Electronic Supplementary Material (ESI) for Environmental Science: Processes & Impacts.

Supporting Information

Compound-Specific Chlorine Isotope Fractionation in Biodegradation of Atrazine

Christina Lihl[a,b], Benjamin Heckel[a,b], Anna Grzybkowska[c], Agnieszka Dybala-Defratyka[c], Violaine Ponsin[d,e], Clara Torrentó[d,f], Daniel Hunkeler[d], Martin Elsner[a,b,*]

[a] Institute of Groundwater Ecology, Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

[b] Chair of Analytical Chemistry and Water Chemistry, Technical University of Munich, Marchioninistraße 17, 81377 Munich, Germany

[c] Institute of Applied Radiation Chemistry, Faculty of Chemistry, Lodz University of Technology, Zeromskiego 116, 90-924 Lodz, Poland

[d] Centre of Hydrogeology and Geothermics (CHYN), University of Neuchâtel, 2000 Neuchâtel, Switzerland

[e] Département des sciences de la Terre et de l'atmosphère, Université du Québec à Montréal, 201 avenue du Président Kennedy, Montréal, QC, Canada

[f] Grup MAiMA, Departament de Mineralogia, Petrologia i Geologia Aplicada, Facultat de Ciències de la Terra, Universitat de Barcelona (UB), C/ Martí i Franquès s/n, 08028, Barcelona, Spain.

* Corresponding Author: Phone: +49 89/2180-78231. E-mail: m.elsner@tum.de

Summary: 11 pages, 3 tables, 5 figures

Gradient programs for HPLC analysis

***A. aurescens* TC1: Separation of atrazine and 2-hydroxyatrazine.** The compounds were separated by using a gradient elution at a flow rate of 1.0 mL/min. The initial conditions (20 % acetonitrile, 80 % buffer of 2 mM K_3PO_4, pH 7.0) were immediately followed by a linear gradient to 65 % acetonitrile within 9 min. These conditions were maintained isocratic for 2 min. A subsequent gradient led back to the initial conditions of 20 % acetonitrile within 1 min, which was maintained for 5 min.

***Rhodococcus* sp. NI86/21: Separation of atrazine, desethylatrazine and desisopropylatrazine.** The compounds were separated by using a gradient elution at a flow rate of 0.8 mL/min. The initial conditions (5 % acetonitrile, 95 % buffer of 2 mM K_3PO_4, pH 7.0) were maintained isocratic for 2 min. Afterwards a linear gradient led to 55 % acetonitrile within 12 min followed by another linear gradient which led to 75 % acetonitrile within 2 min. These conditions were maintained isocratic for 2 min. A subsequent gradient led back to the initial conditions of 5 % acetonitrile within 2 min, which was maintained for 5 min.

Chlorine isotope analysis via GC-qMS according to Ponsin et al.[1] – Method Optimization

For method optimization standards in the range of 1 - 200 mg/L were measured ten times each at three different dwell times (30/60/100 ms) for defining the linearity range and the uncertainty of the method. Furthermore, a long-term stability test over 50 days was conducted.

