Supporting information for

Rate-limiting mass transfer in micropollutant degradation revealed by isotope fractionation in chemostat

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SUMMARY

9 pages, 6 figures and one table

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Supporting Experimental Section

Media preparation

For the mineral salt media, MilliQ water with a low total organic carbon content of <10 µg/L was used. First 100X and 1000X solutions of the respective salts were prepared. 136 g KH_2PO_4 and 178 g $Na_2HPO_4 \cdot 2\ H_2O$ were dissolved in 1 L water and the pH was adjusted with NaOH to pH = 7.2 and autoclaved to give the 100X buffer solution. Furthermore 5 g $MgSO_4 \cdot 7\ H_2O$ and 1.32 g $CaCl_2 \cdot 2\ H_2O$ were dissolved in 1 L water and autoclaved to give the 100X mineral solution. To give the 1000X trace element solution, 2.86 g H_3BO_3, 1.54 g $MnSO_4 \cdot H_2O$, 0.039 g $CuSO_4 \cdot 5\ H_2O$, 0.021 g $ZnCl_2$, 0.041 g $CoCl_2 \cdot 6\ H_2O$, and 0.025 g $Na_2MoO_4 \cdot 2\ H_2O$ were dissolved in1 L water and autoclaved. Furthermore, a 1000X iron solution was prepared by dissolving 0.514 g $FeCl_3 \cdot 6\ H_2O$ in water and filter sterilization (0.22µm, PES). Empty 5 L bottles containing only a magnetic stir bar were autoclaved. Then, 15 mL of a solution of 10 g/L atrazine in ethyl acetate were added to each bottle and the ethyl acetate was evaporated under a sterile nitrogen stream to leave 150 mg solid atrazine. Afterwards, the solid atrazine was dissolved again in 5 L autoclaved water by stirring 48 h on a magnetic stirrer. This 30 mg/L atrazine solution was filter sterilized (0.22 µm, PES) to remove remaining atrazine particles. Finally, 50 mL 100X buffer solution, 50 mL 100X mineral solution, 5 mL 1000X trace element solution, and 5 mL 1000X iron solution were added.

Chemostat cultivation

The culture was stirred vigorously at 600 rpm with a magnetically coupled Rushton impeller. The dissolved oxygen content was monitored with a 235 mm DO sensor and kept constant at 50 % saturation by sparging with sterile air or nitrogen when necessary. The pH was monitored by a 235 mm pH Sensor. The pH stayed constant at 7.20 ± 0.01 during the whole cultivation time – no pH control by acid or base was necessary. A 200 mm Pt-100 temperature sensor was used to monitor the temperature inside the bioreactor. The bioreactor was kept at room temperature (25 °C). The culture volume was kept constant at 2000 mL with a level sensor.

Determining concentrations with HPLC-UV-DAD

Atrazine and 2-hydroxyatrazine concentrations in the bioreactor were measured using a Prominence HPLC system (Shimadzu Corp., Japan) together with a 100 x 4.6 mm Kinetex 5 µ Biphenyl 100 Å column equipped with a SecurityGuard ULTRA Biphenyl cartridge (both Phenomenex Inc., USA). The injected sample volume was 50 µL. Peak separation was achieved by 1 mL/min isocratic flow of 51 % 5 mM KH_2PO_4 buffer pH 7 and 49 % methanol for 9 min. The compounds were detected by UV absorbance at 222 nm and the peaks were quantified using LabSolutions V 5.71 SP2 (Shimadzu Corp., Japan). HPLC standards contained atrazine and 2-hydroxyatrazine (Riedel-de Haën, supplied by Sigma Aldrich, Germany) dissolved in 25 % methanol and 75 % water. Standard concentrations were 10.5, 21, 63, and 210 µg/L for atrazine and 7, 14, 42, and 140 µg/L for 2-hydroxyatrazine.

Cell counting by flow cytometry

To count the cells, they were first fixed with 2.5 % glutaraldehyde, then stained with SYBR Green I (total cells) and propidium iodide (dead cells). To compare measurements over the long cultivation period, reference beads (Trucount™ Absolute Counting Tubes, BD Bioscience, USA) were added to the samples in two technical replicates. The cells were counted on a Cytomics FC 500 flow cytometer (Beckmann Coulter, Germany) equipped with a 488 nm (40 mW) and a 638 nm (25 mW) laser. For detection following parameters were applied: SYBR Green I: discriminator FL1 (green fluorescence) /0, forward scatter 178 V/

gain 2.0, side scatter 624 V/ gain 2.0, FL1 397 V/ gain 1.0, and FL3 (red fluorescence) 572 V/ gain 1.0. Propidium iodide: discriminator FL3 /1, forward scatter 745 V/ gain 1.0, side scatter 693 V/ gain 2.0, FL1 350/ gain 1.0, FL2 (yellow fluorescence) 527 V/ gain 1.0, and FL3 517 V/ gain 1.0. The data was analyzed with CXP software (version 2.2; Beckmancoulter, Germany).

