# *SUPPLEMENTARY INFORMATION*

## Analysis of carbon and hydrogen stable isotope ratios of phenolic compounds: method development and biodegradation application

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## 1. MATERIALS

The following chemicals were used (all of analytical grade): o- and p-cresol, phenol (Merck, for synthesis; ≥ 99%), benzene (Merck, ≥ 99.7 %), dichloromethane (Merck, for spectroscopy, ≥ 99.8%), methanol (Merck, for spectroscopy, ≥ 99.9 %), hydrochloric acid (Merck, 6 N and 0.05 N), sodium hydroxide (Merck, ≥ 97 %), and trifluoroacetic anhydride (Merck, for derivatization, ≥ 99 %). Phenolic compound stock solutions were prepared using milli-Q ultrapure water, dichloromethane or benzene.

The following international stable isotope reference materials were used for calibration and normalization of raw isotope data (Reston Stable Isotope Laboratory, U.S. Geological Survey, Reston, VA, USA):

- Caffeine (USGS63; δ2HVSMOW-SLAP= +174.5±0.9 ‰; δ13CVPDB-LSVEC = -1.17±0.04 ‰);
- *n*-hexadecane (USGS67; δ2HVSMOW-SLAP= -166.2±1.0 ‰; δ13CVPDB-LSVEC = -34.50±0.05 ‰);

24 • Icosanoic acid methyl ester (USGS71; δ2HVSMOW-SLAP= -4.9±1.0 ‰; δ13CVPDB-
25 LSVEC = -10.50±0.03 ‰).

26 ## 2. METHOD DESCRIPTION

27 **SPE extraction procedure**

28 Firstly, cartridges were conditioned by rinsing three times with 3 mL DCM, then three times with
29 3 mL methanol and four times with 3 mL 0.05 N hydrochloric acid. Secondly, the milli-Q ultrapure
30 water containing phenol or cresol isomers was pumped through the SPE cartridges with an
31 approximate flow rate of 20 mL/min. Afterwards, the cartridge packing was dried by flushing with
32 nitrogen for 30 min. Finally, the analytes were desorbed from the cartridge polymer by flushing
33 with 10 ml DCM. The extracts were dried with sodium sulfate, concentrated to 1 mL in a gentle
34 stream of nitrogen at 40°C, transferred to 2 ml screw cap glass vials and were finally stored at 4°C
35 in the fridge until analysis.

36 **Quantification of phenolic compounds**

37 Concentrations of phenolic compounds were determined by GC-MS analysis. A Zebron ZB-1
38 capillary column (60 m length x 0.32 mm ID, 1 µm film thickness; Phenomenex, Germany) was
39 used for separation. One µL extract was injected by an auto sampler (Combi PAL, CTC Analytics
40 GmbH, Switzerland) in 1:5 or 1:10 split ratio modes. The injector temperature was adjusted to 250
41 °C and helium was used as a carrier gas at a constant rate of 1 mL/min. Different temperature
42 programs were used for dichloromethane (DCM) and benzene extracts. Benzene extracts
43 (derivatized and non-derivatized) were analyzed using an oven temperature program starting from
44 45 °C for 5 min, followed by a temperature increase at a rate of 10 °C/min to 250 °C. DCM extracts
45 were analyzed using an oven temperature program starting from 60 °C for 5 min, followed by a
46 temperature increase at a rate of 15 °C/min to 250 °C. Analyzes were performed in full scan mode
47 with m/z range from 50 to 500. Standard solutions of analytes dissolved in DCM or benzene were

48 used for calibration at seven concentrations, each concentration was analyzed by injecting three
49 technical replicates. Water samples after SPE or liquid-liquid extractions (milli-Q ultrapure water
50 or culture media) were analyzed following the principle of identical treatment. Standard deviations
51 of the GC-MS measurements were calculated from the three data points of each sample and did
52 not exceed 10%.

