Supporting Information

Monitoring microbial mineralization using reverse stable isotope labelling analysis by mid-infrared laser spectroscopy

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= -9.7 ‰); (b) ^{13}C-enriched sample (x(^{13}C) = 10.0 %). The peak areas labeled as (13)CO_2(1) and CO_2(1) were used to determine the $^{13}C/^{12}C$ isotopic ratios in this study.

EXPERIMENTAL SECTION

Sample preparation for carbon isotope ratio measurements. We adopted a procedure which is used to analyze carbon isotope ratios in natural water samples by taking liquid samples and liberate CO_2 using phosphoric acid as reported previously.[1] (1) For GC-IRMS measurements, 0.5 mL liquid samples were directly taken from the culture bottles and immediately injected into 15 mL serum bottles sealed with butyl rubber stoppers. The vial had been pre-filled with 4.5 mL phosphoric acid (1M) and flushed with nitrogen or helium gas. (2) For IRIS measurements, 50 µL of 85% phosphoric acid were introduced into an uncapped 12 mL Labco Exetainer vial (Labco Limited, United Kingdom). Then, the vial was closed with screw-capped pierceable butyl rubber septa and flushed with CO_2-free synthetic air via an ASX-7100 autosampler (Teledyne CETAC Technologies, Omaha, USA) for two minutes. Afterwards, an aliquot (0.5 mL) from the culture bottle was taken and injected into the vial through the septum. Then, we left the sample to equilibrate at stable room temperature overnight to liberate all CO_2 into the headspace.

Isotope Ratio Mid-Infrared Spectroscopy (IRIS). IRIS is capable of measuring both carbon and oxygen isotope compositions of CO_2 in air. The instrument's analyzer uses a tunable diode laser absorption (TDLA) technique operated at a mid-infrared wavelength of 4.3 µm. During analysis, the laser scans over the absorption lines for the various CO_2 isotopologues of the sample (i.e. $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$, and $^{12}C^{16}O^{18}O$) and fits the average signal spectrum to a reference to simultaneously quantify CO_2 isotope ratios.

DIC content calibrations by IRIS. DIC standard solutions were made of normal $NaHCO_3$ (s) and Millipore water and used to calibrate the DIC content from 5 to 40 mM *versus* "Total CO_2 Volume" (i.e. the absolute amount of CO_2 in the vial) results from Qtegra™ Intelligent Scientific Data Solution software. It shows an excellent linear relationship (correlation

coefficient R^2 = 0.9967, $y = 10.017m + 6.5638$) between DIC contents (m) and total CO_2 volume (y).

Preparation of ^{13}C-enriched DIC samples. In order to prepare bicarbonate solutions with $x(^{13}C)$ values ranging from 6 to 12% (see Figure 2), we produced 30 mM stock solutions of regular sodium bicarbonate ($x(^{13}C)$ = 1.11%, Sigma-Aldrich Co. LLC) and ^{13}C-labelled sodium bicarbonate ($x(^{13}C)$ = 98%, Sigma-Aldrich Co. LLC) and then added 0.5 mL aliquots into 12 mL Labco Exetainer vials.

Calculations of CO_2 production. The conversions of $x(^{13}C)$ changes to CO_2 production were based on isotope mass balance equations as follows.

(1). CO_2 production under anoxic conditions was calculated following two mass balance equations,

$$CO_{2_{total}} = CO_{2_{background}} + CO_{2_{produced}} \quad (1)$$

$$CO_{2_{total}} \times x(^{13}C)_{total} = CO_{2_{background}} \times x(^{13}C)_{background} + CO_{2_{produced}} \times x(^{13}C)_{produced} \quad (2)$$

where $CO_{2_{total}}$ is the final amount of total inorganic carbon in the system (mmole), $CO_{2_{background}}$ is the initial total inorganic carbon in the system (mmole), $CO_{2_{produced}}$ is the total released CO_2 from microbial mineralization of aromatic compounds (mmole), $x(^{13}C)_{total}$ is the final carbon isotope ratio of total inorganic carbon in the system, $x(^{13}C)_{background}$ is the initial carbon isotope ratio of total inorganic carbon in the system, $x(^{13}C)_{produced}$ is the carbon isotope ratio of CO_2 released from microbial production, assumed to be 1.1%. $CO_{2_{background}}$ can be calculated from the initial total DIC content in the