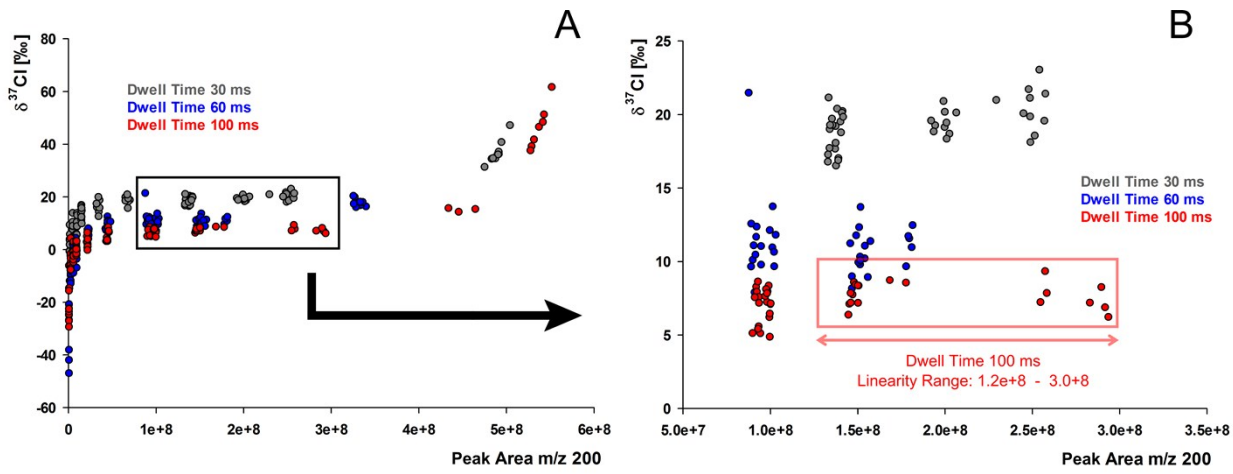

Figure S1. Analysis of different dwell times (30 ms grey, 60 ms blue, 100 ms red) and concentrations (corresponding to different peak areas) in order to define the optimal dwell time and the linearity range of analysis.

Table S1. Ten-fold standard injection at dwell time 100 ms at different concentrations and resulting standard deviations. Grey shaded lines are located inside the linearity range.

Concentration	Standard Deviation s
1 mg/L	7.2 ‰
5 mg/L	4.4 ‰
7 mg/L	5.8 ‰
10 mg/L	6.8 ‰
20 mg/L	2.0 ‰
30 mg/L	2.0 ‰
40 mg/L	1.4 ‰
50 mg/L	1.1 ‰
75 mg/L	0.7 ‰
100 mg/L	1.0 ‰
200 mg/L	16.7 ‰

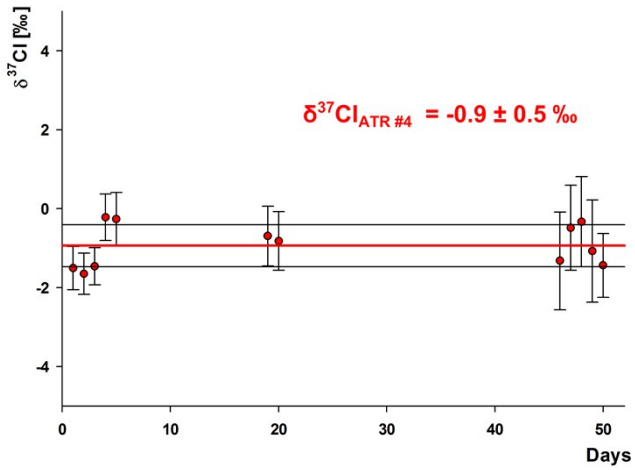

Figure S2. Analysis of the atrazine standard Atr #4 over a period of 50 days. Red dots represent the mean of a ten-fold measurement while error bars illustrate the standard deviations. The mean over all measurements is given as value and as red line, while the standard deviation is given as value and as black line.

As illustrated in Figure S1, a dwell time of 100 ms was chosen as method parameter for analysis and the linearity range for analysis was defined as the peak area (m/z 200) of 1.2 – 3.0 x 10^8. Inside the linearity range, the precision of the method is associated with a maximal uncertainty of ± 1.1 ‰ (see Table S1). The final concentration of standards and samples for analysis should be approx. 75 mg/L, which corresponds to a peak area (m/z 200) of approx. 1.7 x 10^8. The long-term stability test (see Figure S2) resulted in chlorine values of standard injections (Atr #4, 75 mg/L, dwell time 100 ms) which showed no significant differences over the tested period of 50 days.

