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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 (λ_{ex} 251 nm, λ_{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-

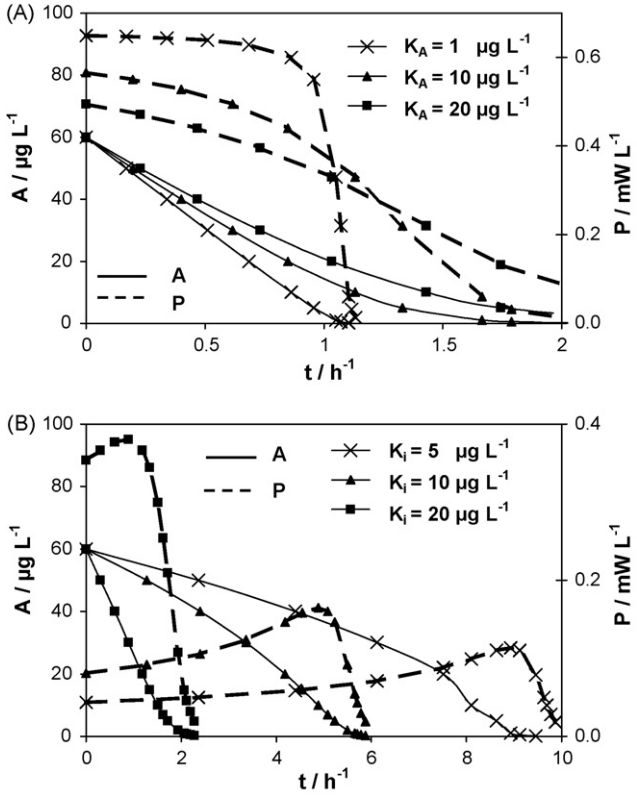

Fig. 1. Expected evolution of substrate concentration (*A*) and heat production rate (*P*) for (a) non-inhibiting and (b) inhibiting substrates. The calculations were done for $r_A^{Max} = 60\ \mu g\ L^{-1}\ h^{-1}$, $A_0 = 60\ \mu g\ L^{-1}$ and $K_A = 10\ \mu g\ L^{-1}$ (for b).

iment, the bacterial suspension was either killed with $HgCl_2$ (0.55 mM) or replaced by PBS containing $HgCl_2$ (0.55 mM). Experimental runs were electrically calibrated at the beginning and the end of each titration experiment to account for small heat flux drifts. Electrical calibration, automatic titration and stirring were controlled by the software "Thermometric Digitam 3". The substrate anthracene was dissolved in a mixture of 15% (v/v) DMSO and PBS to obtain a highly concentrated homogeneous solution and to prevent any loading of the surface of the syringe and cannula materials. A concentrated anthracene solution (6 mg/L) was added pulse-wise (30 μL) to give initial concentrations of 60 μg/L at each pulse. The next addition occurred as soon as the heat production rate approached the base line indicating the consumption of the anthracene. The whole thermal reaction was monitored with a sample frequency of 0.2 Hz. The DMSO concentrations in the syringe and the calorimetric vessel were the same to prevent disturbance by the known high heat of mixing DMSO and water. The possibility of DMSO biodegradation or inhibition of the bacterial metabolism by DMSO were excluded in preliminary tests.

2.4. Theory

Extraction of biodegradation parameter from a thermal signal requires a thermokinetic model correlating heat production rate p (W) with reaction progress $\xi(t)$ (mol L^{-1} s^{-1}). The correlation between p and $\xi(t)$ is linear with the slope of the degradation enthalpy change $\Delta_D H$ (kJ mol^{-1}) and the volume of the calorimeter content V_C (L) (Eq. (1)).

$$p = \xi(t)\Delta_D H V_C \tag{1}$$

The degradation enthalpy can be substituted by the combustion enthalpy $\Delta_C H$ (kJ mol^{-1}), if (i) the substrate is completely mineralized, (ii) growth does not occur, (iii) the biomass composition is not changing and (iv) no exudates are formed. Combustion enthalpies are tabulated [5,22] or can be estimated applying the rule of Thornton [23] or the oxycaloric equivalent [24]. Otherwise the degradation enthalpy can be calculated from the thermal signal p and the concentration difference before and after degradation ΔA (mol L^{-1}) (Eq. (2)).

