Hydrogen isotope fractionation as a tool to identify aerobic and anaerobic PAH biodegradation

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Abstract

Aerobic and anaerobic polycyclic aromatic hydrocarbon (PAH) biodegradation was characterized by compound specific stable isotope analysis (CSIA) of the carbon and hydrogen isotope effects of the enzymatic reactions initiating specific degradation pathways, using naphthalene and 2-methylnaphtalene as model compounds. Aerobic activation of naphthalene and 2-methylnaphthalene by *Pseudomonas putida* NCIB 9816 and *Pseudomonas fluorescens* ATCC 17483 containing naphthalene dioxygenases was associated with moderate carbon isotope fractionation (ε_C = -0.8 ± 0.1 ‰ to -1.6 ± 0.2 ‰). In contrast, anaerobic activation of naphthalene by a carboxylation-like mechanism by strain NaphS6 was linked to negligible carbon isotope fractionation (ε_C = -0.2 ± 0.2 ‰ to -0.4 ± 0.3 ‰). Notably, anaerobic activation of naphthalene by strain NaphS6 exhibited a normal hydrogen isotope fractionation (ε_H = -11 ± 2 ‰ to -47 ± 4 ‰) whereas an inverse hydrogen isotope fractionation was observed for the aerobic strains (ε_H = +15 ± 2 ‰ to +71 ± 6 ‰). Additionally, isotope fractionation of NaphS6 was determined in an overlaying hydrophobic carrier phase, resulting in more reliable enrichment factors compared to immobilizing the PAHs on the bottle walls without carrier phase. The observed differences especially in hydrogen fractionation might be used to differentiate between aerobic and anaerobic naphthalene and 2-methylnaphthalene biodegradation pathways at PAH-contaminated field sites.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds with two or more fused aromatic rings which pose serious environmental problems for terrestrial and marine environments at a global scale.[1-4] Naturally occurring in coal, crude oil, asphalt or creosote, PAHs are also produced by anthropogenic processes such as the use and disposal of petroleum products.[5, 6] Caused by the hydrophobic nature of PAHs most of them usually adsorb to solid soil particles.[7-9] The hydrophobicity and persistence of PAHs increases with their molecular mass.[9] PAHs are known to be toxic as well as potential carcinogenic.[10, 11] Based on their abundance and toxicity, 16 PAHs have been included by the U.S. EPA to the list of priority pollutants.[12] Although limited by generally low bioavailability of PAHs in the environment, several PAHs have been described to be degradable by microorganisms. This microbial degradation is one of the major mechanisms leading to a sustainable restoration of PAH contaminated sites. *In situ* biodegradation of the poorly water-soluble PAHs is difficult to monitor due to the generally limited bioavailability and associated slow reactions kinetics of PAHs. For monitoring and evaluating environmental PAH biodegradation processes, knowledge about responsible organisms and biochemical pathways is important and necessary to understand *in situ* biodegradation processes. Various studies revealed that microorganisms are able to use low-molecular mass PAHs such as naphthalene and 2-methylnaphthalene as sole carbon and energy source under oxic conditions.[8, 13] Both compounds are activated by the addition of molecular oxygen leading to the formation of the respective *cis*-1,2-dihydrodiols[1] catalyzed by naphthalene dioxygenase (NDO).[13-17] Under anoxic conditions, the microbial degradation of naphthalene and 2-methylnaphthalene was demonstrated with nitrate,[5, 18, 19] sulfate,[20-26] ferric iron[27, 28] or carbonate[29-31] as terminal electron acceptors. Recently, a naphthalene carboxylase-like enzyme

has been described catalyzing the first step of anaerobic naphthalene degradation in the sulfate-reducing enrichment culture N47.[32]

Identifying and quantifying biodegradation of a defined organic pollutant in contaminated environments is still challenging. For a reliable assessment of *in situ* degradation, several independent methods may be combined to reduce the uncertainty and to clearly demonstrate ongoing biodegradation processes. Compound specific isotope analysis (CSIA) received increasing attention in the last years since this method can be used to directly assess natural attenuation of contaminants *in situ*.[33] The method is based on the principle that transformation reactions proceed via formation or cleavage of bonds leading to an isotope fractionation. Kinetic isotope effects (KIEs) during these bond changes are typically caused by the preferred reaction of the lighter isotopes (e.g., ^{12}C, ^{1}H) compared to the heavier ones (e.g., ^{13}C, ^{2}H) due to different activation energies. Hence, heavier isotopes are usually enriched in the residual fraction of the reactant and depleted in the product fraction.[34, 35] In general, KIEs are subdivided into two groups, primary and secondary KIEs. Primary KIEs arise from direct bond transformation while secondary ones evolve during hybridization and hyperconjugation processes.[36-38] Secondary KIEs are typically one order of magnitude smaller than the primary KIEs. Nevertheless, due to the large mass difference of hydrogen isotopes, secondary KIEs can reach significant values upon hydrogen isotope fractionation.[39] Since non-destructive processes like dilution, sorption or volatilization generally do not cause a significant isotope fractionation, CSIA can be used to trace *in situ* biodegradation by determining the isotopic signatures of certain contaminants in field samples, either over time or spatially along the contamination plume.[40, 41] However, isotope fractionation can be influenced by processes prior to the isotope sensitive bond transformation assumed to be virtually non-fractionating (e.g., transport or binding of the substrate); the resulting apparent kinetic isotope effects (AKIEs) are often smaller than their corresponding intrinsic

KIEs,[35, 42] thus leading to a masking of isotope fractionation.[43, 44] Previous work suggest that non-fractionating masking effects can be cancelled out by two-dimensional compound specific stable isotope analysis (2D-CSIA).[35, 45] Here, the changes in isotope ratios of two elements are compared and expressed as the lambda value (Λ) which is – in case of carbon and hydrogen isotopes – defined as the slope of the linear regression for hydrogen versus carbon isotope signatures ($\Lambda^{H/C}$).[46] $\Lambda^{H/C}$ values have been shown to be linear for a wide range of carbon-hydrogen bond cleavage reactions,[42, 43, 46, 47] indicating that they are a sensitive and reproducible data evaluation tool to specify initial reactions of hydrocarbon degradation pathways.[43, 46, 47] The dual correlation of hydrogen and carbon fractionation factors to obtain $\Lambda^{H/C}$ may not be applicable in case of very strong hydrogen isotope fractionation as recently shown by Dorer and colleagues for the anaerobic hydroxylation of ethylbenzene catalyzed by ethylbenzene dehydrogenase,[47, 48] or in case of non-detectable carbon and/or hydrogen isotope fractionation.[44] Carbon isotope enrichment factors (ε_C) of naphthalene biodegradation have been reported to be different for two sulfate-reducing cultures (ε_C = -0.3‰ and ε_C = -2‰) whereas hydrogen isotope enrichment factors (ε_H) were similarly strong (ε_H = -43‰ and ε_H = -59‰), resulting in significantly different $\Lambda^{H/C}$ values for both cultures (107 ± 45 versus 29 ± 8).[34] Additionally, small carbon isotope fractionation of naphthalene has been determined *in situ* at two anoxic PAH-contaminated field sites,[40, 49] indicating that analyzing only carbon isotopes is not conclusive for proving anaerobic PAH biodegradation by CSIA.

