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Mass Transfer Limitation during Slow Anaerobic Biodegradation of 2-Methylnaphthalene

Sviatlana Marozava,[†] Armin H. Meyer,[†] Alfredo Pérez-de-Mora,[†] Mehdi Gharasoo,[†,‡] Lin Zhuo,[†] He Wang,[†] Olaf A. Cirpka,[§] Rainer U. Meckenstock,[∥] and Martin Elsner*[,†,⊥]

[†]Institute of Groundwater Ecology, Helmholtz Zentrum München-German Research Center for Environmental Health, Ingolstädter Landstrasse 1, D-85764 Neuherberg, Germany

[‡]University of Waterloo, Department of Earth and Environmental Sciences, Ecohydrology, 200 University Avenue West, Waterloo, Ontario N2L 3G1, Canada

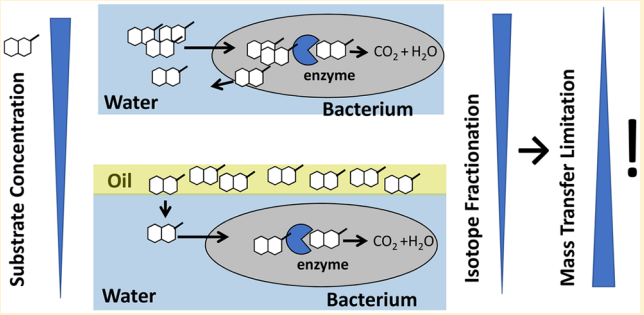
[§]University of Tübingen, Center for Applied Geoscience, Hölderlinstrasse 12, 72074 Tübingen, Germany

[∥]University Duisburg-Essen, Biofilm Centre, Universitätsstrasse 5, D-45141 Essen, Germany

[⊥]Technical University of Munich, Chair of Analytical Chemistry and Water Chemistry, Marchioninistrasse 17, 81377 Munich, Germany

Supporting Information

ABSTRACT: While they are theoretically conceptualized to restrict biodegradation of organic contaminants, bioavailability limitations are challenging to observe directly. Here we explore the onset of mass transfer limitations during slow biodegradation of the polycyclic aromatic hydrocarbon 2-methylnaphthalene (2-MN) by the anaerobic, sulfate-reducing strain NaphS2. Carbon and hydrogen compound specific isotope fractionation was pronounced at high aqueous 2-MN concentrations (60 μM) (ε_{carbon} = −2.1 ± 0.1‰/$\varepsilon_{hydrogen}$ = −40 ± 7‰) in the absence of an oil phase but became significantly smaller (ε_{carbon} = −0.9 ± 0.3‰/$\varepsilon_{hydrogen}$ = −6 ± 3‰) or nondetectable when low aqueous concentrations (4 μM versus 0.5 μM) were in equilibrium with 80 or 10 mM 2-MN in hexadecane, respectively. This masking of isotope fractionation directly evidenced mass transfer limitations at (sub)micromolar substrate concentrations. Remarkably, oil−water mass transfer coefficients were 60−90 times greater in biotic experiments than in the absence of bacteria (k^{org}_{-aq}2-MN = 0.01 ± 0.003 cm h^{-1}). The ability of isotope fractionation to identify mass transfer limitations may help study how microorganisms adapt and navigate at the brink of bioavailability at low concentrations. For field surveys our results imply that, at trace concentrations, the absence of isotope fractionation does not necessarily indicate the absence of biodegradation.



INTRODUCTION

Numerous chemicals regularly enter aquatic systems as a result of human activity. The main sources of pollution are pharmaceuticals or consumer care products from wastewater, pesticides from agriculture, and petroleum or chlorinated hydrocarbons from landfills or accidental spills.[1] For all of these anthropogenic pollutants and many more, biodegradation can be a cost-efficient and sustainable strategy for cleanup. Yet, organic chemicals are frequently detected in the environment at very low concentrations (ng L^{-1} to μg L^{-1})[2,3] and biodegradation activity has been found to critically depend on the substrate concentration available to degrading organisms.[4] The reason why biodegradation at low concentrations is limited in the environment has been a matter of intense debate.[5] Possible drivers are the absence of essential nutrients,[6] the presence of toxic cocontaminants,[7,8] intrinsically slow biochemical transformation of recalcitrant substances[9] or the notion that enzymes responsible for biodegradation are no longer induced at low concentrations.[10] In the absence of toxic substances and when essential nutrients are not lacking, the remaining crucial question is that of bioavailability: (i) is mass transfer of organic contaminants toward microbial cells limiting (ii) or does physiological adaptation take place so that enzymatic turnover in biodegradation becomes intrinsically slow at low concentrations and further degradation is prevented or reaches an asymptotic state?

Compound specific isotope analysis (CSIA) has recently been brought forward to enable a direct observation of bioavailability limitation.[11] In the enzymatic reaction, bonds containing light isotopes (e.g., ^{12}C, ^{1}H) are typically transformed more rapidly than those containing a heavy isotope (e.g., ^{13}C, ^{2}H). Hence, the

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molecules which have not yet been degraded contain increasingly more ^{13}C and ^{2}H as the reaction progresses. If mass transfer is not limiting, molecules inside and outside the cell are in rapid equilibrium, which ensures that the changes in isotope values occurring inside the cell can also be observed outside the cell in solution. If mass transfer toward and into the cells becomes rate-limiting, however, essentially all substrate molecules that reach the interior of the cell are quantitatively transformed. Changes in isotope values as a result of the enzyme reaction will, therefore, no longer be observable outside the cell where samples are taken for analysis (masking of the isotope effect). Therefore, decreased observable isotope fractionation at low substrate concentrations is a potentially powerful, yet little explored, indicator of bioavailability limitation at low concentrations. We recently studied isotope fractionation during rapid biodegradation by microorganisms adapted to low substrate concentrations in chemostat cultivations[12] and have indeed observed that isotope fractionation in aerobic atrazine degradation decreased when steady-state concentrations reached a low $\mu g\ L^{-1}$ level (15−30 $\mu g\ L^{-1}$). Hence, CSIA has been confirmed to reveal mass transfer limitations at concentrations close to those encountered in the environment.[12]

In the environment, however, the bacterial cell membrane is not the only interface for mass transfer at low concentrations. Further bottlenecks to mass transfer may be limited substrate transport toward cells,[11,13,14] sorption and desorption to sediment, slow aqueous diffusion through stagnant water layers,[9] or limited substrate mass transfer from an oil phase (in the case of an oil spill) to the liquid medium with bacteria. Aeppli et al.[15] observed that introduction of a non-aqueous-phase liquid (NAPL) to microbial reductive trichloroethene (TCE) dehalogenation significantly decreased observable carbon isotope fractionation in the laboratory, indicating that biodegradation became mass transfer limited at aqueous TCE concentrations of 130 μM. However, this experiment and another study on mass transfer effects on isotope fractionation by Kampara et al.[11] were conducted with microorganisms facilitating degradation at relatively fast rates (1−3 days of biodegradation). In the case of anaerobic biodegradation of polycyclic aromatic hydrocarbons (PAHs), in contrast, breakdown of as little as 10 $mg\ L^{-1}$ (80 μM) of PAHs can take months under laboratory conditions.[16,17] For such recalcitrant pollutants, this leads back to the original question: is biodegradation in the field slow due to mass transfer limitation (e.g., as a result of desorption from a sorptive phase), due to bacterial adaptation (i.e., because microorganisms downregulate their catabolism at low substrate concentrations), or simply due to the recalcitrant nature of the PAH structure?