$$\Delta_D H = \int_{t_0}^{t^0} p(t)\,dt\;\Delta A V_C \tag{2}$$

where t_0, t^0 stand for the injection time and the time of returning to the baseline, respectively. $\xi(t)$ is correlated with any rate via the stoichiometric coefficient ν_A, i.e. $\nu_A = -1$ in case of biological anthracene combustion (Eq. (3)).

$$\xi(t) = -\frac{dA}{dt} = -r_A(t) \tag{3}$$

Mathematical expressions describing the biodegradation rate, r_A (mol L^{-1} h^{-1}) as a function of the substrate concentration A (mol L^{-1}) for non-inhibitory (Eq. (4a)) or inhibitory substrates

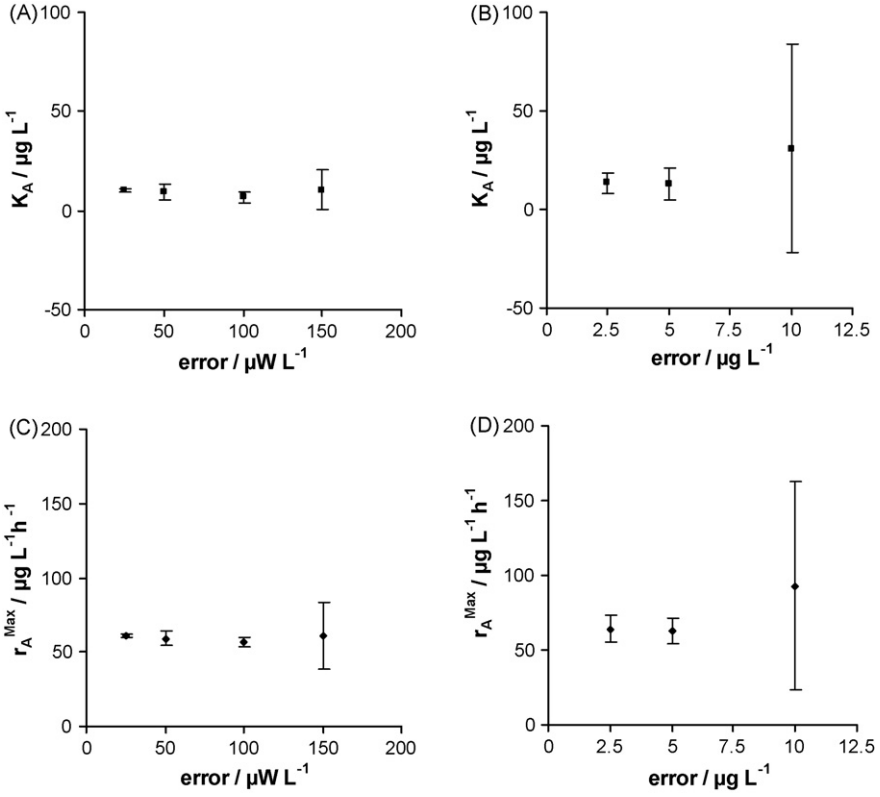

Fig. 2. Influence of the error of chemical (A and B) and calorimetrical (C and D) analysis on the precision of r_A^{Max} (A and C) and k_A (B and D). The parameters for the simulations were A_0 = 60 μg/L, r_A^{Max} = 60 μg L^{-1} h^{-1} and k_A = 10 μg/L.

