Kinetics and Yields of Pesticide Biodegradation at Low Substrate Concentrations and under Conditions Restricting Assimilable Organic Carbon

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The fundamentals of growth-linked biodegradation occurring at low substrate concentrations are poorly understood. Substrate utilization kinetics and microbial growth yields are two critically important process parameters that can be influenced by low substrate concentrations. Standard biodegradation tests aimed at measuring these parameters generally ignore the ubiquitous occurrence of assimilable organic carbon (AOC) in experimental systems which can be present at concentrations exceeding the concentration of the target substrate. The occurrence of AOC effectively makes biodegradation assays conducted at low substrate concentrations mixed-substrate assays, which can have profound effects on observed substrate utilization kinetics and microbial growth yields. In this work, we introduce a novel methodology for investigating biodegradation at low concentrations by restricting AOC in our experiments. We modified an existing method designed to measure trace concentrations of AOC in water samples and applied it to systems in which pure bacterial strains were growing on pesticide substrates between 0.01 and 50 mg liter⁻¹. We simultaneously measured substrate concentrations by means of high-performance liquid chromatography with UV detection (HPLC-UV) or mass spectrometry (MS) and cell densities by means of flow cytometry. Our data demonstrate that substrate utilization kinetic parameters estimated from high-concentration experiments can be used to predict substrate utilization at low concentrations under AOC-restricted conditions. Further, restricting AOC in our experiments enabled accurate and direct measurement of microbial growth rates at environmentally relevant concentrations for the first time. These are critical measurements for evaluating the degradation potential of natural or engineered remediation systems. Our work provides novel insights into the kinetics of biodegradation processes and growth yields at low substrate concentrations.

The global use of pesticides to increase crop yields and control unwanted organisms has resulted in extensive pollution to water resources. This pollution can impair water quality (1, 2) and lead to significant economic effects when pollutant concentrations warrant closure or mandated remediation of a contaminated drinking water source (3, 4). Exploitation of microbial processes has long been considered an attractive option to remediate contaminated water resources (5). However, there are unique challenges inherent in developing remediation strategies for pesticide-contaminated waters. One major challenge is the occurrence of most pesticides at low (sub-microgram/liter) concentrations (6); the fundamentals of growth-linked biodegradation occurring at these low concentrations remain poorly understood.

Substrate utilization kinetics and microbial growth yields are two critically important biodegradation process parameters. Previous research has reported that substrate utilization kinetics can differ significantly at substrate concentrations below a certain threshold (on the order of 100 μg liter⁻¹) compared to high concentrations (7–10), a phenomenon attributed to the presence of at least two separate, concentration-dependent uptake and transformation systems (7, 10). The consequence of these multiphasic kinetics is that extrapolation of kinetic data measured at higher concentrations may significantly underestimate observed kinetics at low, environmentally relevant concentrations (10, 11). Comparatively few studies have investigated microbial growth and yield of bacterial strains at low substrate concentrations (10, 13). Several authors have reported threshold concentrations (typically less than 10 μg liter⁻¹) below which degradation continues in a nongrowth regime (9, 11, 14), but these conclusions are based solely on observed first-order substrate utilization kinetics; direct measurements of microbial growth or yield at these low concentrations have not been reported.

A major limitation in interpreting previous studies on biodegradation at low concentrations is the ubiquitous presence of contaminating assimilable organic carbon (AOC) in experimental systems. Even with extreme preparative measures, it is not possible to limit AOC concentrations in experimental systems to below 5 to 10 μg liter⁻¹ (13). Experience in our lab suggests that experiments conducted with mineral medium in glassware treated according to standard laboratory practices can contain up to 100-fold more AOC than experiments conducted with more rigorously treated glassware (15). In fact, it can be assumed that growth experiments that were conducted in glassware and mineral medium prepared according to standard laboratory procedures may have contained up to 1 mg liter⁻¹ of contaminating AOC as the extent of growth in negative controls could not be distinguished from growth in experiments conducted with up to 1 mg liter⁻¹ of substrate (12, 16, 17).
In effect, this implies that any biodegradation assay conducted at low substrate concentrations (below approximately 1 mg liter$^{-1}$) is a mixed-substrate assay, which will inevitably have profound effects on both substrate utilization kinetics (18–20) and yield measurements (12, 21). Therefore, experiments designed to quantify substrate utilization kinetics and microbial growth yields at low substrate concentrations should include appropriate measures to minimize the interfering effects of contaminating AOC.

