High permeation rates in liposome systems explain rapid glyphosate biodegradation associated with strong isotope fractionation

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Abstract: Bacterial uptake of charged organic pollutants such as the widely used herbicide glyphosate is typically attributed to active transporters, whereas passive membrane permeation as an uptake pathway is usually neglected. For 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomes, the pH-dependent membrane permeation coefficients ($P_{\text{app}}$) of glyphosate, determined by nuclear magnetic resonance (NMR) spectroscopy, varied from $P_{\text{app}}(\text{pH } 7.0) = 3.7 \ (\pm \ 0.3) \times 10^{-7} \text{ m} \cdot \text{s}^{-1}$ to $P_{\text{app}}(\text{pH } 4.1) = 4.2 \ (\pm \ 0.1) \times 10^{-6} \text{ m} \cdot \text{s}^{-1}$. The magnitude of this surprisingly rapid membrane permeation depended on glyphosate speciation and was, at physiological pH, in the range of polar, non-charged molecules. These findings point to passive membrane permeation as potential uptake pathway during glyphosate biodegradation. To test this hypothesis, a Gram-negative glyphosate degrader, *Ochrobactrum sp.* FrEM, was isolated from glyphosate-treated soil and glyphosate permeation rates inferred from the liposome model system were compared to bacterial degradation rates. Estimated maximum permeation rates were, indeed, two orders of magnitudes higher than degradation rates of glyphosate. In addition, biodegradation of millimolar glyphosate concentrations gave rise to pronounced carbon isotope fractionation with an apparent kinetic isotope effect, AKIE$_{\text{carbon}} = 1.014 \pm 0.003$. This value lies in the range typical of unmasked enzymatic isotope fractionation demonstrating that glyphosate biodegradation was little mass transfer-limited and glyphosate exchange across the cell membrane was rapid relative to enzymatic turnover.
INTRODUCTION

Glyphosate (N-(phosphomethyl)glycine) is a systemic, post-emergent, non-selective herbicide widely used in agriculture because of its ability to effectively control a broad range of weeds.\textsuperscript{1-3} One component of its success has been the introduction of transgenic, glyphosate-resistant crops.\textsuperscript{4} The worldwide market share of glyphosate is estimated at USD 5.6 billion, with the USGS estimating glyphosate use at more than 130,000 tons in 2015 alone in the USA.\textsuperscript{2, 6, 7} Historically, the acute toxicity of glyphosate was considered to be low;\textsuperscript{3} it appears, however, that the impact of glyphosate on the environment has been underestimated.\textsuperscript{8-10} Most importantly, the ubiquitous use of glyphosate has been found to affect biodiversity,\textsuperscript{11} which is aggravated by increased usage due to the planting of glyphosate-resistant crops.\textsuperscript{12, 13} The effect of glyphosate on human health is currently disputed. After the World Health Organization classified glyphosate as “probably carcinogenic” to humans (Group 2A),\textsuperscript{14} discussion has continued on whether or not glyphosate use poses a cancer risk.\textsuperscript{15, 16} In addition, the detection of glyphosate and its metabolite aminomethylphosphonic acid (AMPA) in surface waters and groundwaters at increasing frequencies lends urgency to the need to more thoroughly explore its environmental fate.\textsuperscript{17-19} In particular, an improved understanding is warranted on the key drivers that limit its natural microbial degradation, because biodegradation represents the most effective glyphosate remediation pathway.\textsuperscript{20-23}

Recent work highlights the particular role of pollutant mass transfer into microbial cells as a rate-limiting step for biodegradation, especially at low pollutant concentrations.\textsuperscript{24, 25} The mass transfer of polar and charged species (e.g., zwitterionic glyphosate\textsuperscript{26}) into bacterial cells is currently assumed to occur by active transport.\textsuperscript{27, 28} Little is known whether charged molecules can directly permeate the cell membrane as non-polar pollutants do,\textsuperscript{29, 30} and if so, to what extent the
bacterial membrane as diffusion barrier constitutes an even stronger bioavailability limitation for these charged molecules than for non-charged pollutants.\textsuperscript{31} Thus, it is important not only to investigate the membrane permeation rate but also to identify whether the rate of glyphosate is determined by the enzymatic reaction or by slow mass transfer of the herbicide across the cell envelope\textsuperscript{28, 32} (where mass transfer can occur by either membrane permeation\textsuperscript{31} or active transport\textsuperscript{33}).

To investigate membrane permeation processes, different model systems, ranging from the $n$-octanol-water distribution coefficient as a surrogate to partitioning to membrane lipids to more complex systems like lipid discs and black lipid membranes, to synthetic membranes\textsuperscript{34, 35} are used to study the diffusion of drugs and cosmetics through human epithelia.\textsuperscript{36-38} However, these model systems typically contain non-natural lipid phases or non-natural lipid-water interfaces. Therefore, membranes resembling biological lipid bilayers (e.g., liposomes with natural lipid composition) are currently the best model to approximate permeation rates valid for natural systems.\textsuperscript{39, 40} Here, we used unilamellar liposomes composed of a single zwitterionic phospholipid, 1-palmitoyl-2-oleoyl-$sn$-glycero-3-phosphocholine (POPC), as a simplified system to investigate the permeation of glyphosate across phospholipid bilayers.\textsuperscript{41} The POPC vesicles have a gel-to-liquid crystalline phase transition temperature of $–2 \, ^\circ C$ and under our experimental condition are in the liquid crystalline phase, resembling the dominant state of membranes of many bacteria.\textsuperscript{42, 43} This model system lacked additional membrane constituents (e.g., anionic lipids, proteins, lipopolysaccharides) and cell envelope structures (phase-segregated domains in membranes, double membrane in Gram-negative bacteria, peptidoglycan cell wall) that are present in bacterial cells.\textsuperscript{44} We note that porins in the Gram-negative bacterial outer membrane permit passage of
hydrophilic molecules with molecular masses $\lessapprox 600$ Da\textsuperscript{29} and the large pores of peptidoglycan do not restrict pollutant permeation.\textsuperscript{45}

Permeation of the phospholipid bilayer leads to chemical exchange between the outside and the inside of the liposomes. Nuclear magnetic resonance (NMR) spectroscopy offers a direct approach to quantify the permeation process based on the following principle. A nucleus gives rise to an NMR signal at a chemical shift that reflects its chemical environment. Liposomes prepared and suspended in the same solution have roughly equivalent chemical environments inside and outside the liposome. Addition of a non-permeable chemical shift agent (such as a lanthanide ion) to the solution either interior or exterior to the liposome alters the chemical environments on the inside vis-à-vis the outside of the liposome (as the shift agent cannot cross the lipid bilayer) and results in distinct peaks in the NMR spectrum for the nucleus inside and outside the liposomes.\textsuperscript{37, 41, 46}

(In the present study, praseodymium (III) ions (Pr$^{3+}$) were added to the solution external to the liposomes as a non-permeating chemical shift agent.) When the apparent exchange rate constant ($k_a$) between the two chemical environments (here, inside and outside the liposomes) is smaller than the observed frequency difference between the two states ($\Delta\nu$), dynamic exchange of nuclei between chemical environments at equilibrium leads to line broadening.\textsuperscript{47} Lineshape analysis subsequently allows quantification of chemical exchange of between both environments based on the evaluation of associated line broadening in the NMR spectrum.\textsuperscript{47}