(Eq. (4b)) are derived from simple enzyme kinetics [25,26].

$$r_A = r_A^{Max} \frac{A}{k_A + A} \tag{4a}$$

$$r_A = r_A^{Max} \frac{A}{k_A + A} \frac{k_i}{k_i + A} \tag{4b}$$

The parameters of the model are the specific maximum degradation rate, r_A^{Max}, the substrate saturation constant, k_A and the inhibition constant, k_i. The usual biodegradation rate measure, q_A^{Max} describes the ratio of r_A^{Max} to the concentration of biomass or protein in order to make different measured parameters comparable. The analytical integration of the differential Eq. (3) using the respective kinetic expression (Eq. (4a)) or (Eq. (4b)) yields the time dependency of the concentration (Eq. (5a)) or (Eq. (5b)).

$$t = -\frac{k_A \ln(A/A_0) + (A - A_0)}{r_A^{Max}} \tag{5a}$$

$$t = -\frac{k_A \ln(A/A_0) + ((k_A + k_i)/(k_i)(A - A_0)) - (1/2k_1)(A^2 - A_0^2)}{r_A^{Max}} \tag{5b}$$

where A_0 stands for the anthracene concentration at the beginning of each titration. Eqs. (5a) and (5b) are highly convenient even to anyone who uses classical analytical techniques. Adjusting the parameter r_A^{Max}, k_A and k_i of Eqs. (5a) and (5b) directly to the experimental data excludes the error introduced by numerical integration.

With Eqs. (1), (3) and (5a) or (5b) the heat production rate can be predicted or parameters can be adjusted to the thermal signal. Fig. 1 illustrates expected concentration and heat production rates for non-inhibitory (A) or inhibitory (B) substrates.

For parameter fits, the program Berkeley Madonna™ (developed by Robert Macey and George Oster; University of California at Berkeley, USA) was used before a more convenient program based on MATLAB® (The MathWorks, Inc., Natick, MA, USA) was written. The fitting results were the same. To estimate the expected quality of the kinetic parameters, the influence of the different measurement errors was investigated. For that simulation, the exact model lanes were superimposed by a random function. The parameters were fitted to this artificially induced noise. Fig. 2 shows the influence of noise on the extracted kinetic parameters.

This simulation demonstrates clearly the theoretically higher precision of ITC in comparison to the reference method. The manufacturer's specification of the titration mode (170 μW L^{-1}) corresponds to an approximately five times higher precision in the determination of r_A^{Max} or k_A in comparison with our HPLC reference method (error approx. 10 μg L^{-1}). This error estimation does not include the systematic error of the reference method due to surface loading and error reduction resulting from repeated measurements in ITC.

3. Results and discussion

A 180 ng anthracene dissolved in 30 μL 15% DMSO/water mixture was repeatedly added (every 90 min) to the calorimetric vessel to test the proposed method. To maximize the signal, the energy flow into anabolic reactions was reduced by washing the bacteria before use and by adding bacteria suspended in a DMSO/buffer mixture without any nutrients. Fig. 3 compares the calorimetric signal for a bacterial suspension (A) with a reference experiment (B) without bacteria and poisoned buffer.

The calorimetric signals for sterile medium and deactivated bacteria ($HgCl_2$) were the same (results not shown) indicating the absence of heat production due to anthracene/bacterial surface interactions. Both signals showed only short disturbances at each time of injection into the calorimetric vessel. These disturbances could be caused by an imperfect equilibration of the volume added or by a small heat of mixing. To distinguish between both reasons a simple water to water titration experiment was performed. The result was similar to the experiment with sterile medium. Furthermore, the heat of mixing anthracene dissolved in 15% DMSO/water with pure 15% DMSO/water-mixture was calculated using COSMO-RS [27,28]. The calculated heat of mixing (−1.85 kJ/mol) [29] of 1.87 μJ is approximately 1800 times smaller than the measured