liquid ($DIC_{measured}$) and the volume of 20% CO_2 gas in the headspace ($0.2 \cdot n_{gas,headspace}$, confirmed by measured values):

$$CO_{2_{background}} = DIC_{measured} + 0.2 \cdot n_{gas,headspace} \quad (3)$$

Thus, the total CO_2 production can be obtained via Equation 5 which is based on the combination of Equations 1 - 3:

$$CO_{2_{produced}} = \frac{(DIC_{measured} + 0.2 \cdot n_{gas,headspace}) \times (x(^{13}C)_{background} - x(^{13}C)_{total})}{x(^{13}C)_{total} - x(^{13}C)_{produced}} \quad (4)$$

For data interpretation, we evaluated isotope ratios of CO_2 from the liquid phase without further correction, since equilibrium carbon isotope fractionation between the carbonate species acts on all samples in the same way and cancels out in the comparison between standard and samples. Therefore, the carbon isotope ratio of DIC can be taken as representative of the whole closed system:

$$x(^{13}C)_{total} = x(^{13}C)_{total\ DIC} \quad (5)$$

$$x(^{13}C)_{background} = x(^{13}C)_{background\ DIC} \quad (6)$$

(2). We calculated CO_2 production under oxic conditions based on the same mass balance equations as under anoxic conditions (Equations 1 and 2). As negligible amounts of CO_2 exist in the headspace compared to that in the solution at the beginning, $CO_{2_{background}}$ can be calculated using different equation:

$$CO_{2_{background}} = CO_{2_{\text{bicarbonate solution}}} + CO_{2_{natural}} \quad (7)$$

where $CO_{2_{\text{bicarbonate solution}}}$ is the amount of inorganic carbon in the added bicarbonate solution (mmole) and $CO_{2_{natural}}$ is the amount of inorganic carbon in the natural water

(mmole). Thus, the total CO_2 production can be obtained via Equation 5 which is based on the combination of Equations 1, 2 and 7:

$$CO_{2_{produced}} = \frac{\left(CO_{2_{natural}} + CO_{2_{\text{bicarbonate solution}}}\right) \times \left(x(^{13}C)_{background} - x(^{13}C)_{total}\right)}{x(^{13}C)_{total} - x(^{13}C)_{produced}} \quad (8)$$

$CO_{2_{natural}}$ can be obtained via the following equation:

$$CO_{2_{background}} \times x(^{13}C)_{background} = CO_{2_{\text{bicarbonate solution}}} \times x(^{13}C)_{\text{bicarbonate solution}} + CO_{2_{natural}} \times x(^{13}C)_{natural} \quad (9)$$

where $x(^{13}C)_{\text{bicarbonate solution}}$ is the carbon isotope ratio of the added bicarbonate solution and $x(^{13}C)_{natural}$ is the carbon isotope ratio of the natural water (assuming that the carbon isotope ratio of the natural water is 1.1%).

RESULTS AND DISCUSSION

Are $\delta^{13}C_{DIC}$ values = $\delta^{13}C_{CO2(g)}$ in 12 ml vials after acidification? During sample measurement by IRIS using 12 ml vials, concentrated phosphoric acid reacts with liquid samples and converts all DIC species into minority aqueous CO_2 and majority gaseous CO_2. The equilibration of stable carbon isotopes will therefore occur between aqueous and gaseous phases. The question remains if equilibrium fraction corrections are needed for the final carbon isotopic ratios of DIC. The carbon isotope fractionation effect associated with the $CO_{2(g)}$-$CO_{2(aq)}$ partition was discussed in detail previously when samples are acidified with H_3PO_4. The fractionation factors are directly related to the volume of DIC water samples and DIC concentration. According to the equations provided by these authors,[2, 3] when we inject 0.55 mL samples in 12 mL glass vials, it will provide CO_2 molar fractions of 95% for $CO_{2(g)}$

and 6% for $CO_{2(aq)}$. The $\delta^{13}C$ correction will only be between 0.04 to 0.08 ‰. The differences in the carbon isotopic values between the DIC and $CO_{2(g)}$ are quite low and under the precision of 5 ‰ for samples highly enriched in ^{13}C when measured by IRIS. It is reasonable to assume that $\delta^{13}C_{DIC} = \delta^{13} C_{CO2(g)}$. Besides, Assayag et al. also pointed out that the carbon isotopic ratios of these acidified samples could be stable for up to 6 months.[4]

