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PhD Dissertation 03/2008

Electro-bioremediation of Hydrophobic Organic Soil-Contaminants: Effects, Mechanisms, and Interactions

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ISSN 1860-0387

Electro-bioremediation of Hydrophobic Organic Soil-Contaminants: Effects, Mechanisms, and Interactions

Faculty of Biology, Pharmacy and Psychology

University Leipzig

accepted

DISSERTATION

To obtain academic grade

doctor rerum naturalium

Dr. rer. nat

presented

by Master of Science Lei Shi

born on July 7th, 1978 in Hebei, China

Defence: Leipzig, on Feb 29th, 2008

Electro-bioremediation of Hydrophobic Organic Soil-Contaminants: Effects, Mechanisms, and Interactions

Der Fakultät für Biowissenschaften, Pharmazie und Psychologie

der Universität Leipzig

genehmigte

D I S S E R T A T I O N

zur Erlangung des akademischen Grades

doctor rerum naturalium

Dr. rer. nat.

vorgelegt

von Master of Science Lei Shi

geboren am 07.07.1978 in Hebei, China

Verteidigung: Leipzig, den 29.02.2008

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UNIVERSITÄT LEIPZIG

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To my father, I know he would have felt proud

To my mother and sister, do not worry, I've grown up

To Xin, for being always there

To all my dear friends, you are in my heart

Abstract

During successful bioremediation microorganisms convert pollutants to less harmful compounds. The implementation of bioremediation for soil contaminated with hydrophobic organic compounds (HOC) though is restricted by their low bioavailability mainly due to their low water solubility and strong interaction with the soil matrix. This results in a limited mobility of HOC in the soil and, concomitantly, a low rate at which HOC become available for microbial transformation. To achieve substantial HOC biodegradation, microorganisms must come into contact with HOC, although they are often immobilised in soil. In recent years there has been increasing interest in employing electro-bioremediation, a hybrid technology of bioremediation and electrokinetics for the treatment of contaminated soil. Electro-bioremediation has the potential to mobilize both bacteria and their substrates by electrokinetic transport processes, such as electrophoresis and/or electroosmosis. Present electro-bioremediation approaches however mainly aim at the centimeter- to meter-range transport of hydrophilic chemicals and often overlook the role of direct current (DC)-electric fields for the bacterial dispersion and HOC-release at the micrometer-scale. This is a serious gap of our current knowledge hampering the improvement of the electro-bioremediation methodology.

The aim of this thesis hence was (i) to develop a conceptual framework in order to describe the potential effects of DC-electric fields on the microscale interactions governing HOC-bioavailability in soil (Chapter 2), (ii) to assess the effect of weak DC-electric fields typical for electro-bioremediation measures on microbial physiology (Chapter 3), (iii) to determine the mechanisms governing the electrokinetic subsurface dispersion of bacteria (Chapter 4), and (iv) to quantify the effect of electroosmosis on the release of (model) soil-bound HOC (Chapter 5). *To achieve these goals*, well-defined experimental systems were used to obtain reproducible results. In these systems polycyclic aromatic hydrocarbons (PAH)

were used as model HOC and the bacterium *Sphingomonas* sp. LB126 as representative PAH-degrading microorganism.

The main outcome of this thesis is: (i) A theoretical framework (electro-bioremediation tetrahedron) conceptualizing the DC – soil – microorganisms – compound interactions during electrokinetic treatment was developed in order to identify present knowledge and research gaps (Chapter 2). The electro-bioremediation tetrahedron represents the theoretical structure of this thesis. (ii) Weak DC-electric fields as normally used in electro-bioremediation have no negative effect on the viability, culturability and activity of pollutant-degrading bacteria as assessed by both bulk- and single cell-based approaches (Chapter 3.2). In Chapter 3.1 the limits of the cell viability indicator propidium iodide (PI) for environmental (oligotrophic) bacteria are described. This finding is critical for the correct interpretation of the PI-based assessments of the influence of DC-electric fields on the vulnerability of bacteria as described in Chapter 4. (iii) Electrokinetic phenomena lead to an increased dispersion, yet an unchanged deposition efficiency of bacteria in (model) aquifer. Bacteria are transported by both electrophoretic and electroosmotic processes depending on their physico-chemical surface properties (Chapter 4.2). Electrokinetic dispersion has no negative effect on the vulnerability of bacteria (Chapter 4.1). (iv) The electroosmotic flow (EOF), i.e. the surface-induced movement of pore fluids in an electric field, improves the release of HOC bound to model polymer release system mimicking natural organic matter and thus increases the HOC-bioavailability. The EOF-induced release flux is up to 120-fold higher than at stagnant pore water conditions (Chapter 5).

This thesis showed that electro-bioremediation is able to mobilize both soil-bound HOC and HOC-degrading microbes at the micro- to centimeter scale and hence to increase the local HOC-bioavailability in soil matrix and the HOC bioremediation efficiency. It improved our knowledge on electro-bioremediation methodology and thus contributed to the implementation of this environmentally sound biotechnology at the field scale.

Zusammenfassung

Durch mikrobiellen Schadstoffabbau können toxische und umweltgefährdende Kontaminanten in unschädliche Verbindungen umgewandelt werden. Die Implementierung der Bioremediation zur Sanierung von mit hydrophoben organischen Kohlenwasserstoffen (HOK) kontaminierten Böden ist jedoch aufgrund der sehr geringen Bioverfügbarkeit dieser Verbindungen limitiert. Die sehr geringe Wasserlöslichkeit der Schadstoffe und starke Interaktionen mit der Bodenmatrix führen zu einer begrenzten Mobilität der HOK in der ungesättigten Bodenzone, so dass die Schadstoffe nur mit einer sehr geringen Rate zur mikrobiellen Umsetzung zur Verfügung stehen. Um die Biodegradation der HOK zu gewährleisten, müssen die Schadstoffe mit den oftmals ebenfalls immobilisierten Bodenmikroorganismen in Kontakt gebracht werden. In diesem Zusammenhang hat sich seit einiger Zeit gesteigertes Interesse an der Anwendung von Verfahren der Elektro-Bioremediation entwickelt. Es handelt sich hierbei um eine Technologie, die sich die Kombination aus Elektrokinetik und Bioremediation für die Behandlung kontaminierter Böden zunutze macht. Hierbei bewirken elektrokinetische Prozesse wie Elektrophorese und/oder Elektroosmose eine Mobilisierung von Bakterien und den entsprechenden Substraten (Schadstoffen). Aktuelle Studien zu diesem Thema fokussieren hauptsächlich auf den Transport von hydrophilen Chemikalien im Bereich von Zentimetern bis Metern. Welchen Einfluss der Gleichstrom auf die Verteilung der Mikroorganismen sowie der Freisetzung der HOK auf der Mikroskala hat, ist dabei bisher wenig untersucht. Der daraus resultierende Forschungsbedarf behinderte die weitere Überführung dieser Methode in der Sanierungspraxis.

Das Ziel dieser Promotionsarbeit war (i) ein Konzept zu entwickeln, um den potentiellen Einfluss von Gleichstrom auf die Bioverfügbarkeit der HOK im Boden auf der Mikroskala zu beschreiben (Kapitel 2); (ii) die Auswirkungen von Niederspannung (schwacher Gleichstrom), typisch für Elektro-Bioremediationsverfahren, auf die Physiologie

der Mikroorganismen (Kapitel 3) zu untersuchen; (iii) die Parameter zu ermitteln, die die Verteilung der Bodenmikroorganismen im elektrischen Feld bestimmen (Kapitel 4); und (iv) den Effekt der Elektroosmose auf die Freisetzung der am (Modell-) Boden gebundenen Schadstoffe (HOK) zu quantifizieren (Kapitel 5). Um diese Ziele zu erreichen, wurden eindeutig definierte Modellsysteme in Laborexperimenten genutzt, um reproduzierbare Ergebnisse zu erhalten. Für diese Laborversuche dienten polyzyklische aromatische Kohlenwasserstoffe (PAK) als Model HOK und der Mikroorganismus *Sphingomonas* sp. LB126 als repräsentatives aerob PAK-abbauendes Bakterium

Die wichtigsten Ergebnisse dieser Arbeit sind: (i) die Entwicklung eines Konzeptes (Elektro-Bioremediations-Tetraeder), das die Wechselwirkung zwischen Boden – Mikroorganismen – Schadstoff im elektrischen Feld (Gleichstrom) beschreibt, um den aktuellen Stand des Wissens darzustellen und daraus resultierenden Forschungsbedarf abzuleiten. Der Tetraeder veranschaulicht daher in grafischer Weise die theoretische Struktur der Promotionsarbeit. (Kapitel 2). (ii) Wie in umfangreichen Laborversuchen an Einzelzellen und Populationen gezeigt werden konnte, hat schwacher Gleichstrom, wie er typischerweise im Elektro-Bioremediationsverfahren eingesetzt wird, keinen negativen Einfluss auf die Lebensfähigkeit, Kultivierbarkeit und Aktivität der schadstoffabbauenden Mikroorganismen (Kapitel 3.2). Zusätzlich beschreibt Kapitel 3.1 die Grenzen für den Einsatz von Propidiumiodid (PI) zur Unterscheidung von lebenden und toten Zellen oligotropher Umweltmikroorganismen. Diese Erkenntnis hat große Bedeutung für die korrekte Interpretation der PI-basierten Untersuchungen zur Schädigung der Mikroorganismen unter Einfluss von Gleichstrom im elektrischen Feld (Kapitel 4). (iii) Elektrokinetische Phänomene führen zu einer erhöhten Dispersion der Bakterien in Model-Aquiferen. Ein Einfluss auf die „Depositionseffizienz“ wurde jedoch nicht gefunden. Bakterien werden in Abhängigkeit von ihren physikochemischen Eigenschaften (Oberflächenbeschaffenheit) durch elektrophoretische und elektroosmotische Prozesse transportiert (Kapitel 4.2). Die

elektrokinetische Verteilung wirkt sich hierbei nicht negativ auf die Bakterien aus (Kapitel 4.1). (iv) Der elektroosmotische Fluss (EOF), die oberflächeninduzierte Bewegung von Porenwasser in einem elektrischen Feld, verbessert die Freisetzung der an die organische Substanz (Modellpolymere) gebundenen HOK, wodurch die Bioverfügbarkeit der HOK erhöht wird. Verglichen mit stagnanten Porenwasserbedingungen ist die EOF-induzierte Freisetzung bis um das 120fache erhöht (Kapitel 5).

Die vorliegende Arbeit zeigt, dass Verfahren der Elektro-Bioremediation in der Lage sind, matrixgebundene HOK und HOK-abbauende Bakterien auf der Mikrometer- bis Zentimeter Skala zu mobilisieren. Das führt zu einer erhöhten HOK-Bioverfügbarkeit in der Bodenmatrix und damit zu einer verbesserten Sanierungseffizienz HOK kontaminierter Böden. Mit den Ergebnissen dieser Arbeit wurden neue Erkenntnisse über das Verfahren der Elektro-Bioremediation gewonnen. Damit konnte ein Beitrag für die Implementierung dieses biotechnologischen Verfahrens in der Sanierungspraxis geleistet werden.

Table of Contents

1 General Introduction

1.1 Hydrophobic organic compounds (HOC) in soil

1.2 In-situ bioremediation of soil contaminated by HOCs

1.3 HOC-bioavailability in soil

1.4 Electrokinetics

1.5 Electro-bioremediation

1.6 Outline of the thesis

General Introduction

1.1 Hydrophobic organic compounds (HOC) in soil

During the last few decades there has been an exponential growth in the production of synthetic organic chemicals and the use of fossil fuels, with the result that numerous organic compounds have been introduced into soil in vast quantities (1). One large group of these contaminants is referred to as hydrophobic organic compounds (HOC). According to the frequency, toxicity and potential for human exposure, HOCs are found throughout various lists for priority hazardous substances such as those compiled by the U.S. EPA and the Agency for Toxic Substances and Disease Registry (2). In addition, many of them are considered to be mutagenic and/or are known carcinogens (3,4). Among the large number of HOCs present in the environment, polycyclic aromatic hydrocarbons (PAH) are of particular interest to many environmental chemists and engineers and are often considered as representative HOCs (5,6) due to their hazardous properties, abundance and ubiquitous distribution in the environment. Human activities such as the combustion of fossil fuels, the gasification and liquefaction of coal, the incineration of wastes, wood treatment processes, and the accidental spilling of oils are the major entry paths of PAHs into the environment (7-10). Soils can be contaminated with between 1 $\mu g\ kg^{-1}$ and 300 $g\ kg^{-1}$ PAHs (11), depending on the source of contamination (e.g. old coal gasification sites have the highest levels reported). The knotty situations to remediate such highly contaminated soils result from both the hydrophobic characteristics of the PAHs and the heterogeneous structure of the soil matrix.

PAHs are neutral, non-polar molecules comprised of two or more benzene rings, which are fused together through two or more carbon atoms (1). Although each PAH-compound has specific characteristics, general trends can be perceived. As the number of fused benzene rings and thus the molecular weight of the molecules increases, the PAHs become less water soluble and more lipophilic, recalcitrant and genotoxic. The main properties of PAHs, such as

the low aqueous solubility, high affinity to organic and solid phase (e.g. octanol-water partition constant), and high melting point, lead to the particular partition patterns and high sequestration of PAHs observed in soil systems (Figure 1). This consequently makes soil-PAHs especially persistent to remediation treatment. The above mentioned properties of several representative PAHs with particular environmental concerns are listed in Table 1.

Soils are both structurally and compositionally extremely variable and complex environmental media (12). They consist of mainly three constituents: (i) soil pore water, (ii) soil mineral particles of different sizes and shapes and (iii) soil organic matter (SOM) (12) (Figure 1). Soil heterogeneity has been observed from the micro- to the macro-scale, and the differences in the structural and chemical properties of the soil constituents generally lead to different interactions with HOCs. These chemicals often enter the subsurface either as non-aqueous phase liquids (NAPL) or as combustion residue particulates such as soot (13). After years or decades of ageing (or weathering) process, HOCs become increasingly sequestered and less available for treatment. This is due to adsorption to the surface of particles such as clay/silt-sized particles (clay < 2 μm, 2 μm < fine silt < 20 μm) (14-17), absorption into the matrix such as SOM (18) and diffusion into micro- /nano-pores (19) (Figure 1).

1.2 In-situ bioremediation of soil contaminated by HOCs

The persistence of HOCs has made them notoriously challenging to remediate, and as a result, they tend to remain concentrated in soil and have become extremely widespread environmental contaminants. Although HOCs are relatively immobile compared to other more soluble contaminants, they still pose a major threat to the environment due to the long-term contamination problems with slow leakage into groundwater or surface water and the potential of acute release as a result of disasters such as flooding. Excavation of tons of soil materials highly contaminated by HOCs, followed by ex-situ treatment such as landfills or bioreactors, is often economically unfeasible for contaminated sites covering large areas and

is compromised by the possible release or spreading of contaminants during the excavation and transport process (20). Therefore, intensive research and investigations into soil remediation are currently focusing on in-situ technologies as more energy-efficient alternatives. Conventional soil washing/flushing with water is facilitated by additives to enhance the solubility and mobility of HOCs. These additives include surfactant/solvent system (21,22) or polymeric nano-particles (23). Soil in-situ ozonation uses ozone to chemically degrade PAHs and has been found to be effective for the degradation of PAHs in unsaturated soils (24). Phytoremediation for the removal of PAHs from contaminated soils in conjunction with physical or photochemical pre-treatments has also been found to be a feasible option (25). Above all, in-situ bioremediation, which can be described as 'the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state' (26), is believed to be the most cost effective and sustainable approach.

Several important groups of HOCs have been reported (27-30) as being biodegradable with the possibility of mineralization occurring under aerobic condition. Although PAH degradation in absence of oxygen has also been reported (31), most attention has been given to aerobic biodegradation. There are three fundamentally different mechanisms in the aerobic metabolism of PAHs by microorganisms (11). The basis of all these mechanisms is the oxidation of the aromatic ring followed by the systematic breakdown of the compound to PAH metabolites and/or carbon dioxide. PAH-degrading microorganisms are ubiquitously distributed in the natural environment, including soils (bacteria and non-ligninolytic fungi) and woody materials (ligninolytic fungi) (11). However, the bioremediation of soil-PAHs always requires long treatment time and ends in high residual concentrations (32). Huesemann (33) pointed out that the extent of hydrocarbon biodegradation in contaminated soils is critically dependent on four factors: "(i) the presence of hydrocarbon degrading bacteria, (ii) the creation of optimal environmental conditions to stimulate biodegradative

activity, (iii) the predominant petroleum hydrocarbon types in the contaminated matrix, and, finally, (iv) the bioavailability of the contaminants to degrading bacteria."

1.3 HOC-bioavailability in soil

A universally accepted working definition of the bioavailability of soil contaminants has been a hot scientific debate for decades. Semple et al. attempted to classify contaminants from soil and sediments into three fractions, a bioavailable, a bioaccessible and a non-bioaccessible fraction (34). Bioavailable compound is defined as that freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time. Bioaccessible compound is however only available to cross an organism's cellular membrane from the environment over acceptable timescales and the non-bioaccessible fraction is the highly occluded compounds (34). As to the distribution of HOCs in soil illustrated in Figure 1, only aqueous phase dissolved HOCs, which in direct contact with microbes are considered as bioavailable. Based on the level of HOC-sequestration and their consequent release rates, sorption-retarded HOCs (e.g. from NAPL, particle surface, SOM or micropores) can be taken as either bioaccessible or non-bioaccessible for a defined time scale. Besides the sorption-retarded HOC-release, the patchy distribution (Figure 1) of soil bacteria relative to their substrate (HOCs) and their restricted mobility (35) are the other main causes of limited HOC-bioavailability. Only part of soil microbes stay suspended (Figure 1) in soil pore water and are transported freely. Most of them either accumulate on solid surfaces and form biofilms or hide in micro-pores to avoid predation (12) (Figure 1). In order to achieve substantial biodegradation, microorganisms must further come into contact with the frequently soil-bound contaminants.

The concept of bioavailability has been differently applied in different research fields. Toxicologists are more interested in the size of the bioavailable fraction at a given site, which poses distinct threat to human and/or ecological receptors (36). In contrast, remediation

scientists are more concerned about what is bioaccessible over time (37). There are treatment remedies that rely heavily on either decreasing or increasing bioavailability. Sediment capping, for example, is one typical approach for reducing bioavailability to protect bottom-dwelling organisms from a contaminant pool (38). Engineered bioremediation, in contrast, attempts to increase the biodegradability of pollutants, such as HOCs, by enhancing bioavailability via physical or chemical means. Examples of the physical means include grinding or mixing to decrease diffusional path lengths and increasing the temperature of the solid phase to increase mass transfer rates. From an efficiency perspective, the excavation of large quantities of soil materials and their movement for the purpose of homogenizing small quantities of contaminants, microbes and nutrients is far from optimal (20). Chemical means include using surfactants, cosolvents, or chelating agents to increase mass transfer by enhancing the solubility of HOCs (23,39,40). These have the drawback of introducing the potential toxicity of these additives into "cleaned" soils. Gentler and more energy-efficient in-situ technologies to enhance HOC-bioavailability to stimulate bioremediation within economically acceptable time frames have recently attracted more attention (20). The principle of electro-bioremediation, as a hybrid technology of bioremediation and electrokinetics, is to make bioaccessible HOC-fractions bioavailable by mobilizing both soil-bound HOCs and microorganisms.

1.4 Electrokinetics

Electrokinetic phenomena encompass both the motion of suspended species and fluid that result from or produce an electric potential difference. It can be classified as shown in Table 2. The main focus of this thesis is DC-electrokinetics, which includes three main mechanisms (Figure 2), electromigration, electrophoresis and electroosmosis. Suspended species possessing a charge undergo electromigration or electrophoresis in the presence of DC-

electric fields, however their final migration velocity should always be considered together with the electroosmosis flow (EOF) of liquid in the background.

Electromigration and electrophoresis. Electromigration is the transport of a charged ion in solution towards an electrode with opposite charge. On an analogous manner, electrophoresis is the migration of electrically charged particles such as colloids or microbes (41). Electrophoresis is used in capillary gel electrophoresis for the fractionation of DNA (42), in microfluidic chips for the hybridization of DNA (43), in capillary zone electrophoresis for the separation of chemical species (44), and in Doppler electrophoretic light scattering analyzers for the surface charge characterization of colloids or microbes (40). In the pore fluid of a soil matrix, indigenous soil bacteria or augmented HOC-degrading bacteria are normally negatively charged (45), and consequently tend to move towards the anode in presence of a DC-electric field due to electrophoresis (Figure 2).

Electroosmosis. Electroosmosis was recognized as a distinct physical phenomenon by Wiedemann in 1852 (46) and it refers to the movement of liquid relative to a stationary charged surface in presence of an electric potential difference. When an electrolyte and a charged solid surface are brought into contact, oppositely charged ionic species from the liquid are attracted by the charged surface forming an electric double layer (EDL) (Figure 3a). The cations are arranged in two distinct layers (Figure 3a), a fixed layer (the stern layer) and a diffuse layer, which together are termed as the EDL (47). At a location just outside of the stern-diffuse layer interface a plane of shear is established and the potential at this boundary is known as the zeta potential (47) (Figure 3b). The electrical potential within the diffuse layer falls exponentially to zero, and the distance from the soil particle surface over which the potential drops by e^{-1} is referred to as the thickness of EDL (48,49) (Figure 3b). Soil mineral particles are normally negatively charged (50) and attract cations available in the soil pore water (Figure 2). When an electric potential is applied across the soil capillary pores, the cations in the diffuse layer migrate toward the cathode, dragging with them their solvation

shells. Due to frictional forces among the water molecules, the bulk liquid also moves. Since the flow essentially originates from the solid surface, it is assumed to have a near-plug-like flow profile (Figure 2 and Figure 3c) (51). EOF plays a central role in many analytical, mechanical, and environmental processes. Capillary electro-chromatography uses EOF as a means of injection and transport instead of pressure-driven flow for the separation of non-charged species (52,53). There are two main advantages of EOF over pressure-driven flow: (1) less band broadening due to the more homogeneous distribution of the flow velocity across the capillary in the presence of the near-plug-like flow profile compared to more parabolic ones (54); and (2) the possibility of using smaller packing particles with large porous networks to enhance the separation efficiency due to the considerable higher permeability in presence of EOF than pressure-driven flow (54-58).

Electrokinetic soil remediation. The application of electrokinetics in soil treatment almost certainly finds its origin in the work of Casagrande et al. (58) for dewatering and stabilizing soils. Intensive research on electrokinetic soil remediation since the 70s have mainly dealt with the long distance extraction of heavy metals (59-62) and organic contaminants with relatively high solubility, such as phenol and TCE (63-66). In contrast, due to their limited solubility much smaller number of successful studies can be found in literature about the electrokinetic extraction of PAHs (67). Alternative methods combining electrokinetics with additives to enhance PAHs solubility (e.g. surfactants) have been discussed (68).

1.5 Electro-bioremediation

Electrokinetic remediation has a great potential for use in the decontamination of fine-grained and heterogeneous soils (69), where the low hydraulic conductivity makes hydraulic flow difficult to establish. Furthermore, it is relatively more cost-effective when no additional use of conducting pore fluid or surfactant is needed. The requirement of the contaminants to be

solubilised to ensure long distance transport (70), however, strongly limits its application, especially with respect to HOC decontamination. The main thrust of future research appears to lie in the development of hybrid technologies which combine electrokinetics with processes such as bioremediation. Electro-bioremediation is a generic name for a large group of cleanup methods that use microbiological phenomena for the degradation of various contaminants in the soil and electrokinetic phenomena for the acceleration and orientation of transport of the contaminants (or their derivatives) and contaminant-degrading microbes at a site (71). For *in-situ* electro-bioremediation small scale dispersion of HOC is intended rather than 'macroscopic' HOC-transport (HOC-extraction), as bacteria are ubiquitous in soil. Assuming average separation distances of $\leq$ 100 microns (72) between individual bacterial micro-colonies, short distance transport may drastically improve the contact between pollutant molecules and bacteria and, concomitantly, biodegradation (73,74). However, no data are available on local bioavailability changes acting via DC-driven effects on organism–compound and organism–soil interactions. This is a serious gap of our current knowledge hampering the improvement of the electro-bioremediation methodology. The successful implementation and commercialization of the technology requires an understanding of the controlling mechanisms, the development of process optimization schemes, pertinent design and analysis of construction guidelines through the critical assessment of carefully conducted pilot-scale studies, and complementary analysis of the results of theoretical models.

1.6 Outline of the thesis

The aim of this thesis hence was (i) to develop a conceptual framework in order to describe the potential effects of DC-electric fields on the microscale interactions governing HOC-bioavailability in soil (Chapter 2), (ii) to assess the effect of weak DC-electric fields typical for electro-bioremediation measures on microbial physiology (Chapter 3), (iii) to determine the mechanisms governing the electrokinetic subsurface dispersion of bacteria (Chapter 4),

and (iv) to quantify the effect of electroosmosis on the release of (model) soil-bound HOC (Chapter 5). *To achieve these goals*, well-defined experimental systems were used to obtain reproducible results. In these systems polycyclic aromatic hydrocarbons (PAH) were used as model HOC and the bacterium *Sphingomonas* sp. LB126 as representative PAH-degrading microorganism. The knowledge obtained is expected to improve the mechanistic understanding and the implementation of electro-bioremediation technology as a sound basis for the treatment of HOCs in soil.

In addition to the general introduction (Chapter 1) and the summary and concluding remarks (Chapter 6), this thesis concerning electro-bioremediation is divided into four chapters. A theoretical framework (electro-bioremediation tetrahedron (Figure 4)) conceptualizing the DC – soil – microorganisms – compound interactions during electrokinetic treatment was developed in order to identify present knowledge and research gaps (Chapter 2). The tetrahedron's base triangle (Figure 4A) represents the interactions among soil, microorganisms, and a hypothetical (hydrophobic organic) compound that govern its bioavailability or bioaccessibility. Side triangles B and C (Figure 4) illustrate the impact of DC on the physiology and physico-chemistry of organism-compound and organism-matrix interactions that may influence microbial HOC uptake and community dispersion, respectively. Side triangle D (Figure 4) relates DC to abiotic compound-matrix interactions potentially leading to electrokinetic mobilization of HOC and concomitant improvement of local HOC-bioavailability. Chapter 3-5 approach the primary open questions regarding the influence of DC-electric fields on HOC-bioavailability from the aspect of the three side triangles (B-D) displayed in the electro-bioremediation tetrahedron. To support the discussion of bacterial viability or vulnerability in presence of DC-electric fields, in Chapter 3.1 the feasibility of propidium iodide (PI) staining analyzed by flow cytometry was first verified as viability indictor for environmental (oligotrophic) bacteria. Chapter 3.2 discusses the influence of DC-electric field on microbial physiology (viability, culturability and activity).

The factors influencing the electrokinetic dispersion of microbes and their fitness during this dispersion were investigated in a model aquifer and presented in Chapter 4. Finally, Chapter 5 discusses the influence of DC-electric field on the release kinetics of HOCs from model soil matrix.

Figure Legend

Figure 1. Illustration of the patchy distribution of HOCs and HOC-degrading microbes in soil. In soil there are normally: (i) mineral particles with pores of different sizes; (ii) soil organic matter (SOM); (iii) soil pore water; and (iv) non-aqueous phase liquid (NAPL). Only a very small portion of HOCs in soil (i) dissolve in pore water but mostly associated with: (ii) the solid surface as adsorbed-HOCs; (iii) NAPL as organic-dissolved-HOCs; (iv) SOM as absorbed-HOCs; and (v) micro-pores (< 1 μm) without accessibility to any microbes. In contrast, microbes in soil are either (i) suspended in pore water, (ii) accumulates at the solid surface to form biolfilm, or (iii) hide in micro-pores (< 10 μm, >1 μm) from predators. Only aqueous phase dissolved HOCs, which are in direct contact with microbes, are considered as bioavailable. The double arrow bars represent the sorption-release processes of HOCs into or out of solid surface, SOM and NAPL. Dashed lines indicate the possible diffusion path of HOCs to their degraders.

Figure 2. Illustration of DC-electrokinetics in the conducting pore fluid of a soil particle, including: (1) electromigration of ions to electrodes with opposite charge; (2) electrophoresis of normally negatively charged microbes or colloids to the anode; and (3) electroosmosis of water fluid to the cathode with its origin from the electrical double layer (EDL) and with its near-plug-like flow profile.

Figure 3. Illustration of electric double layer (EDL), zeta potential and electroosmotic flow (EOF) profile. a) Distribution of ions around a charged solid surface in the background electrolyte theoretically separated into: (1) stern layer; (2) diffuse layer; (3) bulk solution; (4) EDL; and (5) surface of shear. b) Electric potential distribution over the distance from the solid surface. The electric potential at the interface between the solid and liquid (φ_0) decreases

linearly in the stern layer to (φ_d), and further decreases exponentially in the diffuse layer. The distance from the soil particle surface over which the potential drops by e^{-1} is referred to as the thickness of EDL (ξ). The electric potential at the surface of shear is the zeta potential (ζ) of the solid surface in specific electrolyte. c) Flow profile of EOF (near-plug-like) over the distance from solid surface.

Figure 4. Electro-bioremediation tetrahedron, discussing the interactions among soil, microorganisms, and a (hydrophobic organic) compound in presence of DC-electric fields. The tetrahedron is comprised of four triangles: (A) bioavailability vs. bioaccessibility; (B) influence of DC-electric fields on microorganism-compound interactions; (C) influence of DC-electric fields on soil-microorganism interactions; and (D) influence of DC-electric field on soil-compound interactions. Solid lines with double arrows represent the interactions. Solid lines with one arrow represent the influence of DC-electric fields on each component. Dashed lines with one arrow represent the influence of DC-electric fields directly on interactions between either two components. A more detailed discussion on electro-bioremediation tetrahedron is given in Chapter 2.