Microscopy

The fixed cells were analyzed on agar glass slides by light microscopy with an Axioscope 2 Plus microscope (Carl Zeiss AG, Germany). For imaging, pictures were taken with the digital camera AxioCam HRm (Carl Zeiss AG, Germany) and the software AxioVision (Version 4.8.2; Carl Zeiss AG, Germany).

Determination of carbon and nitrogen enrichment factors

The GC-IRMS system consisted of a TRACE GC Ultra gas chromatograph (GC; Thermo Fisher Scientific, Milan, Italy) linked to a Finnigan MAT 253 isotope ratio mass spectrometer (IRMS) (Thermo Fisher Scientific, Germany) by a Finnigan GC Combustion III Interface (Thermo Fisher Scientific, Germany). The emission energy was set to 1.5 mA for carbon isotope analysis and 2 mA for nitrogen isotope analysis. Helium (grade 5.0) was used as carrier gas and the injector was controlled by an Optic 3 device (ATAS GL, distributed by Axel Semrau, Germany). The samples were injected using a GC Pal autosampler (CTC, Switzerland) onto a 60-m DB-5 (60 m × 0.25 mm; 1 µm film; Restek GmbH, Germany) analytical column. An on-column liner (custom made by a glassblower) was pressed directly onto a CS-fused-silica-methyl-sil retention gap (3 m × 0.53 mm inner diameter) (Chromatographie Service GmbH, Germany).

Isotope values were determined as $\delta^{13}C$ and $\delta^{15}N$ values in per mill relatively to Vienna PeeDee Belemnite (V-PDB) (2), and Air-N_2 (3). The $\delta^{13}C$ and $\delta^{15}N$ values were assessed in relation to a monitoring gas (CO_2 and N_2, respectively) which was measured alongside each run at the beginning and the end. Calibration of monitoring gases was performed in a Finnigan MAT Delta S isotope ratio mass spectrometer with dual inlet system (Thermo Fisher Scientific, Germany). The gases were measured against V-PDB and air, respectively, by use of international reference materials: the CO_2 gases RM 8562, RM 8563, and RM 8564 for CO_2 and NSVEC (N_2 gas) for N_2. Reference standards were provided by the IAEA.

The GC oven started at 35 °C (hold 30 s), ramp 5 °C/min to 80 °C to ensure complete solvent evaporation during the transfer of the sample from the retention gap to analytical column. This was followed by a temperature ramp of 100 °C/min to 160 °C, a ramp of 10 °C/min to 220 °C, then a ramp 2 °C/min up to 250 °C. The initial injector temperature at the Optic 3 was set to 40 °C, 300 s hold, then ramped to 250 °C at 2 °C/s. The initial column flow was set to 0.3 mL/min (hold 120 s), then ramped to 1.4 mL/min within 120 s so that a flow of 1.4 mL/min was established before the GC temperature was raised.

Isotopic enrichment factors were determined by determining the difference of the isotope ratios between the inflow and the outflow: Alternating measurements of the inflow and the bioreactor multiple times allowed the determination of the difference without additional uncertainty of the instrument. The obtained values for the replicates at each residual atrazine concentration were compared by a two-sample t-test. For statistically indistinguishable replicates ($p > 0.1$) the individual values were combined and the average, standard error of mean, and the 95 % confidence intervals were calculated.

Growth in fed batch

Empty 200 mL erlenmayer flasks were autoclaved and 150 µL of a 10 g/L atrazine solution in ethyl acetate were added each. The ethyl acetate was evaporated under a sterile nitrogen stream to yield 1.5 mg solid atrazine in each flask. 75 mL of mineral salt media (see above) were added to the first flask and the

atrazine was dissolved by shaking for 24 h. A fresh culture of *Arthrobacter aurescens* TC1 was used to inoculate with a known amount of biomass. The degradation was followed by HPLC concentration measurements and cell counting by flow cytometry (see above). Upon atrazine consumption, the media was transferred to a new flask for two times. After both, atrazine and 2-hydroxyatrazine, had been consumed, the biomass was determined.