Effect of isotope fractionation on CO_2 quantification. Microbial degradation studies of diverse organic substrates have shown that biodegradation can go along with kinetic isotope fractionation effects.[5, 6] Changes in the carbon stable isotope ratio can for example be used to estimate biodegradation in contaminated aquifers. Consequently, the released CO_2 does not have a constant stable isotope ratio ($x(^{13}C)_{produced}$) but undergoes a steady shift towards more positive values during the course of degradation experiments. However, commonly observed carbon isotope fractionation factors ε for aromatic hydrocarbons are in the per mille range, for example only -0.7 to -5.08‰ for naphthalene degradation under sulfate-reducing conditions.[5, 6] This means that the largest isotope ratio changes of the released CO_2 ($\Delta x(^{13}C)_{produced}$) are quite small compared to the range of the measurements mentioned above (within the experimental error $x(^{13}C) = 0.05\%$). Furthermore, the effect of observable carbon isotope fractionation is expected to be even less pronounced for larger molecules.[5-7] Therefore, we can assume no isotopic fractionation occurs and $x(^{13}C)_{produced}$ stays constant during microbial mineralization.

Upon anaerobic mineralization of aromatic compounds in closed serum bottles, the corresponding released CO_2 will partition both into the liquid phase as DIC and into the headspace as gaseous CO_2. The question remains if we need to take equilibrium isotope fractionation into account for the final carbon isotope ratios of DIC, since it can affect the calculated CO_2 results. During the biodegradation processes reported here, the cultures were

uniformly cultivated at 30 ^{o}C and pH values did not change significantly (7.2-7.4, slightly alkaline). At slightly alkaline pH, DIC would be present in the form of $CO_{2(aq)}$, CO_3^{2-} and mainly HCO_3^-. According to the empirical function of fractionation factors reported by Zhang et al.,[8] the equilibrium fractionation factor α for DIC and $CO_{2(g)}$ is around 1.008, corresponding to a per mille fractionation ε_{DIC-g} = ~8‰. This is again quite small compared to the high ^{13}C enrichments ($\delta^{13}C \approx +10{,}000‰$) and significant shifts in δ^{13}C values as high as -2737‰ ($x(^{13}C)$ = 2.44%) as observed during the biodegradation of naphthalene (Figure 3). For all practical purposes it is, therefore, reasonable to consider $x(^{13}C)$ values of $CO_{2(g)}$ in the headspace as the same as the ones of DIC. To confirm this, we took both DIC and headspace samples from the anaerobic toluene degradation experiments with *T. aromatica* at the last time point of the incubation (Figure 1d). Results showed that the divergences between measured $x(^{13}C)$ values for DIC and CO_2 in headspace samples were as small as $x(^{13}C)$ = 0.0674% (Table S2), which is less than the experimental error of the experiments. This confirmed that the isotopic signature differences between DIC and headspace samples are negligible in comparison to the experimental errors and the extent of biodegradation in our experiments. However, when less ^{13}C-labelled bicarbonate (e.g. $x(^{13}C)$ = 2%) is used and the degradation rate is extremely small, this will not lead to large isotopic ratio changes as shown here. Under such circumstances, only semi-quantitative evidence for microbial degradation may be achieved.

Effect of isotope fractionation caused by methanogenesis on the RIL method.