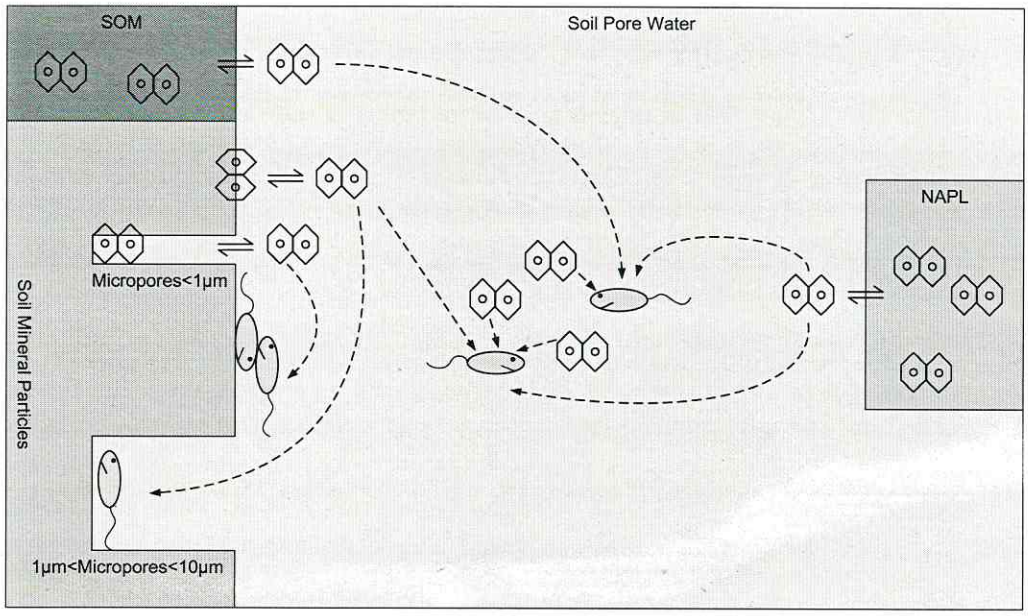
SOM
Soil Pore Water
NAPL
Micropores<1μm
Soil Mineral Particles
1μm<Micropores<10μm

Figure 1.

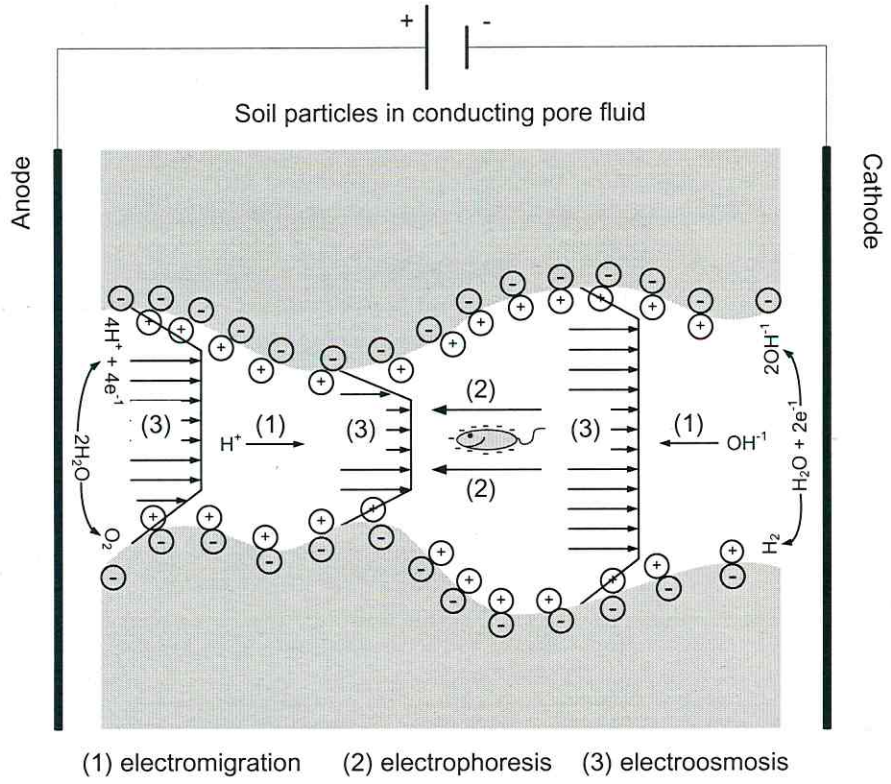
+
-
Soil particles in conducting pore fluid
Anode
Cathode
4H+ + 4e-1
2H2O
O2
(3)
H+
(1)
(3)
(2)
(2)
(3)
(1)
OH-1
2OH-1
H2O + 2e-1
H2
(1) electromigration
(2) electrophoresis
(3) electroosmosis

Figure 2.

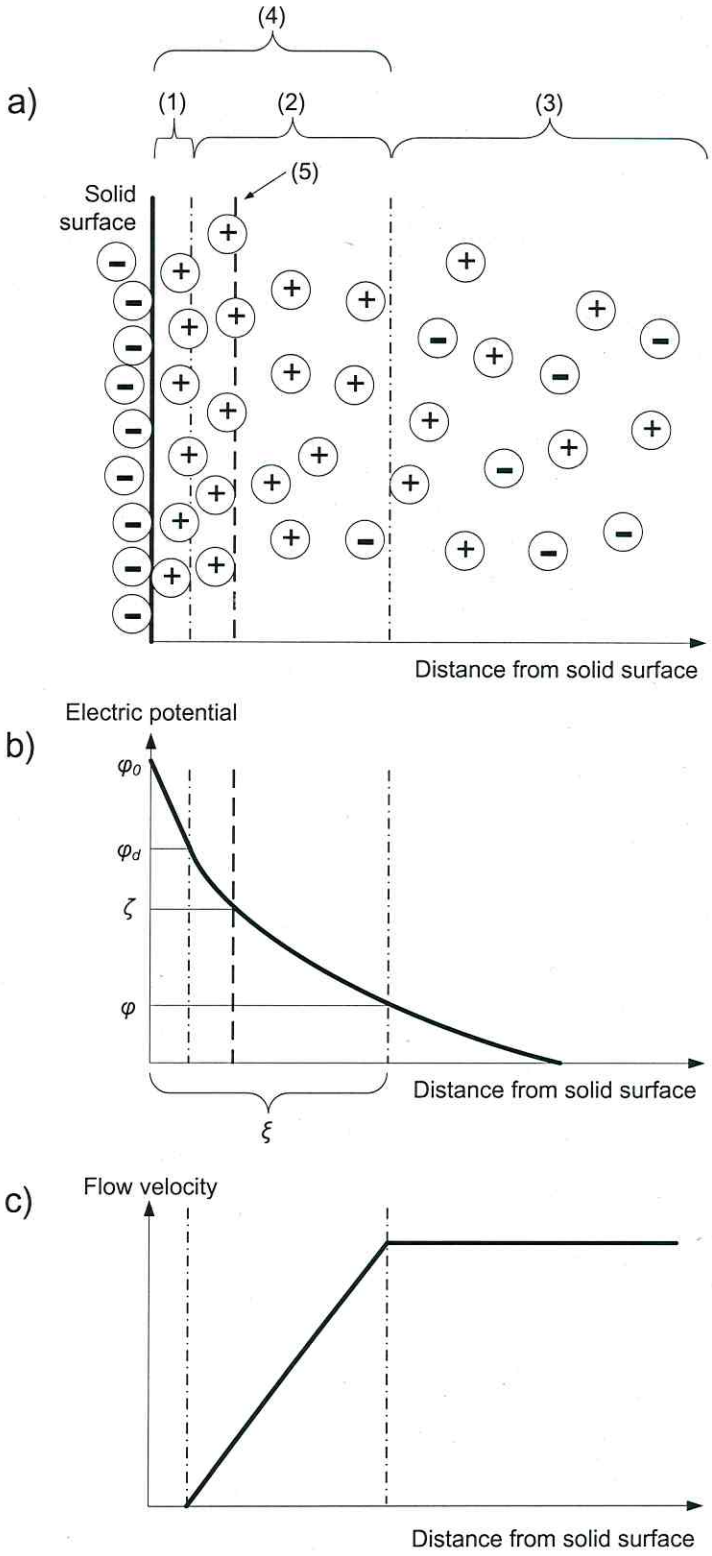
a)
(4)
(1)
(2)
(3)
(5)
Solid surface
Distance from solid surface
b)
Electric potential
φ_0
φ_d
ζ
φ
Distance from solid surface
ξ
c)
Flow velocity
Distance from solid surface

Figure 3.

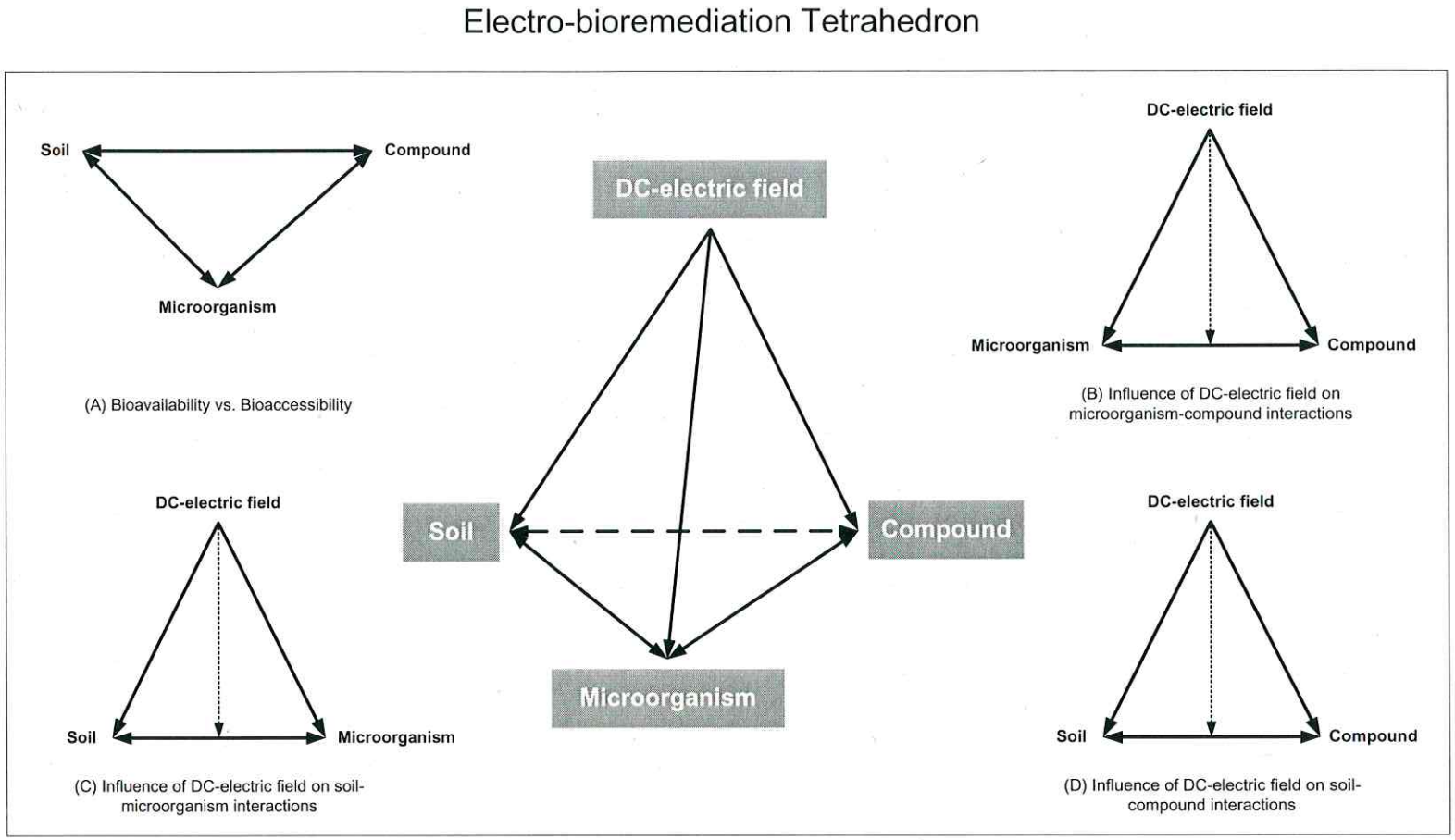
Electro-bioremediation Tetrahedron
Soil
Compound
Microorganism
(A) Bioavailability vs. Bioaccessibility
DC-electric field
Soil
Compound
Microorganism
DC-electric field
Microorganism
Compound
(B) Influence of DC-electric field on microorganism-compound interactions
DC-electric field
Soil
Microorganism
(C) Influence of DC-electric field on soil-microorganism interactions
DC-electric field
Soil
Compound
(D) Influence of DC-electric field on soil-compound interactions

Figure 4.

Table 1. Selected properties of several representative PAHs (1)

Representative PAH	**Molecular weight** ($g\ mol^{-1}$)	**Aqueous solubility** ($mg\ L^{-1}$, at 25°C)	**Octanol-water partition constant** ($-lgK_{iow}$, at 25°C)	**Melting point** (°C)
Naphthalene	128.2	32.2	3.33	80.2
Fluorene	166.2	1.9	4.32	116.0
Phenanthrene	178.2	1.1	4.57	101.0
Pyrene	202.3	0.1	5.13	156.0

Table 2. Classification of electrokinetic phenomena

Type of Force	**In presence of electric field**		**Generating electric field**
	DC-electrokinetics	AC-electrokinetics	
Surface force on fluid	Electroosmosis	AC electroosmosis	Streaming potential
Force on suspended particles	Electrophoresis Electromigration	Dielectrophoresis	Sedimentation potential

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Electro-bioremediation Tetrahedron

2

Electro-bioremediation of hydrophobic organic soil contaminants:
A review of fundamental interactions

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Electro-bioremediation of hydrophobic organic soil-contaminants: A review of fundamental interactions

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Received 5 October 2005; received in revised form 21 December 2005; accepted 8 March 2006
Available online 24 October 2006

Abstract

In recent years, there has been increasing interest in employing electro-bioremediation, a hybrid technology of bioremediation and electrokinetics for the treatment of soil contaminated with hydrophobic organic compounds (HOC). Present electro-bioremediation approaches however mainly aim at pollutant extraction through transport over large distances and tend to neglect both the impact of direct current (DC) on organism–soil interactions and microscale HOC release rates. Accordingly, no data are available on bioavailability changes acting via DC-driven effects on organism–compound and organism–soil interactions. This is a serious gap of our current knowledge hampering the improvement of the electro-bioremediation methodology. This review establishes a conceptual framework of the various influences of DC on processes governing HOC-bioavailability in soil, in particular the DC influence on microbial physiology and the physico-chemistry of organism–soil and organism–compound interactions.

Keywords: Adhesion; Bioremediation; Direct current; Electrokinetics; Electroosmosis; Hydrophobic organic contaminant; Mass transfer; Microorganism; Physiology; Soil

1. Introduction

One of the major challenges in environmental biotechnology is the improvement of in situ soil decontamination-efficiency. Present bioremediation of contaminated soil and groundwater is an empirical business often lacking an environmentally sound methodology that is based on mechanistic knowledge of the rate-limiting microscale processes. In recent years, there has been increasing interest in employing electro-bioremediation, a hybrid technology of bioremediation and electrokinetics for the treatment of soil contaminated with hydrophobic organic compounds (HOC). Several studies have indeed demonstrated improved removal of organic pollutants such as gasoline hydrocarbons, aromatic compounds, herbicides or trichloroethylene in electric fields [1–6] applied to soil. In electro-bioremediation, electrokinetics is the use of weak electric fields (ca. 0.2–2 V cm^{-1} [7]) to soil. It can be used in situ and is particularly effective in (saturated and unsaturated [8]) fine-grained soils of low hydraulic conductivity, which are normally difficult to treat by other in situ methods [9]. Several comprehensive articles have summarized the state of the knowledge with foci on physical [10], physico-chemical [11], engineering [12] and remediation aspects [3,8,13] of electrokinetics in soil clean-up. However, sound data on the effect of weak electric fields on HOC-degrading soil microorganisms is missing [14]. This is surprising since (engineered) bioremediation of polluted soil significantly depends on microbial activity and aims at the stimulation of indigenous microorganisms capable of degrading the pollutants [15]. The 'electro-bioremediation-tetrahedron' depicted in Fig. 1a and b epitomize (· · ·) the influences of DC on the relevant bioremediation processes of soil-bound HOC, i.e. its impact on the soil matrix, HOC, microorganisms and their mutual interactions: the tetrahedron's base triangle A represents the interactions of soil, microorganisms and a (hydrophobic organic) compound that govern its bioavailability; triangle B relates direct current (DC) to abiotic compound–matrix interactions potentially leading to electrokinetic movement of HOC and concomitant improvement of HOC-bioavailability; whereas triangles C and D illustrate the impact of DC on the physiology, chemistry and physico-chemistry of organism–compound, and

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doi:10.1016/j.electacta.2006.03.117

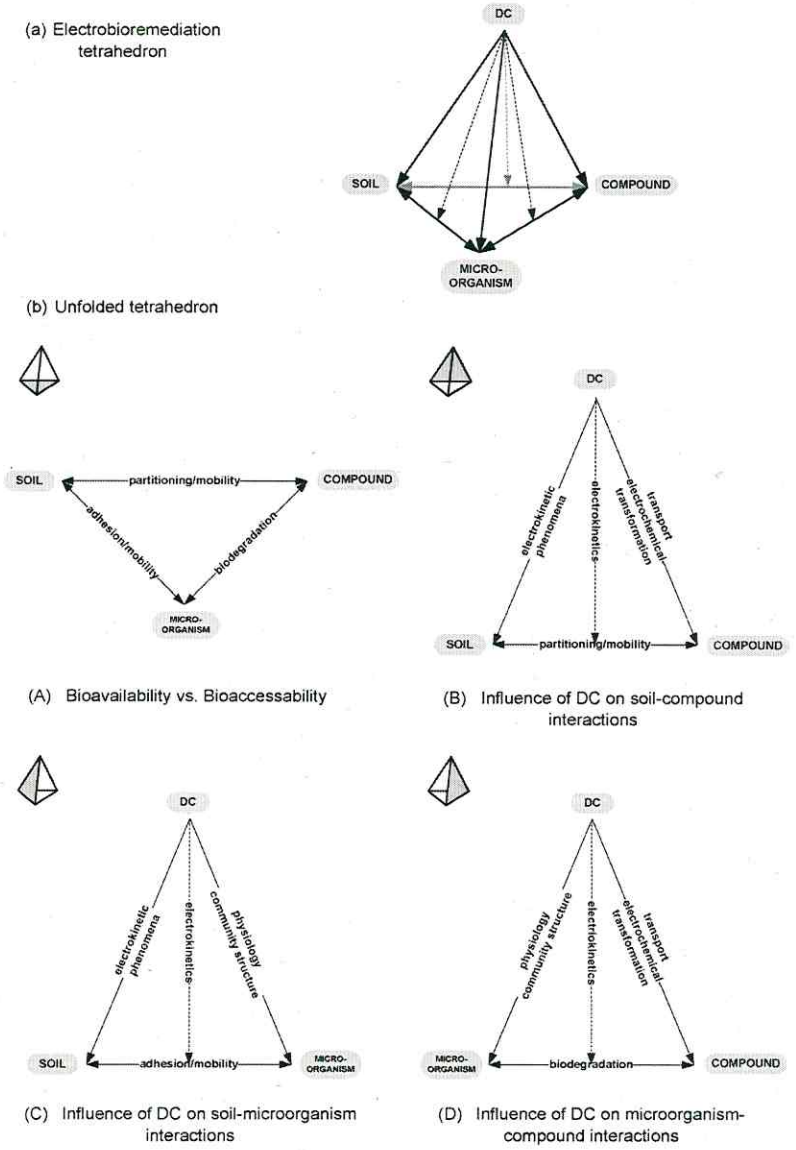

Fig. 1. Visualization of the critical factors influencing the bioavailability and biodegradation of (hydrophobic) organic compounds during electrokinetic treatment of soil ('electro-bioremediation-tetrahedron'). The figure depicts the approach of this paper using different interactions and processes of bioavailability potentially influenced by DC. Present electro-bioremediation approaches include the processes of association and dissociation (triangle B). They solely relate HOC concentrations and HOC-transport over large distances and neglect the impact of DC on microorganisms–matrix interactions (triangle C) as well as physiological and biochemical HOC-transformation processes (triangle D).

organism–matrix interactions that may influence microbial HOC uptake and community dispersion, respectively.

The idea of electrokinetics is to stimulate mobilization of soil components, contaminants and/or microorganisms in order to either physically remove or to disperse them. For in situ electro-bioremediation small scale dispersion of HOC is intended rather than 'macroscopic' HOC-transport (HOC-extraction), as bacteria are ubiquitous in soil [16]. Assuming average separation distances of $\leq$100 μm [17] between individual bacterial micro-colonies, short-distance transport may drastically improve the contact between pollutant molecules and bacteria and, concomitantly, biodegradation [18,19]. However, present electro-

bioremediation approaches mainly aim at pollutant transport over large distances and tend to neglect both the impact of DC on organism–soil interactions and microscale HOC release rates. Accordingly, no data are available on bioavailability changes acting via DC-driven effects on organism–compound and organism–soil interactions. This is a serious gap of our current knowledge hampering the improvement of the electro-bioremediation methodology. This review will therefore try to establish a conceptual framework of the various influences of DC on processes influencing HOC-bioavailability in soil, in particular the DC influence on microbial physiology and the physico-chemistry of organism–matrix and organism–compound interactions.

1.1. Pollutant bioavailability as a limiting factor of soil bioremediation

Various studies have shown that the sequestration of organic contaminants in the solid soil phase by sorption and entrapment reduces their bioavailability depending on the contaminant's physico-chemical characteristics [19–21]. Due to their high solid-water distribution ratios (K_d) HOC typically are associated with carbon phases of particles and are very slowly released from soil, sediments and aquifer solids by diffusive transport processes [19,22]. A sequence of three potential bottlenecks of diffusive mass-transfer controlling HOC release have been described: (i) diffusion of the sorbate within the molecular nanoporous network of natural organic matter (OM) [20], (ii) pore or surface diffusion in aggregated minerals constituting natural particles [21], and (iii) diffusion of the sorbate across an aqueous boundary layer at the exterior of soil particles [23]. As a consequence of progressive binding with soils, residual HOCs may become less leachable and thus unavailable to microbes as bacteria appear to degrade chemicals only in proportion to their dissolved phase chemical activity [24–28]. Two factors have been considered necessary to determine a chemical's bioavailability: (i) the rate of mass transfer of the compound from the soil to the catabolically active cells and (ii) the rate of the compound's uptake and metabolism by the cells, which in turn acts as driving force for the mass transfer to the cell. The bioavailability of a chemical has been defined as the rate of mass transfer relative to the intrinsic activity of the soil biota [17,18] and thus may vary between different species [29–31]. Semple et al. define the term 'bioavailability' as the fraction of a chemical in a soil that can be taken up or transformed immediately by living organisms, whereas the term 'bioaccessibility' refers to the fraction of a substance that could become available with time [32]. Experimental evidence and theoretical considerations [28] show that better substrate bioavailability can be achieved by enabling faster contaminant transport to the bacteria or by mobilizing the bacteria. Theoretically, only short-distance movement of either the pollutant or the bacteria within the soil would be needed to overcome mass-transfer limitations. The principle of electro-bioremediation is thus to make bioaccessible HOC-fractions bioavailable by dispersing both soil-bound microorganisms and HOC.

1.2. Influence of DC on physico-chemical soil–compound interactions

1.2.1. Physico-chemical phenomena

When a DC field is applied to a wet soil matrix, it invokes electroosmosis, electromigration and electrophoresis (for a review cf. [4,33]). Electroosmotic flow emerges because excess ions migrate in a plane parallel to particle surfaces toward the oppositely charged electrode. As they migrate, they transfer momentum to the surrounding fluid molecules via viscous forces. Consequently, electroosmosis is the mobilization of surface-near pore fluids in an electric field, usually from the anode toward the cathode (because cations accumulate near particle surfaces of negative charge). Electroosmotic flow is able to take free-phase dissolved matter toward the cathode [11]. For non-ionic substances, electroosmosis is the predominant transport mechanism. Electroosmotic flow rates vary from 10^{-4} to $10^{-6}\,cm^2\,V^{-1}\,s^{-1}$, depending on the morphological properties of the porous medium, such as the porosity, flow path radius, pore width, as well as the physico-chemical properties of the solid and the liquid phases. In general electroosmosis is more efficient in fine-grained soils (with μm or smaller pores) and at higher water contents [34]. Electromigration is the active movement of ionic species in a DC field to the electrode of opposite charge [35]. Since the electromigration rate is at least one order of magnitude higher than the electroosmotic flow [8], electromigration generally dominates mass transport of ions and charged molecules. Electrophoresis is defined as the transport of charged particles such as clay platelets or bacterial cells toward the electrode opposite in polarity in a DC field. Furthermore, a competition between buoyancy, hydrodynamics, and electroosmosis/electromigration may influence the microfluidics and lead to distinct flow regimes and concomitant solute transport whithin pores [36]. Electrokinetically induced flow processes may further result in steepened chemical concentration gradients and concomitantly enhanced transfer of surface-associated contaminants. The effective chemical diffusion in general, depends on the molecular diffusion coefficient, the concentration gradient, and the porous medium micromorphology (porosity and tortuosity).

Besides these transport processes many other reactions may occur in a DC field such as desiccation due to heat generation, gas generation due to electrolysis of water, decomposition or precipitation of salts and minerals, ion exchange, development of pH gradients, sorption processes and electrochemical transformations. Electrokinetically induced soil–contaminant interactions are particularly important in fine-grained soils because of the large surface to volume ratio and the large specific surface area. Such interactions include: (1) change of Zeta potential (ζ) at the soil particle/pore fluid interface; (2) the capacity to buffer pH changes; and (3) sorption/desorption of reactive contaminants onto or from the soil particle surface and precipitation/dissolution of metallic contaminants in the pore fluid. Electrolysis of pore fluids at both electrodes in wet soils according to the reactions: $2H_2O \rightarrow 4H^+ + O_2(g) + 4e^-$ (at the anode) and $2H_2O + 2e^- \rightarrow 2OH^- + H_2(g)$ (at the cathode) has important secondary effects [11]. The anode reaction will generate an acid front, while the reduction at the cathode produces alkalinity.

These acids and bases will advance through the soil matrix by diffusion, electromigration and/or electroosmosis, and thus may locally change soil pH [8] potentially influencing biodegradation of organics in soils. Concomitant electrochemical redox-reactions may lead to a series of electrochemical reactions, such as the production of reactive oxygen species and other energy-rich intermediates. Previous studies have further indicated the significance of the Zeta potential of soil on electrokinetic remediation efficiency [37–39]. Zeta potentials of kaolinite, montmorillonite and quartz powder with Li^+, Ca^{2+}, Cu^{2+}, Pb^{2+} and Al^{3+} in the presence of anionic surfactants were found to be negative, whereas non-ionic and cationic surfactants produced both positive and negative ζ potentials depending on soil type and ions present in the system [39]: ζ potentials of kaolinite and quartz powder in presence of surfactants showed similar trends, with the absolute magnitude of ζ of quartz powder being higher than that of kaolinite. The ζ potential of montmorillonite commonly shows a different trend from those of kaolinite and quartz powder. Based on the test results, these authors recommended that ζ potential of soils should be determined before the electrokinetic decontamination in order to maximize the efficiency of the technique.

1.2.2. Effect of DC on compound mass transfer processes

It has been demonstrated manifold that electroosmosis efficiently removes water-dissolved phenol, *o*-nitrophenol [33], hexachlorobenzene [4], benzene, toluene, ethylene and xylene (BTEX) [40,41], hexane, isooctane and trichloroethylene (TCE) from clay [2]. It appears, however, that electroosmotic long-distance transport of HOC is significantly enhanced when they are present as droplets, or colloidal particles [7,42,43] or when solubilization is assisted by surfactants, cyclodextrins or chelating agents [7,44–49] (for a comprehensive review on electrokinetic removal of soil-bound HOC cf. [11]). Estimations by Bruell et al. [40] showed that electroosmotic removal efficiencies of organic chemicals from a given kaolin clay depend on the compound's Freundlich constant (K_d (mL g^{-1})) and, to a lesser extent, to its aqueous solubility (c_w^{sat} (mol L^{-1})) leading to the following electrokinetic mobilization efficiency: toluene ($-\log c_w^{sat} = 2.25$; $K_d = 0.6$) > TCE ($-\log c_w^{sat} = 2.04$; $K_d = 0.84$) > benzene ($-\log c_w^{sat} = 1.64$; $K_d = 0.89$) > *m*-xylene ($-\log c_w^{sat} = 2.77$; $K_d = 1.6$) > hexane ($-\log c_w^{sat} = 3.15$; $K_d = 2.5$) > ($-\log c_w^{sat} = 4.52$; $K_d = 24$). It should be noted however, that equilibrium isotherm models, such as the Freundlich isotherm, are based on the assumption that the contaminant is sorbed to the soil particle surface and that the dynamic chemical equilibrium in the dissolved phase is reached instantaneously at all times. This may be adequate when the flow velocity of the groundwater is low. However, as the electrokinetically driven migration velocity of the contaminant is relatively high, the validity of using equilibrium isotherms to describe sorption/desorption processes may not be adequate. Other studies have described the electrokinetic transport of PAHs in coal tar contaminated soil covering a wide range of aqueous solubility ($-\log c_w^{sat} = 3.6$–8.22) [50,51] with PAH removal efficiencies of 44–70% [50] within 21 days and ≤90% [51] within 23 days, respectively. Some selectivity was observed in the transport of PAH compounds in clayey specimens, probably owing to the preferential retention of some compounds by clay [50]. Interestingly, there was little or no selectivity in the transport of PAHs in granular specimens. Naphthalene was the most consistently transported compound while the lowest removal rate was observed with chrysene [50]. To our knowledge however, no mechanistic studies on the impact of DC on the microscale flow and desorption processes of HOC exist for soil environments. Electrokinetically enhanced mass transfer in situ may be relevant at the micro and the macroscale. On the macroscale, electrokinetically enhanced introduction of nutrients, co-substrates, water, electron acceptors or bacteria into soil [2,52–55], pollutant-degrading bacteria into polluted areas [56], and inversely, mobilization of contaminants into biologically active treatment zones [1,2,57] have been described. On the microscale, intra-particle diffusional mass-transfer resistances often impose serious limitations on the rate biotransformation in soil. Electrically driven mass-transfer processes such as electromigration of charged species and electroosmotic solute transport may be convenient and efficient to stimulate chemical fluxes in soil matrices. Particularly, electroosmosis is likely to overcome mass-transfer bottlenecks in low permeable soil matrices by (i) increasing the release of sorbates by inducing liquid flow at the immediate exterior of soil particles, (ii) creating flow in nanopores in the organic sorbent phase, which are inaccessible by hydraulic flow, and (iii) influencing the pore or surface diffusion among aggregated minerals.