53 **Origin of microbial strains and cultivation procedures**

54 *Pseudomonas pseudoalcaligenes* was purchased from the NCIMB culture collection (NCIMB Ltd,
55 Scotland). Cells were cultivated in Brunner mineral salt medium as described elsewhere[1] with *p*-
56 cresol as sole substrate (see below). *Aromatoleum aromaticum* strain EbN1 (DSM 19032) was
57 taken from the strain collection of the Helmholtz Centre for Environmental Research. Originally,
58 the strain was kindly provided by Prof. Dr. Johann Heider (University of Marburg, Germany). *A.*
59 *aromaticum* was cultivated under nitrate-reducing conditions in a mineral salt medium described
60 elsewhere[11] with *p*-cresol as sole substrate (see below). *Desulfosarcina cetonica* was purchased
61 from the DSMZ and cultivated under sulfate-reducing conditions in a mineral salt medium as
62 described elsewhere[2] with phenol as sole substrate (see below). All used strains were cultivated at
63 25 °C on a horizontal shaker (Kühner Switzerland ISF-1-W) at 75 rpm.

64 For aerobic cultivation of *P. pseudoalcaligenes*, 240 ml serum bottles (Glasgerätebau Ochs,
65 Bovenden, Germany) were filled with 90 ml mineral salt medium and 10 ml inoculum. For
66 anaerobic cultivation of *D. cetonica* and *A. aromaticum*, 110 ml serum bottles (Glasgerätebau
67 Ochs, Bovenden, Germany) were filled with the same amount of an anoxic mineral salt media and
68 inoculum, respectively, in the absence of oxygen in a glove box (95% $N_2$/5% $H_2$ in normal
69 operation, Coy Laboratory Products, United States). Stock solutions of *p*-cresol or phenol (100
70 mM) were added in each bottle to reach final concentrations of 0.25 mM (in case of carbon stable
71 isotope fractionation by *P. pseudoalcaligenes*) or 1 mM (in the other fractionation experiments).

Each bottle was closed with a Teflon-coated butyl septum and fixed by an aluminum cap (Glasgerätebau Ochs, Bovenden, Germany).

**Calculation of isotope signatures**

The resulting raw data for carbon and hydrogen stable isotope analysis were normalized via two-point calibration with USGS63 ($\delta^2$H = 174.5 ± 0.9 mUr; $\delta^{13}$C = -1.17 ± 0.04 mUr) and USGS67 ($\delta^2$H = -166.2 ± 1.0 mUr; $\delta^{13}$C = -34.50 ± 0.05 mUr) to compensate for scale compression of the mass spectrometer and to correct $\delta^2$H and $\delta^{13}$C values following the procedure described elsewhere[3]. The international standard USGS71 ($\delta^2$H = -4.9 ± 1.0 mUr; $\delta^{13}$C = -10.50 ± 0.03 mUr) was used as an additional reference material for quality control.

Carbon and hydrogen stable isotope ratios were reported relative to Vienna-Pee Dee-Belemnite (V-PDB) and Vienna Standard Mean Ocean Water (VSMOW) scale, respectively (Eq. 1).

$$\delta^{13}\text{C or } \delta^{2}\text{H [mUr]} = \frac{R_{sample}}{R_{standard}} - 1 \qquad (1)$$

in which $R_{sample}$ and $R_{standard}$ represent the $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios of the sample and of the standard, respectively. Isotope ratios were expressed as $\delta^{13}$C and $\delta^2$H values in milli-Urey (mUr). Bulk carbon or hydrogen enrichment factors ($\varepsilon_C$ or $\varepsilon_H$) were determined from the slope of the regression curve according to the simplified Rayleigh equation:

$$\varepsilon_{bulk,\ C\ or\ H} = \frac{\ln\left({}^{R_t}/_{R_0}\right)}{\ln\left({}^{C_t}/_{C_0}\right)} \qquad (2)$$

where $R_0$ and $R_t$ refer to the isotope ratios and $C_0$ and $C_t$ refer to the concentrations of the phenolic compounds at the beginning and at a certain time point of the experiment, respectively.

The apparent kinetic isotope effect (AKIE) was calculated as described by Eq. (3):

$$AKIE = \frac{1}{1 + \left(\frac{n}{x}\right) * z * \varepsilon_{bulk}} \qquad (3)$$

where $n$ is the number of carbon or hydrogen atoms of phenol or *p*-cresol, x is a number of atoms involved in the reaction, z is a number of indistinguishable reactive positions.

