In situ Bioremediation of Organic Contaminants: Constraints and Novel Approaches

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Når det kjem til stykket

År ut og år inn
har du site bøygd yver bøkene,
du har samla deg meir kunnskap
enn du treng til ni liv.
Når det kjem til stykket, er det
so lite som skal til, og det vesle
har hjarta alltid visst.
I Egypt hadde guden for lærdom
hovud som ein ape.

Olav H. Hauge

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In liebevoller Erinnerung an meinen Vater

Contents

Abstract

In situ bioremediation is an efficient and sustainable technology for the removal of organic pollutants from contaminated soil and groundwater. A comprehensive set of measures is available to support the natural capability of microorganisms to transform and mineralise various types of hydrocarbons, including petroleum constituents, polycyclic aromatic and chlorinated aliphatic compounds.
These biological technologies, while being operable at lower economic and energetic expense than conventional measures, require the sound knowledge of specific biodegradation mechanisms and the manifold interactions between soil constituents, pollutants and microbes. Contaminant bioavailability, the competence of the microbial population for pollutant biotransformation as a function of the soil environment and the availability of reaction partners for contaminant oxidation and reduction play pivotal roles in the determination of the contaminants' fate in the subsurface and may severely inhibit biodegradation. In the following, the objective of the present work is to improve the understanding of constraints to *in situ* bioremediation and the development of novel, environmentally sensible approaches for their characterisation and overcoming.
The superposition of multiple effects of soil composition, influencing microbial proliferation and compound bioavailability, is reflected in the dissimilar biodegradation behaviour of petroleum hydrocarbons in different soil matrices (Paper I). Along this line, sorption modelling and multivariate statistical analysis reveal aromatic functional groups of soil organic matter to govern alkane sorption to soil (Paper II). Novel methods for the characterisation and for the overcoming of hydrocarbon bioaccessibility are introduced in Paper III. Eventually, microbial limitations to biodegradation independently from bioavailability considerations are in the focus in the final section (Paper IV), where a new approach to assist chlorinated hydrocarbon biodegradation in the saturated zone is proposed.

Keywords: *in situ* bioremediation, hydrocarbons, bioavailability, biodegradation

Kurzfassung

Biologische *in situ* Sanierung ist eine effiziente und nachhaltige Technologie für die Entfernung von organischen Schadstoffen aus kontaminiertem Boden und Grundwasser. Ein umfassender Maßnahmenkomplex dient der Unterstützung der natürlichen Fähigkeit von Mikroorganismen, unterschiedliche organische Schadstoffe, wie Mineralöl-, Polyzyklische Aromatische und Chlorkohlenwasserstoffe, zu transformieren und zu mineralisieren.
Diese biologischen Maßnahmen können unter geringerem finanziellem und energetischem Aufwand betrieben werden als konventionelle Technologien, setzen jedoch umfassendes Wissen sowohl über Abbaumechanismen sowie über die vielfältigen Wechselwirkungen zwischen Bodenbestandteilen, Schadstoffen und Mikroorganismen voraus. Die Bioverfügbarkeit von Schadstoffen, die Fähigkeit der Mikroorganisms, Schadstoffe zu transformieren als Funktion der Milieubedingungen im Untergrund, und die Verfügbarkeit von Reaktionspartnern für die Oxidation oder Reduktion von Schadstoffen spielen eine Schlüsselrolle in Bezug auf den Abbau im Untergrund und können ihn wesentlich behindern. Das Ziel der vorliegenden Arbeit ist es, ausgewählte Limitationen des biologischen Abbaues im Untergrund zu charakterisieren und neuartige, ökologisch unbedenkliche Lösungsansätze für deren Überwindung zu entwickeln.
Die Superposition der vielfältigen Einflüsse des Bodens, die sowohl die mikrobiologische Leistungsfähigkeit als auch die Schadstoffverfügbarkeit wesentlich bestimmen, spiegelt sich im unterschiedlichen Abbauverhalten von Mineralölkohlenwasserstoffen in unterschiedlichen Böden wieder (Paper I). Mit Hilfe von multivariater statistischer Analyse kann nachgewiesen werden, dass das Sorptionsverhalten von Alkanen im Boden wesentlich vom Vorhandensein von aromatischen funktionellen Gruppen der organischen Bodensubstanz bestimmt wird (Paper II). Neuartige Methoden werden auch für die Bestimmung und Überwindung der Bioverfügbarkeit von Kohlenwasserstoffen im Boden vorgestellt (Paper III). Schließlich wird ein effektives Verfahren zur Ermöglichung des vollständigen Abbaues von chlorierten Kohlenwasserstoffen unter anaeroben Bedingungen vorgeschlagen (Paper IV).

Schlagworte: biologische *in situ* Sanierung, Kohlenwasserstoffe, Bioverfügbarkeit, biologischer Abbau

Part I

Introduction

Chapter 1

Organic Pollutants in the Environment

1.1 Sources and Extent of Contamination

Accidental spills, careless handling or the incomplete assessment of hazardous effects on environment and human health have lead to the extensive release of organic contaminants to the environment in the past and is continued in the present. In the year 2008, over 50.000 sites were registered as old deposits and old industrial sites in Austria, with different classes of organic pollutants constituting the most prevalent pollutant group in soil and groundwater (Weihs and Siller, 2008). Accordingly, hydrophobic organic contaminants (HOC) such as chlorinated aliphatic (CAH), petroleum (PHC) and polycyclic aromatic hydrocarbons (PAH) is the most abundant contaminant group found at contaminated sites. Their great number demonstrates the need for efficient, economic and environmentally friendly technologies for pollutant control and reduction.
Biologically based *in situ* technologies, effecting contaminant transformation to innocuous products, are ideal candidates for this task. The in-depth understanding of the underlying processes is indispensable for the design of a remediative measure, owing to the complexity of biological processes in the subsurface. The effective management of *in situ* bioremediation is based on the characterisation of obstacles to the biological transformation and mineralisation of xenobiotics and on the elaboration of efficient approaches for their circumvention, providing the motivation for the work presented here.

1.2 Recognition of Toxic Potential

In spite of the large scale release of xenobiotic organic substances to the environment in the course of the past two centuries, from soot and other combustion residues originating from coal pyrolysis, the spilling of oil during

transportation and production commencing in the first half of the nineteenth century, to the development and widespread use of chlorinated hydrocarbons and pesticides in the post-World War II era, the harmful effect of many of these substances on human health and environment were long not recognized to their full extent.

In the year 1775, assumedly the first environmental scientist, Sir Percival Pott, suspected the carcinogenic potential of benzo[a]pyrene from chimney soot, which was proven in 1933 (Harvey, 1991) and resulted eventually in the definition of 16 priority pollutant PAH in the 1980's (ATSDR, 1990). Similarly, chlorinated aliphatic hydrocarbons with Perchloroethene (PCE) and 1,1,1 Trichloroethane as the most prominently used compounds, were employed in many industrial branches as detergents and degreasing agents, including metalworking, textile and colouring industry as well as defence forces, after WWII. While the evidence for CAH toxicity was accumulating since the 1940s, the carcinogenic potential of PCE was recognized only in 1977 (NCI, 1977), but the substance was being sold by retail until the 1980s. Public concern on possible detrimental effects on human health and environment caused by the deliberate or accidental release of largely unknown chemicals to the environment was raised only tardy. Eventually, in 1962, the publication of a book containing an unprecedented data compilation on the severe acute and chronic detrimental effects of pesticides and other xenobiotics on human health and wildlife (Carson, 1962), helped initiate the political environmental movement in the USA. In the wake of the foundation of the US Environmental Protection Agency (EPA) in 1970, private and public organisations pursuing similar purposes were installed in many countries.

Environmental pollution, however, remains a worldwide issue of concern for human health and ecosystems and requires sustainable solutions for its mitigation.

Chapter 2

Remediation of Contaminated Sites

Due to the lack of knowledge or in disregard of the potentially adverse effects of organic chemicals, contaminated soils and aquifers can be presently found worldwide. These sites are subsequently requiring remediation in order to restore their original functions in the ecosystem or for human use.

2.1 Conventional Approaches

There are numerous physically or chemically based soil and groundwater treatment technologies available to reduce contamination. These methods may be applied *in situ*, with the bulk contaminated mass remaining in place, or *ex situ*, characterised by displacement and surface treatment of contaminated mass. These technologies include *Pump & Treat* systems for contaminated groundwater, *Soil Vapour Extraction* for volatile compounds, *In Situ Chemical Oxidation* or *Reduction* (*ISCO* or *ISCR*) or excavation of contaminated soil followed by soil washing, thermal desorption, incineration or mere disposal without further treatment, and several more. These technologies mostly rely on the chemical destruction and / or the extraction of the contaminant or of the bulk contaminated medium.

2.2 Principles of Bioremediation

Bioremediation, by contrast, relies on supporting the natural capability of microorganisms to transform contaminants to less harmful organic or, most desirable, innocuous mineral compounds, i.e. water and carbon dioxide, and is potentially applicable at many sites. Microorganisms are utilizing the contaminant directly as a source of carbon and / or energy, or the compound may be transformed in a co-metabolic process (Alef, 1994). The initiation or acceleration of these naturally occurring degradation processes

can be performed using one or more of a considerable set of possible measures, and may be done so *in* or *ex situ*. *In situ* measures hold, while being of higher complexity and sensitivity than *ex situ* measures, several advantages, including operation under perpetuation of the soils' multiple functions of interest to the stakeholders, e.g. as building lots. The effective implementation of a bioremediation measure generally requires, in contrast to conventional technologies, lower financial and energetic expense, but the sound understanding of the complex cause-and-effect relations governing the efficient microbial metabolisation and eventually mineralisation of organic pollutants.

2.3 Current State of Knowledge and Technologic Potential

The pioneering case for a targeted promotion of microbial contaminant degradation was reported for the Sun Oil Spill in Amber, Pennsylvania, USA, in the year 1972 where the addition of oxygen and fertilizers induced a significant decrease in subsurface PHC content (Lee and Ward, 1985). Since then, bioremediation technology has experienced significant advances, not least by the advent of sophisticated technologies for the characterisation of soil constituents, microbes, soil-pollutant interactions, the modelling of subsurface processes and more, and have rendered it a practical and effective alternative remediation technology. On a large scale, the most prominent boundary conditions to bioremediation and, as a result, the constraints to its effective implementation, are known to date. The full set of interactions occurring in a soil-pollutant-microbe system, however, is still awaiting its ultimate disclosure. In addition, the development of advanced technologies to overcome limitations to biodegradation requires in-depth research.

Chapter 3

Constraints & Novel Approaches

3.1 Key Issues

The occurrence and extent of organic pollutant biodegradation in the environment is dependent on a large set of factors, with the soil environment, the history and type of contamination obtaining key positions in determining contaminant fate. These factors can be classified into biotic and abiotic factors. Where biotic factors determine the specific microbial community, e.g. in its diversity, size or its capability to resist environmental stresses, abiotic factors include the characteristics of the contaminant and its accessibility to soil organisms. Some soil constituents exert a multiple influence, most prominently soil organic matter (SOM), which plays a pivotal role in both determining contaminant availability and habitat conditions for soil microorganisms. For biodegradation to proceed effectively, all of these factors require to be within a certain, optimal range. In contaminated soil and groundwater, this is only the case exceptionally, thus inhibiting naturally occurring or *intrinsic* biodegradation.

3.2 *Biostimulation & Bioaugmentation*

Bioremediation requires the identification of the presently limiting factor(s) and is realised in the application of adequate measures to transcend these constraints, in order to provide for conditions allowing for contaminant breakdown in the subsurface.
The technologies comprising *engineered bioremediation* aim at establishing optimum degradation conditions, e.g. by adjusting nutrient ratios, by supplementing electron donors or acceptors, or, if adjustable, soil water content, pH and other parameters (*biostimulation*) or adding competent microorganisms (*bioaugmentation*). Some limitations can not, up to present, be over-

come, be it due to the lack of in-depth knowledge or suitable technologies (Alef, 1994, Scherr et al., 2006).

3.3 Biodegradability of Organic Pollutants

3.3.1 Molecular Considerations

The biodegradability of a chemical describes its inherent recalcitrance to biological transformation processes. Independently from environmental conditions or from bioavailability, the recalcitrance of a compound is determined on a molecular level, and is dependent on elemental composition, the presence and type of functional groups and the contaminant's molecular conformation (Sollins et al., 1996). The presence of functional groups such as hydroxy- or carboxy-groups or carboxylic acid esters indicate facile biodegradation (Loonen et al., 1999, Tunkel et al., 2000). Their common occurrence in the uncontaminated environment is resulting in the widespread capability of microorganisms to produce processing enzymes (Schwarzenbach et al., 2003) and contributes to the ready biodegradability also of xenobiotic substances containing such groups. Chlorine and nitrogen substituents appear to discourage biodegradability such as for some hetero-PAH, crude oil resins or halogenated aliphatic hydrocarbons (Venkateswaran et al., 1995, Bouwer and McCarty, 1983, Meyer and Steinhart, 2000).

3.3.2 Degradability of PHC, PAH and CAH

The biodegradability of unsubstituted petroleum hydrocarbons is dependent on the chain length and molecular size, the degree of branching and the occurrence of functional groups. Petroleum constituent degradability follows the order (from high to low): n-alkanes > alkylated cyclohexanes > alkylated benzenes > acyclic isoprenoids > alkylnaphthalenes > bicyclic alkanes > alkylated phenanthrenes > steranes > hopanes (George et al., 2002, Alef, 1994, Peressutti et al., 2003, Bartha, 1986).
With increasing ring number, PAH become more recalcitrant to biodegradation (Heitkamp et al., 1988, Bossert and Bartha, 1986, Shuttleworth and Cerniglia, 1995). Only few microorganisms are identified that can grow exclusively on PAH with more than four rings (Kanaly and Harayama, 2000). PAH bioaccessibility and water solubility appear to be too low to meet the microorganisms' energy requirements and may have thus prevented the evolutionary development of suitable enzymes for contaminant breakdown (Johnsen et al., 2005).
The biodegradation of chlorinated aliphatic hydrocarbons is less readily performed than of their unsubstituted congeners (Aulenta et al., 2006). The main obstacle to quick CAH biodegradation is exerted, however, by the change from reducing to oxidising environmental conditions deemed neces-

sary for their rapid and complete biodegradation as well as by the scarcity of microorganisms able to perform the full set of enzymatic activities for contaminant mineralisation (Maymo-Gatell et al., 1999).

3.4 Microbial Nutrient Demand

The demand for macronutrients is proportional to the amount of organic carbon (C) to be metabolised (Leys et al., 2005). Naturally occurring concentrations of macronutrients, mainly of nitrogen (N), phosphorus (P) and potassium (K), do often not suffice for the conversion of the commonly high concentrations of pollutant carbon. For aerobic degradation, optimum molar nutrient ratios are around C:N:P:K=100:10:1:1 (Leys et al., 2005, Atagana et al., 2003), but good degradation performance was also achieved at lower ratios (Aichberger et al., 2005). Anaerobic biodegradation requires lower nutrient input than aerobic degradation (McCarty, 1997, Aulenta et al., 2006). The dispersion of dissolved macronutrients and other amendments by *biostimulation* is facilitated in the saturated zone by transportation with the groundwater stream, while achieving an adequate distribution in the vadose zone forms a more sophisticated task.

3.5 Influence of Soil Constituents on Contaminant Behaviour

3.5.1 Microbial Communities in a Contaminated Environment

In historically contaminated soils, the microbial community is commonly well-adapted to the prevailing contamination (Van Herwijnen et al., 2006) and may be supported to increase biodegradation by *biostimulation*. In some cases, e.g. if an adapted microbial community is not present in the soil or aquifer, a suitable laboratory-cultivated community or individual strains may be introduced by *bioaugmentation*. This procedure is also a commonly used approach where highly specialized microorganisms are required for contaminant breakdown, such as for polychlorinated biphenyls (Di Toro et al., 2006). Eventually, the capability of a microbial community for contaminant breakdown and foremost, contaminant bioavailability will determine the extent of biodegradation for most hydrophobic contaminants (Huesemann et al., 2004).

3.5.2 Effect on the Microbial Population

The composition of contaminated environmental matrices exerts a twofold effect on the biodegradation of hydrophobic organic contaminants: besides

governing contaminant sorption and thus bioavailability, it will also influence microbial population density and diversity (Dunger and Fiedler, 1997), with SOM holding a pivotal role in both respects. Particle size distribution and soil carbon content are contributing to the determination of the available surface area for growth, of pore volume and distribution, soil water content, aeration profile and nutrient binding capability. Sandy soils, low in surface-bound organic matter, have a higher share of macropores, providing for good soil vapour exchange even at elevated water content. Water holding capacity, nutrient presence and binding capacity are rather low for sandy soils. On the other hand, clayey soils with elevated contents of SOM have the highest absolute pore volume, however, a low share of macropores, and are inclined to form microaerobic or microanaerobic zones already at low water content, entailing decreased microbial activity. Nutrient abundance is, by contrast, higher for clayey soils. These effects result in different soil microbial characteristics for different soils (Scheffer and Schachtschabel, 2002, Dunger and Fiedler, 1997).
Where bioavailability will, due to low contamination maturity or low abundance of sorption sites, determine biodegradation to a lesser extent, the microbial degradative capacity will do so. For field cases, commonly historically contaminated soils, bioavailability is the parameter governing biodegradation as opposed to microbial degradative capacity. The effect of soil composition on PHC biodegradation is characterised in Paper 1 (Scherr et al., 2007). The degradative capability of the indigenous aquifer population detached from contaminant bioavailability, is observed for CAH in Paper 4 (Scherr et al., 2008).

3.5.3 Contaminant Sorption by Geosorbents

There is common agreement that soil organic matter (SOM) is governing extent, progress and hysteresis of sorption of hydrophobic organic contaminants in soil (Luthy et al., 1997, Jonker and Koelmans, 2002, Kubicki, 2006), and thus contributes in determining their availability to biological processes. The sorption behaviour of organic contaminants largely depends on SOM type, contaminant type and age of contamination. Sorption mechanisms of the numerous PHC constituents were explored on a large scale (Salanitro et al., 1997, Endo et al., 2008) but lack some in-depth knowledge as to compound-specific behaviour. De Jonge and co-authors (1997) showed that PHC sorption and desorption are primarily controlled by diffusion and compound solubility.

3.5.4 Identification of Alkane Sorption Sites

The subject of PHC behaviour in soil is continued in Paper 2, where a new approach for the elucidation of HOC sorption, comprised by the statistical

correlation of sorption parameters and SOM functional group chemistry is demonstrated for a petroleum alkane. Different conceptual models for the description and ultimately the prediction of the sorption behaviour of a hydrophobic contaminant in the subsurface are available. Sorption isotherms may be determined according to different conceptual models, including Freundlich and Langmuir functions (Oleszczuk, 2009, Rhee and Thompson, 1992, Schwarzenbach et al., 2003). The distributed reactive domain model (DRM), developed and refined by Walter Weber and co-authors between 1992 (Weber et al., 1992) and 2002 (Weber et al., 2002) aims at a more differentiated characterisation of the dissimilar uptake and release characteristics of different types of soil organic matter. Soil sorption sites are being subdivided into mineral surfaces and *amorphous* and *hard carbon* components of SOM, adjoined by the designation of particular sorption characteristics to each domain. The choice of the most appropriate model is, however, far from obvious and depends largely on contaminant and SOM chemistry, as shown in Paper 2.
By contrast to SOM, the characteristics of HOC sorption towards soil mineral matter are far less explored and its influence is perceived differently (Beck and Jones, 1996, Kleineidam et al., 1999). PHC sorption to organic and mineral soil constituents, its progression and effect on biodegradation is an additional issue in Paper 1 (Scherr et al., 2007).

3.6 Bioavailability, Bioaccessibility and Chemical Activity

Several competing conceptual approaches for the characterisation of HOC bioavailability in the environment have been raised and discussed in recent years (Semple et al., 2004, Reichenberg and Mayer, 2006, Alexander, 2000, Huesemann et al., 2004, Kelsey and Alexander, 1997, Reid et al., 2000a). There is common agreement, however, that its definition is complicated (Semple et al., 2004) and its perception is being different for different scientific approaches.
In order to characterise the bioavailability of organic contaminants in the subsurface, a viable approach for the delineation of influences of each the chemical's characteristics and the soil environment has been made recently (Reichenberg and Mayer, 2006). Two incommensurable factors that are independently contributing to contaminant bioavailability are distinguished. While a compound's *chemical activity* describes its potential to undergo spontaneous physicochemical reactions, e.g. sorption and diffusion, and can be precisely defined, its *bioaccessibility* is dependent on the microenvironment, and describes the contaminant mass that can become available for biological processes, including uptake and biodegradation, over time. Summarizing, the bioavailable fraction of an organic pollutant depends on

several factors, including contaminant properties, soil characteristics, persistence time and the receptor organism, and site-specific assays are required to determine pollutant availability to the target organisms (Alexander, 2000).