Significance of the maintenance term on model results

Here, we confirm that the effects due to maintenance were negligible on the obtained carbon isotopic signatures. Solutions of Eqs. (2a) to (2e) of companion paper

$$\frac{d[^{12}S]}{dt} = r_D([^{12}S_{in}] - [^{12}S]) - k_{tr}([^{12}S] - [^{12}S_{bio}]) \tag{2a}$$

$$\frac{d[^{13}S]}{dt} = r_D([^{13}S_{in}] - [^{13}S]) - k_{tr}([^{13}S] - [^{13}S_{bio}]) \tag{2b}$$

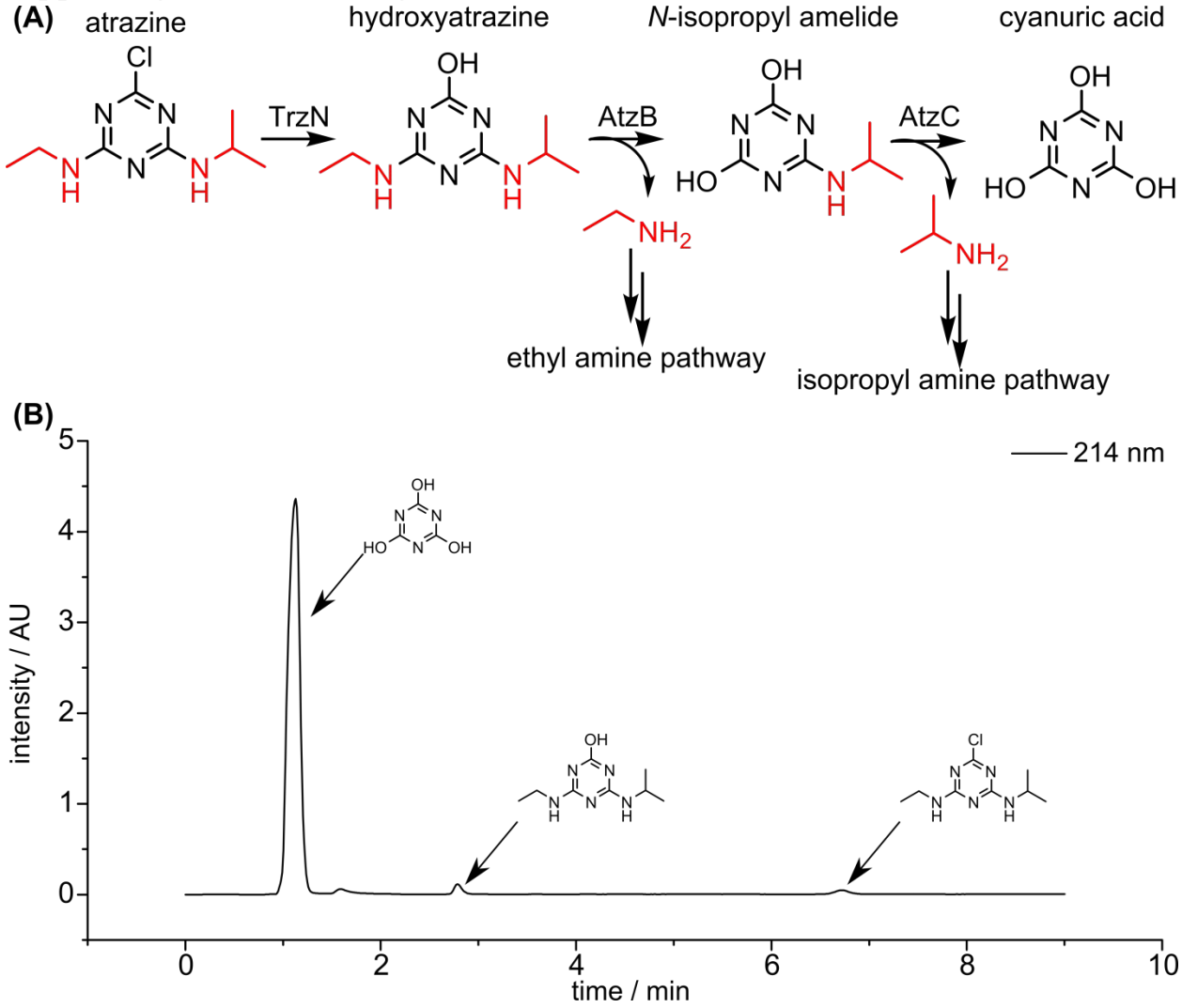
$$\frac{d[^{12}S_{bio}]}{dt} = +k_{tr}([^{12}S] - [^{12}S_{bio}]) - \frac{q_{max}[X][^{12}S_{bio}]}{[^{12}S_{bio}] + [^{13}S_{bio}] + K_m} \tag{2c}$$

$$\frac{d[^{13}S_{bio}]}{dt} = +k_{tr}([^{13}S] - [^{13}S_{bio}]) - \frac{\alpha q_{max}[X][^{13}S_{bio}]}{[^{12}S_{bio}] + [^{13}S_{bio}] + K_m} \tag{2d}$$