In this study, we analyzed the carbon and hydrogen isotope fractionation of aerobic and anaerobic PAH biodegradation using naphthalene and 2-methylnapthalene as model compounds and the aerobic bacterial strains *Pseudomonas putida* NCIB 9816 and *Pseudomonas fluorescens* ATCC 17483 as well as the highly enriched sulfate-reducing culture NaphS6 as model cultures. The aerobic strains contain the well-investigated and widely distributed NDO as PAH-activating

enzyme;[8, 13, 50-53] in contrast, strain NaphS6 activates naphthalene most probably by carboxylation.[26] Furthermore, we studied the influence of three different cultivation techniques on the resulting enrichment factors in order to understand the effects of reduced PAH bioavailability on isotope fractionation. To this end, the sulfate-reducing culture NaphS6 was cultivated (1) with an overlaying 2,2,4,4,6,8,8-heptamethylnonane (HMN) phase serving as a carrier phase for the poorly water soluble naphthalene, (2) with solid naphthalene immobilized as a thin film on the walls of the cultivation bottles and (3) with completely dissolved naphthalene in concentrations lower than its solubility in water. For all incubations, carbon and hydrogen isotope fractionation were measured and compared.

Material and Methods

Cultivation of bacteria

Pseudomonas putida NCIB 9816 (DSM 8368)[54, 55] and *Pseudomonas fluorescens* ATCC 17483 (DSM 6506)[52] were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The strains were cultivated in mineral salt medium[56] amended with naphthalene or 2-methylnaphthalene as sole carbon and energy source. Due to the poor water solubility, PAHs were supplied in an acetone stock solution (naphthalene: 125 mM; 2-methylnaphthalene: 98 mM), of which 1 ml was added to 1 l medium. To ensure that the PAHs are completely dissolved, the medium was ultrasonicated two times for 15 min. Subsequently, the medium was distributed in portions of 90 ml in 250 ml glass bottles which were sealed gastight to avoid any evaporation of the PAHs. The total molar amount of the respective PAH per bottle was 11.3 µmol for naphthalene and 8.8 µmol for 2-methylnaphthalene. Prepared bottles were inoculated with 10 ml of a preparatory culture. Cultures were incubated at

28 °C and 120 rpm in the dark. PAH degradation was monitored by GC-FID (see below). Single cultures were sacrificed by adding 1 ml concentrated HCl at different stages of degradation. The naphthalene-degrading, sulfate-reducing culture NaphS6 was cultivated as described elsewhere.[26] Substrate amendment was established by three different approaches. For the first approach, naphthalene was added as solid crystals directly to the 1 l medium yielding an initial naphthalene concentration of 94 µM. To achieve complete dissolution of naphthalene, the medium was afterwards shaken for three weeks at 120 rpm. Subsequently, the medium was distributed in portions of 90 ml, sealed gastight and inoculated with 10 ml of a preparatory culture. The initial total molar amount of naphthalene was approximately 8.5 µmol per bottle. For the second approach, a volume of 10 ml of a growing culture was added to 90 ml of an anoxic artificial sea water medium.[26] Prepared cultures were overlaid with 1 ml anoxic 2,2,4,4,6,8,8-heptamethylnonane (HMN) containing naphthalene with a concentration of 156 mM or 391 mM yielding a total molar amount of 156 µmol or 391 µmol naphthalene per bottle, respectively. For the third approach, solid naphthalene was immobilized as a thin film on the walls of the cultivation bottles. Therefore, an acetone stock solution containing naphthalene (156 mM or 391 mM, respectively) was prepared and 1 ml of this solution was added to sterile bottles. The solution was carefully rinsed over the walls of the bottles. Afterwards, the acetone was evaporated by a gentle stream of sterilized nitrogen. Such prepared bottles were filled with 90 ml anoxic artificial sea water medium and inoculated with 10 ml active culture previously grown with naphthalene. The final total molar amount of naphthalene per bottle was 156 µmol or 391 µmol, respectively. The naphthalene amounts in the second and third approach were above the water solubility limit. Each culture was incubated at 28 °C. Growth was monitored by measuring the sulfide production. Single cultures were sacrificed by adding 1 ml 10 M NaOH at different stages of degradation. In cultures containing naphthalene dissolved mainly in HMN or directly in

157 the medium, the concentration of the remaining naphthalene was determined by GC-FID. In
158 cultures containing immobilized naphthalene without carrier phase, naphthalene concentrations in
159 the liquid phase were measured by GC-MS after extraction in *n*-hexane.
160
161 *Chemical analyses*
162 Aqueous concentrations of naphthalene and 2-methylnaphthalene were determined by automated
163 headspace chromatography (Varian 3800; Varian, Germany) coupled with a flame ionization
164 detector. Compounds in aqueous samples were separated on a CP SIL 5 CB capillary column (25
165 m x 0.12 mm x 0.12 µm; Varian, Germany) with the following temperature program: 120 °C for
166 5 min, 60 °C/min to 220 °C, and a hold for 2 min. Before analysis, samples (0.5 ml) were mixed
167 with 9.5 ml H_2SO_4 (1.6 mM) in 20 ml glass vials and closed gastight. In cultures containing a
168 HMN phase, 20 µl aliquots of the HMN phase were taken, transferred in 20 ml glass vials and
169 closed gastight. Aqueous and HMN samples were each incubated for 30 min at 70 °C in an
170 agitator (rotation regime of 250 rpm for 5 s and no rotation for 2 s) prior to analysis. One
171 milliliter of each sample's headspace was injected. For calibration, diluted standards of
172 naphthalene or 2-methylnapthalene prepared from stock solutions were treated in the same way
173 as the samples. The stock solutions were prepared in pure methanol or in pure HMN,
174 respectively.
175 For cultures containing immobilized naphthalene, the remaining naphthalene was extracted with
176 1 ml *n*-hexane by continuous shaking at 100 rpm and 20 °C for at least 96 h. Aliquots of these *n*-
177 hexane extracts were analyzed using a HP 6890 gas chromatograph coupled with a HP 5973
178 quadrupole mass spectrometer (Agilent Technologies, Palo Alto, USA). Compounds were
179 separated by a Zebron BPX-5 column (length, 30 m; inner diameter, 0.32 mm; film thickness,
180 0.25 µm; Phenomenex, Torrance, USA) using the following temperature program: 60 °C for 1

min, 25 °C/min to 105 °C, 8 °C/min to 110 °C, 25 °C/min to 300 °C, and a hold for 10 min. For calibration, diluted standards of naphthalene prepared from stock solutions were treated in the same way as the samples. The stock solutions were prepared in *n*-hexane.

For anaerobic degradation experiments sulfide was measured photometrically as colloidal copper sulfide.[57] Complete naphthalene oxidation by strain NaphS6 is illustrated by the following equation: $C_{10}H_8 + 6SO_4^{2-} + 2H^+ + 6H_2O \rightarrow 10HCO_3^- + 6H_2S$.[26]

Analysis of stable isotopes and isotopic calculations

Naphthalene or 2-methylnaphthalene in cultures without HMN phase was extracted by adding 1 ml *n*-hexane. For extraction, the bottles were shaken at 100 rpm and 20 °C for at least 96 h. Aliquots (1 to 5 µl) of those extracts were used for isotope analyses. In case of cultures with HMN, 1 to 5 µl of the respective HMN phase were directly analyzed without *n*-hexane extraction.