Complementary to these model systems, we recently advanced compound-specific isotope analysis (CSIA) as an analytical approach to trace limitations of mass transfer across the cell envelope directly \textit{in vivo} while pollutant biodegradation is ongoing.\textsuperscript{25, 31} The underlying principle is the kinetic isotope effect of the associated enzymatic reaction. As the activation energy during a biochemical reaction is higher for bonds containing a heavy isotope, the turnover of molecules
with a heavy isotope in the reactive position is slower. Therefore, as the enzymatic reaction proceeds, molecules containing heavy isotopes become enriched in the residual (non-reacted) substrate relative to those with light isotopes. This trend can be evaluated by relating the change in isotope ratio \((R_t/R_0)\) to the fraction of the remaining pollutant \(f\) according to the Rayleigh equation\(^{49, 50}\)

\[
\ln \left( \frac{R_t}{R_0} \right) = \ln \left( \frac{\delta^{13}C_t + 1}{\delta^{13}C_0 + 1} \right) = \varepsilon \times \ln(f)
\]

(1)

where the enrichment factor \(\varepsilon\) describes how much slower heavy isotopes react compared to light isotopes. Here, the carbon isotope values \(\delta^{13}C_t\) and \(\delta^{13}C_0\) at time \(t\) and at the beginning of a reaction, respectively, are expressed relative to an international reference material \(\delta^{13}C_{\text{sample}} = (R_{\text{sample}} - R_{\text{Reference}})/R_{\text{Reference}}\) to ensure comparability between laboratories. Thullner et al. delineated a new angle to use the change in isotope ratio as a diagnostic tool to directly observe mass-transfer limitation: strong isotope fractionation is observable, only if the pollutant exchange across the cell envelope is faster than its enzymatic turnover. Otherwise substrate molecules which experience the isotopic discrimination during the enzymatic reaction in the cytosol are used up completely so that they do not return to the bulk solution where the isotope ratio is assessed.\(^{51-53}\) As a consequence, the enzymatic isotope fractionation that is observable in solution becomes masked in the presence of mass transfer limitations – i.e., when active transport (or passive membrane permeation) into and out of the cell is the rate-determining step in biodegradation.\(^{25, 33}\)

For this study, we used a combined approach to gain insight into the role of passive permeation for biodegradation of the zwitterionic pollutant glyphosate, which carries either one (pH < 6) or two (pH > 6) net negative charges at circumneutral pH. First, an NMR study was conducted to experimentally determine pH-dependent passive membrane permeation of glyphosate in phosphatidylcholine liposomes as model system. Second, passive permeation rates were
extrapolated and compared to biodegradation rates of different glyphosate degraders to elucidate the role of passive membrane permeation of glyphosate for nutrient uptake. To this end, *Ochrobactrum sp.* FrEM, a new glyphosate degrader, was isolated from a vineyard soil treated with glyphosate, characterized, and used for degradation experiments. The isotope fractionation measured during glyphosate biodegradation by *Ochrobactrum sp.* FrEM was explored as a diagnostic tool to directly observe the presence or absence of mass transfer limitations and, thus, to validate the assessment based on the results of the liposome model system and our theoretical considerations.

**EXPERIMENTAL SECTION**

**Chemicals.** A list of chemicals used can be found in the Supporting Information (SI).

**Liposome preparation and characterization.** A 25 mg·mL⁻¹ solution of 1-palmitoyl-2-oleoyl-
*sn*-glycero-3-phosphocholine (POPC, transition temperature –2 °C) in chloroform was prepared, and 50 mg POPC (2 mL of the POPC solution) was added to a 3 mL screw cap glass vial tested prior to the experiment to withstand exposure to liquid nitrogen (see below). The chloroform was evaporated under a N₂ stream, and the lipid film was dried with vacuum for at least 12 h. The dried lipids were hydrated with 1 mL of 20 mM glyphosate in D₂O containing a small amount of 3-(trimethylsilyl)-2,2,3,3-tetradeteropropionic acid (TSP) as internal reference for NMR. The pH of the solution, ranging from pH 4.1 to pH 7.8, was adjusted prior to hydration with 1 M sodium hydroxide (in D₂O). (Effective solvent suppression (*vide infra*) resulted insignificant increase in the HOD peak due to NaOH addition.) The vial was vortexed thoroughly, liposomes formed, and the suspension was subjected to three freeze-thaw cycles (freeze in liquid nitrogen for 5 min, thaw in 40 °C water bath for 5 min, and vortex for 30 s) followed by extrusion to yield unilamellar liposomes of uniform size distribution.⁵⁴,⁵⁵ The liposomes were extruded in 1000 µL syringes with
11 passages through a 0.2 µm polycarbonate filter with an Avanti Mini-Extruder (Avanti Polar Lipids, Inc., USA). The hydrodynamic size and the zeta potential of the vesicles were determined by dynamic light scattering and laser Doppler electrophoresis with a ZetaSizer Nano ZS (Malvern Instruments Ltd., United Kingdom) in dilutions of 2 µL liposome solution in 800 µL D₂O. The temperature of the measurement cell was 25 °C. Ten measurements were averaged for each technical replicate (six replicates for dynamic light scattering and five replicates for laser Doppler electrophoresis).

**Nuclear magnetic resonance spectroscopy.** All measurements were carried out on an Avance III 500 MHz spectrometer equipped with a BBFO+ smartprobe (Bruker, USA) at a sample temperature of 25 °C. NMR spectra were recorded with TopSpin 3.5.6 (Bruker, USA). Apodization, Fourier transformation, phase and baseline corrections, absolute referencing on TSP, spectra analysis, and peak fitting was carried out with MestReNova 11.0.3 (Mestrelab Research, Spain). Standard Bruker pulse sequences were used and the spectra collection parameters are summarized in SI Table S1.

**Assessing the line broadening due to chemical exchange across the liposome membrane.** First, a standard ¹H spectrum of 550 µL glyphosate liposome solution was collected to assess the pH-dependent chemical shift of the HOD peak and the chemical shift of the phosphorus nucleus was determined by ³¹P{¹H}. Then, a proton spectrum with phosphorus decoupling ¹H{³¹P} and solvent suppression was recorded. We added 5.5 µL of a 50 mM PrCl₃ solution in D₂O to the NMR tube up to a final concentration of 0.5 mM PrCl₃. Another ¹H{³¹P} spectrum with solvent suppression was recorded and the glyphosate peaks prior to and after PrCl₃ addition were compared by fitting of the peaks. The strong ²J₉HP coupling of 12.4 Hz between the phosphorus and adjacent protons led to splitting of the peak at 2.99 ppm into a doublet in the ¹H NMR spectrum.
(Figure 1). This doublet, however, complicated peak shape analysis to quantify the rate of glyphosate permeation across liposomes. In the absence of Pr$^{3+}$, the shape of the separated doublet peaks could be fit. However, addition of Pr$^{3+}$ led to line broadening due to chemical exchange between the inside and the outside of the liposomes. Thus, the individual peaks of the doublet signal overlapped with each other, rendering peak shape analysis unreliable. We therefore used a $^1$H-NMR pulse sequence that combined solvent suppression (watergate W5) with phosphorus decoupling. As a result, the doublet peak collapsed to a well-resolved singlet that was distinguishable from the POPC liposome signals (Figure 2A).

**Bacterial isolation and characterization.** For bacterial isolation from soil, mineral salt medium at pH 7.0 containing 60 mM sodium glutamate as carbon source and 38 mM ammonium chloride as nitrogen source. Glyphosate (3 mM) was the sole phosphorous source. A detailed description of the bacterial isolation from vineyard soil can be found in the SI.