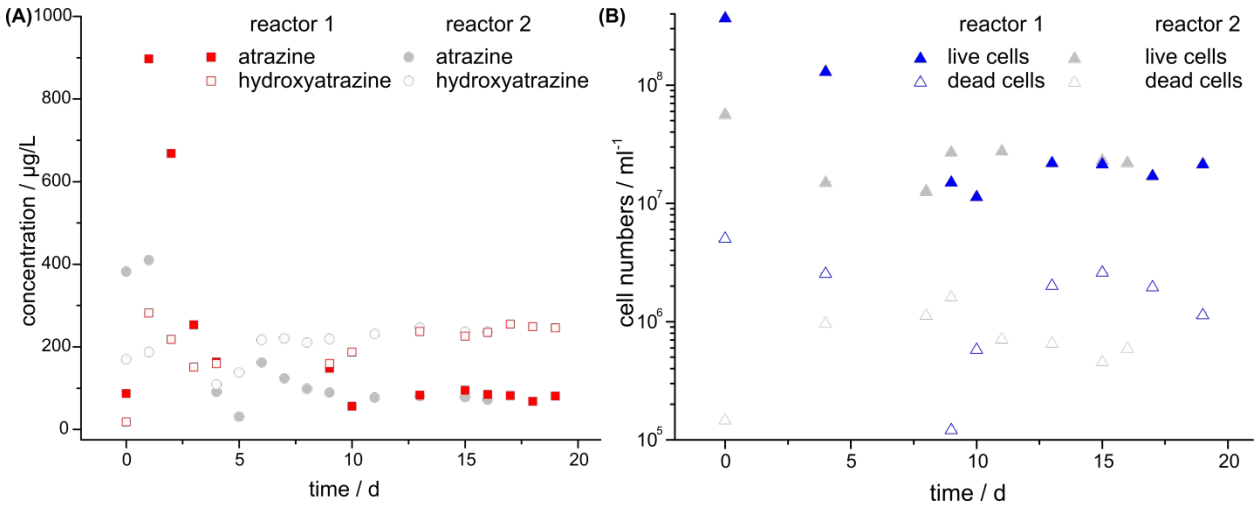
$$\frac{d[X]}{dt} = \frac{q_{max}[X]Y([^{12}S_{bio}] + \alpha[^{13}S_{bio}])}{[^{12}S_{bio}] + [^{13}S_{bio}] + K_m} - m[X]Y - r_D(1-f)[X] \tag{2e}$$

with the parameter values listed in TableSI2 led to similar results for concentrations and $\delta^{13}C$ values with and without the maintenance term (m) included. As shown in Figs. S7 and S8, changes were predominantly observed in biomass. The results indicate that the inclusion of the maintenance term does not affect the phenomenon that isotope fractionation is masked at low substrate concentrations and justify the simplification that maintenance energy is neglected in the companion modeling paper.