3.6.1 Bioavailability of Hydrocarbons

While there are competing concepts for its description, the role of contaminant bioavailability, however, is evident when considering bioremediation, since contaminant availability to the microbial population will determine the extent of biodegradation, provided compound degradability is positive and environmental conditions are within a certain range allowing for microbial transformation and decomposition. Bioavailability plays a decisive role particularly in determining the efficiency of bioremediation for hydrophobic contaminants such as PAH and PHC which can become strongly sequestered to the soil organic matter over time. PAH sorption is mediated by interactions of π-electron clouds of sorbent and sorbate, as well as by sorption in narrow pores (Brändli et al., 2008, Luthy et al., 1997, Jonker and Koelmans, 2002). These sorption processes are increasing in strength with the molecular volume of the compound, its aromaticity and with contact time (Jonker and Koelmans, 2002, Kubicki, 2006). By contrast, biodegradation of aliphatic chlorinated hydrocarbons is dependent to a greater extent on environmental conditions, such as hydrogen supply and the microbial population, than on bioavailability (Lovley and Goodwin, 1988, Lee et al., 2007).

3.6.2 Assessment of HOC Bioavailability

Similarly to the variety of concepts for its definition, numerous methods for the determination of the bioavailability of hydrophobic contaminants in the environment are available. Commonly used approaches for the determination of contaminant bioavailability include biological tests, e.g. the observation of earthworms and insects exposed to xenobiotics (Robertson and Alexander, 1998, Kelsey and Alexander, 1997), the correlation of contaminant sequestration with physicochemical soil properties (Chung and Alexander, 2002) or the analysis of dissolved contaminant concentrations in soil pore water (Bondarenko et al., 2007, Ronday et al., 1997). Chemical or biomimetic analytical techniques include sequential supercritical fluid extraction (SSFE) (Szolar et al., 2004, Bogolte et al., 2007), equilibrium partitioning methods (Ma et al., 1998) and the use of semipermeable membranes (Sun et al., 2008).

3.6.3 An Alternative Method for the Determination of PAH Bioaccessibility

As an alternative to these extractive schemes, the use of cyclodextrin for the complexation of desorbed contaminants is a profitable new approach for

HOC bioavailability determination. Since this method of depletive sampling responds to accessibility and desorption kinetics within the matrix rather than to the strength of a solvent (Reid et al., 2000b, Reichenberg and Mayer, 2006), it is expected to give a good estimation of the contaminant's accessible fraction in a given environment. This issue is picked up in Paper 3 (Scherr et al., 2009), where the applicability of a novel, cyclodextrin-based PAH bioaccessibility assay is investigated, and enables for the assessment of the viability of a novel method to increase bioaccessibility in a highly useful fashion.

3.6.4 Improvement of PAH Bioaccessibility using Plant Oil

Bioavailability will, if limited, restrict the efficiency of bioremediation measures. It can not, up to present, be increased efficiently in large scale, and with ecologically sensible methods.
Conventional *ex situ* measures include the use of harsh organic solvents for soil washing or extraction but are rather transferring contamination, with the residual soil depleted of soil organic matter and nutrients (Alef, 1994), and constituting a disposal problem due to solvent residues. The addition of a biocompatible but non-degradable solvent phase to increase mass transfer from soil, rendering lipophilic contaminants more available to microorganisms (Bouchez-Naitali et al., 1999, Jimenez and Bartha, 1996), was shown to increase PAH biodegradation (Villemur et al., 2000), however the tasks of solvent disposal and the expense for bioreactor operation remain unsolved. The adoption of this concept to enhance *in situ* biodegradation would require the solubilising agent to be non-toxic, completely biodegradable and to be efficiently supporting hydrophobic contaminant biodegradation via increasing their bioaccessibility.
Vegetable oil such as sunflower, peanut and canola oils appear to combine these features (Gong et al., 2006, Pannu et al., 2003, Zhao et al., 2005), however with several modes of microbial support possible. While the mere support of the microbial community e.g. by acting as growth substrate, would not increase the total extent of degraded contaminant but rather increase the degradation rate of the readily available fraction, an increase of the available fraction by plant oil addition would significantly improve bioremediation efficiency and decrease residual concentrations in soil. The first evidence of the occurrence of the latter mechanism, i.e. an increase in bioaccessibility, is described in Paper 3 (Scherr et al., 2009), where the addition of canola oil to previously bioaccessibility-limited, PAH-contaminated soils was shown to substantially decrease residual contaminant fractions.

3.7 Abundance of Electron Donors & Acceptors

The supplementation of electron donors and acceptors to contaminated soil and groundwater holds a key position in *engineered bioremediation*, since their natural abundance is in many cases insufficient to satisfy the mass requirements for the oxidation or reduction of high contaminant concentrations.
Comparably reduced organic contaminants such as PHC or PAH are preferably but not exclusively degraded under aerobic conditions. The oxygen demand for contaminant mineralisation increases with contaminant concentration and the extent of competing oxidative processes (Madsen et al., 1996, Tabak et al., 2003). *Bioventing* for the vadose zone and *biosparging* for the saturated zone are common technologies to introduce electron acceptors where needed (Leeson and Hinchee, 1997).
Highly oxidised compounds, from per- to tri-chlorinated aliphatic hydrocarbons are preferentially reduced. Common biodegradation pathways include stepwise reductive dechlorination and similar processes (Aulenta et al., 2006, Fathepure and Boyd, 1988, DiStefano et al., 1992), most of which require the input of hydrogen in molecular or bound form (Lee et al., 2007). The mineralisation of compounds with less than three chlorine ligands remaining attached to the carbon structure may continue slowly under anaerobic but preferably and more readily under aerobic conditions. Since the change of reducing to oxidising subsurface conditions is technically difficult and connected to great expenses, alternative methods are sought to support anaerobic CAH metabolisation.

3.7.1 A Novel Approach to Enable CAH Mineralisation Under Anaerobic Conditions

The introduction of organic hydrogen carriers into the saturated zone to initiate anaerobic CAH biodegradation is a promising remediative approach. Upon carrier degradation, hydrogen is released and can be used by highly specialised organisms (Maymo-Gatell et al., 1999, He et al., 2005) for contaminant biodegradation. Critical optimisation parameters are hydrogen donor type and concentration (Fennell et al., 1997, Aulenta et al., 2005, Lee et al., 2007) and kinetics of hydrogen production (Lovley and Goodwin, 1988, Smatlak et al., 1996, Yang and McCarty, 1998). The application of natural catalysers may help to complete CAH biodegradation under fully anaerobic conditions (Lovley et al., 1996, Bradley et al., 1998). Based on these considerations, a sustainable and sensible remediation procedure for CAH in groundwater was developed, based on the addition of a set of innocuous groundwater amendments, and is described in Paper 4 (Scherr et al., 2008).

Chapter 4

Conclusion

The pollution of soil and groundwater with organic contaminants is a severe hazard to human and environmental receptors and is of worldwide occurrence. The inherent potential for the transformation or mineralisation of different types of hydrocarbons by natural, microbe-mediated processes can be employed efficiently in order to restore the desired quality of contaminated sites and groundwater bodies. *Engineered bioremediation* subsumes the set of environmental technologies available for the support of natural attenuation processes apt for the mitigation of risks from contaminates sites and aiming at the restoration of the natural functions of soil and groundwater.
The successful implementation of *in situ* bioremediation relies on the correct assessment of site-specific obstacles to contaminant biodegradation and on the availability of efficient technologies to overcome these constraints. Knowledge of fundamental processes governing the biodegradation of petroleum constituents, polycyclic aromatic and chlorinated aliphatic hydrocarbons is established and effective measures for their support are in operation. However, the in-depth characterisation of constraints and novel approaches for their surpassing are important aspects of environmental protection and restoration. Advances in the field of soil-microbe-pollutant interactions, the recognition of specific sorption mechanisms and the development and optimisation of novel approaches to increase bioavailability and biodegradation of hydrophobic organic contaminants are contributing to the improvement of *in situ* bioremediation as an efficient tool for the decontamination of polluted sites.

Chapter 5

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Part II

Included Papers

Chapter 1:

SCHERR, K., AICHBERGER, H., BRAUN, R. & LOIBNER, A. P. (2007) Influence of soil fractions on microbial degradation behaviour of mineral hydrocarbons. *European Journal of Soil Biology*, 43, 341-350.

Chapter 2:

EHLERS, G. A. C., FORRESTER, S. T., SCHERR, K., LOIBNER, A. P. & JANIK, L. J. Influence of the nature of soil organic matter on the sorption behaviour of pentadecane as determined by PLS analysis of mid-infrared DRIFT and solid state ^{13}C-NMR spectra. Submitted to *Environmental Pollution.*

Chapter 3:

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Chapter 4:

SCHERR, K., NAHOLD, M. & LOIBNER, A. P. (2008) Addition of Whey, Lactose and Humic Matter Effects Complete Removal of PCE: Transferring Lab-Scale Observations to in situ Validation. IN KALOGERAKIS, N., FAVA, F. & BANWART, S. A. (Eds.) *Proceedings of the 4th European Bioremediation Conference.*

Chapter 1

Influence of Soil Fractions on Microbial Degradation Behaviour of Mineral Hydrocarbons

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Influence of soil fractions on microbial degradation behavior of mineral hydrocarbons

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Abstract

Various interactions occurring between organic chemicals and soil constituents participate in the determination of the fate of these pollutants, including their biodegradability. These relations need to be characterized in order to design and successfully implement a bioremediation application. In the present study, biodegradation of spiked and aged crude oil contamination in two dissimilar soils was related to their composition. GC-FID analysis of bulk soil samples as well as sand- and <63 μm fractions showed considerable differences in contaminant distribution and degradation behavior within these fractions. Whereas a freshly spiked silty soil showed reasonable degradation (51%), degradation was not significant after ageing. By contrast, a sandy soil was degraded by 25% (recently contaminated) and 19% (aged). Biodegradation occurred in the fine fraction only, with a comparably high content of organic carbon whereas hydrocarbon concentration remained constant in the sand fraction. This was correlated with sorption to the fine fraction where hydrocarbon concentrations were higher by over an order of magnitude compared to the sand fraction. Soil composition, biology and chemistry exert a pronounced influence on microbial degradation in respect to (i) contaminant availability and (ii) the structure and density of the microbial community.

Keywords: Crude oil; Bioremediation; Biodegradation; Soil fractions; Particle-size

1. Introduction

Petroleum hydrocarbons and their derivatives are naturally occurring chemicals that are exploited for a wide range of purposes. Their release into the environment poses a grave environmental problem due to sustained contamination of air, water and soil.

Bioremediation technologies aim at the enhancement, promotion and acceleration of the intrinsic microbiological restoration potential for the decontamination of polluted sites. As such, these technologies are exploiting, as opposed to most conventional physical / chemical treatment technologies, the soil's natural capability for contaminant attenuation and self-restoration [1–3]. Different *in situ* technologies are designed to overcome the multiple restraints to biotransformation of hazardous compounds. These technologies include single or combined measures such as aeration, supplementation of water, nutrients, trace elements or enzymes and the introduction of suitable microbial cultures into the soil (e.g. [1,4]).

The design of an *in situ* remediation application requires sound knowledge of the mechanisms and

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processes governing the fate of these pollutants; likewise, the accurate assessment of the impact of these mechanisms on biodegradation is essential for its successful implementation. Amongst the more prominent factors governing pollutant breakdown are the properties of the contaminant, the soil environment and soil fractions, such as the surface area, composition of surface fractions and reactivity [3,4]. Soil organic matter (SOM) is referred to as the soil component dominating sorption and desorption of hydrophobic organic chemicals (HOC) [5], with regard not only to its level but also its source and maturity (e.g. [6]). As such, the pool of SOM is regarded as being highly heterogeneous in terms of sorption capacity and strength [7], with impacts on bioaccumulation and bioavailability [8] as well as biodegradability [9] of organic pollutants. SOM also largely contributes to the various processes related to the ageing of HOC, which is adjoined by an increase in sorption to the soil matrix and a decrease in the rate and extent of biodegradation [10–14]. The manifold implications of these interactions on the environmental behavior of organic contaminants have recently been extensively reviewed [6]. By contrast, the impact of mineral surfaces on HOC sorption, availability and degradability is not thoroughly characterized, with organic carbon components removed in various microscale studies yielding contradictory results [15,16].

Contrasting biodegradation trends in soils of different composition are related to dissimilar microenvironments affecting contaminant availability as well as abundance and diversity of the microbial community (e.g. [17]). Poor microbial proliferation and diversity are typical for soils with sandy texture and low organic carbon, which is adjoined by lower degradation rates as compared to clay loam and loam [9,18]. The lower abundance of degraders in the sand fraction of contaminated soils is correlated to a higher C/N ratio and lower internal surface [19]. It appears that the soil microbial community benefits from high levels of SOM in its size, diversity and ability to recover from environmental and contamination stresses [20,21]. Contrary to this, SOM content was found to be inversely proportional to biodegradation rates [13] and sorption of contaminants to SOM is suggested to reduce metabolization [22].

However, it has been put forth that in most cases the mass transfer of a chemical to the cell and not the mechanisms of microbial activity, i.e. rates of uptake and metabolism, limits contaminant breakdown [11]. In practice, these two aspects are intrinsically linked to each other as they are governed by the microenvironmental conditions prevailing in the soil matrix.

It was within the scope of our work to determine the extent and nature of the influence the soil constituents have on the degradability of a complex hydrocarbon mixture by soil microorganisms. Crude oil was chosen as a contaminant as it represents a broad range of petroleum-derived products. Based on the laboratory-scale assessment of the degradation performance within dissimilar bulk soils and their fractions in different stages of ageing, it was aimed to gain further understanding of the various interactions governing the breakdown of mineral hydrocarbons and their influence on remediation applications.

2. Materials and methods

2.1. Soils and contaminant

Two soils without contamination history were collected in Lower Austria. Characterization of the homogenized fraction (<2 mm) was performed using standard techniques (Table 1). Soil E is a dark soil taken from a depth of 0–85 cm below surface with a high content of silt- and clay sized particles. Soil F, sampled from below 85 cm, is a light, sandy soil with a low content of organic carbon.

A paraffin-based crude oil (crude *P*, OMV Vienna, Austria) from the Vienna Basin served as contaminant for all degradation tests.

Table 1
Background characteristics of soils E and F

Characteristics	Soil E (%)	Soil F (%)
Soil type[a]	Silty, sandy	Fine sand
Dry matter	94.2	95.6
Maximum water holding capacity (WHC_{max})[b]	0.458	0.302
Total N	0.11	0.05
Total C	1.76	2.83
C/N[c]	16.0	56.6
$CaCO_3$	0.63	1.49
Abundance of particle sizes		
Sand, SF (<2 mm)	56.8	80.2
Silts (2 mm–63 μm)	32.5	14.2
Clay (<2 μm)	10.7	5.6
Silt and clay, FF (<63 μm)	43.2	19.8
Total organic carbon (TOC) content		
Bulk soil (<2 mm)	1.1	0.4
Sand, SF (2 mm–63 μm)	0.6	<0.0005
Silt and clay, FF (<63 μm)	1.7	1.5

[a] Soil classification at sampling.
[b] in g H_2O g^{-1} of soil DM.
[c] in % $\%^{-1}$.

2.2. Stimulation of biodegradation

For biodegradation experiments, a microbial consortium was isolated from an industrial site contaminated with crude oil and enriched at 20 °C on a shaker operating at 100 rpm. The medium used for sub-cultivating consisted of (g L^{-1}): 1 K_2HPO_4, 0.5 KH_2PO_4, 0.5 $NaNO_3$, 0.1 $MgSO_4 \cdot 7H_2O$, and 0.1 NaCl with 1‰ (v/v) of a trace element solution containing (g L^{-1}): 0.5 H_3BO_3, 0.04 $CuSO_4$, 0.1 KJ, 0.2 anhydrous $FeCl_3$, 0.1$(NH_4)_6Mo_7O_2 \cdot 4H_2O$ and 0.4 $ZnSO_4 \cdot 7H_2O$. This medium was amended with 4 ml L^{-1} of paraffin-based and naphthene-based crude oils (OMV Vienna, Austria). Every other week, 3 ml of the microbial suspension were transferred (3 times in total) to 50 ml fresh medium containing 4 ml L^{-1} of each crude *P* and naphthene-based crude oil. For the preparation of master and working cell banks, the microbial suspension was centrifuged, washed at least three times, and resuspended in fresh mineral medium containing 15% glycerol, yielding a final concentration of 1.1×10^9 colony forming units (CFU) per ml (working cell bank). Prior to further manipulation, isolated microbes were stored in cryo-vials at −80 °C. The consortium was added to the microcosms in an inoculum size of 1.32×10^9 CFU kg^{-1} of soil DM.

The contaminated soils were incubated at 60% maximum water holding capacity, amended with trace elements in solution according to [23]. In addition, NH_4NO_3 and H_2KPO_4 were added, yielding a C:N:P ratio of 100:10:1, with C based on the soil TOC content plus the carbon supplied by the contaminant.

Control microcosms were prepared where the inoculum was substituted with mercuric chloride ($HgCl_2$ by Fluka, Buchs, Switzerland) as bio-inhibitor in a concentration of 800 mg kg^{-1} of soil DM following recommendations by [24]. In all other respects, the control tests were treated similarly to the inoculated.

2.3. Soil microcosms

The soils were sieved to 2 mm and spiked with crude P to a target measurable concentration of 5000–6000 mg TPH (Total Petroleum Hydrocarbons) kg^{-1} of soil DM. All tests were conducted at similar ambient conditions. The biodegradation experiments were conducted in 2 L borosilicate beakers, containing 2 kg of spiked soil and other additives, such as nutrients, trace elements, inoculum or sterilant. Oxygen was introduced three times per week by mixing with a clean metal spatula.

Samples of bulk soil and two fractions were collected to determine introductory and final TPH concentration and composition and during incubation at intermediate dates.

Aliquots of spiked soils were stored for 116 days in plastic buckets at 4 °C in the dark, referred to as aged soils. Although most polluted field soils have been exposed to contamination over a longer period of time when remediation is being effected, ageing effects have been observed being completed after intervals ranging from 4 months up to years, strongly depending on the soils' and contaminants' nature [8]. Afterwards, degradation tests, were conducted. The microcosms were kept for 128 (non aged) and 64 days (aged tests) at 20 ± 2 °C in the dark, respectively.

All tests were conducted on spiked soils E and F. Tests were designated according to the specific treatments:

- E-I, F-I: recent contamination plus adapted culture, trace elements, nutrients, oxygen
- E-C, F-C: recent contamination plus bio-inhibitor, trace elements, nutrients, oxygen
- E-A, F-A: aged soils, as analyzed after storage
- E-AI, F-AI: aged contaminated soil plus adapted culture, trace elements, nutrients, oxygen
- E-AC, F-AC: aged contaminated soil plus bio-inhibitor, trace elements, nutrients, oxygen

2.4. Soil fractionation

Soil samples were separated into sand (SF, 2000–63 μm) and silt/clay (FF, <63 μm) fractions. In order to disrupt aggregates 10 g soil was weighed into a glass beaker and suspended in 25 ml 0.1 M $Na_4P_2O_7 \cdot 10H_2O$ for 8 h. The suspension was diluted with water up to 200 ml and shaken on an orbital shaker at 100 rpm for 6 h. Thereafter the suspension was passed through a 63 μm sieve and rinsed with water. Soil fractions were freeze-dried at −2 °C and 0.47 mbar in a model Beta 1-16 freeze drier from Christ (Osterode am Harz, Germany). Dried proportions were extracted and analyzed for TPHs. Distribution factors were defined as the percentage of TPH concentrations in silt/clay ($f_{d,FF}$) and sand fractions ($f_{d,SF}$), respectively, to bulk soil.