Recent results from a long-term experiment by Kümmel et al. (where degradation was observed over 60 days (with degradation rates of approximately 2−7 $\mu M\ day^{-1}$))[18] suggest that slow anaerobic naphthalene degradation was *not* mass transfer limited. Observable isotope fractionation was not reduced when naphthalene was released from a hydrophobic carrier phase in experiments that were conducted at relatively high naphthalene levels corresponding to calculated aqueous concentrations between 50 and 100 μM. For extremely slow growing organisms such as anaerobic PAH degraders, biodegradation rates are very low. For example, in the study of Kümmel et al.[18] 130 μM of naphthalene was anaerobically degraded in 60 days. In contrast, aerobic naphthalene degradation is typically faster (for example, aerobic seawater strain *Cycloclasticus* sp. degrades 40 μM of naphthalene completely in 7 days (6 $\mu M\ day^{-1}$).[19]

If the enzyme reaction is rate-limiting, bioavailability limitation may never be observed even under conditions of slow mass transfer. Therefore, it is still unclear whether mass transfer limitation for slow anaerobic PAH degraders can become a limiting factor—and if yes, at which substrate concentration this becomes relevant.

In order to investigate whether or not mass transfer limitation of slow anaerobic PAH mineralization at extremely low PAH concentrations takes place, we studied degradation of 2-methylnaphthalene (2-MN) by the pure sulfate-reducing culture NaphS2 in batch cultures and determined isotope fractionation of both C and H. To mimic slow-release conditions similar to those encountered in natural systems (e.g., dissolution from oil spills or desorption from sediment), a two-phase system was chosen with an overlaying carrier hexadecane phase delivering 2-MN to the aqueous phase where biodegradation took place. In this system we investigated the two conflicting hypotheses: (1) that isotope fractionation during slow anaerobic biodegradation of 2-MN by the sulfate-reducing strain NaphS2 would remain pronounced, analogous to observations in the recent study by Kümmel et al.,[18] or (2) that decreased (or absent) substrate isotope fractionation would be observed as an indicator of mass transfer limitations when aqueous 2-MN concentrations become low (~4 μM versus 0.5 μM in equilibrium with 80 and 10 mM of 2-MN in a hexadecane phase, respectively). This experimental system was chosen to mimic situations in the field, where PAHs are dissolved in an oil phase or adsorbed to organic matter in sediment and only slowly released, resulting in relatively low concentrations in water.

MATERIAL AND METHODS

Chemicals. Information about the used chemicals can be found in the Supporting Information.

Cultivation Conditions and Degradation Experiments. The 2-methylnaphthalene-degrading pure culture NaphS2 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ strain 14454) and was cultivated as described in Galushko et al.[20] with a marine medium based on Widdel and Bak.[21] Degradation experiments were carried out under anoxic conditions with 10 mM sodium sulfate as an electron acceptor. Either aqueous medium alone was used (one phase) or a two-phase system was employed with hexadecane as an inert overlaying carrier phase that is not biodegradable by NaphS2. (Hexadecane was chosen as a donor phase, as its boiling point is 286.8 °C and it would not interfere with gas chromatographic analysis of 2-MN, the boiling point of which is 241 °C. Moreover, no toxic effect of hexadecane on the activity of NaphS2 has been reported.) To prepare the one-phase system, pure 2-MN crystals (9 $mg\ L^{-1}$) were placed in 1 L bottles with 850 mL of anoxic artificial seawater medium and 150 mL of CO_2/N_2 (20/80 v/v) headspace and the crystals were stirred until complete dissolution. The final 2-MN concentration was 60 μM, corresponding to a high concentration (about half of the expected saturation), so that sufficient substance was available for isotope analysis when samples were taken to follow degradation over time. In the two-phase systems, two different concentrations of 2-MN in hexadecane were prepared, 80 and 10 mM, with the aim to investigate mass transfer limitations at different low concentrations of 2-MN. A 10 mL portion of the respective carrier phase was placed in 220 mL bottles with 180 mL of anoxic artificial seawater medium and

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10 mL of CO_2/N_2 (20/80 v/v) headspace. All cultivation bottles were sealed with 1 cm thick Viton stoppers (Maag Technik, Dübendorf, Switzerland) and inoculated with 10% inoculum. In the one-phase system, aqueous samples were withdrawn (0.8 mL for concentration analysis and 12−37 mL for isotope analysis) with a syringe through the stopper, whereas in the two-phase system, samples were taken from the hexadecane phase (175 μL for isotope analysis and 18 μL for concentration analysis). All incubations were performed in triplicate and with two abiotic controls at 30 °C, in the dark. All bottles (including abiotic controls) were shaken at 52 rpm. In the control experiments, an autoclaved culture solution was added. Abiotic experiments for determination of hexadecane−water mass transfer coefficients in the absence of bacteria were performed in 1 L custom-made glass bottles. An 850 mL portion of anoxic artificial seawater medium was overlaid with 60 mL of hexadecane phase with 80 or 10 mM of 2-MN, and samples for concentration were withdrawn from both phases (the sample amounts are stated above). Sampling from the aqueous phase was performed from an additional horizontal port located 6 cm above the bottle bottom but below the hexadecane phase. The port was sealed with a similar Viton stopper. Care was taken that the hexadecane phase did not touch the stopper.

Analytical Methods. *Concentration Analysis.* For the analysis of 2-MN concentrations in the one-phase system (aqueous phase only), samples were taken from the water phase, whereas in the two-phase systems, the oil phase was sampled. Water samples from one-phase systems were extracted with cyclohexane, whereas hexadecane samples from the two-phase system were diluted with cyclohexane and used directly for analysis (see the Supporting Information for more details). For all approaches, concentration analysis of extracted samples was performed immediately after preparation. Determination of 2-MN concentrations was carried out on an Agilent GC 7890A gas chromatograph attached to a 5975C inert XL EI/CI MSD detector (Agilent Technologies, Waldbronn) using a fused silica HP-5MS column (30 m × 0.250 mm, film thickness 0.25 μm) (see further details in the Supporting Information).

