Bioavailability aspects of hydrophobic contaminant degradation in soils

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Front Cover: Soil microbiology in laboratory- and field scales at the laboratory of Finnish Forest Research Institute and Kurkisuo Landfill area.

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To my father

Contents

Abstract

This thesis concentrates on bioavailability of organic soil contaminants in the context of bioremediation of soil contaminated with volatile or non-volatile hydrophobic pollutants. Bioavailability and biodegradation was studied from four viewpoints: (i) Improvement of bioavailability and biodegradation of volatile hydrocarbons in contained bioremediation systems at laboratory - and pilot-scale. (ii) Improvement of bioavailability of non-volatile, hydrophobic compounds in such systems. (iii) Biodegradation of a non-volatile hydrophobic compound in soil organic matter in microcosms. (iiii) Bioavailability of nitrogen in an open, full-scale bioremediation system.

It was demonstrated that volatility of organic compounds can be controlled by amending the soil with adsorbents. The sorbed hydrocarbons were shown to be available to soil microbiota. As the result, biodegradation of the volatile hydrocarbons was greatly favored at the expense of volatilization.

PAH compounds were shown to be mobilized and their bioavailability improved by a hydrophobic, non-toxic additive, vegetable oil. Bioavailability of the PAHs was recorded as an increased toxicity of the soil. In spite of the increased bioavailability, biodegradation of the PAHs decreased.

In microcosms simulating boreal forest organic surface soil, PAH-compound (pyrene) was shown to be removed from soil biologically. Therefore hydrophobicity of the substrate does not necessarily mean low availability and biodegradation in organic soil.

Finally, in this thesis it was demonstrated that an unsuitable source of nitrogen or its overdose resulted in wasteful spending of this nutrient and even harmful effects on soil microbes. Such events may inhibit rather than promote the bioremediation process in soil.

List of original publications

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

I. **Peltola, R.**, Salkinoja-Salonen, M. 2003. Improving biodegradation of VOCs in soil by controlling volatilization. *Bioremediation Journal* 7: 129 – 138.

II. Koivula, T., Salkinoja-Salonen, M., **Peltola, R.**, Romantschuk, M. 2004. Pyrene degradation in forest humus microcosms with or without pine and its mycorrhizal fungus. *Journal of Environmental Quality* 33: 45 - 53

III. **Peltola, R.**, Salkinoja-Salonen, M., Pulkkinen, J., Koivunen, M., Turpeinen, A-R., Aarnio, T., Romantschuk, M. 2006. Nitrification in polluted soil fertilized with fast- and slow-releasing nitrogen: a case study at a refinery landfarming site. *Environmental Pollution* 143: 247 - 253.

IV. **Peltola, R.**, Maunuksela, L. Salkinoja-Salonen M. 2008. Mobilizing PAHs with vegetable oil - effects on biodegradation and soil toxicity. Submitted manuscript.

The author´s contribution

Paper I:

Rainer Peltola designed the experiments, performed all experimental work, interpreted the results and wrote the manuscript jointly with M. Salkinoja-Salonen.

Paper II:

Rainer Peltola designed the experiments in co-operation with Teija Koivula under supervision of M. Romantschuk. He carried out part of the experimental work and participated in writing of the paper.

Paper III:

Rainer Peltola designed the experiments under supervision of M. Romantschuk and performed all experimental work except the NO_3-N and NH_4-N analyses. He wrote the manuscript jointly with M. Salkinoja-Salonen.

Paper IV:

Rainer Peltola designed the experiments and performed experimental work in co-operation with Liisa Maunuksela. He interpreted the results and wrote the manuscript jointly with Liisa Maunuksela and M. Salkinoja-Salonen.

Abbreviations

K_i	Partition constant of compound *i*
Kow	Octanol-water partition coefficient
K_{ilipw}	Lipid-water partition coefficient of compound *i*
K_{ioc}	Organic matter-water partition coefficient of compound *i*
K_{itriow}	Triolen-water partition coefficient of compound *i*
K_{ih}	Henry´s law constant of compound *i*
C_i	Concentration of compound *i*
ph	Phase
D_{iw}	Diffusion coefficient of compound *i* in water
η	Viscosity
$\overline{V}_i$	Molar volume of compound *i*
Bp	Boiling point
$t_{½}$	Half-life time
σ_x	Diffusion distance
k	Rate constant
OC	Organic compound
DOC	Dissolved organic carbon
COD	Chemical oxygen demand
VOC	Volatile organic compound
PAH	Polyaromatic hydrocarbon
OC	Organic compund
MTBE	Methyl-*tert*-butyl ether
L	Diffusion distance
t_{diff}	Diffusion time

1. Background

Anthropogenic environmental contamination has been part and parcel of the mankinds way of life in the industrialized world. The 19th century industrial revolution brought not only material welfare but also emissions of harmful substances to the environment. These emissions have led to local and global deterioration of the environment when the contaminants have accumulated in air, water, sediments, soils and biota, including man (Schwarzenbach et al. 2003).

According to current thinking all naturally occurring organic compounds may ultimately be degradable by microorganisms under favorable conditions (Leung et al. 2007). The metabolic diversity of natural microbial communities has, so far, saved mankind from self-intoxication. As nature´s self-purifying characteristic is composed of many abiotic and biotic factors, understanding these factors is essential to avoid exceeding nature´s self-purifying capability.

Hydrophobic organic compounds are a major group of environmental contaminants. Those considered to be harmful in the environment, are usually acutely or chronically toxic and recalcitrant. Hydrophobicity means low water solubility and may be the major factor behind these properties (Stokes et al. 2006). This work concentrates mainly on the factors affecting the biodegradation of hydrophobic organic compounds in soil environment.

2. Review of the literature

2.1. Contaminant biodegradation - definitions and determinations

Biodegradation can be defined as the process by which organic substances (or in context of bioremediation, contaminants) are decomposed by micro-organisms or their extracellular enzymes into simpler substances (OECD 2002A). Biodegradation may or may not occur when microbial activity is present, i.e. in water, soils, sediment and organisms. Mineralization or "ultimate" biodegradation (OECD 2001A), the process preferred for environmental remediation, means conversion of organic compounds to inorganic. Organic compounds are used by microbes as carbon and electron source. Biotransformation means conversion of organic compounds into other organic compound(s). Unlike in mineralization, the products of biotransformation can be even more harmful than the starting compounds (Alexander 1999). Biodegradation and biotransformation can therefore be also a detrimental process for soil biota or humans.

To predict the environmental fate of an organic compound, several standardized methods for determining biodegradation have been developed for water, sediment and soil environments (Tables 1-5.). In these methods biodegradation is classified as "ready", if the compound undergoes rapid ultimate degradation in most environments including biological sewage treatment plants. "Inherent" biodegradation means that the compound has the potential to be biodegraded. "Simulation tests" aim at examining the rate and the extent of biodegradation in a laboratory system representing environmental conditions of interest (OECD 2003).

The ISO and OECD biodegradation tests sharing the same cell in tables 1-5 have similar principles and technical procedures as compared to each other. If the studied compound is proven to be recalcitrant, there is rather limited number of OECD tests for evaluating the effects of such compounds in the environment. ISO has wider selection of tests when, for example, ecotoxicological properties of an organic compound is the matter of interest.

Table 1. Standardized methods for the determination of ready biodegradation in aqueous environment.

Standardized method	Test conditions	Measurement	Duration	Inoculum
SFS-EN ISO 7827 / OECD 301A: Water quality. Evaluation in an aqueous medium of the "ultimate" aerobic biodegradability of organic compounds. Method by analysis of dissolved organic carbon (ISO 1994A / OECD 1992A)	Agitation in dark, aerobic conditions at 20-24^{o}C. Test material concentration 10-40 mg DOC l^{-1}	DOC removal	28 d	Micro-organisms (~10^{7}-10^{8} cells l^{-1}) from surface water or activated sludge
SFS-EN ISO 10707: Water quality. Evaluation in an aqueous medium of the "ultimate" aerobic biodegradability of organic compounds. Method by analysis of biochemical oxygen demand (closed bottle test) (ISO 1994B)	Agitation in dark, aerobic conditions at 20-24^{o}C. Test material concentration 10-40 mg l^{-1}	O_2 consumption	28 d	Micro-organisms (~10^{4}-10^{6}) from surface water or sewage treatment works effluent
SFS-EN ISO 14593 / OECD 310: Water quality. Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium. Method by analysis of inorganic carbon in sealed vessels (CO_2 HS test) (ISO 1999A/OECD 2006A)	Agitation in dark, aerobic conditions at 20-24^{o}C. Test material concentration 2-40 mg C l^{-1}	CO_2 production	28 d	Micro-organisms (~10^{7}-10^{8} cells l^{-1}) from surface water or activated sludge
SFS-EN ISO 9408/OECD 301F: Water quality. Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer (ISO 1999B/OECD 1992B)	Agitation in dark or diffuse light, aerobic conditions at 20-25^{o}C. Test material concentration 100 mg l^{-1}	O_2 consumption	28 d	Micro-organisms (~10^{2}-10^{5} cells l^{-1}) from surface water or activated sludge
SFS-EN ISO 9439 / OECD 301B:Water quality. Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium. Carbon dioxide evolution test (ISO 1999C / OECD 1992C)	Agitation in dark or diffuse light, aerobic conditions at 20-24^{o}C. Test material concentration 10-20 mg DOC l^{-1}	CO_2 production	28 d	Micro-organisms (~10^{7}-10^{8} cells l^{-1}) from surface water or activated sludge
OECD 301C: Modified MITI test (OECD 1992D).	Agitation in dark, aerobic conditions at 20-24^{o}C. Test material concentration 100 mg l^{-1}	O_2 consumption	28 d	Micro-organisms (~10^{4}-10^{6}) from surface water or sewage treatment works effluent

Table 2. Standardized simulation tests for determination of biodegradation in water and sediment

Standardized method	Test conditions	Measurement	Duration	Inoculum
OECD 306. Biodegradability in seawater (OECD 1992E)	Agitation in dark or diffuse light under aerobic conditions at 15-20^{o}C. Test material concentration 5-40 mg DOC l^{-1}	DOC removal	< 60 days	Microrganisms in seawater
SFS-EN ISO 11734 / OECD 311: Water quality. Evaluation of the "ultimate" anaerobic biodegradability of organic compounds in digested sludge. Method by measurement of the biogas production (ISO 1995 / OECD 2006B)	Batch culture in dark under anaerobic cultures at 35^{o}C . Test material concentration 20-100 mg OC l^{-1}	Gas (CH_4+CO_2) production	< 60 days	Washed anaerobic digester sludge (total solids 1-3 g l^{-1})
SFS-EN ISO 11733 / OECD 303A Water quality. Determination of the elimination on biodegradability of organic compounds in an aqueous medium. Activated sludge simulation test (ISO 2004A / OECD 2001B)	Activated sludge plant model (Hussman unit) or porous pot unit at 20 - 25^{o}C. Test material concentration 20 mg l^{-1}	Elimination of test compounds	< 12 weeks	Aerobic sewage

Table 2. (Continues). Standardized simulation tests for determination of biodegradation in water and sediment