2.5. TPH extraction and analysis

Extraction and analysis were performed according to the International Standard Method DIN ISO DIS 16703 (2001). Soil samples were amended with 10 ml distilled water, 20 ml acetone p.a. and 10 ml of internal RTW standard (*n*-decane (30 μL L^{-1}) and *n*-tetracontane (30 mg L^{-1}) in *n*-heptane), followed by 60 min

ultrasound assisted extraction. The extracts were cleaned by adding sodium sulfate and magnesium silicate. Analyses were performed on a HP 5890 Series II, Hewlett Packard Gas Chromatograph (Hewlett Packard, Palo Alto, CA, USA) with a FID detector at 360 °C. Separations were carried out on a Hewlett Packard HP-5MS capillary column with a stationary phase of phenyl-methylpolysiloxane (30 m × 0.25 mm i.d.) at a He flux of 1.8 ml min^{-1}. The temperature program was as follows: 60 °C for 1 min, followed by a 20°C min^{-1} ramp to 340 °C, then held at this temperature for 10 min. All samples were collected and analyzed in triplicate. Chemicals were at least of ''Baker analyzed'' grade and used as received from J.T. Baker (Deventer, Netherlands).

3. Results

3.1. Hydrocarbon removal

Significant differences in the biodegradation performance between the particular soils and stages of ageing were observed throughout the tests (Table 2, Fig. 1). TPH in non-aged soil E were degraded by over 50% within 128 days, compared to close to one quarter in sandy soil F. In all tests, corresponding gas chromatograms revealed visible depletion in *n*-alkanes.

After ageing, biodegradation in soil E was diminished to a level below significance, while sandy soil F remained nearly unaffected (Table 2). Ageing was accompanied by prominent changes in GC-FID appearance (Fig. 2a–c). Whereas soil E (Fig. 2b) is almost depleted in *n*-alkanes, such effects are visible but to a lesser extent for soil F (Fig. 2c).

3.2. TPH distribution and degradation within soil fractions

It has to be noted that after lyophilization a considerable loss of lower weight hydrocarbons was detected. This was caused by instrument management difficulties resulting in higher temperatures, which led to evaporation of more volatile compounds. Consequently, soil fractions were analyzed for TPH in a range between *n*-C15 to *n*-C40, designated $TPH_{>15}$. Nevertheless, full recovery of soil fraction TPHs was not achieved.

Analysis exhibited appreciable differences in pollutant distribution patterns among the investigated soils. Both sand fractions exhibited very little capacity to accumulate organic pollutants, and most $TPH_{>15}$ was found in the silt/clay fraction. Biodegradation in all tests was limited to the fine fraction, containing the major portion of organic carbon (E: 1.7%, F 1.5% TOC), with degradation curves in parallel to the respective bulk soils (Fig. 3a–d). The sand fraction is characterized by low to negligible TOC (E: 0.6%, F: <0.05%), with soil organic matter mainly composed of particulates.

Partitioning patterns resulting from the fractions' properties are depicted by the means of the distribution factor, f_d, in Table 3. Sand fractions of E-I and F-I contained about 10% ($f_{d,SF}$) of the respective bulk soils' $TPH_{>15}$. This behavior is sharply contrasted by the ten- (E-I) to over twentyfold (F-I) $TPH_{>15}$ content in the fine fractions. Interestingly, ageing did not exert an influence on the partitioning behavior to this organic-rich fraction, but did so on the sand fraction, expressed by an $f_{d,SF}$ elevated by a factor of about two.

3.3. TPH Fractionation by equivalent carbon number (ECN)

The hydrocarbon fractionation approach based on equivalent carbon number ranges was employed as descriptive tool for the interpretation of GC-FID signals to identify hydrocarbon distribution patterns within the degradation tests.

The fractionation approach uses TPH compound grading by equivalent carbon number index (ECN) which is based on equivalent retention times separated by respective boiling points on a GC column, normalized

Table 2
Reduction of TPH content in inoculated bulk soil during degradation tests and ageing

Reduction in biodegradation tests until						Reduction during ageing[a]		
Day 64			Day 128			Day 116		
Test	%	mg TPH kg^{-1} d^{-1}	Test	%	mg TPH kg^{-1} d^{-1}	Test	%	mg TPH kg^{-1} d^{-1}
E-I	48.8	46.6	E-I	51.2	24.5	E-A	34.3	13.9
F-I	18.7	12.4	F-I	24.7	8.2	F-A	16.4	6.6
E-AI	n.s.	n.s.	E-AI	–	–			
F-AI	19.0	11.7	F-AI	–	–			

n.s., not significant.
[a] In cold storage.

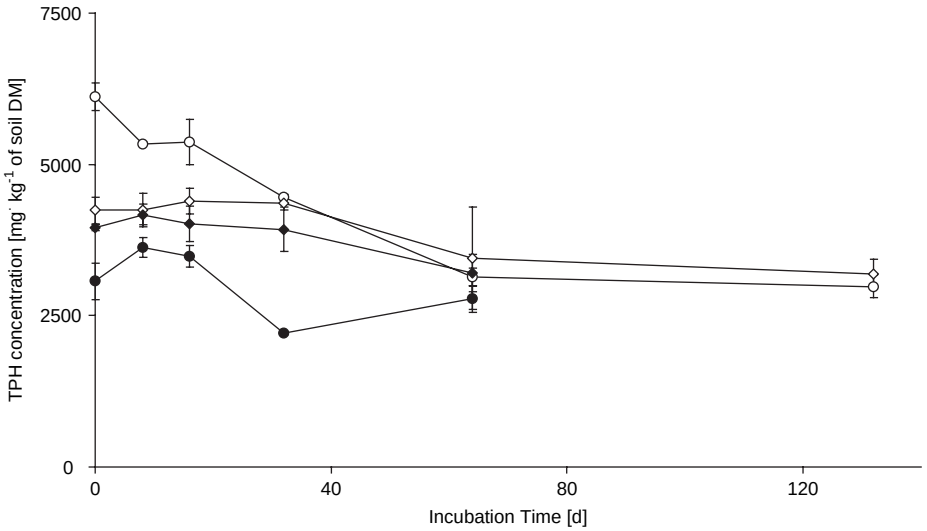

Fig. 1. TPH concentrations during incubation in bulk soils in biostimulation and bioaugmentation treatments: E-I (○), F-I (◇), E-AI (●) and F-AI (◆). Bars represent standard deviations ($n = 3$). Some bars are smaller than the symbols.

to *n*-alkanes [25]. The definition of TPH fractions by ECN is displayed in Table 4. The magnitude of removal (Fig. 4) due to each treatment is calculated as the difference of final to initial abundance (Table 4). Fraction #6 could not be detected after treatment in 50% of the samples and is therefore not included in the discussion.

Removal rates from all tests are displayed in Fig. 4. In all treatments, hydrocarbon fraction #2 proved most recalcitrant to degradation. This phenomenon was more pronounced for soil F than E. Ageing effected an entirely different distribution pattern with a nearly even dissipation in all fractions.

Metabolization of organic compounds was detected in all tests but most pronounced in E-I and F-I. Except for #2 in soil F all compounds groups were degradable with removal in E-I exceeding that in F-I (a difference of 30-43% in ECN > 15–34 but only 11% for ECN > 10–15). It was noted that the percentage of microbial degradation for #1 hydrocarbons in E-I is smaller (13.5%) than in F-I (32.1%), expressed by the difference to the control. Treated aged soils E-AI and F-AI exhibited similar but less pronounced distribution patterns in higher molecular fractions commencing with ECN > 15.

4. Discussion

In this study, soil composition has been found to exert a pronounced influence on the biological breakdown of crude oil. Differences in SOM composition and abundance are likely to influence microbial community structure and density since it has effects on substrate availability and nutrient status—although as to what extent changes in structure impact changes in the enzymatic activity necessary for the decomposition of recalcitrant compounds has not yet been shown [20]. Community structure and density have been shown to be inversely related to soil C/N ratio [19,20,26] and its density has been shown to be proportional to the internal surface of soil particles, applicable to a study involving PAH degraders [19,27]. This indicates that soil F, with its high content of sand, high C/N ratio and low TOC content poses an environment comparably less favorable for microbial proliferation than loamy soil E. Whether SOM has a beneficial [21] or unfavorable effect [13,22] on biodegradability in respect to its binding characteristics towards organic contaminants, is disputable. Our results show that SOM content alone does not suffice as a sole basis for conclusions as to the biodegradability of contaminants within a certain soil matrix. This was demonstrated by the contrasting impact of contact time contaminant/soil on the contaminant's successive biodegradability that was observed for the two soils. These differences were effective after an ageing time as short as 4 months.

In addition to soil chemistry, hydrocarbon pollution itself exerts an impact on microbial populations, leading to selection and acclimatization (e.g. [19,27,28]). It has been observed by Bundy et al. [29] that the composition of bacterial communities in diesel contaminated soils strongly depends on the soil type, with each soil supporting a different microbial community. Accordingly,

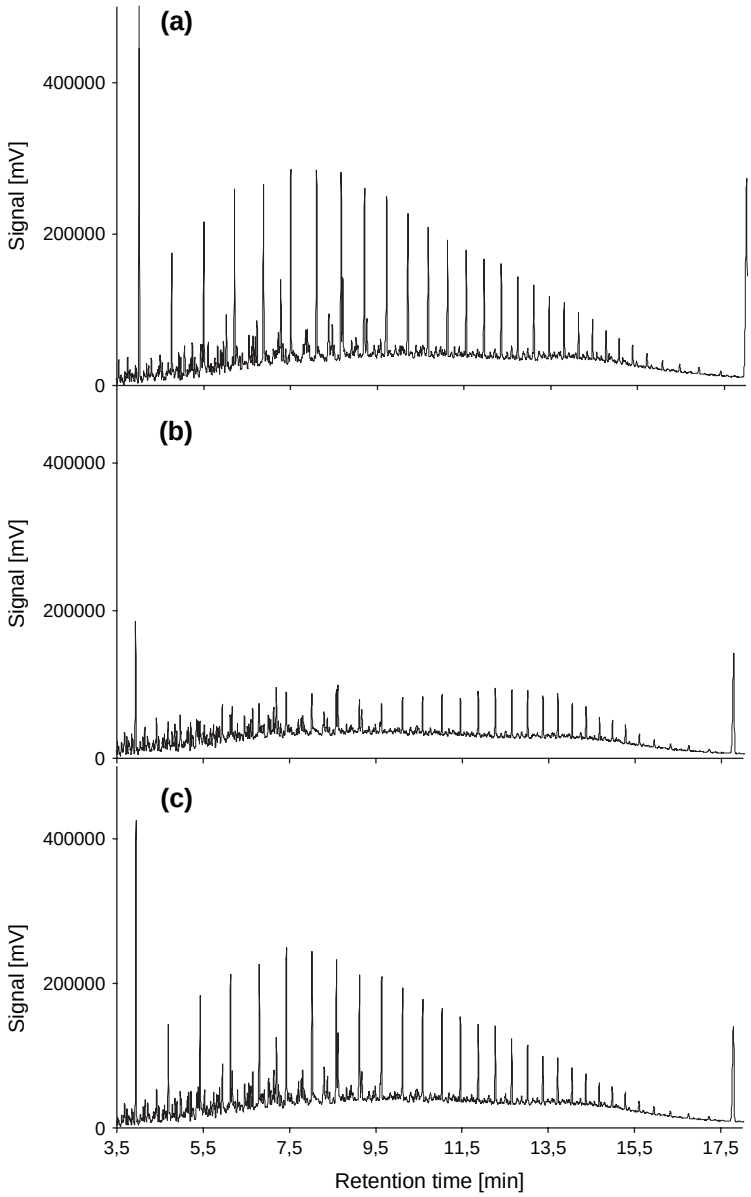

Fig. 2. Chromatograms as determined by GC-FID: preceding ageing (a), aged soil E-A (b), aged soil F-A (c).

it was indicated in the present study that each soil composition favored a particular community structure and density in dependence on the prevailing microenvironmental conditions, and potentially providing favorable growth conditions for particular degrading strains. As such, differences in the communities' metabolic capacities are substantiated in dissimilar degradation capabilities. Possibly, such unlike communities may be effecting visibly disparate degradation patterns, such as were presently observed.

In addition to the direct effect on hydrocarbon decomposition, soil constituents were found to influence contaminant distribution and availability. A more detailed elucidation of the soil constituents' impact on hydrocarbon mineralization was allowed for by the fractionation of bulk soil into sand and fine fractions. The two soil fractions can be classified largely in respect to $TPH_{>15}$ concentration and biodegradability by their respective TOC content: where the sand fractions of both soils coincide in low concentration and resistance to microbial breakdown, the fine fractions are more organic which is accompanied by a ten- to twenty-fold increase in hydrocarbon concentration and enhanced degradability. It was not attempted to establish

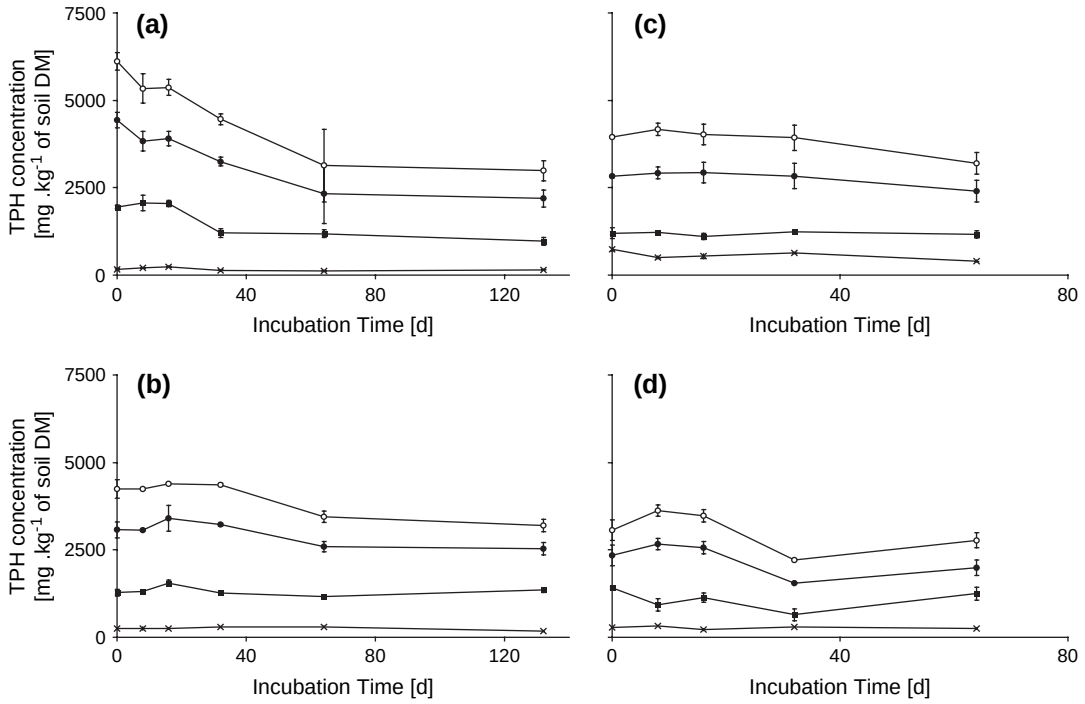

Fig. 3. TPH and $TPH_{>15}$ concentrations during incubation in bulks soils and soil fractions in biostimulation and bioaugmentation treatments: (a) E-I, (b) F-I, (c) E-AI, (d) F-AI. Displayed: TPH bulk soil (○), $TPH_{>15}$ bulk soil (●), $TPH_{>15}$ fine fraction (■), $TPH_{>15}$ sand fraction (×). Bars represent standard deviations ($n = 3$); some bars are smaller than the symbols.

a further correlation with the TOC content, since the SOM parameter, expressed as a weight percentage, is known to not sufficiently describe hydrocarbon uptake and release by SOM and its influence on the biodegradability of organic contaminants (e.g. [5,6,8]).

However, SOM was separated by size and thus distributed unevenly within the fractions. Large size fractions and particulate organic matter (POM) were shown earlier to sorb PAH in higher concentration than organic matter associated with clay minerals [7]. Though a similar characterization presently applied to the constituents of the sand fraction, its $TPH_{>15}$ concentration was lower by about one order of magnitude. This may be due to the low TOC content and masking by other binding effects in the sand fraction. However, the similarity of extracted concentrations (Table 3) is in disagreement with dissimilar sand-fraction TOC (Table 1), pointing towards the influence of mineral surfaces as a sorption site, similarly to [15]. In addition, prolonged contact time to contaminants increased sorption to sand particles, indicated by an elevated $f_{d,SF}$ after ageing. However, care must be taken when interpreting these results since moderate recovery rates were achieved.

Table 3
Distribution factors (f_d) of soil fractions

Test	Distribution factor f_d (%)	
	$f_{d,SF}$	$f_{d,FF}$
E-I	9.5 (2.0)	107.1 (15.9)
F-I	10.4 (1.6)	225.3 (32.4)
E-AI	23.1 (5.5)	110.2 (29.1)
F-AI	26.8 (5.7)	221.8 (23.6)

SF, sand fraction; FF, fine fraction. Values represent means of all samples of a test, standard deviation in brackets ($n = 33$ non-aged, $n = 27$ aged).

Table 4
Definition of TPH fractions based on equivalent carbon number index (ECN)

Fraction	Boiling point ranges normalized to *n*-alkanes, ECN	Abundance in paraffinic crude oil (crude *P*), %
#1	>10–15	28
#2	>15–20	28
#3	>20–25	19
#4	>25–30	14
#5	>30–35	7
#6	>35–<40	4

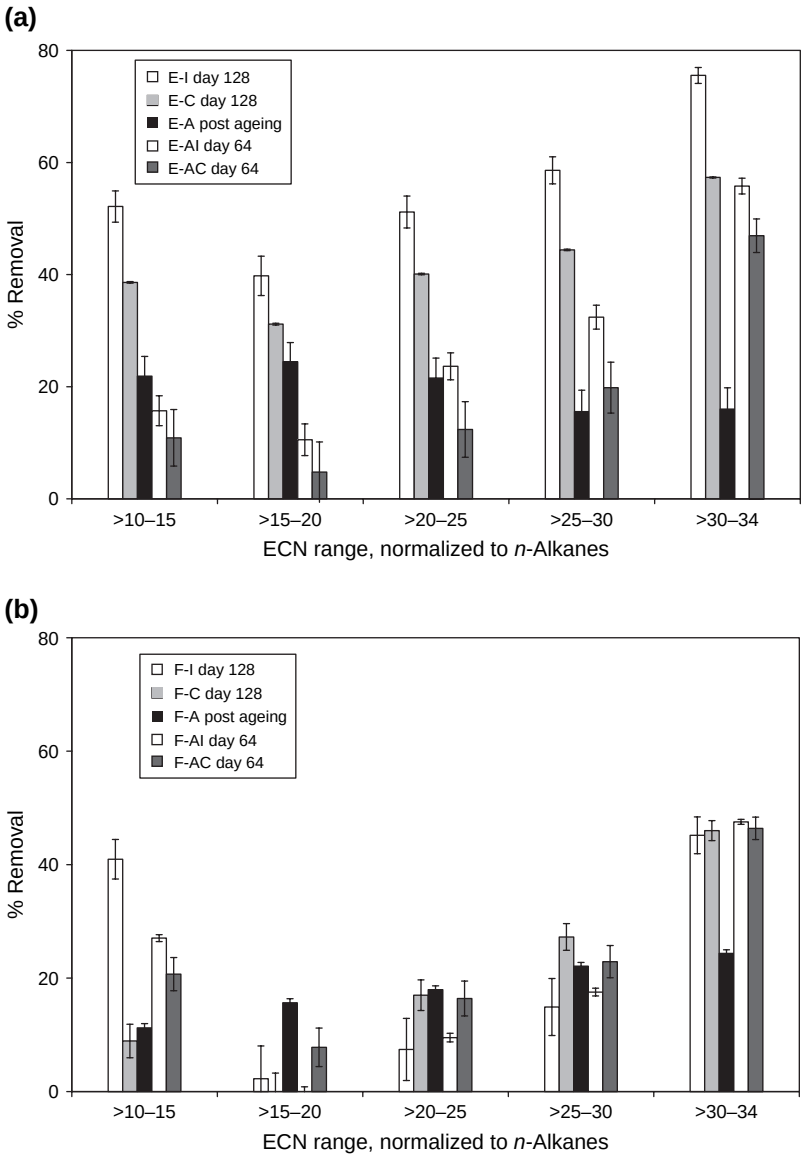

Fig. 4. TPH fractionation of GC-FID signals by equivalent carbon number (ECN). Removal of TPH fractions subsequent to treatments of soil E (a), soil F (b). Percentage removed is given as follows: for recent contamination and post-ageing (-I, -C, -A) as removal from non-aged, non-incubated paraffin-based crude oil composition, and for aged, incubated (-AI, -AC) from post-storage composition (-A). Bars represent standard deviations ($n = 3$).