1.3. Influence of DC on microorganism–soil interactions

Capillary electrophoresis nowadays is an established tool for the separation and analysis of bacteria in the laboratory (for a review, see ref. [58]). Over the last few years several studies have demonstrated that microorganisms can be moved electrokinetically through soil [56,59–61], but little is known on the mechanisms by which microorganisms are electrokinetically dispersed in soil matrices. For bioremediation this is of great importance as most soil bacteria are normally immobilized in situ, i.e. they are attached to soil particles [62] and form microcolonies rather than releasing single cells into the soil water. This leads to the largely heterogeneous micro-scale-distribution of soil bacteria known to limit pollutant bioavailability [17]. Even macroscale (up to 0.4 m) electrophoretic transport of bacteria and NAPL-degrading yeast cells through sand, soil and aquifer sediments to the anode has been described. Depending on the microorganism and the subsurface matrix, electrophoretic transport rates (v) ranged from 0.019 to 0.023 cm^2 h^{-1} V^{-1} for yeast cells [61] and 0.14 to 4 cm^2 h^{-1} V^{-1} for bacteria [56,59]. In other studies however, electroosmotic transport of pollutant-degrading bacteria was the predominant mobilization mechanism with electrophoretic transport accounting for less than 20% of the observed bacterial dispersion in a soil matrix [59,60]. This is in good agreement with the observation that *Escherichia coli* moved in capillaries exclusively by electroosmosis at electrical field strengths of >0.3 V cm^{-1} over a wide range of pH values and ionic strengths tested [63]. The same investigators also found that bacterial motility is the predominant

mode of motion at voltages below 0.2 V cm^{-1}, with bacteria moving at different mean speeds toward anode and cathode due to superimposed effects of electroosmotic flow, electrophoresis, galvanotaxis [64], and random motility. In model aquifer systems up to 90% of weakly negatively charged ($\zeta = -7$ mV) and moderately adhesive *Sphingomonas* sp. L138 was transported by electroosmosis, whereas 0–20% were transported by electrophoresis. Electroosmotically transported bacteria ($v = 0.1 - 0.4\,\mathrm{cm^2\,h^{-1}\,V^{-1}}$) were 50% slower than injected conservative tracers [59]. Poor electrokinetic transport of strongly charged ($\zeta < -32$ mV) and highly adhesive *Mycobacterium frederiksbergense* LB501T [65,66] however occurred in the different model aquifers consisting of alluvial sand, clayey soil or glass beads pointing at a dominant influence of bacterial retention by the solid phase [59]. The strong affinity of the bacteria for these solid matrices could be partially overcome by treating the bacteria with the non-ionic surfactant Brij35 leading to up to 80% enhanced electrokinetic dispersion of both strains [59]. It thus can be concluded that the extent of the retardation in the subsurface depends on both the physics and the chemistry of the subsurface (e.g., the pore size distribution and the ionic strength of the pore water) as well as on biophysical factors (e.g., cell size, cell shape, cell surface charge and/or hydrophobicity).

No data however, are available on the influence of DC on the adhesion and detachment of bacteria in soil matrices. Some knowledge exists on bacterial attachment to medical devices under influence of DC. Several authors have described the electrokinetic inhibition of bacterial colonization on surfaces at low electric currents (17.2 μA cm^{-2}) [67]. A series of fundamental studies have been performed in parallel plate flow chambers [68], studying the influence of electric potential, electric current, and electric charge transfer on both bacterial deposition to and detachment from electrode surfaces. An external electric potential can be applied to a cell suspension in absence of electric current, when the surface in contact with bacterial suspension of at least one electrode consists of non-conductive material such as glass. No effect of the surface potential of glass surfaces on bacterial adhesion was found [69]. In other experiments both positive and negative electric currents were used to detach bacteria from salivary conditioning film on indium tin oxide (ITO) electrodes in order to compare the desorption forces acting parallel (electroosmosis) to forces acting perpendicularly, such as electrophoresis, electrostatics to the electrode surfaces [70]. From their experiments the authors concluded that parallel forces desorb bacteria more effectively than perpendicular forces. It was also reported that current induced the detachment of 10 different bacterial strains. Although initial detachment rates increased with increasing electric current, the final extents of detachment were similar [71]. Bacterial adhesion to a semi-conducting ITO electrode changed the electric potential of the electrode without changing its capacitance, which was explained by charge transfer during bacterial deposition [72]. An average charge of about 10^{-14} C per bacterium was found to be exchanged during initial adhesion corresponding to only a few percent of the total surface charge of a bacterium. Bacterial adhesion to the substrata with different specific resistivity where bacteria either donated or accepted electrons, led to conclude that bacteria adhered more strongly when they donated electrons to the substratum rather than inversely [73]. In conclusion, reported effects of electric fields on bacterial adhesion, detachment and biofilm formation to electrodes are thus likely to be attributed to the electric current rather than to the electric potential applied. As a consequence of bacterial adhesion, charge transfers were also observed between bacteria and conductive or semi-conductive surfaces, and meanwhile the direction of electron transfer was also demonstrated to affect the stability of bacterial adhesion.

1.4. Influence of DC on microorganism–compound interactions

A series of studies has reported stimulation of bioremediation by electrokinetic processes [1,2,5,6,52–55,57,60]. Optimal biodegradation activity requires that DC has no negative effect on the physiology of the biocatalysts, i.e. the indigenous HOC-degrading bacterial communities in soil. To date only one work on the effect of DC on the soil microbes (i.e. the composition and structure of DC-exposed soil microbial communities) has been published [74]. In this study no direct effect of the applied current (0.314 mA cm^{-2}) on soil bacteria could be ascertained [74]. The application of DC to soil however, altered both the physico-chemical characteristics of the soil and changed the microbial community close to the anode due to anodic soil acidification. The authors concluded that, provided that factors such as soil pH and temperature were controlled, electrokinetics had no negative effect on 'soil health' [74]. Earlier studies in subsurface systems have shown that the application of 20 mA cm^{-2} to soils stimulated the activity of sulphur-oxidising bacteria [75] or the biological denitrification of nitrate-contaminated groundwater [55] due to the production of H_2 and pH-changes, respectively.

When electric current is applied to living microbial cells, different responses, such as toxic electrode-effects, metabolic stimulation due to enhanced substrate mass-transfer processes to the cell and electrokinetic removal of inhibitory products [76], sublethal injuries [77] or changes in the physico-chemical surface properties [57,78] can be observed depending on the amperage, treatment time, cell type and medium characteristics [14]. Influences on cell-surface characteristics are of special interest in soil bioremediation as they influence bacterial adhesion to surfaces and concomitantly their mobility in the subsurface [79]. Over the last years several studies have investigated the effects of high- and low-amperage electric current on the inactivation of microorganisms for food processing or for wastewater hygenization. There are a few reports on antimicrobial activity of low amperage per se. It is postulated that the mechanism of action may be the disruption of bacterial membrane integrity, mechanical stress due to hydrodynamic drag [80] or the electrolysis of molecules on the cell surface. At properly selected conditions [81], the application of short electric pulses (electroporation [82]) causes transient or reversible membrane permeability changes that allow the electrorelease (electrolesion) of cell ingredients, such as intracellular proteins [83] or the uptake of foreign molecules, such as DNA, proteins or substances to which the cells is poorly or non-permeable. Matsunaga et al. [84,85] established that the electrochemical destruction of

microbial cells was based on the direct electron transfer between cells and an electrode, and demonstrated that coenzyme A was oxidized to dimers. The same authors also showed that the resulting inhibition of respiratory activity caused microbial cell death. The cytoplasm of cells is electrically conducting whereas the lipid bilayer of the cell membrane can be considered as dielectric. The application of electric fields to cells thus causes the buildup of electrical charge at the cell membrane, and consequently a change in voltage across the membrane. For externally applied electric fields leading to a transmembrane potential difference, $\Delta\Phi_m > 70$–100 mV, a value observed in eukaryotic cells in the absence of DC, the membrane permeability is expected to increase leading to a reversible or irreversible 'dielectric' breakdown [86] of $\Delta\Phi_m$. When the transmembrane potential differences exceed a critical value of usually 1 V, electromechanical cell membrane break down and concomitant cell death occurs [80].

Furthermore, it has been found that exponentially growing cells are usually more sensitive to electric field treatment than cells in the stationary phase. Inactivation rates also depended on the medium's conductivity and osmotic properties. However, it is believed that adverse electrochemical reactions are the main reasons for antimicrobial activity of low DC fields, especially in the vicinity of the electrodes [87]. For instance, no inhibition zone around the cathode was observed under anaerobic conditions as no H_2O_2 could be produced in the absence of oxygen and electrochemical reactions of chloride appeared to play a minor role [87]. Other studies have linked the antimicrobial activity of DC to electrochemically formed toxic substances, such as free chlorine [88], H_2O_2 [87,89] and metallic ions [90]. Some investigators [91] have attributed the lethal effect of electricity to a simple thermal effect called ohmic heating, whereas others excluded ohmic heating and production of H_2O_2 as causes for the lethal effect of electric treatment of *Saccharomyces cerevisiae* [92]. Low-field DC (1.5–30 V cm^{-1}) with current densities of 15 μA cm^{-2} to 2.1 mA cm^{-2} have been further shown to lead to a greatly increased antimicrobial activity of antibiotics against bacterial biofilms [93]. Relevant for bioremediation however may be electrokinetically enhanced mass-transfer processes due to the combined effect of electrophoresis and electroosmosis: in laboratory tests a growth stimulation of immobilized cell by 140% [94] due to the electrokinetic removal of inhibitory products and electroosmotically enhanced glucose-supply has been reported. It thus appears that a number of useful preparative electrochemical transformations can be accomplished by suitable selection of microorganisms and reaction conditions; Enhanced biotransformation of benzonitrile to benzoic acid and ammonia [95], electrically enhanced ethanol fermentation [96], reductive carboxylations [97], reduction of chloropyruvate to chloroacetate catalyzed by a mediator-dependent D-lactate dehydrogenase or the regeneration of pyridine nucleotides [98] have been reported. A recent study has further presented experimental observations that the natural electromagnetic field at the earth's surface may be used by *E. coli* as supplemental energy source to sustain growth [99]. The authors argue that protons in liquid water can be excited at their natural resonance frequencies through Langmuir oscillations. By using electromagnetic energy the microorganisms thus may obtain enough kinetic energy to charge the transmembrane potential and, concomitantly may be capable of converting electromagnetic waves into chemical energy.

2. Outlook

The basic principles of electrokinetic extraction and the electrokinetically enhanced bioremediation of contaminants from fine-grained soil have been experimentally proven to be feasible. Although empirical electro-bioremediation appears to be safe, effective and economically interesting compared to other remediation techniques there is still a need for mechanistic understanding of the molecular processes affecting the release and transport of HOC in soil as well as the dispersion and physiology of HOC-degrading microorganisms before the technology can be fully exploited: Fig. 2 (adapted from [32]) summarizes the molecular processes and interactions of bioavailability potentially influenced by DC: **I** represents the release of a chemical from an organic or inorganic matrix to a more accessible form and, inversely the adsorption of HOC to soil matrices; Processes **II–IV** describe the HOC's transport to and uptake through the membrane of an organism and the processes of microbial

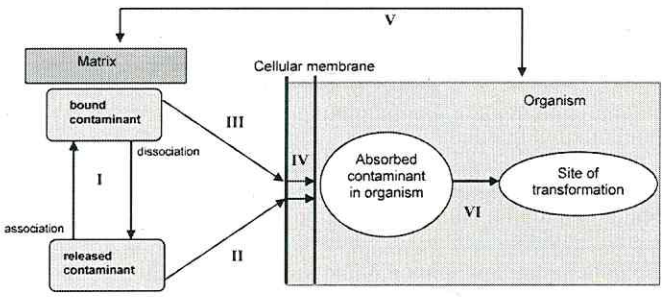

Fig. 2. The influence of direct current (DC) on bioavailability of HOC as determined by various interactions of a compound or a mixture with the solid phase matrix and the receptor organism (adapted from [32]): **I** represents the release of a chemical from an organic or inorganic matrix to a more accessible form and, inversely the formation of non-extractable so-called bound residues; **II–IV** describe the organism–compound and organism–matrix interactions in terms of transport to and uptake by the membrane of an organism and the processes of microbial adaptation to hydrophobic compounds; **III** and **V** describe interactions of the microbes with the matrix; whereas processes **IV** and **VI** include DC-related physiological and biochemical HOC-uptake and transformation processes.

adaptation to hydrophobic compounds; **III** and **V** describe interactions of the microbes with the soil matrix, such as microbial translocation processes or the active improvement of bioavailability by altering bacterial cell surface properties. Up to date no data are available on the mechanistic understanding of the present electro-bioremediation approaches including the processes of association and dissociation of HOC from soil matrix (**I**), or to relate the HOC concentrations to the HOC release and transport over large distances. These studies furthermore neglect processes **IV** and **VI**, i.e. the influence of DC on bacterial cell surfaces, microscopic mass transfer at the cell surfaces and/or the bacterial physiology including the metabolism and degradation at the site of response within the cells. The main thrust of future research consequently lies in the establishment of a profound, conceptual and theoretical fundament for the processes taking place at a molecular and microscale level in soil environments.

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3 Influence of DC-electric field on microorganism-compound interaction

3.1 Limits of propidium iodide (PI) as a cell viability indicator for environmental bacteria

3.2 Activity and viability of PAH-degrading *Sphingomonas* sp. LB126 in a DC-electrical field typical for electro-bioremediation measures

ORIGINAL ARTICLE

Cytometry
PART A

Limits of Propidium Iodide as a Cell Viability Indicator for Environmental Bacteria

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Received 25 July 2006; Revision Received 12 November 2006; Accepted 15 November 2006

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Published online 9 April 2007 in Wiley InterScience (www.interscience.wiley.com)

Part of this manuscript was presented at the 11th Leipziger Workshop, April 27–29, 2006, BioCity Leipzig, Germany. Available at www.leipziger-workshop.de.

DOI: 10.1002/cyto.a.20402

Abstract

Viability measurements of individual bacteria are applied in various scopes of research and industry using approaches where propidium iodide (PI) serves as dead cell indicator. The reliability of PI uptake as a cell viability indicator for dead (PI permeable) and viable (PI impermeable) bacteria was tested using two soil bacteria, the gram$^-$ *Sphingomonas* sp. LB126 and the gram$^+$ *Mycobacterium frederiksbergense* LB501T. Bacterial proliferation activities observed via DAPI and Hoechst 33342 staining were linked to the energy charge and the proportion of dead cells as obtained by $diOC_6$ (3)-staining and PI-uptake, respectively. Calibration and verification experiments were performed using batch cultures grown on different substrates. PI uptake depended on the physiological state of the bacterial cells. Unexpectedly, up to 40% of both strains were stained by PI during early exponential growth on glucose when compared to 2–5% of cells in the early stationary phase of growth. The results question the utility of PI as a universal indicator for the viability of (environmental) bacteria. It rather appears that in addition to nonviable cells, PI also stains growing cells of *Sphingomonas* sp. and *M. frederiksbergense* during a short period of their life cycle. © 2007 International Society for Analytical Cytology

Key terms

multiparametric flow cytometry; bacterial viability; population dynamics; live/dead staining; propidium iodide

STUDIES on the activity of environmental bacterial species often lack detailed information on the physiological states and viability of individual bacteria. By applying cell fluorescence-based methods (for overview see Ref. 1), flow cytometry allows to gain better information on the activity of single cells. Viability tests have been found to be relevant in medicine, biotechnology, and food industry, because of their power to assess the susceptibility of bacteria against antibiotics (2–4), their analysis of the physiological and metabolic states during product formation processes (5,6), and their ability to recognize the cell death of production strains or infection by living pathogens (7–9). Although vast literature is available about organisms of biotechnological and medical relevance, only little is known about the single cell viability of environmental communities with most of the existing literature focusing on waste water treatment plants (10). This is a serious gap of knowledge as (oligotrophic) environmental bacteria are supposed to be key players in biogeochemical and pollutant transformation processes. The viability of environmental bacteria is often assessed with commercially available kits that rely on the propidium iodide (PI)-based assessment of membrane integrity. However, many users are unaware of the facts that such tests have been validated for a very limited number of bacterial species, only (11). Nevertheless, the use of PI-based kits for determining the viability of unknown environmental species is seductive because of their easy and quick application. Here, the reliability of PI as an indicator for the viability of two polycyclic aromatic hydrocarbon (PAH)-degrading soil bacteria, the gram$^-$ *Sphingomonas* sp. LB126 and gram$^+$ *Mycobacterium frederiksbergense* LB501T (12) was assessed in

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detail. The two strains were grown either in the presence of PAH (fluorene for strain LB126 and anthracene for strain LB501T) or glucose as sole carbon and energy sources. The numbers of PI stained cells from different growth phases were determined by flow cytometry. Additional physiological information was obtained by applying DAPI, Hoechst 33342, and $diOC_6$ (3) fluorescent dyes and flow sorting to link the ability of individuals to form colonies on agar plates to the results obtained from the staining procedures.

Materials and Methods

Cell Cultivation

The gram$^-$ *Sphingomonas* sp. LB126 and the gram$^+$ *Mycobacterium frederiksbergense* LB501T (12) were grown aerobically at 20°C and pH 7.5 in 500 ml shake flasks with 300 ml minimal medium at 150 rpm for batch experiments. The carbon and energy sources were either glucose (1 g l^{-1}) or PAHs, such as crystalline anthracene (2 g l^{-1}; ≥96.0% (GC), Fluka Chemie GmbH) for *M. frederiksbergense* and crystalline fluorene (2.7 g l^{-1}; ≥99% (HPLC), Fluka Chemie GmbH) for *Sphingomonas* sp. Glucose was filtered through sterilized 0.2-μm filters (VWR International) before use and the carbon and energy sources were added separately at the beginning of the cultivation. The composition of the mineral medium and growth on crystalline PAH are described elsewhere (13). The strains were regularly cultivated on LB-agar (Lennox, Carl Roth GmbH; Agar from Difco) or on minimal-medium-agar. *E. coli* was grown aerobically at 30°C and pH 7.5 in 500 ml shake flasks at 150 rpm for batch experiments on 300 ml peptone media (l^{-1}): 5 g peptone from meat (pancreatic), 3 g NaCl, 2 g K_2HPO_4, 10 g meat extract, 10 g yeast extract, 5 g glucose. Growth was measured spectrophotometrically by monitoring absorption at 578 nm ($OD_{578\ nm,\ 10\ mm}$).

Cell Preparation and Staining Procedures

Cell preparation. The harvested cells were centrifuged at 3,200*g* for 5 min, if necessary fixed with 10% NaN_3, and stored at 4°C. This procedure was found to preserve the cells for at least 3 months. For flow cytometric measurements, either living or preserved cells were centrifuged again, washed in NaCl-phosphate buffer (0.4 M Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.2), and resuspended at a concentration of 3×10^8 cells ml^{-1}.

Staining. DNA patterns of preserved cells were obtained in the following way: 2 ml of diluted cell suspension were treated with 1 ml solution A (2.1 g citric acid/0.5 g Tween 20 in 100 ml bidistilled water) for 10 min, washed, and resuspended in 2 ml solution B (0.24 μM 4′,6-diamidino-2′-phenylindole [DAPI, SIGMA], 400 mM Na_2HPO_4, pH 7.0) for at least 20 min in the dark at room temperature using a modification of a standard procedure (14). DNA contents of living cells were analyzed by using Hoechst 33342 (2,5′-Bi-1H-benzimidazole; Molecular Probes, OR) as described earlier, yet without the Tween 20 treatment (15).

To assess the membrane potential-related fluorescence intensity (MPRFI), living cells were resuspended in 20 mM imidazole buffer (pH 7.0), and immediately adjusted to 3×10^8 cells ml^{-1}. The composition of the staining solution was taken from Shapiro (16). For optimal alignment, the MPRFI was defined by testing different dye concentrations, staining times, and the action of antibiotics (gramicidin and valinomycin) on exponentially growing cells. Accordingly, 15 μl (0.39 μM) and 5 μl (0.13 μM) of the dye stock solution were added to 2 ml of 3×10^8 cells ml^{-1} for 3 and 4 min to either *Sphingomonas* sp. or *M. frederiksbergense*. All measurements were carried out at 20°C. In addition, the measurements were daily equalized by using YG beads (0.5 μm, FluoSpheres 505/515, F-8813, Molecular Probes/Invitrogen, USA).

To determine membrane integrity, the living cells were washed and resuspended in 2 ml PBS (cell concentration of 3×10^8 cells ml^{-1}) and stained by 20 μl PI (Sigma–Aldrich, Steinheim, Germany; final concentration: 1 μM, stock solution: 0.07 mg ml^{-1}, in PBS, pH 7.2) for 10 min.

Combined double staining of living and dead cells was done by applying Hoechst 33342 and PI in the following way: 2 ml of a harvested and PBS washed cell suspension (3×10^8 cells ml^{-1}) were incubated with 4 μl Hoechst 33342 for 35 min (final concentration: 3.24 μM). Then, 20 μl PI were added and the sample analyzed after additional 10 min.

Flow Cytometry and Cell Sorting

Multiparametric flow cytometry. Flow cytometric measurements were carried out using a MoFlo cell sorter (DakoCytomation, Fort Collins, CO) equipped with two water-cooled argon-ion lasers (Innova 90C and Innova 70C from Coherent, Santa Clara, CA). Excitation of 580 mW at 488 nm was used to analyze the forward scatter and side scatter as trigger signal at the first observation point. DAPI was excited by 180 mW of ML-UV (333–365 nm) at the second observation point. The orthogonal signal was first reflected by a beam-splitter and then recorded after reflection by a 555 nm long-pass dichroic mirror, passage by a 505 nm short-pass dichroic mirror and a BP 488/10. DAPI fluorescence was passed through a 450/65 band pass filter, green fluorescence through BP 520/15 and, red fluorescence through BP 620/45. Photomultiplier tubes were obtained from Hamamatsu Photonics (models R 928 and R 3896; Hamamatsu City). Amplification was carried out at linear or logarithmic scales, depending on the application. Data were acquired and analyzed using Summit software (DakoCytomation, Fort Collins, CO). Fluorescent beads (Polybead Microspheres: diameter, 0.483 μm; flow check BB/Green compensation Kit, Polyscience, USA) were used to align the MoFlo. Also, an internal DAPI-stained bacterial cell standard was introduced for tuning the device up to a CV value not higher than 6%.

Cells from three independent cultures of *Sphingomonas* sp., *M. frederiksbergense*, and *E. coli* were treated with PI and sorted using the most accurate sort mode (single and one drop mode: highest purity 99%) channeling PI fluorescent or PI nonfluorescent cells onto LB agar plates in triplicate. Finally, each agar plate contained a grid of 96 (12 × 8) spots harboring 1 and 2 cells, respectively. After incubation at room temperature (*Sphingomonas* sp., *M. frederiksbergense*) or 37°C

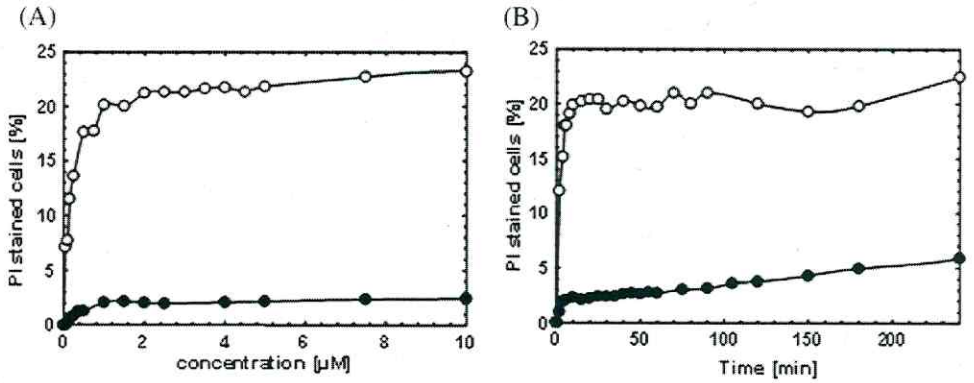

Figure 1. Time and concentration dependencies for *Sphingomonas* sp. (filled circle) and *M. frederiksbergense* (open circle). Stable staining equilibrium reached at (**A**) PI concentration 1 µM for both strains and (**B**) staining time 10 min.

(*E. coli*), the numbers of colonies were counted. The culturability of PI fluorescent and PI nonfluorescent cells was calculated as the ratio of the numbers of colony forming units (CFUs) to the numbers of cells spotted. Bacterial survival of the sort procedure was tested as described elsewhere (15).

Microscopy. To verify reliable staining, the cells were subjected to image analysis (camera: DXC-9100P; software: Openlab 3.1.4., Improvision, Lexington, MA) using ultraviolet light from a 100 W mercury arc lamp. Fluorescence filters used were as follows: Zeiss filter set 02 for blue fluorescence (excitation G 365, BS 395, emission LP 420), Zeiss filter set 09 for green fluorescence (excitation BP 450-490, BS 510, emission LP 515), and Zeiss filter set 15 for red fluorescence (excitation 546/12, BS 580, emission LP 590).

Results

Calibration of the Staining Procedures

All calibrations of the PI staining procedure were done with cells grown on glucose and harvested at the early stationary phase of growth (150 h of cultivation for *M. frederiksbergense* and 24 h for *Sphingomonas* sp.). Time and concentration dependencies were tested for both strains (Fig. 1). A stable staining equilibrium was obtained under conditions described in Materials and Methods, labeling up to 2.8% *Sphingomonas* sp. and 20.5% *M. frederiksbergense* cells.

The same conditions were used for the double staining of Hoechst 33342/PI. Calibration was necessary to prevent toxic effects of the dye combinations on cell viability. The commonly applied surfactant Tween 20 (81.4 nM) affected the membranes of *Sphingomonas* sp. and led to an increase of PI uptake to nearly 100% for high-exponential and to 38% for cells in the mid-exponential and early stationary growth phase, an effect that has been described already for other cells (17). Therefore, Tween 20 was omitted during further labeling procedures of both species.

MPRFI was calibrated by using $diOC_6$ (3) for viable cells harvested at the early exponential growth phase. Stable staining conditions were found after application of 15 µl (0.39 µM) per 2 ml diluted *Sphingomonas* sp. cells within a time range of 3 min, as after longer exposure two apparent subpopulations of clearly differing fluorescence intensities were observed. For *M. frederiksbergense* 0.13 µM $diOC_6$ (3) were necessary for reliable and constant staining within 4 min. The response of the fluorescence intensity of the cell-bound dye to changes in the membrane potential was verified by analyzing the effects of the ionophores valinomycin and gramicidin. Cells were treated with different concentrations of the two ionophores immediately after the staining procedure. Low concentrations of gramicidin (2–3 µM) decreased the MPRFI of both strains and led to an increase of cellular debris as analyzed by flow cytometry, whereas higher concentrations of valinomycin (20–30 µM) were necessary to depolarize the cells.

Course of Cell Growth Followed by DAPI, Hoechst 33342, $diOC_6$ (3), and PI

To determine the most active growth states, cells were analyzed with regard to their DNA contents (by DAPI) while cultivated on the following carbon and energy sources: glucose (both strains, up to 120 h), anthracene (*M. frederiksbergense*, up to 900 h), and fluorene (*Sphingomonas* sp., up to 300 h). The *Sphingomonas* sp. presented up to three subpopulations with different chromosome contents referred to as C_{1n}, C_{2n}, and C_{4n}, all standing for multiples of chromosomes. The chromosome nomenclature was already explained by Müller and Babel (18). On glucose *Sphingomonas* sp. showed no C_{1n} cells yet an up to 50% increase of C_{4n} cells during the exponential growth phase though up to 90% C_{2n} cells dominated the stationary phase of growth. On fluorene, the proliferation behavior was very similar with, however, only 20% of total cells in the C_{4n} subpopulation during the exponential growth phase. Also, a minor content of C_{1n} cells (up to 10%) was additionally observed for a short time period. Growth of *M. frederiksbergense* on glucose presented up to 50% C_{2n} cells during exponential growth and an increase in C_{4n} and even C_{8n} cells during stationary growth probably due to their clotting beha-

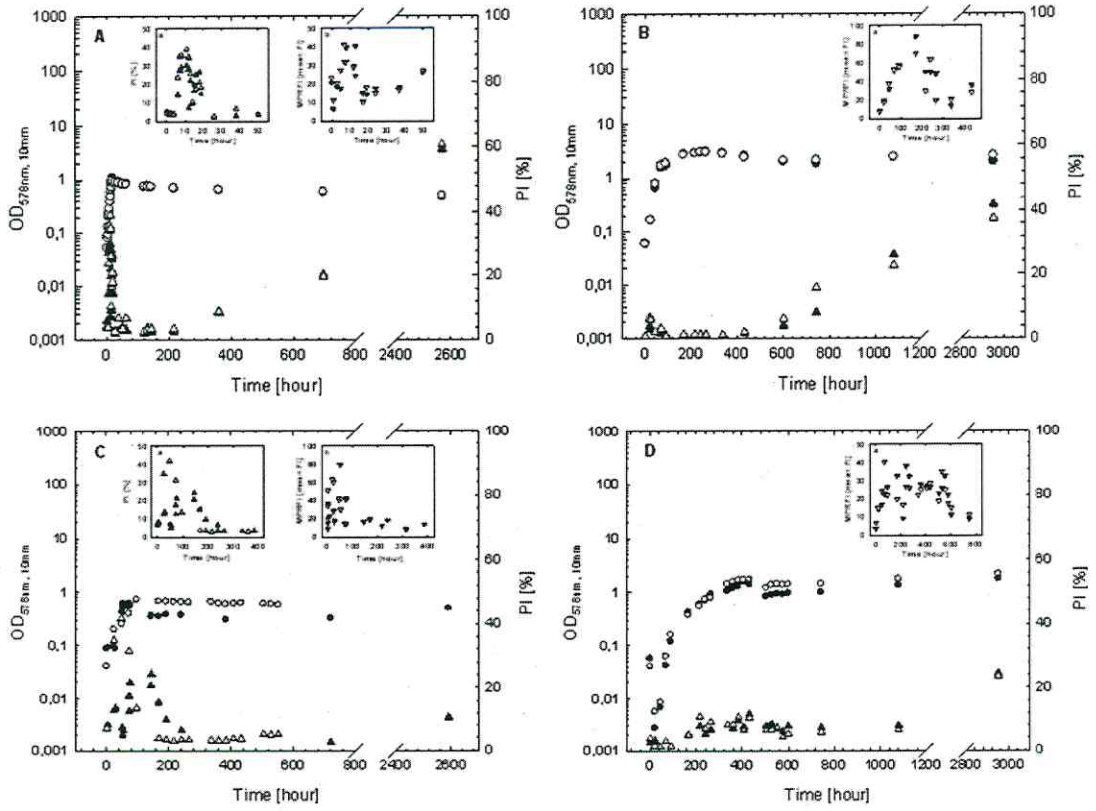

Figure 2. Growth of *Sphingomonas* sp. on 1 g l^{-1} glucose (**A**) and on 2.7 g l^{-1} fluorene (**B**), of *M. frederiksbergense* on 1 g l^{-1} glucose (**C**) and on 2 g l^{-1} anthracene (**D**) with OD (circles), PI-% (triangles) and MPRFI (mean values, reversed triangles). Open and filled markers represent results obtained from independent duplicate batches. Inset (a) and inset (b) are the PI-% and MPRFI during early growth phase, respectively.

vior as well as the formation of small hyphae-like morphology under limiting growth conditions. During growth on anthracene C_{2n} subpopulations remained constant at about 50% during the whole observation period with always only low numbers of C_{8n} cells. Since the anthracene was given in crystalline form because of its low aqueous solubility, even after 900 h of cultivation, a constant dissolution-limited anthracene flux to the cells was provided and no classical stationary phase was observed within this time period of cultivation (13).