The goal of this work was to measure substrate utilization kinetics and microbial growth yields of pesticide biodegradation under AOC-restricted conditions. With respect to substrate utilization kinetics, we hypothesized that previously reported multiphasic kinetics can also be explained as the result of a shift from single-substrate utilization to mixed-substrate utilization. Therefore, we surmised that minimizing the concentration of interfering AOC in our experimental system would help to resolve these kinetic issues. With respect to microbial growth yields, we expect that restricting the AOC in our experiments will allow us to accurately and directly measure yield at environmentally relevant concentrations for the first time. To meet this goal, we modified an existing method designed to measure trace concentrations of AOC in water samples and applied it to systems where pure bacterial strains were growing on pesticide substrates at concentrations between 0.01 and 50 mg liter$^{-1}$. We simultaneously measured substrate concentrations by means of high-performance liquid chromatography with UV detection (HPLC-UV) or mass spectrometry (MS) and cell densities by means of flow cytometry. The parameters were used to simulate substrate utilization at lower concentrations and were compared directly to measured data. Microbial growth yields were likewise calculated over a range of concentrations. Here, we present a novel methodology for investigating biodegradation at low concentrations, and the results provide novel insights into the kinetics of biodegradation processes and growth yields at low substrate concentrations.

**Materials and Methods**

**Chemicals.** All chemicals were reagent grade. Linuron (99.5% purity) was purchased from Ehrenstorfer GmbH (Augsburg, Germany). Carbofuran (99.9% purity) was purchased from Sigma-Aldrich (Seelze, Germany). Spike solutions were maintained in AOC-restricted mineral medium at concentrations of 50 mg liter$^{-1}$ for linuron and 100 mg liter$^{-1}$ for carbofuran. Chemical structures along with relevant physicochemical and thermodynamic properties are provided in Table 1.

**Strains.** The bacterial strains investigated included the linuron degrader *Variovorax* sp. strain SRS16 and the carbofuran degrader *Novosphingobium* sp. strain KN65.2. These strains were selected as candidates for engineered biological processes targeting two specific pesticide pollutants typically found at trace concentrations in groundwater, a primarily AOC-restricted habitat (22). *Variovorax* sp. strain SRS16 was received as a streak on R2A agar plates from S. Sørensen (Department of Geochemistry, Geological Survey of Denmark and Greenland). *Novosphingobium* sp. strain KN65.2 was received as a streak on an R2A agar plate from D. Springael (Department of Earth and Environmental Sciences, Katholieke Universiteit [KU] Leuven). For each strain, a single colony was picked from the agar plate and added to 5 ml of sterilized LB medium. Cells were grown to early stationary phase and diluted to approximately 15% glycerol (0.5 ml of 50% sterile glycerol added to 1 ml of cell culture) and stored at $-80^\circ$C.

**AOC-restricted mineral medium.** Experiments were conducted in a synthetic, AOC-restricted mineral medium containing 3.73 g liter$^{-1}$ KH$_2$PO$_4$, 2.24 g liter$^{-1}$ Na$_2$HPO$_4$·2H$_2$O, 120 mg liter$^{-1}$ (NH$_4$)$_2$SO$_4$, 70 mg liter$^{-1}$ MgSO$_4$, and 1 mg liter$^{-1}$ Ca(NO$_3$)$_2$ at a pH of 6.5. Trace elements were added to final concentrations of 100 μg liter$^{-1}$ H$_2$BO$_3$, 2.5 mg liter$^{-1}$ FeSO$_4$·7H$_2$O, 750 μg liter$^{-1}$ MnSO$_4$·H$_2$O, 1.3 mg liter$^{-1}$ ZnSO$_4$·7H$_2$O, 250 μg liter$^{-1}$ CuSO$_4$·5H$_2$O, 300 μg liter$^{-1}$ Co(NO$_3$)$_2$·6H$_2$O, 150 μg liter$^{-1}$ Ni(NO$_3$)$_2$·6H$_2$O, and 10 μg liter$^{-1}$ NiSO$_4$·6H$_2$O. The mineral medium recipe was modified from recipes previously described for cultivation of each strain on its target substrate (23; T. P. O. Nguyen, D. E. Helbling, K. Bers, T. T. Fida, R. Wattiez, H. P.-E. Kohler, R. De Mot, D. Springael, submitted for publication).