Little quantitative data is available on the biodegradability of crude oil compound groups. However, chemical and physical properties such as solubility, emulsification and surface tension (e.g. [1,30]) influence degradation to a great extent. With increasing chain length, additional effects become important, such as the steric hindrance of large paraffinic molecules (>*n*-C28) to bacterial enzymes [31].

Degradation of such high molecular weight hydrocarbons has rarely been observed. Yet, Heath et al. [30] reported significant degradation of aliphatics between triacontane up to tetracontane. Similarly, substantial depletion of hydrocarbon compounds of ECN > 20–35 were observed in the present study.

Distinct oil compositions evolved after incubation of each a recent contamination and 4 months' storage for both soils. Such dissimilar oil composition patterns can be used for the identification of the contribution of various attenuation processes [32]. We suggest further research to allow for the attribution of hydrocarbon

removal patterns to the responsible process out of the range of mechanisms to be considered such as biodegradation, evaporation or sequestration.

The present study has shown that soil composition greatly influences the efficiency of a combined biostimulation and bioaugmentation treatment aimed at enhancing the biological removal of crude oil. The extent of metabolization was found to depend strongly on soil type and age of contamination. We suggest that soil type influences the prerequisites for microbial breakdown in two interrelated respects: (i) the bioavailability of the contaminants and (ii) the structure and density of the degrading community. The results underline the importance of estimating the impact of soil composition, biology and chemistry for the success of remediative actions.

Acknowledgments

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Chapter 2

Influence of the Nature of Soil Organic Matter on the Sorption Behaviour of Pentadecane as Determined by PLS-Analysis of Mid-Infrared DRIFT and Solid State ^{13}C-NMR Spectra

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Influence of the nature of soil organic matter on the sorption behaviour of pentadecane as determined by PLS analysis of mid-infrared DRIFT and solid state ^{13}C-NMR spectra

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Abstract

The nature of soil organic matter (SOM) functional groups associated with sorption processes was determined by correlating partitioning coefficients with solid-state ^{13}C-NMR and diffuse reflectance mid-infrared spectral features using partial least squares (PLS) regression analysis. Sorption coefficients for pentadecane were determined for three models: the Langmuir, the Dual Distributed Reactive Domain and the Freundlich model. The Freundlich model was found to be the most appropriate as supported by analysis of residuals and coefficients of determination (R^2, 0.48-0.99) and variation (CV, 8-12%). The most likely nature of the functional groups in SOM associated with pentadecane sorption coefficients (K_F) was shown to be aromatic, possibly porous soil char, rather than aliphatic organic components. High PLS cross-validation correlation suggested that the model was robust for the purpose of characterising the functional group chemistry important for pentadecane sorption.

Capsule

NMR/IR spectroscopy and chemometrics reveal the aromatic fraction of soil organic matter being responsible for alkane sorption.

Keywords:

petroleum hydrocarbons, alkanes, soil organic matter, sorption models,

NMR spectroscopy, infrared spectroscopy, chemometrics

Introduction

Contamination of soil by petroleum hydrocarbons (PHC) is a world-wide problem and a great number of studies have been reported on the remediation of contaminated sites (e.g. Aichberger et al., 2005; Cram et al., 2004; Huesemann et al., 2004). Bioremediation is widely accepted as a cost-effective and promising tool for the treatment of soils and aquifers contaminated by petroleum products, although limitations in the effective clean up of soils and sediments still impede success rates. Most commonly used PHC (e.g. gasoline, diesel, and lubricating oil) consist of a very large number of compounds with the exact composition depending on the source of the crude oil and how it was refined (Dobson et al., 2004; Huesemann, 1997). The composition of the various petroleum products also differs due to seasonal variability, and to variability between manufacturers and countries (Kaplan et al., 1997). An estimation of the PHC fraction that is readily available and biodegradable in soil environments is, therefore, complicated by such variability in PHC composition.

The extent of PHC biodegradation in soils generally dependent on four main factors; namely the optimal environmental conditions which underpin biodegradative activity, the presence of hydrocarbon degrading micro-organisms, the predominant PHC types sorbed into the soil matrix which could render particular hydrocarbon constituent compounds recalcitrant, and the bioavailability of the contaminants to the microbial population (Reid et al., 2000; Scherr et al., 2007; Stroud et al., 2008). Slow mass transfer rates, often responsible for reduced bioavailability, can control biodegradation processes where the PHC compounds are not toxic to soil micro-organisms or recalcitrant to biodegradation, or if environmental parameters negate biodegradation (Huesemann, 1995; Salanitro et al., 1997).

The interdependent link that exists between the structure and physico-chemical properties of soils, and the uptake of hydrophobic organic contaminants (HOC) upon interaction with solid matrices, has been established. The conclusions derived from

numerous studies, however, have not been actively incorporated into remediation protocols and risk assessment strategies. Legislators invariably prefer to adopt a conservative approach when the potential of contaminants to be released from soil matrices is evaluated (Ehlers and Loibner, 2006). As a consequence, standardised methods for the determination of contaminant bioavailability in soils are deemed a prerequisite for the incorporation of these concepts into risk assessment (Harmsen, 2007).

The interaction of HOC with soils is governed by variations in concentration and specific properties of soil organic carbon (SOC) constituents and by the properties of some soil mineral components. Where organic carbon content is present above >0.01-0.2%, overall transport and binding reactions have been reported to be affected by the total concentration of soil organic matter (SOM) (Chiou et al., 1998; Huang et al., 2003) and its chemical composition and structure (Perminova et al., 1999). SOM type and quantity has been found to influence polycyclic aromatic hydrocarbon (PAH) sorption to mineral-bound humic matter (Lahlou et al., 1999) as well as particulates such as charred wood material (James et al., 2005). Substantial sorption of numerous organic pollutants in soils and sediments has also been attributed to specific SOM components, particularly to carbonaceous materials such as the black carbon fraction (e.g. Nam et al., 2008; Oen et al., 2006).

The advent of spectroscopic techniques such as mid-infrared diffuse reflectance infrared Fourier-transform (DRIFT) and solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy have made it possible to rapidly characterise the SOC chemistry and determine the molecular properties that play pivotal roles in HOC sorption (Janik et al., 2007; Skjemstad et al., 1994). In recent work using FTIR synchrotron beam-line microscopy, the sorption of hydrophobic PAH into sediments has been found to be controlled largely by the presence of aromatic charcoal organic components (Ghosh et al., 2000). The findings of their work was supported recently by Müller et al. (2007) who

assumed a pore-filling process to micropores formed by clay aggregates although they rated a decreased or unfavourable partitioning into the adjacent water in quartz–montmorillonite structures. Similar dependence of the sorption of hydrophobic *n*-alkanes on the presence of aromatic soil char material and clay-mineral surfaces may also occur.

Various SOM constitutional descriptors have been used to evaluate the relationships between sorption coefficients (K_D, K_F, K_{DOM}, K_{OC}) and the molecular structures of sorbents (Gunasekara and Xing, 2003; Kile et al., 1999; Perminova et al., 1999; Rutherford et al., 1992; Stuer-Lauridsen and Pedersen, 1997; Xing et al., 1994). Such descriptor factors include atomic ratios derived from the elemental content of C, H, N and O of natural or fractionated soils and sediments (Haitzer et al., 1999; Kukkonen and Oikari, 1991; Rutherford et al., 1992; Xing et al., 1994). Quantification of these descriptor factors by ^{13}C-NMR spectroscopy has led to molecular expressions revealing the basis of interaction reactivity with SOM (Abelmann et al., 2005; Ahmad et al., 2001; Gunasekara and Xing, 2003; Kile et al., 1999; Perminova et al., 1999). Data from ^{13}C-NMR spectra has also been used to determine the relationship between sorption coefficients and the soil chemistry responsible for sorption processes by using a multivariate analytical approach (Thomsen et al., 2002).

In order to relate the chemistry of the SOM with sorption capacity, multivariate regression models such as partial least squares (PLS) regression can be used to link partitioning coefficient data from batch equilibrium isotherm tests with solid state ^{13}C-NMR and infrared spectroscopy. PLS is a bilinear modelling method where spectral and reference data are projected onto a small number of latent variables (PLS loadings). In this way, the major chemistry of SOM which is responsible for sorption into soils can be described. While near-infrared (NIR) reflection spectroscopy with PLS modelling has been used to predict the sorption and leachability of pesticides in soils (Bengtsson et al., 1996, 2007), methods such as mid-infrared DRIFT and ^{13}C-NMR

spectroscopy can be used, not only to for quantitative prediction, but also to examine the nature of the chemistry of the various organic components of SOM contributing to the sorption/desorption process.

DRIFT spectroscopy, in particular, offers a simple and rapid method for the characterisation of both the SOM and the soil mineral matrix and avoids many of the disadvantages of the traditional mid-infrared KBr pellet pressing technique. It is more rapid, since spectra of powdered samples can be obtained in less than one minute, little sample preparation is required, and it enhances the less-intense peaks in soil spectra relative to the stronger peaks (Nguyen et al., 1991). In addition, KBr matrix contamination and interferences due to water bands overlapping with soil spectra have been overcome by DRIFT spectroscopy (Capriel et al., 1995; McCarty et al., 2002; Reeves and Delwiche, 1997).

As a start to assessing PHC performance in soil, this paper sets out to test the effectiveness of a batch equilibrium isotherm test and partitioning coefficients to model the sorption of *n*-C_{15} PHC into a small set of highly variable soils, and to determine the chemical characteristics of SOM components responsible for, or correlating with, sorption behaviour using solid-state ^{13}C-NMR and mid-infrared DRIFT spectroscopy.

Materials & Methods

Soils

Six European soils with widely different physico-chemical properties were used in this study (Table 1). Soils were sampled (0-20 cm depth), air-dried and sieved to pass 2 mm before the sorption studies. Particle size determination was performed according to ÖNORM (Austrian Standards Institute) L 1061 (2002). Elemental analysis (total C, H and N) of the soils was determined using a Euro EA Elemental Analyser. The sieved soils were dried at 105^{o}C to remove water prior to analysis and then sealed and cooled to room temperature for analysis. Inorganic carbon (IC) content was determined by

volumetric measurement of evolved CO_2 gas with the Scheibler apparatus (ÖNORM L 1084, 1989). The total organic carbon content (TOC) was calculated by subtraction of IC from total carbon.

Mid-infrared analysis

Sample preparation and mid-infrared DRIFT spectroscopy were performed as described by Janik and Skjemstad (1995) and Janik et al., (1998). Spectra were recorded on samples in a Pike AutoDiff™ (Pike, USA) infrared diffuse reflectance accessory using a Perkin Elmer Spectrum One spectrometer from 7800 to 450 cm^{-1} at a resolution of 4 cm^{-1}, but only the mid-infrared portion from 4000 to 500 cm^{-1} was used for PLS analysis. Prior to multivariate analysis, the spectra of the mineral component of the whole soils were obtained by ignition of the sample at 350ºC for 24 h. The soil mineral component spectra were then subtracted from the unheated whole soil spectra to derive the spectra of the SOM (Nguyen et al., 1991; Rumpel et al., 2001). The assignments of FTIR spectra were chosen according to a number of mid-infrared soil studies (Bracewell et al., 1989; Janik and Skjemstad 1995; Janik et al., 1998, 2007; McCarty et al., 2002; Parfitt et al., 1997; Van der Marel and Beutelspacher, 1976; Skjemstad and Dalal, 1987). All spectra were individually adjusted for a quadratic baseline correction prior to carrying out the PLS analysis, since it was thought that a quadratic correction could improve the PLS models. This was considered to be important given the small number of samples in the current study, as non-systematic variation in any of the spectra could easily introduce a perturbation of the PLS model due to spectral outliers caused by baseline variation. The DRIFT spectra were resolved by fitting individual peaks to the spectra using the array basic curve-fitting application *CURVEFIT.AB* (GRAMS-AI, Thermo NH ,Ver-6).

^{13}C-NMR analysis

Prior to NMR analysis, soil samples were treated with hydrofluoric acid (HF) to concentrate the SOM and remove siliceous and ferrous paramagnetic materials (Skjemstad et al., 1994). Finely ground (< 0.2 mm) soil samples (5 g) were shaken with 50 ml of 10 % HF for 1 hour in polyethylene bottles. After centrifugation at 2000 g for 10 minutes, the supernatants were siphoned off and discarded. The procedure was repeated five times at room temperature and the remaining soil washed five times with 40 ml deionised water and freeze-dried. Single contact time cross-polarisation with magic angle spinning (CPMAS) solid-state ^{13}C-NMR spectra were obtained on a *Varian Unity-200* spectrometer. Freeze-dried and powdered soil samples were placed in a 7 mm diameter cylindrical zirconium dioxide rotor with KEL-F end-caps and spun at 5000 Hz in a Doty Scientific MAS probe. The magnetic field was 4.7 T, giving a ^{13}C resonance frequency of 50.3 MHz. CPMAS spectra were obtained using a standard pulse sequence and a pulse sequence with a 1 ms contact time and 0.3 s recycle delay (Wilson, 1987). Hartman-Hahn conditions and the magic angle were optimized using hexamethylbenzene. The relative intensities of different signal areas were determined by integration of defined chemical shift areas from 300 to 0 ppm according to Wilson (1987).

Sorption isotherm procedure

All sorption experiments were operated according to the batch equilibrium technique (Xing, 2001) in 30 ml screw-cap Teflon-backed glass vials. The background solution was 0.01 M $CaCl_2$ in distilled water with 200 mg l^{-1} $HgCl_2$ as a biocide to minimize microbial activity. The stock solution was prepared by spiking 10 ml of liquid pentadecane into the background solution (1 L) and shaking the mixture on a horizontal shaker overnight. The solution was then poured into a separating funnel and allowed to stand for a further 48 h at 20 $^{\circ}$C (McAuliffe, 1966). Clear samples, drained from the bottom of the funnels, were separated into seven reducing concentration fractions for

the sorption tests. Sorption tests were made in triplicate for each concentration fraction, with soil to solution ratios between 1:50 and 1:100 (dry weight) for the different soils to maintain uptake above 50 % (Boesten, 1994). The samples were allowed to equilibrate at room temperature for 48 h during continuous mixing on a rotary shaker at 70 rpm. After the equilibration period, the samples were centrifuged for 30 min at 1800*g* and allowed to stand for 10 minutes. Supernatants were carefully collected and weighed for solvent extraction and *n*-C_{15} quantification. Mass balances were determined by extracting soil at particular concentration levels. Recovered *n*-C_{15} was found to vary between 67.3 and 114.1%. The liquid-liquid extraction procedure was adapted from the DIN ISO DIS 16703 (2002).

Data analysis

Sorption data were fitted using three alternative sorption isotherm models:

(i) The logarithmic form of the Freundlich equation:

$$\log C_S = \log K_F + N \log C_W \qquad [1],$$

(ii) The reciprocal form of the constant-energy limited-site Langmuir model:

$$\frac{1}{C_{s,max}} = \left(\frac{1}{C_{s,max} K_L} \right) \frac{1}{C_w} + \frac{1}{C_{s,max}} \qquad [2], \text{ and}$$

(iii) The dual distributed reactive domain model (DRDM), which involves a pair of sorption mechanisms involving partitioning (i.e. a linear isotherm with the partitioning coefficient, K_D and a Langmuir limited-site non-linear adsorption isotherm (Weber et al., 1999) :

$$C_s = K_D C_w + \frac{C_{s,\,max} K_L C_w}{1 + K_L C_W} \qquad [3]$$

Where C_S (mg/kg) is the amount of *n*-C_{15} sorbed per unit mass of sorbent (soil); C_w (mg/L) is the aqueous equilibrium concentration in solution; the Freundlich coefficient

(K_F) [(mg/kg).(mg/L)$^{-1}$] which is an index of sorption capacity of the sorbent; *N* is the Freundlich exponent which denotes the degree of deviation from isotherm linearity; $C_{s,max}$ represents the capacity factor (mg/kg); K_L is the Langmuir coefficient (L/kg); and K_D (L/kg) is the distribution coefficient of the linear component of the DRDM.

Freundlich and Langmuir models were determined using linear regression, and the DRDM model by non-linear regression, through minimising the cumulative squared residuals between experimental and calculated values of C_s in equations 1, 2 and 3 using the *Solver* application in *Microsoft Excel*™. The coefficient of determination (R^2) and coefficients of variation (CV) were computed to assess the quality of the regression. Serial correlation in the residuals from the three models' fit was tested via Durbin Watson statistic in *Statgraphics Plus 5.1.* Test parameters were one regressor, with *N* ranging from 19 to 21 at α=0.05.

PLS analysis was carried out using *The Unscrambler*® (CAMO AS, Norway) software package using full leave-one-out cross validation. The procedure for PLS analysis adopted here is similar to that described by Haaland and Thomas (1988) and Esbensen (2002), and later implemented by Janik and Skjemstad (1995) and Janik et al. (1998, 2007) specifically for soils. The first few loading weights, and also the PLS regression coefficients, can be used to qualitatively assess the relationships between the spectral information over the frequency range and the K_F values, leading to the primary spectral signatures of the soil components most strongly correlated with the property of interest i.e. K_F. This procedure assisted in the identification of the factors which ultimately underpinned *n*-C_{15} sorption mechanisms in this study.

Results and Discussion

Soil Characterisation

Peaks for O- and N-alkyls including acetal and ketal carbon in the 110-45 ppm chemical shift region of the NMR-spectra dominated all soils (Table 2), followed by

alkyl-C (45-0 ppm) and aryl-C (160-110 ppm). With a prominent alkyl-C peak centred around 33 to 30 ppm, as can be seen for Anmoor, Norway and Askov soils (see supporting information), a dominance of CH_2 groups is expected (Ahmad et al., 2001; Baldock et al., 1990). In contrast, polar structures such as carboxyl and amide carbon (190 -160 ppm) generally contributed the smallest proportion to SOM. NMR-derived constitutional descriptors, as described by Abelmann et al. (2005) and Perminova et al. (1999) were calculated using defined chemical shift areas (Table 2). Generally, variability in aromaticity ($C_{aryl\text{-}H,C}/\Sigma C$) was low for all soils (0.11-0.15) with the highest value observed for Anmoor (0.15) whereas Waschbach, the soil exhibiting the highest sorption coefficient, revealed a relatively low aromaticity of 0.12. As to the polarity index, again a low variability was observed (1.61-2.06 for Askov and Waschbach, respectively) and no clear relationship with sorption coefficients could be established. Further details are provided in the supporting information.

Difference mid-infrared DRIFT spectra of the SOM components in the six soils are presented in Figure 1. Eighteen peaks were fitted to the spectra of the soils between 3500 and 1100 cm^{-1} of which nine proposed assignments were made. The spectra were characterised by peaks due to hydroxyl (-OH at 3500-3000 cm^{-1}), alkyl (–CH_2 at 2930, 2850 and 1470 cm^{-1}), carboxyl (-COO^-) (1600 cm^{-1} and 1400 cm^{-1}), aromatic groups (-C=C-) (1600-1570 cm^{-1}) and carboxylic acids, ketones and acyclic aldehydes (C=O at 1740-1720 cm^{-1}) (Capriel et al., 1995; Coates, 2000; Ellerbrock and Gerke, 2004; Janik et al., 1998).

To determine the degree that NMR and DRIFT spectroscopy support one another, linear regressions were performed and correlations determined for particular corresponding spectral peaks. These were: 130 ppm (aryl-C) and 1582 cm^{-1} (aromatic –C=C-) (R^2, 0.75; $p < 0.05$); 173 ppm (carboxyl C) and 1730 cm^{-1} (carboxylic acids –C=O) (R^2, 0.73); 103 ppm (hydroxyl) and 3114 cm^{-1} (hydroxyl –OH) (R^2, 0.65; $p < 0.05$); and 32 ppm (alkyl C) and 2926 cm^{-1} (alkyl –CH_2) (R^2, 0.36). This indicated a

reasonable measure of agreement between FTIR and ^{13}C-NMR characterisation methods for the soils.