Standardized method	Test conditions	Measurement	Duration	Inoculum
OECD 308: Aerobic and anaerobic transformation in aquatic sediment systems (OECD 2002B)	Static test with natural water and sediment in dark at 10 - 30^{o}C. Test material concentration of interest. Use of ^{14}C-labelled model compound is recommended	Formation of $^{14}CO_2$ or chemical analysis of transformation products.	< 100 days	Microorganisms in sediment
OECD 309. Aerobic mineralisation in surface water (OECD 2004)	Agitation in dark or diffuse light, aerobic conditions at temperature of interest or 20-24^{o}C. Test material concentration 1 - 100 µg l^{-1}. Use of ^{14}C-labelled model compound is recommended.	Reduction of the test compound or formation of $^{14}CO_2$	< 90 days	Microorganisms in surface water
ISO 14592-1: Water quality. Evaluation of the aerobic biodegradability of organic compounds at low concentrations Part 1: Shake-flask batch test with surface water or surface water/ sediment suspensions (ISO 2002A)	Agitation in dark or diffuse light, aerobic conditions at temperature of interest or 20-25^{o}C. Test material concentration 1–100 µg $^{-1}$. Use of ^{14}C-labelled model compound is recommended.	Reduction of the test compound or formation of $^{14}CO_2$	No fixed duration	Microorganisms in surface water and/or sediment
ISO 14592-2: Water quality. Evaluation of the aerobic biodegradability of organic compounds at low concentrations Part 2. Continuous flow river model with attached biomass. (ISO 2002B)	Flow through system under natural diffuse daylight or constant illumination of artificial white light Test material concentration < 200 µg $^{-1}$.	Reduction of the test compound	< 60 days	Microorganisms in surface water

Table 3. Standardized methods for determination of inherent biodegradation in water

Standardized method	Test conditions	Measurements	Duration	Inoculum
ISO 9888 / OECD 302B: Water quality. Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium. Static test (Zahn-Wellens method) (ISO 1999D/OECD 1992F)	Aerated batch culture in dark or diffuse light at 20 - 25^{o}C. Test material concentration 50 - 100 mg l^{-1}.	DOC or COD removal or specific analysis for primary transformations	28 days	Activated sludge (200 - 1000 mg l^{-1} total solids)
SFS-EN ISO 9887/OECD 302A Water quality. Evaluation of the aerobic biodegradability of organic compounds in an aqueous medium. Semi-continuous activated sludge medium (SCAS) (ISO 1992/OECD 1981A)	Repeated aeration interrupted by settling period during which sampling and addition of fresh sewage and test chemical in semi-continuous activated sludge unit at 20-25^{o}C. Test material concentration 20 mg DOC l^{-1}.	DOC removal	Months (at least 12 weeks)	Settled activated sludge
OECD 306. Zahn-wellens / EMPA test (OECD 1992F)	Agitation in dark or diffuse light under aerobic conditions at 20-25^{o}C. Test material concentration 50-400 mg DOC l^{-1}	DOC removal	< 28 days	Microrganisms activated sludge
OECD 302C MITI (I) (OECD 1981B)	Agitated batch culture in dark at 23 - 27^{o}C. Test material concentration 30 mg l^{-1}	O_2 consumption	14 - 28 days	Aerobic, specially grown mixed, unadapted microorganisms 3 x 10^{7} - 3 x 10^{8} cells l^{-1}

Table 4. Standardized simulation tests for determination of biodegradation in soil

Standardized method	Test conditions	Measurement	Duration	Inoculum
OECD 307: Aerobic and anaerobic transformation in soil (OECD 2002C)	Soil batch tests in constant moisture at temperature of interest. Use of ^{14}C-labelled model compound is recommended.	Formation of $^{14}CO_2$ or chemical analysis of transformation products.	< 120 days	Indigenous soil microbes

Table 5. Standardized methods for determination of inherent biodegradation in soil

Standardized method	Test conditions	Measurement	Duration	Inoculum
ISO 14239 / OECD 304A. Soil quality. Laboratory incubation systems for measuring the mineralization of organic chemicals in soil under aerobic conditions (ISO 1997A / OECD 1981C)	Soil (alfisol, spodosol or entisol spiked with ^{14}C-labelled model compound) batch tests in dark at 20 - 24^{o}C.	Formation of $^{14}CO_2$ or chemical analysis of transformation products.	< 64 days	Indigenous soil microbes
ISO 11266 Soil quality. Guidance on laboratory testing for biodegradation of organic chemicals in soil under aerobic conditions (ISO 1994C)	Static test with soil, test material concentration and temperature of interest in dark.	Formation of $^{14}CO_2$ or chemical analysis of parent compound or transformation products	< 120 days	Indigenous soil microbes
ISO 15473 Soil quality. Guidance on laboratory testing for biodegradation of organic chemicals in soil under anaerobic conditions (ISO 2002C)	Static test with soil, test material concentration and temperature of interest in dark. Anaerobicity is checked with occasional redox measurements.	Formation of $^{14}CO_2$/$^{14}CH_4$ or chemical analysis of parent compound or transformation products.	< 100 days	Indigenous soil microbes

Standardized tests measuring ready or inherent biodegradation can give only crude indications of the fate of the investigated organic compound in soil. Most tests are targeted for water environments and the standardized conditions of tests may remarkably deviate from the real-life conditions. For example, the temperature typical for the biodegradation tests is above 20^{o}C, which seldom prevail in subsurface soil. Simulation tests give more liberty for choosing the conditions of interest.

Another element causing uncertainty to the biodegradation estimations based on tests presented in Tables 1-5 is how the compound is introduced to the matrix. In soils the biological removal of a compound varies considerably depending on the period of time that soil has been exposed to the compound, as many contaminants degrade slowly. For testing biodegradation, the soil (or water or sediment) is spiked with the compound of interest at the onset of the test period. The duration of the test seldom exceeds a few months (see Tables 1-5). Such a spiking practice differs from the real-world, in which the compound of interest remains in the environment for years or decades.

Microbes have had several billions of years to develop enzymatic apparatuses for degrading compounds emanating from natural processes (Leung et al. 2007). In contrast, the chemical industry has discharged its products to the environment for only about one hundred years. Many of the man-made "xenobiotic" compounds possess molecular structures not found in natural chemicals and therefore are foreign to the microbial degrader enzymes. Greater difference in the structure of a xenobiotic as compared to a naturally occurring substance often predicts lower likelihood for extensive biodegradation. The chemical structure of a xenobiotic compound often mimicks that of the "natural" molecules, but the substituents, called "xenophores", are physiologically rare or entirely non-physiological. This may result in a poorly biodegradable compound. Typical xenophores are halogens, NO_2, SO_3H, CN and CF_3 when directly bonded to carbon atoms (Alexander 1999).

Xenobiotics may be biodegraded when (i) compatible with the catabolic enzymatic apparatus of a degrader microbe (Alexander 1999, Leung et al. 2007), (ii) the enzymatic apparatus of a microbe has a wide specificity (Hesselsoe et al. 2005, Baldrian 2006), (iii)

genetic adaptation occurs in a microbe leading to a new catabolic pathway for the xenobiotic (Janssen et al. 2005).

2.2. Soil compartments and their properties

Organic compounds, when released in soil, face a heterogeneous environment composed of soil minerals, soil organic matter, soil water and soil vapors. Figure 1 is a simplified presentation of soil compartments. In reality, the soil environment is a three-dimensional labyrinth of water- or gas-filled pores and soil particles of different sizes, forms and compositions. The dimensions of particles and pores vary from several centimeters to nanometers. The small scales and spatial heterogeneity of soil makes the estimations of physical and physicochemical conditions that surround organic compounds and soil microorganisms extremely challenging (Chenu & Stotzky 2002).

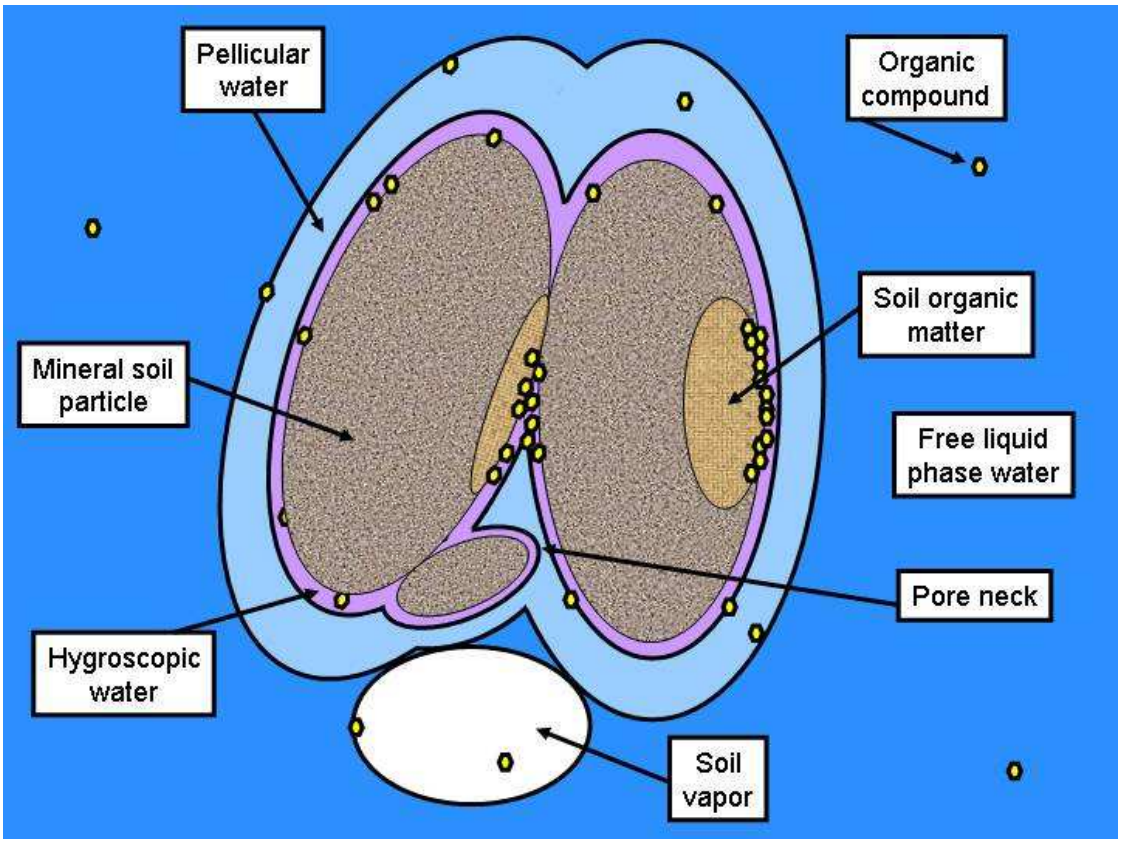


Figure 1. Schematic presentation of soil compartments

Mineral soil particles originate from rock which has undergone physical or chemical weathering. Particle size distribution determines mineral soil texture (stones > 2 mm, sand grains 0.05 - 2 mm, silt 0.002 - 0.05 mm, clay particles < 0.002 mm) and is often used in the classification of soil (McRae 1988, Ehrlich 2002). A decrease of particle size increases soil surface (Table 6). In most soils the sand and silt consist largely of grains of resistant minerals, mainly quartz. The clay is made up of clay minerals, which usually have silicate structures. Surfaces and edges of soil inorganic particles are covered with negative electric charges (McRae 1988).

As can be seen from the table 6, share of soil external surface area decreases with decreasing particle size. This is due to increasing share of soil internal surface. Clay particles are composed of sheet-like crystal layers separated by interlayer spaces. This interlayer area is called soil (clay) internal surface. Diameter of the interlayer space is, depending on clay type, between 1 - 2 nm (10 - 20 Å) (Hartikainen 2001), making it inaccessible to soil microbes and poorly accessible to large organic molecules.

Table 6. Selected characteristics of soil with different mineral soil textures (Chenu & Stotzky 2002)

Characteristic	Dominant texture		
	Sand	Silt	Clay
Total surface area (m^2g^{-1})	3	55	208
External surface area (m^2g^{-1})	3	17	60
% of external surface area covered by soil bacteria (*)	2.26	0.40	0.11
% of total surface area covered by soil bacteria	2.26	0.12	0.03

(*) Assuming 10^{10} bacteria g^{-1} population with cells being 1 µm long and 0.5 µm in diameter.