Carbon and hydrogen isotopic signatures of PAHs were analyzed by an Agilent Technologies 7891A GC system (Agilent, Germany) coupled with a MAT 253 stable isotope ratio mass spectrometer (Thermo Fisher Scientific Germany Ltd. & Co. KG, Germany). For carbon isotope analysis the temperature of the combustion oven was adjusted to 1000 °C. The temperature of the interface for pyrolysis was adjusted to 1420 °C for hydrogen isotope analysis. Samples were separated on a Zebron ZB-1 column (60 m x 0.23 cm x 1 µm; Phenomenex, Germany) at a flow of 2.0 ml/min for carbon and 1.2 ml/min for hydrogen isotope analysis with the following temperature program: 120 °C for 5 min, 4 °C/min to 220 °C, 20 °C/min to 300 °C, and a hold for 5 min.

Isotope ratios were expressed as delta notation ($\delta^{13}C$ and $\delta^{2}H$) according to equation 1:

$$\delta^{13}C \text{ or } \delta^{2}H = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \quad (1)$$

R_{sample} and $R_{standard}$ are the ratios of $^{13}C/^{12}C$ or $^{2}H/^{1}H$ of the sample or the corresponding ratio of a standard, respectively. The isotope composition was reported relative to the Vienna Pee Dee Belemnite (VPDB) for carbon isotopes and the Vienna Standard Mean Ocean Water (VSMOW) for hydrogen isotopes, respectively. Due to the typically small variations in carbon and hydrogen isotope ratios, δ-values are reported in parts per thousand (‰).

Each sample was measured in at least three technical replicates to secure accuracy and reproducibility of the measurement. The standard deviation was maximal 0.5 ‰ for $\delta^{13}C$ and maximal 5 ‰ for $\delta^{2}H$, respectively.

Quantification of isotope fractionation

For quantification of isotope fractionation the linearized Rayleigh equation was used:[58]

$$\ln \frac{\delta_t}{\delta_0} = \varepsilon * \ln \frac{C_t}{C_0} \quad (2)$$

δ_t, C_t and δ_0, C_0 are the isotope composition and concentration of the substrate at a given point in time t and at the beginning of the reaction, respectively. The enrichment factor (ε) combines the change in stable isotope ratio with the change in concentration. To determine the lambda value ($\Lambda^{H/C}$), the hydrogen versus carbon isotope signatures were plotted and the $\Lambda^{H/C}$ value was derived from the slope of linear regression according to equation 3:

$$\Lambda^{H/C}{}_{bulk} = \frac{\Delta\delta^{2}H_{bulk}}{\Delta\delta^{13}C_{bulk}} \quad (3)$$

The uncertainty of the enrichment factors and the $\Lambda^{H/C}$ values, given as the 95% confidence interval (CI), was derived from regression analysis.[59] Moreover, quality of enrichment factors was verified by determining the correlation between PAH concentration and isotope signature

(R^2). In case of correlations lower than 0.8 the respective fractionation was regarded as not significant.

AKIE values

The AKIE characterizes the isotope effect of the atoms at the reactive position of a molecule. Consequently, isotope fractionation factors need to be corrected for the number of atoms located at not reactive positions which are not effected by bond changes.[35] Therefore, bulk isotope compositions (δ_{bulk}) are converted into isotope compositions of the reactive position ($\delta_{reactive\ position}$):

$$\delta_{\text{reactive position}} = \delta_{\text{bulk}} * \frac{n}{x} \quad (4)$$

Here, n is the total number of atoms of an element and x is the number of atoms at the reactive position of the molecule (Figure 1). For naphthalene activation by a dioxygenase, 8 out of 10 carbon atoms

$$\delta^{13}C_{\text{reactive position}} = \delta^{13}C_{\text{bulk}} * \frac{10}{8} \quad (5)$$

and 8 out of 8 hydrogen atoms are located at the reactive position

$$\delta^{2}H_{\text{reactive position}} = \delta^{2}H_{\text{bulk}} * \frac{8}{8} \quad (6)$$

Regarding the activation of 2-methylnaphthalene by a dioxygenase, only 2 out of 11 carbon atoms

$$\delta^{13}C_{\text{reactive position}} = \delta^{13}C_{\text{bulk}} * \frac{11}{2} \quad (7)$$

and 2 out of 10 hydrogen atoms are at the reactive position

$$\delta^{2}H_{\text{reactive position}} = \delta^{2}H_{\text{bulk}} * \frac{10}{2} \quad (8)$$

For a naphthalene activation catalyzed by a carboxylase-like enzyme, the following corrections were used for carbon fractionation:

$$\delta^{13}C_{\text{reactive position}} = \delta^{13}C_{\text{bulk}} * \frac{10}{4} \quad (9)$$

and for hydrogen fractionation:

$$\delta^{2}H_{\text{reactive position}} = \delta^{2}H_{\text{bulk}} * \frac{8}{4} \quad (10)$$

Based on this correction the enrichment factors of the reactive position ($\varepsilon_{\text{reactive position}}$) were determined using the linearized Rayleigh equation as described above.

A second correction was used due to the location of various non-discriminable atoms of an element at the reactive position. The AKIE value was calculated by equation 11:

$$\text{AKIE} = \frac{1}{1+z*\varepsilon_{\text{reactive position}}/1000} \quad (11)$$

z is the amount of non-discriminable atoms at the reactive position. For naphthalene activation catalyzed by a dioxygenase, z is 4 for both carbon and hydrogen fractionation assuming a concerted incorporation or, z is 8 for a stepwise incorporation of the oxygen atoms. Concerning naphthalene activation by a carboxylase, z is 4 for both carbon and hydrogen fractionation. For the reaction of 2-methylnaphtalene catalyzed by a dioxygenase, z is 1 for both carbon and hydrogen fractionation in case of a concerted reaction, or z is 2 in case of a stepwise incorporation of the oxygen atoms, respectively (Figure 1).

The uncertainties associated with AKIE values were estimated by error propagation as given in equation 12.

$$\text{error of AKIE} = \left|\frac{\partial \text{AKIE}}{\partial \varepsilon_{\text{reactive position}}}\right| * \text{error of } \varepsilon_{\text{reactive position}} \quad (12)$$

Results

Carbon and hydrogen stable isotope fractionation during the degradation of naphthalene and 2-methylnaphthalene

The enrichment factors for carbon (ε_C) during aerobic naphthalene and 2-methylnaphthalene degradation ranged between -0.8 ± 0.1 ‰ and -1.6 ± 0.2 ‰ for *P. putida* and *P. fluorescens* (Figure 2A; degradation kinetics are shown in SI Figure 1). Notably, an inverse hydrogen fractionation was obtained for naphthalene and 2-methylnaphthalene degradation (Figure 2B). *P. putida* produced ε_H values of +54 ± 4 ‰ for naphthalene and +71 ± 6 ‰ for 2-methylnaphthalene degradation, whereas ε_H values of +21 ± 3 ‰ for naphthalene and +15 ± 2 ‰ for 2-methylnaphthalene degradation were observed for *P. fluorescens*. In each individual experiment, a high correlation between PAH concentrations and isotope signatures with R^2 values between 0.94 and 0.99 was observed (Figure 2A and B, Table 1) demonstrating the applicability of the Rayleigh equation for assessing the respective enrichment factors.