**Biodegradation of glyphosate by *Ochrobactrum sp. FrEM.*** The biodegradation of glyphosate by *Ochrobactrum sp. FrEM* was carried out in two biological replicates. We inoculated 50 mL of medium (see SI) with *O. sp. FrEM* and incubated the culture at 30 °C at 160 rpm overnight. Cells were harvested by centrifugation (2100 g, Heraeus Megafuge 1.0R, Germany), washed twice with medium, and transferred to 50 mL fresh medium lacking phosphorus for phosphorus depletion. After incubation at 30 °C for 48 h, cells were harvested by centrifugation (2100 g, Heraeus Megafuge 1.0R, Germany) and used to inoculate 150 mL of medium containing 120 µM glyphosate as the only phosphorus source. Bacterial growth was monitored at OD$_{600}$ with a Cary 50 Bio UV-Vis spectrometer (Varian Medical Systems, Inc., USA). During the biodegradation, samples for isotope analysis (10 mL) were taken and the reaction was stopped by adding 1 mL of 2 M sodium hydroxide. The samples were lyophilized and reconstituted in water. The water
volume for reconstitution was decreased from 5 mL to 2 mL as glyphosate was consumed for
glyphosate preconcentration to be within the working range of the isotope measurements (see
below). The isotope ratio in the delta notation ($\delta^{13}$C in per mil relative to Vienna PeeDee Belemnite
(V-PDB)) and the concentration of glyphosate were determined by liquid chromatography Isolink-
isotope ratio mass spectrometry (LC-IRMS) (Thermo Fisher, Germany). The method used for
carbon isotope analysis of glyphosate was modified from Kujawinski et al.56 as follows: A mixed-
phase Primesep 100 column 100 x 5.6 mm, 3 µm particle size (SIELC Technologies, USA) was
used as stationary phase and 2.5 mM phosphate buffer at pH 3.1 was used as mobile phase.
Separation was achieved with 300 µL·min$^{-1}$ isocratic flow. The injection volume was 25 µL. The
reagents for the chemical conversion to CO$_2$ at 99.9 °C were 1.5 M phosphoric acid and 0.84 M
peroxodisulfate at a flow rate of 50 µL·min$^{-1}$ each. The helium (grade 5.0) flow rate in the
separation unit was set to 2.3 mL·min$^{-1}$. The glyphosate concentration was determined with the
area of the glyphosate CO$_2$ peak in the LC Isolink-IRMS chromatogram via external calibration
with glyphosate standards in water (0.03, 0.06, 0.12, and 0.30 mM).

RESULTS AND DISCUSSION

Praseodymium(III) ions interact with glyphosate as well as the liposome surface. The
liposome preparations were of a uniform size with a hydrodynamic diameter of 204 ± 5 nm
(median ± standard deviation) ranging from 194 nm to 239 nm. The median polydispersity index
was 0.093 indicating a uniform and narrow size distribution of the individual liposome
preparations. The neutral zeta potential of the liposomes composed of lipids bearing zwitterionic
phosphatidylecholine headgroups57 changed to +29 ± 6 mV upon PrCl$_3$ addition, because the
strongly positively charged Pr$^{3+}$ associated with the negatively charged phosphate group of POPC.
Praseodymium(III) was added to produce a chemical environment outside the liposomes differing from that inside to allow glyphosate exchange across the membrane to be quantified. Indeed, addition of Pr$^{3+}$ resulted in an interaction of glyphosate with the chemical shift agent which led to a position-specific downfield shift $\Delta \delta_H$ of the glyphosate $^1$H-NMR signals: The chemical shift change produced by a 1 mM PrCl$_3$ solution was $\Delta \delta_H = 0.06$ ppm for the PO$_3^{2–}$-CH$_2$-NH$_2^+$-CH$_2$-COO$^–$ protons and $\Delta \delta_H = 0.16$ ppm for the PO$_3^{2–}$-CH$_2$-NH$_2^+$-CH$_2$-COO$^–$ protons in the $^1$H-NMR spectrum of glyphosate (SI Figure S1). The phosphorus peak was shifted downfield by $\Delta \delta_P = 1.29$ ppm in the $^{31}$P{1H} spectrum (SI Figure S1). That the chemical shift change was strongest for the phosphorus peak and weakest for the protons adjacent to the carboxyl group indicated that Pr$^{3+}$ directly interacted with the negatively charged phosphate group and not with the negatively charged carboxyl group of the zwitterionic glyphosate.

The addition of praseodymium(III) and subsequent peak shape analysis quantified chemical exchange of glyphosate. Subsequent addition of 0.5 mM Pr$^{3+}$ to a glyphosate solution without liposomes moved the chemical shift of the collapsed singlet of the PO$_3^{2–}$-CH$_2$-NH$_2^+$-CH$_2$-COO$^–$ protons downfield from 2.99 ppm to 3.06 ppm. Interaction with the paramagnetic Pr$^{3+}$ changes in the local magnetic field, leading to the shift in frequency; the association/dissociation of Pr$^{3+}$ and glyphosate produced line broadening (Figure 2B). Relying on this approach, we observed a similarly strong chemical shift change also when adding Pr$^{3+}$ to a glyphosate solution containing liposomes (Figure 2C). While the non-permeable Pr$^{3+}$ interacted with glyphosate outside of the liposomes influencing the chemical shift, the shift agent could not enter the liposomes as demonstrated by the observation that inside the glyphosate the chemical shift was left unchanged. As a consequence, two distinct peaks appeared in the spectrum, and the glyphosate peak outside the liposomes was well-resolved from the peak inside. This indicated that the
exchange was slow on the NMR timescale; that is, the ratio \( k_t / \Delta \delta_H \) is smaller than one (\( k_t / \Delta \delta_H < 1 \)),

where \( k_t \) is the apparent rate constant of exchange and \( \Delta \delta_H \) is the chemical shift difference in \(^1\text{H}-\)

NMR.\(^{47}\) The glyphosate exchange across the liposome bilayer was fast enough, however, to lead
to considerable line broadening, \( \Delta \nu \), of the inside peak. The line broadening \( \Delta \nu \) depends on the
apparent rate constant of exchange \( k_t \) according to equation (2)\(^{37}\) and ranged from \( \Delta \nu = 2.6 \text{ Hz} \) at
neutral pH to \( \Delta \nu = 40.8 \text{ Hz} \) at pH 4.

The glyphosate peaks inside the liposomes were fitted to determine the peak width prior to \( (\nu_0) \)

\[
\Delta \nu = \frac{k_{tr}}{\pi}
\]  

(2)

and after addition of \( \text{PrCl}_3 \) \( (\nu_{ex}) \). The resultant line broadening \( \Delta \nu = \nu_{ex} - \nu_0 \) (Figure 2C) was used
to calculate \( k_t \) for each liposome preparation.