Sorption studies

Pearson's correlation coefficients (R) for linear sorption model fits showed weak relationships between C_S and C_w for Anmoor, Waschbach and Norway (0.28, 0.47 and 0.43, respectively) and moderate relationships for Kettering, Askov and IFA (0.6, 0.74 and 0.76, respectively). Since it is expected that sorption to natural soils would in fact exhibit non-linear behaviour, characteristic of heterogeneous sorbents (Huang et al., 1997; LeBoeuf and Weber, 2000), the use of isotherms which model non-linear sorption reactions were applied. Non-linear sorption processes may be modelled with a series of any number of different models including the Freundlich and Langmuir models (Schwarzenbach et al., 2003; Weber et al., 1992, 1999).

The R^2 and coefficients of variation (CV) for the three isotherm fits are presented in Table 3 with R^2 varying between 0.41 and 0.93 (Langmuir fit), 0.41 and 0.76 (DRDM fit) and 0.48 and 0.99 (Freundlich fit). In order to estimate the degree of dispersion of the data points relative to the least squared line, the CV was calculated for the overall fits. CV percentages were markedly higher for the Langmuir and DRDM models and, given the fact that a higher degree of relative dispersion is deduced from these fittings, the applicability of these equations to model the sorption behaviour of pentadecane was considered to be lower than for Freundlich. In addition, the use of the Freundlich model was supported by testing for autocorrelation in the residuals via Durbin Watson statistic. Possible autocorrelation was detected only for one soil using Freundlich model fit residuals whereas the test indicated significant or probably significant correlation for several soils using Langmuir model (three soils) and DRDM (four soils) fit residuals. The multiple sorption processes which occur concurrently in heterogeneous sorbents involving different sites of different energies can be estimated by the Freundlich model as representative of a summation of various sorption mechanisms (Schwarzenbach et

al., 2003; Weber et al., 1999; Xing and Pignatello, 1997). Accordingly, the Freundlich model was considered as the most appropriate for describing the observed sorption trends.

The Freundlich parameters K_F and *N* provided insight into the sorption behaviour of *n*-C_{15} to SOM. Comparing sorption capacity (K_F) between the soils provided information regarding the influence that TOC content has on the binding capacity of the different soils. As Weber et al. (1992) pointed out, the fact that sorption capacities varied widely amongst the six soils, and also exhibited a range of nonlinearities, could indicate the heterogeneous nature of the organic matter of these soils. Sorption isotherms and parameters from the Freundlich regression are shown in Figure 2 and Table 4, respectively. Values of R showed a moderate to strong linear relationship between C_s and C_w for the soils (R values between 0.72 and 0.87). Sorption coefficients ranged from 226 to 1400 L/kg with the Waschbach and IFA soils exhibiting the highest K_F values. The ranking order of the soils in terms of their sorption coefficients was: Waschbach > IFA > Norway > Anmoor > Askov > Kettering.

The Freundlich exponent *N* is described as an index of free energies associated with sorption processes by multiple components of a heterogeneous sorbent in that it shows the degree of deviation from linearity (Schwarzenbach et al., 2003). The Anmoor, Waschbach, Norway and Askov soils presented a concave downward ($N<1$) trend thus indicating that added *n*-C_{15} molecules were bound with weaker free energies. IFA soil, however, exhibited a convex upward ($N>1$) trend from which it can be inferred that more sorbate presence could have induced changes in the SOM structure and enhanced free energies for further sorption (Schwarzenbach et al., 2003; Sharmasarkar et al., 2000). Accordingly, the convex curvatures may result from a swelling of the organic matter content of the SOM due to sorbed pentadecane which triggers enhanced partitioning of additional *n*-C_{15} molecules. Such an enhanced

cosorptive process is, therefore, suggested for IFA in particular, given the high *N* value (1.66), and suggests a greater partitioning ability of *n*-C_{15} with this soil.

DRIFT and NMR-PLS

The results of the PLS analysis must be considered with due regard to the small number of samples and the high risk of heavy leverage by any one of the samples. PLS was applied for the sole purpose of identifying the chemistry of the SOM in these particular soils which supported *n*-C_{15} sorption and was, therefore, not employed with the intention to establish a calibration set to predict K_F values of other unknown soils through spectroscopic analysis. It is conceivable that conclusions about organic carbon chemistry derived from PLS regressions performed in this study may not be extended to other soils. Hence, a further study using a larger set of soil samples and sorption coefficient data will be undertaken to confirm conclusions drawn from this study.

Before carrying out PLS prediction, the measured K_F values were transformed with a square-root function in order to reduce the large range of K_F values for the PLS regression model. This improved the accuracy of the PLS cross-validation and also had the effect of reducing the cross-validation curvature, further improving the PLS model. It was also advantageous to reduce the infrared frequencies used for PLS modelling to those with possible organic contributions, e.g. alkyl, carboxyl and aromatic. One PLS factor was sufficient to achieve an R^2 of 0.96 and a root mean square error of cross-validation (RMSECV) of 84 $K{\cdot}L^{-1}$. The regression is illustrated in Figure 3a. Two factors would have improved the regression but with increased risk of overfitting the model due to the small number of samples in the calibration.

Figure 3b shows details of the PLS regression coefficients. Peaks in the regression coefficients can be used to explain the correlations between the spectra and K_F, with positive peaks corresponding to positive correlations and negative peaks to negative correlations. The first PLS loading accounted for 47% of the spectral variability. Positive regression coefficients were observed at 1590 and 1364 cm^{-1} due to aromatic

species such as in charcoal and graphitic structures (Janik et al., 2007; Tung et al., 2004) and a negative weight peak at 2858 cm^{-1} due to alkyl-CH_2. The very weak intensity of the alkyl peak suggests that involvement of $–CH_2$ functional groups was not a major contributor to sorption of *n*-C_{15} for these soils. It was outweighed by sorption onto aromatic structures.

For NMR-PLS analysis, two PLS factors were required to achieve an optimum calibration with an R^2 of 0.96 and RMSECV of 86 $K{\cdot}L^{-1}$. The first and second loading weights accounted for 79 and 11% spectral variability, respectively. Again, the NMR chemical shift regions were reduced to only those shifts identified with organic functionalities (alkyl, carboxyl, aromatic and amide groups) and those regions that did not contribute to improving the R^2 significantly were omitted. The result of the PLS cross-validation regression is shown in Figure 4a and the regression coefficients plotted in Figure 4b. Positive regression coefficients were observed at 99 ppm (ketal carbon) and 139 ppm (aryl H and aryl C carbons). Two peaks near 213 and 225 ppm were unidentified. These positive regression coefficients were supported when omitting the Kettering sample from the PLS regression model. This model required only one PLS factor for optimum calibration.

The conclusions of the DRIFT-PLS is supported, to some extent, by the ^{13}C NMR analysis in that sorption of *n*-C_{15} was located on aromatic structures and little or no indication of adsorption to aliphatic soil organic matter was shown. In addition, the PLS analysis showed that carbonyl functionalities such as in hydrophilic esters and ketones also appeared to have had an affect on sorption of the compound. The results suggest that PLS regression can be used as a tool to identify the components of SOM which supported sorption of pentadecane in these soils. Although overfitting of the PLS models, and hence instability in the regressions, was reduced by using a minimum number of latent variables, a further study using a larger set of soil samples and sorption coefficients is required.

Sorption of a range of HOC classes has been shown to be located in the aromatic domains of SOM, constituents of SOM and dissolved organic matter. These include chlorinated aliphatics (e.g. trichloroethene), PAH and pesticides (e.g. carbaryl, diuron, esfenvalerate) (Abelmann et al., 2005; Ahangar et al., 2008; Ahmad et al., 2001; Thomsen et al., 2002). Sorption to aliphatic moieties was shown in a study by Chefetz et al. (2000). The authors postulated that non-aromatic structures (alkyl and O-alkyl C) of SOM constituents (humin, lignin, peat and lignite) can significantly contribute to sorption of pyrene. In this study, however, there was no evidence for sorption of pentadecane to aliphatic moieties possibly due to the different physical-chemical characteristics of the contaminants used. The outcome of the present study seems to indicate that the aromatic component of SOM is a good indicator of a soil's capacity to adsorb alkane PHC.

The relationships established with DRIFT and ^{13}C-NMR spectral data versus K_F provide a means to identify the key structural parameter of SOM and ascertain its ability to bind *n*-C_{15}. A larger soil data set is currently being employed in sorption tests to confirm the outcome of this study. This may afford the possibility to establish calibration regression curves to predict binding affinity for alkane PHC to soils of unknown origin and molecular structure based on SOM characterisation by means of spectroscopic analysis without having to perform laborious sorption isotherms.

The structure-activity-prediction relationship proposed by Ehlers and Loibner (2006) incorporating sorbent characteristics, sorption activity of a contaminant and a prediction tool such as PLS regression could be a viable approach to base site management decisions on. Although it must be recognised that there are uncertainties associated with a number of the methods employed (e.g. sorbent characterisation techniques, sorption tests) it represents a potent methodology to assess a complex set of mechanisms and interactive processes in the soil environment.

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Table 1. Some physicochemical properties of the soils used in the sorption isotherms

Soils	Origin	Texture	pH	Sand (%)	Silt (%)	Clay (%)	TOC (%)	N (%)	H (%)	C (%)
Anmoor	Austria	Silty loam	7.2	8.0	62.0	30.0	5.3	0.5	0.8	11.4
Waschbach	Austria	Silty loam	7.3	11.0	63.0	26.0	2.6	0.3	0.7	3.1
Kettering	UK	Sandy loam	6.7	48.0	27.0	25.0	2.0	0.2	0.7	2.0
Norway	Norway	Loamy sand	4.4	56.0	33.0	11.0	5.8	0.3	0.9	5.8
Askov	Denmark	Loamy sand	6.3	67.0	23.0	10.0	1.5	0.1	0.3	1.6
IFA	Austria	Loamy sand	7.7	48.0	35.0	17.0	1.8	0.2	0.5	2.5

Table 2 Integrated peak intensities of ^{13}C-NMR analysis and descriptor factors derived from the integrated peaks

Soils	^{13}C-NMR spectroscopy									
	Chemical shifts (ppm) and relative intensities (%)					^{13}C NMR-derived constitutional descriptors				
	190-160 ppm[a]	160-140 ppm[b]	140-110 ppm[c]	110-45 ppm[d]	45 - 0 ppm[e]	$\Sigma C_{aryl}/\Sigma C_{alkyl}$ [f]	Polarity[g]	$C_{alkyl-O,N}/C_{alkyl-H,C}$	$C_{aryl-H,C}/\Sigma C$[h]	$C_{aryl-O,N}/C_{aryl-H,C}$
Anmoor	13.2	6.4	15.1	34.4	22.1	0.38	1.61	1.56	0.15	0.42
Waschbach	12.9	5.1	12.4	44.5	19.5	0.27	2.06	2.28	0.12	0.41
Kettering	10.8	4.8	12.3	45.4	20.7	0.26	1.96	2.19	0.12	0.39
Norway	7.9	5.9	11.6	45.4	25.4	0.25	1.66	1.79	0.12	0.51
Askov	10.0	5.1	11.1	43.1	26.3	0.23	1.61	1.64	0.11	0.46
IFA	12.4	6.4	13.9	40.2	19.9	0.34	1.86	2.02	0.14	0.47

[a] Carboxyl, amide C
[b] O,N-substituted aryl C ($C_{aryl-O,N}$)
[c] H,C-substituted aryl C ($C_{aryl-H,C}$)
[d] O,N-substituted alkyl C ($C_{alkyl-O,N}$)
[e] H,C-substituted alkyl C ($C_{alkyl-H,C}$)
[f] $\Sigma C_{aryl} = C_{aryl-O,N} + C_{aryl-H,C}$; $\Sigma C_{alkyl} = C_{alkyl-O,N} + C_{alkyl-H,C}$ (Perminova et al., 1999)
[g] Polarity index according to Abelmann et al. (2005)
[h] ΣC = total carbon in main structural fractions integrated from ^{13}C NMR spectra

Table 3 Coefficients of determination (R^2) and coefficients of variation (CV %) for the regression fits of the sorption data to the three isotherm models.

Soils	Langmuir		DRDM		Freundlich	
	R^2	CV (%)	R^2	CV (%)	R^2	CV (%)
Anmoor	0.48	80.0	0.41	60.0	0.48	10.4
Waschbach	0.93	28.6	0.69	41.6	0.71	7.7
Kettering	0.41	92.0	0.51	55.1	0.52	11.7
Norway	0.72	73.8	0.55	50.2	0.60	8.4
Askov	0.57	65.6	0.70	39.7	0.99	8.8
IFA	0.55	81.8	0.76	36.6	0.65	9.8

Table 4 Freundlich parameters from the fit of the sorption data

Soils	Log K_F[a] (log L/kg)		CV(%)	N[a]		CV(%)	K_F (L/kg)
Anmoor	2.61	(0.18)	6.8	0.64	(0.16)	24.7	407
Waschbach	3.15	(0.06)	1.9	0.75	(0.12)	15.4	1400
Kettering	2.35	(0.18)	7.4	1.03	(0.23)	21.9	226
Norway	2.84	(0.1)	3.6	0.58	(0.11)	18.8	697
Askov	2.59	(0.05)	2.1	0.48	(0.06)	12.8	392
IFA	3.00	(0.06)	2.1	1.66	(0.28)	16.7	1010

[a]Values in parenthesis are standard errors calculated for the Freundlich parameters

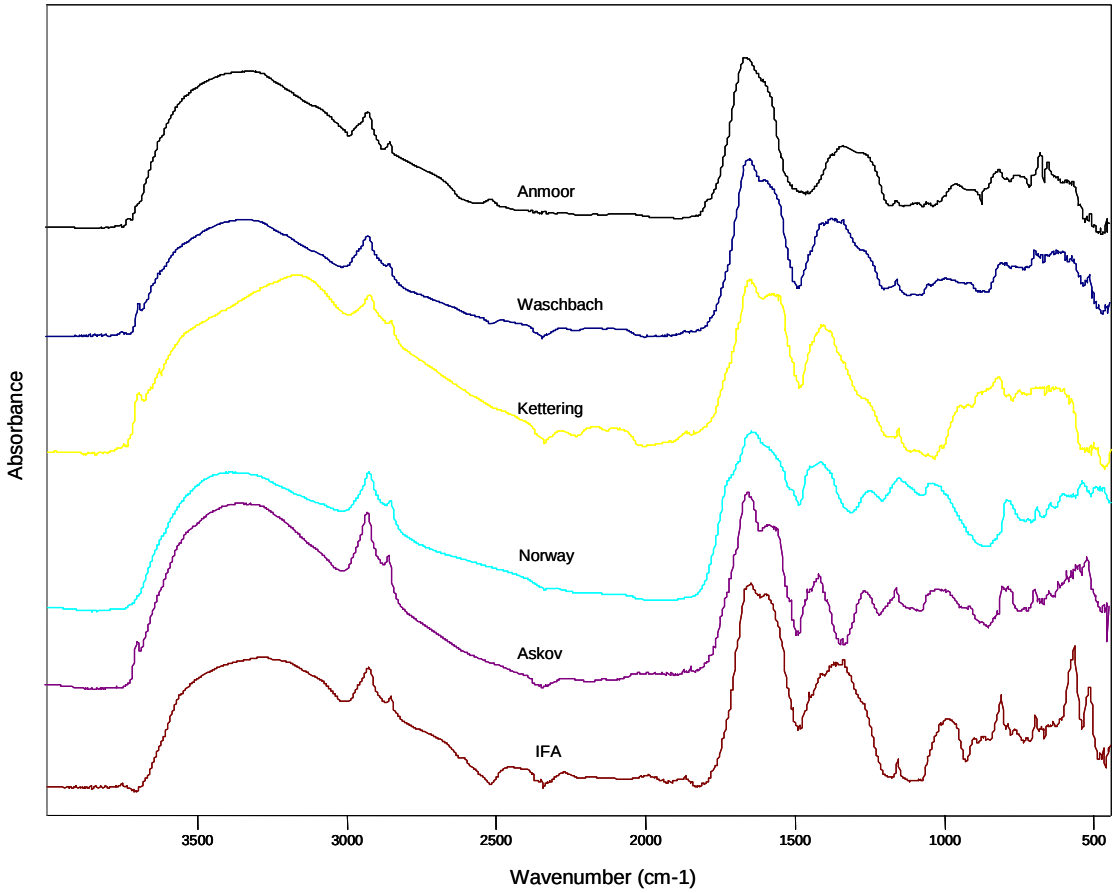

Figure 1 MIR DRIFT difference spectra of the six soils.

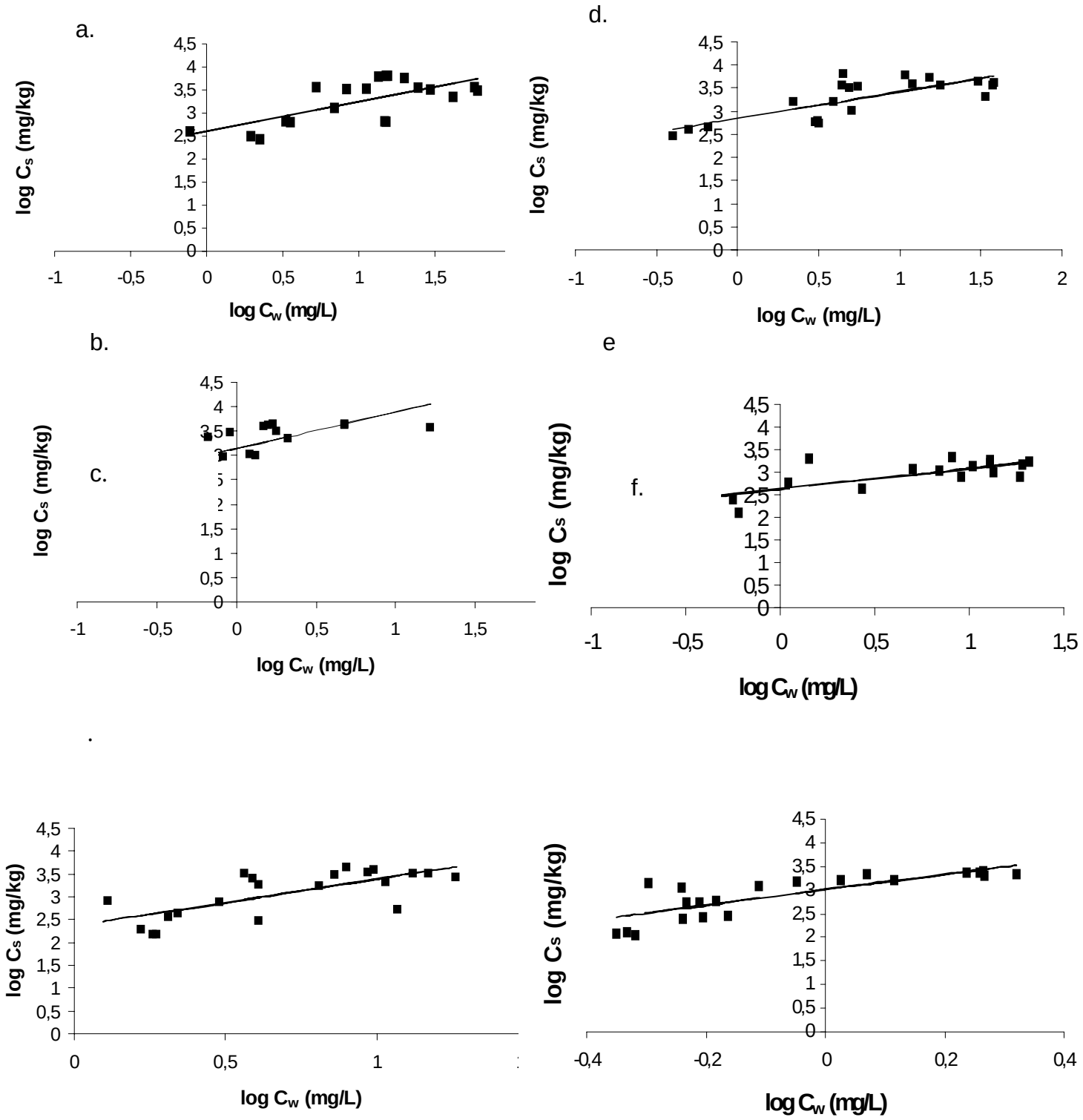

Figure 2 Experimental data and Freundlich isotherm model fits for (a) Anmoor, (b) Waschbach, (c) Kettering, (d) Norway, (e) Askov and (f) IFA soils

