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# The kinetics of polycyclic aromatic hydrocarbon (PAH) biodegradation assessed by isothermal titration calorimetry (ITC)

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**Abstract**

Interest in the biodegradation mechanisms and environmental fate of hydrophobic organic compounds (HOC) such as polycyclic aromatic hydrocarbons (PAHs) is motivated by their ubiquity in the environment, their persistence and their potentially deleterious effect on human health. Due to their high hydrophobicity, PAH tend to interact with non-aqueous phases and natural organic matter and, as a consequence, are poorly bioavailable for microbial degradation. Here, a novel calorimetric approach was developed and successfully tested in order to gain real time information on the kinetics and the physiology of PAH bioconversion in aqueous systems. Anthracene-degrading soil bacterium *Mycobacterium frederiksbergense* LB501T was exposed to a pulsed titration of dissolved anthracene and the resulting thermal reaction monitored. The heat flux signals of the biodegradation of 180 ng anthracene were interpreted in terms of the Michaelis-Menten kinetics and the parameters $K_A$ and $r_A^{max}$ of anthracene degradation were derived. The comparison with a conventional method shows the suitability of isothermal titration calorimetry (ITC) to extract kinetic degradation parameters of organic trace pollutants from simple ITC experiments.


*Keywords:* Isothermal titration calorimetry (ITC); Bioavailability; HOC/PAH biodegradation; *Mycobacterium frederiksbergense* LB501T; Modeling

## 1. Introduction

Bioremediation of polluted soil mostly makes use of indigenous microorganisms. However, despite a potentially metabolically active biomass, in situ bioremediation of soils polluted with hydrophobic organic pollutants (HOC) frequently results in slow pollutant degradation rates and, as a consequence, in limited clean-up efficiencies [1,2]. It is generally observed that HOC strongly accumulate in the solid or non-aqueous liquid phases of soils [2]. By contrast, microorganisms appear to degrade chemicals only when they are dissolved in water [3] and, hence, limited access controls the biodegradation of poorly soluble chemicals [4]. A sensitive, non-invasive and universally applicable analysis of the kinetics of mass transfer-limited biodegradation processes would be ideal for assessing the fate of HOC in the environment. Common methods for the determination of the whole-cell kinetic biodegradation parameters of one class of HOC, the polycyclic aromatic hydrocarbon (PAH) either rely on continuous assessment of oxygen consumption and/or $CO_2$-production or on the chemical analysis of the PAH-removal rates. In the latter approach, problems arise from the difficult handling and error-prone extraction of the extremely hydrophobic PAH and the requirement for extremely sensitive analytics. Calorimetry by contrast, appears to be a convenient and powerful alternative of sufficient sensitivity that overcomes some of the problems since PAH biodegradation rates are directly followed as absolute values of the reaction heat production in the bioreactor [5]. The achievable thermal sensitivity between few nW and 1 μW corresponds to a degradation rate of few $\mu g\,L^{-1}\,h^{-1}$ and is sufficient to follow the biodegradation of ultra traces in the magnitude of few ng.

Microcalorimetric quantification of enzyme activities and the stoichiometry and kinetics of whole-cell biocatalysis have been successful in the last decades [6–9]. The reproducibility of calorimetric measurements can be further increased when adding well defined volumes of reactants to a biocatalyst suspension that is already in thermal equilibrium. This technique called isothermal titration calorimetry (ITC) connects extremely sensitive thermal measurement equipment (approx. 20–100 nW) with an automatic syringe able to add reactants with a precision of few nL to the solution. Furthermore, by the multiple injection method several calorimetric measurements per experiment are possible [10]. The reproducibility can be increased substantially this way. This extreme sensitivity allows the use of

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ITC for studying biological interactions with weak heat production (or release) such as protein–protein and protein–peptide interactions in such diverse processes as cell signaling [11], Alzheimer disease [12], transcription [13] and protein repair with chaperones [14]. Recent applications showed the utility of the technique to study protein–drug [15], drug–DNA [16] and protein–DNA interactions. Although ITC to quantify enzyme activities is already well established in the literature, there are no reports of its application to whole cell biotransformation. The ultra-sensitive ITC should be very useful especially to follow the biodegradation of trace concentrations of substrates. The goal of this study was thus to extend ITC to the investigation of microorganisms degrading poorly bioavailable substances such as PAHs and to develop mathematical tools for extraction of kinetic parameters.

## 2. Experimental

### 2.1. Organisms and culture conditions

*Mycobacterium frederiksbergense* LB501T, an aerobic, rod-shaped bacterium isolated from PAH-contaminated soil is capable of degrading and growing on anthracene [17]. The bacteria were cultivated at room temperature in Erlenmeyer flasks on a gyratory shaker at 150 rpm. We used a minimal medium [18] that contained 1.5 g/L solid anthracene (>98%, Fluka, Buchs, Switzerland) as sole carbon and energy source and was amended with 0.5% trace element solution [19]. The biomass concentration was determined photometrically at 578 nm (Cary400Scan, Varian Deutschland GmbH, Darmstadt).