**Glyphosate permeation of lipid bilayers depends strongly on pH.** Because \( k_t \) strongly
depends on the surface area and on the size of the liposomes, \( k_t \) is not suitable to compare the
chemical exchange of different liposome preparations and at different pH values. Therefore, Males
et al. derived the apparent permeation coefficient \( P_{app} \) [m‧s\(^{-1}\)] by including the inner liposome
volume and the volume-to-surface ratio according to equation (3),\(^{37}\) where \( d_{lip} \) is the diameter of
the respective liposome and \( \delta \) is the membrane thickness (4 nm); note that this membrane thickness
\( \delta \) should not be confused with the chemical shift \( \delta_H \) (or \( \delta_P \)) in the NMR spectrum or the isotope
value \( \delta^{13}\text{C} \). Our NMR approach is not subject to aqueous boundary layer effects (i.e., the
permeation coefficient so determined is that of the lipid bilayer alone). The NMR experiments
measured a dynamic exchange process under equilibrium conditions. The \( k_t \) does, however,
include a contribution from glyphosate diffusing a short distance through water to arrive at the
lipid bilayer surface. We therefore refer to our rate constant of exchange as an apparent value, and
the permeation coefficient derived from it as an apparent permeation coefficient, \( P_{app} \):
The permeation coefficient describes how rapidly glyphosate permeates a hypothetical two-dimensional POPC membrane sheet and was much higher than expected (Figure 3A). At circumneutral pH the apparent permeation coefficient of glyphosate (double negatively charged, molecular weight $\text{MW} = 167 \text{ g} \cdot \text{mol}^{-1}$) $P_{\text{app}}(\text{pH } 7.0) = 3.7 (\pm 0.3) \times 10^{-7} \text{ m} \cdot \text{s}^{-1}$ was considerably higher than the one of maleate$^{46}$ (double negatively charged, $\text{MW} = 114 \text{ g} \cdot \text{mol}^{-1}$) and in the same range as the permeation coefficient of the polar, neutral serotonin species ($\text{MW} = 176 \text{ g} \cdot \text{mol}^{-1}$).$^{58}$

With decreasing pH, the permeation rate increased, with an apparent permeation coefficient of $P_{\text{app}}(\text{pH } 4.1) = 4.2 (\pm 0.1) \times 10^{-6} \text{ m} \cdot \text{s}^{-1}$ at pH 4.1. The pH-dependence correlated linearly with the average degree of ionization and thus the average charge of glyphosate (Figure 3B). The net charge of $-2$ (one positive and three negative charges) of glyphosate at neutral pH slowed passive membrane permeation. Protonation of the phosphate group at pH 4.1 reduced the net charge of glyphosate to $-1$ and, consequently, accelerated membrane permeation. This also indicates that the effect of $\text{Pr}^{3+}$ on the measured permeation coefficient is negligible. If the change towards positive liposome surface potential due to $\text{Pr}^{3+}$ addition ($\text{vide supra}$) facilitated permeation due to attraction of the strongly negatively charged glyphosate, the doublely negatively charged glyphosate species would permeate faster. However, the opposite is the case. Furthermore, that two distinct glyphosate peaks appear in the NMR spectrum ($\text{vide supra}$) demonstrates that $\text{Pr}^{3+}$ does not enter the liposomes together with glyphosate which could lead to changed permeation characteristics.

Therefore, we hypothesize that the zwitterionic structure of glyphosate facilitates glyphosate permeation, whereas the increased negative charge at neutral pH slowed passive permeation of glyphosate.
Membrane permeation can lead to considerable glyphosate uptake into bacterial cells. The entry of non-polar pollutants into bacterial cells by passive permeation of the cell envelope is well recognized,\textsuperscript{30, 59} and charged, polar molecules like glyphosate are commonly assumed to be taken up almost exclusively by active transport or porin-assisted permeation.\textsuperscript{60, 61} Contrary to this expectation, our observations in a liposome model system that lacked transporters or porins gave membrane permeation coefficients of glyphosate in the same range as those of non-charged molecules (see above).\textsuperscript{58} This observation suggests that passive membrane permeation of glyphosate mono- and dianions may provide sufficient influx into bacterial cells for it to serve as phosphorus source. Because the diffusion through water of glyphosate is fast compared with the diffusion through the lipid membrane,\textsuperscript{62, 63} the membrane as significant barrier influences how fast the molar amount of substrate outside the bacteria $n_{\text{out}}$ is reduced via passive membrane permeation at the rate $(dn_{\text{out}}/dt)_{\text{permeation}}$. This process is driven by the concentration gradient across the membrane and is defined by the linear exchange term in equation (4) as proposed by Males \textit{et al.}\textsuperscript{64}

$$\left(\frac{dn_{\text{out}}}{dt}\right)_{\text{permeation}} = -(k_{tr}K_{lip-w}[S_{\text{out}}]) + (k_{tr}K_{lip-w}[S_{\text{in}}])$$  \hspace{1cm} (4)

Here, $K_{lip-w}$ is the membrane lipid-water partitioning coefficient, $[S_{\text{out}}]$ and $[S_{\text{in}}]$ are the glyphosate concentrations outside and inside the bacterial cell, whereas $K_{lip-w}[S_{\text{out}}]$ and $K_{lip-w}[S_{\text{in}}]$ are the concentrations within the lipid membrane (outside and inside), respectively. With the definition of the diffusion coefficient across the membrane (lipid bilayer) $D_{lip}$ (5), the apparent rate constant of exchange $k_{a}$ can be calculated for a single bacterial cell by equation (6)

$$D_{lip} = \frac{P_{app} \times \delta}{K_{lip-w}}$$  \hspace{1cm} (5)
where \( A_{\text{cell}} \approx 3 \mu m^2 \) is the estimated surface area of one bacterial cell and \( \delta \) is the membrane thickness (one 4 nm membrane in Gram-positive and two 4 nm thick membranes which equals 8 nm in total in Gram-negative bacteria). Together with equation (7), a term is obtained for the concentration gradient-dependent glyphosate influx of a single bacterial cell:

\[
\left( \frac{dn_{\text{out}}}{dt} \right)_{\text{cell-permeation-max}} = - \left( P_{\text{app}} A_{\text{cell}} [S_{\text{out}}] \right) + \left( P_{\text{app}} A_{\text{cell}} [S_{\text{in}}] \right)
\]

The glyphosate influx is at its maximum \( \left( \frac{dn_{\text{out}}}{dt} \right)_{\text{cell-permeation-max}} \) when the concentration gradient is large \( ([S_{\text{in}}] = 0) \). We compared this maximum permeation rate with the glyphosate degradation rate of \textit{Achromobacter sp.} MPS 12A described by Sviridov et al.\textsuperscript{22} The glyphosate degradation rate of a single \textit{Achromobacter sp.} MPS 12A cell \( (dn/dt)_{\text{deg-cell}} = -1.4 \times 10^{-21} \text{mol} \cdot \text{s}^{-1} \cdot \text{cell}^{-1} \) at a concentration of 3 mM\textsuperscript{22} was estimated by correlating the number of cells with the optical density OD\textsubscript{600} and the bulk glyphosate degradation rate. While this correlation strongly depends on the strain and the growth conditions, the previously reported value of \( 8 \times 10^8 \text{cells} \cdot \text{mL}^{-1} \cdot \text{OD}_{600}^{-1} \) provides a good first estimate.\textsuperscript{65, 66} The comparison showed that the calculated maximum membrane permeation rate at pH 7 \( (dn_{\text{out}}/dt)_{\text{cell-perm-max}} = -1.9 \times 10^{-18} \text{mol} \cdot \text{s}^{-1} \cdot \text{cell}^{-1} \) was two orders of magnitude higher than the degradation rate. As a consequence, even though glyphosate has a net charge of –2 at pH 7, its passive membrane permeation is predicted to be fast enough to provide enough influx for bacterial biodegradation and to serve as phosphorus source. This hypothesis clearly warrants further testing. If true, it should be possible to confirm it (a) by the observation of similarly rapid biodegradation per cell in a different strain and (b) by applying compound-specific isotope fractionation as a diagnostic tool to observe the absence of mass transfer limitations directly. If permeation is indeed faster than enzymatic conversion, glyphosate molecules inside
and outside the cell are expected to be in rapid equilibrium. Thus, glyphosate molecules enriched in heavy isotopes due to the enzymatic reaction in the cytosol will get out of the cell into the bulk solution. This would lead to the isotope effect of the enzyme reaction being observable outside the cell, resulting in strong isotope fractionation during biodegradation. A new bacterium was, therefore, isolated from soil, and isotope fractionation was measured during glyphosate degradation.