Isotope Analysis. For C and H isotopic analyses of 2-MN in the one-phase system samples were taken from the aqueous phase, and in the two-phase system they were taken from the hexadecane phase; the samples were stored at −20 °C prior to isotope analysis according to Elsner et al.[22] Carbon and hydrogen compound-specific isotope ratios of 2-MN were measured using a TRACE GC Ultra gas chromatograph (GC) (Thermo Fisher Scientific; Milan, Italy), coupled to a Finnigan TM MAT253 IRMS (Thermo Fisher Scientific; Bremen, Germany).

Carbon isotope ratios were expressed in the delta notation ($\delta^{13}C$) in per mille according to

$$\delta^{13}\mathrm{C\ compound} = \frac{(\delta^{13}\mathrm{C}/^{12}\mathrm{C})\ \mathrm{compound}}{(\delta^{13}\mathrm{C}/^{12}\mathrm{C})\ \mathrm{reference}} \quad (1)$$

where $^{13}C/^{12}C$ compound and $^{13}C/^{12}C$ reference are the ratios of the heavy isotope (^{13}C) to the light isotope (^{12}C) in the sample and in the international reference material, respectively. A laboratory CO_2 standard was used as the calibration gas. This laboratory standard had been calibrated to the international standard V-PDB by reference CO_2 standards (RM 8562, RM 8563, RM 8564).

Hydrogen isotope analysis of 2-MN was performed using a laboratory H_2 monitoring gas, which had not been calibrated against an international standard. For this reason, changes in hydrogen isotope ratios are given as relative differences according to

$$\Delta\delta^2\mathrm{H} = \delta^2\mathrm{H}_t - \delta^2\mathrm{H}_0 \quad (2)$$

where δ^2H_t is the ratio $^2H/^1H$ in a sample at the corresponding time of sampling and δ^2H_0 is the mean isotope value of the control bottles at time point zero.

Isotope analyses were performed in duplicate for C and in triplicate for H. Reproducibility for $\delta^{13}C$ was always better than 0.5‰ and for δ^2H better than 5‰.

Quantification of Isotope Fractionation. The linearized Rayleigh equation was used to evaluate isotope fractionation:[18]

$$\ln\frac{R_t}{R_0} = \varepsilon \ln\frac{C_t}{C_0} \quad (3)$$

where R_t and R_0 describe the average isotope ratio of the heavy isotope to the light isotope in a compound at a given time and at the beginning of the reaction (i.e., when nothing has been degraded so far), respectively, and C_t and C_0 are concentrations of a compound at a given time and at the beginning of the reaction, respectively. The enrichment factor ε links the shifts in isotope ratios to the extent of compound degradation and is a measure of the bulk fractionation: that is, the isotope fractionation in the molecular average of a compound.

A more detailed description of the experimental setup and degradation experiments, as well as of carbon and hydrogen isotope analyses of 2-MN, is provided in the Supporting Information.

Determination of AKIE. Position-specific kinetic isotope effects (KIEs) are used to describe a position-specific fractionation at the reactive position of a substrate molecule. KIE values are referred to as intrinsic if they directly reflect isotope fractionation in an elementary chemical reaction.[23] In contrast, the term "apparent kinetic isotope effect" (AKIE) is used when intrinsic KIEs are masked by steps such as desorption, transport into the cell, or attachment to the enzyme, which precede the chemical reaction.[24] Calculation of position-specific isotope effects was performed according to Elsner et al.[24] Here, the bulk enrichment factor ε_{bulk} can be used to estimate an isotope effect at the reactive position $\varepsilon_{\mathrm{reactive\ position}}$ according to eq 4

$$\varepsilon_{\mathrm{reactive\ position}} \approx \frac{n}{x}\varepsilon_{\mathrm{bulk}} \quad (4)$$

where n is the number of atoms of a specific element in 2-MN (for carbon $n = 11$, for hydrogen $n = 10$) and x is the number of atoms in a reactive position. As the first step in anaerobic 2-MN degradation is fumarate addition to the methyl group,[25] only one carbon atom ($x = 1$) but three hydrogen atoms ($x = 3$) are in the reactive methyl group. Conversion of $\varepsilon_{\mathrm{reactive\ position}}$ to AKIE can be calculated by eq 5,

$$\mathrm{AKIE} \approx \frac{1}{1 + z\varepsilon_{\mathrm{reactive\ position}}} \quad (5)$$

where z is the number of atoms competing for the reaction. In the case of carbon there is just one ($z = 1$) carbon atom in the methyl group. However, in the case of hydrogen three C−H bonds compete for the reaction ($z = 3$).

Data Evaluation and Mathematical Model. In abiotic experiments in the absence of biodegradation, 2-MN diffuses from the oil phase and dissolves into the water phase. Due to the

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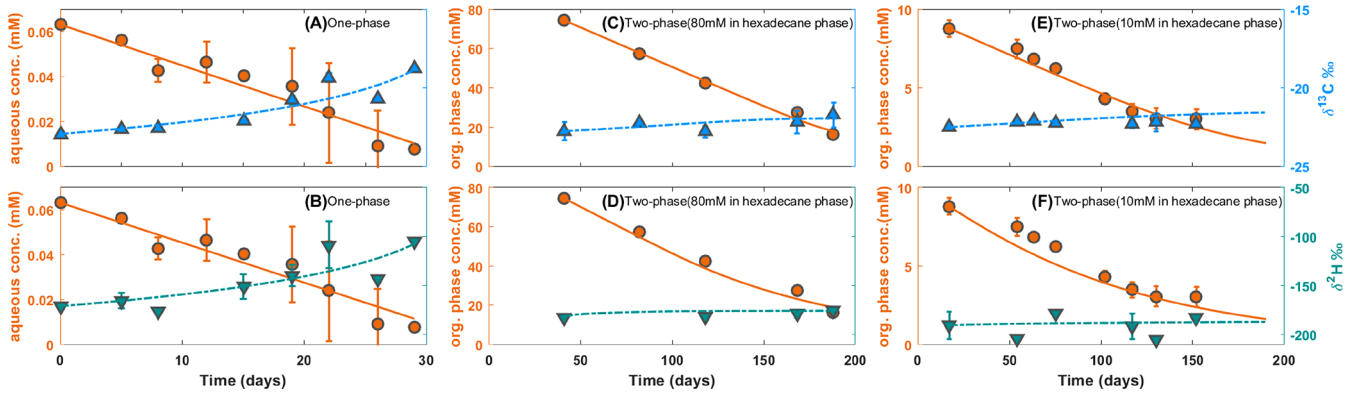