**Inoculum preparation.** A sterile loop was used to inoculate frozen cells maintained in 15% glycerol at $-80^\circ$C into 5 ml of sterilized 10-fold-diluted LB growth medium amended with approximately 10 mg liter$^{-1}$ of the target substrate. Cells were grown at 30°C to late exponential phase, at which point 1 ml of culture was sampled, centrifuged at 5,000 × g for 5 min, carefully decanted, and resuspended in an equal volume of AOC-restricted mineral medium. This washing procedure was repeated three times. The final cell density was determined by means of flow cytometry. Cells were subsequently inoculated at 10$^6$ cells ml$^{-1}$ into 20 ml of mineral medium amended with approximately 10 mg liter$^{-1}$ of the target substrate. Cells were cultivated until the chemical was completely degraded (monitored by HPLC-UV), at which point 1 ml of culture was sampled, centrifuged at 5,000 × g for 5 min, carefully decanted, and resuspended in an equal volume of AOC-restricted mineral medium. This washing procedure was again repeated three times to ensure complete removal of residual AOC. The final inoculum concentration was determined by means of flow cytometry.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Linuron</th>
<th>Carbofuran</th>
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<td>CAS no.</td>
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<td>1563-66-2</td>
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<td>Molecular formula</td>
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<td>29.48</td>
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<tr>
<td>Theoretical $Y_{GC}$</td>
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<td>0.41</td>
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</table>

$^a$ $K_{ow}$ is the octanol-water partition coefficient, estimated from KOWWIN, version 1.67 (46).

$^b$ Water solubility at 25°C from WSKOW, version 1.41 (46).

$^c$ Henry’s constant from HENRYWIN, version 3.10, from ChemAxon (Cambridge, MA).

$^d$ Gibbs energy of formation of each substrate estimated according to the group contribution approach (47, 48).

$^e$ Gibbs free energy of the electron donor half-reaction for each substrate.

$^f$ Theoretical yield ($Y_{GC}$) calculated as mol of C in cells per mol of C in substrate was estimated using the McCarty efficiency approach (32, 37–39).

$^g$ CAS, Chemical Abstract Services.

**TABLE 1 Chemical structures and relevant physicochemical and thermodynamic properties**
Incubation experiments. All experiments were designed based on methods previously established for measuring bacterial growth on trace concentrations of AOC (15). Briefly, experiments were conducted in 40-ml carbon-free borosilicate glass vials. All glassware and caps were treated rigorously to remove residual AOC as previously described (15, 24, 25). Microbial growth and substrate utilization were measured in 20 ml of AOC-restricted mineral medium amended with the target substrates. A series of three incubation experiments was designed to address three main objectives. First, high-concentration incubation experiments were conducted in triplicate at approximate initial conditions of 10^3 cells ml^-1 and 50 mg liter^-1 of the target substrate to confirm growth-linked substrate utilization for each strain-substrate pair. Second, low-concentration incubation experiments were conducted under a matrix of initial cell densities (10^0, 10^1, 10^2, and 10^3 cells ml^-1) and substrate concentrations (10, 0.1, 0.03, and 0.01 mg liter^-1) to systematically investigate the effects of stepwise changes in initial conditions on microbial growth and substrate utilization. Third, a series of incubation experiments was conducted at an initial cell density of 10^5 cells ml^-1 and a range of initial substrate concentrations from 1 to 50 mg liter^-1 to obtain an optimal data set for estimation of kinetic parameters. All reaction mixtures were incubated at 30°C until cells attained stationary phase or until the measured substrate concentration was below the limit of detection. Samples were taken at the time of inoculation and periodically thereafter to measure cell growth by means of flow cytometry and substrate concentration by means of HPLC-UV or HPLC-MS. As a positive control, 10^3 cells ml^-1 was inoculated into 5 ml of sterilized LB medium, and growth was confirmed by visual inspection of changes in optical density. As negative controls, incubation experiments were run in the absence of either cells or substrate.