Hydrogenotrophic methanogenesis leads to the consumption of carbon dioxide. In this case, carbon isotope fractionation factors between CH_4 and CO_2 are in the per mille range from −79‰ to about −28‰. [9]

In order to illustrate the effect of such fractionations on the carbon isotope ratios of CO_2 in RIL experiments, we took the data of the anaerobic naphthalene degradation experiment

shown in Figure 3 as an example. Assuming that the same amount of naphthalene (1.19 mM) was degraded during fermentation (Equation 10), this would lead to the same decrease of the carbon isotope ratio (^{13}C fractions) from 11.89% to 9.45% of the total inorganic carbon in the system. Due to the CO_2 release in this process, the total inorganic carbon in the system would increase from the initial concentration 40.75 mM to 52.62 mM.

$$C_{10}H_8 + 20H_2O \rightarrow 10CO_2 + 24H_2 \quad (10)$$

Furthermore, CO_2 would be used for hydrogenotrophic methanogenesis (Equation 11) and we assumed that all electrons released during fermentation go to methane. This is not possible in reality because some electrons are used for biomass production, but we calculated it as an extreme example. This process would lead to a release of 7.12 mM CH_4 and a consumption of 7.12 mM CO_2. The final concentration of the total inorganic carbon in the system would be 45.50 mM.

$$6CO_2 + 24H_2 \rightarrow 6CH_4 + 12H_2O \quad (11)$$

In methanogenesis, the amount of ^{13}C in the initial total inorganic carbon ($CO_{2_{total-initial}}$) equals the amount of ^{13}C in the produced methane ($CH_{4_{produced}}$) plus the amount of ^{13}C in the remaining total inorganic carbon ($CO_{2_{total-end}}$) (Equation 12).

$$CO_{2_{total-initial}} \times x(^{13}C)_{total-initial} = CO_{2_{total-end}} \times x(^{13}C)_{total-end} + CH_{4_{produced}} \times x(^{13}C)_{produced} \quad (12)$$

where $x(^{13}C)_{total-initial}$ is the initial carbon isotope ratio of total inorganic carbon in the system, $x(^{13}C)_{total-end}$ is the final carbon isotope ratio of total inorganic carbon, and $x(^{13}C)_{produced}$ is the carbon isotope ratio of CH_4.

The carbon stable isotope fractionation between produced CH_4 and remaining CO_2 can be described by Equation 13.[10]

$$\varepsilon_{CH_4} \approx \delta CH_{4_{produced}} - \delta CO_{2_{total-end}} \quad (13)$$

where ε_{CH_4} is the stable isotope fractionation factor, $\delta CO_{2_{total-end}}$ is the final carbon isotope ratio of total inorganic carbon in the system, and $\delta CH_{4_{produced}}$ is the carbon isotope ratio of CH_4. The conversion of delta values δ to atom fractions $x(^{13}C)$ can be found in the main text.

We chose the biggest fractionation factor between CH_4 and CO_2 ($\varepsilon_{CH_4} = -79‰$) to see the biggest possible ^{13}C isotopic ratio shifts of total inorganic carbon caused by methanogenesis. Following the above isotope mass balance equations, the total inorganic carbon would be enriched from $x(^{13}C) = 9.45\%$ to $x(^{13}C) = 9.46\%$ by stable isotope fractionation. This little change of only 0.01% is much less than the one caused by the fermentation of the naphthalene, and release of CO_2, in the RIL set up ($\Delta x(^{13}C) = 2.44\%$). This extreme example demonstrates that the effect of CO_2 consumption by methanogenesis on the results of an RIL experiment can be neglected.

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Table S1 Stoichiometric calculations of the measured and the theoretical ratios between the reduction of electron acceptors or donors and CO_2 production at the last sampling point for Figures 1a-c.

	Figure 1a	Figure 1b	Figure 1c
	Toluene oxidation: CO_2 production	Sulfate reduction: CO_2 production	Iron reduction: CO_2 production
Theoretical ratios	0.17	0.61	4.80
Calculated ratios	0.59	0.31	5.76

Table S2 Comparison of ^{13}C atom fractions of DIC and headspace samples measured by Isotope Ratio Mid-Infrared Spectroscopy. Offset = $x(^{13}C)_{DIC}$ - $x(^{13}C)_{headspace}$.