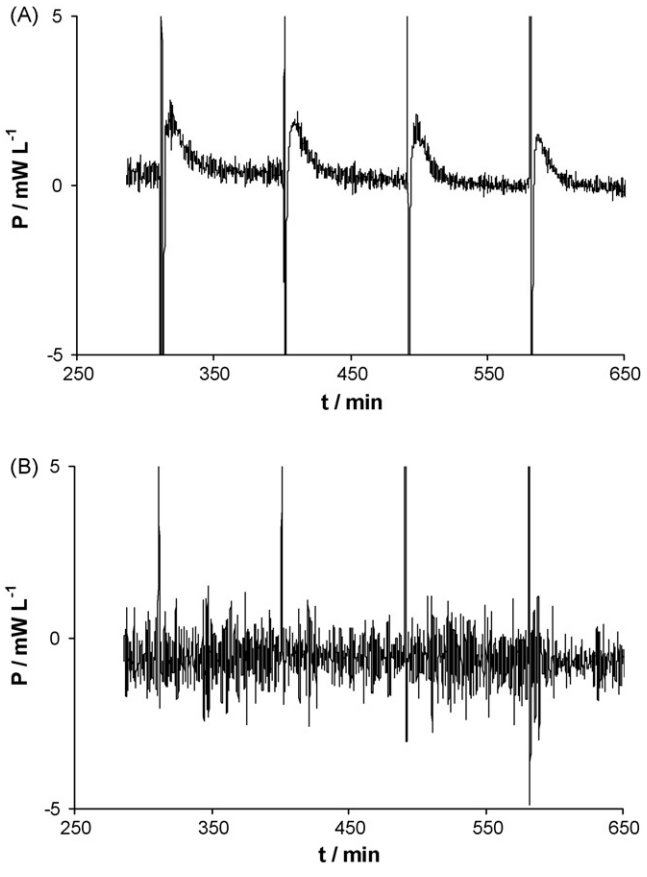

Fig. 3. Heat flux (*P*) response by bacteria (A) and by a sterile reference solution (B) upon addition of 180 ng anthracene.

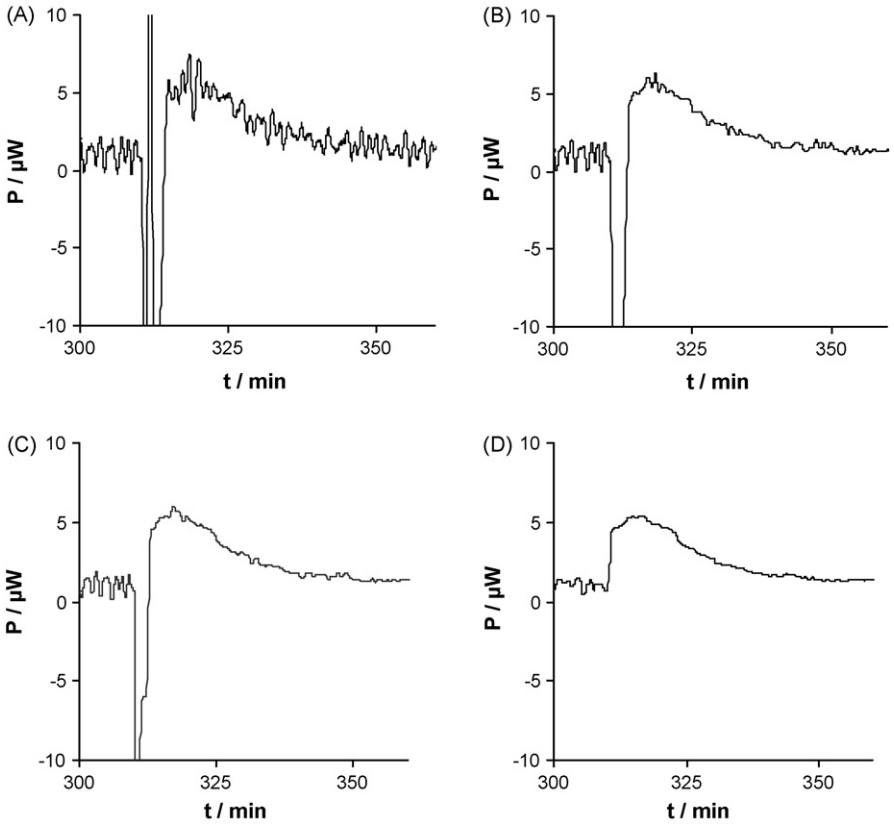

Fig. 4. Effect of smoothing data with the median. The median was calculated from (A) 1 (original data), (B) 25, (C) 40 and (D) 80 measurements.