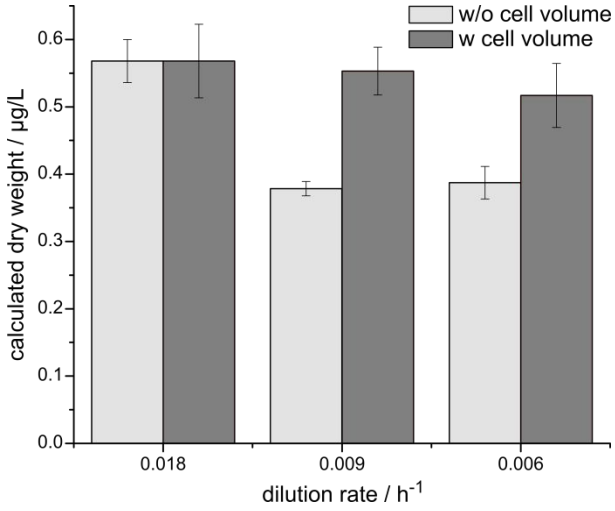
Supporting Information Figures



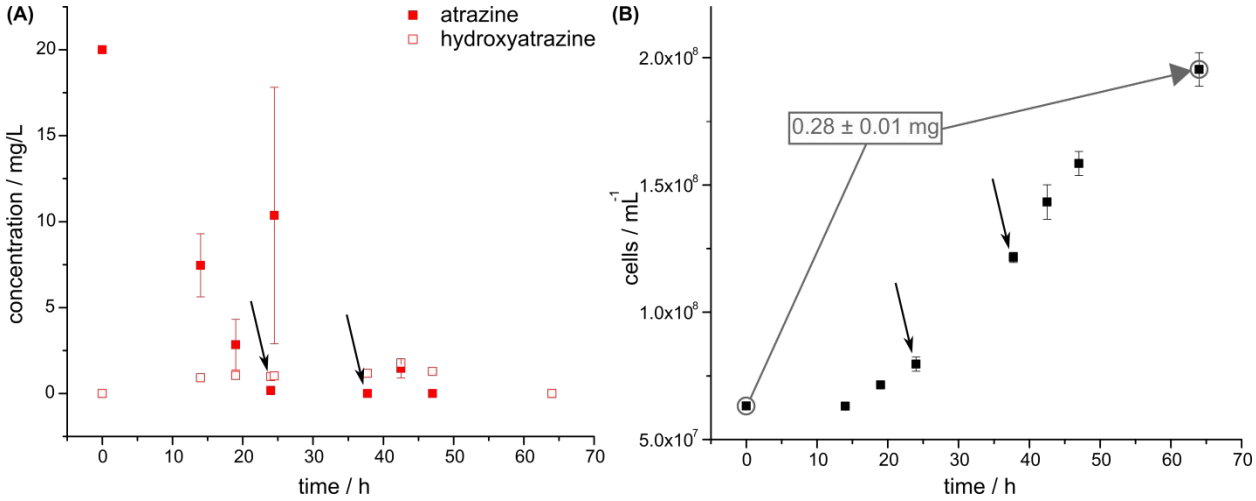
SI Figure S1. Atrazine metabolism of *Arthrobacter aurescens* TC1. (A) Atrazine is first hydrolyzed by TrzN to hydroxyatrazine. Then, ethyl amine is cleaved of by hydrolysis with AtzB and AtzC catalyses the hydrolysis to cyanuric acid and cleaves of isopropylamine. Both, ethyl amine and isopropyl amine, are further metabolized (1). Cyanuric acid is not further degraded and accumulates in the medium. **(B)** High pressure liquid chromatography (HPLC) chromatogram of bioreactor samples with detection of analytes by their UV absorbance at 222 nm. The atrazine of the inflow is almost quantitatively degraded to cyanuric acid (>99.5 % degradation). Hydroxyatrazine (retention time 2.7 min) and atrazine (retention time 6.8 min) are detected and can be quantified via external calibration.



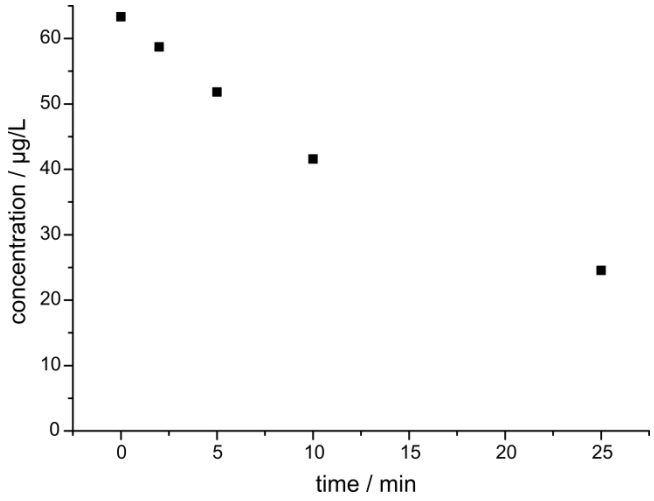
SI Figure S2. Growth of *Arthrobacter aurescens* TC1 in aerobic, atrazine limited chemostats during initial high dilution rate. (A) Residual concentrations of atrazine and the first metabolite 2-hydroxyatrazine. The reactors achieved steady state after 13 and 10 days, respectively, with approximately identical atrazine and hydroxyatrazine concentrations. Samples for isotope analysis were taken at the end of the cultivation (replicate 1: day 19; replicate 2: day 16) (B) cell numbers during cultivation in chemostats.



