

Bias and uncertainty of $\delta^{13}\text{C}$ isotopic mixing models

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Abstract Patterns in the isotopic signal (stable C isotope composition; $\delta^{13}\text{C}$) of respiration ($\delta^{13}\text{C}_\text{R}$) have led to important gains in understanding the C metabolism of many systems. Contained within $\delta^{13}\text{C}_\text{R}$ is a record of the C source mineralized, the metabolic pathway of C and the environmental conditions during which respiration occurred. Because gas samples used for analysis of $\delta^{13}\text{C}_\text{R}$ contain a mixture of CO_2 from respiration and from the atmosphere, two-component mixing models are used to identify $\delta^{13}\text{C}_\text{R}$.

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Measurement of ecosystem $\delta^{13}\text{C}_\text{R}$, using canopy airspace gas samples, was one of the first applications of mixing models in ecosystem ecology, and thus recommendations and guidelines are based primarily on findings from these studies. However, as mixing models are applied to other experimental conditions these approaches may not be appropriate. For example, the range in $[\text{CO}_2]$ obtained in gas samples from canopy air is generally less than $100 \mu\text{mol mol}^{-1}$, whereas in studies of respiration from soil, foliage or tree stems, the range can span as much as $10,000 \mu\text{mol mol}^{-1}$ and greater. Does this larger range in $[\text{CO}_2]$ influence the precision and accuracy of $\delta^{13}\text{C}_\text{R}$ estimates derived from mixing models? Does the outcome from using different regression approaches and mixing models vary depending on the range of $[\text{CO}_2]$? Our research addressed these questions using a simulation approach. We found that it is important to distinguish between large ($>1,000 \mu\text{mol mol}^{-1}$) and small ($<100 \mu\text{mol mol}^{-1}$) ranges of CO_2 when applying a mixing model (Keeling plot or Miller-Tans) and regression approach (ordinary least squares or geometric mean regression) combination to isotopic data. The combination of geometric mean regression and the Miller-Tans mixing model provided the most accurate and precise estimate of $\delta^{13}\text{C}_\text{R}$ when the range of

CO_2 is $\geq 1,000 \mu\text{mol mol}^{-1}$.

Keywords Carbon · Respiration · Soil · Regression · Isotope

Introduction

Determination of the stable C isotope composition of respired CO_2 ($\delta^{13}\text{C}_\text{R}$) is becoming a powerful tool in ecological studies. In most cases it is difficult if not

impossible to acquire samples of pure, respired $\delta^{13}\text{C}_R$; instead, respired CO_2 almost always occurs as a mixture with atmospheric CO_2 in gas samples used for analysis of the $\delta^{13}\text{C}$ of respiration ($\delta^{13}\text{C}_R$). Therefore, two-end-member mixing models are generally used to estimate $\delta^{13}\text{C}_R$. Much attention has been given recently to the statistical approaches that are used to quantify $\delta^{13}\text{C}_R$. However, these analyses have focused almost entirely on the single experimental situation that involves samples of air from the airspace within canopies to determine $\delta^{13}\text{C}_R$ of whole ecosystems. However, the results of these analyses may not be valid for other experimental situations—especially those in which the proportion of respired CO_2 relative to atmospheric air is much greater, and therefore both the absolute concentrations as well as the range of $[\text{CO}_2]$ in the air samples are much greater. Does a larger range in $[\text{CO}_2]$ influence the precision and accuracy of estimates of $\delta^{13}\text{C}_R$ derived from mixing models? Does the outcome from using different regression approaches [ordinary least squares (OLS) vs. geometric mean regression (GMR)] and mixing models (Keeling plot or Miller-Tans) vary depending on the range of $[\text{CO}_2]$ in the sample dataset? We addressed these questions using a simulation approach.

Two mixing models are commonly used to estimate $\delta^{13}\text{C}_R$, the Keeling plot (Pataki et al. 2003) and the Miller-Tans model [Miller and Tans (2003); these models are further described in "Materials and methods"]. Likewise, two regression approaches are commonly used in conjunction with these models, the OLS and GMR to determine the parameters of the mixing equation. The statistical implications of the choices in mixing models and regression approaches have been analyzed in detail for the most common applications involving $\delta^{13}\text{C}_R$ determination of whole ecosystems using gas samples from the canopy airspace (Pataki et al. 2003; Zobitz et al. 2006).

In the canopy airspace, the proportion of respired CO_2 relative to the atmospheric background is small; thus the range of $[\text{CO}_2]$ ($[\text{CO}_2]_{\text{range}}$) in gas samples collected in any given period is usually $<100 \text{ umol mol}^{-1}$. Rigorous comparisons have been conducted to determine whether one or the other mixing model is preferable when applied to these conditions of $[\text{CO}_2]_{\text{range}}$; in most cases the uncertainty and precision of the two models are similar (Zobitz et al. 2006), and by convention Keeling plots are primarily used (Tu and Dawson 2005). However, Keeling plots are also applied to regimes where the $[\text{CO}_2]_{\text{range}}$ can extend from around 500 to $30,000 \text{ umol mol}^{-1}$ depending on the experimental conditions: foliar respiration (Xu et al. 2004), soil respiration (Ohlsson et al. 2005; Mortazavi et al. 2004; Kayler et al. 2008), or studies concerning human respiration and car exhaust (Affek and Eiler 2006). Because both the $[\text{CO}_2]_{\text{range}}$ as well as the sample values of $[\text{CO}_2]$ vary dramatically in these different applications, and because

the $[\text{CO}_2]_{\text{range}}$ may affect the uncertainty and precision of regression analyses, we distinguish between "small" $[\text{CO}_2]_{\text{range}}$, for $[\text{CO}_2]_{\text{range}} < 100 \text{ umol mol}^{-1}$, and "large" $[\text{CO}_2]_{\text{range}}$ regimes, for $[\text{CO}_2]_{\text{range}} > 1,000 \text{ umol mol}^{-1}$. Rigorous comparisons of the models have not been conducted for cases when the range of CO_2 values is broad.