Soil organic matter is composed of plant and animal debris and intermediates and end-products of the decomposing debris (McRae 1988). Soil biota is not considered to be a part of the soil organic matter (Hartikainen 2001). The rate of decomposition depends on the origins of the debris and the conditions in soil. Eventually an amorphous substance which has lost all its original structure, humus, is formed. The humus fraction, which is

soluble in or extractable into aqueous base (and insoluble to organic solvents), is commonly referred to as humic substances. The water insoluble and organic solvent soluble part of humus is referred as humin or kerogen (Schwarzenbach et al. 2003). The humic substances are further divided into humic acids that precipitate at acid pH and fulvic acids which do not (Ehrlich 2002, Schwarzenbach et al. 2003). Humus contains numerous oxygen-containing functional groups including carboxy-, phenoxy-, hydroxy- and carbonyl substituents. Depending on the type of humus, the number of such polar groups may vary significantly, affecting the polarity of humus. Highly polar fulvic acids may have oxygen-to carbon (O/C) molar ratios of near 0.5, whereas humin/kerogen has O/C ratios around 0.2 to 0.3 (Schwarzenbach et al. 2003). In general, less polar humus fractions (low O/C ratio) are located on surfaces of soil mineral particles, and water-soluble fractions of humus can be dissolved in soil water. The share of organic matter in soil varies, peat soils are practically 100% organic, whereas deep subsurface layers of moraine soil are practically 100% inorganic. Organic matter surface area depends on its particle or aggregate size (Table 7).

Table 7. External surface area of soil organic matter (Chenu & Stotzky 2002)

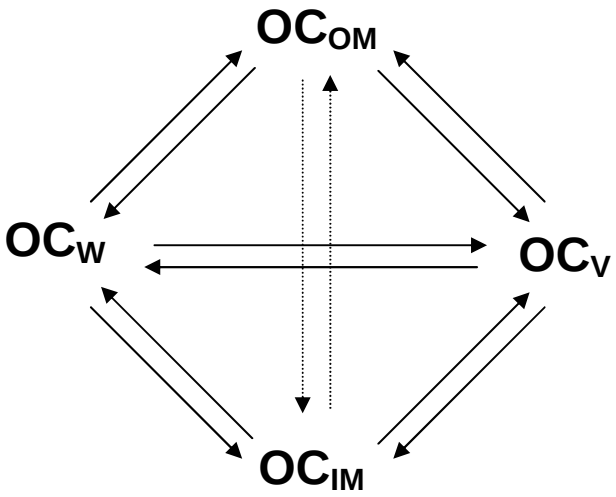
	Organic matter particle size		
	> 50 µm	0.2 - 2 µm	< 0.2 µm
Surface area (m^2g^{-1})	0.9 - 8.3	24 - 42	48 - 73

Solid matter constitutes about 50% of the volume of mineral soil, the other 50% is pore space occupied by soil gases or water (Ehrlich 2003). As soil particles are negatively charged and water molecules are dipoles, electro-molecular forces between the soil and water molecules create a thin layer of water with ordered molecules called **hygroscopic water** surrounding soil particles (McRae 1988, Ehrlich 2002). The thickness of the hygroscopic water layer depends on the size of the particle it surrounds, for sand particles it is about 30 nm and clay particles 3 nm (Kuznetsov et al. 1963). This water does not move as a liquid. Hygroscopic water is surrounded by a layer of **pellicular water**, which may move from one soil particle to another by intermolecular attraction, but not by gravity nor hydrostatic pressure (Kuznetsov et al. 1963, Ehrlich 2002). **Free liquid phase water**

or gravitational water moves freely in soil pores (transmission pores) by capillary forces or gravity.

The soil pores, which are not water filled, are occupied by **soil vapor**. As soil pores are not connected to open atmosphere, the composition of soil vapors is regulated by gaseous diffusion and soil respiration. Therefore soil vapor composition varies, but typically the CO_2 concentration is 0.5 - 5.0% and the O_2 concentration 15 - 20 % (McRae 1988).

An organic compound that enters the soil can remain as a free phase or it can be distributed among the soil compartments described above. It can be adsorbed to inorganic particles or to organic matter surfaces, sorbed to organic matter, dissolved to soil water or vaporized in soil air. This distribution is a dynamic state, in which an individual molecule transfer constantly from one soil compartment to another, as shown in Figure 2.



W = soil water
OM = soil organic matter
V = soil vapor
IM = soil inorganic matter.

Figure 2. Possible locations and dynamics of an organic compound (OC) in soil.

In principle, organic compound transfer directly between organic and inorganic matter (Fig. 2, dotted lines) is possible by solid phase diffusion, but this process is slow (Schwarzenbach et al. 2003). In static conditions an equilibrium between the

concentrations of the organic compound located in different soil compartments is finally achieved, which means that there is no net flow of the compound from one phase to another. The equilibrium can be described by the equilibrium partition constant K_i, calculated with Equation 1:

$$K_i = \frac{C_{iph1}}{C_{iph2}} \quad \text{(Eq. 1)}$$

C_{iph1} = Concentration of compound *i* in phase 1
C_{iph2} = Concentration of compound *i* in phase 2

Generally the transfer of an organic compound from one phase to another is controlled by the dissolved species of the compound in soil environments when water is present (Schwarzenbach et al. 2003).This is because soil particles are always surrounded by water films. Organic molecules may move by advection dissolved in the free liquid phase water or by diffusion in the hygroscopic and the pellicular waters. Diffusion is the only mode of molecular transfer in pores, which are separated from free liquid water by connecting pores, **pore necks**, smaller than 0.3 µm (Standing & Killham 2007). Soil bacteria are actually "aquatic" organisms in the sense that they rely on organic and inorganic compounds dissolved in soil water for their nutrition (Chenu & Stotzky 2002). Therefore water filled transmission pores which offer rapid transfer of dissolved compounds is the major location for microbial activity in soil (Standing & Killham 2007).

2.3. Bioavailability and biodegradation

In order to degrade an organic compound the degrading microbe or its extracellular enzymes need a physical *contact* with the compound (Rosenberg et al. 1992). To attain this contact, the compound has to be *available* for the degrading microbe (Reid et al. 2000, Semple et al. 2003). Generally it is postulated that only the molecules dissolved in soil water are biodegraded (White & Alexander 1996, Cornelissen et al. 1998, Gomez-Lahoz & Ortega-Calvo 2005). This non-bioavailability of sorbed compound to soil microbes is valid whether the sorbent is a soil particle (Ehlers & Loibner 2006) or an artificial sorbent, such as activated carbon (Aktas & Cecen 2007). In addition to bioavailability of the compound to be degraded, also other factors (electron acceptor, inorganic nutrients) required for biodegradation have to be available. The soil water dissolved molecules can also be poorly or not bioavailable if the molecule is dissolved in non-advecting water. Such water is hygroscopic or pellicular or water located in a soil pore with a neck pore diameter smaller than 0.3 µm (Standing & Killham 2007). Generally, the contact between a hydrophobic compound and microbial cell may be improbable even in absence of physical obstacles because soil microbes can occupy only small fraction of the soil surface area (Table 6).

The accessibility of a compound to a biological entity, i.e. bioavailability is one of the key factors affecting contaminant biodegradation in soils (Mihelcic et al. 1993, Reid et al. 2000, Semple et al. 2003). Unlike biodegradation, there is no general definition for bioavailability. There are numerous different definitions varying in details and complexity, the shortest being “Bioavailability is the flux of contaminants to biota”, as reviewed by Semple et al. (2007). Due to lack of consistency of clear definition, bioavailability measurements are not as standardized as biodegradation measurements. Numerous approaches have been developed to estimate sequestration and bioavailability of a contaminant in soil. Often these methods are based on liquid- or solid-phase extraction of contaminants from soil, aimed to mimic cellular uptake, but also living organisms are used. Some examples of such methods are presented in Table 8. Estimation of bioavailability is of great importance because it is a major factor determining not only

biodegradation, but also toxicity and ecological risk of an organic compound in soil. However, there are great variations in estimations of bioavailability depending on the selected test method (Sun & Li 2005, Bergknut et al. 2007).

Table 8. Some bioavailability determination methods.

Extraction vehicle	Reference
Supercritical CO_2 in direct contact with soil	Weber & Young 1997
Liquid (water and/or organic solvent) in direct contact with soil	Kelsey et al. 1997
Liquid (cyclodextrin solution) in direct contact with soil	Reid et al. 2000
C_{18} membrane disk in direct contact with soil	Tang et al. 1999
Liquid (water) in contact with soil mediated by dialysis membrane	Woolgar & Jones 1999
Earthworm (*Eisenia foetida*) in direct contact with soil	Belfroid et al. 1995

2.4 Factors affecting contaminant bioavailability and biodegradation in soil

2.4.1. Impact of the contaminant water solubility on its bioavailability and biodegradability

The aqueous solubility of an organic compound is the maximum concentration of the given compound that can be dissolved in pure water at a given temperature and pressure (Schwarzenbach et al. 2003). As the water molecule is a strong dipole, forming intermolecular hydrogen bonds, while hydrophobic (literally "water-fearing") compounds are only weak dipoles or apolar, they can not interact with polar water molecules and tend therefore to escape from water to more hydrophobic environments.

In the natural soil environment, where water is always present, water solubility is one of the key factors determining the fate of an organic compound in soil. As previously mentioned, phase transfers in soil are controlled by the dissolved species of a chemical. Water miscible, electrically neutral or negatively charged compounds remain in the soil water phase and move in soil with the advection of soil water. Positively charged organic compounds may interact with negative charges of soil particles through cation exchange, but these interactions are readily reversible (Li et al. 2000). As the major fraction of soil microbes are attached to surfaces of wider soil pores in microcolony- or biofilm-like structures (Standing & Killham 2007, van Elsas et al. 2007), water movement in these pores promote contacts between the dissolved organic molecules and degrading microbes. Therefore inherently biodegradable, water soluble organic compounds are usually bioavailable and not persistent in soil if other conditions favor biodegradation (Semple et al. 2003).

When an organic compound is hydrophobic, i.e. its water solubility is low, partition of the compound between aqueous and water-immiscible bulk liquid can be used to describe its behaviour in aqueous environments. The water-immiscible liquid most widely used is *n*-

octanol. The partition constant of an organic compound, *i*, between *n*-octanol and water phases, the octanol-water coefficient (*K*ow), is calculated with Equation 2:

$$K_{iow} = \frac{C_{io}}{C_{iw}} \qquad \text{(Eq. 2)}$$

K_{iow} = Octanol-water coefficient for compound *i*
C_{io} = Concentration of compund *i* in *n*-octanol phase
C_{iw} = Concentration of compound *i* water phase

Kow is usually presented as the logarithmic value of the coefficient, denoted as log Kow or Pow. For example, log Kow of anthracene is 4.68, meaning that the concentration of anthracene in *n*-octanol is 48 000 - fold higher than in water in a two-phase system. As Kow can be determined with numerous methods (Table 9), significant variations in the Kow values found for a single compound in the literature are not uncommon.

Table 9. Some Kow measuring and estimation methods.

Experimental methods	Shake flask method (OECD 1995)	Partitioning of compound between *n*-octanol and water phases in closed vessel placed in a mechanical shaker, suitable for $-2 < \log K_{ow} < 4$
	Slow-Stirring method (OECD 2006C)	Partitioning of compound between *n*-octanol and water phases in closed stirring-vessel, suitable for $\log K_{ow} < 8.2$
	Generator column method (U.S. EPA 1996)	Partition of compound between solid support sorbed *n*-octanol and eluting water, suitable for $1.0 < \log K_{ow} < 6.0$
	Chromatographic method (OECD 1989)	Retaining of compound in hydrophobic stationary phase of HPLC column, suitable for $0 < \log K_{ow} < 6.0$
Structure-based estimations	Fragment method (Rekker 1977)	
	Computerized fragment method (CLOGP, Chow & Jurs 1979)	Values for "fundamental" fragments of molecule are given from experimental Kow values via multiple linear regressions and summarized with correction factors.
	Atom/fragment contribution method (AFC, Meylan & Howard 1995)	

A hydrophobic molecule can leave soil water by volatilization or sorption. The sorption of an organic compound from water to soil solid matter is usually directly proportional to its Kow (Schwarzenbach et al. 2003). As mentioned in section 2.2., sorbed molecules are not bioavailable and therefore non-biodegradable. Therefore high Kow values indicate limited bioavailability and biodegradation of an organic compound in soil (Cerniglia et al. 1992, Reid et al. 2000, Semple et al. 2001, 2003).