**Isolation and glyphosate degradation activity of *Orchrobactrum sp. FrEM***. Repeated subculturing of an inoculum from soil samples in a medium containing 3 mM glyphosate as sole phosphorus source resulted in the isolation of a bacterial strain with glyphosate-degrading activity. Glyphosate was only used as phosphorus source. Shushkova et al. faced difficulties when isolating a strain with glyphosate as carbon and phosphorus source. The bacteria were rod-shaped as observed by light microscopy (**SI Figure S2**). Sequence alignment (BLAST) of the 16S rRNA showed a 99% homology with *Ochrobactrum anthropic, O. rhizosphaerae, O. pituitosum,* and *O. intermedium,* which all belong to the family of Brucellaceae of Alphaproteobacteria, and 70% homology with *Ochrobactrum haematophilum.* The strain was termed *Ochrobactrum sp. FrEM*** (**SI Figure S3**). The fastest glyphosate degradation by *O. sp. FrEM* occurred after 4.5 days when the cell density was high (OD$_{600}$ ≈ 0.8). Within 12 h, the glyphosate concentration decreased from 104 mM to 55 mM equaling a maximum glyphosate degradation rate ($dn/dt)_{deg-cell} = -1.7 \times 10^{-21}\text{ mol} \cdot \text{s}^{-1} \cdot \text{cell}^{-1}$ (**Figure 4A**) which was as high as that of *Achromobacter sp. MPS 12A* (see above). Furthermore, just as for *Achromobacter sp. MPS 12A,* the calculated maximum membrane permeation rate at pH 7 ($dn_{out}/dt)_{cell-perm-max} = -7.5 \times 10^{-20}\text{ mol} \cdot \text{s}^{-1} \cdot \text{cell}^{-1}$ at a concentration of 0.12 mM was larger than the degradation rate indicating that passive permeation
of the cell envelope is likely not rate limiting for glyphosate biodegradation. We subsequently aimed to verify this hypothesis by compound-specific isotope fractionation analysis.

**Carbon isotope fractionation revealed rapid glyphosate mass transfer across the cell wall.**

Glyphosate biodegradation by *O. sp.* FrEM (Figure 4A) was accompanied by significant carbon isotope fractionation. Carbon isotope values $\delta^{13}C$ of glyphosate increased from $-28 \pm 0.5$‰ in the beginning to $-19 \pm 0.5$‰ after 90% glyphosate conversion reflecting an enrichment of $^{13}C$ over $^{12}C$. The corresponding enrichment factor $\varepsilon^{13}C = -4.5 \pm 0.5$‰ was determined according to the Rayleigh equation (Figure 5, and equation (1)). The primary apparent kinetic isotope effect AKIE, a measure for the isotope effect at the reactive position, allows the direct comparison of isotope effects of different reactions and reactants and was calculated according to equation (8)\(^{68}\)

$$AKIE_{carbon} = \frac{1}{\frac{n}{x}^{13}C + 1}$$  \(8\)

where $n$ denotes the total number of carbon atoms and $x$ the number of carbon atoms at the reactive position. With $n = 3$ and $x = 1$, the primary apparent kinetic isotope effect for glyphosate degradation (via breaking a single bond between carbon and phosphorous) was $AKIE_{carbon} = 1.014 \pm 0.003$, which is in the range of chemical reactions that involve breaking a single bond to a carbon atom.\(^{68,69}\)

This suggests that any additional rate determining steps like active transport\(^{33}\) or slow passive membrane permeation\(^{31}\) masked the enzymatic isotope fractionation only to a small extent, if at all. As a consequence, we conclude that, indeed, glyphosate exchanged rapidly across the cell envelope consistent with our hypothesis that passive permeation across the cell envelope may be an important, and until now underestimated, driver to facilitate biodegradation of glyphosate or other charged pollutants (C, N, P sources). Future research should not only address the role of pH.
in the permeation of whole cells during biodegradation but also elucidate the possible role of transporters or porins (e.g., by studying isotope fractionation during glyphosate degradation in cell free extracts of *O. sp*. FrEM or with liposomes containing the degrading enzyme).

**Possibility of mass transfer limitations at low concentrations in the environment.** While passive membrane permeation has previously been associated with only non-polar molecules, our results suggest that also charged species like glyphosate can enter the bacterial cell not only assisted by proteins, e.g. by active transporters\textsuperscript{70, 71} or facilitated diffusion via porins,\textsuperscript{29, 72} but also by passive permeation of the cell membrane more rapidly than commonly thought. This can facilitate glyphosate biodegradation and lead to rapid turnover at high concentrations in water and soil.\textsuperscript{10} A different situation must be considered, however, if the concentration gradient across the cell envelope is shallower, that is, when the outside concentration is lower. While the degradation-associated isotope fractionation was determined at high concentrations (> 25 µM) the concentration in soil and ground- and surface waters is much lower (< 2 µM, < 15 nM, and < 0.5 µM respectively).\textsuperscript{18, 73} We recently demonstrated that mass transfer across the cell membrane becomes rate-limiting for atrazine biodegradation at trace concentrations.\textsuperscript{25} Similarly, at a glyphosate concentration of 1 µM, the calculated maximum membrane permeation rate is reduced to only $\left(\frac{dn_{out}}{dt}\right)_{cell-perm-max} = -6.5 \times 10^{-22} \text{ mol} \cdot \text{s}^{-1} \cdot \text{cell}^{-1}$, which is lower than the respective degradation rate per cell. At these concentrations, acceleration of cell wall transfer of glyphosate with high affinity active transporters may become necessary to boost biodegradation. Interestingly, Pipke *et al.* described such an active glyphosate transporter with an uptake rate of $\left(\frac{dn_{out}}{dt}\right)_{cell-transport} = -1.8 \times 10^{-21} \text{ mol} \cdot \text{s}^{-1} \cdot \text{cell}^{-1}$ which is just in the range of observed glyphosate degradation rates.\textsuperscript{74} However, its affinity constant $K_M = 0.125$ mM for glyphosate uptake is rather high, resulting in low transporter activity at trace concentrations. This increased mass transfer limitation
at trace concentrations may cause biodegradation to stall and might explain the frequent detection
of glyphosate in the environment.17

ASSOCIATED CONTENT

A list of chemicals, media composition, and strain isolation, figures of the glyphosate spectra,
Micrograph of Ochrobactrum sp FrEM, phylogenetic tree, LC-IRMS chromatogram, and a table
summarizing spectra collection parameters (PDF). This information is available free of charge via
the Internet at http://pubs.acs.org.