Comparison of the GC-qMS methods for chlorine analysis of this study and Ponsin et al.[1]

Both the GC-qMS method optimized here and that shown by Ponsin et al.[1] can be used to measure chlorine isotope values of atrazine. The main difference between the two methods is the the amount of atrazine injected on the analytical column (and the corresponding optimum concentration): this study: 150 ng on column (75 mg/L), Ponsin et al.[1]: 10 ng on column (5 mg/L). For 5 mg/L (corresponding to 10 ng on column in our method), we observed a strong dependency between peak area (concentration) and chlorine isotope values. Additionally, very large variations leading to large standard deviations (> 4 ‰) were observed (see Table S1). However, 5 mg/L was outside of our defined linearity range. The linearity range was defined at higher concentrations, between 50 and 100 mg/L (see Table S1), and thus samples were only measured within this range. Ponsin et al.[1] measured their samples at lower concentrations, but due to their requirement that standards and samples had to have the same concentration (20 % tolerance), chlorine isotope values could be corrected, leading to accurate results. An advantage of the method of Ponsin et al.[1] is that lower concentrations can be measured. However, regarding the precision our method seems to be more optimized. We observed a maximal standard deviation s of ± 1.1 ‰ (for an atrazine concentration of 50 mg/L) while Ponsin et al.[1] reported a standard error σ_s of ± 1 ‰ (n = 10) corresponding to a standard deviation of approx. ± 3.2 ‰, for the atrazine concentration range between 10 and 30 mg/L.

Table S2. Comparison of the method parameters of this study and the study of Ponsin et al.[1].

	Ponsin et al.[1]	This study
Injectionvolume/-temperature	1 µL / 250 °C	2 µL / 220 °C
Analytical Column	DB-17 MS	DB-5 MS
GC Temperature Program	60 °C (1 min), 30 °C/min to 190 °C (3 min), 3 °C/min to 210 °C (3 min)	65 °C (1 min), 20 °C/min to 180 °C (10 min), 15 °C/min to 230 °C (8 min)
Column Flow	1.2 mL/min	1.4 mL/min
Split Flow	splitless	1 min splitless, then split mode (split ratio 1:10)
Temperature MS Quad/MS Source	150 °C / 230 °C	150 °C / 230 °C
Dwell Time	30 ms	100 ms
Concentration Optimum → Amount on Analytical Column	10 ppm → 10 ng	75 ppm → 150 ng
Amount Dependency	Measurement of standards with similar concentration as samples, 20 % tolerance between sample and standard concentration	Defining linearity range, where no amount dependency is observed (peak area between $1.2x10^8$ and $3.0x10^8$)

Consideration of interfering fragments

The constructive-critical comments of a reviewer pointed out that H-transfer reactions can occur from one fragment to the other in the ions source during GC-qMS analysis. It was questioned whether this could bias chlorine isotope measurements. Specifically, (I) ions that do *not* contain ^{37}Cl may contribute to m/z 202; (II) ions may turn up as m/z 200 even though they *do* contain ^{37}Cl.

Case (I). In combination with the substitutions of ^{13}C and ^{15}N, H-transfer can lead to the formation of fragments with m/z 202 ($[^1H_{12}{}^{12}C_6{}^{13}C^{14}N_5{}^{35}Cl]^+$, $[^1H_{12}{}^{12}C_7{}^{14}N_4{}^{15}N^{35}Cl]^+$) which may add up to the "correct" peak of ($[^1H_{11}{}^{12}C_7{}^{14}N_5{}^{37}Cl]^+$ and, therefore, interfere with chlorine CSIA. The probability of occurrence of these fragments was calculated by using the ratio of the peaks of m/z 200 ($[^1H_{11}{}^{12}C_7{}^{14}N_5{}^{35}Cl]^+$) and m/z 201 ($[^1H_{11}{}^{12}C_6{}^{13}C^{14}N_5{}^{35}Cl]^+$, $[^1H_{11}{}^{12}C_7{}^{14}N_4{}^{15}N^{35}Cl]^+$, $[^1H_{12}{}^{12}C_7{}^{14}N_5{}^{35}Cl]^+$) in the atrazine mass spectrum (see Figure S3).