2.4.2. Impact of the contaminant molecular size on its bioavailability and biodegradability

With increasing molecular size, the boiling point, Kow and sorption of the compound to soil particles increases. These trends are obvious in homologous molecular series, such as aromatic hydrocarbons (Table 10).

Table 10. Boiling points (Bp), log Kows and diffusion coefficients (D_{iw}) of selected aromatic compounds

Compound	Aromatic subunits	Molecular weight (D)	Molar volume ($cm^3\ mol^{-1}$)	Bp [(2)] (^oC)	D_{iw} [(3)] cm^2s^{-1}	log Kow [(4)]
Benzene	1	78.1	71.6	80.1	10.7×10^{-6}	2.17
Naphthalene	2	128.2	108.5	218.0	8.4×10^{-6}	3.33
Anthracene	3	178.2	145.4	341.0	7.0×10^{-6}	4.68
Pyrene	4	202.3	158.5	403.0	6.7×10^{-6}	5.13
Benzo(a)pyrene	5	252.3	215.0	496.0	5.6×10^{-6}	6.13

[(1)] Calculated value (Schwarzenbach et al. 2003)
[(2)] Schwarzenbach et al. 2003
[(3)] Calculated value (Eq. 2)
[(4)] Schwarzenbach et al. 2003

The molecular size of an organic compound determines to some extent also its migration in soil pores. Organic molecules may diffuse also in the smallest pores of soil, including the residual pores (pore diameter < 0.3 µm), which are not accessible for the degrading microbes. The diffusion coefficient (D_{iw}) of a molecule in water can be calculated using Equation 3 (Othmer & Thakar 1953, Hayduk & Laudie 1974, Scwarzenbach et al. 2003):

$$D_{iw}(cm^2 s^{-1}) = \frac{13.26 \times 10^{-5}}{\eta^{1.14} \overline{V}_i^{0.589}} \quad \text{(Eq. 3)}$$

D_{iw} = Diffusion coefficient for compound *i* in water
η = Water viscosity (1.002 x 10^{-2} g $cm^{-1}s^{-1}$ at 20°C)
$\overline{V}_i$ = Molar volume of the compound (cm^3mol^{-1})

Einstein-Smoluchowski equation (Schwarzenbach et al. 2003) is used to calculate transport time by diffusion:

$$t_{diff} \approx \frac{L^2}{D_{iw}} \quad \text{(Eq. 4)}$$

L = Diffusion distance
t_{diff} = Diffusion time

It can be seen from Table 10 and Equation 4 that the time required for benzo(a)pyrene to diffuse over constant distance in water is about two-fold as compared to benzene.

The molecular size is a major factor determining the volatility of an organic compound. Volatility is often described as the boiling point or the vapor pressure, which are measures of the volatility of the condensed, pure compound. However, these parameters give only a rough estimation of the compounds´ behavior in soil, since water is practically always involved in soil environments. A better parameter under such conditions is the Henry´s Law Constant, which describes partition of a compound *i* between water dissolved and air phases and can be presented as a dimensionless variable calculated with Equation 5:

$$K_{ih} = \frac{C_{ia}}{C_{iw}} \quad \text{(Eq. 5)}$$

K_{ih} = Henry´s Law Constant for compound *i*
C_{ia} = Concentration of *i* in air phase
C_{iw} = Concentration of *i* water phase

As can be seen from Equation 5, Henry´s law constant decreases when water solubility increases. Benzene, for example has higher boiling point (80.1°C) than MTBE (55.2°C),

making MTBE more volatile than benzene when present as pure compound. However, as the water solubility of benzene (1.79 g l^{-1}) is lower than MTBE (42 g l^{-1}), the dimensioless Henry´s law constant for benzene (0.224) is greater than that for MTBE (0.029). The low Henry´s law constant combined with poor biodegradability of MTBE has made it persistent in gasoline-contaminated soils (Davis & Erickson 2004, Iturbe et al. 2005, Häggblom et al 2007).

Volatility of a hydrophobic compound makes it usually rather non-persistent in soil due to volatilization. The uncontrolled volatilization of VOCs is not a preferred process, as VOCs represent a direct (Hutcheson et al. 1996) and an indirect human health hazard because they enhance ozone formation in the troposphere (Olivotto & Bottenheim 1998).

2.4.3. Impact of the contaminant toxicity on its biodegradability

Hydrophobic organic compounds are often toxic due to interaction of these compounds with the cellular membranes and membrane constituents (Sikkema et al. 1995). The partition of a hydrophobic compound *i* between water and cellular membranes can be calculated with Equation 6 (Schwarzenbach et al. 2003):

$$\log K_{ilipw} = 0.91 \times \log K_{iow} + 0.50 \quad \text{(Eq. 6)}$$

K_{ilipw} = partition coefficient between water and cellular membrane for compound *i*

A high octanol-water coefficient of a hydrophobic organic compound thus indicates favored partition from water to biological membranes and increased membrane toxicity. Increase in K_{ilipw} indicates increased biomagnification characteristics of the organic compound in water or in soil/water environments (Fisk et al. 1998, Armitage & Gobas 2007). However, the range where Equation 6 can be applied is limited, as the partition of a hydrophobic organic compound to cellular membranes is most preferred when Kow is 1.5 - 4.0, as reviewed by Sikkema (1995) and Ramos et al. (2002). This is due to the multiphase nature of water-membrane lipid bilayers, which differs from water-octanol two-phase system. The water-lipid bilayer system has a hydrophilic interfacial phase

which creates surface tension between water and lipid "bulk" phases, whereas the water-octanol system has water and "bulk" (octanol) phases only (De Young & Dill 1988).

Toxicity of an organic contaminant can limit biodegradation if the toxic effect of the compound is strong enough to limit the microbe´s degrading activity (Alexander 1999). There are numerous standardized microbial toxicity assays, but only few of them are meant for the soil environments. Most tests are intended for testing of aqueous elutriates or other extracts (Ahtiainen 2002). Some solid phase microbial toxicity tests used for sediment and soil testing are presented in Table 11. There are numerous other soil toxicity tests in which the target organism is a plant or soil animal. These tests are meant for general ecotoxicological evaluation of soil properties (ISO TC 190).

Table 11. Microbial toxicity tests applicable for soil environment

Test	Principle	Microbes involved
Luminescent bacteria flash test (Lappalainen et al. 1999)	Kinetic measurement of luminescence inhibition during exposure	*Vibrio fischeri*
Toxi-Chromo Pad test (Kwan 1995)	β-galactosidase synthesis inhibition after/during exposure	*Escherichia coli*
B. cereus contact test (Rönnpagel et al. 1995)	Inhibition of dehydrogenase activity	*Bacillus cereus*
OECD 216: Soil microorganisms: Nitrogen transformation test (OECD 2000A).	Nitrate evolution from organic substrate	Indigenous nitrifying microbes
OECD 217: Soil microorganisms: Carbon transformation test (OECD 2000B)	CO_2 evolution from spiked glucose or O_2 consumption	Indigenous heterotrophic microbes
ISO 14238: Soil quality: Biological methods. Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes (ISO 1997B).	Nitrate evolution from organic substrate	Indigenous nitrifying microbes
ISO 15685: Soil quality: Determination of potential nitrification and inhibition of nitrification. Rapid test by ammonium oxidation. (ISO 2004)	Nitrite evolution from ammonium	Indigenous nitrifying microbes

If a major part of organic carbon in soil comes from the contaminant, the carbon transformation test (Table 11) is poorly applicable especially with long-term contamination. A non-polluted control is usually required when performing toxicity tests. The indigenous microbes in soil are adapted to such contamination and it is impossible to have a non-polluted control soil with identical soil properties.

2.4.4. Impact of the soil type and soil constituents on contaminant bioavailability and biodegradability

As explained in section 2.2., an organic compound may be dissolved in soil water, volatilized into soil vapor or sorbed to soil minerals or organic matter. When soil mineral particle size decreases, the soil surface area increases, creating more surface for adsorption. Small soil particles decrease soil pore volumes and reduce the hydraulic conductivity ("filtration speed") allowing more time for dissolved molecules to interact with soil particles and microbes.

Table 12. Filtration speeds of advecting water in different soils (McRae 1988)

Texture	Indicative hydraulic conductivity (cm h^{-1})
Coarse sand, gravel	> 50
Fine sand	12 – 25
Silt	2-6
Clay	0.5 – 2
Heavy clay	< 0.25

The soil organic matter in soil is usually hydrophobic, because hydrophilic materials leach with advecting water from the soil. Therefore the dissolved hydrophobic organic molecules sorb readily to hydrophobic soil organic matter. This sorption may be adsorption to the surfaces of soil organic matter particles or absorption into the organic matter, depending on the properties of the soil organic matter. It is generally assumed that

sorption of an organic compound to soil organic matter plays a significant role in sorption for soils (Schwarzenbach et al. 1981, Murphy et al. 1990, Doucette 2003), although some contradicting claims have been proposed (Ran et al. 2003). The sorption coefficients for hydrophobic organic compounds onto purified soil organic materials can be several orders of magnitude greater than those measured for mineral model sorbents (Celis et al. 2006). Therefore it can be expected that organic matter is generally the major contributor of bioavailability of a hydrophobic organic compound in soil.

The partition of an organic compound between soil water and soil organic matter can be estimated with the Equation 7 (Schwarzenbach et al. 2003):

$$K_{ioc} = a \times \log K_{iow} + b \quad \text{(Eq. 7)}$$

K_{ioc} = Organic carbon sorption coefficient for the compound *i*
a, b = constants

As can be seen from equation 7, the sorption of an organic compound to organic carbon (soil organic matter) will depend greatly on its octanol-water coefficient. The slope *a* and intercept *b* are compound-group specific constants, which can be determined experimentally (Schwarzenbach et al. 2003).

In addition to the hydrophobic characteristic of a compound, soil organic matter quality also has an effect on the solid-water distribution and the bioavailability of the compound. It has been shown that sorption of a hydrophobic organic to soil organic matter may be controlled by aromatic carbon (Perminova et al. 1999, Abelmann et al. 2005), aliphatic carbon (Simpson et al. 2003, Kang and Xing 2005, Chen et al. 2007), or polarity of the soil organic matter (Tanaka et al. 2005).

The particle- or aggregate state of the organic matter includes portions with both fluid and rigid characters referred to as "rubbery" and "glassy", respectively (Leboeuf & Weber 1997, Xing & Pignatello 1997). Hydrophobic organic compounds may thus both adsorb onto surfaces and micropores of "glassy" and absorb into "rubbery" portions of soil organic matter, resulting in different sorption kinetics (Schwarzenbach et al. 2003, Pan et al. 2007).

Sorption of a compound to soil is time-dependent. Long exposure time results in pronounced sequestration of the compound, since molecular diffusion to the smallest pores of soil particles takes time, as reviewed by Pignatello et al. (1996). Desorption of these sequestered molecules is time consuming or nonexisting. This results in decreased biodegradation of an organic compound when soil contact time increases (McLeod & Semple 2000).

Sorption and desorption processes may differ in extent or time as reviewed by Doucette (2003). This difference, hysteresis, has been proposed to be caused by the entrapment of molecules in soil nanopores or different sorbent properties of "rubbery" and "glassy" portions of organic matter (LeBoeuf & Weber 1997, Luthy et al. 1997, Weber et al. 1998). The sorption-desorption process is assumed to be fast in "rubbery" domains and slow in "glassy" domains. It has been suggested that soil organic matter may change its conformation between "rubbery" and "glassy" when, for example, pH changes (Feng et al. 2006). If such a change in environmental conditions of soil occurs, the conformation change of the organic matter may result in different desorption as compared to sorption if an organic molecule has already sorbed onto it.