The dual-isotope approach was used for calculation of Λ values by regression of measured data for carbon and hydrogen isotopes (equation 4).

$$\Lambda = \frac{\Delta\delta^2 H}{\Delta\delta^{13}\mathrm{C}} \quad (4)$$

where $\Delta\delta^{13}C$ and $\Delta\delta^{2}H$ are the absolute fractionation ratios of carbon and hydrogen, respectively.

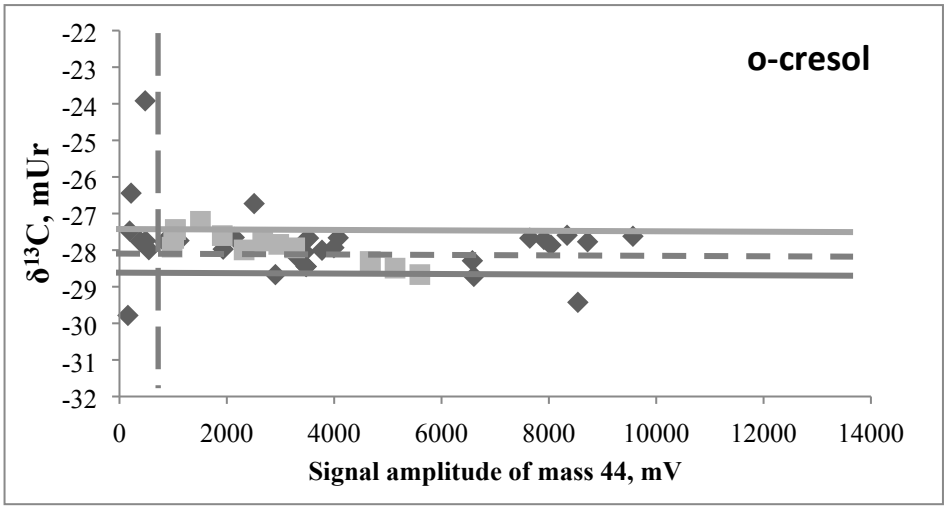
## 3. RESULTS

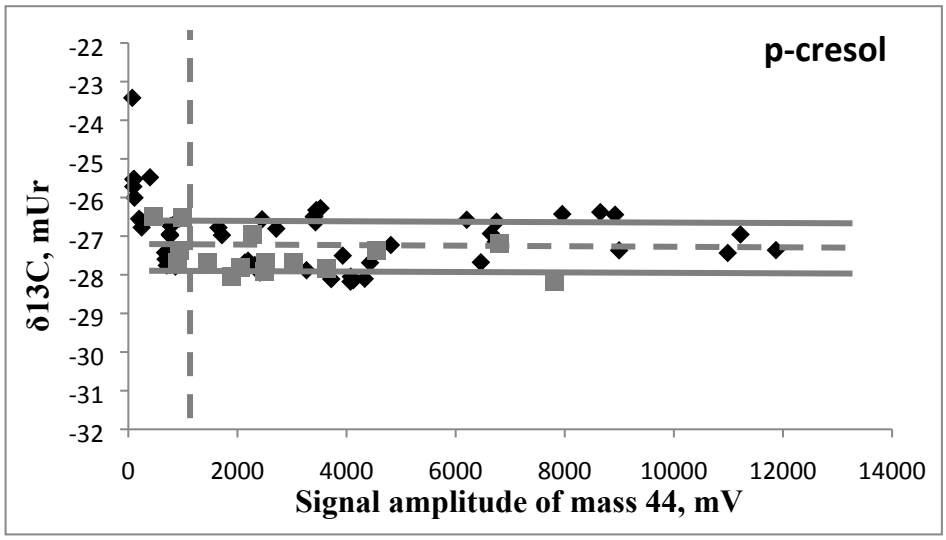
**Table SI 1:** SPE extraction efficiency

| Concentration in water (mg/L) | Extraction efficiency (%) | | |
|---|---|---|---|
| | *p*-cresol (n = 3) | *o*-cresol (n = 3) | phenol (n = 3) |
| 1 | 49 ± 2 | 50 ± 6 | 39 ± 2 |
| 2 | 61 ± 2 | 50 ± 6 | 60 ± 2 |
| 3 | 65 ± 2 | 68 ± 3 | 77 ± 5 |
| 5 | 68 ± 2 | 59 ± 3 | 66 ± 3 |
| 8 | 67 ± 5 | 65 ± 4 | 69 ± 3 |