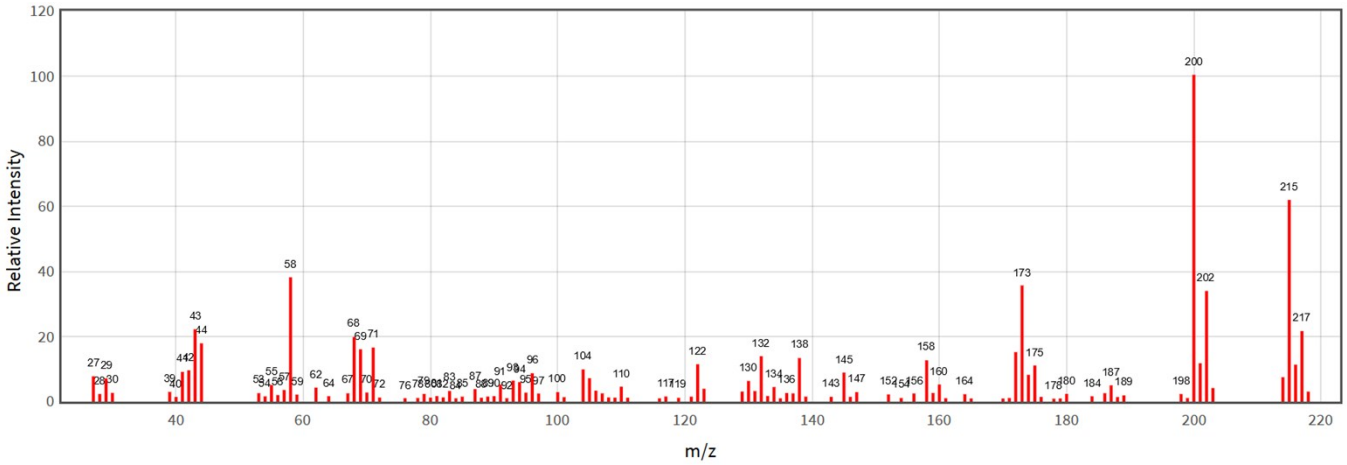

Figure S3. Mass spectrum of atrazine (taken from NIST 2020)[2].

By using the relative intensities shown in Figure S3, it can be observed that the probability for a fragment with m/z 201 (that is, the mass of a fragment that has received a hydrogen atom) is approx. 11.8 %. However, ions at this mass may also represent "true" ^{13}C and ^{15}N isotopologues of mass m/z 201. Taking into account the natural abundance of ^{13}C and ^{15}N, as well as the number of C and N atoms in the molecule, it becomes clear that, indeed, most of this abundance is attributable to "true" ^{13}C and ^{15}N isotopologues $[^{1}H_{11}{}^{12}C_{6}{}^{13}C^{14}N_{5}{}^{35}Cl]^{+}$ = 7.7%, $[^{1}H_{11}{}^{12}C_{7}{}^{14}N_{4}{}^{15}N^{35}Cl]^{+}$ = 1.8%, and only a minor fraction is attributable to artefacts from hydrogen transfer: $[^{1}H_{12}{}^{12}C_{7}{}^{14}N_{5}{}^{35}Cl]^{+}$ = 2.3% (see Table S3).

Table S3. Probability of occurrence of fragments with m/z 201.

Fragment with m/z 201	**Probability of occurrence***
$[^{1}H_{11}{}^{12}C_{6}{}^{13}C^{14}N_{5}{}^{35}Cl]^{+}$	□ 7.7 %
$[^{1}H_{11}{}^{12}C_{7}{}^{14}N_{4}{}^{15}N^{35}Cl]^{+}$	□ 1.8 %
$[^{1}H_{12}{}^{12}C_{7}{}^{14}N_{5}{}^{35}Cl]^{+}$	11.8 % - 7.7 % - 1.8 % ≈ 2.3 %

*based on natural abundance for each stable isotope considered (^{13}C: 0.011056, ^{15}N: 0.00366)[3]