Using the same batch-cultures, the quantity of dead cells was determined as the fraction of PI permeable cells. However, unexpectedly, the quantities of PI positive cells were always found to be very high during the early growth phase. These results were surprising and demanded a closer investigation. Therefore, cultivation duration was extended to up to 2,600 h for glucose and over 3,000 h for both fluorene and anthracene (Fig. 2). The data showed an obvious increase in PI stained *Sphingomonas* sp. cells up to 40% during exponential growth on glucose (see also the inset in Fig. 2A) and up to 8% on fluorene (Fig. 2B). The same behavior was observed for *M. frederiksbergense* on its two substrates (Figs. 2C and 2D). The temporarily high PI uptake leveled down to nearly 2–5% on all given substrates of the two species quickly afterwards, only to increase after about 400 h cultivation for *Sphingomonas* sp. and notedly after 2,600 h of cultivation for *M. frederiksbergense.*

To demonstrate the viability of PI-stained cells in the early exponential growth phase, the MPRFI related stain $diOC_6$ (3) was applied to provide further information on viability of the cells. Changes in energy charge were followed over the same time ranges in coevally and independently harvested samples. As expected, the energy charge-related fluorescence intensities were homogeneously distributed and went along with high PI uptake (Fig. 2, insets b).

Hoechst 33342 was used to elucidate which cell states of the cell cycle might have captured PI maximally. The data for *M. frederiksbergense* are presented in Figure 3. A clear assignment was difficult to make due to dye pumping (Fig. 3A, 48 h, gate 1) and quenching of the Hoechst 33342 fluorescence intensities when high amounts of PI (Fig. 3a, 48 h, gate 2)

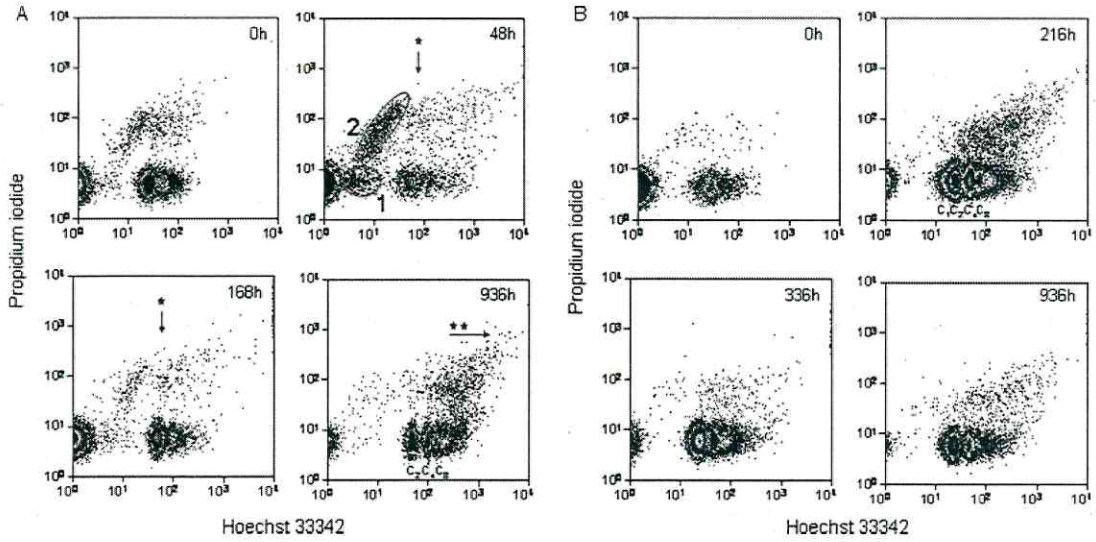

Figure 3. *Mycobacterium frederiksbergense* LB501T grown on glucose (**A**) and anthracene (**B**) stained with Hoechst 33342 (45 min; 3.2 μM) and PI (10 min; 1 μM) up to nearly 1,000 h. The gates indicate either PI uptake (2) or Hoechst 33342 pumping (1). Arrows represent Hoechst 33342 stained cells with PI fluorescence.

entered the cells. Such a quenching was already described in double stained Hoechst 33342/PI yeast cells (19). However, referring to the part of unaffected glucose grown cells, those with the lowest DNA contents had the highest quantities of PI stained cells during the first 48 and 168 h of cultivation (arrows with one star), yet at the end of cultivation there was a higher number of PI cells with higher chromosome contents, even though the C_{2n} was the dominant subpopulation (arrow with two stars). Growth on anthracene revealed a different behavior of the population, no pumping and nearly no quenching was observed, also the quantities of dead cells were nearly equally distributed between the subpopulations.

Viability of PI Stained Cells

The viability of PI stained cells of *M. frederiksbergense*, *Sphingomonas* sp., and *E. coli* (as control) was determined as their culturability on LB agar. The culturability was calculated as the ratio of the number of CFUs to the number of cells spotted; the culturability of PI treated but unstained cells was also analyzed for comparison.

Initial experiments revealed remarkable species-specific differences of culturabilities even if cells had never been treated with PI with 96% for *E. coli*, 84% for *M. frederiksbergense*, and 20% for *Sphingomonas* sp. (inset of Fig. 4). Similar degrees of culturability were observed with PI treated but unstained cells (Fig. 4). Hence, the mere presence of PI did not apparently affect cellular growth.

In contrast, the culturability of fluorescent cells was reduced. This was most obvious with *E. coli*. Only 4% of fluorescent *E. coli* cells were culturable, indicating a large majority of 96% dead bacteria among the PI stained cell population. Exponentially grown and PI stained cells of the two soil bacteria displayed a different behavior: almost 60% of *M. frederiksbergense* and 10% of *Sphingomonas* sp. cells grew to colonies. Taken into account the species-specific cultivabilities, 50–75% of all PI stained cells were culturable. In other words, the PI-stained population consisted of more culturable than dead cells.

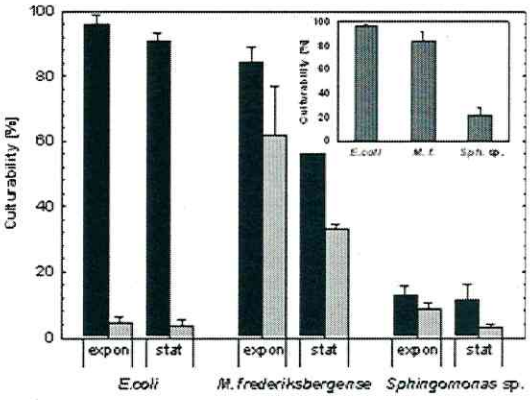

Figure 4. Culturability (%) of *E. coli*, *Sphingomonas* sp., and *M. frederiksbergense*, upon PI treatment. Cells from exponential (expon) and stationary phase (stat) were harvested, diluted in PBS-buffer, treated with PI, sorted, and spotted onto LB-agar (black bars, cells PI treated but unstained; grey bars, cells PI treated and stained). Error bars represent the standard deviations from triplicate measurements. The inset shows the different general culturability (%) of exponentially grown bacteria not exposed to PI.

Bacteria from the late stationary phase showed reduced viability upon PI staining. Only 34% of cells of *M. frederiksbergense* and 3% of *Sphingomonas* sp. formed colonies. This indicated a large proportion of dead bacteria, even so, the number of culturable cells was unexpectedly high. Hence, both with exponentially and stationary phase cells, PI staining did not reliably indicate death of *M. frederiksbergense* and *Sphingomonas* sp.

Discussion

As bacteria are of outstanding relevance in medicine, biotechnology, and environmental microbiology, the identification of their activity at given conditions requires reliable and quick staining-based viability assessments. The application of such staining cocktails needs detailed knowledge of the dye's effects as well as the physiology and structure of the microorganisms studied. As PI has been described manifold in literature to penetrate injured membranes and cell walls, the dye is often applied to distinguish living from dead microorganisms. However, although PI uptake was described to occur in compartmented *Bifidumbacterium* species (20), producing segregate double stained cFDA/PI cells, large quantities of these cells were reproducibly culturable on agar plates, a phenomenon attributed to transiently injured or dead (compartmented, probably as part of a hyphae-like morphology), but recovering cells.

As many commercially available viability kits are developed for medically relevant strains, such as *E. coli*, PI-staining of exponentially growing *E. coli* was included in this study and compared to the staining behavior of environmental isolates. In contrast to the latter, growing *E. coli* cells were found to take up negligible amounts of PI (1–2%, not shown). However, up to 40% of the environmental isolates were stained by PI during the exponential growth phase and large quantities of PI-stained cells of both *Sphingomonas* sp. and *M. frederiksbergense* remained culturable on LB-agar plates after cell sorting. This applied also, yet to minor quantities, to PI stained and sorted cells from the stationary growth phase (Fig. 4). The presence of viable cells was corroborated by the observation that the exponentially growing cells showed cell cycle activities (analyzed by DAPI), performed pumping of Hoechst 33342 and also had a high and stable energy charge as analyzed by MPRFI.

Although these data clearly question the utility of PI as universal indicator for cell death, the question why the cell walls of some actively growing cells became permeable for PI remains unanswered. Generally, there are three main protein families responsible for the bacterial SEDS (shapes, elongation, division, and sporulation) functioning: PBPs (penicillin binding proteins, peptidoglycan synthesis complex), Fts-proteins and RodA (tubulin homologues, responsible for the cell division site), and MreB (actine homologue and probably responsible for cell elongation). The loss of peptidoglycan stability has been described to be connected to the functioning of these molecules. For gram$^-$ bacteria it was assumed that the membrane integrity is endangered if subunits of glycan strands are inserted into broken single cell wall glycan-layers during cell growth (see for review Ref. 21). Additionally, the cell membrane integrity is also known to be temporarily affected during periods of fast cell size growth and to be particular sensitive to a variety of antibiotics due to physical cell wall reconstruction at new cell poles or sites of cell-division (22). The significant differences of PI-uptake between fast growing *E. coli* cells (μ = 0.347 h^{-1}) and slowly proliferating *Mycobacteria* (glucose: μ = 0.027 h^{-1}; anthracene: μ = 0.013 h^{-1}) and *Sphingomonas* species (glucose: μ = 0.046 h^{-1}; fluorene: μ = 0.01 h^{-1}) may thus be explained by the inability of the latter to quickly close broken glycan strands by highly efficient enzymes.

Mycobacteria are further known to exhibit porin-mediated transport of hydrophilic compounds across the outer membrane. Although the porin MspA is described to be at least 1,000-fold less efficient than those of gram$^-$ bacteria (23), its expression was found both to increase the glucose uptake and to accelerate growth. Consequently, Mailaender et al. (24) discussed porins as the mediator for a tenfold increased uptake of the fluorescent dye Syto 9 due to presumed local membrane interruptions upon expression of *mspA*.

In conclusion, the data presented question the utility of PI as universal indicator for the viability and culturability of environmental bacteria. Careful testing of the reliability of PI-based viability assays thus is recommended for their application outside of standardized procedures.

Acknowledgments

The authors thank Christine Süring and Helga Engewald for skilled technical assistance.

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Microbial Biotechnology (2008) 1(1), 53–61 doi:10.1111/j.1751-7915.2007.00006.x

Activity and viability of polycyclic aromatic hydrocarbon-degrading *Sphingomonas* sp. LB126 in a DC-electrical field typical for electrobioremediation measures

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Summary

There has been growing interest in employing electrobioremediation, a hybrid technology of bioremediation and electrokinetics for the treatment of contaminated soil. Knowledge however on the effect of weak electrokinetic conditions on the activity and viability of pollutant-degrading microorganisms is scarce. Here we present data about the influence of direct current (DC) on the membrane integrity, adenosine triphosphate (ATP) pools, physicochemical cell surface properties, degradation kinetics and culturability of fluorene-degrading *Sphingomonas* sp. LB126. Flow cytometry was applied to quantify the uptake of propidium iodide (PI) and the membrane potential-related fluorescence intensities (MPRFI) of individual cells within a population. Adenosine tri-phosphate contents and fluorene biodegradation rates of bulk cultures were determined and expressed on a per cell basis. The cells' surface hydrophobicity and electric charge were assessed by contact angle and zeta potential measurements respectively. Relative to the control, DC-exposed cells exhibited up to 60% elevated intracellular ATP levels and yet remained unaffected on all other levels of cellular integrity and functionality tested. Our data suggest that direct current ($X = 1$ V cm^{-1}; $J = 10.2$ mA cm^{-2}) as typically used for electrobioremediation measures has no negative effect on the activity of the polycyclic aromatic hydrocarbon (PAH)-degrading soil microorganism, thereby filling a serious gap of the current knowledge of the electrobioremediation methodology.

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Introduction

In recent years there has been increasing interest in employing electrobioremediation, a hybrid technology of bioremediation and electrokinetics for the treatment of contaminated soil. Several studies have demonstrated improved biodegradation of organic pollutants such as gasoline hydrocarbons, aromatic compounds or trichloroethylene in weak electric fields (*ca.* 0.2–2 V cm^{-1}; Probstein and Hicks, 1993) applied to soil (Wick *et al.*, 2007). Present electrobioremediation practice is an empirical business lacking detailed knowledge of interactions among the environmental matrices, contaminants and biocatalysts in the presence of weak electric fields. Most likely, the stimulation of biodegradation originates from the DC-induced movement of water, contaminants and microorganisms, leading to an overall homogenization of the reaction partners (Harms and Wick, 2006). Optimal biodegradation activity requires that the application of direct current (DC) has no negative effect on the biocatalysts, i.e. the indigenous contaminant-degrading bacterial communities in soil. To date only limited information on the effect of DC on pollutant-degrading soil microorganisms is available (Lear *et al.*, 2007). In a recent study (Lear *et al.*, 2004), no effect of the applied current *per se* (0.314 mA cm^{-2}) on the composition and structure of soil microbial communities could be ascertained and observed microbial community changes close to the electrodes were attributed to DC-induced changes of the soil pH and physicochemical soil structure. In other studies pH changes and the production of H_2 close to the cathode were found to stimulate the activity of sulfur-oxidizing bacteria (Jackman *et al.*, 1999) at a current density of 20 mA cm^{-2} or the biological denitrification of nitrate-contaminated groundwater (Hayes *et al.*, 1998). When living microbial cells were exposed to high electric fields, different effects, such as changes in the cell membrane integrity (Zimmermann *et al.*, 1974), sublethal injuries by reactive oxygen species (Liu *et al.*, 1997; Li *et al.*, 2004) or ohmic heating (Palaniappan *et al.*, 1992), changes in the physicochemical surface properties (Shimada and Shimahara, 1985; Luo *et al.*, 2005), and metabolic stimulation due to electrokinetically induced

Table 1. Fluorene degradation kinetics and initial physiological characteristics of fluorene-degrading *Sphingomonas* sp. LB126 suspended cells in pH- and temperature-stable batch systems.

X^a (V cm^{-1})	J^b (*m*A cm^{-2})	$N_{initial}{}^c$ (×10^8 cells ml^{-1})	$PI_{initial}{}^d$ (%)	$MPRFI_{initial}{}^e$ (mean FI)	$ATP_{initial}{}^f$ (×10^{-18} mol cell^{-1})	$q_{initial}{}^g$ (×10^{-20} mol s^{-1} cell^{-1})
0	0	1.0–8.0	2–3	130–170	0.15–0.9	2.2–2.4
1	10.2	1.0–8.0	2–3	130–170	0.15–0.9	1.9–2.4

a. Electric field strength.
b. Electric current density.
c. Total number of cells assessed by flow cytometry.
d. Fraction of the propidium iodide (PI)-stained cells relative to total number of cells.
e. Mean membrane potential-related fluorescence intensity (MPRFI).
f. Adenosine triphosphate (ATP) contents.
g. Fluorene biodegradation rate.

substrate transfer processes (Pribyl *et al.*, 2001) or oxygen or hydrogen generation at the electrodes (She *et al.*, 2006), were described. The extent of the electrical impacts depended on the amperage, treatment time, cell type and medium characteristics (Velizarov, 1999). Here we present work aimed at studying the effect of direct current on the physiology of fluorene-degrading *Sphingomonas* sp. LB126. Strain LB126 was chosen as a representative of polycyclic aromatic hydrocarbon (PAH)-degrading organisms, as sphingomonads are important degraders of PAHs in soil (van Herwijnen *et al.*, 2003). In order to characterize the physiological state of strain LB126 exposed to weak DC-electric fields typical for electrobioremediation measures (Luo *et al.*, 2006; Niqui-Arroyo *et al.*, 2006), a series of indicators of cellular integrity or functionality (Hewitt and Nebe von Caron, 2004) was employed. Flow cytometry was applied to quantify total cell numbers, propidium iodide (PI) uptake and membrane potential-related fluorescence intensities (MPRFI) of individuals. In addition, bulk adenosine triphosphate (ATP) contents and fluorene biodegradation rates were determined and expressed on a per cell basis. Possible influences of DC on the physicochemical cell surface properties such as surface hydrophobicity and charge were investigated by contact angle and zeta potential measurements.

Results

The effect of weak direct current (1 V cm^{-1}; J = 10.2 mA cm^{-2}) on the physiology of *Sphingomonas* sp. LB126 was determined in batch systems during the degradation of 100 mM potassium phosphate buffer (PB)-dissolved fluorene at 25 ± 1°C. In order to characterize the physiological state of DC-exposed LB126, a series of indicators of cellular integrity or functionality was employed including PI uptake and MPRFI of individuals, bulk ATP contents and fluorene biodegradation rates as well as selected physicochemical cell surface properties.

Influence of DC on the PI uptake and ATP contents

Multiparametric flow cytometry revealed no impact of an electric field [X = 1 Vcm^{-1}; J = 10.2 mA cm^{-2} (Table 1)] on the total cell counts (N_t) that might have resulted from DC-induced cell lysis or dispersion of aggregates (Fig. 1A). Similarly, no significant impact of DC treatment on the fraction of PI-stained cells was observed as the fraction remained below 4% during DC exposure as in control experiments (Fig. 1B). The discrimination of PI-permeable and PI-impermeable (intact membranes) bacteria was used as an indicator for compromised cell membranes. In order to compare the cells' membrane integrity with their culturability, cells were cultivated on Luria–Bertani agar plates and the fraction of colony-forming units (cfu) relative to flow cytometric counts N_t quantified. The culturability of strain LB126 was very low (0.02–0.5%) and not correlated with PI-based membrane-related cell integrity. No apparent difference in the culturability of DC-treated and control cells was observed, i.e. culturability upon both treatments decreased by 20–30% (data not shown). The intracellular ATP levels of cells were 40% elevated after 40 min of DC exposure whereas control cells exhibited a nearly 20% reduced ATP content during the observation period (Fig. 2A). Increased ATP values by contrast were not paralleled by MPRFI (Fig. 2B) which did not change in DC-treated and control cells.

Influence of DC on fluorene biodegradation kinetics

The influence of DC on the fluorene biodegradation rate (q) of strain LB126 was investigated by exposing suspended cells to saturated fluorene solutions and analysing fluorene depletion in the presence or absence of the weak electric field. Fluorene biodegradation rates in the presence of a weak electric field were corrected for the apparent abiotic losses (such as electrochemical degradation, evaporation and sorption losses). The apparent q of fluorene biodegradation at various substrate concentrations (Fig. 3) was similar in the presence and the absence of DC and attained its maximum $q_{initial}$ of 1.9–2.4 × 10^{-20} mol s^{-1} cell^{-1} above

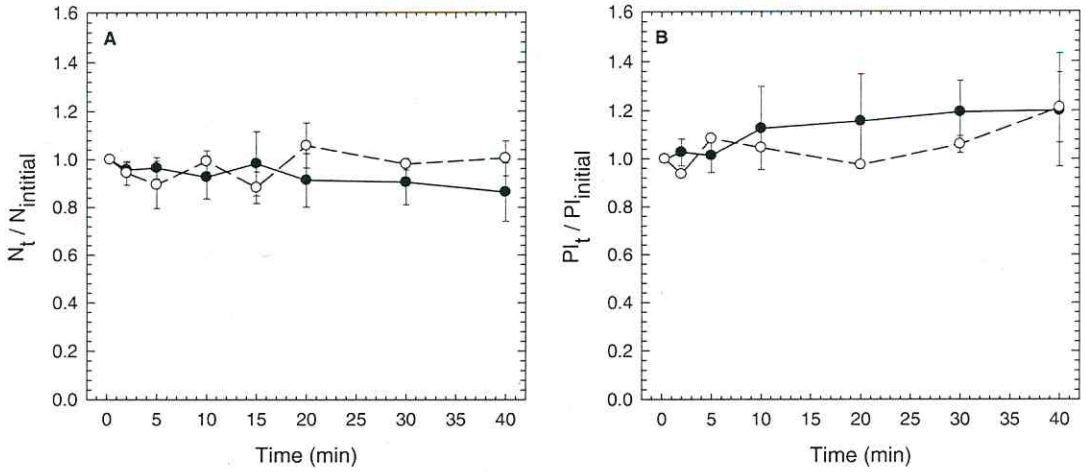

Fig. 1. Time-dependent fractions of total (N_t; A) and PI-stained (PI_t, B) *Sphingomonas* sp. LB126 cells in the presence (filled circles, 1 V cm^{-1}) and absence (open circles) of DC. Data are normalized relative to initial conditions ($N_{initial}$; $PI_{initial}$).

fluorene concentrations of approximately 6–10 μM. The assumption that fluorene depletion reflected biodegradation was checked by O_2-respiration measurements. O_2-respiration rates in the range of 0.6–1.8 × 10^{-19} mol s^{-1} cell^{-1} at all time points suggested the utilization of fluorene in DC-treated and control cells.

Influence of DC on cell size and physicochemical cell surface properties

No effects of DC on bacterial cell size as measured by multiparametric flow cytometry (data not shown) and physicochemical cell surface properties (Fig. 4) were observed. Water contact angles (θ_W) and zeta potentials (ζ) were used to observe possible influences of DC on the physicochemical surface properties of *Sphingomonas* sp. LB126 (Fig. 4). Strain LB126 was poorly negatively charged ($\zeta = 2.3 \pm 1$ mV) and hydrophobic ($\theta_W = 80° \pm 6°$).

Discussion

DC-induced influences on cell physiology

The cytoplasm of a cell is electrically conducting whereas

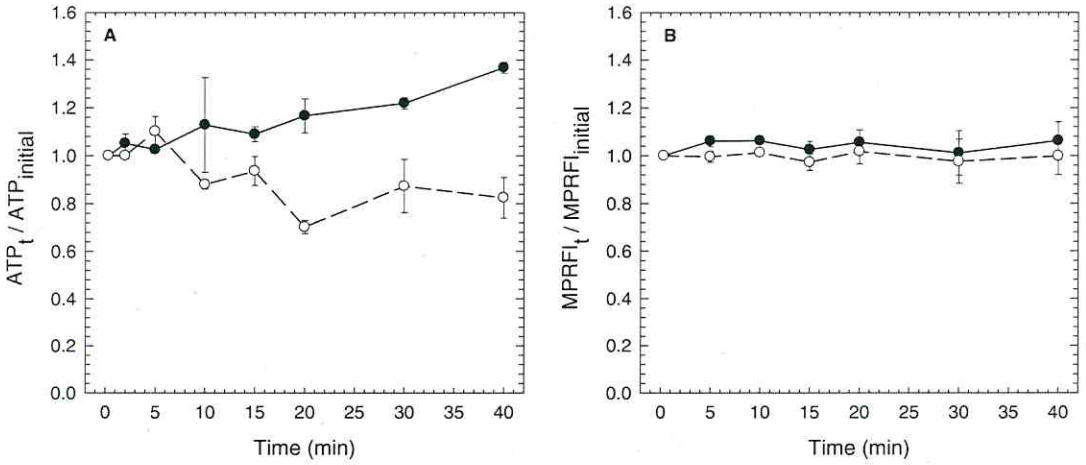

Fig. 2. Normalized fractions of intracellular ATP contents (A) and cell membrane potential-related fluorescence intensity (MPRFI) (B) in the presence (filled circles, 1 V cm^{-1}) and absence (open circles) of DC. Data are normalized relative to initial values.

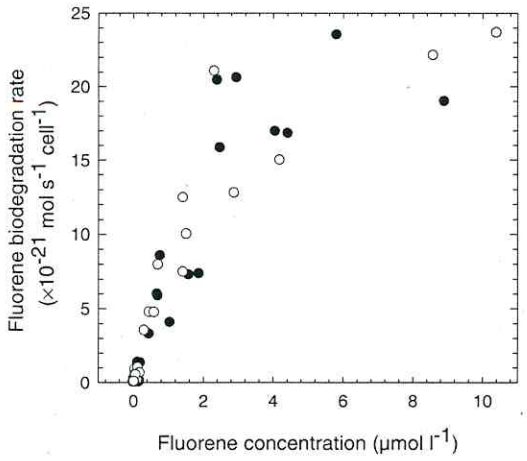

Fig. 3. Representation of the apparent fluorene biodegradation rates of *Sphingomonas* sp. LB126 relative to measured dissolved bulk fluorene concentrations in the presence (filled circles, 1 Vcm^{-1}) and absence (open circles) of DC. Fluorene biodegradation rates in the presence of DC were corrected for apparent abiotic (electrochemical) losses as described in *Experimental procedures*.

the lipid bilayer of the cell membrane can be considered as dielectric. The application of electric fields to cells thus causes the build-up of electrical charge at the cell membrane, especially at the membrane areas pointing at the cathode and anode, and thus induces a change in voltage across the membrane (Zimmermann *et al.*, 1974). For externally applied electric fields above 1 kV cm^{-1} leading to a transmembrane potential difference ($\Delta\Phi_m$) of > 70– 250 mV, the membrane permeability is expected to increase with the consequence of a reversible or irreversible 'dielectric' breakdown of $\Delta\Phi_m$ (Zimmermann *et al.*, 1974) and concomitant cell death (Grahl and Markl, 1996). Local instabilities in the membranes due to electromechanical compression and electric field-induced tension (Ho and Mittal, 1996; Weaver and Chizmadzhev, 1996) have been proposed to be the reason for the often observed electropermeabilization (electroporation) of cells exposed to pulses of high electric fields. Biotechnological electroporation has found extensive application for the electrorelease of cell ingredients and the uptake of foreign molecules, such as DNA, proteins or substances for which the cells is poorly permeable or non-permeable (Turgeon *et al.*, 2006). Permanent cell membrane breakdown by contrast was used to inactivate microbes, for example for the preservation of food (Elez-Martinez *et al.*, 2004). Both effects have been demonstrated by increased PI uptake (Wouters *et al.*, 2001; Aronsson *et al.*, 2005; Garcia *et al.*, 2007), loss of culturability (Yao *et al.*, 2005) or ATP leakage (Sixou *et al.*, 1991; Wouters *et al.*, 2001; Aronsson *et al.*, 2005).