Figure 1. Changes in concentration and carbon and hydrogen isotope fractionation associated with the anaerobic degradation of 2-MN by the sulfate reducing culture NaphS2. Averaged data from two or three replicates are shown. Lines depict simulations using the model and parameters described in the methods section. (A) 2-MN concentration (circles) and carbon isotope ratios (triangles) in the purely aqueous system starting with an initial concentration of 60 μM and (B) 2-MN concentration (circles) and hydrogen isotope ratios (triangles). (C) 2-MN concentration and carbon isotope ratios in a two-phase system with 80 mM 2-MN dissolved in hexadecane phase (squared norm of residuals of fitting 16.77 with $R^2 = 0.9975$) and (D) 2-MN concentration and hydrogen isotopes (squared norm of residuals of fitting 196.27 with $R^2 = 0.9980$). (E) 2-MN concentration and carbon isotope (squared norm of residuals of fitting 1.95 with $R^2 = 0.9862$) in a two-phase system with 10 mM of 2-MN dissolved in hexadecane phase and (F) 2-MN concentration and hydrogen isotopes (squared norm of residuals of fitting 1.90 with $R^2 = 0.9900$).

absence of bacteria in the water phase, the only process of importance is the substrate exchange across the oil−water interface described by the linear driving force model

$$\frac{d[S_0]}{dt} = -{}^{a}k_{tr}\left(\frac{[S_0]}{p_c} - [S_w]\right) \quad (6a)$$

$$\frac{d[S_w]}{dt} = {}^{a}k_{tr}\left(\frac{[S_0]}{p_c} - [S_w]\right) \quad (6b)$$

where ${}^{a}k_{tr}$ is the mass transfer coefficient controlling the exchange rate between the two phases in an abiotic experiment ($k^{org\text{-}aq}{}_{2\text{-MN abiotic}}$), p_c is the partitioning coefficient (L_w to L_o^{-1}), and S_0 and S_w are the 2-MN concentrations in the oil and water phases, respectively. Equations 6a and 6b were then fitted to the concentrations of 2-MN in the water phase to estimate the values of $k^{org\text{-}aq}{}_{2\text{-MN abiotic}}$ and L_w to L_o^{-1} for abiotic experiments (Figure 3).

One-phase abiotic experiments were performed in the absence of an oil phase, where 2-MN was initially dissolved in water and then a microbial inoculum was added. Biodegradation of light and heavy fractions of 2-MN (L_S and H_S, respectively) follows Michaelis−Menten kinetics[26] and is described by the set of equations[27]

$$\frac{d[L_S]}{dt} = -\frac{q_{max}[L_S]}{[L_S] + [H_S] + K_m} \quad (7a)$$

$$\frac{d[H_S]}{dt} = -\frac{\alpha q_{max}[H_S]}{[L_S] + [H_S] + K_m} = -\frac{(\varepsilon + 1)[H_S]}{[L_S] + [H_S] + K_m} \quad (7b)$$

where α is the isotopic fractionation factor, ε is the isotopic enrichment factor, q_{max} is the maximum degradation rate, and K_m is the half-saturation constant of the Michaelis−Menten equation. Parameter estimates of K_m, q_{max}, α_C, and, α_H were derived by fitting eq 7 simultaneously to both 2-MN concentration and isotopic data (Figure 1 panels A and B).

In two-phase experiments in the presence of degrading bacteria, eqs 6 and 7 were merged. Heavy and light isotopologues (L_{S_0}, H_{S_0}, L_{S_w}, and H_{S_w}) of 2-MN were considered to be dissolved in two distinguishable phases (oil and water) where the exchange rate between the oil and water phases was controlled by the mass transfer limiting coefficient k_{tr} ($k^{org\text{-}aq}{}_{2\text{-MN biotic}}$)[13,15,28−30]

$$\frac{d[L_{S_0}]}{dt} = -k_{tr}\left(\frac{[L_{S_0}]}{p_c} - [L_{S_w}]\right) \quad (8a)$$

$$\frac{d[L_{S_w}]}{dt} = k_{tr}\left(\frac{[L_{S_0}]}{p_c} - [L_{S_w}]\right) - \frac{q_{max}[L_{S_w}]}{[L_{S_w}] + [H_{S_w}] + K_m} \quad (8b)$$

$$\frac{d[H_{S_0}]}{dt} = -k_{tr}\left(\frac{[H_{S_0}]}{p_c} - [H_{S_w}]\right) \quad (8c)$$

$$\frac{d[H_{S_w}]}{dt} = k_{tr}\left(\frac{[H_{S_0}]}{p_c} - [H_{S_w}]\right) - \frac{\alpha q_{max}[H_{S_w}]}{[L_{S_w}] + [H_{S_w}] + K_m} \quad (8d)$$

The values of $p_c = 21000$, $\alpha_H = 0.96$, and $\alpha_C = 0.998$ were taken as constants from the previous fits to abiotic and one-phase experimental data according to eq 3 where $\alpha = \varepsilon + 1$ (see the Results and Discussion). Using a modified version of ReKinSim,[31] the set of eqs 8a−8d were solved and fitted to the results of the experiments performed under biotic conditions in the presence of an oil phase (Figure 1 panels C, D, E, and F).

RESULTS AND DISCUSSION

Kinetics of Bacterial Degradation Is Slowed Down in the Presence of an Organic Donor Phase. In a one-phase system where 2-MN was dissolved in the aqueous medium, complete degradation of 60 μM 2-MN by the sulfate-reducing strain NaphS2 took 33 days, which led to the degradation rate of 1.8 μm day^{-1} (Figure 1). In contrast, when 2-MN was released into aqueous solution from a hexadecane donor phase (10 and 80 mM), the degradation was considerably longer: 70% and 80% of 2-MN were degraded after 153 and 188 days, respectively

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Table 1. Parameters Estimated from the Fitting of the Models Described in Data Evaluation and Mathematical Model (Eqs 6-8) to the Experimental Data:[a] Fraction of the Substrate in the Water Phase (f), Hexadecane Water Partition Constant at 25 °C (L_w to L_h^{-1}), Hexadecane−Aqueous Phase Mass-Transfer Coefficients ($k^{org\text{-}aq}$), Half-Saturation Constants (K_M), Conversion Rates (q_{max}), and Isotope Enrichment Factors (ε)