2.4.5. Soil microbial populations

The diversity of soil microbial communities is enormous. It has been proposed that soil may contain 10^9 - 10^{10} microbial cell cm^{-3}. Estimations about the number of distinct genomes vary from 10^4 to 10^6 different genomes g^{-1} of soil (Torsvik et al. 2002, Gans et al. 2005, Roesch et al. 2007). The vast majority of this diversity is uncharacterized. These estimations are based on direct analyses of soil DNA and RNA. Information on the physiological properties of the non-cultured soil bacteria is limited. A good example on this is the bacterial phylum *Acidobacteria*, which dominates in many molecular soil surveys (Kuske et al. 1997, Dunbar et al. 1999, McRae et al. 2000). However, at the time of this writing the most recently described genus, *Terriglobus* with *Terriglobus roseus* defined as the type species, is only the fourth described member of *Acidobacteria* phylum (Eichorst et al. 2007).

The microbial populations of soil capable of degrading the organic contaminants have received a substantial amount of research, as reviewed by El Fantroussi & Agathos (2005). The soil microbiology pioneers stated already at the turn of the 19th to the 20th century that "everything is everywhere, and the environment selects" (O´Malley 2007). In other words, if the contaminants are natural products, like crude oil or compounds resembling natural products, the spontaneous development of a microbial population capable of degrading it in soil is only a matter of time. Bioaugmentation, which is a bioremediation protocol in which the degrader microbes are added to the soil faces serious challenges because the inoculant microbes are likely to be affected by the stressful conditions in soil to which they are not adapted (van Elsas et al. 2007). Some success has been achieved when the microbial inoculum has been applied in intensively controlled conditions like above-ground bioreactors under controlled conditions (Alexander 1999). Successful bioaugmentations even in in situ conditions have also been performed when the conditions of the environment in which the inoculum is supposed to function have carefully been taken into account, as reviewed by Jørgensen (2007).

As mentioned, most of the soil microbes are attached to soil particle surfaces as microcolonies or biofilms. Microbial adhesion depends on surface properties of the cells and extracellular polymers, anchoring the cells to surfaces (Chenu & Stotzky 2002). Attached cells depend on substrates dissolved in soil which move with advecting water or by diffusion. Sorbed substrate molecules are not available for microbes even in close proximity without desorption. Microbes can enhance desorption of hydrophobic substrates by producing biosurfactants. Biosurfactants are amphiphilic compounds that reduce surface tension of water and form micelles, thus increasing mobilization and bioavailability of the hydrophobic organic compounds. Biosurfactants can be extracellular or remain attached to the cell (Lang & Philp 1998). Biosurfactants are grouped as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric and particulate compounds (Mulligan 2005). Addition of surfactants to soil contaminated by hydrophobic organic compounds may increase biodegradation of the contaminant as reviewed by Mulligan (2005), but also opposite effects have been observed (Vipulanadan & Ren 2000, Wong et al. 2004, KyungHee et al. 2005). If a hydrophobic contaminant is

present as a bulk phase or it is dispersed as droplets in soil, a direct contact between degrading bacteria and the contaminant phase is possible depending on the surface properties of the microbial cell. In general, it seems that under such conditions bacteria with hydrophilic surfaces produce biosurfactants, whereas bacteria with hydrophobic surfaces act by direct contact to the free contaminant phase (Bouchez-Naitali et al. 1999).

2.4.6. Impact of the soil moisture on contaminant bioavailability and biodegradability

Water is the most significant feature of soil as the habitat for microbial life (Standing & Killham 2007). As every other form of life, soil microbes require water. Gases, heat, microbes, predators and nutrients move with water, but water also acts as a barrier especially in transport of gases. The diffusion coefficient of O_2 in water is 1.8×10^{-4} cm^2 s^{-1}, meaning that it takes over one hour for oxygen to diffuse through a 1 cm water layer (eq. 4). Soil pores in subsurface layers of soil do not have a direct contact to the atmosphere. Therefore, the aerobic activity in soil subsurface layers is totally dependent on oxygen dissolved in advecting water. The availability of oxygen may be rather limited, as water solubility of oxygen is low (0.24 mmol l^{-1} at 20^{o}C). When oxygen is not available, alternative electron acceptors, such as NO_3^- or SO_4^{2-} may be used (Table 13). As mentioned in table 13, an organic contaminant may act also as an electron acceptor.

Table 13. Main redox couples and associated microbial processes (Schwarzenbach et al. 2003, Standing & Killham 2007, Kuchovsky & Sracek 2007). OC = organic compound

Terminal electron acceptor	Final product	Microbial process	Redox potential E^0_H (V)
O_2	H_2O	Aerobic respiration	0.81
NO_3^-	N_2	Denitrification	0.74
Mn^{4+}	Mn^{2+}	Manganese reduction	0.53
Fe^{3+}	Fe^{2+}	Iron reduction	-0.05
SO_4^{2-}	H_2S	Sulphate reduction	-0.27
Halogenated OC	Reduced OC	Reductive dehalogenation	- 0.27 - - 0.43 (chlorinated ethenes)
CO_2	CH_4	Methanogenesis	-0.43

Energy yield from degradation of an organic molecule is the highest when oxygen is used as the terminal electron acceptor. In bioremediation processes, especially in in situ applications, oxygen has been delivered to soil subsurface layers by injecting oxygen-saturated water, air or pure oxygen to the contaminated soil (Jørgensen 2007). Also oxygen releasing compounds have been used. Hydrogen peroxide (H_2O_2) dissolved into water can deliver oxygen to subsurface layers of soil, as hydroge peroxide decomposes spontaneously into water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$). It has been observed that petroleum-degrading soil bacteria can tolerate H_2O_2 concentrations up to 1000 mg l^{-1} (Brown & Norris 1994). Such a concentration of H_2O_2 theoretically releases 29 mmol oxygen l^{-1} of water. Another oxygen releasing compound is magnesium peroxide (MgO_2), an insoluble powder which releases oxygen when hydrated ($MgO_2 + 2H_2O \rightarrow Mg(OH)_2 + H_2O_2$; $2H_2O_2 \rightarrow 2H_2O + O_2$). Magnesium peroxide has been used to construct "oxygen release barriers" by inserting MgO_2-filled polyester filter socks in ground water wells in in situ bioremediation (Odencrantz et al. 2006). Such a solid-phase barrier may release oxygen for several months.

Anaerobic contaminant degradation has been enhanced by injecting nitrate, sulphate or hydrogen releasing compounds to soil. Energy yield from nitrate reduction is almost as high as with O_2 reduction. The availability of nitrate as a terminal electron acceptor can be enhanced much more effectively than oxygen, as reviewed by Wilson & Bouwer (1997). This is due to the high water solubility of nitrate. For example, subject to an initial concentration of 4000 mg kg^{-1} hydrocarbons of soil, in the case of nitrate only 80 m^3 of injection water is required for bioremediation of 1 m^3 of soil, if the nitrate concentration of water is 500 mg l^{-1}. The quantity of water required for remediation of the same amount of soil comes to about 3000 m^3 if the water is saturated with gaseous oxygen (Battermann & Meier-Löhr 1995). Sulphate has been used to facilitate anaerobic degradation of benzene in situ. Sodium sulphate solution (1.14 gl-$^{-1}$) was injected to benzene-contaminated groundwater (4.9 mg of benzene l^{-1}) resulting to benzene reduction below detection limit (Anderson & Lovley 2000).

In reductive dehalogenation, the contaminant itself acts as an electron acceptor. To facilitate dehalogenation, availability of electron donor has to be facilitated. Electron donor (hydrogen) can be injected directly to contaminated plume, or hydrogen releasing compound (fermentable carbon source) like lactate, fumarate or methanol can be used as reviewed by Scow & Hicks (2005) and Jørgensen (2007).

Water content of soil affects the concentration of an organic compound in water. As partition coefficients (Eq 1) are based on concentrations of compound in the different phases, an increase in the total water content of soil leads to an increased desorption of an organic compound from the soil particles into the water phase where they are bioavailable (White & Alexander 1996).

2.4.7. Impact of the soil temperature on contaminant bioavailability and biodegradability

The influence of temperature on the rate of chemical reactions can be described by the Arrhenius equation (Equation 8):

$$\ln k = \ln A - \frac{E_a}{RT} \quad \text{(Eq. 8)}$$

k = rate constant
A = constant
E_a = activation energy (J mol^{-1})
R = gas constant (8.3 J K^{-1})
T = temperature (K)

As can be seen from equation 8, an increase in the temperature results in an increase of the rate constant. Biodegradation of an organic compound in soil is a chemical reaction catalyzed by enzymes in soil. However, since this reaction is catalyzed by soil microbial enzymes, the temperature dependence of biodegradation in soils is not as straightforward as expressed by the Arrhenius equation. The soil microbes can be divided into psychrophilic, mesophilic, thermophilic and hypothermophilic microbes according to their temperature optima (Standing & Killham 2007). The minimum and the maximum temperatures allowing growth for an individual group are typically within a range of 20 - 30 ^{o}C. Temperatures outside this range make the microbes inactive. Within the

temperature region suitable for growth of mesophilic microbes, it is estimated that there is an approximate doubling of the rate of biochemical activity with every 10°C rise between 0°C and 30°/35°C (Gounot 1991, Standing & Killham 2007).

Biodegradation of organic contaminants in soils has been observed to occur in conditions ranging from psychrophilic to hypothermophilic conditions, but the residual concentration of the hydrophobic contaminant seems to be higher in cold than in warmer conditions (Margesin 2000, Kosegi et al. 2000, Ferguson 2003, Feitkenhauer & Markl 2003, Iqbal et al. 2007, Perfumo et al. 2007). This is probably due to decreased bioavailability of a non-gaseous hydrophobic organic compound in a cold environment, as the water solubility, diffusivity and desorption from surfaces decreases when the temperature decreases (Nedwell 1999, Iqbal et al. 2007).

2.4.8. Nutrients

Organic contaminants are mainly composed of carbon (typically 70-80% of molecular weight). A large input of these compounds leads to depletion of the available pools of major inorganic nutrients, such as nitrogen and phosphorus (Morgan & Watkinson 1989), in addition to depletion of electron acceptors. This imbalance can be corrected by biostimulation, which means addition of these nutrients, usually as a commercial fertilizer (Alexander 1999). Biostimulation has been shown to enhance biodegradation of organic compounds when the input of contaminant is high and/or the natural reservoir of inorganic nutrients is low. These conditions prevail especially in marine beaches (Bragg et al. 1994, Swanell et al. 1996, Menendez-Vega et al. 2007) and in landfarming applications (Maila & Cloete 2004).

Inorganic nutrients, as well as organic carbon sources, must be available for the microbes. If nutrient is added in readily soluble form it may be lost by leaching. This can be expected especially in marine beaches which are flooded by tidal water even twice per day (Fernández-Alvarez et al. 2006, Li et al. 2007). In inland areas nitrification, the process in which ammonium is oxidized to nitrite and further to nitrate, may limit the availability of nitrogen. Both NH_4^+ and NO_3^--N can be used by soil microbes, but NO_3^- leaches readily

with advecting water. Leaching losses can be reduced by using slow-release fertilizers, which supply a sustained release of nutrients from insoluble reservoir. These slow-release fertilizers have proved to be usable especially in marine beach bioremediations (Xu et al. 2005A and 2005B).