Propidium iodide, a cationic dye, is believed to enter cells with compromised cytoplasmic membranes. Because cells exhibiting PI-permeable membranes often happen to be injured or dead, PI is also commonly used in commercially available viability assays to distinguish dead from viable cells, especially for medical relevant strains of *Escherichia coli* (Comas and VivesRego, 1997; Virta *et al.*, 1998; Lehtinen *et al.*, 2004). Propidium iodide-based viability assessments of environmental bacteria however have to be interpreted with great care, as the PI

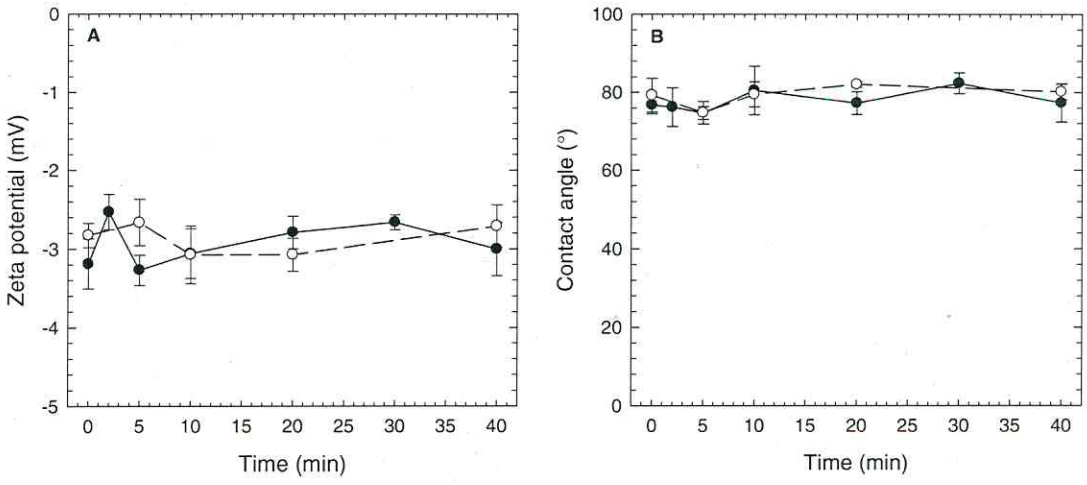

Fig. 4. Time-dependent physicochemical cell surface properties of *Sphingomonas* sp. LB126 exposed to DC. (A) and (B) reflect the water contact (θ_w) and the zeta potential (ζ), respectively, in the presence (filled circles, 1 V cm^{-1}) or absence (open circles) of DC.

uptake was demonstrated to depend on the physiological state of *Sphingomonas* sp. LB126 cells and did not correlate to their culturability (Shi *et al.*, 2007). For instance, up to 40% of LB126 cells were stained by PI during early exponential growth on glucose as compared with 2–5% of cells in the early stationary growth phase. This observation was explained by a transient permeabilization of the cell membrane during rapid growth of the cell body on energy-rich and easily available growth substrates (Shi *et al.*, 2007). In this study, bacteria from early stationary growth phase were used to exclude cell growth-related PI uptake. The fraction of PI-stained cells did not change during the DC treatment and hence indicates the absence of an electropermeabilization of the cell membrane of strain LB126. This finding is corroborated by the observation of unchanged cell sizes of both DC-treated and control cells excluding electromechanical cell elongation induced by electroporation of cell membranes (Ho and Mittal, 1996; Weaver and Chizmadzhev, 1996).

The time-dependent culturability of DC-treated and control cells was followed as cfu on LB agar. The culturability of fluorene-grown strain LB126 was low and highly variable between replicate experiments with only 0.02–0.5% of the cells counted by flow cytometry forming colonies on LB agar. However, no differences of the culturability between DC-treated and control cells were observed. Supported by the data for *Sphingomonas* sp. LB126 and earlier observation of unaffected anthracene degradation by DC-exposed *Mycobacterium frederiksbergense* LB501T (Wick *et al.*, 2004), it can be concluded that mild DC treatment has no negative effect on the culturability of these PAH-degrading bacteria.

Electric fields have been described to stimulate ATP synthesis and induce cellular damages. Initial ATP pools of *Sphingomonas* sp. LB126 were in the range of 1.5–9.0×10^{-10} nmol per cell, being values typically found for metabolically active cells (Holms *et al.*, 1972; Müller *et al.*, 2000; Loffhagen *et al.*, 2006). Petersen *et al.* have demonstrated that cells at a given growth phase may have a constant ATP pool controlled by steady-state interactions of ATP synthesis and ATP consumption (Petersen and Moller, 2000). As growth was excluded in our experiments, different intracellular ATP pools in DC-exposed and control cells may reflect influences on the rates of ATP synthesis and ATP consumption. It is not daring to attribute the gradual decline of the ATP pool in the nutrient-deprived control cells to reduced ATP synthesis. Explaining the significant, gradual increase of ATP in DC-treated cells is more complicated. While similar MPRFI of DC-treated and control cells point at similar ATP synthesis, the unaffected catabolic activity (fluorene biodegradation rates and oxygen consumption) and similar culturability suggest the absence of DC-induced stress. In a recent study Zanardini and colleagues (2002) have found an approximately threefold increase of the ATP content of a mixed culture in wastewater exposed to 40–200 mA DC for 10 days (Zanardini *et al.*, 2002). Several studies have furthermore reported stimulated ATP synthesis by both oscillating (Tsong *et al.*, 1989) and high-voltage pulsed ($X = 1–6$ kV cm^{-1}) electric fields (Teissie, 1986). Although an external DC field was applied in our study, individual cells may have been exposed to fluctuating electric fields due to their rotation relative to the DC field in the stirred cell suspension. Increased ATP levels thus may have been the result of cumulative effects of DC-promoted transmembrane pH-gradients and/or membrane potential differences, which were not maintained during the manipulation of cells needed for the flow cytometric analysis of the MPRFI.

Implications for electrobioremediation

Although electrobioremediation appears to be effective, optimization of the technology requires mechanistic understanding of the processes affecting the activity and dispersion of hydrophobic organic contaminants (HOC)-degrading microorganisms. Electrokinetic biodegradation enhancement was mostly attributed to the homogenization of nutrients or otherwise immobile pollutants and the directed electrokinetic transport of bacteria. For bioremediation this is of great importance as most soil bacteria are quite immobile because they are attached to soil particles or form microcolonies in the soil matrix. This leads to the largely heterogeneous microscale distribution of soil bacteria known to limit pollutant bioavailability (Bosma *et al.*, 1997). Electrokinetic influences on cell surface characteristics thus are of special interest in soil bioremediation as they potentially revert bacterial adhesion to surfaces and thus increase bacterial mobility (Redman *et al.*, 2004). In this study, no influence of DC on the surface charge and surface hydrophobicity of strain LB126, i.e. properties known to influence subsurface transport, was observed. The applicability of electrokinetics in PAH bioremediation is supported by (i) unchanged fluorene biodegradation activities, (ii) the maintenance of intact, PI-impermeable cell membranes, (iii) the cells' unchanged membrane potential and (iv) low yet similar culturability on LB agar plates in the presence and absence of DC. To our knowledge, this is the first assessment of the impact of DC on the activity and viability of PAH-degrading cells at the single-cell level. Future work can thus focus on the effects of electrokinetics on the ecology of microbial communities in the subsurface as well as on its influence on substrate mass transfer to bacteria (or vice versa) in electrobioremediation regimes.

Experimental procedures

Organism and culture conditions

Fluorene-degrading strain *Sphingomonas* sp. LB126 (van

Herwijnen *et al.*, 2003) was cultivated in minimal medium (Harms and Zehnder, 1994) in the presence of 2.7 g l^{-1} solid fluorene (≥ 99%, Fluka, Buchs Switzerland; crystals taken as obtained by the provider) as sole carbon and energy source. Cultures were grown at room temperature (20°C) on a gyratory shaker at 150 r.p.m. in 500 ml Erlenmeyer flasks containing 300 ml of medium. Growth together with cell cycle (i.e. DNA pattern), cell activity (i.e. MPRFI) and membrane integrity (i.e. PI uptake) was assessed by flow cytometry as described earlier (Shi *et al.*, 2007). For degradation assays cells were harvested in the early stationary phase after about 50–72 h because at that time they had relative high activity while showing low PI uptake. The cells were washed twice in cold 100 mM PB at pH = 7.0 and re-suspended in PB to obtain suspensions with an optical densities at 578 nm (OD_{578}) of about 10.

Determination of culturability

Bacteria were quantified as cfu on LB agar (2% w/v; Lennox, Carl Roth GmbH; agar from Difco) using the drop plate method (Chen *et al.*, 2003) with six replicates per sample. Colony-forming units were counted after 10 days of incubation at room temperature and the percentage of culturable bacteria determined as the ratio of the numbers of cfu relative to the numbers of cells (N_t) as quantified by multiparametric flow cytometry.

Analysis of fluorene degradation kinetics with suspended cells

The kinetic parameters of fluorene degradation by suspended cells were determined in batch systems consisting of rectangular cuvettes [8 (length) × 8 (depth) × 3 cm (width)] containing 100 ml of fluorene-saturated 100 mM PB at 25 ± 1°C. The stability of the temperature was assessed by a multifunction electrode (ECM Multi, Dr. Lange) in separate experiments. Saturated aqueous fluorene solutions were prepared by adding excess crystalline fluorene to 100 mM PB in screw cap Erlenmeyer flasks in the dark as described earlier (Wick *et al.*, 2004). Degradation experiments were performed in the presence and the absence (control cells) of direct current (1 V cm^{-1}; J = 10.2 mA cm^{-2}) through titanium-iridium electrodes (Electro Chemical Services International SARL, Châtelaine, Switzerland). Degradation experiments were started by adding 1–2 ml of fluorene-grown cell suspension in PB to fluorene-saturated PB to give OD_{578} of 0.10–0.14 and initial samples taken immediately after the addition of the bacteria. Frequently, 5 ml of samples were taken for chemical and microbial analyses. The pH value was measured during degradation experiments by pH paper indicator (pH 4.0–7.0 and 7.5–14, Merck). Samples for fluorene analysis (1 ml) were filtered through a 0.2-μm regenerated cellulose filter (RC 58, Schleicher & Schuell, Dassel, Germany) in a stainless steel filter holder (Millipore, Bedford, MA, USA) in order to remove suspended bacteria. Fluorene concentrations were analysed by high-performance liquid chromatography (HPLC) (Shimadzu Class-VP) on a RP-18 column (Nucleosil 100-5 C18 4 mm inside diameter) using an isocratic mobile phase [MeOH/water (90:10 v/v); flow: 1 ml min^{-1}] and fluorescence detection (λ_{ex} = 270 nm, λ_{em} = 340 nm). The apparent overall fluorene biodegradation rate (q_{app}; mol s^{-1} l^{-1}) was corrected for abiotic fluorene depletion due to sorption, volatilization and (in case of DC treatment) electrochemical fluorene degradation according to Eq. 1:

$$q_{app} = \frac{d[C]}{dt} - k_{app}[C] \quad (1)$$

where k_{app} (s^{-1}) is the apparent first-order fluorene abiotic depletion rate and $[C]$ (mol l^{-1}) the measured fluorene concentration at time (t). Abiotic fluorene depletion followed apparent first-order kinetics with $k_{app,DC}$ of 1.8×10^{-4} s^{-1} and $k_{app,control}$ of 9.7×10^{-5} s^{-1} in the presence and absence of DC respectively. The q_{app} was normalized by the number of cells N_t to obtain an average per cell value of fluorene biodegradation rate q (mol s^{-1} $cell^{-1}$).

Multiparametric flow cytometry analyses

Flow cytometry measurements were carried out using a MoFlo cell sorter (DakoCytomation, Fort Collins, CO, USA) equipped with a water-cooled argon-ion laser (Innova 70°C from Coherent, Santa Clara, CA, USA). Excitation of 580 mW at 488 nm was used to analyse the forward scatter (FSC) and side scatter (SSC) as trigger signal at the first observation point. Green and red fluorescence was analysed by using a BP520/15 and a BP620/45 filter respectively. Amplification was carried out at logarithmic scales. Data were acquired and analysed using Summit software (DakoCytomation).

Cell counting. The numbers of cells were counted accurately (and with negligible deviation) using flow cytometry. Fluorescent beads [FluoSpheres® polystyrene microspheres 1.0 μm in diameter, yellow-green fluorescent (505/515), Invitrogen] were mixed with 1 ml of samples taken from fluorene degradation experiments as described above. Dot plots were gated with regard to cell populations and beads, and then the numbers of cells were calculated (Vogt *et al.*, 2005).

Membrane integrity. Cells with compromised membranes were visualized by flow cytometry with nucleic acid-specific fluorochrome PI. The detailed calibrations of the staining procedure have been presented elsewhere (Shi *et al.*, 2007). Ten microlitres of PI (SIGMA-ALDRICH, Steinheim, Germany; final concentration 1.05 μM, stock solution 0.07 mg ml^{-1} PBS, pH 7.2) was mixed immediately with 1 ml of samples taken from fluorene degradation experiments (directly out of the cuvettes). After 10 min of staining, samples were measured by flow cytometry. The fractions of red PI-stained cells (%) were determined.

Membrane potential-related fluorescence intensity (MPRFI). Cell MPRFI was determined by flow cytometry using dihexyloxacarbocyanine dye $DiOC_6(3)$ (Aldrich) (Müller *et al.*, 1999). In order to assess the MPRFI, living cells were re-suspended in 20 mM imidazole buffer (pH 7.0) and immediately adjusted to 3×10^8 cells ml^{-1}. The composition of the staining solution was taken from Shapiro (1988). For optimal alignment, the MPRFI was defined by testing different dye concentrations, staining times and the action of antibiotics (gramicidin and valinomycin) on exponentially growing cells (calibration performed by Shi *et al.*, 2007).

Accordingly, 7.5 µl of the dye stock solution was used to stain 1 ml of 3×10^8 cells ml^{-1} cell suspension for 3 min. All measurements were carried out at 20°C. The cell size was obtained from the FSC signals.

Quantification of the ATP

Samples containing 1 ml of bacterial suspension were removed from fluorene degradation experiments, transferred immediately to 2 ml Eppendorf tubes containing 0.5 ml of 23 mM ethylenediaminetetraacetic acid (EDTA) dissolved in ice-cold 1.3 M perchloric acid, and stored at 4°C until ATP analysis. Shortly, samples were centrifuged at 4°C for 15 min at 10 000 r.p.m. Cell supernatants (500 µl) were neutralized to pH 7.7 with 300 µl of 0.72 M KOH containing 0.16 M $KHCO_3$ and ATP contents of the supernatants measured by a luciferin-luciferase bioluminescence assay using a Wallac Multilabel Counter 1420 (Turku, Finland) as described earlier (Loffhagen *et al.*, 2006).

Determination of the O_2-respiration rate

The oxygen concentration of air-saturated PB was measured using the method of Robinson and Cooper (1970). The respiration of whole-cell suspensions was measured in a reaction chamber (volume 1–5 ml, Cyclobios Oxygraph; A. Paar, Austria), and signals from the polarographic oxygen sensor were digitally stored and analysed by the Cyclobios program DatGraf v. 2.0 as described earlier (Loffhagen *et al.*, 2006). Samples of 10 ml were taken instantly during fluorene degradation experiments, then washed and re-suspended in 2.5 ml of 100 mM PB of pH 7.0. Such suspended cells of 2.2 ml were added to the reaction chamber while stirring and then incubated at 25°C for respiration measurements. Endogenous respiration was first monitored for 15 min and then 10 µl of fluorene-DMSO (stock concentration 2.53×10^{-3} mol l^{-1}) was added, after which the fluorene-dependent oxygen consumption rate was measured.

Determination of physicochemical cell surface properties

Cell surface hydrophobicities of bacteria taken from the fluorene degradation experiments were derived from the static contact angles (θ_w) of small water droplets placed on filters covered with layers of bacteria. Measurements were performed with a goniometer microscope (Krüss GmbH, Hamburg, Germany) as described before (Wick *et al.*, 2002). Contact angles of at least 10 droplets of 1 µl were measured for each organism. The zeta potential was approximated from the electrophoretic mobility measured by a Doppler electrophoretic light scattering analyser (Zetamaster, Malvern Instruments, Malvern, UK) according to the method of Helmholtz-Smoluchowski (Hiementz, 1986).

Acknowledgements

The authors wish to thank H. Engewald, K. Lange, J. Reichenbach, R. Remer, C. Süring and B. Würz for skilled technical help.

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Influence of DC-electric field on soil-microorganism interaction

4

4.1 Effect of electrokinetic transport on the vulnerability of PAH-degrading bacteria in a model aquifer

4.2 Factors influencing the electrokinetic dispersion of PAH-degrading bacteria in a laboratory model aquifer

Environ Geochem Health
DOI 10.1007/s10653-008-9146-0

ORIGINAL PAPER

Effect of electrokinetic transport on the vulnerability of PAH-degrading bacteria in a model aquifer

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Received: 13 August 2007 / Accepted: 8 October 2007

Abstract There has been increasing interest in employing electro-bioremediation, a hybrid technology of bioremediation and electrokinetics, to overcome the low bioavailability of hydrophobic organic contaminants (HOC) by homogenizing sorption-retarded HOC and immobilised microorganisms. Present electroremediation approaches mainly aim at macroscale pollutant extraction and tend to neglect possible impacts of direct current (DC) on the physiology of microorganisms. The effect of weak electric fields ($X = 1$ V cm^{-1}) on the fitness of electrokinetically dispersed fluorene-degrading *Sphingomonas* sp. LB126 in bench-scale model aquifers was investigated by flow cytometry using propidium iodide (PI) as an indicator that distinguishes between PI-permeable (cells with porous membranes, i.e. dead or vulnerable) and PI-impermeable bacteria. After 15.5 h of DC treatment 56% of all cells recovered were dispersed at the centimetre scale relative to 29% in the absence of DC. There was no overall negative effect of the 15.5-h DC treatment on cell vulnerability, as 7.0% of the DC-treated bacteria exhibited PI-staining compared to 6.5% of the control population. Minor differences were observed in the subpopulation that had been mobilised by electroosmosis with an approximately twofold increase in the percentage of PI-stained cells relative to the control. Enhanced PI staining did not correlate with reduced culturability of the cells on rich-medium agar plates. Relative to the control, DC-treated cells mobilised by electroosmosis were threefold more culturable, confirming earlier data that that PI-cell membrane permeability does not always indicate reduced viability of oligotrophic environmental bacteria. Our findings suggest that electrokinetics is a valuable mechanism to transport viable and culturable polycyclic aromatic hydrocarbon (PAH)-degrading bacteria in soil or sediments.

Keywords Bioavailability · Electro-bioremediation · Electrokinetics · Flow cytometry · Propidium iodide · Cell viability · Polycyclic aromatic hydrocarbons

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Introduction

The heterogeneous distribution of hydrophobic organic contaminants (HOC) and HOC-degrading organisms in soil limits the bioavailability and potential bioremediation of these pollutants (Johnsen et al. 2005). There is increasing interest in employing electro-bioremediation, a hybrid technology that combines bioremediation and electrokinetics, to overcome the low bioavailability of HOC by homogenizing both immobilised microorganisms and sorption-retarded HOC (Harms and Wick 2006). Optimal biodegradation

activity requires that the application of direct current (DC) has no negative effect on the viability of the biocatalysts, i.e. the indigenous HOC-degrading soil bacteria (Wick et al. 2007). When DC is applied to living microbial cells, various responses, such as toxic electrode effects, metabolic stimulation due to enhanced substrate mass transfer (Pribyl et al. 2001), sublethal injuries (Guillou et al. 2003), irreversible dielectric cell membrane breakdown (Zimmermann et al. 1974), or changes in the physicochemical surface properties (Luo et al. 2005) can be observed depending on the applied current, treatment time, cell type and medium characteristics (Velizarov 1999). Earlier studies using soil have shown that the application of 20 mA cm^{-2} DC current stimulated the activity of sulphur-oxidising bacteria (Jackman et al. 1999) or the biological denitrification of nitrate-contaminated groundwater (Hayes et al. 1998) due to the production of H_2 and subsequent pH changes. A study by Lear et al. (2005), analysing the effect of DC on the structure of soil microbial communities, has suggested that there is no negative effect of the applied current density ($J = 0.31$ mA cm^{-2}) on soil health in the absence of DC-induced changes in soil pH and temperature. Here we present a study aimed at analysing the influence of weak electric fields (1 V cm^{-1}) on the viability of electrokinetically dispersed fluorene-degrading *Sphingomonas* sp. LB126 (van Herwijnen et al. 2003) injected into laboratory model aquifer systems under constant-temperature constant-pH conditions. Flow cytometry was used to assess the total number of cells and to analyse propidium iodide (PI) uptake as an indicator of membrane damage and potential cell vulnerability to DC at the single-cell level.

Materials and methods

Organisms and culture conditions

Sphingomonas sp. LB126 was cultivated in a minimal medium (Harms and Zehnder 1994) on 1 g l^{-1} glucose, harvested in the early stationary phase after 24 h of growth, and washed prior to use as previously described (Wick et al. 2004). The cell surface of *Sphingomonas* sp. LB126 is poorly charged ($\zeta = -7.7 \pm 1.4$ mV) and has low hydrophobicity ($\Theta_w = 34 \pm 10°$) (Bastiaens et al. 2000). Culturability of cells was assessed by the drop plate method (Chen et al. 2003), using Luria-Bertani (LB) agar plates (Lennox, Carl Roth GmbH; Agar from Difco). Colony forming units (CFU) were counted after 7–10 days incubation at room temperature and percentage culturability calculated as the ratio of CFU to total number of cells measured by multiparametric flow cytometry analysis.

Electrokinetic experiments

The model aquifer chamber (Fig. 1) and the electrokinetic system used here, with 0.05 M Tris-acetate-buffer (TA) of pH 7 as the electrolyte and 1 mm glass beads as packing material, has been described in detail earlier (Wick et al. 2004). Duplicate electrokinetic experiments were carried out that applied a constant electric field strength ($X = 1$ V cm^{-1}) and an initial current density (J) between the electrodes of $J = 1.8$ mA cm^{-2}. An identical setup with no DC applied served as a control. No formation of a pH gradient or temperature changes (<2°C) were observed in either setup during the experimental period (15.5 h). One milliliter of a suspension containing about 5×10^9 bacteria ml^{-1} was slowly injected (1 min injection time) at a point that was half the depth of the bed equidistant from the center of the aquifer chamber (Fig. 1). At the end of each experiment, pore water samples were taken simultaneously using 10 ml glass syringes at the point of injection (0 cm) and at distances of 4.5, 9 and 15 cm from the centre of the aquifer chamber in order to avoid cell dispersion artifacts due to consecutive sampling. To assess the total number of cells, the cells adhering to the glass beads were also recovered by taking sample cores (1.6 cm diameter) at given locations as previously described (Wick et al. 2004).

Multiparametric flow cytometry

Flow cytometric measurements were carried out using a MoFlo cell sorter (DakoCytomation, Fort Collins, CO) equipped with a water-cooled argon-ion laser (Innova 70C from Coherent, Santa Clara, CA). Excitation of 580 mW at 488 nm was used to analyse the forward scatter (FSC) and side scatter (SSC) as the trigger signal at the first observation point. Green and red fluorescence was analysed by using BP520/15 and BP620/45 filters, respectively. Amplification

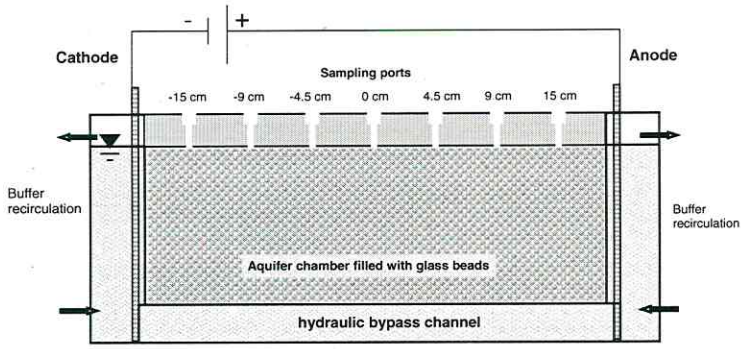

Fig. 1 Schematic view of the electrokinetic apparatus (40 cm × 7 cm × 3.5 cm ID). It is composed of two electrode chambers, a lid-covered model aquifer chamber (35 cm × 4 cm × 3.5 cm ID), and a bypass channel placed below the aquifer chamber connecting both electrode compartments. Titanium–iridium electrodes were inserted 1 cm from both front ends. Membranes were placed 1 cm from the electrodes in order to separate the electrode compartments from the aquifer materials. Sampling locations are indicated and marked as distances from the centre of the aquifer chamber

was carried out on logarithmic scales. Data were acquired and analysed using Summit software (Dako-Cytomation, Fort Collins, CO).

Cell counting

The numbers of cells were counted (with negligible deviation) using fluorescent beads (FluoSpheres® polystyrene microspheres 1.0 μm in diameter, yellow-green fluorescent (505/515), Invitrogen) which were mixed with 1 ml of sample taken from the electrokinetic experiments. Dot plots were gated with regard to cell populations and beads, and the numbers of cells were calculated (Vogt et al. 2005).

PI staining

Cell membrane porosity was analysed by flow cytometry using the nucleic-acid-specific fluorochrome propidium iodide (PI). The results of the staining procedure calibrations have been presented elsewhere (Shi et al. 2007). Ten microliters of a PI solution (Sigma-Aldrich; final concentration 1.05 μM, stock solution 0.07 mg ml^{-1} PBS, pH 7.2) were mixed immediately with 1 ml samples taken from electrokinetic experiments. After 10 min staining, samples were measured by flow cytometry. The fractions of red PI-stained cells (in percentages) were determined.

Results

Electrokinetic transport

Short-term experiments were conducted to analyse the transport of bacteria in glass-bead-filled model aquifers influenced by direct electric current. Non-motile and PAH-degrading *Sphingomonas* sp. LB126 were injected into the center of the packings and exposed to electric fields of 1 V cm^{-1} with $J = 1.8$ mA cm^{-2} for 15.5 h. Total numbers of bacteria were quantified at various locations in the model aquifer by flow cytometry analysis. In the presence of DC, 18% and 39% of the sampled cells had been transported by electrophoresis and electroosmosis, respectively (Fig. 2). Electrokinetic transport velocities were in the range 0.4–0.8 cm h^{-1}. Approximately 43% of all cells remained immobilised at the point of injection. In DC-free controls a symmetrical distribution of the bacteria was found with about 71% remaining immobilised in the center of the aquifer chamber.

Propidium-iodide-based cell vulnerability analysis

In parallel to the distribution, the vulnerability of mobilised and immobilised LB126-cells to direct electric current was analysed. The viability of individual cells was investigated by multiparametric flow cytometry using PI uptake as an indicator for

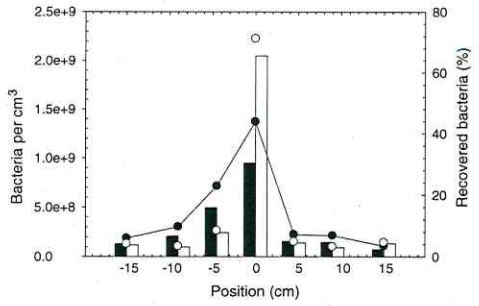

Fig. 2 Representative distribution of *Sphingomonas* sp. LB126 cells injected to a pH- and temperature-stable model aquifer system filled with glass beads after 15.5 h of exposure to $X = 1$ V cm^{-1} (black bars). Black and white bars represent bacterial distribution in the DC-treated and the DC-free control chamber, respectively. The second *y*-axis relates to the circles, reflecting the relative amount of total bacteria recovered at each given position in the presence (filled circles) and absence (open circle) of DC

porous cell membranes. Figure 3 depicts the percentage of PI-stained bacteria sampled from given positions in both the model aquifer and the control after 15.5 h of electrokinetic treatment. No effect of DC on cell membrane porosity was found; 7.0% of the DC-treated bacteria were PI stained relative to 6.5% in the control. This represents a threefold increase relative to the percentage PI staining of the inoculum (2–3%). PI-stained cells were not equally distributed in the chamber. Immobilised bacteria at the point of injection (position 0) had 13.5% PI stained cells in the presence, and 8.7% in the absence of, DC (Fig. 3), whereas the percentage of PI-stained bacteria mobilised by electrophoresis, electroosmosis or diffusion was clearly below that of the inoculum (Fig. 3). A comparison of the percentage of PI-stained cells in the DC treatment to those in the control experiments showed that the percentage of PI-stained cells mobilised in the direction of the cathode (electroosmosis) was about 2.6 times greater than the control, whereas there was no difference for cells mobilised to the anode (electrophoresis).

Discussion

Several studies have demonstrated improved biodegradation of organic pollutants in weak electric fields applied to soil (Wick et al. 2007). The stimulation of biodegradation probably originates from the DC-induced movement of contaminants and microorganisms, leading to an overall homogenisation of the reaction partners (Wick et al. 2004, 2007; DeFlaun et al. 1997; Suni et al. 2004). However, optimal biodegradation activity requires that the application of direct current (DC) has no negative effect on the biocatalysts, i.e. the contaminant-degrading bacterial communities in soil. Here we assessed the effect of electrokinetic transport on the vulnerability of PAH-degrading bacteria at the cellular level. Our data indicated that there was no negative influence of a weak electric field, typically used for electro-bioremediation measures, on the PI permeability of *Sphingomonas* strain LB126; more than 93% of cells in the DC-treated model aquifer remained unstained relative to 94% in the untreated control. This finding is in good accordance with a recent study showing that catabolic activity of PAH-degrading bacteria is unaffected when exposed to an electric field of 1 V cm^{-1} (Shi et al. 2008). It further corroborates data of Lear et al. (2005, 2007) suggesting that weak DC per se has no impact on the composition and structure of soil microbial communities. Closer inspection of the PI data reveals some differences in the distribution of PI-stained cells in the DC-treated and control aquifer chambers. A rough estimation was performed in order to understand these differences. This indicated that changes in local

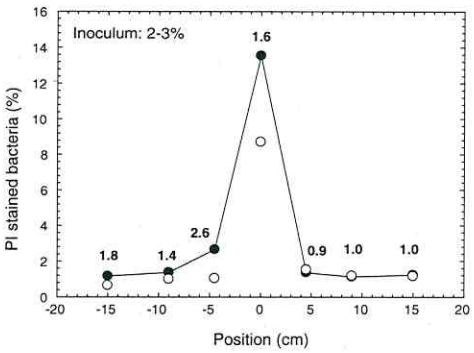

Fig. 3 Representative percentages of PI-stained *Sphingomonas* sp. LB126 cells at each given position in the model aquifer system in the presence (filled circle) and absence (open circle) of an electric field of strength 1 V cm^{-1} after 15.5 h of direct current (DC) exposure (cf. Fig. 2). Numbers reflect the ratio of the PI percentage of cells exposed to DC relative to the control at the locations indicated

staining by PI could be explained by assuming that PI-stained cells are immobilised, thereby concentrating at the injection point, compared to the transport of viable (unstained) cells (Fig. 3). The increased PI sensitivity of immobilised cells is surprising and requires further investigation. In order to assess the relevance of the PI staining as an indicator of cell vulnerability, the viability of cells mobilised by electroosmosis was spot-checked using conventional cultivation. Relative to the control (culturability of about 11%), electrokinetically mobilised cells exhibited an about threefold higher culturability (about 36%) which points to a positive correlation between increased PI permeability and culturability. This data is consistent with recent observations that actively growing *Sphingomonas* sp. LB126 bacteria were stained by PI and remained culturable on LB-agar plates (Shi et al. 2007). The use of PI staining as an indicator of cell death needs to be interpreted with great care (Shi et al. 2007). The 1.2–2.6 times increase in the percentage PI staining of DC-treated cells may not reflect reduced fitness, but may represent electrokinetic activation/stimulation of the bacteria (e.g. by enhanced mass transfer of nutrients or oxygen to the cells (Pribyl et al. 2001). Our data show potential for utilizing catabolically active bacteria under mild electrokinetic conditions. Future work should focus on the effects of electrokinetics on the functioning of microbial communities during pollutant electro-bioremediation regimes.