Sample ID	$x(^{13}C)_{DIC}$ %	$x(^{13}C)_{headspace}$ %	Calculated $x(^{13}C)_{headspace}$ %	Offset %
1	10.189	10.130	10.116	0.058
2	11.973	11.898	11.889	0.075
3	11.032	10.973	10.954	0.058
4	13.056	12.990	12.9661	0.066
5	12.518	12.440	12.431	0.078

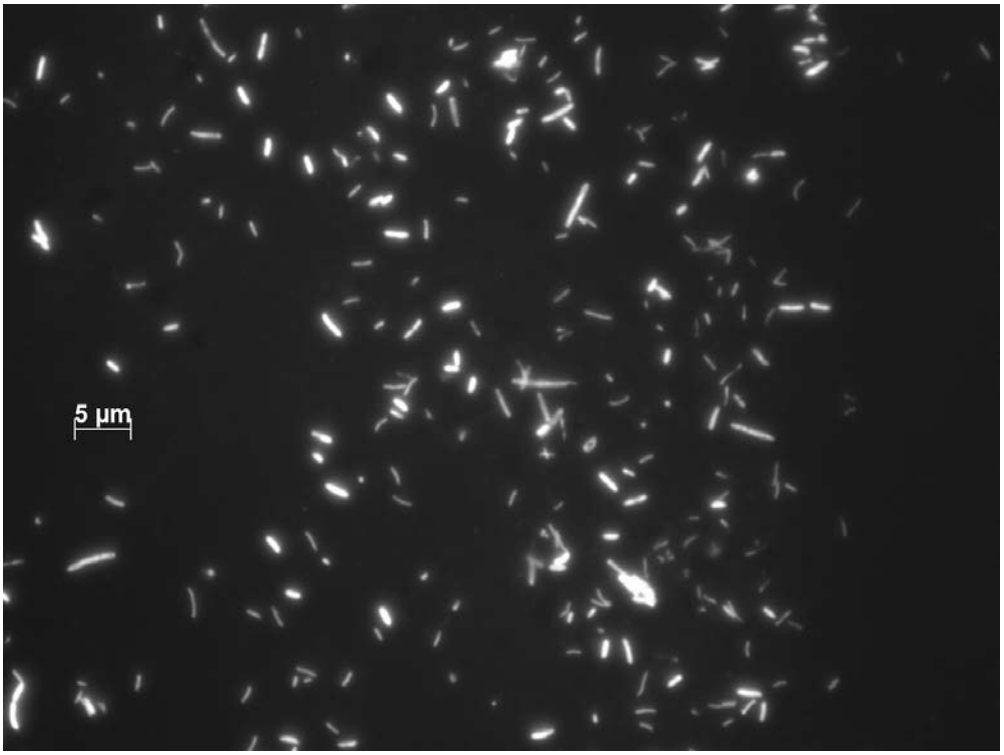


Figure S1 Epifluorescence microscopy (EFM) images of naphthalene-degrading, iron-reducing enrichment cultures after six months' cultivation stained with 4', 6-diamidino-2-phenylindole (DAPI).

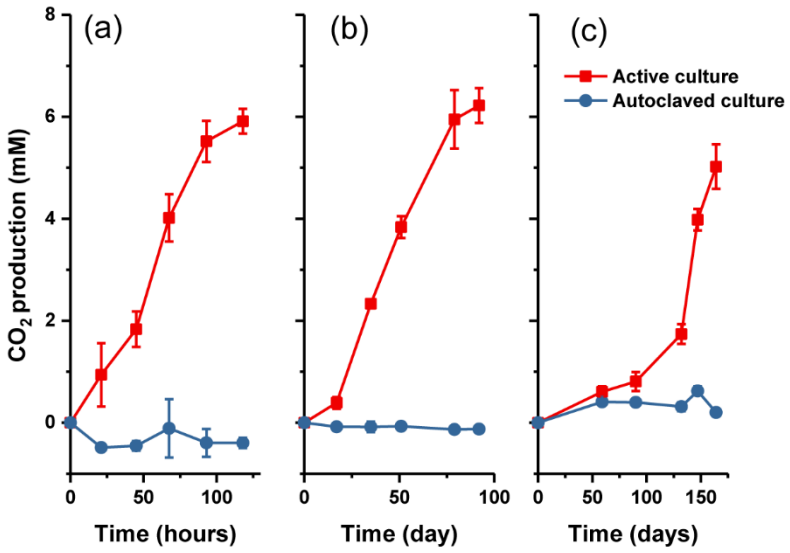


Figure S2 The calculated CO_2 production for anaerobic degradation of (a) toluene by *G. metallireducens* (Figure 1a), (b) 2-methylnaphthalene by the enrichment culture N47 (Figure 1b), (c) naphthalene by an uncharacterized iron-reducing enrichment culture SN (Figure 1c). Data points depict means of two or three parallel incubations measured three times each. Error bars represent standard deviation of the biological replicates.