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Figure 1. $^2J_{HF}$ coupling prevents direct measurement of glyphosate permeation of liposomes with standard $^1$H-spectra. Glyphosate showed one singlet at 3.74 ppm and one doublet at 2.99 ppm in the $^1$H NMR spectrum with solvent suppression (black line). Strong $^2J_{HF}$ coupling led to formation of a doublet centered at 2.99 ppm. Upon Pr$^{3+}$ addition to the liposome suspension, the spectrum changed (red line). The glyphosate peaks outside the liposomes were shifted downfield (doublet at 3.2 ppm and singlet at 3.79 ppm) and the peaks inside the liposomes broadened due to chemical exchange. As a consequence, the individual doublet peaks overlapped, almost coalescing into a singlet and rendering peak shape analysis impossible. Both spectra in this figure were collected at pH 7.5.
Figure 2. Peak shape broadening due to exchange can be quantified by fitting the peaks in $^1H$\text{^{31P}} NMR spectra. (A) Clear separation of the glyphosate signals from the signals of the POPC liposomes in the $^1H$\text{^{31P}} NMR spectrum (black line) enabling reliable peak shape analysis. (B) Spectral region showing glyphosate protons attached to carbon 2 with (red line) and without Pr$^{3+}$ (black line) in the absence of liposomes. The line broadening upon Pr$^{3+}$ addition was caused by the interaction with the paramagnetic Pr$^{3+}$. Even though the signal without Pr$^{3+}$ slightly overlapped with the broad glyphosate signal in the presence of Pr$^{3+}$, both peaks were well resolved. (C) The glyphosate peaks inside and outside the liposomes remained well resolved when POPC liposomes were present. Fitting the peak shape (blue dashed lines) prior (black line) and after (red line) the addition of PrCl$_3$ yielded peak widths and, thus, allowed the line broadening to be quantified. The broadening of the glyphosate peak inside the liposomes (2.99 ppm) was caused by chemical exchange of glyphosate between the inside and the outside of the liposomes, because the non-permeable Pr$^{3+}$ was not able to interact with glyphosate inside the liposomes. All spectra in this figure were collected at pH 7.5.
Figure 3. The pH-dependence of the permeation coefficient $P_{\text{app}}$ (black squares) correlated with the net charge of glyphosate. (A) $P_{\text{app}}$ depended strongly on the pH of the liposome solution. The permeation at neutral pH ($P_{\text{app}}(\text{pH } 7.0) = 3.7 \pm 0.3 \times 10^{-7} \text{ m} \cdot \text{s}^{-1}$) was one order of magnitude lower than at slightly acidic pH ($P_{\text{app}}(\text{pH } 4.1) = 4.2 \pm 0.1 \times 10^{-6} \text{ m} \cdot \text{s}^{-1}$). (B) Permeation correlated with the ionization of glyphosate which can be explained by two different permeation coefficients of the two different glyphosate species (two negative and one negative charge at the phosphate group, respectively). Both panels show the mean and the error bars depicting the standard deviation.
Figure 4. Glyphosate biodegradation was accompanied by growth and strong isotope fractionation. (A) Glyphosate degradation by *Ochrobactrum* sp. FrEM. Consumption of glyphosate (blue triangles) as source of phosphorous led to bacterial growth (red circles). (B) During this biodegradation, $^{13}$C/$^{12}$C ratios of glyphosate increased, as indicated by less negative $\delta^{13}$C values. All graphs show the mean and the error bars indicating the range of two biological replicates. The exclamation marks (!) above two isotope data points indicate that a reliable isotope value could be measured for only one biological replicate at the respective time points. Isotope values were measured in technical triplicates for each sample and are associated with an analytical uncertainty of ± 0.3 ‰.
Figure 5. Pronounced isotope fractionation indicated rapid glyphosate exchange across the bacterial cell envelope. The carbon isotope fractionation factor ($\varepsilon^{13}C = -4.5 \pm 0.5 \%$) was determined according to the Rayleigh equation (equation (1)). The corresponding $\text{AKIE}_{\text{carbon}} = 1.014 \pm 0.003$ (see equation (8)) was in the range of typical carbon isotope effects. This indicated that the enzymatic isotope fractionation was not masked by mass transfer limitations and that exchange of glyphosate across the cell envelope was comparatively rapid during bacterial degradation by *Ochrobactrum sp.* FrEM.
1. Franz, J. E.; Mao, M. K.; Sikorski, J. A., *Glyphosate: a unique global herbicide*. American Chemical Society: 1997.

2. Dill, G. M.; Sammons, R. D.; Feng, P. C. C.; Kohn, F.; Kretzmer, K.; Mehrsheikh, A.; Bleeke, M.; Honegger, J. L.; Farmer, D.; Wright, D.; Haupfear, E. A., Glyphosate: Discovery, Development, Applications, and Properties. In *Glyphosate Resistance in Crops and Weeds*, John Wiley & Sons, Inc.: 2010; pp 1-33.

3. Duke, S. O.; Powles, S. B., Glyphosate: a once-in-a-century herbicide. *Pest Management Science* 2008, 64, (4), 319-325.

4. Dill, G. M., Glyphosate-resistant crops: history, status and future. *Pest Management Science* 2005, 61, (3), 219-224.

5. Bohn, T.; Cuhra, M.; Traavik, T.; Sanden, M.; Fagan, J.; Primicerio, R., Compositional differences in soybeans on the market: Glyphosate accumulates in Roundup Ready GM soybeans. *Food Chemistry* 2014, 153, 207-215.

6. Woodburn, A. T., Glyphosate: production, pricing and use worldwide. *Pest Management Science* 2000, 56, (4), 309-312.

7. USGS Estimated Annual Agricultural Pesticide Use. https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2015&map=GLYPHOSATE

8. Helander, M.; Saloniemi, I.; Saikkonen, K., Glyphosate in northern ecosystems. *Trends in Plant Science* 2012, 17, (10), 569-574.

9. Relyea, R. A., THE LETHAL IMPACT OF ROUNDUP ON AQUATIC AND TERRESTRIAL AMPHIBIANS. *Ecological Applications* 2005, 15, (4), 1118-1124.

10. Giesy, J. P.; Dobson, S.; Solomon, K. R., Ecotoxicological Risk Assessment for Roundup® Herbicide. In *Reviews of Environmental Contamination and Toxicology: Continuation of Residue Reviews*, Ware, G. W., Ed. Springer New York: New York, NY, 2000; pp 35-120.

11. Sullivan, T. P.; Sullivan, D. S., Vegetation management and ecosystem disturbance: impact of glyphosate herbicide on plant and animal diversity in terrestrial systems. *Environmental Reviews* 2003, 11, (1), 37-59.

12. Mamy, L.; Gabrielle, B.; Barriuso, E., Comparative environmental impacts of glyphosate and conventional herbicides when used with glyphosate-tolerant and non-tolerant crops. *Environmental Pollution* 2010, 158, (10), 3172-3178.

13. Firbank, L. G.; Forcella, F., Genetically Modified Crops and Farmland Biodiversity. *Science* 2000, 289, (5484), 1481-1482.

14. WHO, IARC Monographs: evaluation of five organophosphate insecticides and herbicides. *WHO 2015, 112.*

15. Chang, E. T.; Delzell, E., Systematic review and meta-analysis of glyphosate exposure and risk of lymphohematopoietic cancers. *Journal of Environmental Science and Health, Part B* 2016, 51, (6), 402-434.