study	f_{aq} (%)	L_w to L_h^{-1}	system		isotope	$k^{org\text{-}aq}_{substrate}$ (cm h^{-1})	K_M (μM)	q_{max} (M^{-1} (g biomass)$^{-1}$)	ε (‰)
this work	0.09	20000 ± 2400	two-phase abiotic			0.01 ± 0.003			
			one-phase biotic	60 μM aq	^{2}H		0.007 ± 0.147	0.075 ± 0.01	−40 ± 7
					^{13}C		0.003 ± 0.499	0.077 ± 0.006	−2.0 ± 0.2
			two-phase biotic	80 mM (4 μM aq)	^{2}H	0.59 ± 0.18 (^{2}H)	0.01 ± 0.08	0.017 ± 0.003	−6 ± 3.0
					^{13}C	0.87 ± 0.21 (^{13}C)	0.01 ± 0.08	0.020 ± 0.010	−0.9 ± 0.7
				10 mM (0.5 μM aq)	^{2}H			0.002 ± 0.002	n.d.[b]
					^{13}C			0.004 ± 0.005	n.d.[b]
Aeppli et al.[56]	0.85	400	two-phase abiotic			1.8 ± 0.3			
			one-phase biotic	400 μM aq	^{13}C		70		−18.8 ± 0.6
			two-phase biotic	0.4 mM (130 μM aq)	^{13}C	35	70		−8.5 ± 0.6
Kümmel et al.	3.33	3000	two-phase abiotic						
			one-phase biotic	156 μM aq	^{2}H				−47 ± 4
					^{13}C				−0.4 ± 0.3
			two-phase biotic	156 mM (53 μM aq)	^{2}H				−46 ± 14
					^{13}C				n.d.[b]
				391 mM (132 μM aq)	^{2}H				−44 ± 7
					^{13}C				−0.3 ± 0.6

[a]The aqueous concentrations are indicated in parentheses next to the initial concentrations in the donor phase. For comparison, data from the similar studies of Aeppli et al.[15] and Kümmel et al.[18] are also indicated [b]n.d.: not detected.

(0.01 and 0.03 μM day^{-1}, respectively) (Figure 1). Toxic effects of the overlaying oil phase can essentially be ruled out, because the hexadecane isomers (2-MN and hexadecane) are hardly soluble in water and the strains investigated have even been isolated in the presence of these phases. In contrast, these results are consistent with the well-established understanding that in controlled laboratory systems with no other limiting factors (such as in batch or column experiments) it is the fraction, f, of PAHs available to bacteria that determines apparent turnover rates. For example, it has been shown that the presence of activated charcoal which contains adsorbed substrates leads to lower substrate concentrations available to microorganisms, resulting in slower degradation rates.[32]

Briefly, if the substrate present in a reactive compartment (here, the concentration of substrate in the water phase available to microorganisms $[S]_{aq}$) is considered to be degraded with a first-order rate constant k, this can be described by the equation

$$-\mathrm{d}[S]/\mathrm{d}t = k[S]_{aq} = k\left(\frac{n_{aq}}{V_{aq}}\right) = k\left(\frac{f n_{tot}}{V_{aq}}\right) = kf\left(\frac{n_{tot}}{V_{aq}}\right) = (k \times f)[S]_{tot} \quad (9)$$

where n_{aq} and V_{aq} denote aqueous substrate amount and aqueous volume, respectively. $[S]_{tot}$ is the substrate concentration that would exist if all substrate amount, n_{tot}, was present in the aqueous phase in the absence of a carrier phase. Hence, if the fraction f of substrate present in the bioavailable (aqueous) phase is small and if k is the true rate constant in this phase, the turnover of total substrate as the sum over both compartments is characterized by a much smaller apparent rate constant $k{\cdot}f$. In our study where hexadecane was used as a donor phase to release a PAH compound, the actual concentration of 2-MN in the water phase was small due to the preferential partitioning of 2-MN to hexadecane (partitioning constant $_{hexadecane/water}$(2-MN) = 1.2×10^4)[33] corresponding to a substrate fraction of $f_{2\text{-}MN,aq} \approx 0.1\%$ (Table 1). However, the substrate turnover was reduced only by a factor of about 10 in comparison to the condition where 2-MN was dissolved in the aqueous phase only (Figure 1). This raises questions about the underlying mechanisms: has the enzyme kinetics become so fast that mass transfer became limiting or was mass transfer from the hexadecane phase accelerated in the presence of bacteria?

In a study by Kümmel et al.[18] an analogous two-phase system with a very similar anaerobic sulfate degrader (strains NaphS2 and NaphS6 have sequence similarities of 97.4%[34]) and naphthalene as a model PAH compound was investigated. Although heptamethylnonane was used as a donor phase in the report by Kümmel et al.,[18] its nature is similar to the hexadecane used in our study. However, degradation of naphthalene took 30−40 days (3−6 μM day^{-1} (compared to 6 days in the absence of a donor phase (1.2 μM day^{-1})), while the substrate fraction was relatively high: $f_{naphthalene,aq} \approx 3\%$[18] (Table 1). Here, the aqueous medium was exposed to high donor phase concentrations (156 and 391 mM) and the calculated expected aqueous

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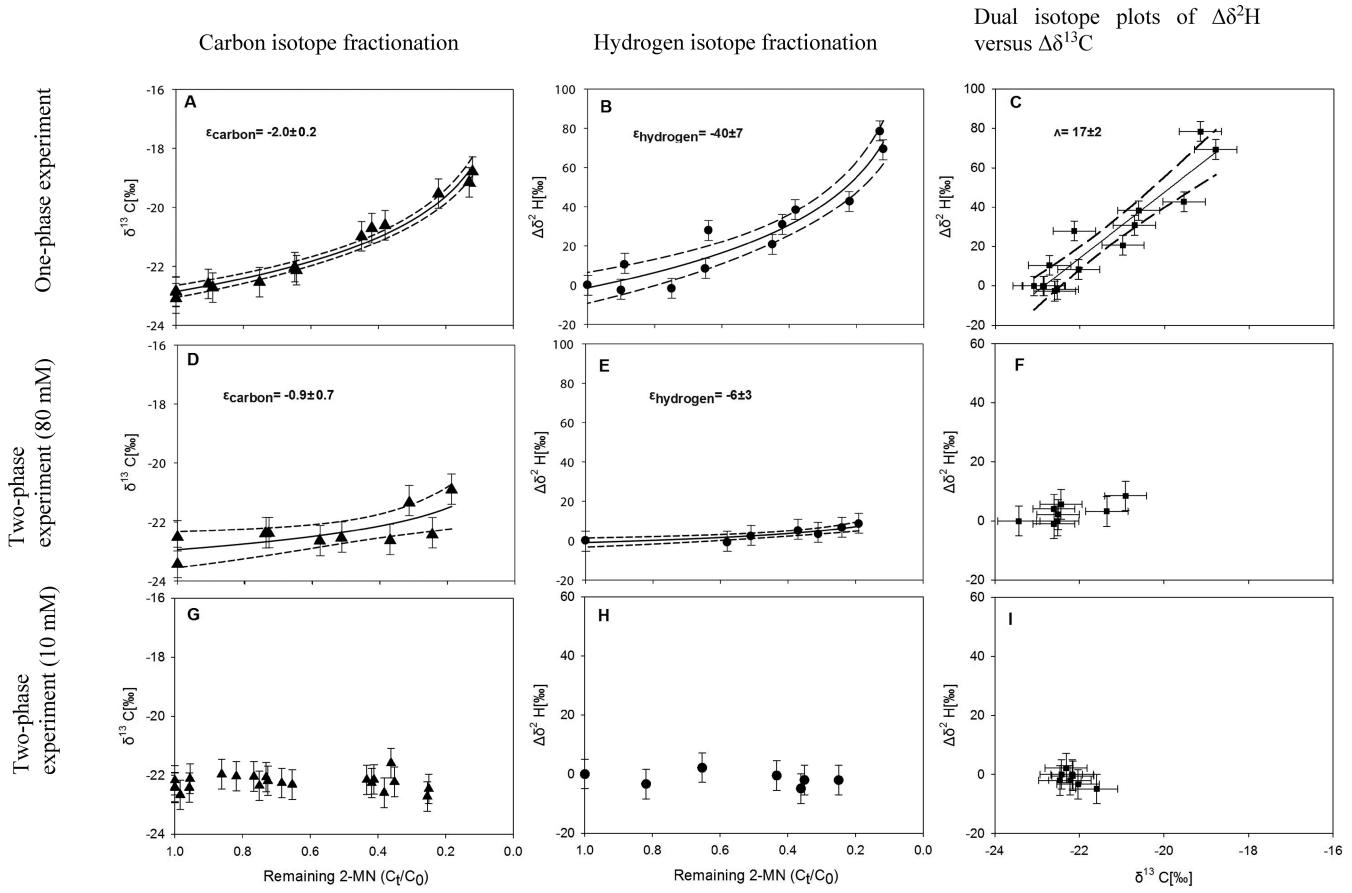