Acknowledgements The authors wish to thank J. Reichenbach, R. Remer, B. Würz, and H. Engewald for skilled technical help.

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Factors influencing the electrokinetic dispersion of PAH-degrading bacteria in a laboratory model aquifer

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Submitted to Applied Microbiology and Biotechnology

Abstract

Despite growing interest in the electro-bioremediation of contaminated soil it is still largely unknown to which degree weak electric fields influence the fate of contaminant-degrading microorganisms in the subsurface. Here we evaluate the factors influencing the electrokinetic transport and deposition of fluorene-degrading *Sphingomonas* sp. LB126 in a laboratory model aquifer exposed to a direct current (DC) electric field (1 V cm^{-1}) typically used in electro-bioremediation measures. The influence of cell size, cell membrane integrity, cell chromosome contents (all assessed by flow cytometry), cell surface charge and cell hydrophobicity on the spatial distribution of the suspended and matrix-bound cells after 15 hours of DC-treatment was evaluated. In presence of DC the cells were predominantly mobilised by electroosmosis to the cathode with an apparent velocity of 0.6 cm h^{-1}, whereas a minor fraction only of the cells augmented was mobilised to the anode by electrophoresis. Different electrokinetic behaviour of individual cells could be solely attributed to intra-population heterogeneity of the cell surface charge. In the absence of DC by contrast, a Gaussian-type distribution of bacteria around the point of injection was found. DC had no influence on the deposition efficiency, as the glass beads in presence and absence of an electric field retained quasi-equal fractions of the cells. Propidium iodide staining and flow cytometry analysis of the cells indicated the absence of negative influences of DC on the cell wall integrity of electrokinetically mobilised cells and thus point at unchanged physiological fitness of electrokinetically mobilised bacteria.

Introduction

Engineered bioremediation of soil relies on the presence of contaminant-degrading bacteria and optimal physical and chemical conditions for their catabolic activity. In order to achieve substantial biodegradation, microorganisms must further come into contact with soil-bound contaminants, such as polycyclic aromatic hydrocarbons (PAHs). This may be a particular problem for bioaugmented organisms, which need to be transported to the zone of contamination (Thomas and Ward 1989). Numerous laboratory (Lahlou et al. 2000), field-scale (Dybas et al. 2002; Major et al. 2002), and modelling studies (Schafer et al. 1998; Shein et al. 2002; Kim 2006) have been performed to understand and predict microbial transport in porous media. The observed inefficiency of bacterial transport was attributed mainly to the extremely low hydraulic conductivity in soil micro-pores (Silliman et al. 2001) and bacterial attachment to the surfaces of soil particles (Baygents et al. 1998). Strategies such as the chemical modification of bacterial surfaces (Gross and Logan 1995) and treatment with surfactants (Jackson et al. 1994; Brown and Jaffe 2001) were proposed to enhance the efficiency of bacterial transport by decreasing bacterial attachment. However, at low hydraulic conductivity, bacterial transport will always be strongly limited (Li et al. 1996). In recent years, there has been increasing interest in electro-bioremediation, a hybrid technology of bioremediation and electrokinetics, for the treatment of soil contaminated with hydrophobic organic compounds (HOC). Electrokinetics has the potential to enhance the contact probability of the bacteria

and their HOC substrates by transporting contaminants to bacteria or vice versa (Wick et al. 2007).

Bacterial transport in porous media driven by electrokinetics has been reported. It is a result of either electrophoretic movement of negatively charged bacteria to the anode (DeFlaun and Condee 1997; Lee and Lee 2001) and/or bacterial migration with the electroosmotic water flow to the cathode (Suni and Romantschuk 2004; Wick et al. 2004). Besides this, differential electrophoresis in capillaries is an accepted method for the separation of bacteria in the laboratory (Rodriguez and Armstrong 2004). In comparison with hydraulic flow restricted by capillary effects, electrokinetics (i.e. electrophoresis and electroosmosis) are quite independent from pore size, and electroosmosis is especially efficient in fine-grained soils (with pores in the μm range or smaller) (Hlushkou et al. 2007). Here we present a study on the influence of an electric field typically used in electro-bioremediation (1 V cm^{-1}) on the transport and deposition of fluorene-degrading *Sphingomonas* sp. strain LB126 (van Herwijnen et al. 2003) in a model bench scale aquifer packed with glass beads. Total numbers of adhered and suspended cells were quantified at distinct distances from the injection point and analysed by flow cytometry for their cell size distribution, their membrane integrity (by propidium iodide (PI) uptake) and their chromosome contents indicating the cell cycle stage (by DAPI staining). Static contact angle and dynamic light scattering experiments were performed to assess the influence of electric field on the physico-chemical cell surface characteristics (cell

surface hydrophobicity and cell surface charge) for the transport behaviour of the bacteria (Redman et al. 2004) .

Materials and Methods

Organisms and culture conditions. *Sphingomonas* sp. LB126 was cultivated in minimal medium (Harms and Zehnder 1994) with 1.0 g L^{-1} glucose as sole carbon and energy source. A previous study (van Herwijnen et al. 2003) has shown that strain LB126 is also able to grow on fluorene. Cultures were grown at room temperature (20°C) on a gyratory shaker at 150 rpm in 500-mL Erlenmeyer flasks containing 300 mL of medium. Growth together with stages in the cell cycle (via DNA contents as a measure of chromosome numbers) and membrane integrity (via uptake of propidium iodide, PI) were assessed by flow cytometry as described earlier (Shi et al. 2007). Cultures were harvested in the early stationary phase after 24 h of growth, washed three times in 50 mM Tris acetate buffer (TA) at pH = 7.0 to prevent further growth on residual glucose, and re-suspended in TA to obtain bacterial inocula with an OD_{578nm} of about 30 for use in electrokinetic experiments. Potential growth of LB126 cells on TA buffer was checked in separate batch experiments, where no change in OD_{578nm} was observed in 24 hours.

Electrokinetic experiments. The apparatus used in electrokinetic experiments has been described in detail earlier (Wick et al. 2004). Shortly, it consists of two electrode chambers (2 x 7 x 3.5 cm) at the ends, a lid-covered model aquifer chamber (35.5 x 4 x 3.5 cm) in between and a bypass channel below the aquifer chamber having hydraulic contact with both electrode chambers to exclude

advective hydraulic water flow through the aquifer chamber. Glass beads of 1 mm in diameter (Roth AG, Reinach, Switzerland) were sterilized, carefully packed into the aquifer chamber and then saturated by TA buffer giving a porosity of 0.39. The use of a model aquifer with well-defined properties (surface charge and ionic strength) is very crucial for the fundamental discussions in this study. One mL of bacterial inoculum was slowly injected during 1 min at half of the bed depth at equal distance from the two electrodes in the centre of the aquifer chamber. A constant electric field strength of 1 V cm^{-1} was applied for 15 hours with an initial current density (J) between the electrodes of J = 1.8 mA cm^{-2}. At the end of each experiment pore water samples were taken simultaneously from the aquifer chamber at the point of injection (0 cm), and at 4.5, 9, and 15 cm towards both electrodes. At each sampling point, 10 mL of pore water and a sample of about 12 mL glass beads were harvested as described earlier (Wick et al. 2004). Harvested glass beads were re-suspended in 20 mL 50 mM TA in a sterilized test tube and sonicated two times for 30 s with a break in between to detach adhered bacteria. After the detachment of bacteria the beads were dried and weighed to calculate exact bead surface areas of the samples. All bacterial suspensions were further analyzed as described below.

The efficiency of detachment was tested in a separate column system. LB126 cells were first pumped through a column packed with the same type of glass beads and the percentage of adhered cells was calculated from the breakthrough curve. Afterwards the glass beads were harvested and cells were detached following the same method as in the electrokinetic experiments. About 24 ± 8% of cells adhered to

glass beads could be detached with above-mentioned method. This detachment efficiency was used to estimate the total amount of cells adhered on glass beads during electrokinetic and control treatments.

Multiparametric flow cytometry analyses. Flow cytometry measurements were carried out using a MoFlo (Dako, Fort Collins, CO) equipped with two water-cooled argon-ion lasers (Innova 90C and Innova 70C from Coherent, Santa Clara, CA). Excitation of 580 mW at 488 nm was used to analyze the forward scatter (FSC) and side scatter (SSC) as trigger signals at the first observation point. The orthogonal signal was first reflected by a beam-splitter and then recorded after reflection by a 555 nm long-pass dichroic mirror, passage by a 505 nm short-pass dichroic mirror and a BP 488/10. DAPI fluorescence was passed through a 450/65 band pass filter, green fluorescence through BP 520/15 and red fluorescence through BP620/45. Photomultiplier tubes were obtained from Hamamatsu Photonics (models R 928 and R 3896; Hamamatsu City). Fluorescent beads (yellow-green fluorescent beads: 2 μm, FluoSpheres 505/515, F-8827, blue fluorescent beads: 1 μm, FluoSpheres 350/440, F-8815, crimson fluorescent beads: 1 μm, FluoSpheres 625/645, F-8816, Invitrogen, USA) were used to align the MoFlo. Also, an internal DAPI-stained bacterial cell standard was used to achieve distributions with a coefficient of variation less than 6%. Amplification was carried out at logarithmic scales. Data were acquired and analyzed using the Summit software (Dako, Fort Collins, CO). ***Cell Counting:*** The numbers of cells were counted accurately (as seen from negligible deviation) using flow cytometry. Fluorescent beads (FluoSpheres®

polystyrene microspheres 1.0 µm in diameter, yellow-green fluorescent (505/515), Invitrogen) were mixed with 1 mL of samples taken from electrokinetic experiments. Dot plots were gated with regard to cell populations and beads, and then the numbers of cells were calculated (Vogt et al. 2005). Cell aggregation was observed neither by microscopy nor flow cytometry, so clearly separated sub-populations were analysed. ***Membrane integrity.*** Cells with compromised membranes were quantified by flow cytometry using the nucleic acid specific fluorochrome propidium iodide (PI). Details of the staining procedure have been presented elsewhere (Shi et al. 2007). Ten microliters PI (SIGMA-ALDRICH, Steinheim, Germany; final concentration 1.05 µM, stock solution 0.07 mg mL^{-1} PBS, pH 7.2) were mixed immediately with 1 mL of samples taken from electrokinetic experiments (directly out of the chamber). After 10 minutes of staining, samples were measured by flow cytometry. The fractions (%) of red PI-stained cells were determined. ***DNA-patterns.*** The harvested cells were centrifuged at 3,200 x g for 5 min, inactivated with 10% NaN_3 and stored at 4°C for at least 2 hours. DNA patterns of preserved cells were then obtained in the following way: 2 mL of diluted cell suspension were treated with 1 mL solution A (2.1 g citric acid/0.5 g Tween 20 in 100 mL bidistilled water) for 10 min, washed and re-suspended in 2 mL solution B (0.24 µM 4',6-diamidino-2'-phenylindole [DAPI, SIGMA], 400 mM Na_2HPO_4, pH 7.0) for at least 20 min in the dark at room temperature using a modification of a standard procedure (Meistrich et al. 1978).

Determination of physico-chemical surface properties of bacterial cells and glass beads. Cell surface hydrophobicities of bacteria taken from electrokinetic experiments were derived from the static contact angles (θ_w) of small water droplets placed on filters covered with layers of bacteria. Measurements were performed with a goniometer microscope (Krüss GmbH, Hamburg, Germany) as described before (Wick et al. 2002). Contact angles of at least 10 droplets of 1 µL each were measured. The zeta potential (ζ) was approximated from the electrophoretic mobility of the bacteria in 50mM TA measured by a Doppler electrophoretic light scattering analyzer (Zetamaster, Malvern Instruments, Malvern, UK) according to the method of Helmholtz-Smoluchowski (Hiementz 1986). The zeta-potential of glass beads was approximated by the same method. Prior to the measurements, the glass beads were levitated to form a colloidal suspension (Qiu and Ling 2006) in 50 mM Tris acetate buffer (TA) at pH = 7.0.

Theory. *Bacterial diffusion*: The Einstein-Smoluchowski law (eq. 1) (Kohlmeier et al. 2005) relates the mean-squared displacement $\langle x^2 \rangle$ (cm^2) over a time interval t (h) to the effective diffusion coefficient D_{eff} ($cm^2\ h^{-1}$).

$$\left\langle x^2 \right\rangle = 2 * D_{eff} * t \qquad (1)$$

The mean transport velocity u_{dif} ($cm\ h^{-1}$) can be estimated by eq. 2:

$$u_{dif} = \frac{\langle x \rangle}{t} \qquad (2)$$

Electrokinetic – transport: The electrokinetic mobility μ_{EK} ($cm^2\ V^{-1}\ h^{-1}$) of bacteria (eq. 3) is the resultant of their electrophoretic (μ_{EP}) (eq. 4) and electroosmotic (μ_{EO}) (eq. 5) mobilities according to

$$\mu_{EK} = \mu_{EO} + \mu_{EP} \quad (3)$$

$$\mu_{EP} = \frac{\varepsilon_r \varepsilon_0 \zeta_{bac}}{\eta} \quad (4)$$

$$\mu_{EO} = \frac{\varepsilon_r \varepsilon_0 \zeta_{soil}}{\eta} n\tau \quad (5)$$

where ε_r is the dielectric constant of water (ε_r = 78.5), ε_0 ($1.15*10^{-2}$ kg cm h^{-2} V^{-2}) is the vacuum permittivity, η ($3.19*10^{-2}$ kg cm^{-1} h^{-1}) is the viscosity of water, ζ_{soil} (V) is the mean zeta potential of the model soil matrix (in our case glass beads) and ζ_{bac} (V) is the mean zeta potential of LB126 cells in 50 mM TA buffer; n (0.39) and τ (1.8) are the porosity and tortuosity (Kohlmeier et al. 2005) of the glass bed, respectively. Using these calculated values of μ_{EP} and μ_{EO}, the theoretical electrokinetic velocity u_{EK} (cm h^{-1}) can be estimated from

$$u_{EK} = \mu_{EK} * E \quad (6)$$

where E (V cm^{-1}) is the electric field strength applied.

Bacterial deposition: The apparent bacterial partition ratio R_d (eq. 7) relates the number of freely suspended cells (C_{pw}) at given distances (d) from the point of injection to the number of deposited cells (C_{gb}) in the corresponding volume of total porous medium.

$$R_d = \frac{C_{pw}(d)}{C_{gb}(d)} \quad (7)$$

Bacterial deposition efficiency (D_d) (eq. 8) is the concentration of deposited bacteria at distance (d) relative to the number of cells having passed this location during the elapsed time (15 hours) of the experiment.

$$D_d = \frac{C_{gb}(d)}{\int_d^{15} (C_{pw}(d) + C_{gb}(d)) dd} \quad (8)$$

Results

Influence of weak DC-electric fields on bacterial transport. When *Sphingomonas* sp LB126 cells were exposed to an electric field of 1 V cm^{-1} directed displacement of the bacteria towards the cathode (Figure 1b) was observed. Estimates based on the peak displacement (9 cm) after 15 hours of DC-application revealed a mean electrokinetic transport velocity of -0.6 cm h^{-1}. By contrast, no directed movement of the bacteria occurred in the absence of DC. The distribution of the cell abundance at the point of injection after an identical time interval (Figure 1a, open circles) was fitted best by a diffusion-driven Gaussian distribution (R^2=0.9832) (Figure 1a, solid line). Assuming Gaussian distribution thus an effective diffusion coefficient (D_{eff}) of 0.15 cm^2 h^{-1} and a mean transport velocity u_{dif} of 0.13 cm h^{-1} can be calculated (eqs. 1 & 2). Figure 1b clarifies the electrokinetic influence on bacterial dispersion by depicting the ratios of electrokinetically- and diffusion-driven cells: Electroosmosis led to an up 24-fold and a two-fold increase of suspended and surface-bound cells relative to the control at the expense of a significant (5-fold of suspended cells) reduction of cells at the injection point. Despite of the electroosmotic predominance, a minor yet statistically significant fraction of the

LB126 cells was mobilised by electrophoresis to the anode as seen by ratios above unity of adhered cells in the anode compartment.

Influence of weak DC-electric fields on bacterial deposition. Significant differences of apparent bacterial partition ratios R_d (eq. 7) of DC-exposed- and control cells were observed (Figure 2a). At the point of injection ($d = 0$), R_d of cells in the control was threefold higher than R_d of DC-exposed cells (Figure 2a). In contrast, at the cathode side an up to fourfold-enhanced R_d (d = – 9 cm) in presence of DC was observed, whereas no differences of R_d of cells at the anode side was observed. In order to account for the fact that R_d is influenced by the deposition history, i.e. that C_{gb} is the reflection of both the deposition efficiency and the elapsed time available for deposition; we further calculated the deposition efficiency (D_d) as explained in eq. 8. Results in Figure 2b show that the elevated R_d of cells transported by electroosmosis (Figure 2a) was not reflected by D_d. The calculated variations of D_d of ranging from 0.11 - 0.21 (Figure 2b) were statistically (student t-test) not significant and thus point at the absence of an effect of electrokinetic mobilisation on bacterial deposition.

Influence of intra-population heterogeneity on electrokinetic dispersion. In order to explain the observation that a fraction of the population of strain LB126 was transported to the anode (visible as a hump-shaped peak in the anode half of Figure 1b), the potential role of intra-population cell heterogeneities on electrokinetic mobilisation was tested. Bacteria were sampled at distinct locations in the model aquifer after electrokinetic treatment and subsequently analysed for their cell sizes,

membrane integrity, chromosome contents, and the charge and hydrophobicity of their cell surfaces. We observed uniform cell surface hydrophobicity (Θ_w = 40.6 ± 4.1) and cells size (length = 1.2 μm, width = 1 μm regardless of the treatment and the location sampled. Likewise, cells from DC and control treatments retrieved from all locations exhibited DNA patterns identical to the inoculum (data not shown). On average 83% and 17% of the cells possessed one chromosome and two chromosome contents, respectively. The mean zeta potential (ζ = -2.9±6.9 mV) of the LB126 inoculum was derived from electrophoretic mobility measurements by dynamic light scattering (DLS). Interestingly, significant differences in the zeta potentials of cells along the aquifer were also found after DC treatment (Figure 3b). Cells transported toward the anode were more negatively charged than cells transported toward the cathode (Figure 3b). In contrast, cells transported purely by diffusion and random movement in control experiment had more or less the same zeta potential and only cells remaining at the injection point presented a significantly higher zeta potential (Figure 3b). No correlation was observed between the cell surface charge and the integrity of the bacteria (inferred from intact membranes resisting against PI uptake (Shi et al. in press)) after 15 h of electrokinetic treatment (Table 1). The average PI-stained fraction (PI-%) of mobilized bacteria was 1.3% irrespective of DC application, which is slightly below the PI-% of the inoculum (2.3%). By contrast, increased (PI-%) of 15 and 7% (Table 1) were found at the injection points after DC-treatment and control experiments, respectively.

Discussion

Effect of the weak DC-electric fields on the dispersion and deposition of *Sphingomonas* sp. LB126. The dispersion of a non-growing population of bacteria in a porous medium around the point of injection is the result of bacterial transport that is counteracted by deposition processes. Transport mechanisms may include bacterial movement, diffusion, and hydraulic advection. In this study, we purportedly excluded hydraulic flow in order to simplify the study of physical factors influencing the electrokinetic transport of *Sphingomonas* sp. LB126. The Gaussian distribution of strain LB126 abundance around the injection point (Figure 1A, solid line) in the absence of a DC-electrical field indicates that diffusive random movement of bacteria were the only translocation mechanisms. At a circum-neutral pH, bacteria (Wilson et al. 2001) and mineral particles (Saichek and Reddy 2005) are typically negatively charged. In an electric field applied to porous media the random movement and diffusion of negatively charged bacteria will thus be superimposed by electrophoresis and electroosmosis acting in opposite directions. The observed mean transport velocity (u_{EK} = -0.6 cm h^{-1}) of LB126 cells in our model soil matrix was clearly dominated by electroosmosis and as a such about five-fold increased relative to diffusion-driven dispersion (u_{dif} = 0.13 cm h^{-1}) yet similar to the electroosmotic movement of uncharged molecular markers (Wick et al. 2004) and the theoretically predicted value (u_{EK}) of -0.82 cm h^{-1}, which was calculated based on the mean zeta potential (ζ_{soil}) of the glass bead surface (ζ_{soil} = -45.7 mV), the LB126 cells (ζ_{bac} = -2.94 mV mV) (eq. 6) , the observed electrophoretic (μ_{EP} =

0.08 $cm^2\ V^{-1}\ h^{-1}$) and the electroosmotic mobility (μ_{EO} = -0.90 $cm^2\ V^{-1}\ h^{-1}$) of LB126 cells (eqs. 4 & 5) at the experimental conditions used, respectively. The bacterial deposition efficiency (D_d) and the apparent bacterial partition ratio (R_d) were further calculated to assess the influences of electroosmotic, electrophoretic and diffusive transport mechanisms on the deposition of mobilised cells. Similar values of D_d of both control and electrokinetically treated LB126 cells (Figure 2b) however, indicated no apparent influence of electrokinetic transport on deposition of strain LB126 relative to purely diffusion-driven transport (D_{eff} of 0.15 $cm^2\ h^{-1}$). D_{eff} is in the range of typical bulk random motility diffusion coefficients derived from capillary experiments (0.005 – 0.248 $cm^2\ h^{-1}$) (Lewus and Ford 2001).

The difference between D_d and R_d can be explained by the fact that R_d is influenced by the deposition history, i.e. that C_{gb} is the reflection of both the deposition efficiency and the elapsed time available for deposition. The high R_d at cathode side in presence of DC represents a snapshot of the efficient electroosmotic displacement of the not-yet-deposited fraction of the injected population as reflected clearly in Figure 1b. Surprising in Figure 1b however free and surface-bound cells in the anode half were nearly as abundant as in the control experiment. Seen the strong overall displacement of the population toward the cathode, this is astonishing and indicates that part of the population may have undergone higher electrophoretic attraction to anode than the mean population. This observation motivated us to analyse the influence of population heterogeneity on the electrokinetic dispersion of bacteria in soil matrix.

Influence of population heterogeneity on electrokinetic transport. Heterogeneity in pure bacterial population and its significance for bacterial transport and attachment in porous media have been recognized and discussed before (Simoni et al. 1998; Bolster et al. 2000; van der Mei and Busscher 2001; Mailloux et al. 2003). For strain L126 we observed uniform cell surface hydrophobicity, cell size, and chromosome content regardless of the treatment and the location sampled. By contrast, significant differences in the zeta potentials of electrokinetically-mobilised cells along the aquifer were detected. Cells transported by electrophoresis toward the anode were more negatively charged than cells translocated by electroosmosis toward the cathode (Figure 3b). No differences in the zeta potential of diffusively transported calls however were observed in the control. The zeta potential of the cells was derived from electrophoretic mobility measurements by dynamic light scattering (DLS). Experimental values were subsequently used in eq. 4 for the calculation of the electrophoretic component of the bacterial transport. Closer inspection of the DLS results of the inoculum (Figure 3A) revealed a Gaussian-type distribution of individual zeta potential signals ranging from ζ_{bac} = -22.1 mV to ζ_{bac} = 18.6 mV and thus points at an intra-population heterogeneity of the cell surface charge of the inoculum. In order to avoid over-interpretation of this finding however, it should be noted that the DLS signal width can also be partially caused by the limitations in the precision of the determination (e.g. the Brownian motion of the cells and their variations in size or cell surface structure) (Xu 1993; van der Mei and Busscher 2001; Dong 2002). Given the intra-population heterogeneity in the zeta

potential and the continuously increasing cell surface charges towards the anode (Figure 3b, filled circles), one may derive that the model aquifer, similar to capillary electrophoresis, separated individual bacteria according to their surface charge during electrokinetic treatment. It thus appears that electrophoresis compensated electroosmosis as mobilising force and consequently resulted in an apparent translocation of more negatively charged bacteria to the anode. In order to theoretically overcompensate electroosmotic forces (eq. 5) by electrophoresis (eq. 4), a zeta potential of -30 mV of the cells would have been required rather than the observed maximum of -22.1 mV. Nevertheless similar fractions of the total population were transported towards the anode in presence or absence of an applied electric field. This indicates that for part of the subpopulation electroosmosis was just compensated by electrophoresis and that diffusion may account for the translocation in our system. It also may be that heterogeneities of the electroosmotic flow in the glass bed allowed electrophoresis to compensate the electroosmotic translocation of part of the more negatively charged subpopulation. It was also interesting to see, that in control experiments allowing for diffusion of the cells only, bacteria with higher negative zeta potentials tended to move further, leaving a subpopulation of less negative zeta potentials at the injection point behind (Figure 3b, open circles). This can be explained by favoured deposition (i.e. more retardation) of less negatively (less electrostatic repulsion) and positively (electrostatic attraction) charged bacteria (Poortinga et al. 2002). Similar to the zeta potential, a clearly enhanced PI-% of LB126 cells at the point of injection was

observed (Table 1). Increases were up to three- and sevenfold in the control and DC-treated experiment, respectively. High PI-% however was observed for cells remaining at the point of injection only. The PI-% of all mobilised (either by diffusion or electrokinetic processes) was consistently smaller than the value of the inoculum (Table 1). This surprising observation can be convincingly explained by a rough mass-balance calculation assuming that: (1) the total number of PI-stainable cells added by the inoculum (2-3%) kept unchanged after 15 hours of electrokinetic experiments; (2) only PI-unstainable cells were transported away from the injection point (for unknown reasons); and (3) thereby simply PI-stainable cells were concentrated at the injection point (Shi et al. DOI 10.1007/s10653-008-9146-0). Interestingly in the control experiment, only attached bacteria exhibited significantly enhanced PI-% (Table 1), which suggests better PI-staining of adhered cells. It should be noted that PI-incorporation into environmental bacteria does not necessarily reflect reduced viability (Shi et al. 2007). We recently observed that even actively growing *Sphingomonas* sp. LB126 bacteria were stained by PI and remained culturable on LB-agar plates (Shi et al. 2007). However, no growth was supposed to be possible during 15 h of experiments in TA buffer and no difference of DNA pattern was observed between the cells harvested at injection point and mobilized cells, and hence the active growth of LB126 cells only at injection point seems less possible. As the overall PI-% of electrokinetically mobilized bacteria was below 2% in this study, it is not daring to state that a weak DC electric field does affect the integrity of electrokinetically transported bacteria of strain LB126.

Relevance for electro-bioremediation. This work demonstrates that electrokinetics in presence of 1 V cm^{-1} of electric field can transport bacteria at velocities up to 0.4-0.8 cm h^{-1} in coarse porous media with transport heavily depending on the physico-chemical properties of both the porous media and the surface properties of individual bacteria. The results also demonstrate that minor heterogeneities of bacterial populations may have an important effect on the distribution of microorganisms. Although a pure culture was used in this study, it mimics the electrokinetic transport behaviour of differently charged subsurface bacteria. Given the right conditions and organisms, electrokinetic dispersion could thus be used to distribute specialized bacteria in hotspots of contamination, e.g. in aquifer sediments. To our knowledge, this is the first study that indicates the absence of electrokinetic effects on the deposition efficiency of bacteria in (model) soil matrices. The observation that electrokinetically transported bacteria of strain LB126 exhibited very low permeability for propidium iodide (as a cell integrity indicator) is promising in the perspective of utilizing catabolically active bacteria under mild electrokinetic conditions.

Acknowledgements

The authors wish to thank H. Engewald, T. Hübschmann, J. Reichenbach, R. Remer, C. Süring and B. Würz for skilled technical assistance.

Figure Legends

Figure 1. (a) Gaussian-fit (solid line) of the relative distribution of *Sphingomonas* sp. LB126 recovered from the model aquifer after the injection of about $3 * 10^{10}$ cells mL^{-1} in the absence of DC (open circles). (b) Numbers of free (filled squares) and surface-bound cells (open squares) of strain LB126 cells after exposure to 1 V cm^{-1} for 15 h relative to free and surface-bound cells recovered in the control experiment depicted in panel A. Negative distances direct towards the cathode while positive to anode. Figure 1 represents average and standard deviations of ≥ 3 independent experiments.

Figure 2. Apparent bacterial partition ratios (R_d, a) and deposition efficiencies (D_d, b) of electrokinetically mobilized (filled symbols and columns) and control cells (open symbols and columns). Negative distances direct towards the cathode while positive to anode. Figure 2 represents average and standard deviations of ≥ 3 independent experiments.

Figure 3. (a) Representative distribution of the zeta potential (ζ) of an axenic culture of *Sphingomonas* sp. LB126 as calculated from dynamic light scattering (DLS) experiments. The distribution is a result of the Brownian motion, heterogeneity of surface structure and surface charge of the bacteria. (b). Representative distribution of the ζ of cells harvested along the aquifer in presence (filled circles) or absence (open circles) of an electric field strength of (E = 1 V cm^{-1}) for 15 h. Negative distances direct towards the cathode while positive to anode.

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Influence of DC-electric field on soil-compound interaction

5

Electroosmotic flow stimulates the release of alginate-bound phenanthrene

Electroosmotic Flow Stimulates the Release of Alginate-Bound Phenanthrene

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Received September 19, 2007. Revised manuscript received December 10, 2007. Accepted December 17, 2007.

There is growing interest in employing electro-bioremediation, a hybrid technology of bioremediation and electrokinetics for the treatment of contaminated soil. Most present applications of electrokinetics aim at pollutant extraction, which requires transport over large distances facilitated by electroosmotic flow (EOF). They do not explicitly account for the possibility that EOF passing along soil particles stimulates the release of hydrophobic organic compounds (HOC) and locally improves pollutant bioavailability. Here, we report on the stimulated release of polycyclic aromatic hydrocarbon (phenanthrene) from model organic matter in the presence of direct current (DC)-electric fields (0.5–2 V cm^{-1}) typically used in electro-bioremediation measures. Alginate beads were employed as a model polymer release system (MPRS) exhibiting similar release behavior as natural organic matter (NOM). In the presence of EOF the phenanthrene release flux from alginate beads was between 1.4- and 1.8-fold higher than under hydraulic flow conditions with equal bulk water velocity and 30—120-fold higher than under stagnant water conditions. Our data suggest that DC-electric fields (0.5–2 V cm^{-1}) can stimulate the release of PAH bound to particles exposed to stagnant water zones often found at hydraulic flow regimes restricted by low permeability.