16. Andreotti, G.; Koutros, S.; Hofmann, J. N.; Sandler, D. P.; Lubin, J. H.; Lynch, C. F.; Lerro, C. C.; De Roos, A. J.; Parks, C. G.; Alavanja, M. C.; Silverman, D. T.; Beane Freeman, L., Glyphosate Use and Cancer Incidence in the Agricultural Health Study. *JNCI: Journal of the National Cancer Institute* 2017, djx233, https://doi.org/10.1093/jnci/djx233.
17. Mamy, L.; Barriuso, E.; Gabrielle, B., Environmental fate of herbicides trifluralin, metazachlor, metamitron and sulcotrione compared with that of glyphosate, a substitute broad spectrum herbicide for different glyphosate-resistant crops. *Pest management science* **2005**, *61* (9), 905-916.

18. Battaglin, W. A.; Meyer, M. T.; Kuivila, K. M.; Dietze, J. E., Glyphosate and Its Degradation Product AMPA Occur Frequently and Widely in U.S. Soils, Surface Water, Groundwater, and Precipitation. *J. Am. Water Resour. Assoc.* **2014**, *50* (2), 275-290.

19. Horth, H.; Blackmore, K., Survey of glyphosate and AMPA in groundwaters and surface waters in Europe. *Report by WRC plc, Swindon, Swindon, Wiltshire, United Kingdom. No.: UC8073 2009*, 2.

20. Ermakova, I.; Kiseleva, N.; Shushkova, T.; Zharikov, M.; Zharikov, G.; Leontievsky, A., Bioremediation of glyphosate-contaminated soils. *Applied Microbiology and Biotechnology* **2010**, *88* (2), 585-594.

21. Pipke, R.; Amrhein, N., Degradation of the Phosphonate Herbicide Glyphosate by Arthrobacter atrocyaneus ATCC 13752. *Applied and Environmental Microbiology* **1988**, *54* (5), 1293-1296.

22. Sviridov, A. V.; Shushkova, T. V.; Zelenkova, N. F.; Vinokurova, N. G.; Morgunov, I. G.; Ermakova, I. T.; Leontievsky, A. A., Distribution of glyphosate and methylphosphonate catabolism systems in soil bacteria Ochrobactrum anthropi and Achromobacter sp. *Applied Microbiology and Biotechnology* **2012**, *93* (2), 787-96.

23. Borggaard, O. K.; Gimsing, A. L., Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. *Pest Management Science* **2008**, *64* (4), 441-456.

24. Bosma, T. N. P.; Middeldorp, P. J. M.; Schraa, G.; Zehnder, A. J. B., Mass Transfer Limitation of Biotransformation: Quantifying Bioavailability. *Environ. Sci. Technol.* **1997**, *31* (1), 248-252.

25. Ehrl, B. N.; Kundu, K.; Gharasoo, M.; Marozava, S.; Elsner, M., Rate limiting mass transfer in micropollutant degradation revealed by isotope fractionation. *Environ. Sci. Technol. submitted, attached as supporting information for review only.*

26. Sprankle, P.; Meggitt, W.; Penner, D., Adsorption, mobility, and microbial degradation of glyphosate in the soil. *Weed Science* **1975**, *23*, 229-234.

27. Button, D. K., Kinetics of nutrient-limited transport and microbial growth. *Microbiol. Rev.* **1985**, (49), 270-297.

28. Ferenci, T.; Robert, K. P., Bacterial Physiology, Regulation and Mutational Adaptation in a Chemostat Environment. In *Advances in Microbial Physiology*, Academic Press: 2008; Vol. Volume 53, pp 169-315.

29. Delcour, A. H., Outer Membrane Permeability and Antibiotic Resistance. *Biochimica et biophysica acta 2009*, **1794**, (5), 808-816.

30. Parales, R. E.; Ditty, J. L., Substrate Transport. In *Handbook of Hydrocarbon and Lipid Microbiology*, Timmis, K. N., Ed. Springer Berlin Heidelberg: Berlin, Heidelberg, 2010; pp 1545-1553.

31. Ehrl, B. N.; Gharasoo, M.; Elsner, M., Isotope Fractionation Pinpoints Membrane Permeability as a Barrier to Atrazine Biodegradation in Gram-negative Polaromonas sp. Nea-C. *Environ. Sci. Technol.* **2018**, *52* (7), 4137-4144.

32. Wick, L. M.; Quadrani, M.; Egli, T., Short- and long-term changes in proteome composition and kinetic properties in a culture of Escherichia coli during transition from glucose-
excess to glucose-limited growth conditions in continuous culture and vice versa. *Environmental Microbiology* 2001, 3, (9), 588-599.

33. Qiu, S.; Gözdereliler, E.; Weyrauch, P.; Lopez, E. C. M.; Kohler, H.-P. E.; Sørensen, S. R.; Meckenstock, R. U.; Elsner, M., Small 13C/12C Fractionation Contrasts with Large Enantiomer Fractionation in Aerobic Biodegradation of Phenoxy Acids. *Environ. Sci. Technol.* 2014, 48, (10), 5501-5511.

34. Beate I. Escher, L. S., Chemical Speciation of Organics and of Metals at Biological Interphases. In *Physicochemical Kinetics and Transport at Biointerfaces*, Hermann P. Van Leeuwen, W. K., Ed. Wiley: 2004; Vol. 9, pp 205-269.

35. Wohnsland, F.; Faller, B., High-Throughput Permeability pH Profile and High-Throughput Alkane/Water log P with Artificial Membranes. *Journal of Medicinal Chemistry* 2001, 44, (6), 923-930.

36. Passeleu-Le Bourdonnec, C.; Carrupt, P.-A.; Scherrmann, J. M.; Martel, S., Methodologies to Assess Drug Permeation Through the Blood–Brain Barrier for Pharmaceutical Research. *Pharmaceutical Research* 2013, 30, (11), 2729-2756.

37. Males, R. G.; Herring, F. G., A 1H-NMR study of the permeation of glycolic acid through phospholipid membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1999, 1416, (1–2), 333-338.

38. Naderkhani, E.; Vasskog, T.; Flaten, G. E., Biomimetic PVPA in vitro model for estimation of the intestinal drug permeability using fasted and fed state simulated intestinal fluids. *European Journal of Pharmaceutical Sciences* 2015, 73, 64-71.

39. Lande, M. B.; Donovan, J. M.; Zeidel, M. L., The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *The Journal of General Physiology* 1995, 106, (1), 67-84.

40. Dietrich, C.; Bagatolli, L. A.; Volovyk, Z. N.; Thompson, N. L.; Levi, M.; Jacobson, K.; Gratton, E., Lipid Rafts Reconstituted in Model Membranes. *Biophysical Journal* 2001, 80, (3), 1417-1428.

41. Xiang, T.-X.; Anderson, B. D., Development of a combined NMR paramagnetic ion-induced line-broadening/dynamic light scattering method for permeability measurements across lipid bilayer membranes. *Journal of Pharmaceutical Sciences* 1995, 84, (11), 1308-1315.

42. Russell, N. J.; Fukunaga, N., A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. *FEMS Microbiology Letters* 1990, 75, (2-3), 171-182.

43. Sharon, V.; Dina, P.; Ehud, P.; H., P. A.; Itzhak, F., Coexistence of Domains with Distinct Order and Polarity in Fluid Bacterial Membranes. *Photochemistry and Photobiology* 2002, 76, (1), 1-11.

44. Epand, R. M.; Epand, R. F., Lipid domains in bacterial membranes and the action of antimicrobial agents. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 2009, 1788, (1), 289-294.