bacterial signal (3.3 mJ). This indicates that imperfect thermal equilibration of the added volume is responsible for the short disturbances. The lane of the bacteria experiment showed clear, quantifiable signals as response to the addition of only tiny traces of the substrate (180 ng anthracene). The definite integrals of each injection response (3.27 ± 0.26 mJ), i.e. the measured heat, were highly reproducible. The difference between the expected heat (7.0 mJ) for the catabolism (complete biological combustion) and the measured heat (3.27 ± 0.27 mJ) may result from ongoing growth, changes in biomass composition and/or formation of exudates. The exudation of organic material was reported for *Mycobacterium frederiksbergense* LB501T [30]. Growth and cell compositional changes caused by only 180 ng anthracene addition are hardly quantifiable by conventional analytics. Although differences in reaction heat spread the calorimetric signal, this spreading can be accounted for by Eq. (2). Thus, the spreading does not influence the kinetic parameters derived from the shape of the thermal signal.

The signal shape was similar to the expectation for non-inhibitory substrates (Fig. 1A). The anthracene concentration is probably too low to influence biodegradation adversely. The raw signal was influenced by thermal disturbances due to injections and by a relatively high random noise. For gaining kinetic information, the lane thus needed to be smoothed and mavericks excluded. The moving median [31] is the best procedure to exclude mavericks and to smooth the data without loss of information. Fig. 4 shows the influence of smoothing data on the analytical information.

The best result was achieved with a moving median of 40 data points. The parameter fitting used the smoothed calorimetric data after recovering from the thermal disturbance. The quality of calorimetric data fitting is shown in Fig. 5.

The fitted curve described the heat production very well. Each injection can be described by the same parameter set ($r_A^{Max} = 740\,mg\,L^{-1}\,h^{-1}$, $K_A = 60\,\mu g\,L^{-1}$), illustrating the good quality of the overall curve fitting. For comparison, Fig. 6 shows the degradation kinetics measured by conventional (HPLC) analytics. The data evolution was similar to the expected one for non-inhibiting substrates (Fig. 1A) and the model described

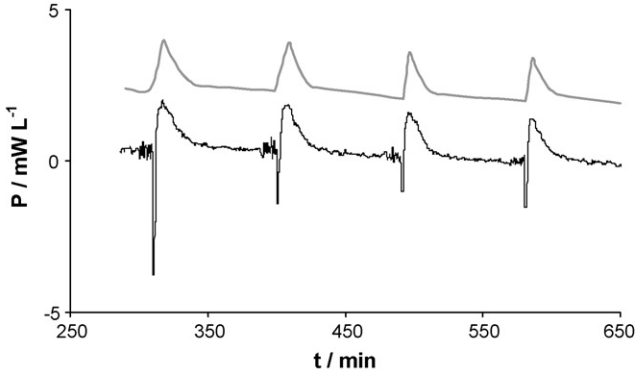

Fig. 5. Kinetic parameter estimation from calorimetric data. The raw line stands for the experiments whereas the smooth line represents the thermokinetic model (with an artificial offset of $2\,mW\,L^{-1}$).

Table 1
Comparison of the kinetic parameter of anthracene biodegradation by *M. frederiksbergense* LB501T determined calorimetrically and by conventional analytics for different salt contents

Salt content ($g\,L^{-1}$)	Calorimetry		Conventional analytics (HPLC)	
	q_{max} ($mol\,mg\text{-}Protein^{-1}\,h^{-1}$)	k_s ($mol\,L^{-1}$)	q_{max} ($mol\,mg\text{-}Protein^{-1}\,h^{-1}$)	k_s ($mol\,L^{-1}$)
0	$(9.0 \pm 0.3) \times 10^{-8}$	$(3.2 \pm 0.3) \times 10^{-7}$	$(12.2 \pm 3.7) \times 10^{-8}$	$(4.6 \pm 0.7) \times 10^{-7}$
20	$(5.3 \pm 0.6) \times 10^{-8}$	$(3.5 \pm 0.3) \times 10^{-7}$	$(8.4 \pm 1.0) \times 10^{-8}$	$(3.3 \pm 0.8) \times 10^{-7}$
40	$(4.3 \pm 0.4) \times 10^{-8}$	$(3.2 \pm 0.1) \times 10^{-7}$	$(3.6 \pm 0.6) \times 10^{-8}$	$(3.1 \pm 0.6) \times 10^{-7}$