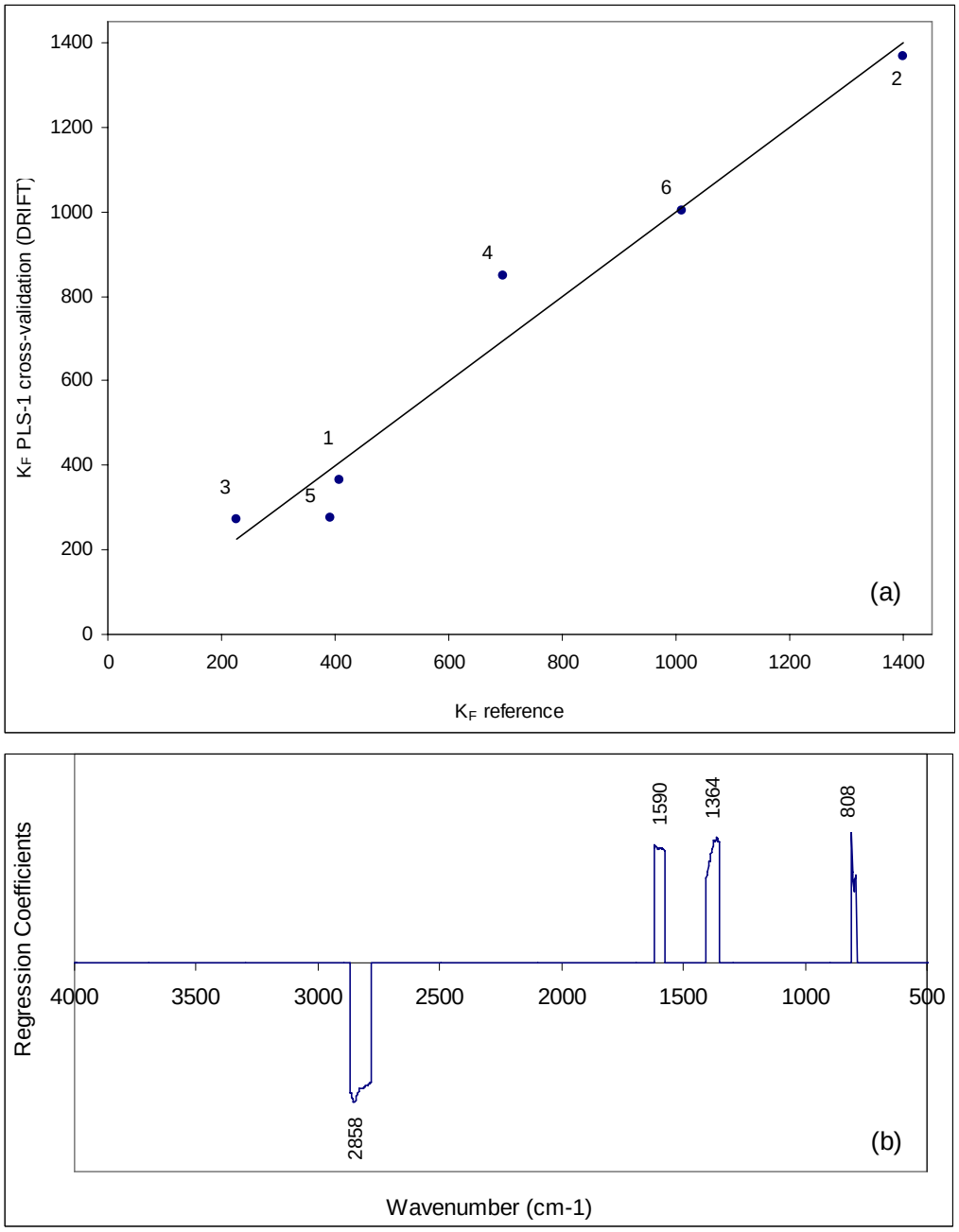

Figure 3 Results of the PLS cross-validation for K_F from mid-infrared DRIFT spectra for all six soils. (a) Cross-validation predicted versus reference K_F regression plot, (b) PLS regression coefficients showing positive and negative peaks corresponding to organic components with high correlations with K_F.

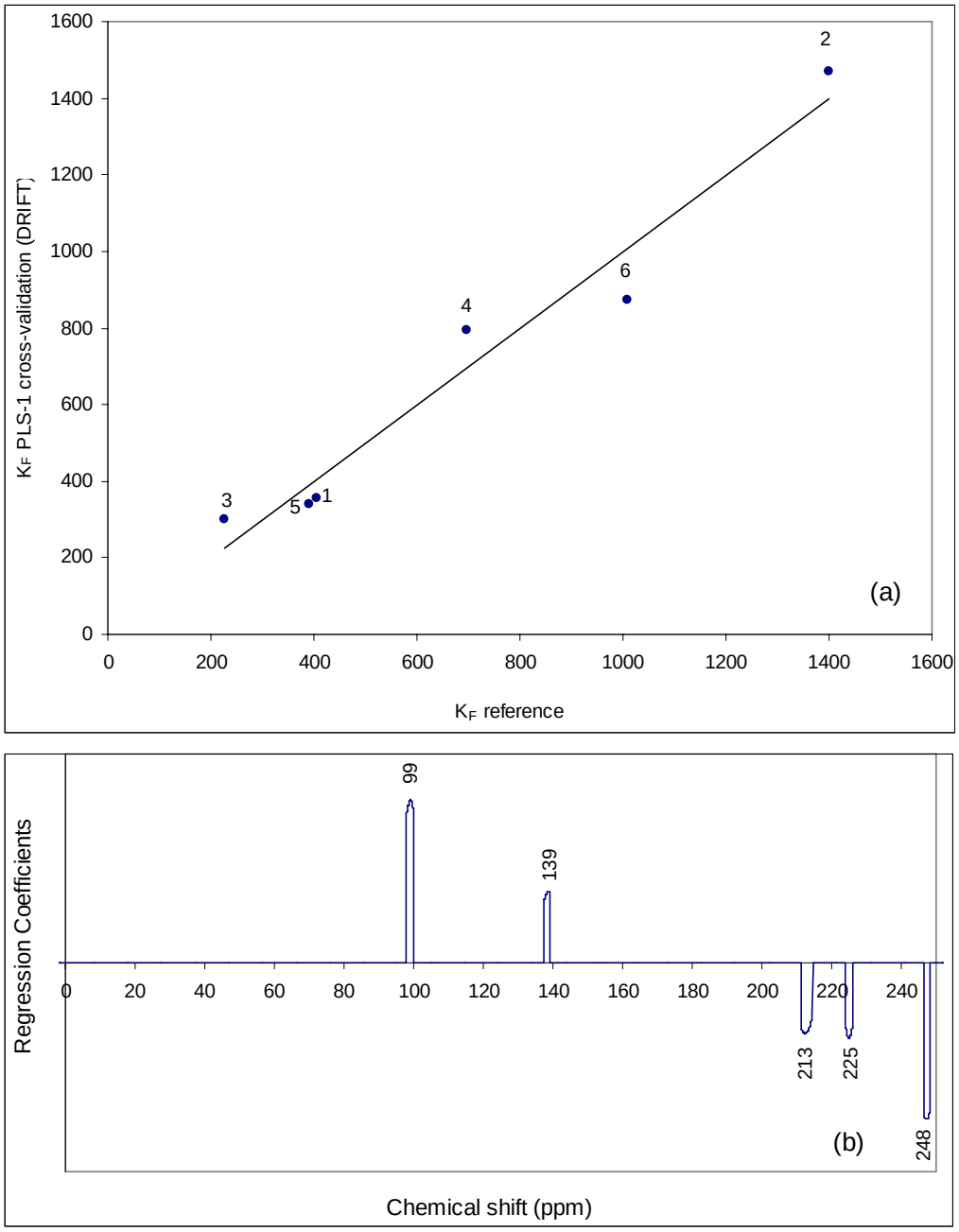

Figure 4 Results of the PLS cross-validation for K_F from solid-state CPMAS ^{13}C-NMR spectra for all six soils. (a) Cross-validation predicted versus reference K_F regression plot, (b) PLS regression coefficients showing positive and negative peaks corresponding to organic components with high correlations with K_F.

Chapter 3

Effect of Vegetable Oil Addition on Bioaccessibility and Biodegradation of Polycyclic Aromatic Hydrocarbons in Historically Contaminated Soils

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Research Article

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Effect of vegetable oil addition on bioaccessibility and biodegradation of polycyclic aromatic hydrocarbons in historically contaminated soils

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Abstract

BACKGROUND: Bioaccessibility is often the limiting factor for the biodegradation of polycyclic aromatic hydrocarbons (PAH) in soils. The present study explores the potential of canola oil amendment, an economically and ecologically attractive soil additive, for the enhancement of bioaccessibility and, in consequence, biodegradation of PAH in historically contaminated, bioaccessibility limited soils.

RESULTS: The amendment of canola oil (1% and 5%, w/w) to contaminated soils increased the bioaccessibility and the subsequent biodegradation of PAH with up to four rings. Residual concentrations of pyrene and fluoranthene in oil-treated soils were 38–53% lower compared to the unamended tests. The continuous removal of bioaccessible PAH with a passive sampling system confirmed that oil amendment indeed increased bioaccessibility, leading to a lower non-accessible PAH fraction. Canola oil amendment did, by contrast, not increase the bioaccessibility of high molecular weight PAH, likely due to their strong binding to soil organic carbon compounds.

CONCLUSION: Canola oil can be used efficiently in low concentrations to render PAH up to four rings accessible for biodegradation in historically contaminated soils. Contaminants remaining in soil after treatment may pose a significantly lowered environmental risk, as is indicated by the lack of mobilisation by a solubilising agent such as canola oil.

Keywords: bioremediation; soil; polycyclic aromatic hydrocarbons; bioaccessibility; canola oil; passive sampling

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are a widely distributed class of hydrophobic organic contaminants in the environment. Owing to the mutagenic and carcinogenic effect that is assigned particularly to high molecular weight congeners,[1,2] PAH pose a serious threat to environmental receptors and human health. In the 1980s, 16 polycyclic aromatic compounds were listed as priority pollutants by the US Environmental Protection Agency (16 EPA PAH[3,4]). The considerable impact of PAH as environmental contaminants has led to the development of sophisticated remediation technologies for the clean-up of contaminated soils and sediments, such as bioremediation, which is based on supporting the natural capability of microorganisms to metabolise organic contaminants. The recalcitrance of PAH to biological degradation processes increases with increasing ring number.[5–7] However, the extent of PAH biodegradation and resulting residual concentrations in the subsurface is strongly dependent on, besides microbial factors, PAH bioavailability, which is often the delimiting parameter for PAH biodegradation in the subsurface. Numerous methodical and systematic approaches for the determination of contaminant bioavailability have been published.[8–13] Recently, the distinction of two incommensurable aspects of bioavailability has been proposed.[14] The chemical activity of a compound, on the one hand, provides a measure for the chemical's potential to undergo spontaneous physicochemical processes such as partitioning and diffusion, and can be precisely defined.[15] The close relationship between the chemical activity and the toxicity of PAH to small soil invertebrates has recently been demonstrated.[16] The bioaccessible quantity of a contaminant, on the other hand, describes the quantity of the contaminant that can be released from the soil and become available for biological processes, such as uptake or biodegradation.[14] Bioaccessibility will always remain operationally defined, since it depends on

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several factors related to the specific environment, amongst them the target organism, the biological process of concern, desorption conditions and the medium for exposure.[9] The chemical activity as well as the bioaccessibility of a soil pollutant decrease with increasing contact time to the matrix[8,17] and can be limited both physically by obstruction of molecules in pores and chemically by sorption to specific soil constituents such as black carbon and similar structures.[8,18,19] These sorption processes are outstandingly strong and increase with molecular volume and aromaticity.[11,13,19–21] Pollutant accessibility cannot, up to now, be increased economically for large scale bioremediation applications.

The application of vegetable oil is deemed a promising approach for the clean-up of PAH-contaminated soil since it is an environmentally friendly, biodegradable and low-cost soil amendment,[22–25] and can be used in both biological and non-biological treatment technologies. Since PAH solubility is higher by three to five orders of magnitude in the free oil phase than in the water phase,[22,26,27] plant oil can be employed as a mild extractant for the physical removal of PAH from soils. Rapeseed, peanut and sunflower oils were successfully applied for the solubilisation of PAH from contaminated soils.[22,23,28,29] Typical oil-to-soil ratios used for extraction are around 1 : 1 (v/w).[23] By contrast, the application of vegetable oil, in concentrations two orders of magnitude lower than those used for extraction, was observed to stimulate the biological degradation of PAH in soils, but the mechanism of support was not identified.[24,25] Due to its mild solvent character, vegetable oil could contribute to enhancing PAH mass transfer from water and soil to the degrading microorganisms. The biodegradation of pyrene was noted to proceed faster by an order of magnitude due to enhanced mass transfer from the water phase, via a xenobiotic organic phase present in the form of droplets of biologically stable solvents, to the degrading microbial population.[30] It is to be expected that also the mass transfer of hydrophobic contaminants from the soil matrix to microorganisms could be accelerated by the introduction of an organic phase. The use of biodegradable vegetable oils for this purpose appears to be an attractive alternative to conventional non-biodegradable solvents. Moreover, the high abiotic extraction efficiency of plant oil for PAH in historically contaminated soils[23,28] indicates a high capacity for contaminant mobilisation, adjoined by the potential to overcome also contaminant binding processes to soil constituents that are otherwise restricting bioaccessibility. The vegetable-oil induced biosurfactant production of specific microorganisms[31–33] may further contribute to lowering the non-accessible PAH fraction. An increase of the bioaccessible PAH fraction resulting from plant oil addition has the potential to substantially increase the efficiency of various bioremediation measures, such as biopiles or bioslurry reactors, and may also be applied *in situ*. However, to our knowledge, the effect of canola oil addition on increasing the bioaccessible PAH fraction in historically contaminated soils has not been investigated yet.

The scope of the present study was to determine the effect of the addition of canola oil on PAH biodegradation performance in bioaccessibility-limited soils. Two historically contaminated soils were used in bioaccessibility and biodegradation tests with and without vegetable oil amendment. The occurrence of a bioaccessibility limitation in unamended soils was investigated by the comparative assessment of non-accessible and non-biodegradable PAH fractions. These data were yielded by a passive sampling device on one hand and by biostimulated degradation tests on the other, and represented residual PAH concentrations, i.e. $c_{(PAH)}$, where $\partial c_{(PAH)}/\partial t \approx 0$. In the following, the relationship of residual PAH concentrations obtained with canola-oil amended (1% and 5% (w/w)) biodegradation tests to the unamended biodegradation tests enabled assessment of the effect of canola oil on achievable residual PAH concentrations. Canola oil concentrations applied were designed (i) to provide sufficient organic phase for contaminant solubilisation, (ii) to be sufficiently low to be retained by the soil[34] and (iii) to be degraded effortlessly by soil microbes,[25,35] aiming at avoiding amendment residues in remediated soil.

EXPERIMENTAL

Soils

Two soils were collected from historically PAH-contaminated sites in Austria: KD08 from a former railroad sleeper preservation site, and soil WG08 from a former manufactured gas plant site. After excavation, soils were sieved to <2 mm and stored in the dark at 4 °C until further use. Characteristic soil parameters are displayed in Table 1. The contamination (16 EPA PAH, ΣPAH[3,4]) was approximately 1400 and 350 mg kg^{-1} for KD08 and WG08, respectively, with fluoranthene and pyrene as main constituents (Table 2). According to US soil taxonomy, KD08 represents a sandy loam whereas WG08 is a loamy sand. WG08 is nearly three times higher in total organic carbon (TOC) than KD08 (Table 2). The visibility of small black particles in the fraction <2 mm in soil WG08 indicates an anthropogenically elevated content of soil organic carbon.

Extraction of PAH and canola oil

The extraction of solid contents of bioslurry flasks and passive sampler devices was performed with an automated Soxhlet device using ethyl acetate (p.A., VWR International, Vienna, Austria) as extracting agent as described by Szolar and co-authors[36] without further sample clean-up. Samples for analysis by high-performance liquid chromatography (HPLC) were diluted with acetonitrile (HPLC gradient grade, VWR International) according to the measurement range, but at least by a factor of three in order to be compliant with HPLC conditions.

Extracts for canola oil analysis for gas chromatography with flame ionisation detection (GC-FID) were concentrated with a TurboVap II evaporator (Caliper Life Sciences, Hopkinton, MA, USA) by a factor of 10 for measurement of oil concentrations lower than 1000 mg kg^{-1} soil dry matter (DM). Samples with higher oil content were analysed directly.

PAH analysis

Liquid chromatography was performed with an HP 1050LC (Hewlett-Packard, Palo Alto, CA, USA) equipped with an HP 1100 series three-dimensional fluorescence detector (3D-FLD). Injection volume was set to 20 µL for standards and samples, respectively, on an 1050 HP autosampler. For separation, an ODS Hypersil guard column (20 × 4 mm, particle size 5 µm, Thermo Fisher Scientific Inc., Vienna, Austria) followed by a C-18 Grace Vydac (Grace Davison Discovery Sciences, Deerfield, IL, USA) separation column (250 × 4.6 mm, particle size 5 µm) was used. The column was heated at 26 °C and operated with an eluent flow rate of 1.5 mL min^{-1}. The eluent gradient profile was set up as follows: 50% acetonitrile/50% Milli-Q® water for 2.5 min, followed by a linear gradient of 9.5 min up to 90% acetonitrile and a linear gradient of 8 min up to 100% acetonitrile, held for 2.5 min. Subsequently,

Table 1. Soil characteristics for soils KD08 and WG08, fraction <2 mm

Soil parameters	Unit	KD08	WG08
DM (dry matter)	%	87.2 (0.31)	80.0 (0.16)
Soil pH	–	7.0 (0.03)	7.4 (0.04)
Particle size distribution			
Sand: 63 µm–2 mm	% w/w	56 (1.15)	77 (0.46)
Silt: 2–63 µm	% w/w	40	20
20–63 µm	% w/w	18 (0.92)	11 (0.20)
10–20 µm	% w/w	9 (0.19)	3 (0.06)
6–10 µm	% w/w	6 (0.38)	2 (0.06)
2–6 µm	% w/w	7 (1.10)	3 (0.09)
Clay: <2 µm	% w/w	4 (0.78)	3 (0.07)
TPH C_{10}–C_{40}[37]	mg kg DM^{-1}	450 (40.8)	1317 (43.7)
OTS (organic total solids)	% w/w	5.5 (0.27)	12.5 (0.32)
TIC (total inorganic carbon)	% w/w	1.1 (0.07)	1.2 (0.03)
TOC (total organic carbon) = TC − TIC	% w/w	2.8 (0.0)	7.7 (0.0)
TC (total carbon)	% w/w	3.9 (0.33)	8.8 (0.59)
Nitrogen	% w/w	0.2 (0.02)	0.21 (0.01)
NO_3-N (available)	mg kg DM^{-1}	1.7 (0.11)	2.4 (0.26)
NH_4-N (available)	mg kg DM^{-1}	<0.05[a]	8.9 (0.20)
PO_4-P (available)	mg kg DM^{-1}	2.3 (0.14)	49.9 (1.99)
Glucose-induced respiration	mg CO_2 g DM^{-1} 4 h^{-1}	0.13 (0.019)	0.08 (0.002)

[a] Below level of detection.
Values in parentheses are standard deviations; $n = 3$

Table 2. PAH contamination profile for soils KD08 and WG08: individual compounds, abbreviations and total concentration (ΣPAH)

Compound	KD08[a]	WG08[a]
Naphthalene (NAP)	17 (1.6)	54 (4.4)
Acenaphthylene (ACY)	n.d.[b]	n.d.[b]
Acenaphthene (ACE)	18 (1.3)	9 (0.5)
Fluorene (FLU)	29 (7.1)	9 (1.1)
Phenanthrene (PHE)	111 (8.6)	39 (1.5)
Anthracene (ANT)	83 (7.7)	7 (0.5)
Fluoranthene (FLT)	519 (45.2)	58 (1.6)
Pyrene (PYR)	326 (25.5)	35 (0.6)
Benzo(*a*)anthracene (BaA)	96 (6.4)	21 (0.5)
Chrysene (CHR)	100 (8.6)	10 (0.5)
Benzo(*b*)fluoranthene (BbF)	43 (1.2)	27 (1.3)
Benzo(*k*)fluoranthene (BkF)	17 (0.6)	11 (0.6)
Benzo(a)pyrene (BaP)	20 (0.4)	26 (1.3)
Dibenz(*a*, *h*)anthracene (DBA)	n.d.[b]	7 (1.2)
Benzo(*g*, *h*, *i*)perylene (BP)	5 (0.4)	16 (0.8)
Indeno(1,2,3-*c*, *d*)pyrene (IP)	7 (0.6)	24 (0.6)
Sum of 16 EPA PAH, ∑PAH	1395 (54.7)	353 (5.7)

[a] Units are mg kg^{-1} DM; values in parentheses are standard deviations; $n = 3$.
[b] Not detected.

a linear gradient (2.5 min) was set back to initial conditions for column conditioning preceding the next run. The 3D-FLD was operated at four different excitation/emissions wavelength pairs: excitation was set to 260 nm, and emission wavelengths were 350, 420, 440 and 500 nm, respectively. Acenaphthylene was measured by UV detection. An external standard mixture of the 16 EPA PAH[3] with 10 mg L^{-1} of each compound dissolved in acetonitrile for HPLC analysis (Supelco, Sigma-Aldrich, Buchs, Switzerland) was used for PAH quantification.