Introduction

Various studies have shown that the sequestration of hydrophobic organic compounds (HOC) in the solid soil phase by sorption and entrapment reduces their bioavailability (*1*, *2*). As a consequence of progressive binding with soils, residual HOCs may become less leachable and thus unavailable for biodegradation as bacteria appear to degrade chemicals only in proportion to their dissolved phase chemical activity (*3*). Three potentially rate-limiting steps of HOC release have been identified: (i) diffusion of the sorbate within the molecular nanoporous network of natural organic matter (NOM) (*4*), (ii) pore or surface diffusion in aggregated minerals constituting natural particles (*5*), and (iii) diffusion of the sorbate across an aqueous boundary layer at the exterior of soil particles (*6*). In recent years, there has been increasing interest in employing electro-bioremediation, a hybrid technology of bioremediation and electrokinetics for the treatment of soil contaminated with HOC. Several studies (for a review, see ref *7*) have demonstrated that electric fields (ca. 0.2–2 V cm^{-1}) applied to soil improve the biodegradation of organic pollutants such as gasoline hydrocarbons and aromatic compounds. HOC mobilization by electroosmotic flow (EOF) was suggested as the principal mechanism promoting bioavailability (*8*). EOF is the surface-induced movement of pore fluids in an electric field, usually from the anode toward the cathode. Due to its surface-dependency it is more efficient in fine-grained soils (with μm or smaller pores) than in coarse-grained soils (*9*). This is of particular technical interest since the conductivity of fine-grained soils for pressure-driven (e.g., hydraulic) water flow is extremely small leading to quasi-stagnant water in micro- and nanopores. Electroosmotically enhanced in situ flushing of HOC contaminated soils gave promising results (*10*), yet to our knowledge no mechanistic studies on the impact of EOF on HOC release from soil particles have been performed. Indications for possible effects come from the field of capillary electrochromatography (CEC) where EOF is used to enhance the transfer of solute between the mobile and stationary phase. As a result, CEC separates uncharged molecules better than high performance liquid chromatography (HPLC) that relies on pressure-driven-flow (*11*).

Here we tested our hypothesis that EOF may overcome mass-transfer bottlenecks (*7*) in soil matrices of low permeability as it moves water along the immediate exterior of soil particles and inside aggregated minerals. We determined the influence of EOF on the release kinetics of phenanthrene from alginate beads in a saturated model soil matrix and compared it with the results of theoretical mass transfer calculations. Alginate beads constitute well-described model polymer release systems (MPRS) reported to exhibit similar PAH-release behavior as complex natural organic matter (NOM) (*12*). Electrical field strengths of 0.5 V cm^{-1}, 1 V cm^{-1}, and 2 V cm^{-1}, typical for electro-bioremediation measures were used to produce EOF at velocities of 1–5 cm h^{-1} through alginate bead packing. Comparison of the effects of EOF with those of hydraulic flow of equal velocity or stagnant conditions revealed that EOF stimulates the release of alginate-sequestered phenanthrene significantly.

Materials and Methods

Alginate Beads. Alginate beads were prepared from a homogeneous mixture of 4% (w:w) alginate (Protanal LF 10/60, FMC BioPolymer, Drammen, Norway), 0.2% (w:w) phenanthrene (Fluka, purum, for fluorescence, 97% (HPLC)), 9.8% (w:w) kaolinite (Fluka, purum, natural), and bidistilled water following the procedure described by Wells et al. (*12*) with a bead maker (encapsulation unit VAR J1, Nisco Engineering, Zurich, Switzerland). Solid phenanthrene was sonicated, fractionated, and repeatedly sieved prior to bead formation. Five groups of 20–80 beads were randomly selected to estimate the effectiveness of phenanthrene loading. The beads were first levigated thoroughly together with sodium sulfate (Merck, for analysis) to deplete water and then extracted with 3 mL of hexane (Merck, for analysis) by shaking for 30 min in the presence of deuterated phenanthrene as an internal standard (10 μg mL^{-1}, phenanthrene D10, 99.5%, Dr. Ehrenstorfer GmbH). The extraction was carried out three times in a 10 mL brown glass vial. The pooled extracts were analyzed by GC-MS. Fifty alginate beads were randomly selected to determine their diameters by image analysis (camera DXC-9100P) using the OpenLab software (Improvision, England).

Characterization of the Surface Charge of Alginate Beads. The zeta-potential of alginate beads was approximated from the electrophoretic mobility measured by a Doppler

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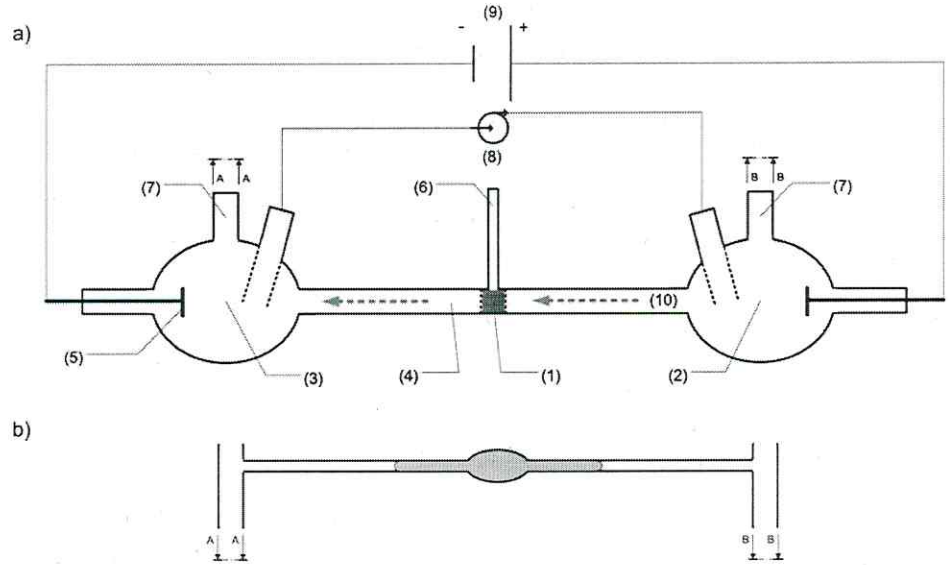

FIGURE 1. Schematic view of the setup used for electrokinetic experiments. (a) A saturated model soil chamber (1 cm i.d., 1 cm (L)) (1) is confined by glass frits (pore size of 160–250 μm) and packed with alginate beads. A glass tube (4) connects the model soil chamber with the electrode compartments (about 150 mL each) containing two titanium-iridium electrodes as the cathode (3) and anode (2). The disk-shaped electrodes (5) are welded onto a connector rod and connected to a DC power pack (9). A peristaltic pump (8) circulates phosphate buffer from the cathode side to the anode side either to provide a hydraulic flow (10) through the bead packing or to serve as a hydraulic bypass, i.e., to diminish the hydraulic head created by electroosmotic flow. Sampling ports allow taking samples from the pore water of the bead packing (6) and from two electrode compartments (7). (b) A removable bridge with a capillary (4 mm i.d.) holding an air bubble (gray shade) is used to measure the velocity of the electroosmotic flow.

electrophoretic light scattering analyzer (Zetamaster, Malvern Instruments, Malvern, UK) according to the method of Helmholtz-Smoluchowski (*13*). Prior to the measurements, the alginate beads were levigated to form a colloidal suspension (*14*) in 10 mM phosphate buffer (PB) with a pH of 6.5–6.8. Twenty of alginate beads were randomly selected and the electric resistance of individual alginate beads measured by FLUKE 185 multimeter.

Analytical Methods. Extracts (hexane samples) were analyzed by GC-MS (Agilent GC6890 and MSD5973N) on an HP5MS column (30 m × 250 μm × 0.25 μm) using helium at a flow rate of 1 mL min^{-1}. Phenanthrene in water samples from batch experiments was analyzed by UV absorbance (Cary 400Scan, Varian, U.S.) at 250 nm in a quartz cuvette (Hellma 104-QS, 10 mm light path). Phenanthrene in water samples from electrokinetic experiment was analyzed by high performance liquid chromatography (HPLC) (Shimadzu Class-VP) on an RP-18 column (Nucleosil 100–5 C18 4 mm ID) using an isocratic mobile phase (MeOH/water (90:10 v/v); flow: 1 mL min^{-1}) and UV detection at 250 nm. UV absorbance of phenanthrene from method (*2*) was also calibrated for phenanthrene concentration by HPLC.

Batch Release Experiments. Alginate beads were incubated in 150 mL PB in 300 mL Erlenmeyer flasks covered by glass stoppers on a gyratory shaker at 150 rpm at room temperature in the dark. Prior to incubation, 0.61 ± 0.02 g (wet weight) of beads were given into a 10 mL sterilized glass vial and washed three times with 8 mL PB. Water samples of 1 mL were repeatedly transferred into a quartz cuvette using plastic pipet tips and analyzed for UV absorbance of phenanthrene at 250 nm. After the first 24 h of phenanthrene release, the beads were washed and transferred to the same amount (150 mL) of fresh PB for another 24 h of release. Release kinetics in the first and second 24 h were calculated and fitted by linear regression.

Electrokinetic Setup and Release Experiments. The electrokinetic apparatus made of borosilicate glass was composed of three compartments: two electrode compartments (about 150 mL each) at both ends and a central buffer-saturated model soil chamber of 1 cm inner diameter and 1 cm length as the central part of a tube connecting both electrode chambers (Figure 1a). Two glass frits (pore size of 160–250 μm) separated the model soil chamber from the connecting tube. The small volume and horizontal extension of the model soil chamber were chosen to mimic a point source of pollutant and to minimize effects of long distance transport (e.g., repeated de- and absorption) on the observed release kinetics. The whole electrokinetic setup was mounted in a frame to keep it precisely horizontal. Titanium-iridium disk-shaped (12 mm in diameter and 1.5 mm thick) electrodes (De Nora Deutschland GmbH) were welded onto connector rods (3.0 mm in diameter and 5 cm length). Electrodes were inserted at 1 cm from both ends and 24 cm apart from each other. The whole electrokinetic setup was filled with PB. The anode and cathode were connected to a PowerPac (P333, Szczecin, Poland) capable of delivering direct current of 0–2 A up to a voltage of 50 V. Two outlets placed at the middle of each electrode compartment (Figure 1a) were used to circulate PB from the "cathode side" to the "anode side" by a peristaltic pump (ISM 935, Ismatec, Glattbrugg, Switzerland) with sterilized tubing (Pharmed, Ismatec, SC0740, ID 0.25 mm). In the absence of DC-fields, pumping was used to create hydraulic PB-flows (8–32 μL min^{-1} depending on experimental conditions applied) through the central model chamber. In the presence of electric fields however, the pumping of PB served as a "bypass function" to remove the hydraulic head at the cathode side created by the electroosmotic flow (EOF). Pumping rates were adjusted in a way that they exactly balanced the EOF and thus did not actively add any water flow other than EOF in the central model soil chamber. This required measuring the EOF velocity in the absence of the pumping bypass. To this end, the pump was replaced by a rigid glass capillary (ID 4 mm; Figure 1b) connecting both electrode compartments as a bridge (Figure

1b), and the displacement of an air bubble filling the whole diameter of the capillary was followed.

About 330 alginate beads corresponding to 0.65 ± 0.05 g were carefully packed into the central model soil chamber through a thin glass tube (2 mm i.d. and 5 cm (L)) within 10 min. The bead packing had a calculated porosity of 0.5. Immediately after packing, 100 μL of pore water was removed with a sterilized 1 mL syringe from the model soil chamber and both electrode compartments. The whole setup was then left for 12 h (i.e., with no pumping, no electric field application, and no sampling). Then, a second set of samples was taken before the hydraulic flow was started. Hydraulic flow was applied alone for 6 h, and then an electric field (0.5 V cm^{-1}, 1 V cm^{-1}, and 2 V cm^{-1}) was applied for another 6 h along with hydraulic pumping at identical flow velocity through the active bypass (Figure 1a) (*8*),) to diminish backward hydraulic flow though the model soil chamber. No displacement of the air bubble in the capillary bridge in reasonable time (e.g., 1 h) was observed when the back pressure created by EOF was counteracted by pumping. Pore water samples were collected for phenanthrene analysis by HPLC at intervals into 2 mL glass vials with 200 μL microinserts. Prior to sample collection, 5 μL of mercury chloride (1 mg mL^{-1}) was added into each insert to prevent any microbial degradation. The pH of water samples was measured by pH indicator paper (Merck, pH 0–6 and 5–10). The temperature was measured in the cathode and anode chambers.

Mass Transfer Calculations. *Mass Balance.* Experimental release rates were used to establish a balance of the phenanthrene release. The mass of phenanthrene released from alginate beads into the bulk solution during a given period (ΔM_{rel}) can be expressed by eq 1:

$$\Delta M_{rel} = \Delta M_{pore} + M_{out} - M_{in} \quad (1)$$

ΔM_{pore} is the change of phenanthrene mass in the aqueous phase of the model soil chamber as given by eq 2:

$$\Delta M_{pore} = \Delta C_{dis} V_{pw} = [C_{dis}(t_2) - C_{dis}(t_1)] V_{pw} \quad (2)$$

M_{out} is the phenanthrene mass washed out from bead packing by either hydraulic or electroosmotic flow. M_{out} can be simplified to the average concentration in two consecutive samplings (eq 3):

$$M_{out} = \int_{t_1}^{t_2} C_{dis}(t) V \mathrm{d}t = \frac{C_{dis}(t_1) + C_{dis}(t_2)}{2} V(t_2 - t_1) \quad (3)$$

M_{in} expresses the phenanthrene mass brought into MPRS packing by either hydraulic flow or electroosmotic flow, which is negligible relatively to ΔM_{pore} and M_{out}. ΔC_{dis} is the change of the pore water phenanthrene concentrations $C_{dis}(t_2) - C_{dis}(t_1)$ measured at two successive time points, V_{pw} is the volume of pore water in the central model soil chamber and V is the flow velocity (μL s^{-1}) driven either by EOF or hydraulic pumping.

Release Flux. Based on measured phenanthrene concentration in pore water samples, the specific release flux (q) can be calculated by eq 4 from $C_{dis}(t_1)$ and $C_{dis}(t_2)$ at successive time points:

$$q = \frac{\Delta M_{rel}}{(t_2 - t_1) \times A} = \frac{[C_{dis}(t_2) - C_{dis}(t_1)] V_{pw} + \frac{C_{dis}(t_1) + C_{dis}(t_2)}{2} V(t_2 - t_1)}{(t_2 - t_1) \times A} \quad (4)$$

where A is the total surface area of all alginate beads in the model soil chamber, C_{sat} is the saturation concentration of phenanthrene at 25 °C in water (1124.4 μg L^{-1}). A has been estimated from the number of alginate beads and the mean radius of these beads.

Diffusive Boundary Layer Thickness. The film mass transfer coefficient (k_L) of phenanthrene from alginate into water can be estimated from q by eq 5:

$$k_L = \frac{q}{(C_{sat} - C_{dis})} \quad (5)$$

k_L was used to estimate the apparent thickness of the diffusive boundary layer (ζ_{DBL}) based on the radial diffusion model from film mass transfer theory (*15*) as in eq 6:

$$\zeta_{DBL} = \frac{4\pi D_{eff} r^2}{k_L A - 4\pi D_{eff} r} \quad (6)$$

where D_{eff} is the diffusion coefficient (1.57 × 10^{-2} cm^2 h^{-1}) of phenanthrene (*16*) in water and r (cm) is the radius of the alginate beads.

Results and Discussion

Characteristics of Alginate Beads Used As Model Polymer Release System. On average, 1.3 ± 0.3 μg of phenanthrene was entrained in each spherical alginate bead with a mean radius of 0.665 ± 0.015 mm. Due to strong cross-linking by $BaCl_2$ (0.3 M) and the kaolinite content, the beads were negligibly swellable (*12*). In earlier work, the alginate matrix was reported to bring about anomalous (non-Fickian) release kinetics that mimic PAH release from natural materials well and especially those observed with natural organic matter (NOM) (*12*). The mean diameter of phenanthrene particles is smaller than 30 μm, and thus ca. 40 times smaller than the diameter of alginate beads. Phenanthrene dissolution kinetics hence could be assumed to be much faster than the diffusion mass transfer to the bead surface (*12*).

The mean zeta potential (ζ_{alg}) of the alginate beads in PB was −45.1 ± 1.8 mV. The electroosmotic mobility μ_{EO} of 1.1 × 10^{-4} m^2 h^{-1} V^{-1} in the bead packing containing PB was calculated by the following equation (*17*):

$$\mu_{EO} = \frac{\epsilon_r \epsilon_0 \zeta_{alg}}{\eta} n\tau \quad (7)$$

where ϵ_r is the dielectric constant of water (ϵ_r = 78.5), ϵ_0 (8.85 × 10^{-12} F m^{-1}) is the vacuum permittivity, η (3.19 kg m^{-1} h^{-1}) is the water viscosity, τ (1.8), and n (0.5) (*18*) are the tortuosity and porosity of the packing, respectively.

Due to the low electric field strengths (0.5–2 V cm^{-1}) used and the use of $BaCl_2$ as cross-linking agent, no apparent changes of size and weight of the alginate beads were observed during the electrokinetic treatment (data not shown). This is in accordance with earlier observations by Zvitov et al. (*19*).

Phenanthrene Release Kinetics in Shaken Batch Systems. Phenanthrene release from alginate beads was first studied in shaken batch systems. Biphasic release kinetics was observed (filled circles in Figure 2). Linear regression-analysis of release kinetics revealed an about 3-fold difference between the initial "fast" release (release rate of 2.63 μg L^{-1} min^{-1} with R^2 of 0.99) and the "slow" release (release rate of 0.99 μg L^{-1} min^{-1} with R^2 of >0.99) after approximately 2 h. After 24 h, the alginate beads were transferred again into fresh buffer. No further changes in the slow release kinetics of pretreated alginate beads were detected (Figure 2, represented by ○). The apparent biphasic fast and slow release demonstrates that the shrinking core model (SCM) of diffusion is appropriate to describe the release of phenanthrene from alginate beads (as well as from some sediments and soils) (*12*). As the soluble phenanthrene diffuses out, more of the entrained solid phenanthrene dissolves and the

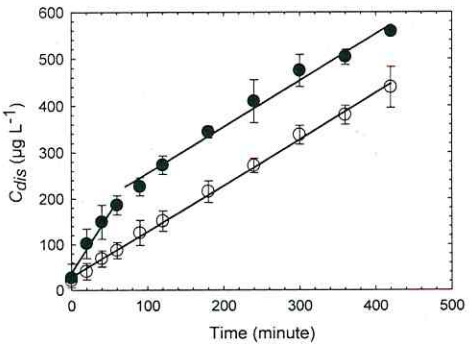

FIGURE 2. Kinetics of phenanthrene release (C_{dis}: phenanthrene concentration in PB) from 0.61 ± 0.02 g alginate beads in 150 mL phosphate buffer (PB) in batch experiments. The initial fast release phase was followed by slow release (●). After 24 h of continued release (not shown), the beads were transferred to the same amount of fresh PB and release resumed for 7 more hours (○). Linear regressions are indicated by straight lines. One standard deviation from n = 4 independent experiments is presented by error bars.

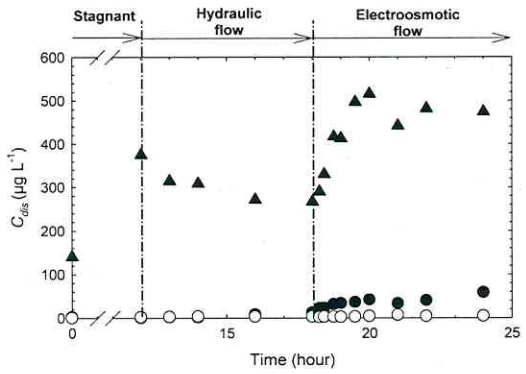

FIGURE 3. Representative phenanthrene release experiment performed in the electrokinetic apparatus. Each experiment was divided into three phases: (1) stagnant condition for 12 h; continued by (2) hydraulic flow (of equal velocity as the subsequent electroosmotic flow) for six hours and at last (3) electroosmotic flow (e.g., at electric field strength of 1 V cm^{-1} as shown in this figure) for 6 hours. The intention of this study was to compare the release kinetics of alginate-bound phenanthrene at different flow conditions (i.e., stagnant, hydraulic flow, and EOF) yet in a quasi-identical experimental setup. Dissolved phenanthrene concentration (C_{dis}) were followed in the pore water of the beads packing (▲), the cathode compartment (●) and the anode compartment (○).

saturated core containing solid phenanthrene gradually shrinks in radius.

Electrokinetic Stimulation of Phenanthrene Release from Alginate Beads. The alginate beads used in electrokinetic release experiments were first washed for 12 h to obtain a constant slow release flux during the entire experiment. The mass of phenanthrene released during the entire experiment was less than 20 µg of the total content of 260 µg of solid phenanthrene. Phenanthrene release before and during a typical electrokinetic treatment at 1 V cm^{-1} is shown in Figure 3. Release experiments comprised three phases: stagnant conditions for 12 h, followed by hydraulic flow for 6 h, and finally EOF for 6 h, to compare the release kinetics of alginate-bound phenanthrene at different flow conditions (i.e., stagnant, hydraulic flow, and EOF) yet in a quasi-identical experimental setup. Under stagnant conditions phenanthrene concentrations in both electrode compartments (Figure 3, ● and ○) remained below the detection limit (<3 µg L^{-1}) due to the absence of advective flow and slow aqueous phenanthrene diffusion, whereas in the pore water of the model soil chamber it rose to 324 ± 47 µg L^{-1}. The application of hydraulic flow decreased the aqueous phenanthrene concentration in the model soil chamber by 25% and led to a continuous increase of the phenanthrene concentration to 12 µg L^{-1} in the cathode compartment. By contrast, the phenanthrene concentration in the anode compartment remained <8 µg L^{-1} due to minimal phenanthrene transport through the hydraulic bypass. In the third phase the hydraulic water flow through the model soil chamber was replaced by EOF of equal bulk flow velocity. An increase of phenanthrene in the cathode compartment and about a 2-fold increase of the pore water phenanthrene concentration in the model soil chamber were observed (Figure 3, ▲). The phenanthrene release depended on the DC-electric fields applied to the beads packing (not shown here, processed data shown in Figure 4). Current densities at 0.5, 1, and 2 V cm^{-1} were 0.78 ± 0.02, 1.51 ± 0.11, and 3.25 ± 0.28 mA cm^{-2}, respectively. During the 6 h of DC treatment, the pH in the model soil chamber remained constant (6.5–6.8) at all field strengths. Minor acidification (at 0.5 V cm^{-1}: pH ≈ 6.5–5.0; 0.1 V cm^{-1}: pH ≈ 6.8–4.5; 2 V cm^{-1}: pH ≈ 6.5–3.0) and alkalinization (no change at 0.5 and 1 V cm^{-1}: and at 2 V cm^{-1}: pH ≈ 6.5–8.5) only were observed in the anode and cathode compartments, respectively. No temperature change was observed in cathode and anode chamber during 6 h of DC treatment.

As the hydraulic and EOF average bulk flow rates were chosen to be equal, EOF appeared to induce a higher phenanthrene release than the hydraulic flow at equal macroscopic flow rates. The specific release fluxes (i.e., the amount of phenanthrene released per unit time and unit bead surface area) were calculated using eqs 1–4 from measured pore water concentrations of phenanthrene (Figure 3) at every two successive time points during the electrokinetic release experiment. In the absence of DC, the release flux and hydraulic flow velocity (0–5 cm h^{-1}) were directly proportional (R^2 > 0.99) (Figure 4A). Similarly, EOF-induced phenanthrene release was directly proportional (R^2 > 0.99) to the EOF and the electric field strengths applied (Figure 4A). Relative to stagnant conditions, the phenanthrene release fluxes in the presence of EOF and hydraulic flow were 30−120-fold and 20−70-fold enhanced, respectively. This drastic increase can be mainly attributed to the advective pore water exchange created by either type of flow and illustrates the strong influence of pore water movement on sorbate release. More important in the context of the present study is the observation that EOF-induced phenanthrene release fluxes were 1.4−1.8-fold higher (Figure 4A) than those in the presence of hydraulic flow of equal bulk velocity (Figure 4A).

Mechanism of Phenanthrene Release under EOF. We assumed that the dissolution of phenanthrene microcrystals inside of the alginate beads does not limit the release, hence leaving slow diffusion of dissolved phenanthrene inside the alginate beads and through the diffusive boundary layer (ζ_{DBL}) (DBL) around alginate beads as the rate-limiting steps (*20, 21*). As to the latter, we further assumed that the peculiarities of EOF as a surface-dependent water movement reduces the effective thickness of DBL. Intra-aggregate EOF in the alginate beads caused by the embedded kaolinite particles is unlikely to happen due to the measured >1000-fold higher resistance of the alginate beads (20–30 MΩ) than in the model soil chamber. Theory predicts for ideal conditions (i.e., a capillary channel with uniform morphology) the hydraulic flow to exhibit a parabolic flow profile (*22*) (Figure 5, curve B). EOF, instead, has a plug-flow profile (Figure 5, curve A) (*22*); The

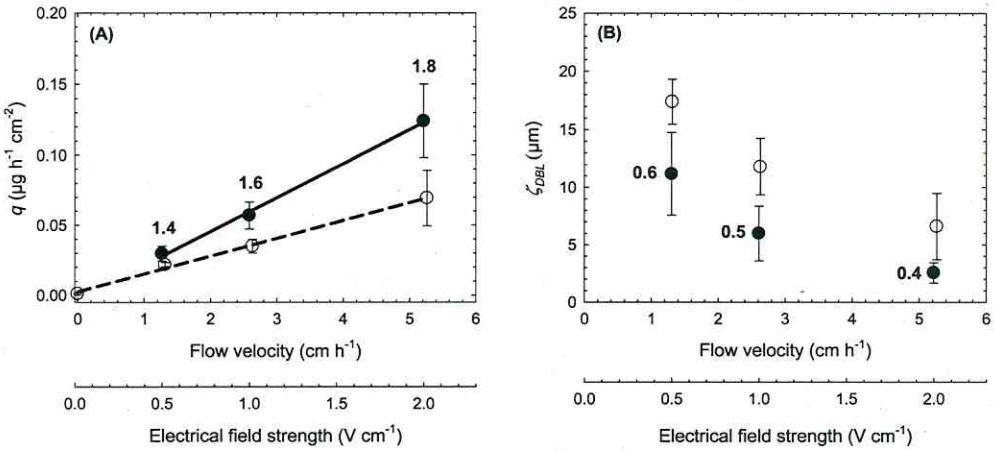

FIGURE 4. (A) Release fluxes (q) from alginate beads during electrokinetic experiments plotted against the velocities of hydraulic (○) and electroosmotic flow (●) and the electrical field strengths corresponding to the latter (linear regressions gave $R^2 > 0.99$). (B) Calculated apparent thicknesses of the diffusive boundary layers (ζ_{DBL}) in the presence of electroosmotic (●) and hydraulic flow (○). The labels represent the calculated ratios of q (Figure 4A) and ζ_{DBL} (Figure 4B) at electroosmotic relative to hydraulic flow conditions. The observed differences are statistically significant (*t*-test). The error bars represent one standard deviation derived from $n \geq 3$ independent experiments.

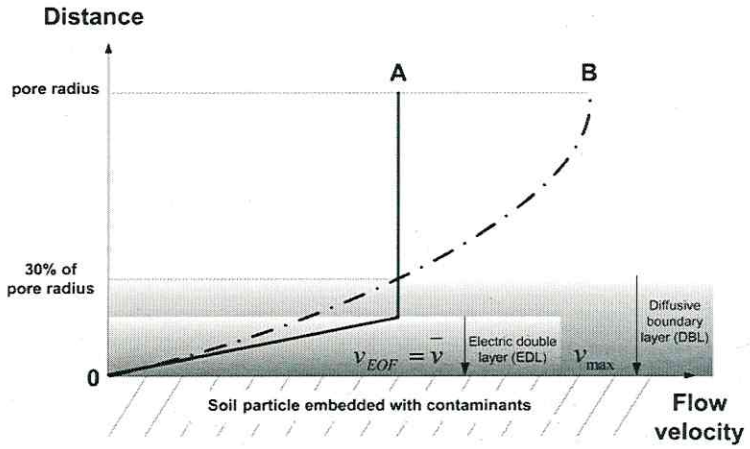

FIGURE 5. The overlay of schematic illustrations of near-particle-surface flow profiles from hydraulic flow and electroosmotic flow (EOF) in ideal condition (i.e., single straight channel with uniform morphology). Under ideal conditions, EOF has a profile deviating from plug flow only inside of the electric double layer (EDL), which is the origin of the EOF (curve A). Hydraulic flow, instead, has a parabolic profile (curve B). Different flow profiles cause that at equal bulk linear flow, hydraulic flow attains equal velocity only at a larger distance from the solid surface (indicated by the arrow). When hydraulic flow is laminar, the release of hydrophobic organic compounds (HOCs) is constrained by diffusion through the diffusive boundary layer (DBL) which ideally does not exist in pure electroosmotic flow. Note that the thickness of EDL and DBL are not represented to scale.

velocity of a parabolic flow of pore water (v) decreases continuously while it approaches the soil surface and becomes smaller than the bulk average flow velocity when the distance to soil surface is smaller than 30% of R, i.e. the pore radius (eq 8):

$$v = \bar{v} = \frac{1}{2}v_{max} = v_{max}\left(1 - \frac{r^2}{R^2}\right) \qquad (8)$$

$\bar{v}$ is the bulk average flow velocity which is the half of maximum flow velocity (v_{max}) at the center of soil pore, and r is the distance from the center. By inserting the measured phenanthrene pore water concentrations into eqs 1–6 a continuously reduced DBL-thickness from 18 to 7 μm (Figure 4B) and an approximately 3-fold enhanced phenanthrene release flux (Figure 4A) were calculated when the hydraulic flow rate was increased from 1 to 5 cm h^{-1}.