Figure 2. Carbon (left-hand panel) and hydrogen (middle panel) isotope fractionation as well as dual element isotope plots of $\Delta\delta^2$H versus δ^{13}C (right-hand panel) associated with anaerobic degradation of 2-MN by the sulfate-reducing culture NaphS2. The upper panels (A−C) depict the pure aqueous system. The middle (D−F) and lower (G−I) panels depict the two-phase system (aqueous/hexadecane) starting with an initial concentration of 80 mM (B) and 10 mM (C) 2-MN dissolved in hexadecane. Carbon and hydrogen enrichment factors ε_{carbon} and $\varepsilon_{hydrogen}$ are derived according to the Rayleigh equation with 95% confidence intervals. The slopes of the solid linear regression line in the dual isotope plots give Λ values, and the dashed lines represent the corresponding 95% confidence intervals. Error bars display the accuracy of δ^{13}C and $\Delta\delta^2$H measurements, which were always better than 0.5% and 5%, respectively.

concentrations of naphthalene were approximately 20−60 times higher than in our study (53 and 132 μM, respectively, assuming a partitioning constant of $_{hexadecane/water}$(naphthalene) of 3000,[33] Table 1). In addition to this thermodynamic consideration—the effect of partitioning equilibria between aqueous and organic phases—the effects of enzyme kinetics (substrate activation, enzyme kinetics) are expected to play a role, and these enzyme kinetics may be expected to be concentration-dependent according to a Michaelis−Menten relationship. In a next step these effects were, therefore, probed by isotope fractionation measurements.

Compound-Specific Isotope Fractionation Is Masked When Low Concentrations Are Released by a Donor Phase. In the one-phase experiment of our study (aqueous solution only), 2-MN degradation was associated with pronounced carbon and hydrogen isotope fractionation leading to $\varepsilon_{carbon/bulk} = -2.0 \pm 0.3‰$ and $\varepsilon_{hydrogen/bulk} = -40 \pm 7‰$. These values correspond to apparent kinetic isotope effects (AKIE) of $AKIE_{carbon} = 1.02 \pm 0.002$ and $AKIE_{hydrogen} = 1.67 \pm 0.15$ (Figure 2A,B and Table 1) that—although clearly masked—are still relatively pronounced considering that the values $AKIE_{carbon} > 1.01$ and $AKIE_{hydrogen} > 2$ are indicative of enzymatic C−H bond oxidation.[24] We conclude that the intrinsic isotope effect of the enzyme reaction was to a great extent represented in the observable isotope fractionation, meaning that mass transfer limitations did not play a dominant role in this case.[11,29] Comparable C and H enrichment factors were obtained for the degradation of naphthalene dissolved in the aqueous phase in the study of Kümmel et al.,[18] suggesting that isotope fractionation of the enzyme reaction was strongly expressed and mass transfer limitations were largely absent. Moreover, the linear regression for dual element carbon and hydrogen isotope fractionation, $\Lambda^{H/C} = 17$ (Figure 2C) was in a range similar to that of reported values of naphthalene degradation.[18,35]

In contrast to the results of Kümmel et al.,[18] the observable isotope fractionation in the two-phase systems of our study (hexadecane/water) was very small (Figure 2D,E) or even not detectable at all (Figure 2G,H). For the experiment with $c(2\text{-MN})_{hexadecane,initial}$ = 80 mM, changes in isotope ratios corresponded to $\varepsilon_{carbon/bulk} = -0.9 \pm 0.7‰$ and $\varepsilon_{hydrogen/bulk} = -6 \pm 3‰$ (Figure 2D and Table 1), giving much smaller apparent kinetic isotope effects of $AKIE_{carbon} = 1.010 \pm 0.007$ and an even greater reduction in $AKIE_{hydrogen} = 1.06 \pm 0.04$. In the experiment with $c(2\text{-MN})_{hexadecane,initial}$ = 10 mM changes in isotope ratios were not even observable (Figure 2G,H). Hydrogen isotope fractionation was more pronounced than carbon isotope fractionation in both cases: when 2-MN was

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dissolved in aqueous phase only; and even in the presence of a donor phase at concentrations of 80 mM. This suggests that hydrogen isotope fractionation may be able to detect degradation in field studies even when carbon isotope fractionation is no longer observed.

Our results provide evidence that isotope fractionation was masked by slow mass transfer in the presence of a hexadecane carrier phase and that this effect became stronger at lower concentrations. These observations are in stark contrast to those of Kümmel et al.,[18] where degradation of naphthalene was also investigated with a slow-growing sulfate-reducing culture. There, similar $\varepsilon_{hydrogen}$ values were obtained, irrespective of whether naphthalene was completely dissolved in aqueous medium ($\varepsilon_{hydrogen} = -47 \pm 4‰$) at a concentration of 85 μM or whether it partitioned into water from an overlaying carrier phase ($\varepsilon_{hydrogen} = -46 \pm 14‰$)[18] to give an aqueous concentration between 53 and 132 μM (Table 1). A decisive difference between the studies is that Kümmel et al. started with similar aqueous naphthalene concentrations in one- and two-phase systems (53−132 μM). This is in contrast to our study, in which the concentration of 60 μM 2-MN in the one-phase system was much greater than the concentration of 0.8 μM expected in equilibrium with 10 mM of 2-MN in hexadecane of the two-phase system (on the basis of the partitioning constant hexadecane/water(2-MN) of 1.2×10^4).[33] Considering that we observed a concentration dependence of isotope fractionation in our two-phase system (Figure 2), we interpret this as evidence that an onset of mass transfer limitations occurred specifically at low concentrations, consistent with our recent observations for rapid aerobic degradation in chemostat studies.[12]

