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

Lei Shi, Susann Müller, Norbert Loffhagen, Hauke Harms and Lukas Y. Wick*

UFZ Helmholtz-Center for Environmental Research, Department of Environmental Microbiology, 04318 Leipzig, Germany.

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.

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 (mA cm^{-2})	$N_{initial}^c$ ($\times 10^8$ cells ml^{-1})	$PI_{initial}^d$ (%)	$MPRFI_{initial}^e$ (mean FI)	$ATP_{initial}^f$ ($\times 10^{-18}$ mol cell^{-1})	$q_{initial}^g$ ($\times 10^{-20}$ 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 $\pm$ 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 $\times$ 10^{-20} mol s^{-1} cell^{-1} above
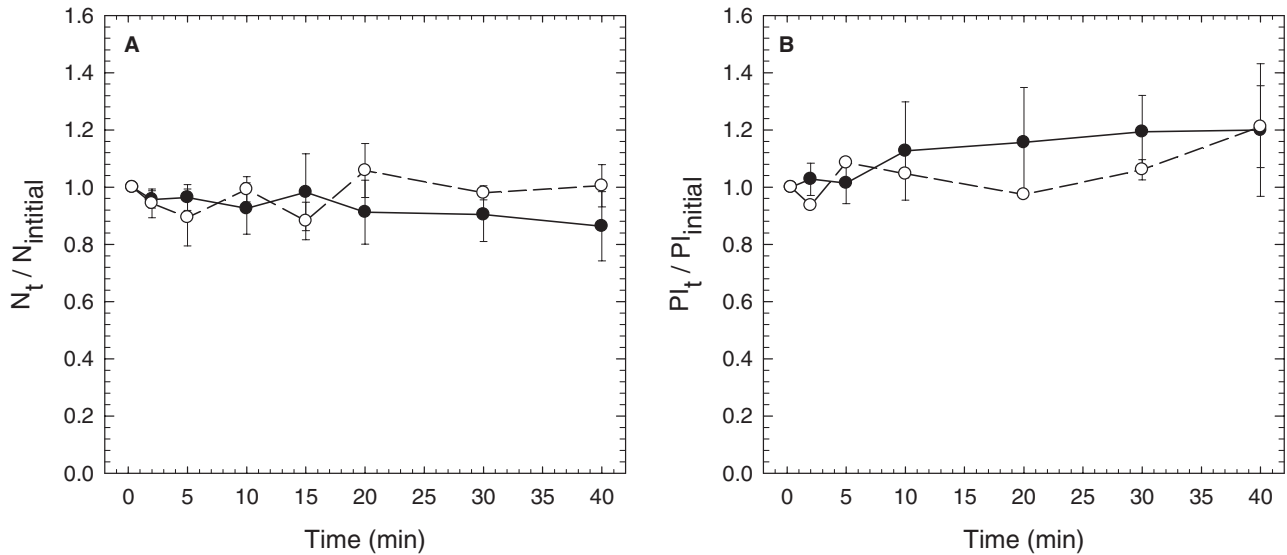
Fig. 1. Time-dependent fractions of total (N_t; A) and PI-stained (PI_t, B) *Sphingomonas* sp. LB126 cells in the presence (filled circles, 1 V cm^{-1}) and absence (open circles) of DC. Data are normalized relative to initial conditions ($N_{initial}$; $PI_{initial}$).

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 $0.6–1.8 \times 10^{-19}$ 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

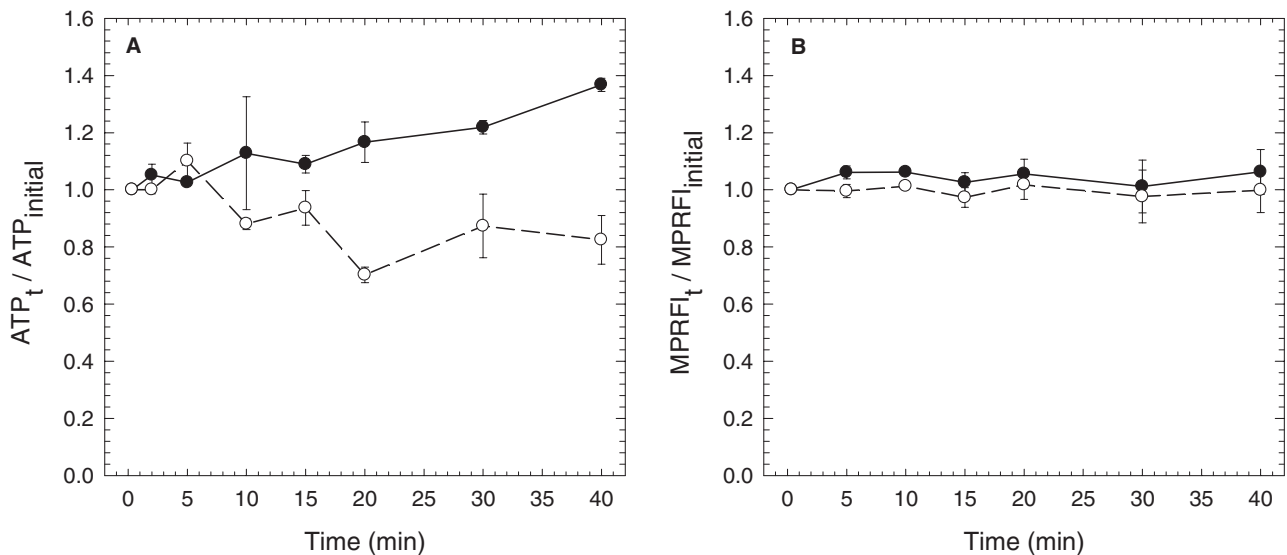The cytoplasm of a cell is electrically conducting whereas