**Table SI 2:** Liquid-liquid extraction efficiency

| Concentration in water (mg/L) | Extraction efficiency (%) | |
|---|---|---|
| | p-cresol (n = 4) | phenol (n = 4) |
| 5 | 41 ± 5 | 5.2 ± 0.3 |
| 10 | 36 ± 3 | 10 ± 1 |
| 100 | 60 ± 4 | 24 ± 3 |



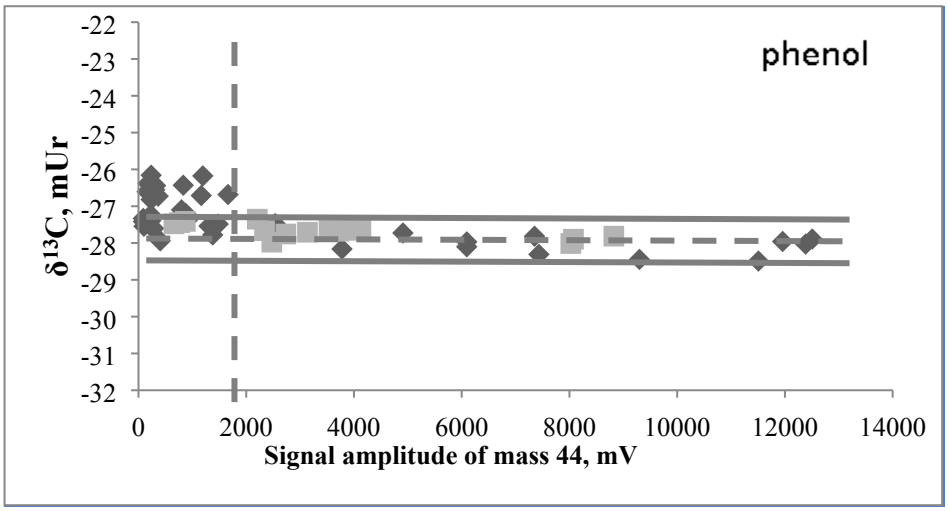




**Figure SI 1:** Correlation of response and carbon isotope values of phenols (linearity plot). The diamonds (♦) and the squares (■) relate to $\delta^{13}C$ values in standard solutions and after SPE, respectively. The solid lines show the interval of the $\delta^{13}C$ mean value ± 0.5‰; the horizontal dashed line shows the mean $\delta^{13}C$

value, the vertical dashed line shows a minimum amplitude of the linear range for standard solution and SPE.

Figure SI 1 shows the dependency of isotope data on the peak intensity precision of the method. A non-linear shift of $\delta^{13}C$-values was observed below m/z 44 signal amplitudes of 900, 900 and 2000 mV for phenol, *o*- and *p*- cresol, respectively. The linearity was satisfactory above these values for all standard solutions.

