laboratory results, bioaugmentation of these soils may be necessary, in order to degrade the pollutant to accepted levels. There are several microorganisms that are associated with PCB degradation, and one or a combination of them could be used. It is, however, important to bear in mind that such an approach has mainly functioned under laboratory conditions, and hasn't been much developed under field scale conditions. It has also been reported that bioaugmentation may not work the same way in the field when competed out by the indigenous microorganisms, as under the most optimal laboratory conditions (Hickman and Reid, 2008). More aggressive stimulation with biosurfactants, mixtures of microorganisms, glucose or biphenyl (Luo *et al.*, 2008; Sobiecka *et al.*, 2009) may also be worth trying, but it should be taken into account that the cost of this kind of an approach would be way higher than the ones tried out in this experiment and the poor degradability of especially chemical surfactants should not be neglected.

Since the PCBs were bioavailable to earthworms, a bioremediation approach with earthworm assistance could also be considered. Earthworms can accumulate the pollutants that are most recalcitrant, and therefore often work as a "finishing tool" after remediation. In some studies earthworms have significantly enhanced the bioremediation (e.g. Singer *et al.*, 2001; Hickman and Reid, 2008), however, more efficiently together with bioaugmentation than alone. More in depth studies on earthworm assisted PCB biodegradation and further validation of the bioavailability method chosen for this study are encouraged. High mortality in the current bioavailability study could partly be explained by some practical difficulties, and therefore the method should be validated. It would also be of interest to study whether temperature has an effect on the bioaccumulation of PCBs, which would then give a better estimate of the bioaccumulation in the field conditions. It is also of interest to monitor the changes in microbial communities in the soils, during future bioremediation trials. More in depth identification of single degraders could also be interesting, but community fingerprinting would be more cost effective and could give a picture of the whole community instead of single organisms.

More in depth studies on the biological parameters of bioremediation would give a clearer picture of the PCB degrading microbial communities and how they react to changes in the environment. It would also be interesting to monitor the changes not only before and after the treatments, but also throughout the whole experiment with regular intervals. In further bioremediation trials, it would be interesting to monitor the microbial community in more detail and take samples during the whole experiment to detect how the community reacts on biostimulation, or any approach that has been chosen. If more detailed information on the PCB degraders is sought after, the amplified DNA extracts could be cloned, sequenced and compared to databases that include information on PCB degrading organisms (Insam, 2001).

It would be interesting to study how the different parameters react to pollution in different Icelandic soil types. This could be studied by sampling soil from different soil type environments but with similar pollution history, e.g from all the previous military areas in Iceland. Laboratory studies with freshly spiked pollutants could also give an important insight into this issue, but more realistic picture of the real field situation would be obtained with samples from aged polluted locations.

In general, establishment of guidelines for polluted soils and the management thereof in Iceland is crucial. As previously stated, it would be beneficial to develop the guidelines in accordance to European legislation, taken into account the Icelandic climatic, geologic and geographical conditions. An inclusive report was written in 1996 by UST, where different critical values for soil and sediment pollution were discussed. Without proper guidelines, any kind of remediation project has problems with deciding on degradation end point goals or simply knowing what should be done. Furthermore, guidelines and management of polluted areas gives a working frame for people operating in the field and makes it more convenient and effective to carry out remediation processes. An important detail to think about in the legislation is what PCB congeners to include. If the more chlorinated congeners are not taken into account (as is the case in the draft from 1996), a large part of the Icelandic PCB pollution is not considered. Aroclor 1260 was the main PCB mixture used by the US Army, and therefore the Icelandic regulation should reflect that. This

applies especially to areas that are going to be used as residential areas in the future.