Also for the mass m/z 202 one can, hence, assume that the probability of a hydrogen transfer from mass 201 is 2.3 %. Hence, this probability still needs to be multiplied with the probability that a ^{13}C and ^{15}N is present in the molecule. This gives the probability of occurrence of the

interfering fragments with m/z 202: $[^{1}H_{12}{}^{12}C_{6}{}^{13}C^{14}N_{5}{}^{35}Cl]^{+}$ = (7.7 % × 2.3 %) = 1.8 ‰, and $[^{1}H_{12}{}^{12}C_{7}{}^{14}N_{4}{}^{15}N^{35}Cl]^{+}$ = (1.8 % × 2.3 %) = 0.4 ‰. Hence, 1.8 ‰ of all ions of m/z 202 are $[^{1}H_{12}{}^{12}C_{6}{}^{13}C^{14}N_{5}{}^{35}Cl]^{+}$ instead of true ^{37}Cl isotopologues, and 0.4 ‰ of all ions of m/z 202 are $[^{1}H_{12}{}^{12}C_{7}{}^{14}N_{4}{}^{15}N^{35}Cl]^{+}$ instead of true ^{37}Cl isotopologues.

These numbers already show that the effect is very small. However, much of this effect can actually be corrected by the identical treatment of standard and sample. The exception is if the standard has a different $\delta^{13}C$ compared to the sample. To estimate the artifact introduced by this difference, one can assume that $\delta^{13}C$ of atrazine would vary by about 20 ‰ when biodegradation occurs. Hence, the artifact of protonated ^{13}C isotopologues *that cannot be corrected by the identical treatment of standard and sample* would be $\Delta[^{1}H_{12}{}^{12}C_{6}{}^{13}C^{14}N_{5}{}^{35}Cl]^{+}$ = 20 ‰ × 1.8 ‰ = 0.036‰. In a next step we therefore need to calculate how much such a variability in Δ[m/z 202] would influence the $\delta^{37}Cl$ measurement.

The natural abundance ratio of $^{37}Cl/^{35}Cl$ and, therefore, of the peaks m/z 202 and m/z 200, is about 0.33 (see Figure S3). Hence, a shift of $\Delta\delta^{37}Cl$ = 1 ‰ would correspond to a change in the relative peak abundance of m/z 202 to 200 of about 0.33 × 1 ‰ = 0.33 ‰. In comparison, the contribution of the variability introduced by the "artefact" peak $\Delta[^{1}H_{12}{}^{12}C_{6}{}^{13}C^{14}N_{5}{}^{35}Cl]^{+}$ to the variability in Δ[m/z 202] (0.036 ‰) is a factor of ten smaller. This artefact, therefore, is expected to affect the measured $\delta^{37}Cl$ values by 0.1 ‰ at most. Therefore, within the precision of our methods, the influence of H-abstraction during chlorine CSIA is negligible.

Case (2). In a similar way, H-atoms can also be cleaved off during GC-qMS analysis. Therefore, a molecular isotopologue of atrazine with m/z 202 ($[^{1}H_{11}{}^{12}C_{7}{}^{14}N_{5}{}^{37}Cl]^{+}$) could transform to m/z 200 ($[^{1}H_{9}{}^{12}C_{7}{}^{14}N_{5}{}^{37}Cl]^{+}$) which could interfere with chlorine CSIA. Since the transformation rate of m/z 202 to m/z 200 corresponds to the transformation rate of m/z 200 to m/z 198, this can be easily investigated by analyzing the peak of m/z 198 in the mass spectrum of atrazine (see Figure S3). Since the relative abundance of the peak of mass m/z 198 is very low (< 2%), it can be concluded that the loss of H-atoms is negligible and thus it does not interfere with chlorine CSIA.