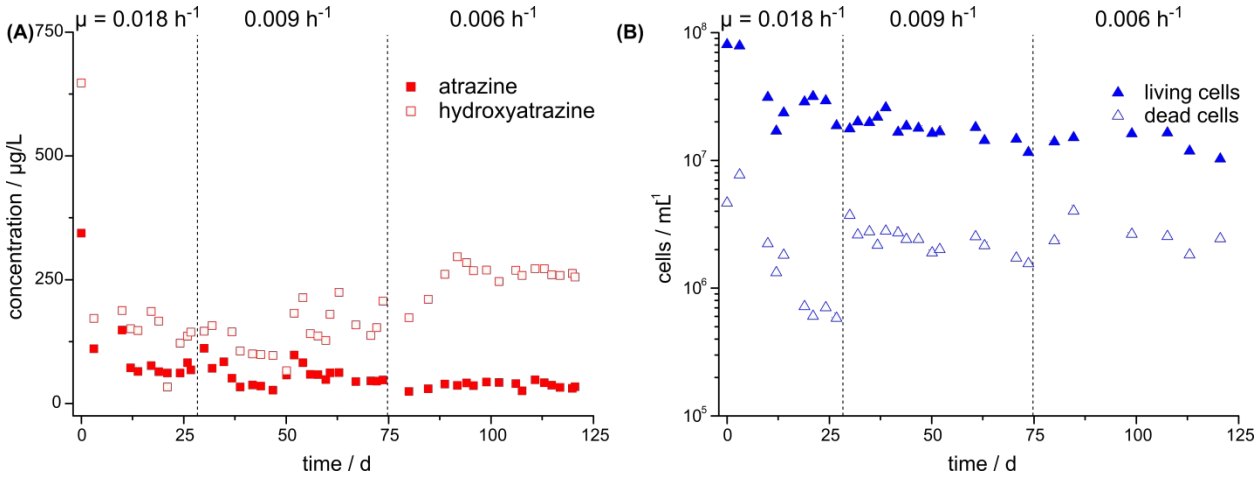
SI Figure S3. Calculated dry weight for three dilution rates (± SD): Since the biomass in the bioreactor was too low for a direct determination, the dry weight was instead calculated (i) by multiplying the cell number with the dry weight per cell reported by Strong *et al.* (1) and (ii) by correcting this number in addition with the observation that cell volumes increased at low dilution rates. According to (i) a decreased cell number at low dilution rates (μ_{low} and μ_{min}) would lead to a decreased biomass ("w/o cell volume"), whereas after consideration of the increased cell volume (ii) the biomass is constant for all dilution rates.



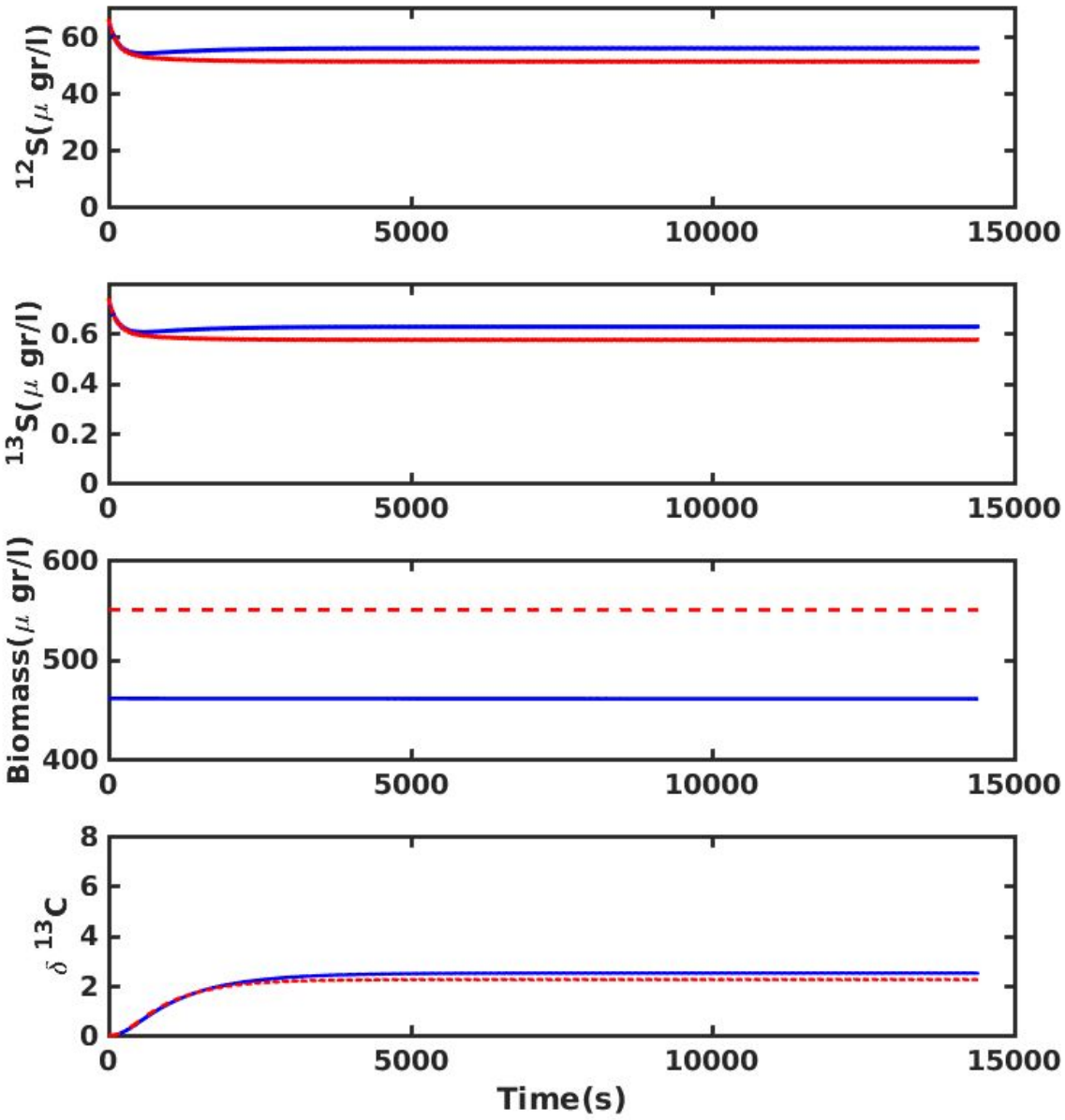
SI Figure S4. Growth of *Arthrobacter aurescens* TC1 on atrazine in fed batch. (± SD) (A) Residual concentration of atrazine and its first metabolite 2-hydroxyatrazine. The black arrows indicate time points at which the media was exchanged. The variability of the atrazine concentrations is quite high due to slightly different atrazine dissolution upon atrazine addition (black arrows). In contrast, the hydroxyatrazine concentration is similar in the replicates indicating similar degradation and metabolic activity and thus similar growth. This is also verified by the cell numbers (B). The dry weight was measured in the beginning and in the end of the cultivation. Degradation of 4.5 mg atrazine equals an uptake of 1.05 mg carbon leading to formation of 0.28 ± 0.01 mg biomass. This corresponds to a yield of Y = 0.27 ± 0.01 $g_{biomass}/g_{carbon}$.