For determining the magnitude of isotope fractionation of anaerobic naphthalene degradation by strain NaphS6 different methods of substrate amendment were used: (1) naphthalene dissolved in an overlaying hydrophobic HMN phase, (2) naphthalene immobilized as a thin film on the walls of the cultivation bottles and (3) naphthalene completely dissolved in the medium. Here, the latter experimental set-up was used as reference experiment to investigate possible influences on isotope fractionation occurring during the phase-transfer steps of the other methods. Due to poor water solubility and high salt content of the medium, the initial naphthalene concentration within this reference experiment was 94 µM yielding a total molar amount of 8.5 µmol naphthalene per bottle. Caused by the intrinsic sulfide concentration of the medium and the low initial naphthalene concentration, determined changes in sulfide concentration were close to the detection limit leading to just a minor correlation between increasing sulfide and decreasing naphthalene concentrations (SI Figure 2A). However, correlation between sulfide and naphthalene concentrations in experiments using naphthalene dissolved in HMN or immobilized naphthalene was in the expected range of a complete naphthalene oxidation by strain NaphS6 (SI

295 Figure 2B, C). As indicated by the increasing sulfide and decreasing naphthalene concentrations,
296 strain NaphS6 showed a significantly faster substrate disappearance when naphthalene was
297 dissolved in HMN (SI Figure 2).
298 In all experiments with strain NaphS6, a negligible carbon fractionation was observed, ranging
299 from -0.2 ± 0.2 ‰ to -0.4 ± 0.3 ‰. Considering the errors and the low correlation between
300 naphthalene concentration and isotope signature (R^2 values between 0.10, 0.33 and 0.76; Figure
301 3A, Table 1), the stable carbon fractionation was regarded to be not significant. In contrast to
302 carbon isotope fractionation, a significant hydrogen isotope fractionation was detected. In
303 cultures containing completely dissolved naphthalene, the ε_H value was -47 ± 4 ‰. For
304 naphthalene dissolved in HMN, ε_H values ranged from -44 ± 7 ‰ to -46 ± 14 ‰, whereas
305 considerably lower and partially non-significant ε_H values were observed in cultures with
306 different amounts of immobilized naphthalene (Table 1). In general, ε_H values for naphthalene
307 dissolved in HMN were comparable to ε_H values determined for naphthalene directly dissolved in
308 the medium, if high initial naphthalene concentrations were provided (Figure 3B, Table 1). No
309 significant time-dependent change in the isotopic signature of the investigated PAHs was
310 observed in biomass-free control bottles containing similar PAH concentrations (data not shown).
311 Apparent kinetic isotope effects for carbon ($AKIE_C$) ranged between 1.004 ± 0.004 to 1.022 ±
312 0.005 for the aerobic dihydroxylation of naphthalene and 2-methylnaphthalene (Table 1). Due to
313 the inverse hydrogen isotope fractionation, the respective $AKIE_H$ were smaller than 1: $AKIE_H$
314 values for *P. putida* ranged between 0.823 ± 0.012 (naphthalene degradation assuming a
315 concerted reaction) and 0.541 ±0.024 (2-methylnaphthalene degradation assuming a stepwise
316 reaction), whereas $AKIE_H$ values for *P. fluorescens* ranged between 0.924 ± 0.010 (naphthalene
317 degradation assuming a concerted reaction) and 0.813 ± 0.057 (2-methylnaphthalene degradation
318 assuming a stepwise reaction). The $AKIE_H$ values for the anaerobic degradation of naphthalene

by strain NaphS6 ranged between 1.097 ± 0.020 and 1.573 ± 0.082 depending on the method of naphthalene application (Table 1). The corresponding $AKIE_C$ values were not calculated due to the insignificant carbon isotope fractionation.

Two dimensional isotope fractionation analysis

Due to the inverse stable hydrogen fractionation upon aerobic naphthalene degradation, the linear regression for carbon and hydrogen discrimination exhibited negative $\Lambda^{H/C}$ values (SI Figure 3, Table 1). The $\Lambda^{H/C}$ values of *P. putida* converting naphthalene ($\Lambda^{H/C}$ = -18 ± 2) and 2-methylnaphthalene ($\Lambda^{H/C}$ = -30 ± 5) were twofold higher than the $\Lambda^{H/C}$ values of *P. fluorescens* for naphthalene ($\Lambda^{H/C}$ = -9 ± 1) and 2-methylnaphthalene ($\Lambda^{H/C}$ = -15 ± 1) conversion, respectively. For NaphS6, $\Lambda^{H/C}$ values were not determined due to the non-significant stable carbon fractionation during anaerobic naphthalene degradation.

Discussion

Stable isotope fractionation accompanying naphthalene and 2-methylnaphthalene dihydroxylation by NDO

Naphthalene and 2-methylnaphthalene dihydroxylation was accompanied by significant carbon and hydrogen isotope fractionation. Notably, a moderate inverse hydrogen isotope fractionation was observed for both investigated strains containing a NDO (Figure 2B, Table 1). The isotope effects of NDO can be explained by taking a closer look into the reaction mechanism. NDO is a part of a multicomponent enzyme containing a NADH oxidoreductase, a ferredoxin and an oxygenase comprising the active centre; the oxygenase is termed NDO.[8] The active site contains a Rieske [2Fe-2S] center and a mononuclear non-heme iron attached. Dihydroxylation of naphthalene is initiated by reactive oxygen species derived from molecular oxygen. Molecular

oxygen is activated by a two-electron transfer from the non-heme Fe(II) in the active centre; the electrons are originally stemming from $NADH+H^+$ and are transferred via the oxidoreductase, the ferredoxin and the Rieske center to the non-heme Fe(II).[60] The two oxygen atoms bound to the non-heme iron react with two carbon atoms of naphthalene in a concerted reaction via the formation of an epoxide leading to the product (1R,2S)-1,2-dihydronaphthalene-1,2-diol. Though, also different mechanisms for the actual oxygen insertion steps such as an step-wise reaction via either cationic or radical intermediates have been proposed;[61] however, in all mechanisms proposed so far no C-H bonds are cleaved upon the reaction. Thus, any changes of the hydrogen isotopic composition during the degradation of the respective substrate should be solely caused by secondary kinetic isotope effects. Those can be expected as during naphthalene dihydroxylation of a π C-C bond (sp^2 hybridization) is converted into a σ C-C bond (sp^3 hybridization) caused by the reaction with reactive oxygen species (Figure 1). At a sp^2 carbon atom the substituted hydrogen atoms have a lower oscillation frequency caused by an increase of the available space for oscillation. Consequently, C-C bonds with sp^2 hybridization in aromatic systems or double bonds are relatively flexible which is generally preferred by lighter isotopes like hydrogen. If a C-C sp^2 bond is converted to a sp^3 hybridized σ C-C bond one further substituent is introduced leading to a decrease in space, a higher oscillation frequency of the substituents and more rigid bonds. This state is preferred by the heavier isotope deuterium.[62] Accordingly, naphthalene or 2-methylnaphthalene isotopologues containing a deuterium atom at the reactive site are probably preferentially transformed by the NDO than isotopologues containing a hydrogen atom at this site, leading to an enrichment of the lighter isotopologues in the residual fraction and consequently to an inverse isotope effect. The AKIE values determined for hydrogen considering a concerted reaction (Table 1) are in the typical range of the secondary inverse isotope effect between 0.7 and 1.[63, 64]