In comparison to the hydraulic flow, EOF has a near-plug-flow profile (Figure 5, curve A) (*22*). EOF originates from the solid surface (*22*) and its flow velocity is supposed to change inside of the electric double layer (EDL) only (Figure 5). From eq 9 a thickness of the ζ_{EDL} of 2.2 nm can be derived.

$$\zeta_{EDL} = \left[\frac{\epsilon_r\epsilon_0 RT}{2IF^2}\right]^{1/2} \qquad (9)$$

In eq 9 R (8.314 J K^{-1} mol^{-1}) is the universal gas constant, F (9.65 × 10^4 C mol^{-1}) the Faraday constant, T the temperature (293.15 K), and I the ionic strength of the

electrolyte. The I of the 10 mM phosphate buffer used in our experiments is 20 mM as derived from eq 10,

$$I = \frac{1}{2}\sum_{i=1}^{n} c_{\mathrm{bulk},i} z_i^2 \qquad (10)$$

with c_{bulk} being the bulk concentration and z is the charge of the individual ions (K^+, HPO_4^{-2}, and $H_2PO_4^-$). Note that ζ_{EDL} is independent from the field strength. This is somewhat contradictory to our results (Figure 4B) proposing a decrease of the effective ζ_{DBL} from 11 to 3 μm depending on the electric field strength (eqs 5–6). This requires explanation as does the calculation that the effective ζ_{DBL} of 3–11 μm was much thicker than the hypothetical ζ_{EDL} of 2.2 nm only. We propose as an explanation that in a porous medium of relatively large pore diameters, local EOF will create hydraulic flow; Once being transported downstream from their place of acceleration by EOF, water molecules in the bulk will move just like water molecules driven by hydraulic flow without remembrance of the original accelerating force. In a packed bed that principally allows hydraulic flow to occur, local EOF will thus predominantly be superimposed by hydraulic flow from upstream with the characteristics of the latter such as reduction of the effective ζ_{DBL} at faster flow rate (e.g., induced by higher electric field strengths). We further propose the nonuniformity of EOF in the pores of packed beds as a second explanation: Numerical simulation by Hlushkou et al. (*23*) revealed that EOF velocity profiles in individual pores of a bead packing deviate from the ideal plug-flow profiles (Figure 5, curve A) in single straight channels due to the nonuniformity of the local electrical field strength, particularly at the solid–liquid interface. Packed beds differ from capillary situations as large fractions of the solid surface of packed beds are not aligned parallel to the direction of the electric field and thus may only contribute to EOF by their local tangential component to the applied electric field. In certain locations for instance, the solid–liquid interface can even be perpendicular to the direction of applied electric field, and hence not contribute to the EOF. The DC-induced reduction of the effective ζ_{DBL} and the resulting release fluxes are thus likely to be caused by EOF-induced changes of the flow velocity-profiles rather than the theoretically predicted EDL thickness in a given electric field.

Relevance for Electro-Bioremediation. This work demonstrates that electroosmosis can stimulate the release of HOC from (model) soil particles up to 30–120 times in comparison with stagnant conditions. The average macroscopic velocity of EOF in the presence of an electrical field strength of 0.5–2.0 V cm^{-1} typical for electro-bioremediation measures was about 1−5 cm h^{-1}. Compared with hydraulic flow of equal linear velocity, the HOC release flux in the presence of EOF was still 1.4−1.8-fold increased. It should be noted that this enhancement was achieved in a system with relatively large pores. In low permeability soils permitting either extremely limited hydraulic flow or none at all (*24*), EOF should be able to more clearly enhance the release of HOC since it would be a way to move stagnant water particularly in small pores. To our knowledge, this is the first study that indicates the stimulation of electroosmotic flow on HOC release from (model) soil particles. Although not addressed here, we expect EOF to enhance the local HOC-bioavailability and as a consequence the bioremediation efficiency. Future studies will address the effect of electroosmosis on the release of PAH sequestered by real soil particles and on HOC bioavailability.

Acknowledgments

We thank J. Reichenbach, R. Remer, C. Süring, and B. Würz for skilled technical assistance and FCM BioPolymer for providing alginate.

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6 Summary and Concluding Remarks

6.1 Summary

6.1.1 Influence of DC-electric field on microorganism-compound interactions

6.1.2 Influence of DC-electric field on soil-microorganism interactions

6.1.3 Influence of DC-electric field on soil-compound interactions

6.1.4 Relevance for electro-bioremediation

6.2 Concluding Remarks

6.2.1 Personal retrospect

6.2.2 My opinion on electro-bioremediation

6.1 Summary

The idea of electrokinetics is to stimulate the mobilization of soil components, contaminants and/or microorganisms in order to either physically remove or to disperse them. For *in-situ* electro-bioremediation small scale dispersion of HOC is intended rather than 'macroscopic' HOC-transport (HOC-extraction), as bacteria are ubiquitous in soil. Assuming average separation distances of $\leq$ 100 microns (1) between individual bacterial micro-colonies, short distance transport may drastically improve the contact between pollutant molecules and bacteria and, concomitantly, biodegradation (2,3). However, present electro-bioremediation approaches mainly aim at pollutant transport over large distances and tend to neglect both the impact of DC on organism-soil interactions and microscale HOC release rates. Accordingly, no data are available on bioavailability changes acting via DC-driven effects on organism–compound and organism–soil-interactions. This is a serious gap of our current knowledge hampering the improvement of the electro-bioremediation methodology. The goal of this study was to gain a better insight into the various influences of DC on processes influencing HOC-bioavailability in soil, in particular the DC influence on microbial physiology and the physico-chemistry of organism–matrix and organism-compound interactions as shown in the electro-bioremediation tetrahedron figure (Chapter 1-Figure 4, Chapter 2). The whole thesis has been structured around this tetrahedron and has approached the primary open questions in electro-bioremediation from three main aspects (Chapters 3-5).

6.1.1 Influence of DC-electric field on microorganism-compound interactions.

Optimal biodegradation activity requires that DC-electric field has no negative effect on the physiology of the biocatalysts. For the electro-bioremediation of HOC-soil contaminants, however, sound data on the effect of weak electric fields on HOC-degrading soil microorganisms is still missing. This is surprising since (engineered) bioremediation of

polluted soil significantly depends on microbial activity and aims at the stimulation of indigenous microorganisms capable of degrading the pollutants.

The influence of DC-electrical fields typical for electro-bioremediation measures (1 V cm^{-1} of electric field strength with 10.2 mA cm^{-2} of current density) on the physiology of PAH-degrading *Sphingomonas* sp. LB126 was presented in Chapter 3.2. Strain LB126 was chosen as a representative of PAH-degrading microorganisms, since sphingomonads are important degraders of PAHs in soil (4). This study was done in a well-defined laboratory setup with inert electrodes and simple phosphate buffer to exclude possible side impacts from the application of DC, such as pH changes, ohmic heating and toxic electrode effects. A series of indicators of cellular integrity or functionality were employed to characterize the physiological state of strain LB126 from several important aspects, such as (i) cell viability indicated by membrane integrity through propidium iodide (PI) staining measured by flow cytometry; (ii) cell catabolic activity indicated by ATP content, transmembrane potential measured by flow cytometry, and fluorene biodegradation via Michaelis Menten kinetics; and (iii) cell reproducibility indicated by cell culturability on solid medium.

No changes of the membrane integrity, the transmembrane potential, the fluorene biodegradation activities and the culturability were observed irrespective of the application of DC-electric fields. DC-exposed cells exhibited an up to 60% elevated intracellular ATP levels relative to the control, which stimulates further research. In the same study, the influence of DC-electric field on cell-surface properties (e.g. hydrophobicity and surface charge) were also investigated as they are highly relevant to bacterial adhesion and consequent mobility in subsurface. No changes in surface charge and hydrophobicity of strain LB126 were observed.

In an accompanying study the feasibility of propidium iodide (PI) as cell viability indicator was assessed. As PI has been described manifold in literature to penetrate injured membranes and cell walls, the dye is often applied as a cell viability indicator to distinguish living from dead microorganisms (5-7). Users for environmental applications are often

unaware that commercially available PI viability kits are only validated for medically relevant strains, such as *E. coli*. Here the reliability of PI as an indicator for the viability of two PAH-degrading soil bacteria, the gram$^-$ *Sphingomonas* sp. LB126 and gram$^+$ *Mycobacterium frederiksbergense* LB501T was assessed and compared to the application of PI for *E. coli*. Surprisingly, up to 40% of both strains LB126 and LB501T were stained by PI during their early exponential growth on glucose but remained largely culturable, as opposed to only 2-5% of PI-stained cells in the early stationary phase of growth. In contrast, growing *E. coli* cells were found to take up negligible amounts of PI (1-2%) and PI-stained *E. coli* were non-culturable. The state of viable cells from early exponential growth phase was corroborated by the observation that the exponentially growing LB126 and LB501T cells showed high cell cycle activities by DAPI staining, performed active pumping of Hoechst 33342 and also had a high and stable energy charge as analyzed by membrane-potential-related-fluorescence-intensity (MPRFI) through $diOC_6(3)$ staining. These data clearly demonstrated that PI can penetrate the membrane of some actively growing cells and consequently question the utility of PI as universal indicator for cell death. The significant differences of PI-uptake between fast growing *E. coli* cells and slowly proliferating *Mycobacteria* and *Sphingomonas* species may be explained by the inability of the latter to quickly close the broken glycan strands by highly efficient enzymes, due to the latter as normal inhabitants of oligotrophic environment. The findings here are especially important for the further application of PI in the study of membrane integrity related cell viability issues in presence of DC-electric fields typical for electro-bioremediation measures.

6.1.2 Influence of DC-electric field on soil-microorganism interactions.

Engineered bioremediation of soil relies on the presence of contaminant-degrading bacteria, optimal physical and chemical conditions for their catabolic activity, and the possibility of contact between the bacteria and their HOC substrates. This is particularly challenging for

bioaugmented organisms, which need to be transported to the zone of contamination (8). Transport, however, often happens to be restricted by the low hydraulic conductivity in soil micro-pores (9). Electrokinetics has the potential to move negatively charged bacteria to anode by electrophoresis (10,11) and/or stimulate bacterial migration with electroosmotic flow (EOF) to the cathode (12,13). In comparison with hydraulic flow restricted by capillary effects, electrokinetics are quite independent from pore size, and electroosmosis is especially efficient in fine-grained soils (with pores in the μm range or smaller) (14). In recent years, there has been increasing interest to use electrokinetics to enhance the contact probability of the bacteria and their HOC substrates by transporting contaminants to bacteria or vice versa. However, it is still largely unknown to which degree weak electric fields influence the fate (transport, deposition and physiological fitness) of indigenous or augmented microorganisms in the subsurface.

The vulnerability of electrokinetically-dispersed PAH-degrading bacteria (Chapter 4.1) and the factors influencing the electrokinetic transport and deposition (Chapter 4.2) of bacteria were studied using fluorene-degrading *Sphingomonas* sp. LB126 in a bench scale model aquifer. A DC-electric field strength (1 V cm^{-1}) typical for electro-bioremediation measures was employed and effects were compared with control experiment without DC treatment. The transport of strain LB126 in presence of 1 V cm^{-1} of DC-electric field in the model aquifer was dominated by electroosmosis to the cathode at velocities up to 0.6 cm h^{-1}. In contrast, the transport of LB126 cells in the control experiment by diffusion and random movement was negligible. Besides the transport to cathode, about one fifth of mobilized bacteria were found close to anode. Significant difference in the mean surface charge (e.g. zeta potential) of bacteria harvested at cathode and anode demonstrated that minor heterogeneities of bacterial populations may have an important effect on the distribution of microorganisms in DC-electric fields. Although a pure culture was used in this study, it mimics the electrokinetic transport behaviour of differently charged subsurface bacteria. DC-

electric field had no influence on the deposition efficiency of strain LB126 relative to the control, as the model soil (glass beads) under both conditions retained equal fractions of the cells. Propidium iodide staining and flow cytometry analysis of the cells indicated the absence of negative influences of DC-electric field on the cell wall integrity of electrokinetically mobilized cells and thus point at unchanged physiological fitness of electrokinetically mobilized bacteria.

6.1.3 Influence of DC-electric field on soil-compound interactions.

The strong sequestration of HOCs in the solid and organic phase of soils (15) and consequent slow release rate reduce their bioavailability significantly. Three potentially rate-limiting steps of HOC release have been identified: (i) diffusion of the sorbate within the molecular nano-porous network of natural organic matter (NOM) (16), (ii) pore or surface diffusion in aggregated minerals constituting natural particles (17), and (iii) diffusion of the sorbate across an aqueous boundary layer at the exterior of soil particles (15). Electroosmotic flow is the surface-induced movement of pore fluids in an electric field, which may overcome the mass-transfer bottlenecks of HOCs in soil matrices of low permeability as it moves water along the immediate exterior of soil particles and inside aggregated minerals. Although electroosmotically enhanced in-situ flushing of HOC contaminated soils gave promising results (18), yet mechanistic studies on the impact of EOF on HOC release from soil particles are scarce.

The stimulated release of PAH (phenanthrene) from model organic matter in the presence of DC-electric fields (0.5-2 V cm^{-1}) typically used in electro-bioremediation was observed and reported in Chapter 5. Alginate beads were employed as a model polymer release system (MPRS) exhibiting similar release behaviour as natural organic matter (NOM) (19). The electrokinetic apparatus used had a horizontal extension and an aquifer chamber with small volume (about 0.8 mL) in the middle to mimic a point source of pollutant and to

minimize effects of long distance transport (e.g. repeated de- and absorption) on the observed release kinetics. EOF was observed to be able to stimulate the release of phenanthrene from (model) soil particles up to 30-120 times as compared to stagnant conditions. The average macroscopic velocity of EOF in the presence of electric field strength of 0.5 - 2.0 V cm^{-1} was about 1-5 cm h^{-1}. Compared with hydraulic flow of equal linear velocity, the phenanthrene release flux in the presence of EOF was still 1.4 to 1.8-fold increased. This enhancement was less than predicted from diffusion calculations, which might be explained by a deviation from pure plug flow type of EOF at the solid liquid interface.

6.1.4 Relevance for electro-bioremediation

For the need of mechanistic understanding of the individual processes involved in electro-bioremediation, representative microbe (*Sphingomonas* sp. LB126), target pollutant (fluorene and phenanthrene as the representative of PAHs), and well-defined model systems were used to study the potential influences of DC-electric fields on each interaction among soil, microorganisms, and HOCs in detail. Although the system used lacked certain complexity to represent a natural subsurface, the knowledge attained here is particularly necessary to open the black box of electro-bioremediation. The findings from this study can be condensed into four statements with relevance to electro-bioremediation:

- The electro-bioremediation tetrahedron concept (Chapter 1, Figure 4) effectively refined the main features and interactions involved in electro-bioremediation into a comprehensive framework. With its support, the potential influences of DC-electric fields on HOC-bioavailability and relevant research gaps become clear and systematic.
- At the single cell level, no negative impacts of DC-electric fields typical for electro-bioremediation measures on the activity and viability of PAH-degrading cells were observed, which points at unaffected biodegradation potential as the prerequisite of bioremediation application. In addition, no changes of cell surface properties (e.g.

hydrophobicity and surface charge) were observed irrespective of the application of DC-electric fields, and hence the influence of DC-electric fields on bacterial transport and deposition via the altered cell surface properties can be neglected.

- We believe that this is the first study that indicates the absence of electrokinetic effects on the deposition efficiency of bacteria in soil matrices, a finding that simplifies the discussion and modelling of the electrokinetic dispersion of bacteria in subsurface. However, a detailed knowledge of the physico-chemical conditions of the soil matrix and the heterogeneity of the surface properties of the specialized bacteria is still very critical to predict the electrokinetic dispersion results of bacteria in hotspots of contamination. The observation that electrokinetically transported bacteria exhibited very low permeability for propidium iodide (as a cell membrane integrity indicator) is promising in the perspective of utilizing catabolically active bacteria under mild electrokinetic conditions. Effective electrokinetic dispersion of HOC-degrading bacteria has the potential to enhance HOC-bioavailability via decreasing the distance between biocatalysts and their substrates.
- We also believe that this is the first study that indicates the stimulation of HOC release from (model) soil particles by electroosmotic flow (EOF) and consequently we expect EOF to enhance the local HOC-bioavailability.

The main thrust of future research lies in the potential influences of electrokinetics on the functioning of microbial communities in pollutant electro-bioremediation regimes, and the effect of electroosmosis on the release of PAH sequestered by real soil particles and on HOC-bioavailability. Based on the detailed knowledge obtained, theoretical models are expected to be constructed and to support further implementation of this technology in pilot and field scale.

6.2 Concluding remarks

6.2.1 Personal retrospect

Looking back, I felt especially lucky to have worked in such an interdisciplinary research field (electro-bioremediation) that offered me enough challenges to integrate diverse interactions into a bigger picture and to approach the open questions from multiple disciplines, such as biology, physics, chemistry, and engineering. After I became much more familiar with electrokinetic soil remediation literature during my first year of PhD, I was astonished that the idea of using electric field for soil dewatering had its root back to 1949 and studies regarding the use of electrokinetics in soil remediation also had a history of about 20 years. I started to question myself where my research locates in such an interdisciplinary field and what I can contribute. Keeping these questions in mind, electro-bioremediation tetrahedron was first developed to identify present knowledge and research gaps. With this framework as the theoretical structure of this thesis, I was confident to proceed further and have a good overview of my study.

When I started to reach out to the open questions from each side-triangle of electro-bioremediation tetrahedron, I found out how many possible research sub-projects were down there. Just take the study regarding the influence of DC-electric fields typical for electro-bioremediation measures on cell activity and viability as an example. The enhancement of ATP contents in presence of 1 V cm^{-1} DC-electric field (Chapter 3.2) and the increased culturability of electroomostically transported cells (Chapter 4.1) require much further investigations to verify the reasons down to the cellular levels. Are these observations general phenomena? What are the fundamental mechanisms behind them? What are their application potentials? There are a lot of exciting questions waiting for answers. I felt fully motivated to continue to search for the answers, but the restricted time, awaiting work to fulfil the tetrahedron as a round story, and my limited knowledge regarding microbiology made it impossible for me to dig this topic further during my PhD.

Serendipity is a major component of scientific discoveries and inventions, which one accidentally discovers something fortunate, especially while looking for something else entirely. Electrokinetics was introduced to me as one of the main core mechanisms in electro-bioremediation to facilitate the mobilization of both microbes and HOCs in soil pores. While getting closer to its physical inherence, electrokinetics startes to reveal its beauty, power and abundant application potentials in front of me. For example, as one of the most powerful microfluidic mechanisms, there are numerous interesting scientific discussions regarding its application in the breakthrough analytical device lab-on-a-chip (20). Electrokinetics has its double sides, either using electricity to creat flow or using flow to produce electricity. Streaming potential induced from the pressure-driven flow through microchannels for example can be used to construct electrokinetic battery (21,22). As a new alternative energy source without pollution, it attracts both scientific and commerical interests. I came for my PhD project to develop more into biological directions, but I found now I am more attracted by the beauty of electrokinetics and further alternative energy, as a broader field it presents in front of me. Result-orientated development of alternative energy would be my future career focus.

6.2.2 My opinion on electro-bioremediation

In this thesis inductive approach was used to study electro-bioremediation, from fundamental mechanistic understanding to field applications. I personally prefer the fundamental approach, with which I have the chance to understand the mechanisms through well-controlled parameters. However, I am not sure how helpful it is to solve real problems in the much more complex nature. From an engineering point of view, a remediation project should ideally be fast, complete, economical and safe. In real field application, however, these goals are normally impossible to reach altogether and trade-offs must be made. Electro-bioremediation as an innovative technology for bioremediation of soil contaminated by HOCs attracts great

scientific and application interests, but it is not the universal method to remediate all contaminated sites. Then where are the application potential and the future of this technology?

- *Specific applications*: From the economical point of view, electro-bioremediation method is less suitable for large field sites due to the consumption of electricity. However, it can be much more competitive for small-scale in-situ applications, such as below buildings and underground services/structures. The application of electro-bioremediation in cold climate areas (e.g. average soil temperature below 10°C over the year) can be also attractive, due to the enhancement of microbial activity by ohmic heating in presence of electric currents.
- *Limited bioavailability*: The potential of electrokinetics to improve local HOC-bioavailability is the main argument to promote the application of electro-bioremediation in the field. However, bioavailability itself is still a concept under intensive scientific debates and lacks of fully established methods to monitor. The experience is also not adequate regarding the application of bioavailability concept in decision-making and risk assessment. For the selection of remediation method, limited bioavailability can result in the use of either natural attenuation or the bioavailability stimulation methods such as electrokinetics. A bioavailability stimulation method however might also appose extra toxic influence on biota when microbial activity is not efficient enough to follow the degradation of available contaminants. All the factors should be carefully considered before a field-scale application of electro-bioremediation.
- *Soil complexity*: Soil is a very complex environmental medium. The interactions among soil matrix, chemicals and microbes are not yet fully exploited. To introduce another feature especially one with its own multiple characteristics, such as electric field, into such a complex system, obviously will increase the challenge to fully understand the mechanisms behind. However, a hybrid technology, like electro-

bioremediation, is the future to solve soil problems, because the complex inherence of soil-contaminants requires the ingenious combination of different technologies.

- *A good platform for science*: I think a lot of research programs based on electro-bioremediation just take it as a good platform, which makes the integration of multiple interdisciplinary concepts possible for scientific discoveries. Although scientists should have their responsibility for application need in mind, the basic understanding of how the nature behaves is their root.

I am very grateful for what I have learned during this project and also very confident to start the next stage of career with the knowledge I have built up.

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Acknowledgement

Many thanks go to Dr. Lukas Wick and Prof. Hauke Harms for offering me the opportunity to join the "electro-bioremediation" project and for their scientific guidance, support and understanding during the last three years. I would like to stress in particular my appreciation to Lukas for not only being my supervisor, but for also being a very special friend, whom I was able to discuss several life and future issues.

I would also like to thank my working group members: Jana Reichenbach, Rita Remer and Birgit Würtz for all the technical helps they gave me during my laboratory work, Dr. Killian Smith and Dr. Martin Thullner for their fruitful scientific discussions, Makeba Kampara, Shoko Fruno and Joanna Hanzel for the nice or hard time we spent together in the lab.

Thanks to the flow cytometry group led by Dr. Susann Müller and the stress physiology group led by Dr. Norbert Loffhagen for the successful collaboration during the project's lifespan. Martina Kolbe and Katrin Lübke for the countless sterilized materials they prepared for my experiments. Peter Portius and Axel Borbonus from the glass studio for their excellent work in realizing the PAH release experiment setup.

In addition, I would like to express my gratitude to an innumerable number of friends with whom I have shared very valuable moments and memories that I will cherish forever. Paula Martinez, Nicole Stelzer and Christian Jung are the closest friends for me in Leipzig. I still remember that I got to know Nicole in January 2005 because she temporarily used the lab of my working group. She started to introduce me to her friends and then slowly I got to know Christian. Paula came to UFZ on March 2005 and it was a very cold winter. In the last three years, step by step we developed our friendship. Friends take a pass into my heart like a little stream. Cannot exactly say since when we started to be friends instead of colleagues, and then since when we started to be best friends instead of normal friends. So many tiny things brought us closer and closer. I cherish this friendship forever. Nicole also helped me on

translating my abstract from English to German. There are also a lot of friends that I would like to thank, such as Monica Rosell Linares, Ivonne Nijenhuis, Fang Liu, Makeba Kampara, Danuta Cichocka, Gwenael Imfeld, Charles Junghanns, Ran Bi and many others. Thank you for sharing my happiness and joys and for making my stay in Leipzig extra special. I am also very thankful to two of my old friends from Hamburg, Asma Alazeib and Pei-chi Hsu. They were always there whenever I asked for helps. I would also like to thank two special friends from Netherlands, Marja and Arnold Goffree. Although we mainly contact each other through e-mails, there are still a lot of things we can chat about and the cares from heart are the most important.

Special appreciation goes to my mother, sister, Niuniu, Zhangpeng and Xin, you are my families. Wherever I go and whatever I do, I know that I will always have a home in Tangshan with my mother and sister and they will always be there for me with arms widely open. To my wonderful nephew Niuniu, I want you to know that your aunt really loves you. Zhangpeng, you are like my real brother. Xin, as the most special person in my life you have my word.

Publications

1. Wick, L.Y., **L. Shi**, and H. Harms (2007) "Electro-bioremediation of hydrophobic organic soil contaminants: a review of fundamental interactions" *Electrochimica. Acta.* (52) 3441-3448.
2. **Shi, L.**, S. Günther, T. Hübschmann, L. Y. Wick, H. Harms and S. Müller (2007) "Limits of propidium iodide (PI) as a cell viability indicator for environmental bacteria" *Cytometry A* (71A) 592-598.
3. **Shi, L.**, S. Müller, N. Loffhagen, H. Harms, and L.Y. Wick (2008) "Activity and viability of PAH-degrading *Sphingomonas* sp. LB126 in a DC-electrical field typical for electro-bioremediation measures" *Microbial Biotechnology* 1 (1) 53-61.
4. **Shi, L.**, S. Müller, H. Harms, and L.Y. Wick (2007) "Vulnerability of electrokinetically-dispersed PAH-degrading bacteria in a model aquifer" *Environmental Geochemistry and Health* (DOI 10.1007/s10653-008-9146-0).
5. **Shi, L.**, S. Müller, H. Harms, and L.Y. Wick (2007) "Factors influencing the electrokinetic dispersion of PAH-degrading bacteria in a model aquifer" *Applied Microbiology and Biotechnology* (submitted).
6. **Shi, L.**, H. Harms, and L.Y. Wick (2007) "Electroosmotic flow stimulates the release of matrix-bound phenanthrene" *Environmental Science & Technology* (accepted).

Oral presentations

1. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM), Jena, Germany March, 19^{th}-22^{nd}, 2006;

Electro-bioremediation: Physiology of Pollutant Degrading Bacteria in Weak Electric Fields

L.SHI, S. MÜLLER, N. LOFFHAGEN, H. HARMS, and L. Y. WICK

2. 5^{th} International Workshop of Slide-based Cytometry, Leipzig, Germany April 27^{th}-29^{th}, 2006;

Flow Cytometry Analysis of the Viability of Pollutant Degrading Bacteria Exposed to Weak Electric Fields

L.SHI, L. Y. WICK, S. GÜNTHER, H. HARMS, and S. MÜLLER

3. International Symposium on Environmental Biotechnology, Leipzig, Deutschland July, 9^{th}-13^{th}, 2006;

Electro-bioremediation: Physiology of Pollutant Degrading Bacteria Exposed to Weak Electric Fields

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4. SETAC Asia/Pacific, Beijing, China, September, 18th-20th, 2006;
Electro-bioremediation: Influence of direct current on the activity and dispersion of PAH degrading bacteria in model soil systems
L.SHI, S. MÜLLER, N. LOFFHAGEN, H. HARMS, and L. Y. WICK

5. 16th Annual Deutsche Gessellschaft für Zytometrie (DGfZ) meeting, Leipzig, Germany, October, 18th-19th, 2006;
Flow Cytometry Analysis of the Viability of Pollutant Degrading Bacteria Exposed to Weak Electric Fields
L.SHI, L. Y. WICK, S. GÜNTHER, H. HARMS, and S. MÜLLER

6. 6th Symposium on Electrokinetic Remediation, Vigo, Spain, June, 12th-15th, 2007;
Influence of Electroosmotic flow on the PAH Release from Model Soil Matrices
L.SHI, H. HARMS, and L.Y. WICK
Electro-bioremediation: Influence of Direct Current on the Physiology and Dispersion of Pollutant Degrading Bacteria in Model Soil
L.SHI, S. MÜLLER, H. HARMS, and L. Y. WICK

Posters

1. AXIOM-Virtual Institute Spring School, 'Microbial Activity at Biogeochemical Gradients', Leipzig, Germany, April, 3rd-6th, 2006;
Electro-bioremediation of poorly bioavailable soil-contaminants
L.SHI, H. HARMS, and L. Y. WICK

2. 4th International Workshop 'Bioavailability of Pollutants and Soil Remediation', Seville, Spain, September, 10th-13th, 2006
Electro-bioremediation: Influence of direct current on the activity and dispersion of PAH degrading bacteria in model soil systems
L.SHI, S. MÜLLER, N. LOFFHAGEN, H. HARMS, and L. Y. WICK

Curriculum Vitae

Short statement

I was born in Tangshan of Hebei province of China on the July 7th, 1978. It is a city famous for the most destructive earthquake in modern history on 1976 (8.2 degree), during which the city was completely flattened and more than 25.5 thousands people died. I grew up with the city together and witnessed its urbanization. After 18 years of childhood and teenage time, I went to Beijing about 300 kilometers away from home for university education. I decided to continue my master training after I got bachelor degree in 2001 not in China but abroad. Both for the abundant and colorful of European cultures and the extensive experience and high reputation of Germany in environmental engineering, I chose Germany as the first destination to set out my foreign journey. Since October 2001, I have been in Germany for six years.

Education

10,2004 - 02,2008	PhD Candidate, Institute of Biology, Pharmacy and Psychology, **Leipzig university**, Germany Researcher as PhD candidate, **Helmholtz Centre for Environmental Research-UFZ**, Germany
10,2001 - 09,2004	Master of Science, Institute of Environmental Technology and Energy Economics, **Technical University Hamburg-Harburg**, Germany **Master thesis**: Applicability of FFF-MALLS/DLS coupling for the determination of size and shape of natural colloids **Project work**: Geotextile and natural zeolite as reactive materials for the subaqueous capping of PAH-contaminated sediment: chemical and toxicological laboratory investigation
09,1997 - 07,2001	Bachelor of Engineering, Department of Environment and Energy, School of Chemical Engineering and Environment, **Beijing Institute of Technology**, China **Bachelor thesis**: Natural manganese mineral---H_2O_2 system degrading organic wastewater at **Chinese Academy of Sciences**, Research Center for Eco-Environmental Sciences, State Key Laboratory of Environmental Aquatic Chemistry (SKLEAC)

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