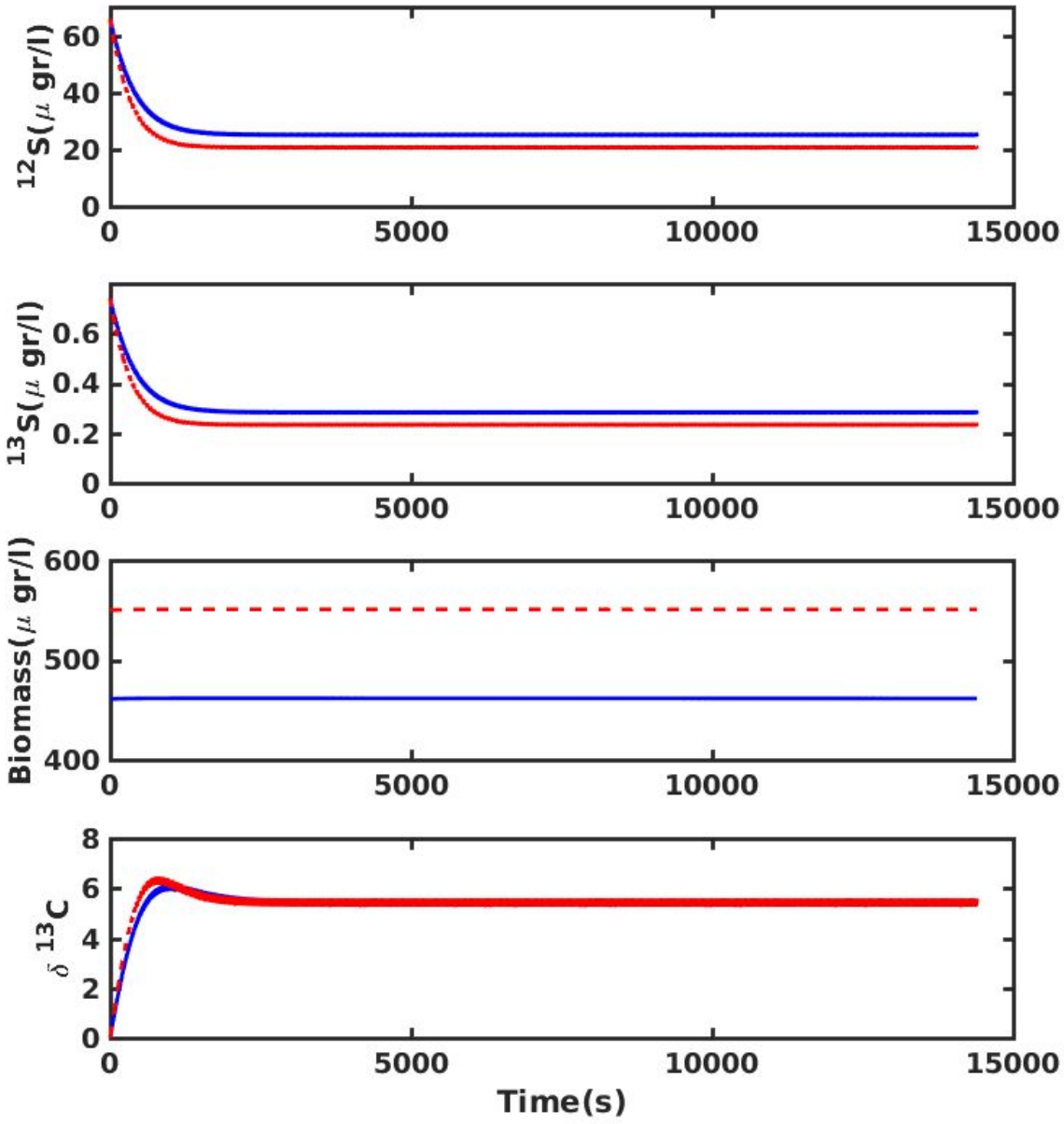
SI Figure S5. Degradation of atrazine in a sample taken from the bioreactor when not sterilized by filtration immediately. This degradation would lead to isotope fractionation and thus a change in the measured concentrations and isotope ratios of bioreactor samples in the time span before filtration. Since samples were filter-sterilized after at most 1 minute, the data indicates that such degradation-induced artefacts were always lower than 5 %.