Correspondingly, inverse hydrogen isotope effects have been recently reported for ring dihydroxylation of ethylbenzene by *P. putida* F1 [47] and chemical hydroxylation of benzene, toluene, ethylbenzene and nitrobenzene by OH radicals.[65] In former studies, a dioxygenation of the ring of monoaromatic compounds (benzene, toluene) by different dioxygenases was reported to produce no significant hydrogen isotope fractionation.[43, 46] Conversely, in the study of Morasch and colleagues,[66] a moderate normal hydrogen isotope effect upon dihydroxylation of naphthalene was observed for the NDO of *P. putida* NCIB 9816; here, a 50:50 mixture of non-labelled and per-deuterated naphthalene-d_8 was used for determining hydrogen isotope fractionation. This might be due steric effects of naphthalene-d_8 slightly inhibiting its conversion since any selectivity of reactions catalyzed by Rieske oxygenases is thought to be determined solely by the specific orientation of the substrate in the binding pocket of the enzyme.[60, 61, 67] Notably, the molecular volume of naphthalene-d_8 is expected to be greater than that of non-labelled naphthalene at the temperatures used in the degradation experiments.[68]

During naphthalene dihydroxylation, a π C-C bond of the aromatic ring is converted to a covalent σ C-C bond forming a dihydrodiol intermediate prior to further reactions. The low $AKIE_C$ values observed in this study for dihydroxylation of naphthalene and 2-methylnaphthalene assuming a concerted reaction mechanism were similar to $AKIE_C$ values previously reported for dioxygenases acting on benzene,[46] toluene[43] or ethylbenzene[47] and matched the previously described interval of 1 to 1.01 for the comparable epoxidation reaction of a C-C double bond.[35] Due to the inverse hydrogen isotope fractionation, $\Lambda^{H/C}$ values were negative for naphthalene and 2-methylnaphthalene degradation initiated by NDO. Notably, $\Lambda^{H/C}$ values of *P. putida* converting naphthalene and 2-methylnaphthalene were two times larger than the respective values of *P. fluorescens*. This diversity in $\Lambda^{H/C}$ values might be caused by slight sequence variations of the NDO between the tested organisms leading to the assumption that the NDO exist naturally in

391 different isoenzymes. Similar variations modifying isotope fractionation have been recently
392 observed for the benzylsuccinate synthase catalyzing the anaerobic activation of toluene.[43, 64] To
393 verify this hypothesis, NDO gene sequences should be correlated with the particular stable
394 isotope fractionation factors in future studies.
395 It can be assumed that due to the inverse hydrogen isotope effect, the 2D-CSIA isotope pattern
396 produced by NDO-like enzymes will be considerably different from other 2-methylnaphthalene
397 transformation reactions not investigated in this study. Aerobic oxidation of methyl moieties of
398 aromatic systems by monooxygenases have been reported to be linked to a moderate to strong
399 normal carbon isotope effect and a moderate to strong normal hydrogen isotope effect,[43, 47, 69, 70]
400 leading to (positive) $\Lambda^{H/C}$ values of > 20. Analogously, the anaerobic fumarate addition to the
401 methyl moiety of 2-methylnaphthalene is expected to produce (positive) $\Lambda^{H/C}$ values due to
402 normal carbon and moderate to strong normal hydrogen isotope effects reported for aromatic and
403 aliphatic hydrocarbons activated by this reaction.[43, 47, 71-74]
404
405 *Stable isotope effects accompanied to anaerobic naphthalene activation*
406 In contrast to the reaction of the NDO, naphthalene degradation by the anaerobic enrichment
407 culture NaphS6 was associated by a normal hydrogen fractionation. NaphS6 is thought to activate
408 naphthalene in a reaction similar to the proposed naphthalene carboxylation by a carboxylase-like
409 enzyme catalyzing the activation of naphthalene in the sulfate-reducing enrichment culture N47,
410 leading to the cleavage of a C-H bond and the formation of a single covalent C-C bond.[32] Thus,
411 any hydrogen and carbon isotope fractionation are expected to be caused by primary kinetic
412 isotope effects. Hydrogen isotope fractionation of strain NaphS6 was significantly higher than
413 carbon isotope fractionation, which was always close to the detection limit under the tested
414 substrate amendment procedures for anaerobic cultivation, excluding the calculation of

statistically significant ε_C and $AKIE_C$. Notably, the magnitude of hydrogen isotope fractionation was strongly influenced by the cultivation conditions: cultures amended with immobilized naphthalene revealed a significant lower hydrogen fractionation compared to cultures in which naphthalene was amended in a HMN carrier phase or completely dissolved in the culture medium in lower concentrations (Table 1). Hence, isotope fractionation in cultures with immobilized naphthalene seemed to be masked by a rate-limiting, isotope insensitive substrate dissolution prior to the isotope sensitive step of the activation reaction. This effect was particularly apparent when the culture was amended with a total molar amount of 156 µmol immobilized naphthalene resulting in a non-significant stable hydrogen isotope fractionation. As in the case of stable carbon isotope fractionation a further data processing for this experiment was not conducted. Generally, masking effects caused by limited bioavailability of the substrate result in a lower magnitude of isotope fractionation and have been also observed e.g., during biodegradation of hydrocarbons under certain conditions.[71, 73, 75] However, ε_H and $AKIE_H$ values for the anaerobic degradation of naphthalene dissolved in HMN (Table 1) were similar to ε_H and $AKIE_H$ values determined in the reference experiment using naphthalene completely dissolved in the medium. Moreover, the determined values were also similar to the ε_H and $AKIE_H$ values determined in a previous study for anaerobic naphthalene degradation of the enrichment culture N47 and the pure culture NaphS2.[34] This indicates that by using HMN as a carrier phase, a significant masking of isotope fractionation could be avoided; the method of applying substrates by a non-polar carrier phase may be generally suitable for determining isotope enrichment factors coupled to the slow degradation of poorly water-soluble compounds. Notably, an extremely low carbon isotope fractionation (ε_C = -0.3 ‰ ± 0.1 ‰) was reported for naphthalene degradation by strain NaphS2,[34] similar to the results for strain NaphS6 in this study. In contrast, carbon isotope fractionation for the enrichment culture N47 was reported to be significantly higher (ε_C ranging