Concentration analysis of atrazine and its metabolites

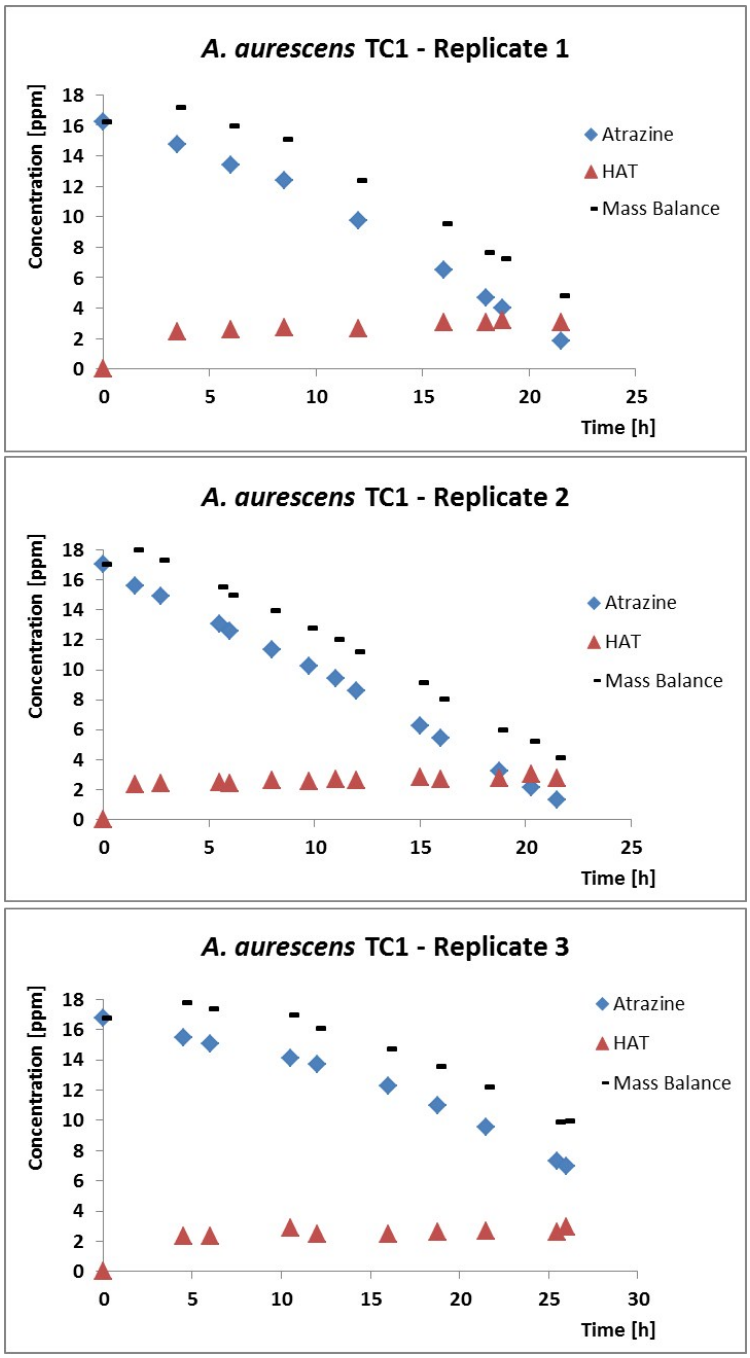

Figure S4. Degradation of atrazine to 2-hydroxyatrazine (HAT) with *A. aurescens* TC1. The mass balance is not closed due to further degradation of HAT[4].