Flow cytometry. Cell densities were measured on a BD Accuri C6 Flow Cytometer (Erembodegem, Belgium). Aliquots of 500 μl from an incubation experiment were combined with 5 μl of SYBR green stain ( Molecular Probes, Basel, Switzerland) diluted 100-fold in dimethyl sulfoxide (Fluka Chemie AG, Buchs, Switzerland) in a 1.5-ml plastic centrifuge tube (Greiner Bio One Frickenhausen, Germany), vortexed briefly, and incubated in the dark at 40°C for 10 min. For cell densities of less than 5 x 10^4 cells ml^-1, samples were measured directly on a BD Accuri C6 Flow Cytometer without dilution. In the case of higher cell densities, samples were appropriately diluted with 0.1-μm-pore-size-filtered bottled mineral water (Evian, France) to achieve a cell density in the range of 3 x 10^3 cells ml^-1 and 5 x 10^5 cells ml^-1. Data were analyzed with the CFlow, version 1.0.227.4, flow cytometry software. For all strains, enumeration was achieved with signals collected on the gated combined 533 nm/670 nm density plot (26).

Chemical analysis. Chemical analysis was conducted on either an HPLC-UV (0.3 to 50 mg liter^-1 experiments) or HPLC-MS (0.01 to 0.3 mg liter^-1 experiments) instrument. HPLC-UV analyses were performed on a Gynkotek system with a Dionex AS100 autosampler and a UVD 340U diode array detector. Compounds were separated on a Nucleosil 100-5 C18 HD column (250 by 4.0 mm) with an octadecyl modified high-density silica stationary phase (Macherey Nagel, Düren, Germany). The mobile phase consisted of nanopure water and HPLC-grade methanol (Acros Organics, Geel, Belgium), each amended with 0.1% (volume) formic acid (98 to 100%; Acros Organics, Geel, Belgium). Samples were injected into the column at 20 μl with an initial mobile phase of 90:10 water/methanol, and elution from the column was achieved with a final mobile phase of 5:95 water/methanol. The QExactive spectrometer was used with electrospray ionization in positive mode. XCalibur, version 2.0.7, software (Thermo, Waltham, MA) was used for chromatogram analysis and interpretation. Limits of quantification for each compound on HPLC-MS were less than 0.001 mg liter^-1.

Dry weight of cells. The dry weight of cells (X_DW) was measured by first collecting stationary-phase cells on an oven-dried 0.2-μm-pore-size Nucleopore polycarbonate track-etched membrane filter (Whatman, Piscataway, NJ, USA). The wetted filter was then dried at 105°C, and the X_DW given as the mass per cell was calculated as:

\[ X_{DW} = \frac{(m_f - m_i)}{V \times X} \]  

where \( m_f \) is the mass of the dry filter after filtration (mg), \( m_i \) is the mass of the dry filter before filtration (mg), \( V \) is the volume of stationary-phase cells filtered (ml), and \( X \) is the stationary-phase cell density determined by means of flow cytometry (cells ml^-1).

Estimation of kinetic parameters. Microbial growth and substrate utilization parameters were estimated from the experimental data by assuming Monod kinetics as given in equations 2 and 3:

\[ \frac{dX}{dt} = \frac{\mu_{max} S}{K_s + S} \]  

\[ \frac{dS}{dt} = -\frac{\mu_{max} S}{Y_{CC} \cdot K_s + S} \]  