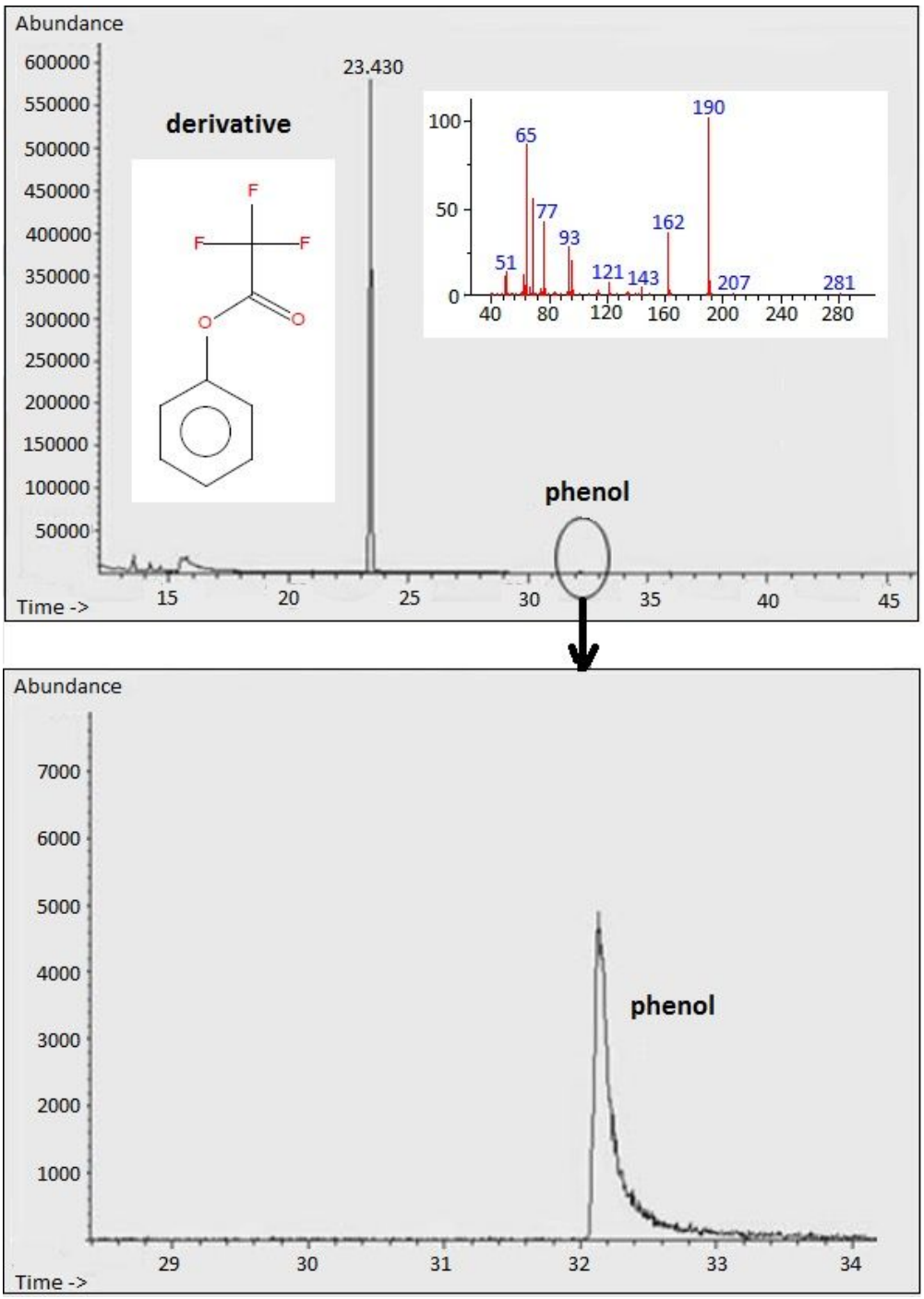
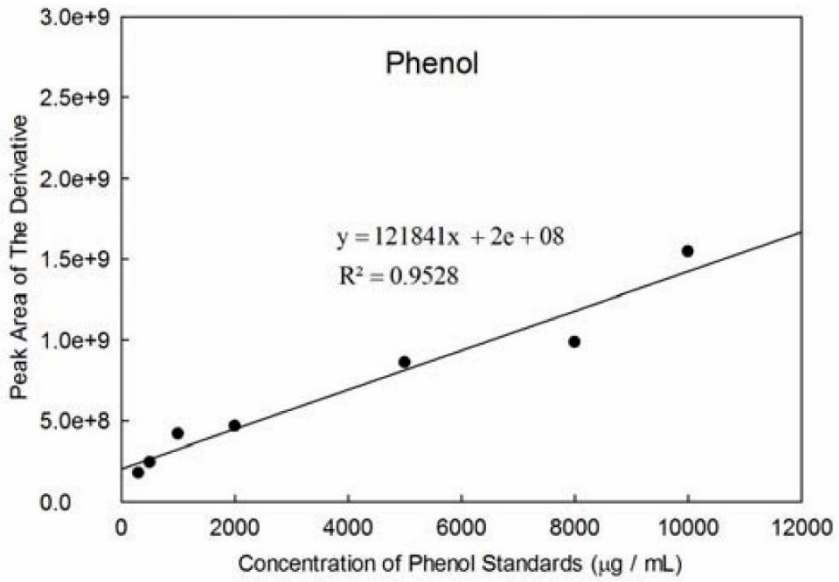