439 from -1.1 ‰ ± 0.4 ‰[40] to -2.0 ‰ ± 0.4 ‰[34]), indicating that the reaction mechanism of
440 naphthalene carboxylase of N47 is slightly different to that of strains NaphS2 and NaphS6.
441
442 *Environmental implications*
443 The results of this study demonstrate that stable hydrogen isotope fractionation has a substantial
444 potential to differentiate between aerobic and anaerobic biodegradation of low molecular mass
445 PAHs in the environment, as aerobic dihydroxylation produced inverse ε_H values and anaerobic
446 activation produced normal ε_H values (Table 1). So far, reports on monitoring the degradation of
447 PAHs directly in the field by determining isotopic signatures are rather scarce and focused on
448 stable carbon isotopes.[40, 49, 76, 77] In two studies was observed that anaerobic naphthalene
449 biodegradation was linked to small carbon isotope fractionation, allowing quantifying
450 naphthalene biodegradation.[40, 49] In contrast, *in situ* naphthalene carbon isotope fractionation was
451 low or below the detection limit at two other anoxic sites.[49, 76] The data indicate that analyzing
452 only carbon isotopes is insufficient for characterizing anaerobic PAH biodegradation by CSIA.
453 Accordingly, a more accurate alternative to trace aerobic and anaerobic *in situ* PAH-
454 biodegradation could be achieved by analyzing stable hydrogen isotope fractionation, as recently
455 already suggested for detecting anaerobic PAH degradation.[34]
456 A main issue regarding the monitoring of the biological fate of PAHs at field sites is the low
457 water solubility and the tendency of PAHs to adsorb to solid soil particles leading to a decreased
458 bioavailability.[7] Consequently, *in situ* isotope fractionation may be masked by non-fractionating
459 processes prior to the isotope sensitive bond transformation (e.g., dissolution of PAHs),
460 illustrated by the considerable lowering of isotope fractionation observed in this study in
461 experiments using solid naphthalene immobilized as a thin film on the walls of the cultivation
462 bottles. The results of this and other studies[34, 40] suggest that the concept of dual isotope analysis

463 which works well for e.g., monitoring the biodegradation of monoaromatic hydrocarbons[43, 45, 47,
464 73] is not generally applicable for PAHs. Additionally, the low water solubility of PAHs may
465 prevent reliable measurements of isotopic compositions caused by insufficient concentrations
466 leading to further challenges for the monitoring of PAH biodegradation in water samples.
467 Accordingly, different non-fractionating extraction strategies have to be considered such as solid-
468 phase extractions[78] or the use of semipermeable membrane devices[79]. Recently described
469 methods for sensitive carbon and/or hydrogen isotope analysis of PAHs may offer chances to
470 isotopically analyze PAHs up to the ng L^{-1} range.[80-82] Further limitations of CSIA can result from
471 mixing effects of different aquifer zones during sampling of contaminated groundwater;
472 especially at the fringe of the plume, reactive zones often occur at fine spatial scale.[83] As a rule of
473 thumb, samples for CSIA or other analyses should be taken from multi-level sampling wells [84]
474 located in the source and the fringe of the plume to prevent any mixing effects. Furthermore,
475 other methods such as signature metabolites analysis (SMA) could be additionally employed for
476 proving ongoing *in situ* PAH biodegradation.[40] A drawback of SMA is that detection of
477 degradation metabolites will indicate for *in situ* PAH biodegradation only qualitatively, and also
478 the absence of any signature metabolite does not inevitably mean that biodegradation does not
479 occur.[85]
480 In summary, stable hydrogen isotope fractionation for low molecular mass PAHs can be a helpful
481 tool for detecting specific biodegradation pathways at field sites, although different redox
482 conditions and bioavailability limitations may result in variable isotopic patterns. Thus, for
483 monitoring the fate of PAHs in nature with an acceptable accuracy also auxiliary approaches
484 have to be taken into consideration.[85]
485

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Supporting Information available.

This material is available free of charge via the internet at http://pubs.acs.org.

Legends of Figures and Tables

Figure 1: Initial reactions of anaerobic naphthalene degradation by strain NaphS6 (A) and aerobic naphthalene and 2-methylnaphthalene degradation by the strains *P. putida* NCIB 9816 and *P. fluorescens* ATCC 17483 (B). It is currently assumed that strain NaphS6 activates naphthalene (I) through a carboxylation-like reaction under strictly anoxic conditions yielding 2-napthoic acid (II). Under oxic conditions, naphthalene and 2-methylnaphthalene (III) are activated by a dihydroxylation reaction catalyzed by naphthalene dioxygenase (NDO). Here, a π C-C bond with sp^2 hybridization is converted to a covalent σ C-C bond with sp^3 hybridization yielding the respective diols (IV).

Figure 2: Carbon (A) and hydrogen (B) fractionation for the aerobic degradation of naphthalene (circles) and 2-methylnaphthalene (triangles) by *P. fluorescen*s (black symbols) and *P. putida* (green symbols).

Figure 3: Carbon (A) and hydrogen (B) fractionation for the anaerobic degradation of naphthalene by strain NaphS6 using a total molar amount of 8.5 µmol (squares), 156 µmol (circles) or 391 µmol (triangles) naphthalene per bottle as initial amendment, respectively. To

study the influence of different cultivation techniques on the resulting stable isotope fractionation the bacterial strain NapS6 was either cultivated with naphthalene directly dissolved in the media (red symbols), dissolved in HMN (green symbols) or immobilized as a thin film on the walls of the cultivation bottles (black symbols). In case of low correlations between naphthalene concentration and isotopic signature (R^2 less than 0.8) the fractionation was regarded to be not significant. Consequently, the respective slopes of linear regression were not included in the graph.

Table 1: Bulk enrichment factors (ε_{bulk}), enrichment factors for the reactive position (ε_{rp}), AKIE and lambda values for the aerobic conversion of naphthalene and 2-methylnaphthalene by *P. fluorescens* and *P. putida* and the anaerobic conversion of naphthalene by strain NaphS6.