Mass-Transfer Rates at the Hexadecane/Water Interface under Abiotic Conditions. To further explore the mass transfer rates that are observed for 2-MN in partitioning from a hexadecane phase to water in the absence of biodegradation, an abiotic experiment was conducted. For this purpose, 2-MN was dissolved in a hexadecane donor phase to final concentrations of 10 and 80 mM, respectively. Aqueous concentrations of 2-MN were monitored until equilibrium (Figure 3A,B), and the resultant data were modeled. As expected, the estimated partitioning constants of hexadecane/water(naphthalene) and the mass transfer coefficients $k^{org\text{-}aq}{}_{2\text{-}MN,abiotic}$ did not differ considerably between the experiments with $c(2\text{-}MN)_{hexadecane,initial}$ = 10 mM and $c(2\text{-}MN)_{hexadecane,initial}$ = 80 mM. The actual concentrations of 2-MN in aqueous solution in the abiotic two-phase systems were slightly smaller than expected values of 0.8 and 6.7 μM according to a published partitioning constant hexadecane/water(2-MN) of 1.2×10^4 [33] (0.5 and 4 μM 2-MN in equilibrium with 10 and 80 mM in the hexadecane phase, respectively, Figure 3A,B), resulting in both cases in partitioning constants of hexadecane/water(2-MN) = $(2.0 \pm 0.2)\cdot 10^4$ (Table 1) that were greater than reported in the literature (partitioning constant hexadecane/water(2-MN) = 1.2×10^4). A likely explanation is the high salt concentration of the bacterial medium used for cultivation of the marine strain NaphS2, which decreased the aqueous solubility of 2-MN. The mass transfer kinetics of both experiments could be described with a coefficient of $k^{org\text{-}aq}{}_{2\text{-}MN,abiotic} = 0.053 \pm 0.015$ h^{-1} or—when the volume of the hexadecane phase (60 cm^3) and its surface area (326 cm^2) are accounted for—$k^{org\text{-}aq}{}_{2\text{-}MN,abiotic}$ can be expressed as 0.01 ± 0.003 cm h^{-1} (Table 1). In a next step, kinetic modeling was conducted to derive mass transfer rates in the presence of bacteria and to compare them to these "abiotic" mass transfer coefficients, as described below.

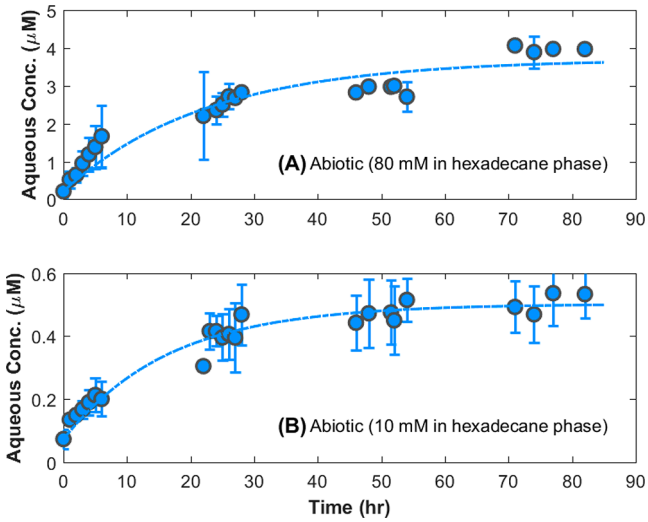

Figure 3. Dissolution kinetics of 2-MN from a hexadecane phase into water. 2-MN was dissolved in a hexadecane phase at concentrations of 80 mM (A) and 10 mM (B). The model for substrate mass transfer through the oil−water interface eq 6 was fitted to the experimental data to give a value estimation for 2-MN mass transfer coefficient (k_{tr}) and partitioning coefficient (p_c).

Modeling of Microbial Growth and Carbon and Hydrogen Isotope Fractionation. Modeling of the biotic experiment was performed to explore whether differences in isotope fractionation between 10 and 80 mM of 2-MN in the oil phase can be explained by considering the influence of mass transfer and enzymatic transformation. Furthermore, the modeling aimed to derive quantitative rate constants for both processes. Finally, we compared the model-derived coefficient to that observed in the abiotic experiment (Table 1).

The following parameters were estimated from biotic experiments: half-saturation constants (K_m), conversion rates (q_{max}), and organic phase−aqueous phase mass transfer coefficients ($k^{org\text{-}aq}{}_{2\text{-}MN,biotic}$) (Table 1). In the one-phase experiment the following values were estimated from carbon and hydrogen isotopic data with $\varepsilon_C = -2.3 \pm 0.01$ and $\varepsilon_H = -41.9 \pm 1.37$, respectively (Table 1): K_m (μM) = 0.0029 ± 0.499 for C and 0.0065 ± 0.147 for H, q_{max} (μM h^{-1} (biomass)$^{-1}$) = 0.077 ± 0.0063 for C and 0.075 ± 0.01 for H. The modeling of C isotopic data and 2-MN concentrations in the oil phase for $c(2\text{-}MN)_{hexadecane,initial}$ = 10 mM led to estimates of $k^{org\text{-}aq}{}_{2\text{-}MN,biotic} = 13.5 \pm 3.2$ or 0.87 ± 0.21 cm h^{-1} and $q_{max} = 0.0022 \pm 0.0016$ μM h^{-1} (biomass)$^{-1}$; for $c(2\text{-}MN)_{hexadecane,\ initial}$ = 80 mM, $q_{max} = 0.017 \pm 0.0026$ μM h^{-1} (biomass)$^{-1}$. The goodness of fit to both C isotopic signatures ($\delta^{13}C$) and 2-MN concentrations in the hexadecane phase can be visually observed in Figure 1C,E. A similar range of values was estimated by the fit to H isotopic signatures (δ^2H) and 2-MN concentrations in the hexadecane phase (Table 1 and Figure 1D,F).

For the modeling of the data (Figure 1), there was no significant difference between kinetic parameters derived from carbon or hydrogen isotope data. In both cases, the constraint from isotope fractionation data allowed us to observe that mass transfer of the substrate to bacterial cells became limiting when concentrations in aqueous solution decreased to the low micromolar range (0.5 and 4 μM).