**Figure SI 2:** Example of the chromatogram and mass-spectra of the phenol derivatization product phenyl trifluoroacetate.



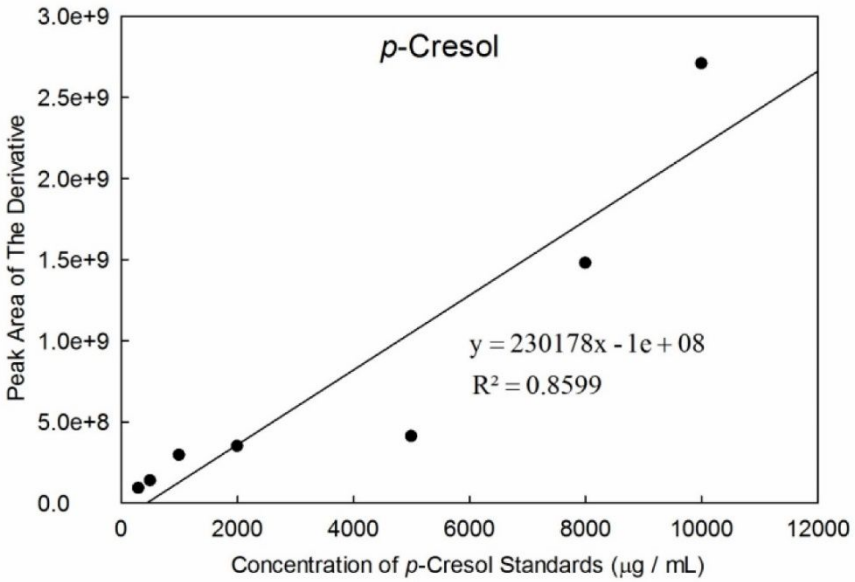
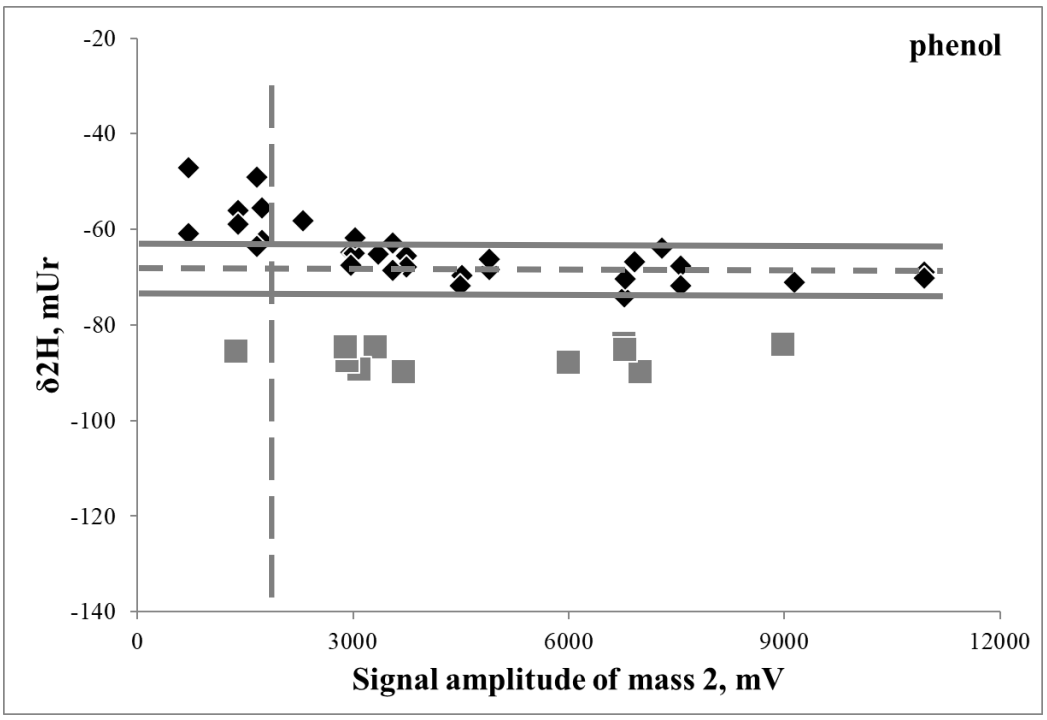