Figure 5.1 below illustrates a simplified suggestion for a schematic plan for future remediation planning in Iceland. In many cases (e.g. UST 1996; Stenuit *et al.*, 2008), remediation actions have been presented as a hierarchical process whereas Figure 5.1 illustrates the process as a continuous circle. This approach highlights the importance of monitoring and continuous remediation process. The remediation process most commonly starts with identification of a polluted site followed by sampling. In order to minimize cross contamination and any other sampling errors, appropriate sampling guidelines (e.g. Karstensen *et al.*, 1997) should be used. In the next step the concentration and toxicity of the pollutant is measured, and compared to critical values. Chemical analysis can give answers to the pollution levels, whereas biosensors, including for example earthworms, can be used to measure the toxicity of the pollutant. In this study, an earthworm test according to Hallgren *et al.* (2006) was used, but many others are available (e.g. U.S. Environmental Protection Agency, 1996; Schaefer, 2003).

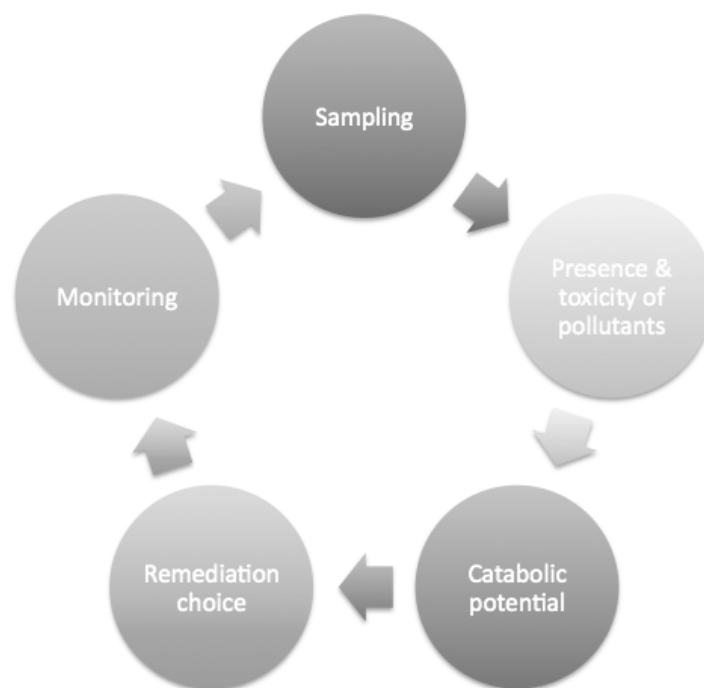


Figure 5.1 A simplified illustration of a suggestion for future remediation site plan.

Before any decision is made about remediation actions, a step with investigation of the catabolic activity (Stenuit *et al.*, 2008) of the soils in question is encouraged as a result of this study. In this phase either specific genes involved in the pollutant degradation process or microorganisms can be investigated from the soils. Thereafter a remediation option can be chosen, based on the catabolic potential of the soils to degrade the pollutant. A bioremediation hierarchy consists of three options: natural attenuation, biostimulation and bioaugmentation (e.g. Nogales *et al.*, 1999; Crawford and Crawford, 2005). The first option is the most cost-efficient and easy to carry out, but also the most time consuming. Several different biostimulation and bioaugmentation options have been described in detail in this study under section 2 State of the Art. It should be borne in mind, that this suggestion does not include other remediation options such as chemical and physical approaches. Those options should be considered when bioremediation is not a realistic option, i.e. when the catabolic potential of the soils is non-existing, or if the timeframe is out of the scope of bioremediation. One of the most important steps in the remediation process is monitoring, i.e. to follow up on the process and also to confirm that the land is safe for the use it has been chosen for. Monitoring may be carried out at different scales and through different approaches. Microbial community structure and dynamics of the soils can be studied through fingerprinting techniques, microbial community functions by studying e.g. the functional genes involved in the degradation and finally chemical analysis can report changes in the pollutant concentrations (Stenuit *et al.*, 2008). If critical values have been exceeded, the process may begin from sampling again. It is crucial that monitoring is not carried out as a one-time check up, but on a regular basis. However, a detailed risk assessment is not taken into account in this simplified illustration, and should be a part of remediation planning. An extensive state of the art study on remediation planning and critical values for pollutants in soils in the EU is recommended before an Icelandic regulation is finalised and published.

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Appendix A

The areal photographs below represents the preliminary studies conducted on the PCB pollution levels at the old NATO base in Keflavik (Almenna, 2008).