SI Figure S6. Growth of *Arthrobacter aurescens* TC1 in aerobic, atrazine limited chemostats during stepwise decrease of the dilution rate - replicate 2. (A) Residual concentration of atrazine and the first metabolite 2-hydroxyatrazine. (B) Cell numbers in chemostats. Both, residual atrazine concentrations and cell numbers are identical to the values of the first biological replicate. In day 46 of the cultivation, we observed that the flow rate was decreased due to a pump failure and thus a dilution rate lower than the desired dilution rate of $\mu = 0.009\ h^{-1}$ was the consequence. Upon adjustment and calibration of the pump, it took two weeks to achieve steady state again.



SI Figure S7: Solution of Eqs. (2a) to (2e) for which the mass-transfer through the cell membrane is present with the parameter values in Table S2. The blue lines show the solution for the case where the maintenance term is considered while the red lines show the solution for the case where maintenance term was neglected. The comparison between these results shows that maintenance had a small effect on all the model estimates except for biomass.



SI Figure S8: Solution of model without mass-transfer limitation through the cell membrane with the parameter values in Table S2. The blue lines show the solution for the case where the maintenance term is considered while the red lines show the solution for the case where the maintenance term was neglected. The comparison between these results shows that maintenance had a small effect on all the model estimates except for biomass.

Supporting Information Table

SI Table S1. Concentration comparison of numerical model with and without a mass transfer term (k_{tr} = 0.0025 s^{-1})

	experimental	model with bioavailabilty term		model without bioavailabitlity term	
dilution rate / h^{-1}	c(atrazine) / µg/L	c(atrazine) / µg/L	offset / µg/L	c(atrazine) / µg/L	offset / µg/L
0.018	61.5	105.4	43.9	45.3	-16.2
0.009	44.5	50.7	6.2	20.6	-23.9
0.006	31.9	33.5	2.4	13.4	-18.9

The residual atrazine concentrations in the chemostats calculated by the numerical model for the different dilution rates are compared with experimental values. The model without mass transfer term overestimates the degradation. In contrast, by including a mass transfer term, the concentrations of the numerical model match the experimental values of the lower dilution rates.

SI Table S2: Model parameter values

Dilution rate (r_D)	0.009 hr^{-1}
Maximum specific conversion rate (q_{max})	6.01 hr-1
Half-saturation constant (K_m)	0.237 mg/l
Yield factor (Y)	0.018
Isotopic fractionation factor (α)	0.09946
Fraction of biomass retained from chemostat (f)	0
Maintenance term (m)	0.1 hr^{-1}
Reactor volume (V)	2000 ml
Mass-transfer limiting coefficient (k_{tr})	0.0025 s^{-1}

Supporting Information References:

1. Strong LC, Rosendahl C, Johnson G, Sadowsky MJ, & Wackett LP (2002) *Arthrobacter aurescens* TC1 metabolizes diverse s-triazine ring compounds. *Appl. Environ. Microbiol.* 68(12):5973-5980.
2. Coplen TB (2011) Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Communications in Mass Spectrometry* 25(17):2538-2560.
3. Coplen TB, Krouse HR, & Boehlke JK (1992) Reporting of nitrogen-isotope abundances. *Pure & Applied Chemistry* 64:907-908.