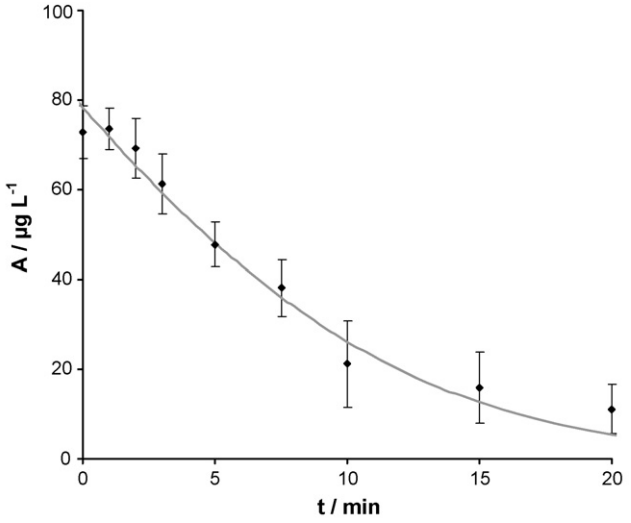

Fig. 6. Kinetic parameter estimation from the reference experiments with HPLC analysis.

the data evolution very well. The absence of adverse effects of $60\,\mu g\,L^{-1}$ anthracene on bacterial degradation found by conventional analysis confirmed the calorimetric findings qualitatively.

Beside this, kinetic parameters obtained by both methods should also agree. Table 1 compares the degradation parameters obtained calorimetrically and by conventional analysis for different media. The accordance between both parameter sets clearly demonstrates the applicability of calorimetry for the quantitative analysis of the biodegradation of trace-amounts of pollutants. This is promising for the development of ITC into a routine method for studying the biodegradation of micropollutants or poorly bioavailable substances.

The media applied for testing the calorimetric method differ in their salinity. It is well known that increased salinity often reduces the maximum degradation rate [32]. This was confirmed by our experiments. The half saturation concentration k_A of $3.2–4.1 \times 10^{-7}\,mol\,L^{-1}$ was in the magnitude of the anthracene solubility and seemingly independent of salinity.

More complex thermal signals are expected for substances where the initial degradation step is not rate-determining. In this case, intermediate pools are intra- or extracellularly formed and the calorimetric signal is also influenced by the degradation of these pools. This deviating behaviour can be accounted for by a more sophisticated kinetic model. Depending on the mathematical structure of this model the analytical integration of Eq. (2) will be either impossible or too difficult. In this case, Eq. (2) has to be integrated numerically in order to be applicable to our method.

We have demonstrated that isothermal titration calorimetry (ITC) is well suited to determine biodegradation parameters even for extreme low concentrations of pollutants. In comparison with conventional methods of determining the biodegradation of trace compounds, ITC offers various advantages.

- The accuracy of the parameter is higher because of the extreme sensitivity of modern calorimetric sensors and the much higher sampling rate.
- The measurement and the reaction occur in the same vessel reducing systematic errors introduced by sample preparation and analysis.
- The on-line calorimetric measurement reduces the man power required to determine kinetic parameter sets.
- Multiple automatic injections allow the rapid repetition of degradation processes and the investigation of concentration dependencies within one experiment.

The prediction of biodegradation parameters in nature is difficult for HOCs because different processes overlap, e.g. phase transitions, reactions on surfaces, adsorption on surfaces, transport processes and the bioconversion. ITC presents an easy way to quantify bioconversions and their dependency on environmental factors, e.g. pH, temperature, salinity and nutrient availability. ITC can thus contribute to the illumination and understanding of complex processes occurring during pollutant degradation. The main thrust of future research will thus be to establish calorimetry for microbial HOC-degradation in heterogeneous multiphase systems.

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