Amending of soil with nutrients is straightforward when contaminated soil is excavated and bioremediated ex situ or when contamination is limited to soil surface. Nutrient addition faces challenges in situ bioremediation as the need for nutrients is often located in groundwater zone or below it, even along bedrock surface (Jørgensen 2007). Typical application in such cases is injecting nutrients as water solution to the contaminated plume through injection wells (Knapp & Faison 1997), but also gaseous form of nitrogen, ammonia (NH_3) has been used (Marshall 1995). Transport of nutrients in soil can be also be enhanced by applying electrical current across an electrode system inserted in contaminated soil, which promotes movement of charged nutrient ions (Acar et al. 1997)

The quantity of N and P to be added is calculated from the amount of C in the material to be degraded. Alexander et al. (1999) calculated theoretical C:N:P ratio to be 100:3:0.6, assuming that 30% of the carbon in the degraded compound is assimilated into biomass. He also stated that such calculations often overestimate the need of N and P, because biomass is itself decomposed, liberating N and P and because the soil always contains sometimes even considerable available N and P for microbial use.

3. Aims of the study

The aim of this thesis was to (i) investigate bioavailability of organic contaminants and inorganic nitrogen in different soil environments and (ii) develop bioremediation processes which would allow for environmentally safe availability of organic contaminants and inorganic nutrient for the degrading microbes.

In addition to bioremediation, bioavailability of a hydrophobic organic contaminant was studied in a microcosm simulating a natural boreal forest.

Detailed aims of the thesis were:

1. To develop a bioremediation method for VOC-contaminated soil which would allow *ex situ* composting with minimal volatilization losses.

2. To evaluate environmental risks following stimulation of contaminant bioavailability in PAH contaminated soil.

3. To estimate biodegradation and bioavailability of pyrene under conditions simulating boreal forest soil.

4. To assess nitrogen cycling relevant to full-scale bioremediation of oil contaminated soil in order to select a suitable form of nitrogen available for the oil-degrading microbes.

4. Materials and methods

4.1 Experimental setup in the laboratory-, pilot- and full-scale

Bioavailability of two different types of organic contaminants, volatile monoaromatic and hydrophobic polyaromatic hydrocarbons were studied in laboratory- and pilot scale. For details of the experimental setup see Papers I and IV.

Mineralization and volatilization of ^{14}C-benzene in soil was measured in laboratory scale (150 g wt.w) in four closed microcosms. Field-contaminated soils (VOC-contamination) in each microcosm were spiked with ^{14}C-benzene and amended with activated carbon (10 g kg^{-1} of soil) and/or an inoculum of *Rhodococcus opacus* GM-14 (Zaitsev et al. 1995) to a density of 10^{10} cfu kg^{-1} of soil. $^{14}CO_2$ evolved from mineralization of the ^{14}C-benzene was collected in a trap of NaOH-solution. ^{14}C-benzene volatilized from the soil was collected in an activated carbon trap. Mass balances between the mineralized and the volatilized fractions were calculated by measuring the radioactivities in both traps over 52 days. Degradation and volatilization of VOCs (toluene, ethylbenzene, xylenes and trimethylbenzene) in field-contaminated soils was studied in pilot-scale (approx. 1 metric ton) in four rotating drums equipped with aeration systems. The exhaust air from the drums was channeled to gas traps holding 40 kg activated carbon. The soils in the drums were amended with activated carbon (9.8 - 14.5 g kg^{-1} of soil) and/or an inoculum of *R. opacus* GM-14 to a density of 2.2 - 7.7 x 10^8 cfu kg^{-1} of soil. Mass balances between the degraded and volatilized VOCs were calculated by measuring the VOC remaining in the soil and accumulated in the active carbon traps during 240 days.

The effects of vegetable oil amendment on PAH bioavailability was studied in pilot-scale (approx. 26 metric tons) in two reactors under controlled temperature and aeration. For details of the experimental setup see Paper IV. The field-contaminated soil (creosote) in one reactor was amended with 1.5 kg of rape seed oil per l m^3, introduced as a 30% (v/v) emulsion in water. Soil in the other reactor was amended with water only. Composting

time in the reactors was 460 days. Soils were periodically sampled and effects of the rape seed oil amendment were determined by comparing the soil concentrations of 16-EPA PAHs, *Vibrio fischeri* toxicity, and phytotoxicity of the vegetable oil amended and non-amended soils. The effects of rape seed oil on the mineralization of ^{14}C-phenanthrene was separately studied in closed systems.

Mineralization and bioavailability of ^{14}C-pyrene in forest humus was studied in eighteen closed microcosms simulating natural pine/mycorrhiza symbiosis using the method described by Finlay & Read (1986). For details of the experimental setup see paper II. Pristine humus and field-contaminated (mineral oil-waste) soils were spiked with ^{14}C-pyrene and placed into the microcosms with or without pine (*Pinus sylvestris*) and its mycorrhizal fungus (*Paxillus involutus*). The incubation time was 180 days. $^{14}CO_2$ evolved in the mineralization process was collected to traps of NaOH-solution. Mass balances between the mineralized, plant assimilated and soil-attached fractions of ^{14}C-pyrene were calculated by measuring the radioactivities in the gas trap, in the soil and in the vegetation.

Availability and transformations of methylene urea and urea as nitrogen sources were studied at a full-scale mineral oil-waste landfarming field located in southern Finland (60^o 15´ N, 25^o 30´ E). For details of the experimental setup see Paper III. The test area was divided into seven experimental plots of 343…544 m^2. Each plot was fertilized with methylene urea or with urea. Follow-up time was 393 days. Plots were sampled and nitrogen transformations and effects of the transformations were determined by measuring concentrations of NH_4^+-N, NO_3^--N, nitrification rate, heterotrophic bacterial numbers and oil hydrocarbon concentrations.

4.2 Analytical protocols

Table 14. Analytical protocols used in this thesis. For the details see papers I to IV

Analyte	Matrix	Protocol / Reference
Radioactivity	NaOH-solution	Scintillation count (Papers I, II and IV)
	Activated carbon	Burning, $^{14}CO_2$ entrapment and scintillation count (Papers I and II)
	Plant material	Burning, $^{14}CO_2$ entrapment and scintillation count (Paper II)
	Oil-waste soil	Burning, $^{14}CO_2$ entrapment and scintillation count (Paper II)
	Pristine humus	Burning, $^{14}CO_2$ entrapment and scintillation count (Paper II)
VOC concentration	VOC soil	Dichloromethane : acetone extraction and GC/MS analysis (Paper I)
	Activated carbon	Dichloromethane : acetone extraction and GC/MS analysis (Paper I)
NH_4-N concentration	Oil-waste soil	Krom 1980 (Paper III)
NO_3-N concentration	Oil-waste soil	Greenberg et al. 1980 (Paper III)
Nitrification rate	Oil-waste soil	Alef 1995 (Paper III)
Hydrocarbon concentration	Oil-waste soil	Dichloromethane extraction, separation in AlO_3 and gravimetric measurement (Paper III)
Number of heterotrophic bacteria	Oil-waste soil	Laine & Jørgensen 1997 (Paper III)
PAH concentration	Creosote soil	Nordtest 1997 (Paper IV)
V. fischeri toxicity	Creosote soil	Lappalainen et al. 1999 (Paper IV)
Inhibition of barley root growth	Creosote soil	Smreczak & Maliszewsa-Kordybach 2003 (Paper IV)
Inhibition of cress seed germination	Creosote soil	Saadi et al. 2007 (Paper IV)
Inhibition of cress root growth	Creosote soil	Plaza et al. 2005 (Paper IV)

5. Results and discussion

5.1 Controlling bioavailability - VOCs

Reduction of volatilization of monoaromatic VOCs from contaminated soil during bioremediation was studied in Paper I and is retrospectively discussed below. Activated carbon amendment was used as a VOC retaining agent in contaminated soil in static microcosms and rotating drums under controlled conditions in laboratory- and pilot-scale.

Activated carbon resembles soil organic matter in many ways: it is an amorphous form of carbon with many oxygen-containing functional groups. Many types of activated carbons - including the coconut shell carbon used in this study - are derived from plant material (Carrott & Carrott 2007), which is also the major precursor of soil organic matter. The amount of activated carbon used in this study to retain VOCs was 10 - 15 g kg^{-1} of soil (1 - 1.5% w/w). The volatilized fraction of VOCs dominated in both laboratory- and pilot scale compostings (Table 15.) when activated carbon was not present. This indicates that the natural soil compartments are poor adsorbents for VOCs as compared to activated carbon. The superior VOC sorbent properties of activated carbon in soil as compared to soil intrinsic compartments have been observed also elsewhere (Lake & Rowe 2005).

Table 15. Final mass balances of U- ^{14}C-benzene spiked to soil after laboratory scale compostings and mixture of monoaromatic VOCs in field-contaminated soil after pilot-scale compostings.

	Fraction sizes of VOCs, calculated from original data in Paper I							
	Laboratory scale compostings, 52 days. 100% = 293 mg kg^{-1} benzene				Pilot-scale compostings, 240 days. 100 % = 2300 mg kg^{-1} aromatic VOC			
Treatment	A	B	C	D	A	B	C	D
Volatilized	83 %	42 %	15 %	4 %	60 %	72 %	3 %	5 %
Biodegraded	15 %	41 %	36 %	6 %	40 %	28 %	86 %	84 %
Retained in soil	2 %	17 %	49 %	90 %	0 %	0 %	11 %	11 %

A = No activated carbon nor inoculum
B = Inoculated with *R. opacus* GM-14
C = Amended with activated carbon
D = Amended with activated carbon and inoculated with *R. opacus*

VOC volatilization from soil in pilot scale compostings was rapid when activated carbon was not present: 90% of VOCs was lost by volatilization within 12 days (Fig. 2). When activated carbon was present, a major part of the VOCs became biodegraded. However, the residual concentration of VOCs, 270 mg kg^{-1}, was still too high for unlimited land-use. The residual concentration was constant for over 200 days, indicating sequestration of VOCs to activated carbon. Sequestration of organic compounds to soil organic matter occurs after prolonged residence time in soil (Ehlers & Loibner 2006). As the diffusion speed of monoaromatics is rather high due to the small molar volume of a monoaromatic molecule, the sequestration of VOCs to activated carbon seemed to occur within 40 days, since after that no reduction of VOCs was observed. Activated carbon can be grouped as "glassy" carbon phase. Sequestration of aromatic VOCs therefore probably occurs through diffusion into nanopores, which is a relatively fast process due to the high diffusivities of monoaromatic organic compounds.

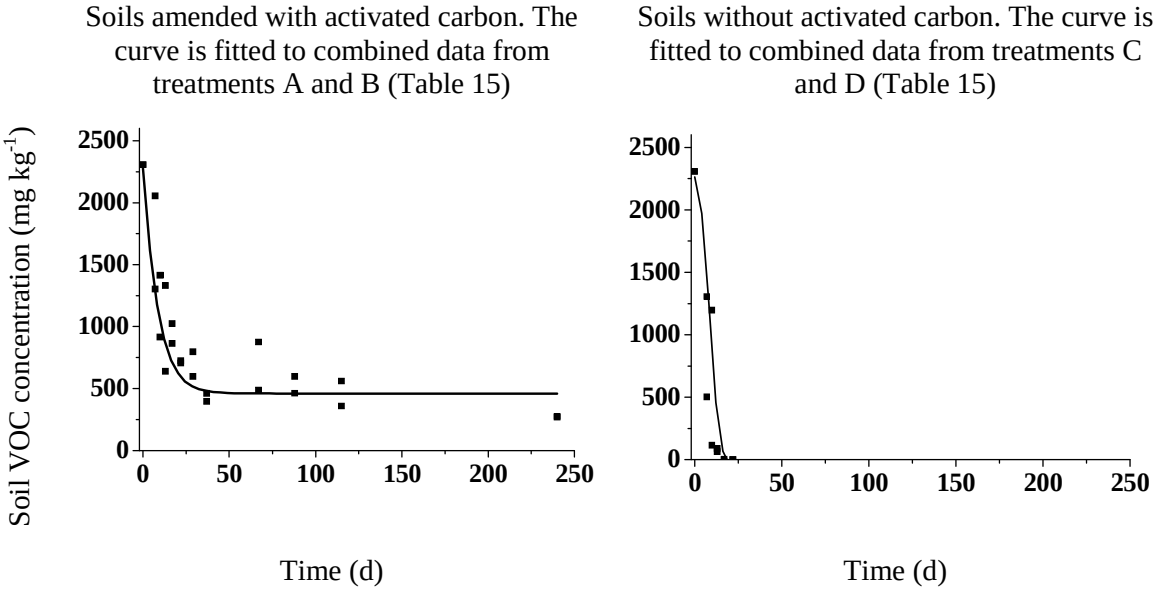


Figure 2. VOC removal from soils in pilot-scale compostings. Re-drawings are based on original data in paper I.