For the degradation experiments, the bacteria were harvested by centrifugation for 5 min at 11,180 × *g* and 20 °C. The cells were washed two times with 0.1 M phosphate-buffered saline (PBS) and then resuspended in PBS to an optical density of about 0.1. The bacteria were stored at 4 °C until the start of the experiment.

### 2.2. Chemical analyses

Anthracene consumption was measured by gas chromatography after each calorimetric experiment. For that purpose anthracene was extracted by two consecutive liquid–liquid extractions with benzene. The dried sample was resuspended in hexane containing the internal standard heneicosane. The GC-MS analysis was performed on a GC-MS system (6890 Series, Hewlett-Packard) using a HP-5MS column. The column temperature was raised from 120 to 280 °C with a slope of 35 grad $min^{-1}$ to achieve an optimal substance separation. Anthracene was identified by the *m*/*z* values 178 and 179, the internal standard by the *m*/*z* values 57 and 71.

Degradation experiments with the reference technique were performed in 300-mL Erlenmeyer flasks. Bacteria were added to 100 mL PBS containing 15% DMSO to an optical density of about 0.1. The bacterial suspension was continuously stirred. The reaction was started by adding anthracene solution in 15% DMSO with a glass syringe. The resulting start concentration was approximately 60 μg/L. Samples were separated from biomass by filtration (0.2 μm regenerated cellulose filter, Schleicher und Schuell Microscience GmbH, Dassel). $HgCl_2$ was added to each sample to exclude later biodegradation. Anthracene was quantified by isocratic HPLC (90:10 MeOH/water, 1 mL $min^{-1}$) using a Nucleosil 100-5-C18 column (Knauer, Berlin, Germany) and detecting anthracene fluorimetrically ($\lambda_{ex}$ 251 nm, $\lambda_{em}$ 450 nm).

### 2.3. Calorimetry

Calorimetric measurements were performed at 30 °C with a 2247 thermal activity monitor (TAM, Thermometric AB, Järfälla, Sweden) fitted with a measuring cylinder 2277–202. The measuring cylinder is constructed in twin form, with two measuring cups. The first cup was fitted with a 4-mL titration unit from the thermometric 2250 micro reaction systems whereas the second cup was used as reference. The reference cup was filled with sterile, poisoned buffer. After electrical calibration, the accuracy of the calorimeter was tested from time to time using the imidazole-catalysed hydrolysis of triacetin [20]. The calorimeter tests followed a suggestion of Chen and Wadsö [21]. The titrations were done using an automatic Lund Syringe Pump 2 equipped with a 250-μL syringe (Thermometric AB, Järfälla, Sweden). The titration vessel was filled with 3 mL of the prepared bacterial suspension in 15% (v/v) DMSO/buffer and stirred at 50 rpm. In the control exper-

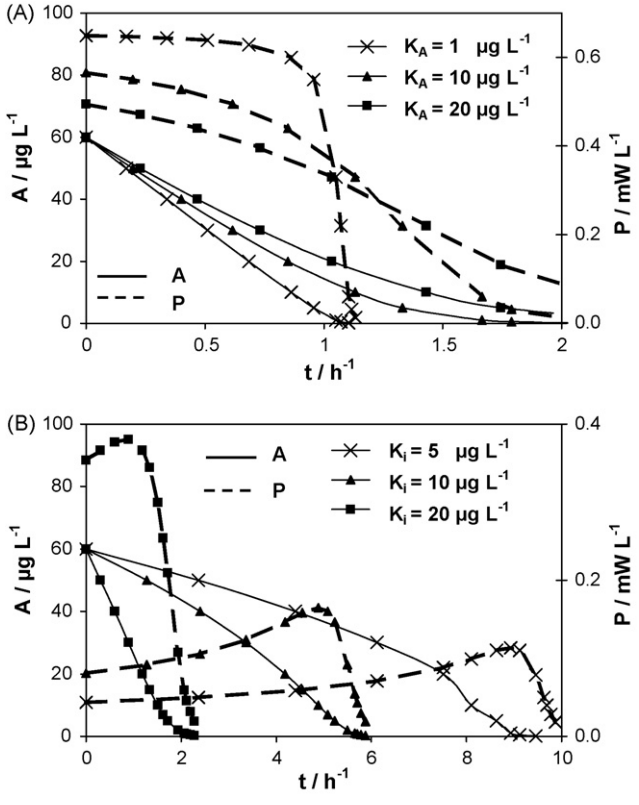


Fig. 1. Expected evolution of substrate concentration (*A*) and heat production rate (*P*) for (a) non-inhibiting and (b) inhibiting substrates. The calculations were done for $r_A^{Max} = 60\,\mu g\,L^{-1}\,h^{-1}$, $A_0 = 60\,\mu g\,L^{-1}$ and $K_A = 10\,\mu g\,L^{-1}$ (for b).