Implications for Mass Transfer Coefficients at the Hexadecane/Water Interface. The estimated mass transfer coefficients of 2-MN in the biotic two-phase system were 60−90

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times greater than in the abiotic system (Table 1). Hence, when applying the slow mass transfer coefficients derived from our abiotic experiments, we would expect that isotope fractionation would be even more strongly masked in the presence of bacteria. Similarly, in the study of Aeppli et al.,[15] the mass transfer coefficient of TCE from an organic phase to the aqueous phase was 20 times higher ($k^{org\text{-}aq}_{TCE}$ = 35 cm h^{-1}) in the biotic relative to the abiotic experiment ($k^{org\text{-}aq}_{TCE}$ = 1.8 cm h^{-1}) (Table 1). In our study, mass transfer coefficients of the two-phase biotic systems were similar irrespective of donor phase concentrations (0.59 ± 18 and 0.87 ± 0.21 cm h^{-1} for 80 mM and 10 mM 2-MN in hexadecane, respectively). In contrast, conversion rates (q_{max}) were approximately 5−8 times lower at 10 mM 2-MN in hexadecane (0.5 μM in aqueous phase) in comparison to 80 mM 2-MN in hexadecane (4 μM in the aqueous phase) (Table 1). This difference is proportional to the equilibrium concentrations of 2-MN in the aqueous phase in the biotic two-phase systems, showing that bacteria adjusted their conversion rates/Monod kinetics to the prevailing concentrations.

A number of studies have reported that bacteria can increase mass transfer of organic chemicals by their consumption so that the concentration of a compound at the bacterial cell is decreased and the concentration gradient is made steeper.[36] It has been modeled and experimentally verified that bacteria increase dissolution of chlorinated solvents[37−40] as well as of PAHs[41,42] from a NAPL phase. Moreover, bacteria can perform different active strategies to increase mass transfer from the organic phase, which include release of biosurfactants[41] or extracellular polymeric substances,[43] adhesion of bacterial cells to the surface of a nonaqueous phase,[44−46] chemotaxis,[47] biofilm formation,[48] or even transport of substrate due to motility.[49] In our study, we observed slight foaming of the nonaqueous phase during bacterial growth as well as some biofilm-like formations at the donor−water phase, which might indicate biosurfactant production or bacterial adhesion. Notably, no foaming has been observed in abiotic controls. A more detailed investigation, however, was beyond the focus of this study.

■ ENVIRONMENTAL SIGNIFICANCE

Bioavailability Limitation at Low Substrate Concentrations. The bioavailability of organic contaminants in the environment, particularly of hydrophobic compounds such as PAHs, plays a crucial role in their limited biodegradability.[50,51] Low water solubility and preferential adsorption of PAHs to soil organic matter turn these contaminants into cumbersome substrates for bacterial accessibility. As a result, the slow diffusion of organic contaminants from solid or oil phases has been hypothesized to be the main reason for their limited biodegradation in the environment, rather than the lack of bacterial degradation capacity.[5] The current study explored the relevance of this process for slow anaerobic degradation in the presence of a non-aqueous-phase liquid (NAPL) where bacteria are exposed constantly to low aqueous concentrations of the substrate delivered from the oil phase. We demonstrate that slow-growing anaerobic bacteria experience not only lower biotransformation rates but also limited biodegradation potential at micro- and submicromolar aqueous concentrations in oil−water systems. Stable isotope fractionation of 2-MN was masked at such low concentrations and, thus, provided evidence for mass transfer limitation as the main cause for the observed phenomena. Remarkably, this masking effect was less pronounced when aqueous concentrations of 2-MN increased in oil−water systems from 0.5 μM in equilibrium with 10 mM in hexadecane phase to 4 μM in equilibrium with 80 mM in hexadecane phase. This suggests a gradual rather than an abrupt change in mass transfer. Biodegradation still took place despite mass transfer limitation, although at reduced rates. Thus, our study shows that bioavailability limitations at low substrate concentrations are relevant not only for aerobic microorganisms with relatively fast growth rates (e.g., *Arthrobacter aurescens* as in the study of Ehrl et al.[29] or *Pseudomonas putida* in the study of Kampara et al.[11] at concentrations of 1 μM) but also for slow-growing anaerobic degraders. In this study we were able to verify mass transfer limitation for low microbial cell numbers (10^6−10^7 cells mL^{-1}) maintained on a low but constant supply of substrates which was originally demonstrated for artificially high biomass (5×10^8 cells mL^{-1}).[11] Our data show that mass transfer to bacterial cells is limited at low substrate concentrations of 2-MN, yet biodegradation occurs. The greater chemical diffusion gradient from the hydrophobic compartment to the aqueous phase caused by microbial activity (in comparison to abiotic controls) brings forward an explanation why biodegradation still occurs beyond mass transfer limitation. Stable isotope fractionation, thus, has been able to pinpoint a concentration range where cell physiological changes related to bioavailability are likely to occur.

Implications for Field Studies. The results of the current study demonstrate that compound-specific stable isotope analysis (CSIA)—used widely to monitor the fate of organic pollutants in the environment—can be masked by mass transfer limitation at very low contaminant concentrations, even for intrinsically slow processes such as anaerobic degradation. This is particularly relevant for oil constituents such as PAHs, because they are barely water soluble and slow to degrade. At trace substrate concentrations the absence of isotope fractionation can, therefore, not necessarily be considered as an indication of the absence of biodegradation (Figure 2F,I).

To asses biodegradation in the field it is, therefore, important to combine CSIA with other field monitoring technologies such as hydrochemical approaches,[52] functional proteomics,[53] investigation of signature metabolites,[54] and other biomarkers.[55]

However, for those scenarios where mass transfer of PAHs is not limited and, thus, changes in isotope fractionation can be observed, our study suggests that H isotope analysis offers better sensitivity than that of C.

Due to the lower H/C ratio of higher molecular mass PAHs, evidence from H isotope fractionation is expected to be even superior in compounds such as phenanthrene. Yet, experiments have to be conducted to investigate whether isotope fractionation occurs or not, as the maximum solubility of phenanthrene in aqueous solution is only about 8 μM: that is, within the same order of magnitude for which isotope fractionation was no longer observed for 2-methylnaphthalene. Future studies need to explore whether this may be the limit set for CSIA as a tool for monitoring PAH biodegradation at contaminated sites.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b01152.

Introduction to the determination of isotope enrichment factors by the Rayleigh equation, list of chemicals used, further detailed description of the experimental setup and

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of concentration and isotope analyses, and results from the concentration analyses (PDF)

AUTHOR INFORMATION

Corresponding Author

*M.E.: tel, +49(0)89 3187 2565; fax, +49(0)89 3187 2565; e-mail, martin.elsner@helmholtz-muenchen.de.

ORCID

Rainer U. Meckenstock: 0000-0001-7786-9546

Martin Elsner: 0000-0003-4746-9052

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

2-MN,2-methylnaphthalene; HMN,2,2,4,4,6,8,8-heptamethylnonane

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