where \( S \) is the concentration of carbon in the substrate (mM \( C_{substrate} \)), \( \mu_{max} \) is the maximum specific growth rate (day^-1), \( K_s \) is the concentration of carbon in the substrate giving one-half the maximum rate (mM \( C_{substrate} \)), \( X \) is the biomass concentration of carbon in cells (mM \( C_{cells} \)), and \( Y_{CC} \) is the mol yield in mol of carbon in cells per mol of carbon in the substrate (mol of \( C_{cells} \) × mol of \( C_{substrate} \))^-1. All concentrations were converted to millimolar carbon for consistency and to follow the previously established convention (30–32). Cell densities measured by means of flow cytometry in units of cells ml^-1 were converted to mM \( C_{substrate} \) by using the measured dry weight of cells (\( X_{DW} \)) and an assumed molecular weight of cells of 22.6 g \( X_{DW} \) C mol^-1 (32) and by assuming that the organic cell formulation CH_1.06O_0.92N_0.02 is 95% of the \( X_{DW} \) (33).

We used R, version 3.0.0 (34), and the flexible modeling environment (FME) package (35) to numerically and simultaneously solve the differential equations provided in equations 2 and 3 and to estimate the values of \( \mu_{max} \), \( K_s \) and \( Y_{CC} \) by minimizing the sum of the squared residuals between measured and modeled data. We then used a Markov chain Monte Carlo (MCMC) method that uses a delayed rejection and adaptive Metropolis procedure (36) to construct a Markov chain and sample from probability distributions over 50,000 iterations to generate a marginal distribution of parameter values to assess uncertainty and drift in the parameter estimates.

Estimation of theoretical and experimental molar yield. The theoretical molar yield for each substrate was estimated by using the efficiency approach of McCarthy (32, 37–39). We further adjusted theoretical molar yield predictions to account for putative oxygenase activation reactions through a previously described method (33). More information on the procedures to estimate theoretical molar yield along with an example calculation for carbofuran is provided in the supplemental material.

Experimental yield is reported as numeric yield (i.e., \( Y_{s/i} \) yield reported as cell numbers per mass of substrate) and as molar yield (i.e., \( Y_{CC} \) yield reported as moles of cell carbon per moles of substrate carbon). Numeric yield was estimated as:

\[ Y_{s/i} = \frac{(S_0 - S_f)}{(X_0 - X_f)} \]  

where \( Y_{s/i} \) is the numeric yield (cells μg substrate^-1), \( S_0 \) is the initial substrate concentration (μg ml^-1), \( S_f \) is the residual substrate concentra-
tion remaining when cells reach stationary phase (µg ml⁻¹), \( X_c \) is the cell density at stationary phase (cells ml⁻¹), and \( X_{ap} \) is the cell density at stationary phase in the no-substrate negative control (cells ml⁻¹). Numeric yield was converted to molar yield by using the dry weight of cells (X_{DW}) and an assumed molecular weight of cells of 22.6 g X_{DW} mol⁻¹ (32) and by assuming that the organic cell formulation CH₁₄O₀.₄N₀.₂ is 95% of the X_{DW} (33).

RESULTS AND DISCUSSION

**AOC concentrations in mineral medium.** The goal of this work was to measure substrate utilization kinetics and microbial growth yields at low substrate concentrations and under AOC-restricted conditions. We therefore first sought to determine the concentration of background AOC in our experimental system. To do this, we inoculated each strain into the mineral medium at initial cell densities of 10⁵ cells ml⁻¹. Cell densities measured after 7 days were 1.3 × 10⁸ and 6.6 × 10⁸ cells ml⁻¹, which corresponds to the nutritional equivalent of 13 and 6.6 µg liter⁻¹ of AOC for strains SRS16 and KN65.2, respectively, using standard yield conversion factors (15). Achieving these low concentrations of background AOC enabled quantification of substrate utilization kinetics and microbial growth yield on target substrates down to approximately 10 µg liter⁻¹; experiments conducted below this limit would be difficult to interpret due to the influence of background AOC.