**Figure SI 3:** Correlation of the peak area of phenyl or methylphenyl trifluoroacetates to the concentrations of phenol (upper figure) and p-cresol (lower figure), respectively.



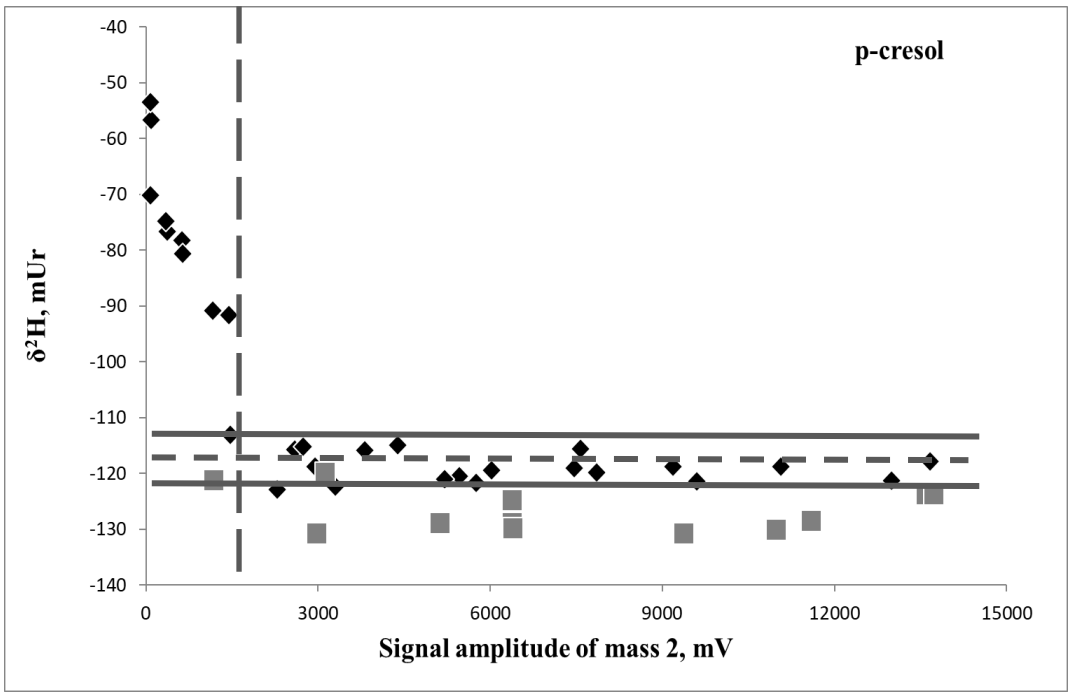




**Figure SI 4:** Correlation of response and hydrogen isotope composition of the trifluoroacetate derivatives of phenol and *p*-cresol (linearity plot). The diamonds (♦) and the squares (■) relate to $\delta^2$H values in standard solutions and after liquid-liquid extraction, respectively. The grey dashed line shows the $\delta^2$H mean value in standard solution, the solid lines show the interval of $\delta^2$H mean value ± 5‰ in standard solution, the vertical dashed line shows a minimum amplitude of the linear range for standard solution and liquid-liquid extraction.