Canola oil analysis

The GC method was set up according to the hydrocarbon index method.[37] The unresolved complex mixture at the end of the chromatogram, making up 95% of the total area, was calculated as area sum representing the total oil concentration. Calibration was performed with canola oil solutions in ethyl acetate between 500 and 5000 mg L^{-1}.

The gas chromatograph used was an Agilent 5890 (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionisation detector. The column used was a DB5-HT (0.25 mm × 30 m × 0.25 µm, Agilent Technologies) and splitless injection volume was 1 µL at 350 °C. The initial temperature was 60 °C held for 5 min; the oven was heated at a rate of 20 °C min^{-1} up to 380 °C and held stable for 10 min. The flame ionisation detector was operated at 400 °C.

Bioslurry treatment

One day prior to experiment start-up, soils were amended with commercially available canola oil. The oil was amended on a mass basis ($w_{oil}/w_{soil\ DM}$), and was added drop-wise to the soil, using a Pasteur pipette while the soil was stirred in a glass beaker. Oil-amended soils were then left to acclimatise overnight. The concentrations of canola oil were adjusted to 1% and 5% (w/w). For each soil, the test set-up was constituted of three parallel aerobic experimental lines differing in canola oil concentration: one unamended and two oil-amended (1% and 5% (w/w), respectively) ones. Batch bioslurries were kept in 100 mL Pyrex® (VWR international, Leuven, Belgium) flasks, each containing 5 g of soil DM and 20 mL nutrient solution, leaving

a headspace of approximately 80 mL. Suspension flasks were covered with punctured aluminium foil to allow for oxygen input and were stored at 20 °C on an orbital shaker (200 rpm). For each sampling day, a triplicate of suspension flasks per parallel was sacrificed. The contents were filtered for at least 2.5 h over paper filters, resulting in a soil DM of about 80%. Soil samples were extracted and analysed for PAH and canola oil content. Oil and PAH concentrations in the slurry supernatant were deemed negligible based on results from preliminary experiments. Soil slurry pH was controlled during incubation time using commercially available test strips and determined to be 6.8 ± 0.1.

Nutrient addition was calculated for each parallel based on the total content of organic carbon (contributions by soil, contaminants and oil amendment) in the soil samples to yield a molar nutrient ratio of C : N : P=100 : 10 : 1.[38] The nutrient solution consisted of NH_4NO_3 (Fluka, Sigma-Aldrich, Buchs, Switzerland), KH_2PO_4 (Merck, Darmstadt, Germany) and Na_2HPO_4 (JT Baker, Deventer, Netherlands), all with purity ≥99%, dissolved in bidistilled water. In addition, 10 mL trace element solution and 1 mL vitamin solution were amended per litre of nutrient solution.

Nutrient concentrations were as follows for the six parallels (KD08, WG08; each unamended, 1% and 5% oil amendment), with: (a) NH_4NO_3, (b) KH_2PO_4 and (c) Na_2HPO_4 (values in mg L^{-1} nutrient solution); for KD08 unamended: (a). 2317, (b) 396 and (c) 411; for KD08, 1% oil: (a) 3091, (b) 528 and (c) 547; for KD08, 5% oil: (a) 6189, (b) 1055 and (c) 1093; for WG08 unamended: (a) 6371, (b) 1086 and (c) 1126; for WG08 1% oil: (a) 7145, (b) 1218 and (c) 1262; for WG08, 5% oil: (a) 10 243, (b) 1744 and (c) 1808. The trace element solution was composed as follows: H_3BO_3 3 mg L^{-1}, $CaCl_2$ 531 mg L^{-1}, $CoCl_2$ 3.9 mg L^{-1}, $CuSO_4.5H_2O$ 17.3 mg L^{-1}, $FeSO_4.7H_2O$ 206 mg L^{-1}, KI 101 mg L^{-1}, $MnSO_4.1H_2O$ 14 mg L^{-1}, $Na_2MoO_4.2H_2O$ 5.3 mg L^{-1}, Na_2SeO_4 0.6 mg L^{-1}, $NiCl_2.6H_2O$ 4.6 mg L^{-1}, $ZnSO_4.7H_2O$ 20.5 mg L^{-1} and H_2SO_4 conc. 1 mL L^{-1}, all purchased from Sigma-Aldrich, purity at least 98%. The vitamin solution was composed as follows: 4-aminobenzoic acid 5 mg L^{-1}, biotin 2 mg L^{-1}, folic acid 2 mg L^{-1}, α-lipoic acid 5 mg L^{-1}, nicotinic acid 5 mg L^{-1}, panthothenic acid calcium salt hydrate 5 mg L^{-1}, pyridoxine hydrochloride 10 mg L^{-1}, riboflavin 5 mg L^{-1}, thiamine hydrochloride 5 mg L^{-1} and vitamin B_{12} 0.1 mg L^{-1}, all purchased from Sigma-Aldrich, purity at least 99%.

An inoculum was enriched from PAH-contaminated soils, activated sludge and compost and cultivated on anthracene oil (voestalpine Stahl, Linz, Austria) as the sole carbon source. 5 µL was added to each suspension flask. Inoculum cultivation and its metabolic capability to degrade PAH with up to six rings to a significant extent were described earlier.[13]

Bioaccessibility assays

In parallel to the biodegradation tests, an experiment to evaluate PAH bioaccessibility in soils KD08 and WG08 was conducted, using a novel passive sampling device (PSD). A PSD consists of a glass beaker with a strong PAH sorbent at its bottom. 5 g soil (dry matter) was weighed into the beaker and 10 mL cyclodextrin solution amended with NaN_3 (Sigma-Aldrich) to prevent degradation was added. This system has been developed at the National Environmental Research Institute (NERI; Roskilde, Denmark) and its working principle, performance and applicability will be described in detail in a companion article.

Cyclodextrin is known to form water-soluble PAH : HPCD (hydroxypropyl-β-cyclodextrin) inclusion complexes[39] that lower aqueous boundary layer resistance[12,40] and thereby accelerate the mass transfer from soil particle surface to the bulk solution and from the bulk solution to the sorbent. This sampling device continuously and efficiently traps all PAH molecules as they are desorbed, resulting in a soil sample containing the non-accessible PAH fraction. For each sampling day, a triplicate of passive sampler devices was sacrificed; i.e., the soil was extracted and analysed for residual PAH concentrations following the same procedure as for the slurry samples.

Statistical analysis

Statistical comparison of residual, i.e., post-biodegradation and non-accessible, PAH fractions was performed using one-way ANOVA, $\alpha = 0.05$, and Tukey's multiple comparison test. Analyses were conducted for comparison of individual PAH and ΣPAH. PAH concentrations were assumed as residual at near-zero decrease rates ($c_{(PAH)}$, where $\partial c_{(PAH)}/\partial t \approx 0$).

All analyses were conducted in triplicate. Measured concentrations refer to soil dry matter, if not indicated otherwise.

RESULTS AND DISCUSSION

In the present study, two types of assays were conducted to assess the effect of plant oil amendment on residual PAH concentrations in historically contaminated, bioaccessibility-limited soils. The non-bioaccessible PAH fraction was determined in unamended soils using an abiotic PSD. PAH biodegradation endpoints were determined in biostimulated soil slurry tests with and without canola oil amendment. Data interpretation is based on the comparison of resulting post-biodegradation (bioslurry tests) and non-bioaccessible PAH concentrations (PSD).

PAH bioaccessibility in unamended soils

In the PSD, contaminant molecules are trapped under formation of PAH : HPCD complexes when released from the soil and are then collected by a highly sorptive matrix. Since this method of depletive sampling responds to accessibility and desorption kinetics within the soil rather than to the strength of a solvent,[39] it is expected to give a good estimation of the contaminant's accessible fraction in a given environment, with the non-accessible fraction remaining in the soil.

The statistical comparison of residual PAH concentrations in unamended bioslurry experiments, and of the non-accessible PAH fraction in unamended soils, as determined by PSD, revealed that the PSD gave a good prediction of post-biodegradation concentrations for four- to six-ring compounds in both soils. For only two out of all detected four-, five- and six-ring compounds in both soils (Table 2) the biodegradable pool was significantly overestimated by the passive sampling device. Thus, the PSD appears to be a proficient tool for assessing PAH bioaccessibility in historically contaminated soils. Moreover, since bioaccessibility as measured using the PSD correlated well with actual biodegradation in the unamended bioslurry tests for both soils, it is indicated that PAH biodegradation was limited by contaminant accessibility in the unamended soils rather than by biological factors. While the similarity of non-degraded and non-accessible PAH concentrations indicates a bioaccessibility limitation, a higher non-degraded PAH fraction would point towards a limitation imposed by other factors, such as adverse biological conditions,[14] on the biodegradation process.

In oil-amended soils, canola oil represents, in addition to the highly sorptive matrix in the PSD, a second hydrophobic, PAH-trapping phase, which is thoroughly intermixed with the soil. Any

PAH solubilised in the canola oil would be co-analysed with the soil contained in the PSD, leading to an overestimation of the non-bioaccessible PAH fraction and rendering use of the PSD for bioaccessibility determination in oil-amended soils unsuitable. Consequently, it was decided to determine the influence of plant oil on PAH bioaccessibility in soils KD08 and WG08 via the comparison of post-biodegradation PAH concentrations in the oil-amended *versus* those in the unamended, bioaccessibility-limited bioslurry system.

Influence of canola oil amendment on PAH degradation

The addition of 5% canola oil resulted for all PAH compounds and in both soils in higher or at least equal residual concentrations as with 1% amendment. Degradation of all detectable EPA-PAH (ΣPAH) in soil KD08 was between 78.7 (±1.2)% and 70.3 (±3.1)% for 1% and 5% oil amended, respectively. Slightly but significantly higher residual concentrations in the 5% amended parallel were detected in soil KD08 for four PAH, namely naphthalene, anthracene, benzo(*k*)fluoranthene and indeno(1,2,3-*cd*)pyrene. The remaining 10 compounds in soil KD08 were degraded to an equal extent with both amendments.

Degradation of ΣPAH was lower in soil WG08 (23.9 ± 3.7% and 27.5 ± 8.2% degradation with 1% and 5% canola oil addition, respectively) than in soil KD08. For soil WG08, there was no significant difference of the 5% oil supplement for any individual residual PAH concentration when compared to the 1% oil amendment. Consequently, a fivefold increase in the oil concentration did not result in a decrease in residual PAH concentrations in the two soils.

In a recent study,[25] rapeseed oil was observed to entail the oxidation of anthracene and benzo(*a*)pyrene in contaminated soils, assumedly by an abiotic process, connected to the activation by photooxidation or fungal enzymes. In the present study, however, no significant effect of oil amendment on residual PAH concentrations of the two above-mentioned substances was observed, either for 1% oil amendment (Figs 1 and 2; see below) or for 5% oil amendment, presumably due to a difference in soils and type of vegetable oil.

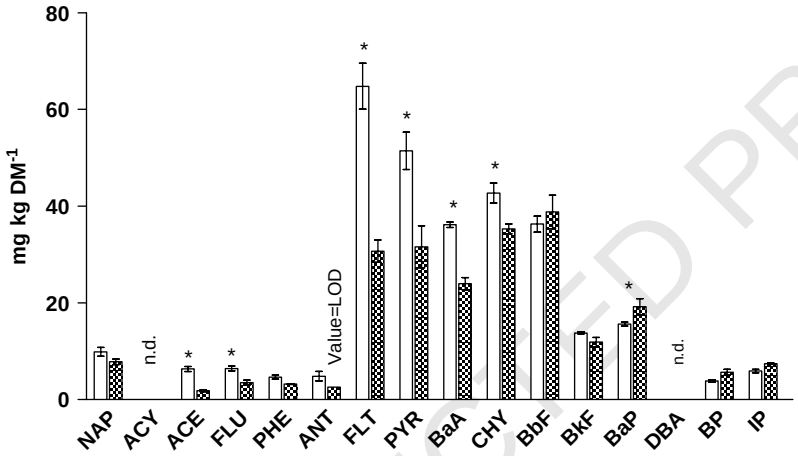

Figure 1. Residual PAH concentrations in soil KD08 after 105 days of incubation. Blank bars: unamended soil slurries; dotted bars: 1% canola oil amendment. Residual anthracene concentration was within the level of detection (LOD) in the 1% amended parallel and thus excluded from statistics. Asterisks indicate statistically significant difference between parallels; error bars represent standard deviation ($n = 3$); n.d., not detected; for abbreviations refer to Table 2.

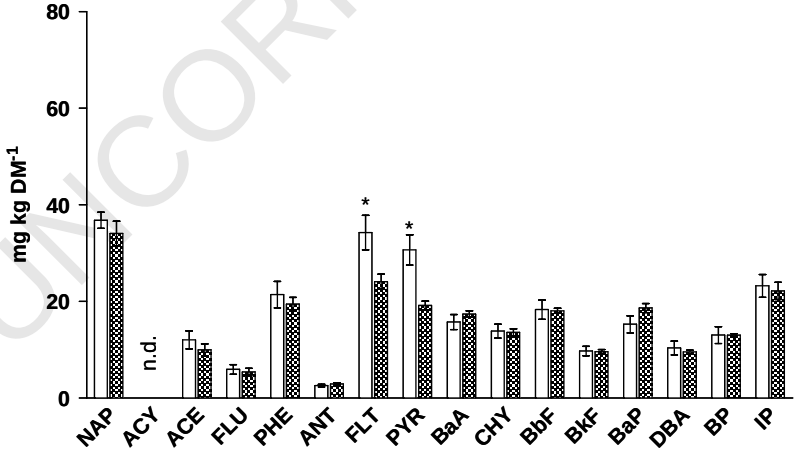

Figure 2. Residual PAH concentrations in soil WG08 after 98 days of incubation. Blank bars: unamended soil slurries; dotted bars: 1% canola oil amendment. Asterisks indicate statistically significant difference between parallels; error bars represent standard deviation ($n = 3$); n.d., not detected; for abbreviations refer to Table 2.

Effect of canola oil on bioaccessibility and biodegradation of four-ring PAH

Figures 1 and 2 show residual, post-biodegradation concentrations of all detected PAH as affected by 1% oil addition in soils KD08 and WG08, respectively, and residual concentrations from unamended slurry experiments. Biodegradation data on the 5% oil amendment are omitted for the lack of significant difference to the 1% oil amendment.

In soil KD08 (Fig. 1), all four-ring compounds plus acenaphthene and fluorene exhibited a significant decrease in their residual concentration when amended with canola oil. For soil WG08 (Fig. 2), significant reductions were observed for fluoranthene and pyrene.

Exemplifying, residual concentrations after bioslurry treatment (unamended, 1% and 5% canola oil amendment) and non-accessible fractions, as determined by PSD assays, of selected four-ring PAH in the two soils are displayed in Figs 3 and 4. Individual post-degradation PAH concentrations in unamended soils were not significantly different from non-accessible fractions (PSD) for either soil (Figs 3 and 4). PAH concentrations were significantly reduced with 1% oil amendment, by approximately 38.6% (KD08) and 37.5% (WG08) for pyrene, and by 52.6% (KD08) and 29.7% (WG08) for fluoranthene when compared to the unamended tests. Chrysene and benzo(*a*)anthracene residual concentrations were significantly lower in the 1% amended tests by 17.3% and 33.7% in soil KD08 than in the unamended tests. In connection with the observation that PAH biodegradation in unamended soils was bioaccessibility limited, it can be concluded that the substantially lower post-biodegradation PAH concentration in oil-amended soils was a result of an increase in contaminant bioaccessibility.

Vegetable oils are water-immiscible mild solvents[29] and may also lower surface tension[25] or stimulate the biological production of surfactants.[31–33] They hold the potential to increase the bioaccessibility of hydrophobic organic contaminants by increasing mass transfer from the solid phase, including the readily accessible portion but also a fraction of the non-bioaccessible contaminants. Inaccessible hydrophobic contaminant molecules partition into the oil phase, where they are present for microorganisms in a well-bioaccessible form,[41] entailing significantly increased contaminant biodegradation.[30]

Solubilisation or emulsification of contaminants by surfactants may also contribute to accelerate PAH biodegradation.[41] It has been verified that the stimulating effect of canola oil on biosurfactant production[31,32] also increases PAH biodegradation by actinomycetes.[33]

Given the solubilising character of plant oil, the extent of increase in contaminant bioaccessibility depends strongly upon the strength of pollutant sorption towards soil constituents and is reflected in the non-uniformly increased PAH biodegradation in the two oil-amended soils. While the impact of vegetable oil amendment on PAH degradation was profound in soil KD08, originating from a railroad sleeper preservation site, the increase in biodegradation was lower in WG08, a soil originating from a manufactured gas plant (MGP) site. Soil WG08 also has a high content of organic carbon (7.7%, Table 1), with black particulates visible, most likely enriched in the industrial history of this site, while this was not observed for soil KD08, which is also markedly lower in TOC (2.8%, Table 1).

Strong PAH sorption was frequently observed in MGP soils, since PAH affinity is high to anthropogenic, MGP-site related soil carbon types.[42–45] The strong sorption of PAH to carbonaceous materials, possibly black carbon-like structures, in soil WG08 is likely to be the reason for the lower decrease of the non-bioaccessible fraction in this soil, with the plant-oil induced PAH mobilisation being limited to less strongly bound contaminants.

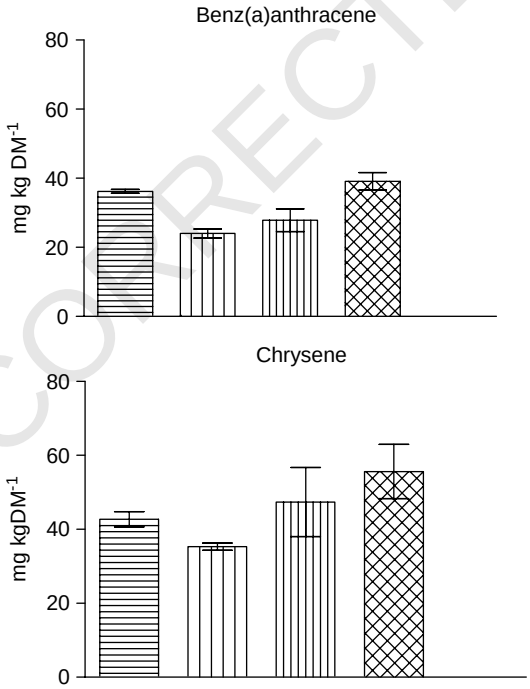

Figure 3. Residual concentrations of benz(*a*)anthracene (top) und chrysene (bottom) in soil KD08: soil slurries, unamended (horizontal stripes), 1% (spaced vertical) and 5% (vertical) canola oil amendment and non-bioaccessible fraction (chequered) as determined with passive sampling device. Error bars represent standard deviation; $n = 3$.