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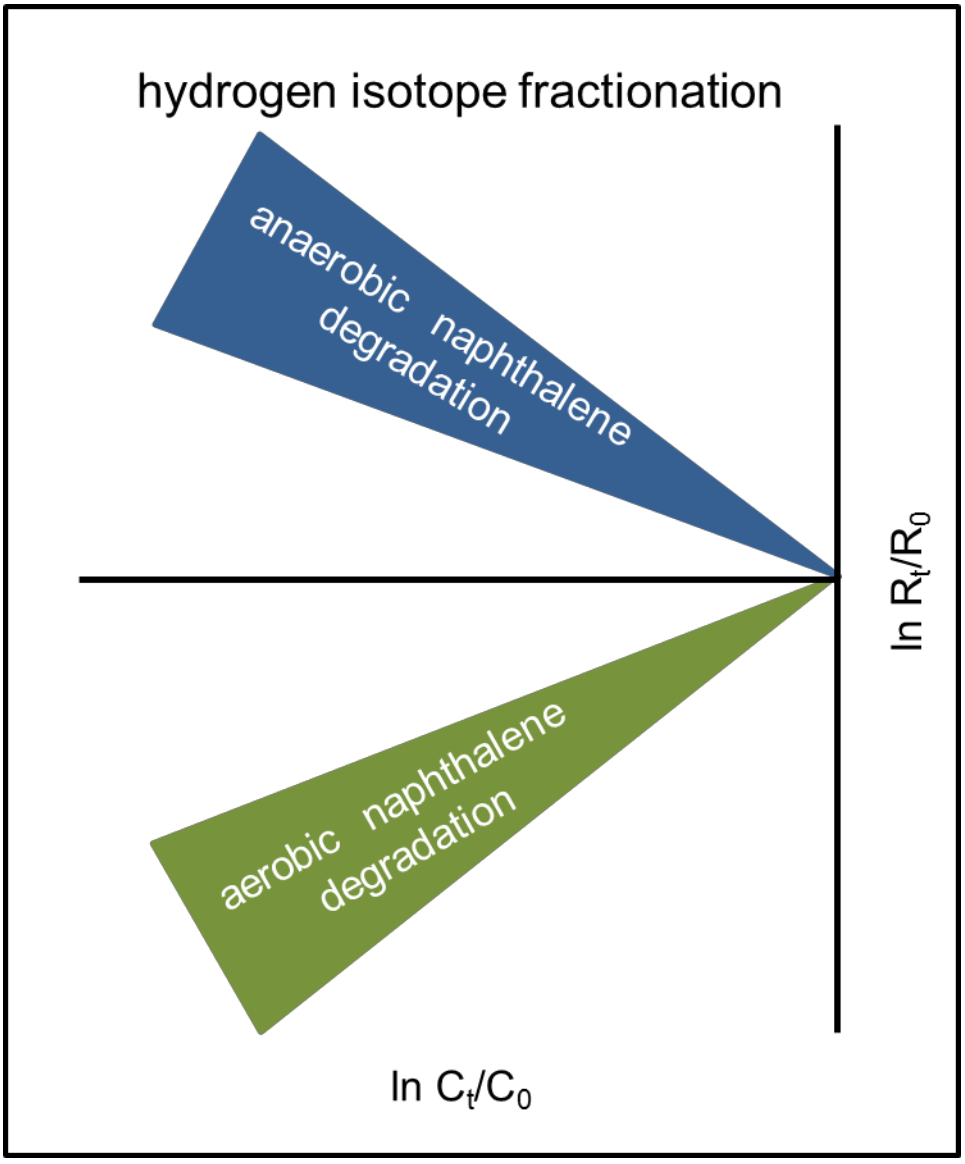
TOC art



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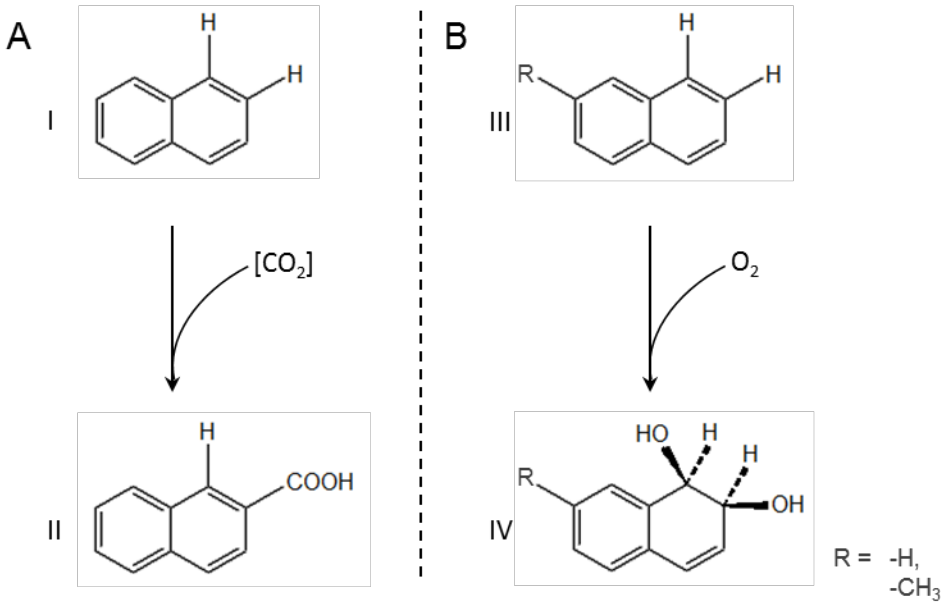

Figure 1

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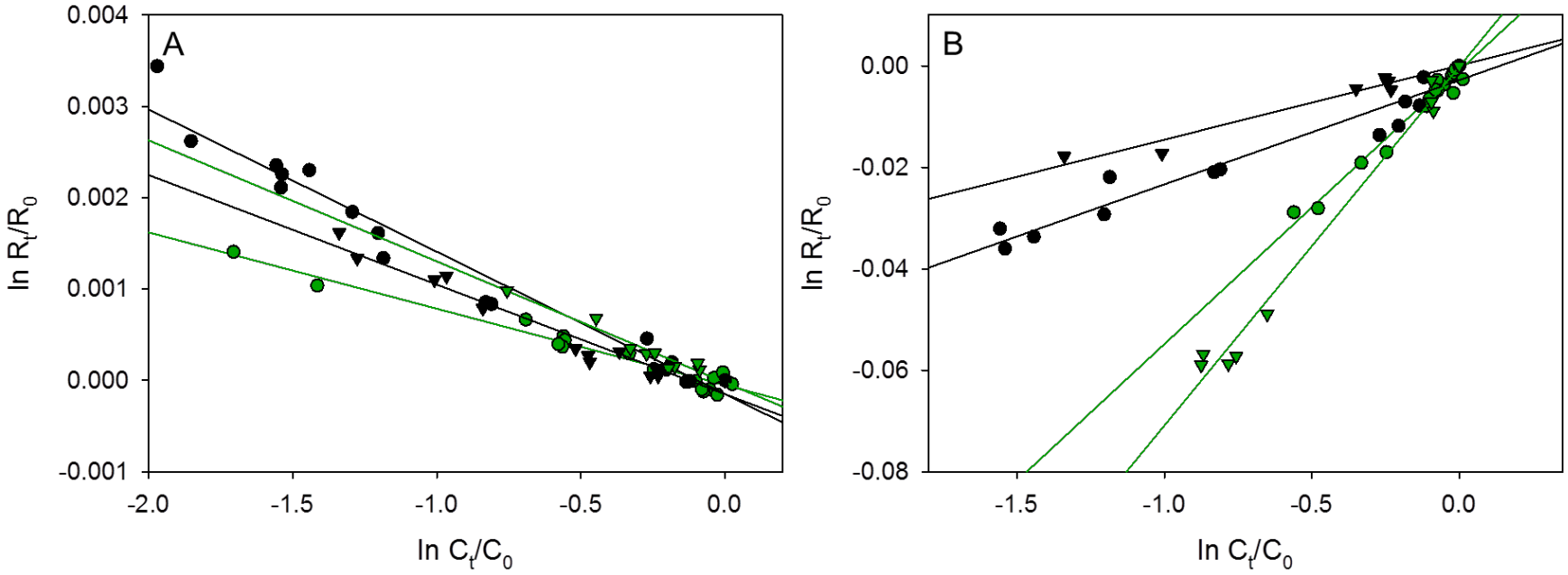
Figure 2