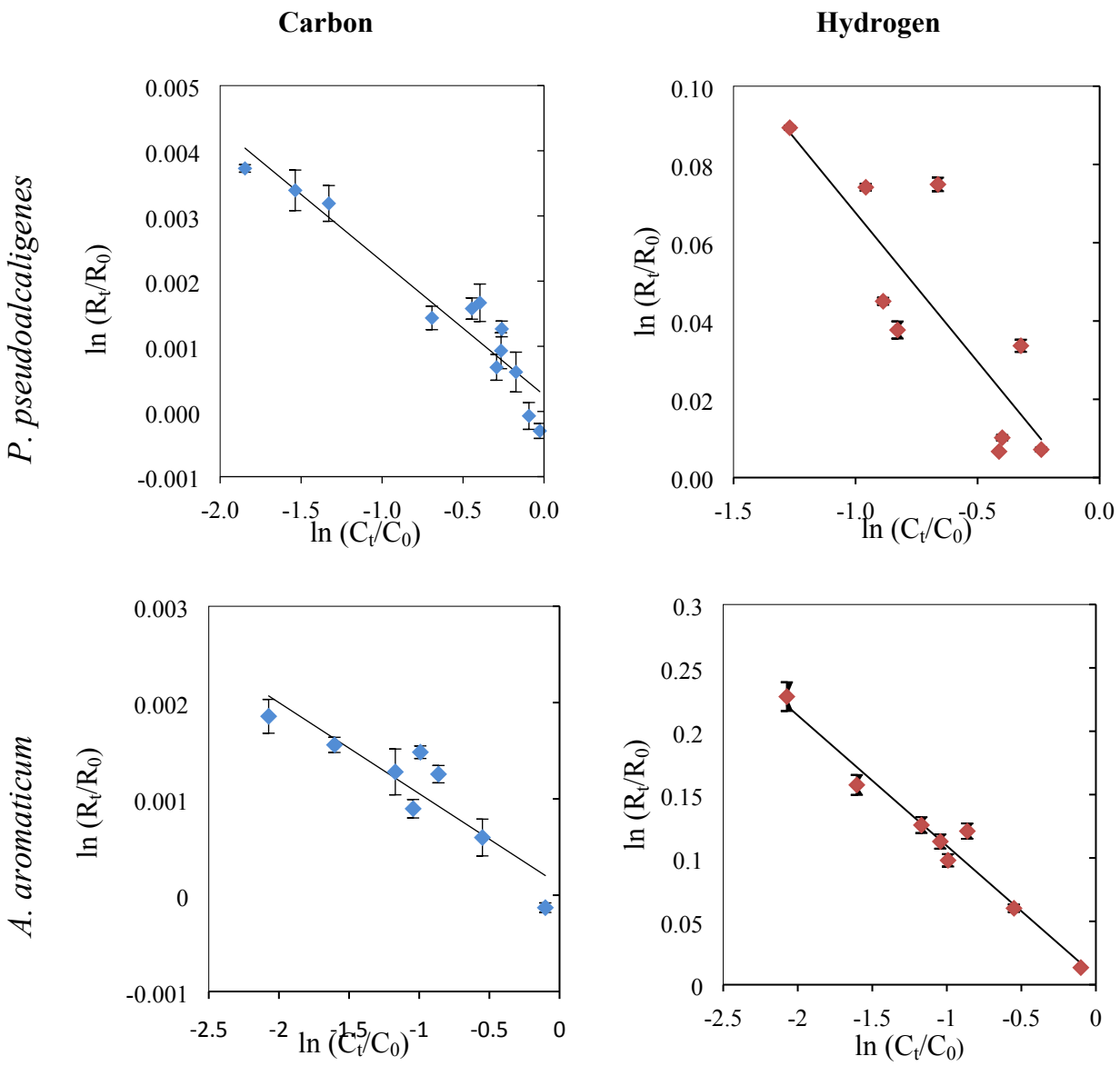



**Figure SI 5:** Rayleigh plots of carbon and hydrogen isotope fractionation during aerobic and anaerobic *p*-cresol degradation by *P. pseudoalcaligenes* and *A. aromaticum*, respectively.

## 4. REFERENCES

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