45. Vollmer, W.; Blanot, D.; De Pedro, M. A., Peptidoglycan structure and architecture. *FEMS Microbiology Reviews* 2008, 32, (2), 149-167.

46. Prestegard, J. H.; Cramer, J. A.; Viscio, D. B., Nuclear magnetic resonance determinations of permeation coefficients for maleic acid in phospholipid vesicles. *Biophysical Journal* 1979, 26, (3), 575-584.

47. Millet, O.; Loria, J. P.; Kroenke, C. D.; Pons, M.; Palmer, A. G., The Static Magnetic Field Dependence of Chemical Exchange Linebroadening Defines the NMR Chemical Shift Time Scale. *J. Am. Chem. Soc.* 2000, 122, (12), 2867-2877.
48. Melander, L.; Saunders, W. H., Reaction rates of isotopic molecules. John Wiley: New York, 1980; p 331.

49. Hoefs, J., Theoretical and Experimental Principles. In Stable isotope geochemistry, 3rd ed.; Wyllie, P. J., Ed. Springer-Verlag: Chicago, 1987; pp 1-25.

50. Schmidt, H. L., Fundamentals and systematics of the non-statistical distributions of isotopes in natural compounds. Naturwissenschaften 2003, 90, (12), 537-552.

51. Thullner, M.; Kampara, M.; Richnow, H. H.; Harms, H.; Wick, L. Y., Impact of Bioavailability Restrictions on Microbially Induced Stable Isotope Fractionation. 1. Theoretical Calculation. Environ. Sci. Technol. 2008, 42, (17), 6544-6551.

52. Kampara, M.; Thullner, M.; Richnow, H. H.; Harms, H.; Wick, L. Y., Impact of Bioavailability Restrictions on Microbially Induced Stable Isotope Fractionation. 2. Experimental Evidence. Environ. Sci. Technol. 2008, 42, (17), 6552-6558.

53. Thullner, M.; Fischer, A.; Richnow, H. H.; Wick, L. Y., Influence of mass transfer on stable isotope fractionation. Applied Microbiology and Biotechnology 2013, 97, (2), 441-452.

54. MacDonald, R. C.; MacDonald, R. I.; Menco, B. P. M.; Takeshita, K.; Subbarao, N. K.; Hu, L.-r., Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. Biochimica et Biophysica Acta (BBA) - Biomembranes 1991, 1061, (2), 297-303.

55. Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R., Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochimica et Biophysica Acta (BBA) - Biomembranes 1985, 812, (1), 55-65.

56. Kujawinski, D. M.; Wolbert, J. B.; Zhang, L.; Jochmann, M. A.; Widory, D.; Baran, N.; Schmidt, T. C., Carbon isotope ratio measurements of glyphosate and AMPA by liquid chromatography coupled to isotope ratio mass spectrometry. Analytical and Bioanalytical Chemistry 2013, 405, (9), 2869-2878.

57. Moncelli, M. R.; Becucci, L.; Guidelli, R., The intrinsic pKa values for phosphatidylethanolamine, phosphatidylethanolamine, and phosphatidylserine in monolayers deposited on mercury electrodes. Biophysical Journal 1994, 66, (6), 1969-1980.

58. Viscio, D. B.; Prestegard, J. H., NMR studies of 5-hydroxytryptamine transport through large unilamellar vesicle membranes. Proceedings of the National Academy of Sciences of the United States of America 1981, 78, (3), 1638-1642.

59. Endo, S.; Escher, B. I.; Goss, K.-U., Capacities of Membrane Lipids to Accumulate Neutral Organic Chemicals. Environ. Sci. Technol. 2011, 45, (14), 5912-5921.

60. Kallimanis, A.; Frillingos, S.; Drainas, C.; Koukkou, A. L., Taxonomic identification, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading Arthrobacter sp. strain Sphe3. Applied Microbiology and Biotechnology 2007, 76, (3), 709-717.

61. Kell, D. B.; Dobson, P. D.; Oliver, S. G., Pharmaceutical drug transport: the issues and the implications that it is essentially carrier-mediated only. Drug Discovery Today 2011, 16, (15–16), 704-714.

62. Culbertson, C. T.; Jacobson, S. C.; Michael Ramsey, J., Diffusion coefficient measurements in microfluidic devices. Talanta 2002, 56, (2), 365-373.

63. Worch, E., A new equation for the calculation of diffusion coefficients for dissolved substances. In [Water], Fachgruppe Wasserchemie In Der Gesellschaft Deutscher, C., Ed. 1993; Vol. 81, pp 289-297.

64. Males, R. G.; Phillips, P. S.; Herring, F. G., Equations describing passive transport through vesicular membranes. Biophysical Chemistry 1998, 70, (1), 65-74.
65. Volkmer, B.; Heinemann, M., Condition-Dependent Cell Volume and Concentration of Escherichia coli to Facilitate Data Conversion for Systems Biology Modeling. *PLOS ONE* 2011, 6(7), e23126.

66. Kopinke, F.-D.; Georgi, A.; Roland, U., Isotope fractionation in phase-transfer processes under thermodynamic and kinetic control – Implications for diffusive fractionation in aqueous solution. *Science of The Total Environment* 2018, 610-611, (Supplement C), 495-502.

67. Shushkova, T. V.; Ermakova, I. T.; Sviridov, A. V.; Leontievsky, A. A., Biodegradation of glyphosate by soil bacteria: Optimization of cultivation and the method for active biomass storage. *Microbiology* 2012, 81(1), 44-50.

68. Elsner, M.; Zwank, L.; Hunkeler, D.; Schwarzenbach, R. P., A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants. *Environ. Sci. Technol.* 2005, 39(18), 6896-6916.

69. Mancini, S. A.; Hirschorn, S. K.; Elsner, M.; Lacrampe-Couloume, G.; Sleep, B. E.; Edwards, E. A.; SherwoodLollar, B., Effects of Trace Element Concentration on Enzyme Controlled Stable Isotope Fractionation during Aerobic Biodegradation of Toluene. *Environ. Sci. Technol.* 2006, 40(24), 7675-7681.

70. Muller, R. H.; Hoffmann, D., Uptake kinetics of 2,4-dichlorophenoxyacetate by Delftia acidovorans MC1 and derivative strains: Complex characteristics in response to pH and growth substrate. *Bioscience Biotechnology and Biochemistry* 2006, 70(7), 1642-1654.

71. Nichols, N. N.; Harwood, C. S., PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from Pseudomonas putida. *Journal of Bacteriology* 1997, 179(16), 5056-61.

72. Button, D. K.; Robertson, B.; Gustafson, E.; Zhao, X. M., Experimental and theoretical bases of specific affinity, a cytoarchitecture-based formulation of nutrient collection proposed to superecede the Michaels-Menten paradigm of microbial kinetics. *Applied and Environmental Microbiology* 2004, 70(9), 5511-5521.

73. Sanchis, J.; Kantiani, L.; Llorca, M.; Rubio, F.; Ginebreda, A.; Fraile, J.; Garrido, T.; Farre, M., Determination of glyphosate in groundwater samples using an ultrasensitive immunoassay and confirmation by on-line solid-phase extraction followed by liquid chromatography coupled to tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* 2012, 402(7), 2335-2345.

74. Pipke, R.; Schulz, A.; Amrhein, N., Uptake of Glyphosate by an Arthrobacter sp. *Applied and Environmental Microbiology* 1987, 53(5), 974-978.