**High-concentration incubation experiments.** The first set of incubation experiments were designed to confirm growth-linked substrate utilization for both strain-substrate pairs under AOC-restricted conditions; both of the strains selected for this work have previously been shown to mineralize a target pesticide over a wide range of concentrations (40; Nguyen et al., submitted), but so far only limited data on growth and yield have been reported (see the supplemental material for the discussion on each strain). The results of these experiments are presented in Fig. 1. High-concentration incubation experiments were conducted at initial conditions of approximately 50 µg liter⁻¹ of substrate and 10⁵ cells ml⁻¹. Under these conditions, strains SRS16 and KN65.2 grew as shown in Fig. 1a and b, respectively. Stationary phase was attained within 9 days, and cell densities exceeded 10⁷ cells ml⁻¹ in experiments with strain SRS16; stationary phase was attained within 4 days, and cell densities exceeded 10⁸ cells ml⁻¹ in experiments with strain KN65.2. No residual linuron or carbofuran concentrations were measured. In negative controls, there was no measurable growth in the AOC-restricted mineral medium in the absence of the pesticide substrate (growth on the background AOC was less than the initial cell density of these experiments) and pesticides did not disappear in the absence of cells (Fig. 1, dashed lines).

**Low-concentration incubation experiments.** In the second set of incubation experiments, we aimed to measure substrate utilization and microbial growth over a range of initial substrate concentrations and cell densities to elucidate the effects of stepwise changes in initial conditions on substrate utilization kinetics and microbial growth yields. We designed a matrix of 28 incubation experiments investigating seven initial substrate concentrations and four initial cell densities. We selected initial substrate concentrations between 0.01 and 10 mg liter⁻¹ to test for shifts in substrate utilization kinetics below previously reported thresholds for multiphasic kinetics on the order of 100 µg liter⁻¹ (7–10). We selected initial cell densities between 10³ to 10⁶ cells ml⁻¹ to likewise test for shifts in substrate utilization kinetics in potential growth (high ratio of substrate concentration to cell density) and nongrowth (low ratio of substrate concentration to cell density) regimes (9, 11, 14). A summary of substrate utilization observed in these incubation experiments is provided in Fig. 2, where the fraction of the initial substrate concentration remaining after incubation periods of 3 and 28 days is reported.

The effect of lowering initial substrate concentrations and cell densities on the extent and kinetics of substrate utilization varied considerably between the two strains. In experiments with strain SRS16, the extent of substrate utilization was unaffected by lower initial substrate concentrations and cell densities; we observed partial substrate utilization in all incubations after an incubation period of 3 days (Fig. 2a) and nearly complete substrate utilization after an incubation period of 28 days (Fig. 2b). In experiments with strain KN65.2, substrate utilization was apparently affected by both lower initial substrate concentrations and cell densities. We observed nearly no substrate utilization after an incubation period of 3 days (Fig. 2c) and a bipartite substrate utilization pattern after an incubation period of 28 days (Fig. 2d); complete substrate utilization was observed for most incubations with the...
exception of those at initial cell densities of $10^3$ and $10^4$ cells ml$^{-1}$ and initial substrate concentrations below 1 mg liter$^{-1}$ and 0.1 mg liter$^{-1}$, respectively, where we observed nearly no substrate utilization.

**Incubation experiments for kinetic parameter estimation.**

Estimating parameters to describe Monod growth and substrate utilization kinetics from experimental data is problematic due to the potential colinearity of $\mu_{\text{max}}$ and $K_s$ (41). Other investigators have demonstrated that optimal experimental conditions can be established to limit the colinearity of $\mu_{\text{max}}$ and $K_s$, thus rendering those parameters identifiable (42, 43). Optimal conditions are based on ratios between the initial cell and substrate concentrations and the magnitude of $\mu_{\text{max}}$ and $K_s$. Because we had no a priori knowledge of the magnitude of $\mu_{\text{max}}$ and $K_s$, we designed a series of independent incubation experiments to obtain an optimal data set for estimation of kinetic parameters. These experiments were conducted at an initial cell density of $10^5$ cells ml$^{-1}$ and at initial substrate concentrations between 1 and 50 mg liter$^{-1}$ to obtain substrate utilization and microbial growth data encompassing a wide range of ratios between initial cell densities and substrate concentrations. We then selected the experimental conditions that produced the lowest colinearity between $\mu_{\text{max}}$ and $K_s$ and highest identifiability of all parameters based on an analysis of Markov chain traces and pairs plots. The optimal data set for kinetic parameter estimation was identified at an initial cell density of $10^5$ cells ml$^{-1}$ and initial substrate concentrations of 1 mg liter$^{-1}$ and 10 mg liter$^{-1}$ in experiments with strains SRS16 and KN65.2, respectively (see supplemental material for details). The model fits to the experimental data for the optimal data sets are provided in Fig. 3. The estimated parameter values and standard deviations of the marginal distributions are provided in Table 2.