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.
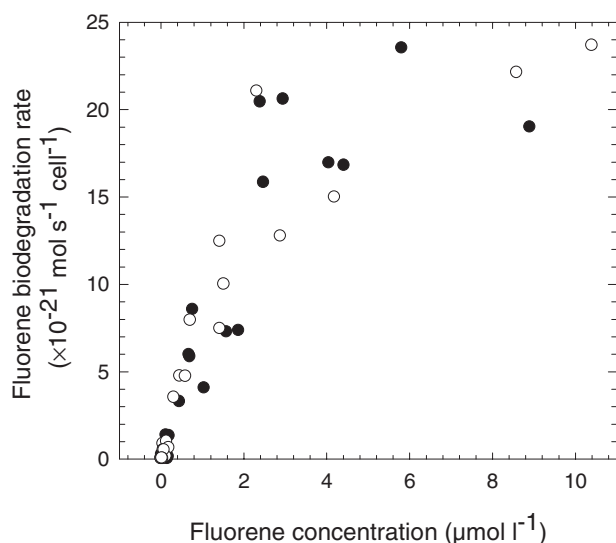
Fig. 3. Representation of the apparent fluorene biodegradation rates of *Sphingomonas* sp. LB126 relative to measured dissolved bulk fluorene concentrations in the presence (filled circles, 1 Vcm⁻¹) and absence (open circles) of DC. Fluorene biodegradation rates in the presence of DC were corrected for 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
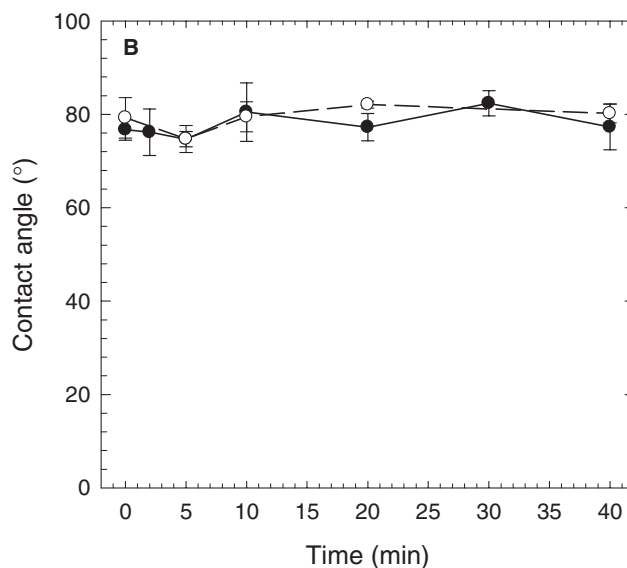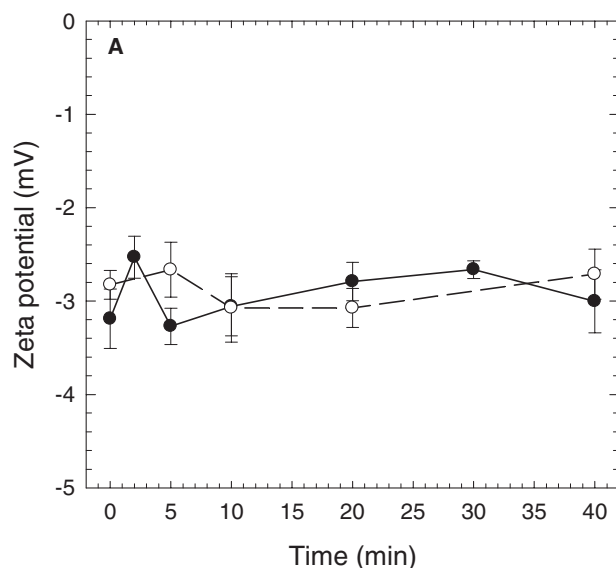

Fig. 4. Time-dependent physicochemical cell surface properties of *Sphingomonas* sp. LB126 exposed to DC. (A) and (B) reflect the water contact (θ_w) and the zeta potential (ζ), respectively, in the presence (filled circles, 1 V cm⁻¹) or absence (open circles) of DC.

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 $1.5–9.0 \times 10^{-10}$ 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 ($\geq$ 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) $\times$ 8 (depth) $\times$ 3 cm (width)] containing 100 ml of fluorene-saturated 100 mM PB at 25 $\pm$ 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:

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

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

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