Activated carbon is widely used as a sorbent for harmful organic compounds. Purification of water and air are typical applications. Biodegradation of organic compounds sorbed to activated carbon, bioregeneration, is a known phenomenon in water treatment systems (Aktas & Cecen 2007). As water is always present in natural soils bioregeneration of

activated carbon in water- and soil water environments must have similarities. Bioregeneration of activated carbon in water environment can be enhanced by inoculating it with an individual strain (Jones et al. 1998) or a bacterial consortium (Caldeira et al. 1999). In this study, inoculation of activated carbon by *Rhodococcus opacus* GM-14 dramatically decreased the volatilization of benzene when studied at laboratory scale. However, decrease of volatilization did not result in an improved mineralization. *R. opacus* strains are known to use the dioxygenase pathway for degradation of benzene (Na et al. 2005). The dioxygenase pathway proceeds with catechol as an intermediate. Catechol, however, oxidizes abiotically in aerobic environments into water insoluble polymers (Colarieti et al. 2002). Such polymers may bind to activated carbon irreversibly, as reviewed by Aktas and Cecen (2007). This could explain the lack of volatilization and mineralization in laboratory scale when both *R. opacus* and activated carbon were present. In pilot scale, inoculation had no effect on volatilization nor biodegradation of the aromatic VOCs, indicating superiority of intrinsic degrader microbes as compared to inoculant bacteria. Also the size of inoculum was higher in the laboratory- than in the pilot scale compostings.

5.2 Controlling bioavailability - PAHs

Polyaromatic hydrocarbons have limited bioavailability due to their high hydrophobicity. These limitations may be overcome by addition of a mobilizing agent, such as surfactant or vegetable oil. Rape (*Brassica rapa*) seed oil emulsion was used as an attempt to mobilize aged PAHs in creosote-contaminated soil excavated from timber impregnation field. The effects on mobilization on PAH biodegradation and soil toxicity were studied under controlled aeration and temperature at pilot-scale in Paper IV and is retrospectively reviewed and discussed below.

Mixing PAH-contaminated soil with water emulsion of rape seed oil resulted in an apparent increase of the concentration of PAHs in the soil. This indicates mobilization of sequestered PAHs (Table 16).

Table 16. Effect of rape seed oil emulsion on apparent soil concentrations of pentane extractable PAHs. The data is presented in details in Paper IV.

PAH	K_{ow} [(1)]	Concentration in non-amended soil (mg kg^{-1})	Concentration in soil amended with rape seed oil emulsion (mg kg^{-1})
Naphthalene	3.17	79	156
Acenaphthylene	3.94	13	22
Acenaphthene	4.15	395	551
Fluorene	4.02	341	497
Phenanthrene	4.35	634	911
Anthracene	4.35	136	185
Pyrene	4.35	368	537
Fluoranthene	4.93	535	745
Benzo(a)anthracene	5.52	95	126
Chrysene	5.52	97	135
Benzo(b)fluoranthene	6.11	44	59
Benzo(k)fluoranthene	6.11	38	49
Benzo(a)pyrene	6.11	31	42
Indeno(1,2,3-c,d)pyrene	6.7	8	12
Dibenzo(a,h)anthracene	6.7	14	2
Benzo(g,h,i)perylene	6.7	5	8
Σ [16 U.S. EPA PAH][(2)]		**2833**	**4037**

[(1)] Calculated value: [www.syrres.com/esc/est_kowdemo.htm]
[(2)] Keith & Telliard (1979)

It is our suggestion that PAHs migrated from the soil pores into droplets of rape seed oil emulsion. Transfer of phenanthrene from soil to oil emulsion droplets has been documented previously (Zhao et al. 2005). Chiou (1985) determined the partition coeffients (K_{itriow}) for 38 different hydrophobic organic compounds in water-triolein systems and found them to be slightly greater than the water-octanol coefficient, when log Kow of the compound was ≤ 5.10, and slightly smaller than the water-octanol coefficient, when log Kow of the compound was ≥ 5.58. Organic matter-soil partition coefficients of an organic compound can be calculated by equation 7:

$$K_{ioc} = a \times \log K_{iow} + b$$

For PAHs, the constants *a* and *b* were determined to be 0.98 and -0.32, respectively (Schwarzenbach et al. 2003). Since the K_{ioc} of a PAH is always smaller than the K_{iow} and the K_{itriow} greater than K_{iow} until K_{iow} exceeds 5.58, it can be expected that the partition of a PAH will favor the vegetable oil phase in soil-vegetable oil systems up to K_{iow} values of 5.5 or higher.

The soil concentration (i.e. apparent K_m value, see paper IV, Table 2) required for mineralization of ^{14}C-phenanthrene was clearly higher in soil amended with rape seed oil emulsion. The same amendment immediately increased the toxicity of soil when analysed by direct contact to cress roots and barley roots (Table 17). Rape seed oil emulsion thus increased the bioavailability of soil toxic constituents at least to plant roots. The increased bioavailability did not result in increased biodegradation of PAHs. On the contrary, the apparent substrate concentrations required for mineralization increased when rape seed oil was present (Paper IV, Table 2). Reduction of PAH biodegradation in soil in presence of 1% rape seed oil has been detected also elsewhere with spiked soil (Pizzul et al. 2007). PAH degrading microbes may have preferred vegetable oil as an energy source instead of PAHs. It is also possible that copiotrophic soil bacteria which grow rapidly in presence of easily degradable substrate like vegetable oil colonize the surroundings of rape seed oil droplets. As PAH-degrading bacteria are assumed to be oligotrophic as reviewed by

Johnsen et al. (2005), they are suppressed by such rapidly growing, opportunist soil microbes.

Table 17. Effect of rape seed oil emulsion to toxicity of soil containing aged PAH calculated from original data in paper IV.

Soil toxicity assessed by	Non-amended soil	Soil amended with rape seed emulsion
Inhibition of *V. fischeri* luminescence (%)	84 %	85 %
Inhibition of barley root growth (%)	28 %	69 %
Inhibition of cress root growth (%)	0 %	47 %
Inhibition of cress seed germination (%	3 %	10 %

The International Organization for Standardization (ISO) defines bioavailability in terms of relevant target organisms: bioavailability to organisms able to degrade the contaminant, bioavailability to organisms able to ingest the contaminant, bioavailability to plants and bioavailability to humans (ISO 2003). In this study, increased bioavailability of soil contaminants by rape seed oil emulsion was more relevant for plants than degrading microorganisms. The consequences from the increase of PAH bioavailability after amending the soil with rape seed oil is a good example of different categories of bioavailability.

5.3 Bioavailability of pyrene in humus

As mentioned in section 2.4.4., it is generally assumed that sorption of an organic compound to soil organic matter plays a significant role in sorption for soils. Since sorbed compounds are not bioavailable, it can be assumed that a hydrophobic organic compound is sparingly bioavailable in the organic surface layer of boreal forest soil. To study this, gamma-sterilized humus spiked or not spiked with pyrene and mixed or not mixed with oil contaminated soil was implanted in microcosms with or without pine and its mycorrhizal fungus, *Paxillus involutus*. The results are presented in details in Paper II and reviewed and discussed below.

After mycorrhiza had colonized the implanted regions in microcosms (35 days after implantation) the gamma-sterilized humus spiked with radiolabelled pyrene was added to the microcosms. The CO_2 produced in the microcosms during incubation over 180 days was collected and amount of $^{14}CO_2$ measured. After incubation, ^{14}C assimilated into microcosm vegetation and/or remained in implantations was also measured (Table 18).

Table 18. Mineralization rates and percentages of incorporated ^{14}C-pyrene spiked into humus implants in microcosms with or without pine and mycorrhiza based on original data in Paper II. A = With pine and mycorrhizal fungi, B = Without pine and mycorrhizal fungi

Implantation	Concentration of pyrene (mg kg^{-1})	Average mineralization rate during 180 days [(1)] (µg kg^{-1} d^{-1})		Percentage of input ^{14}C remained in the implant after 180 days	
		A	B	A	B
^{14}C-pyrene spiked humus with additional pyrene	91.2	62	106	80. ±13.3	50.4±8.5
^{14}C-pyrene spiked humus	0.07	$< 3 \times 10^{-3}$	$< 3 \times 10^{-3}$	79.9±9.8	89.9±8.5
^{14}C-pyrene spiked humus mixed with oily soil	0.95	0.5	0.7	71.4±12.6	85.7±9.6

[(1)] Calculated by dividing sum of mineralized and assimilated ^{14}C by incubation time.

The highest mineralization rate and the most effective pyrene removal were observed in the humus implants with the highest input concentrations of pyrene. The experimental K_{ioc} of pyrene is 5.13 (Schwarzenbach et al. 2003). It can therefore be concluded that sorption

of pyrene to humus is strongly favored, leading therefore to decreased availability and biodegradation as reviewed by Johnsen et al. (2005) and Semple et al. (2003). Biodegradation of PAHs in the environment has been studied actively for several dozens of years, as reviewed by Atlas (1981) and Johnsen et al. (2005). A decrease of pyrene degradation in soil when the amount of organic matter increases has been observed (Smith et al. 1997, Cottin & Merlin 2007), but knowledge about pyrene degradation in boreal humus is limited. Kurola (2007) studied mineralization of phenanthrene and pyrene in non-sterile boreal humus with an initial soil concentration of 0.2 – 50 μg cm^{-3}. These authors found phenanthrene and pyrene mineralization percentages below 5 % in 98 days. Bogan & Sullivan (2003) studied phenanthrene mineralization by inoculant bacteria in six gamma-sterilized soils with varying content of organic matter. In their study, contact time of phenanthrene in the gamma-sterilized soils varied from 0 to 120 days prior to inoculation. Interestingly, the mineralization of phenanthrene in soil with the highest organic matter content (25%) after 0 and 40 days of contact time was equally active, indicating no sequestration during the 40 day contact time. In soils with lower organic matter content (2 – 11%), mineralization was clearly lower after 40 days contact time. In the present study, 50% of ^{14}C-pyrene was removed from humus containing 90 mg kg^{-1} of pyrene and 32% organic carbon (w/w, Paper II). These findings contradict the conclusion about low bioavailability and low biodegradation of pyrene in organic soils. However, the humus in the soil in this study as well as in the study of Bogan & Sullivan (2003) was sterile (γ-irradiated) and possibility that chemical properties of the humus were altered due to strong ionizing radiation cannot be excluded.

The presence of pine and pine mycorrhiza decreased pyrene removal from humus when the initial concentration of pyrene was high. After the data in Paper II was published (2003), similar phenomena were reported by Genney et al. (2004) and Joner et al. (2006) with fluorene and anthracene biodegradation in pine/mycorrhiza microcosms. Heinonsalo et al. (2000) observed increase in polarity of hydrocarbons in soil when pine/mycorrhiza combination was present, indicating oxidative attack by mycorrhiza. Fungal enzymes are known to oxidize PAHs (Rodriguez et al. 2004, Baborova et al. 2006) resulting in bound residue formation in soil (McFarland & Qiu 1995). It is thus possible that the presence of mycorrhiza in humus resulted in covalent binding of oxidized PAHs to the humus.