Noteworthy are the magnitude of the estimates for $\mu_{\text{max}}$ and $K_s$ for each strain. The $\mu_{\text{max}}$ estimate for strain SRS16 (1.3 day$^{-1}$) is six times lower than the estimate for strain KN65.2 (7.8 day$^{-1}$). However, the $K_s$ estimate for strain SRS16 (0.0029 mM C or 0.06 mg liter$^{-1}$) is 2 orders of magnitude lower than the estimate for strain KN65.2 (0.54 mM C or 10 mg liter$^{-1}$). Taken together, these parameters suggest that while strain KN65.2 should utilize carbofuran more rapidly than strain SRS16 utilizes linuron at high concentrations, strain SRS16 should utilize linuron more rapidly than strain KN65.2 utilizes carbofuran at low concentrations. These insights are derived from the kinetic parameters estimated from a single set of high-concentration experiments.

**Simulations of substrate utilization at low concentrations.**

We hypothesized that previously reported multiphasic kinetics can be the result of a shift from single-substrate utilization to...
mixed-substrate utilization. Therefore, we expected that restricting AOC in our experimental system would eliminate observed shifts in kinetics and enable extrapolation of kinetic parameters from high-concentration to low-concentration conditions. To test this hypothesis, we used the kinetic parameters reported in Table 2 to simulate substrate utilization under the matrix of initial conditions investigated in the low-concentration incubation experiments. We then compared the measured and simulated data for each set of initial conditions; fits of the raw data are presented in Fig. S4 and S5 of the supplemental material. A plot of the sum of the squared error (SSE) of the residuals measured between measured and simulated data are provided in Fig. 4 as a measure of the goodness of fit. All data are presented in Fig. S4 and S5 as the fraction of substrate remaining as a function of time so that the magnitude of the SSE can be compared across all experiments, regardless of initial substrate concentration.

Examination of Fig. S4 and S5 in the supplemental material reveals that the substrate utilization kinetics measured at high concentrations predict substrate utilization at lower concentrations under AOC-restricted conditions. Multiphasic kinetics were not observed. While our data cannot disprove the existence of multiple, concentration-dependent uptake and transformation systems in bacteria that lead to observed shifts in substrate kinetics at low concentrations, our data are consistent with our hypothesis that observed shifts in kinetics could be the result of shifts from single-substrate utilization to mixed-substrate utilization. Published data suggest that shifts to mixed-substrate utilization can likewise result in shifts in kinetics (18–20, 44).

**Estimation of theoretical and experimental molar yield.** We also expected that restricting AOC in our experiments will enable accurate and direct measurement of yield at environmentally relevant concentrations for the first time. We estimated numeric yields from the high- and low-concentration incubation experiments (when final cell densities were significantly greater than initial cell densities) according to equation 4. Restricting the AOC in our experimental system enabled yield estimates for strains SRS16 and KN65.2 at initial substrate concentrations as low as 0.1 and 0.03 mg liter⁻¹, respectively. This confirms that the bipartite behavior is solely a consequence of substrate utilization kinetics and can be predicted with parameters estimated from high-concentration experiments. In a few cases, measured carbofuran utilization was more rapid than predicted, resulting in rather high SSE values and indicating a relatively poor performance of the model under these few sets of initial conditions. In these cases, the poor performance of the model was at the boundary of the observed bipartite behavior (Fig. 4b).

**FIG 4** Sum of the squared error (SSE) for the fits of the kinetics model to the low-concentration incubation experiment data for *Variorax* sp. SRS16 with linuron (a) and *Novosphingobium* sp. KN65.2 with carbofuran (b).
Table 2 can be used to predict substrate utilization and strain growth under a variety of scenarios.

References