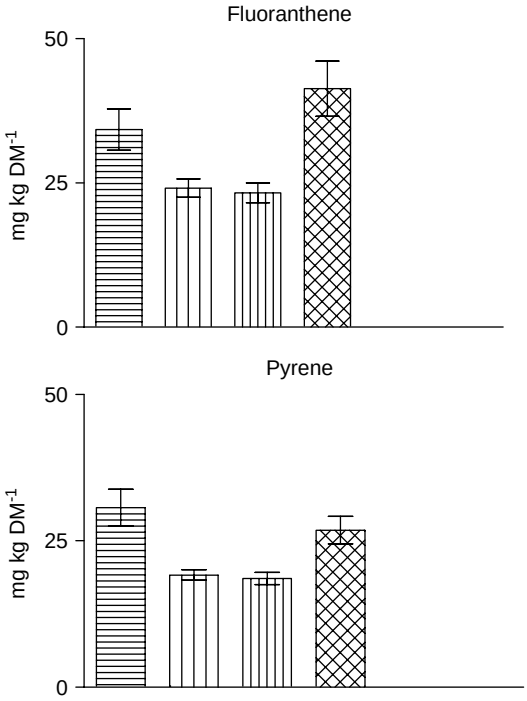

Figure 4. Residual concentrations of fluoranthene (top) und pyrene (bottom) in soil WG08: soil slurries, unamended (horizontal stripes), 1% (spaced vertical) and 5% (vertical) canola oil amendment and non-bioaccessible fraction (chequered) as determined with passive sampling device. Error bars represent standard deviation; $n = 3$.

These results indicate that the addition of canola oil is a proficient tool to increase the bioaccessibility and consequently, biodegradation of PAH with up to four rings in historically contaminated soils. Moreover, the contaminants that remain sequestered under these mildly extractive conditions will have a significantly lowered chemical activity,[13,14] which implies reduced contaminant mobility and exposure and thus a reduced risk for human health and environmental receptors.

Effect of canola oil on bioaccessibility and biodegradation of five- and six-ring PAH

By comparison to four-ring compounds, our results show that biodegradation of five- and six-ring PAH (high molecular weight or HMW compounds) was not increased by the addition of canola oil in the two historically contaminated soils: KD08 and WG08 (Figs 1 and 2). Non-accessible concentrations of HMW compounds determined with the PSD coincided well with residual concentrations after biodegradation in the unamended soils, with slightly but statistically different values for only two out of all detectable five- and six-ring compounds in both soils (Table 2). There was no significant increase in biodegradation in the oil-amended parallels (Figs 1 and 2).

There is little data available on the feasibility of HMW PAH removal from contaminated matrices using vegetable oil. Observations of a vegetable-oil related increase of HMW PAH biodegradation were made with benzo(*a*)pyrene,[24,25] a five-ring compound, but studies did not include bioaccessibility measurements. Soil extraction with vegetable oil was connected to co-extraction of soil organic matter-bound or particle-associated PAH,[22,29] rendering the estimation of the actual extent of HMW PAH solubilisation from these data difficult. It is to be expected that the high affinity of HMW PAH towards carbonaceous geosorbents in historically contaminated soils[8,11,18] would outcompete PAH solubilisation in plant oil, unlike that for lower molecular weight compounds. This is further corroborated by the good sorption of oil-solubilised HMW PAH on activated charcoal.[46] Consequently, we hypothesise that the increased solubility of HMW PAH in lipophilic phases such as vegetable oil does not compensate for their strong retention, being the high molecular attraction of planar, heavy polycyclic contaminants featuring a large surface area, towards condensed aromatic •SOM structures.[8,18,19]

Biodegradation of canola oil in slurry microcosms

The biodegradation of canola oil over incubation time in soils KD08 and WG08 is shown in Fig. 5. For soil WG08, canola oil concentrations after 98 days were equal, irrespective of initial oil concentration, amounting to approximately 3500 mg kg^{-1}, corresponding to 71.5% and 91.1% degradation of 1% and 5% oil amendment, respectively. Remaining oil concentrations in soil KD08 were found after 105 days to be between 50 mg kg^{-1} (1% amendment; 99.94% degradation) and 150 mg kg^{-1} (5%; 99.96%, respectively). Constant bioslurry pH indicated a sufficiently high capacity in both soil systems for buffering possible acidic oil metabolites.

Fatty acids of canola oil comprise (w/w) oleic acid (51–70%), linoleic acid (15–30%) and linolenic acid (5–14%)[47] and main triglycerides were observed to be fully mineralised within 16 days.[48] The complete mineralisation of vegetable oils by soil microorganisms, while not formally determined in the present study, was expected to be effortless since the required enzymes are common to many microbes.[49] Rapid mineralisation of plant oil in soils, however, requires an adequate supply of oxygen and nutrients, which significantly impedes, if not satisfied, microbial activity.[25,35] Anaerobic degradation of vegetable oil may result in a higher transient increase of toxicity.[48,50] The inhibitory effect

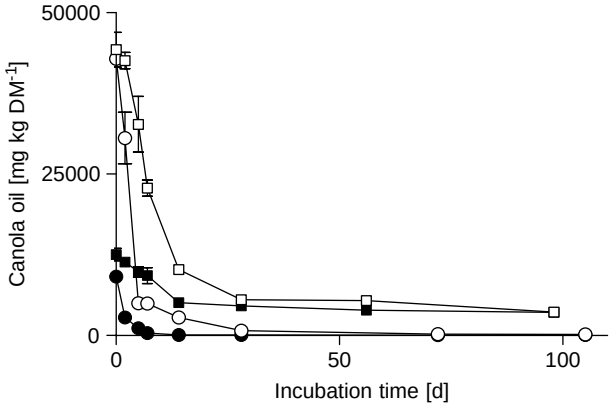

Figure 5. Concentration of canola oil in oil-amended soil slurries over incubation time. Soils and oil concentrations (w/w): KD08, 1% (filled circles) and 5% (empty circles); WG08, 1% (filled squares) and 5% (empty squares). Error bars represent standard deviation; $n = 3$. Some error bars are smaller than the symbols.

caused by canola oil concentrations of the same order as those presently applied on bacteria and sediment-dwelling organisms was observed to be below related toxicity threshold values.[50] Thus the use of canola oil in the same concentration range as applied in the present study is not expected to be of considerable environmental concern.

There is limited knowledge on the effect of canola oil on the microbial community's metabolic capacity for PAH degradation. Upon addition of plant oil, the dominance within the microbial community will shift to those populations best adapted to the new substrate and environmental conditions, and the resulting different enzymatic profile may substantially influence PAH degradation pathways. Further research would be required on characterising the effects of vegetable oil addition on the metabolisation of PAH.

CONCLUSIONS

The use of canola oil as a non-toxic, biodegradable amendment to PAH-contaminated soils is a viable approach to increase the efficiency of bioremediation measures. Oil amendments to soil in concentrations as low as 1% (w/w) were sufficient to significantly lower achievable contaminant concentrations and are expected to be effortlessly degradable by the microbial community. Such low amendments render the collection and regeneration of the oil unnecessary, which is otherwise required when plant oil is used as an extractant for abiotic PAH removal. The results of the present study are, to our knowledge, the first evidence that vegetable oil enhances biodegradation of PAH of up to four rings in historically contaminated soils by increasing contaminant accessibility to the microbial community. Remaining PAH fractions are, owing to their lowered chemical activity, expected to pose a significantly lower environmental risk. Supplementary research is required preceding the exploitation of these observations for remediation purposes, such as the verification of complete PAH mineralisation under plant-oil amended conditions.

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Chapter 4

Addition of Whey, Lactose and Humic Matter Effects Complete Removal of PCE: Transferring Lab-Scale Observations to *in situ* Validation

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ADDITION OF WHEY, LACTOSE AND HUMIC MATTER EFFECTS COMPLETE REMOVAL OF PCE : TRANSFERRING LAB-SCALE OBSERVATIONS TO *IN SITU* VALIDATION

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ABSTRACT

The biodegradation of chlorinated aliphatics in the saturated zone can be supported via the addition of various dissolved and gaseous amendments. However, a successful implementation strongly depends upon the precise delivery of the adequate type and amount of additive. Process parameters for thirteen groundwater amendments supporting PCE biodegradation were optimized in bioreactors using site material. The application of lactose and whey, respectively, followed by the amendment of a soil humic matter fraction, resulted in the complete PCE transformation within 43 days. The *in situ* implementation of these results underlines the importance of pre-optimizing crucial implementation parameters such as electron donor concentration and the arrangement of amendment addition within time and space. Based on the results drawn from the small-scale experiments, it is possible to give a profound estimate of amendment addition efficiency and pre-optimize application parameters in the field scale.

1. INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAH) such as Perchloroethylene (PCE) and its metabolites are soil and groundwater contaminants of major concern. Microbiological remediation of contaminated sites relies on the promotion of naturally occurring microbial dechlorination processes in the subsurface [7]. The addition of electron donors and other dechlorination supporting substances is a promising method to support the mineralization of CAH ([2], [7]).
A major obstacle to the efficient and reliable application of such a treatment is the control of complex subsurface degradation processes, possibly leading to reduced efficiency, mobilization of toxic intermediates and incomplete mineralization. Small scale experiments under controlled conditions prior to *in situ* implementation under site-similar conditions enable for optimization of process parameters, such as amendment type and concentration as well as dimensioning kinetics of application and distribution. The adaptation of these parameters to *in situ* conditions and further optimization in field scale are sensitive tasks.

2. MATERIALS AND METHODS

Prior to field validation, a groundwater amendment procedure using dissolved and gaseous substrates to enhance dechlorination of PCE in the saturated zone was developed and optimized in the lab-scale.
Anaerobic aquifer material and groundwater from prospective validation sites were used to operate slurry bioreactors (500mL). Incubation parameters were: 20°C in the dark, submerged in water, shaken at 80 rpm (orbital). Abiotic and methanogenesis-inhibited parallels were also constructed (using $HgCl_2$ and 2-bromoethanesulfonate, respectively, as specific inhibitors). Gaseous amendments were applied in specially designed saturated/unsaturated bioreactor soil columns (5L) using aquifer material. Thirteen different aqueous organic and gaseous amendments were tested. Chlorinates were analyzed on a Purge & Trap PTA-3000 (Axel Semrau, Sprockhövel, Germany) coupled with an Agilent GC Series 6890 (Restek 624 column coupled with Restek VRX) equipped with a mass selective detector, and operated in SIM mode. Temperature program was 35° (3 min), gradient of 8°C/min to 200°C, held for 5 min. Substrate degradation pattern (VFA) was determined on an Agilent HPLC Series 1100 equipped with an RI detector.

3. RESULTS

3.1. Lab-scale experiments

Bioreactors were treated with selected amendments (single or in combination). Eventually, a two-step amendment treatment was chosen for the field applications.
Lactose (Figure 1) and whey (data not shown) effected the conversion of PCE to *cis*-DCE and VC most efficiently. Hydrogen sources were supplied at super-stoichiometric concentrations with the ratio of electrons amended (eq_{am}) to electrons required (eq_{req}) of 5:1 (thus, eq_{am}:eq_{req}=5:1) based on the theoretical assumption of complete channelling of

reducing equivalents into CAH reduction. Lactose was degraded immediately after addition. In the course of PCE degradation, *cis*-DCE was accumulated intermediately and was only degraded after 150 days (Figure 1, left side), similarly to acetic acid produced from lactose (Figure 1, right-hand). For whey amendment, similar results were recorded (data not shown). A 10-fold increase in electron donor concentration (eq_{am}:eq_{req}=50:1) resulted in a significant reduction of CAH degradation performance in parallel to a massive gas production and the formation of butyric acid.

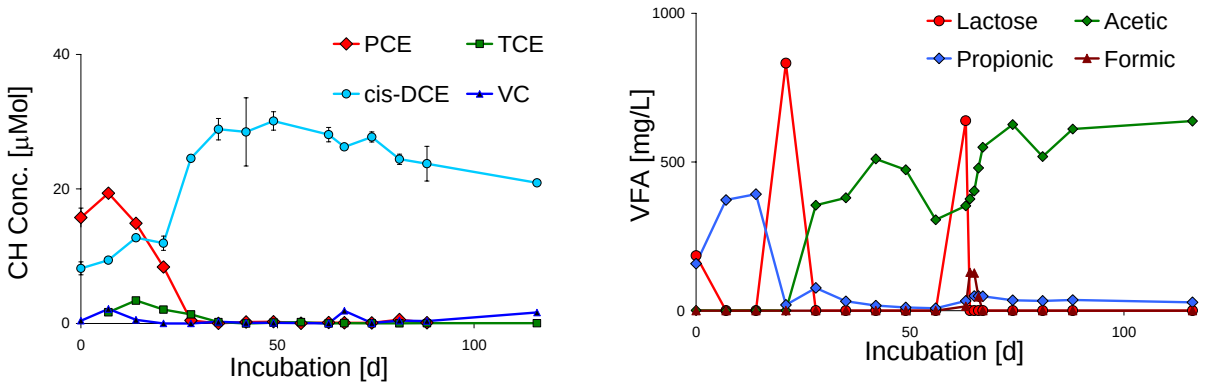

Figure 1 Degradation patterns in CAH-contaminated soil slurry under lactose-amendment: CAH degradation pattern (left) and lactose amendment (days 0, 21 and 63) and VFA production (acetic acid, propionic acid and formic acid) over time (right)

Different amendments were evaluated as to their efficiency in terms of accelerating *cis*-DCE and VC metabolization using slurry transfers from electron donor treatments (e.g. whey, glucose, lactose). Of the amendments used, the addition of different moieties of soil-borne humic compounds (HM, 2 to 100 µMol) resulted in the complete transformation of *cis*-DCE and VC on a time-scale of 15 to 30 days (Figure 2).

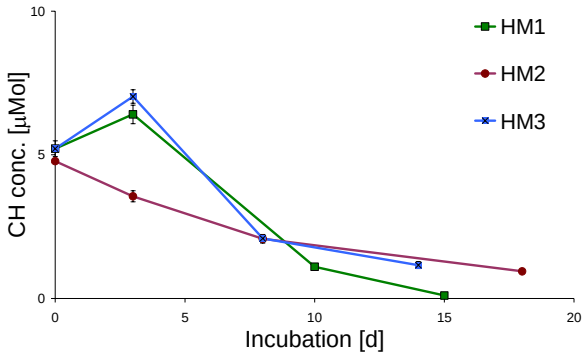

Figure 2 Transformation of cis-DCE to non-chlorinated compounds under amendment of various soil-borne humic compounds (HM1, HM2, HM3, concentrations from 2 to 100 µMol)

3.2. Field implementation – *in situ* Validation

Based on the preliminary amendment-efficiency assessment tests, the pilot-scale field validation was implemented, using lactose and a soil humic matter (HM) extract on site #1 and whey/HM on site #2. Optimizing addition timing, spatial arrangement and concentrations of the groundwater additives are the main assignments. Field scale validation and optimization of the treatments is carried out at two PCE-contaminated sites in Upper Austria.

3.2.1. Site #1: Lactose + HM

Site #1 is a former (in operation from 1968-1982) dry cleaning facility. PCE concentrations in the groundwater have been recorded in the site center (Z8, Figure 3, right) to be up to 40.000 microgram per liter, with degradation products (*cis*-DCE and VC) present. The contaminated area has been estimated to be up to 400 m^2, with a plume length of 400 m. In the year 2000, a groundwater extraction was installed. Hydraulic conductivity in the shallow aquifer is about 10^{-5} to 10^{-6} m/s with an average gradient of 2,5%.

The following validation parameters are used: lactose is infiltrated in the center well (Z8), HM in Z8 and in the additional wells downstream upon detection of *cis*-DCE resulting from upstream lactose injection. Initial lactose amendment is sub-stoichiometric, based on a 1:4 ratio of electron donors amended to required (eq_{am}:eq_{req}=1:4) and a theoretical radius of influence. Lateral dispersion and degradation efficiency monitoring recordings include CAH and

VFA-pattern, nutrient depletion and fluorescent tracer analysis, being feedback data for further amendment dimensioning. Injection concentrations for HM are achieved similarly. Based on these data, the main process parameters, the balance of total groundwater concentration versus injection rates and load, are optimized.

Figure 3 *In situ* validation site #1: aerial photo depicting the site (left), location of wells (Z8-Z11, Z17; colored circles), average contaminant concentration prior to in situ validation (numbers below, in µg CAH l^{-1}), additional injection / monitoring wells (grey circles) and groundwater flow direction (blue arrow, right)

3.2.2. Site #2: Whey + HM

Site #2, a metal working facility, is still in operation. From 1972 to 1992, PCE and 1,1,1 TCA were used as degreasing agents. Maximum CAH concentrations were determined at 10.000 $\mu g l^{-1}$. (Figure 4, right). Biodegradation products are present. The groundwater leaves the site in a radial stream (Figure 4, left) with an average flow velocity of 1 $m d^{-1}$. The site is mostly surrounded by farm land; domestic wells exist in the neighboring village to the south.

In addition to soil vapor extraction, a drainage system coupled with activated charcoal filter is operated. One hot spot has been excavated recently (Figure 4, red circle), leaving a duct (4 by 5 meters) which is presently used for infiltration of groundwater amendments and hydraulic process control. Whey is injected initially at a one-by-four sub-stoichiometric ratio (eq_{am}:eq_{req}=1:4), based on its lactate and lactose content. Further process control is based on feedback of monitoring data similar to site #1.

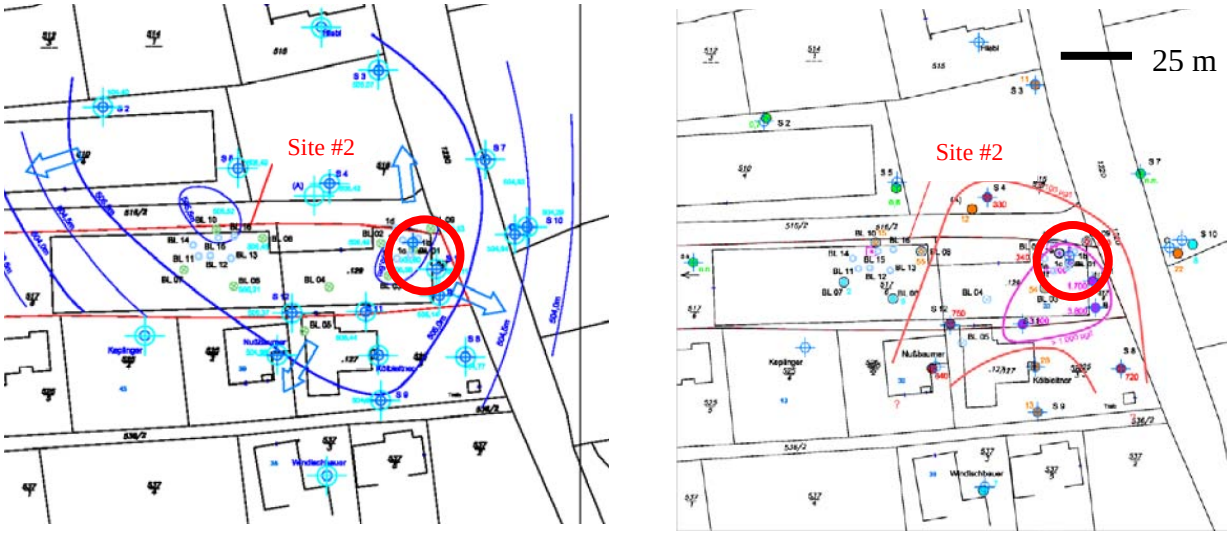

Figure 4 *In situ* validation site #2: groundwater flow regime (left) and CAH contamination isolines (right). Amendments are infiltrated via an excavation duct (red circle)

4. DISCUSSION

Results collected in the field validation of a pre-designed *in situ* treatment on two sites in Upper Austria allow for the assessment of the significance of preliminary laboratory scale tests conducted under site-specific conditions for the prediction of the treatment's success in the field.
Based on the experiences on the two sites, the crucial factors influencing the success of the implementation of a lab-designed remediation process in the field can be identified. Electron donor type ([1], [6]) and concentration are crucial factors influencing degradation performance and specifically the proliferation of the dechlorinating population ([4], [8]). Presently, a surplus of electron donors has been shown to hinder degradation rather than to support it. Spatial distribution of amendments, the kinetics of amendment addition in regard to competition for electron donors and depletion require specific attention in the field. As opposed to the current practice of few but massive donor amendment events, the current results indicate that more dense but low concentration addition could be of advantage concerning process control and efficiency.
The use of soil-borne humic substances to support degradation of low-chlorinated organic contaminants ([3], [5]) is being implemented to our knowledge for the first time in the field. This appears to be a good method to deal with highly toxic and mobile intermediate products such as VC and *cis*-DCE also in the field scale.
After completion of the in situ validation, a deeper understanding of in situ degradation processes can be gained based on the comparison of expected versus observed parameters. This procedure offers the possibility to project findings in small experimental scales into the field scale, thus increasing efficiency of further in situ remedial actions.

5. ACKNOWLEDGEMENTS

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