5.4 Bioavailability of nitrogen in bioremediation

The bioavailability of nutrients is equally important as the bioavailability of carbon and energy source for bioremediation. The fate and bioavailability of fertiliser nitrogen (urea and methylene urea, Fig. 3.) were studied on a landfarming site with mineral oil pollution. The results are presented in details in Paper III and reviewed and discussed below.

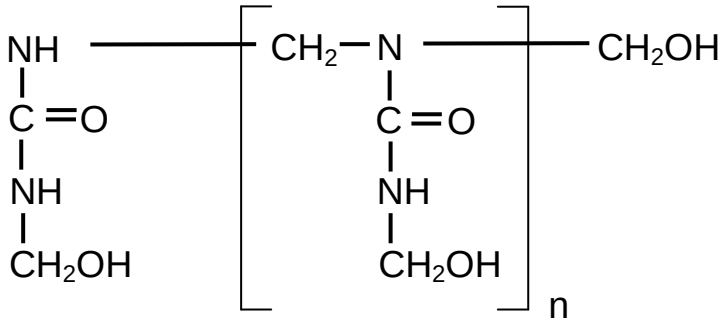


Figure 3. Molecule structure of polymeric form of urea, methylene urea

The landfarming field had received oil waste, mainly sludges, for over 20 years and had been routinely fertilised for more than 10 years with high amounts of urea targeted to give a carbon: nitrogen ratio of 10**:**1. The soil contained 35 – 59 g kg^{-1} of non-polar solvent extractable organic matter of mainly refinery oil waste origin. Seven test plots at the site were fertilised with different doses of methylene urea or urea. The immediate effects of fertilizer nitrogen form and dosage on landframing soil nitrogen transformations, pH and heterotrophic bacterial counts were obvious (Tables 19 and 20), especially with high doses of urea.

Table 19. Effects of the highest applied doses of fertilizer nitrogen in oil waste landfarming soil. The data is presented in details in Paper III.

Fertilization of the plot	Concentration of NH_4-N (mg kg^{-1})		Concentration of NO_3-N (mg kg^{-1})		pH		Heterotrophic bacteria (10^8 $cfug^{-1}$)	
	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17
None	7.5 ± 0.3	10 ± 2.8	224 ± 24	44 ± 6	6.9	7.4	1.6 ± 0.69	3.5 ± 0.34
Methylene urea 889 g N m^{-2}	34.3 ± 7.6	190 ± 42	620 ± 15	1430 ± 141	7.1	6.3	1.3 ± 5.4	15 ± 8.4
Urea 893 g N m^{-2}	296 ± 2.4	5580 ± 594	461 ± 10	380 ± 71	6.9	8.6	2.5 ± 8.9	0.05 ± 0.01

Table 20. Nitrification rates in landfarming soil with the highest fertilizer nitrogen doses. The data is presented in details in Paper III.

Fertilization of the plot	Ex situ nitrifying activities of the soil (mg NO_3-N formed d^{-1} kg^{-1} of soil)				
	Day 0	Day 17	Day 70	Day 287	Day 393
None	0.70 ± 0.23	1.69 ± 0.62	0.71 ± 0.71	0.28 ± 0.05	0.26 ± 0.05
Methylene urea 889 g N m^{-2}	1.60 ± 0.20	8.83 ± 2.63	0.97 ± 0.27	0.53 ± 0.12	0.45 ± 0.11
Urea 893 g N m^{-2}	2.11 ± 0.38	0.53 ± 0.73	0.63 ± 0.07	0.41 ± 0.11	0.33 ± 0.02

High soil concentrations of NH_4-N found in the plots dosed with high amount of urea indicated high ureolytic activity in the landfarming soil. Activity of the subsequent nitrogen transformation, nitrification, was low or not detectable in this plot (Table 20). This explains why NH_4 accumulated. Such a phenomenon has been reported in agricultural soil where nitrification was blocked (Cai et al. 2002) Also soil pH increased resulting in the dominance of NH_3 in the NH_4^+/ NH_3 pool. The gaseous NH_3 may have intoxicated the nitrifying bacteria, but also the numbers of heterotrophic bacteria declined. The numbers of the heterotrophic colony forming units recovered rapidly, but nitrification rates remained low during the whole observation period of almost 400 days (Figures 2 and 4 in Paper III, Table 20). When fertilizing was done with methylene urea, the NH_4 concentration did increase, but not to toxic level, and the number of heterotrophic colonies was stable and nitrification active.

Nitrification in the soil with high concentration of hydrocarbons was as active (Table 20) as reported for agricultural soils (Alef 1995). This should be taken into account when fertilizing bioremediation processes, especially under *in situ* conditions to avoid nitrogen transformation to less or non-bioavailable form. Dosing of nitrogen is usually calculated based on the concentration of carbon in the contaminant pool to be degraded in the soil (Section 2.4.8, Alexander 1999). However, if fertilization is done repeatedly, a clear distinction should be done between the total concentration of carbon and the biodegradable concentration of carbon. In the studied landfarming field the soil

concentration of organic carbon in hydrocarbons was about 30 g kg^{-1} and removal of organic carbon was 8 g kg^{-1} y^{-1} (Paper III). If yearly nitrogen dosing is based on total amount instead of the biodegraded amount target carbon, more than three-fold overdosing occurs. Such overdosing is wasteful since the nitrogen transformation end-product, nitrate escapes from soil by leaching. Overdosing may even be detrimental to soil biota wit high doses, as intermediate product of microbial nitrogen transformation chain, NH_4^+ dissociates to gaseous and poisonous NH_3 in high pH. When the need of nitrogen is high, these effects can be avoided by using slowly soluble forms of nitrogen, for example methylene urea.

6. Conclusions

The following conclusions summarize the results achieved in this thesis:

1. VOC volatilization from contaminated soil during bioremediation can be prevented by adding a small amount of activated carbon (1 %). The major part of VOCs adsorbed to activated carbon are bioavailable and biodegradable. However, the residual concentration of VOCs in pilot-scale composted soil indicates irreversible sequestration of VOCs to activated carbon.

2. Inoculating activated carbon with degrader bacteria reduces volatilization losses further in laboratory-scale, but did not allow scaling up to pilot scale.

3. PAH-compounds in contaminated soil were mobilized by emulsified food grade vegetable oil. This mobilization increased bioavailability of the toxic constituents in soil which could be measured as phytotoxicity, but biodegradation of the PAHs was inhibited. Bioavailability of toxic constituents may increase the ecotoxicogical risk of soil under bioremediation and should be taken into account when manipulating the bioavailability of contaminants in soil.

4. Mobilization of PAHs by vegetable oil lowered the affinity of degrading microorganisms to substrate. One explanation for this is that the degrader organisms may have preferred vegetable oil over the PAHs as an energy source

5. Pyrene can be degraded effectively in an organic matrix (boreal forest humus) without previous exposure to high concentrations of PAHs. The high K_{ow} of a substrate does thus not necessarily mean low availability and biodegradation in organic soil.

6. The presence of pine/root mycorrhiza decreased the mineralization and possibly increased the sequestration of ^{14}C-labelled pyrene into soil humus.

7. Nitrogen transformations can be fully functional in heavily oil-contaminated soil. The nitrogen cycle may become interrupted in excessively fertilized soil when the intermediate product from urea, NH_4^+ dissociates to the volatile NH_3.

8. In bioremediation, nitrogen dosing should be based on biodegradable rather than the total amount of the organic contaminant. Otherwise overdosing of fertilizer may occur which is wasteful and high doses can harm the soil biota.

8. When high doses of nitrogen are needed for bioremediation, slowly soluble forms of nitrogen should be used to avoid the detrimental effects of rapid transformations of nitrogen.

7. Tiivistelmä

Väitöskirjan tavoitteena oli tutkia öljyperäisillä orgaanisilla yhdisteillä pilaantuneen maa-aineksen biologisen puhdistamisen edellytyksiä laboratorio-, pilot- ja täydessä mittakaavassa. Erityistä huomiota kiinnitettiin tekijöihin, jotka vaikuttavat pilaantumista aiheuttavien haitta-aineiden sekä biohajotusprosessissa tarvittavan typen biosaatavuuteen. Tutkittavat haitta-aineet olivat haihtuvia orgaanisia öljyjakeita (VOC), monirenkaisia aromaattisia hiilivetyjä (PAH) ja raskaita mineraaliöljyjakeita.

VOC-yhdisteiden biosaatavuutta parannettiin lisäämällä pilaantuneeseen maahan pieni määrä aktiivihiiltä. Tällä toimenpiteellä ehkäistiin VOC-yhdisteiden haihdunta maasta biologisen käsittelyn aikana. Aktiivihiileen kiinnittyneet VOC-yhdisteet olivat enimmäkseen hajottajamikrobeille biosaatavassa muodossa, koska niiden pitoisuudet laskivat maa-aineksessa ilman että VOC-yhdisteitä karkasi ilmakehään. Jäämäpitoisuudet maassa olivat kuitenkin liian korkeat käsitellyn maan rajoittamatonta loppusijoitusta varten. Osa aktiivihiileen sitoutuneista VOC-yhdisteistä sitoutui siten maa-ainekseen palautumattomasti, mahdollisesti mikrobiologisiksi aineenvaihduntatuotteiksi muuntuneina.

Rypsiöljyemulsion lisääminen pilaantuneeseen maahan paransi PAH-yhdisteiden biosaatavuutta. Kasvisöljy mobilisoi maahan sitoutuneita PAH-yhdisteitä, mikä näkyi PAH-yhdisteiden lisääntyneenä uutettavuutena. Mobilisoitumisesta huolimatta PAH-yhdisteiden biohajotus ei tehostunut eli ne eivät olleet hajottajamikrobeille biosaatavassa muodossa. Sen sijaan biosaatavuus kasveille lisääntyi, mikä näkyi maa-aineksen lisääntyneenä toksisuutena kasvien juurille. Tämä osoittaa, että biosaatavuus kasveille on eri asia kuin biosaatavuus mikrobeille.

PAH-yhdisteiden biohajomista tutkittiin malliekosysteemissä, eli mikrokosmoksessa joka mallinsi pohjoista metsäekosysteemiä pienessä mittakaavassa. Pilaantumattomassa metsämaahumuksesta löytyi selkeä PAH-yhdistettä (pyreeni) biohajottava potentiaali,

vaikka aiempi tutkimus oletti orgaanisen aineen runsauden huonontavan rasvaliukoisten orgaanisten yhdisteiden biosaatavuutta mikrobeille ja siten myös biohajoavuutta. Kun malliekosysteemiin lisättiin kasvavan männyn taimi ja sen juurisieni (mykoritsa), niin pyreenin mineralisoituminen eli hajoaminen hiilidioksidiksi asti väheni. Tämä johtui mahdollisesti pyreenin kemiallisesta sitoutumisesta humukseen juurisienen hapettavien entsyymien katalysoimana.

Maa-aineksen biologisen käsittelyn kannalta välttämättömän typpiravinteen biosaatavuutta tutkittiin täyden mittakaavan biologisella käsittelyalueella, johon oli tuotu öljyisiä lietteitä yli 20 vuoden ajan. Kun lannoitteena oli urea, se hajosi nopeasti ammoniakiksi osoittaen ureolyyttisten mikrobien olleen aktiivisia. Myös nitrifikaatio, eli ammoniakin mikrobiologinen hapetus nitraatiksi toimi öljynpitoisessa maassa tehokkaasti, mutta nitrifikaatio oli hitaampaa kuin urean hydrolyysi mikä johti ammoniumtypen kertymiseen kun lannoitus oli runsas. Tästä seurasi pH:n nousu ja ammoniakin haihdunta, eli typpilannoitetta hukkaantui ilmakehään. Tutkimus osoitti, että hitaasti vapautuvia typen muotoja, kuten metyleeniureaa, onkin syytä käyttää kun typen kokonaistarve on suuri koska ammoniakin kertymistä ei tällöin tapahtunut.

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