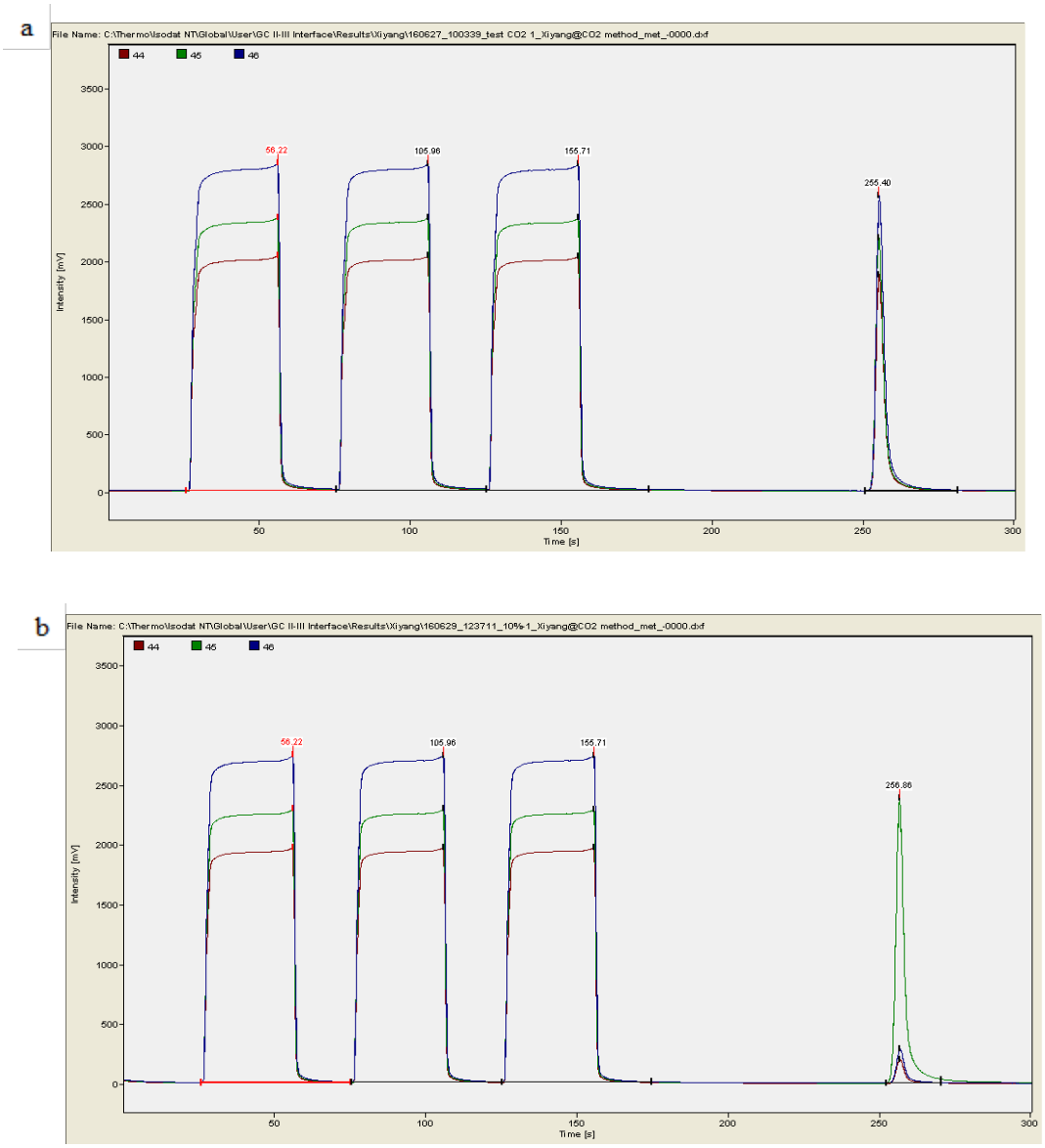
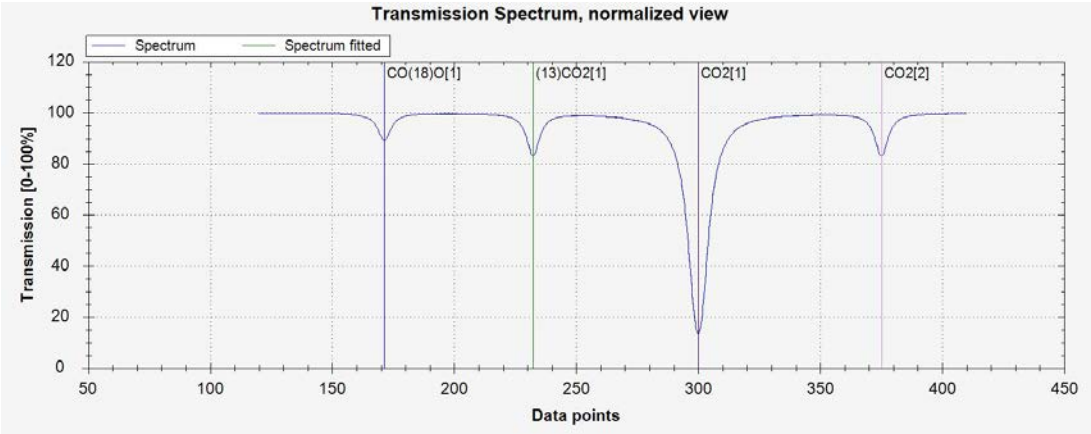