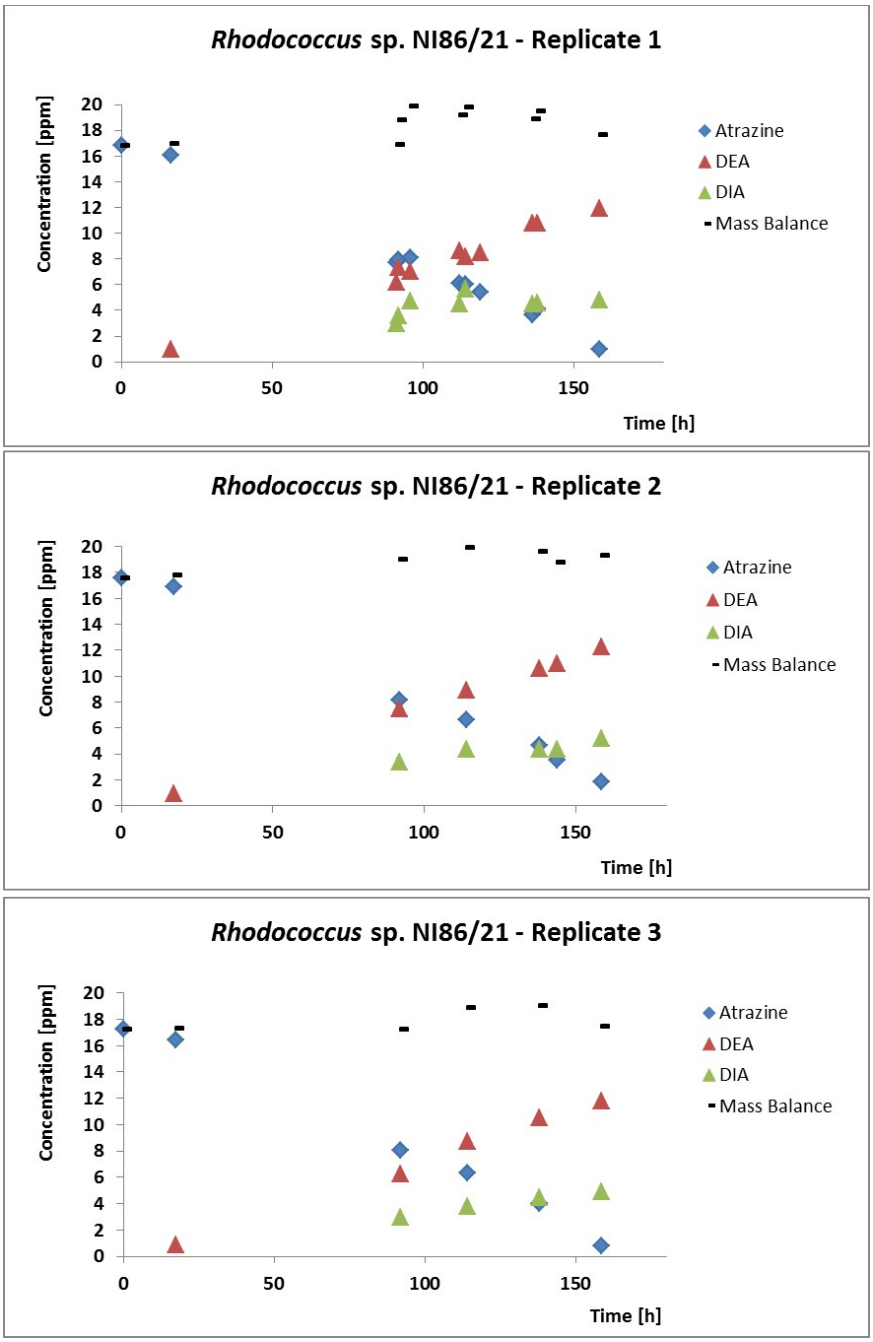

Figure S5. Degradation of atrazine to desethylatrazine (DEA) and desisopropylatrazine (DIA) with *Rhodococcus* sp. NI86/21.

References

(1) Ponsin, V.; Torrentó, C.; Lihl, C.; Elsner, M.; Hunkeler, D. Compound-specific chlorine isotope analysis of the herbicides atrazine, acetochlor and metolachlor. *Anal. Chem.* **2019,** *91*, (22), 14290-14298.

(2) NIST 2020. NIST Chemistry WebBook. www.webbook.nist.gov/chemistry/ (15.01.2020)

(3) Coplen, T. B.; Böhlke, J. K.; De Bievre, P.; Ding, T.; Holden, N. E.; Hopple, J. A.; Krouse, H. R.; Lamberty, A.; Peiser, H. S.; Revesz, K.; Rieder, S. E.; Rosman, K. J. R.; Roth, E.; Taylor, P. D. P.; Vocke, R. D.; Xiao, Y. K. Isotope-abundance variations of selected elements - (IUPAC Technical Report). *Pure Appl. Chem.* **2002,** *74*, (10), 1987-2017.

(4) Meyer, A. H.; Penning, H.; Elsner, M. C and N isotope fractionation suggests similar mechanisms of microbial atrazine transformation despite involvement of different Enzymes (AtzA and TrzN). *Environ. Sci. Technol.* **2009,** *43*, (21), 8079-8085.