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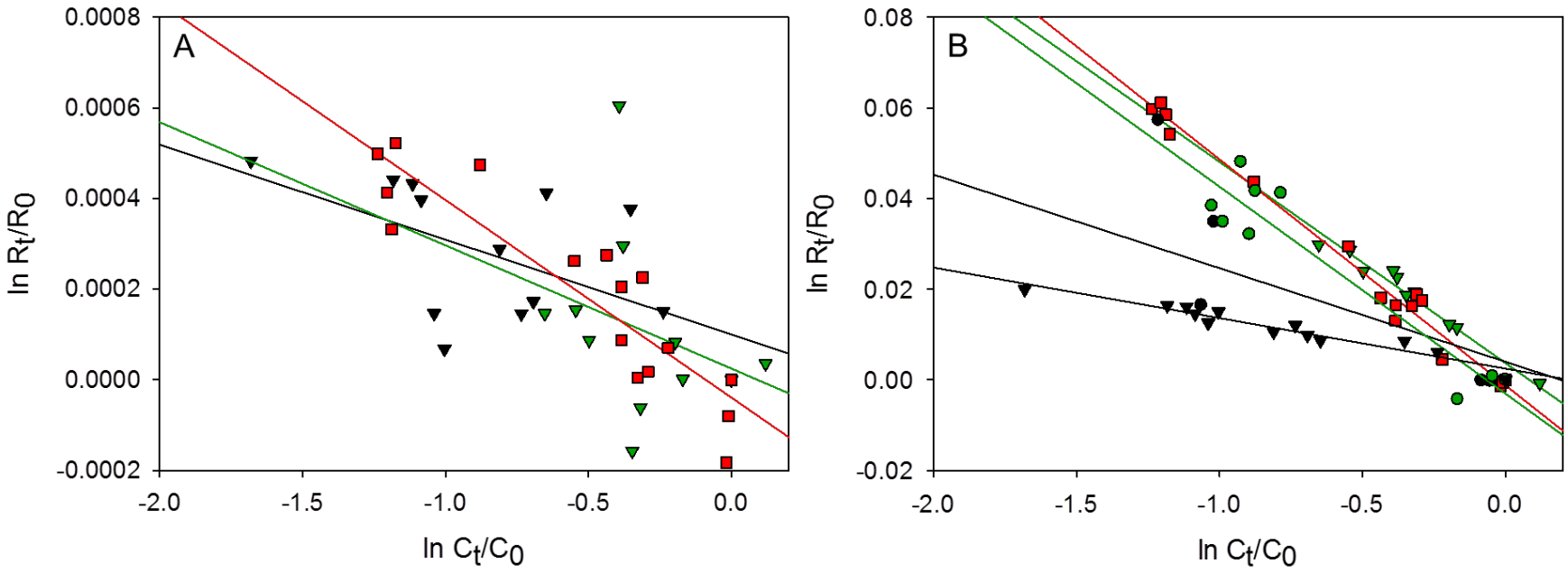

Figure 3

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Table 1 (Kuemmel et al., revision)

Organism and enzymatic PAH activation	Method of PAH application	Substrate	Initial molar amount of PAH per bottle [µmol]	$\varepsilon_{C\ bulk}$ [‰], (R^2)	$\varepsilon_{C\ rp}$ [‰], (R^2)	$AKIE_C$ (concerted reaction[1])	$AKIE_C$ (stepwise reaction[1])	$\varepsilon_{H\ bulk}$ [‰], (R^2)	$\varepsilon_{H\ rp}$ [‰], (R^2)	$AKIE_H$ (concerted reaction[1])	$AKIE_H$ (stepwise reaction[1])	$\Lambda^{H/C}$, (R^2)
P. putida (naphthalene dioxygenase)	Dissolved	Naphthalene	11.3	-0.8 ± 0.1 (0.96)	-1.0 ± 0.1 (0.96)	1.004 ± 0.004	1.008 ± 0.009	+54 ± 4 (0.97)	+54 ± 4 (0.97)	0.823 ± 0.012	0.699 ± 0.017	-18 ± 2 (0.98)
		2-methylnaphthalene	8.8	-1.3 ± 0.2 (0.96)	-10.6 ± 2.6 (0.95)	1.011 ± 0.003	1.022 ± 0.005	+71 ± 6 (0.99)	+424 ± 41 (0.99)	0.702 ± 0.020	0.541 ± 0.024	-30 ± 5 (0.98)
P. fluorescens (naphthalene dioxygenase)	Dissolved	Naphthalene	11.3	-1.6 ± 0.2 (0.97)	-1.7 ± 0.2 (0.95)	1.007 ± 0.001	1.014 ± 0.002	+21 ± 3 (0.94)	+21 ± 3 (0.94)	0.924 ± 0.010	0.859 ± 0.018	-9 ± 1 (0.97)
		2-methylnaphthalene	8.8	-1.2 ± 0.2 (0.95)	-6.7 ± 1.1 (0.93)	1.007 ± 0.001	1.014 ± 0.002	+15 ± 2 (0.97)	+115 ± 43 (0.92)	0.897 ± 0.035	0.813 ± 0.057	-15 ± 1 (0.99)
NaphS6 (putative carboxylation)	Dissolved	Naphthalene	8.5	-0.4 ± 0.3 (0.76)	n.a.	n.a.	n.a.	-47 ± 4 (0.97)	-91 ± 8 (0.97)	1.573 ± 0.082	-	n.a.
NaphS6 (putative carboxylation)	HMN carrier	Naphthalene	156	n.d.	n.d.	n.d.	n.d.	-46 ± 14 (0.89)	-90 ± 28 (0.89)	1.559 ± 0.271	-	n.a.
			391	-0.3 ± 0.6 (0.10)	n.a.	n.a.	n.a.	-44 ± 7 (0.96)	-88 ± 13 (0.96)	1.539 ± 0.127	-	n.a.
NaphS6 (putative carboxylation)	Immobilized	Naphthalene	156	n.d.	n.d.	n.d.	n.d.	-21 ± 18 (0.63)	n.a.	n.a.	n.a.	n.a.
			391	-0.2 ± 0.2 (0.33)	n.a.	n.a.	n.a.	-11 ± 2 (0.93)	-22 ± 4 (0.93)	1.097 ± 0.020	n.a.	n.a.

For calculation of enrichment factors for reactive positions and AKIE, the number of total C and H atoms (n), number of atoms at reactive positions (x) and at intramolecular competing sites (z) were considered as follows: naphthalene activation by a dioxygenase following a concerted reaction, C: n = 10, x = 8, z = 4, H: n = 8, x = 8, z = 4; naphthalene activation by a dioxygenase following a stepwise reaction, C: n = 10, x = 8, z = 8, H: n = 8, x = 8, z = 8; 2-methylnaphthalene activation by a dioxygenase following a concerted reaction, C: n = 11, x = 2, z = 1, H: n = 10, x = 2, z = 1; 2-methylnaphthalene activation by a dioxygenase following a stepwise reaction, C: n = 11, x = 2, z = 2, H: n = 10, x = 2, z = 2; naphthalene activation by a carboxylase-like reaction, C: n = 10, x = 4, z = 4, H: n = 8, x = 4, z = 4.

[1] valid only for the reaction of naphthalene dioxygenase; n.a. = not applicable; n.d. = not detected