Figure S3 Mass spectrum of CO_2 obtained via Isotope Ratio Mass Spectrometry: (a) natural sample with a low ^{13}C abundance ($\delta^{13}C$ = -9.7 ‰); (b) ^{13}C-enriched sample ($x(^{13}C)$ = 10.0 %). CO_2 samples are firstly ionized to different ions mass-to-charge ratios (m/z) at 44, 45 and 46. The first three peaks (square-shaped) belong to working gas while the last one to the sample.

a



b

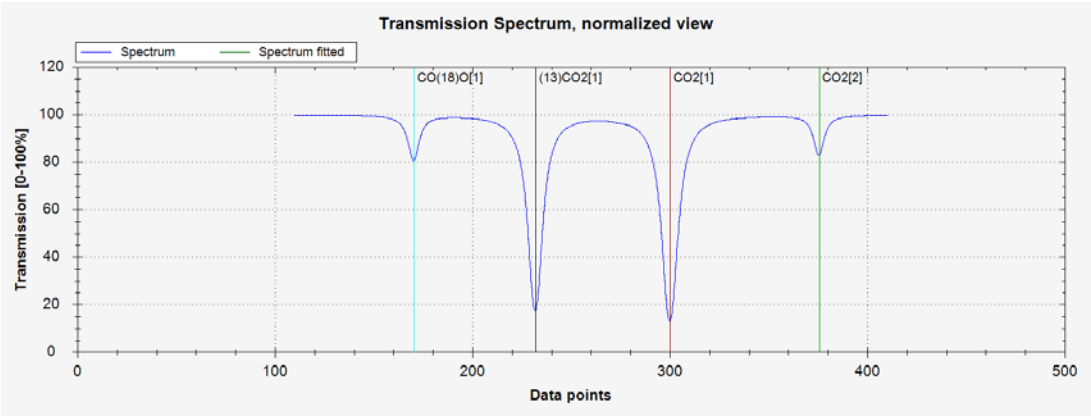


Figure S4 Normalized transmission spectrum of air containing 380 ppm CO_2 obtained via Isotope Ratio Mid-Infrared Spectroscopy: (a) natural sample with a low ^{13}C abundance ($\delta^{13}C$ = -9.7 ‰); (b) ^{13}C-enriched sample ($x(^{13}C)$ = 10.0 %). The peak areas labeled as $(13)CO_2(1)$ and $CO_2(1)$ were used to determine the $^{13}C/^{12}C$ isotopic ratios in this study.