The best combination of mixing model and regression approach will produce the most accurate and precise estimate of $\delta^{13}\text{C}_R$, where accuracy is defined as the nearness of a measurement to the true value, and precision is defined as the degree to which repeated measurements yield the same value (Zar 1999). The accuracy and precision of mixing models applied to large $[\text{CO}_2]_{\text{range}}$ regimes may not be similar to those for small $[\text{CO}_2]_{\text{range}}$ regimes. Previous studies have shown that a larger concentration gradient (i.e., greater range of x values), should reduce the uncertainty of estimates of $\delta^{13}\text{C}_R$ (Ohlsson et al. 2005), but estimates of $\delta^{13}\text{C}_R$ in a large $[\text{CO}_2]_{\text{range}}$ have not been discussed in the context of measurement or sampling error. Furthermore, researchers typically use a much smaller number of samples for each regression analysis in studies of foliar or soil respiration (large $[\text{CO}_2]_{\text{range}}$ regimes) than in studies of ecosystem respiration (Tu and Dawson 2005), allowing them to estimate $\delta^{13}\text{C}_R$ both spatially and temporally. However, this tradeoff between the $[\text{CO}_2]_{\text{range}}$ and the sample size has not been investigated.

Complicating this tradeoff further is the degree of measurement error in both $[\text{CO}_2]$ ($[\text{CO}_2]$ error) and the $\delta^{13}\text{C}$ values ($\delta^{13}\text{C}$ error). The precision of isotopic measurements is largely determined by the method of analysis (i.e., isotope ratio mass spectrometer, tunable diode laser, etc.); and while the precision of each machine varies from laboratory to laboratory, a reasonable upper level estimate of the uncertainty of these instruments is on the order of 0.2‰ or less. The precision of the $[\text{CO}_2]$ measurements by infrared gas analyzers is on the order of $0.1\text{--}1 \text{ umol mol}^{-1}$ (Miller and Tans 2003; Hauck 2006). However, the potential for greater error is likely in high $[\text{CO}_2]_{\text{regimes}}$ for several reasons. First, the gas standards available from suppliers used to calibrate gas analyzers are typically accurate within $>2\%$ of the target value. At best, this error would represent a constant bias, but concentrations in gas tanks are susceptible to fluctuations in temperature, pressure and water vapor. Thus, there exists a random element to the uncertainty in these standards as well. Second, measurement error occurs with sampling in the field, storage in the laboratory, and with instrument analysis. Finally, additional errors can be introduced by small-scale heterogeneity if the $[\text{CO}_2]$ measurements and isotopic measurements are not made on the same air sample. Taken together, these errors could be on the order of 1–2% which could represent several hundred umol mol^{-1} in large $[\text{CO}_2]_{\text{range}}$ regimes and should therefore be included in the analysis of mixing-model performance.

The goal of this study was to evaluate how factorial combinations of two mixing models and two regression approaches (Keeling-OLS, Miller-Tans-OLS, Keeling-GMR, Miller-Tans-GMR) compare in small $[CO_2]_{range}$ versus large $[CO_2]_{range}$ regimes, with different combinations of pertinent variables ($[CO_2]_{range}$, $[CO_2]$ error, $\delta^{13}C$ error and n) that are realistic for experimental applications in each of the two regimes (Fig. 1). Our approach was to conduct a series of simulations using artificial datasets. From these simulations we report: (1) how the bias and uncertainty of estimates of $\delta^{13}C_R$ in large concentration and small concentration regimes differ, (2) which simulation input variables influence $\delta^{13}C_R$ bias and uncertainty, and (3) which mixing and regression model produces the least bias and uncertainty when applied to samples from large $[CO_2]_{range}$ systems.

Materials and methods

Mixing models and regression approaches

The two mixing models we examined are based on the conservation of mass given in Eq. 1.

$$\delta_{obs}[CO_2]_{obs} = \delta_{bg}[CO_2]_{bg} + \delta_s[CO_2]_s \quad (1)$$

The equation describes a gas sample as a mixture of two sources of CO_2 : the background atmosphere and the source of respiration. Where the subscripts obs, s, and bg refer to the observed, source and background values, respectively.

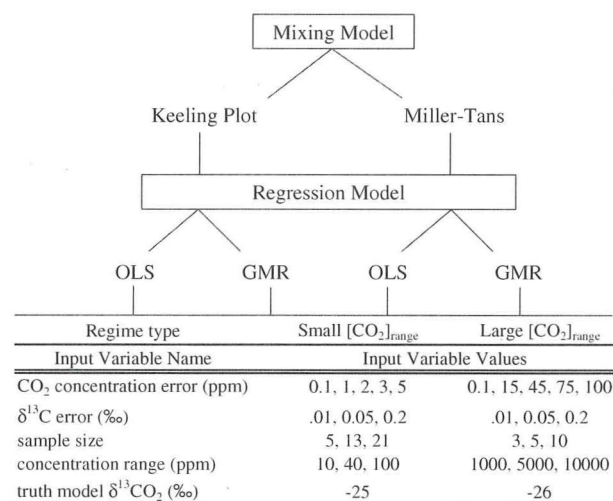


Fig. 1 Diagram illustrating the different mixing and regression model combinations tested for bias and uncertainty. The table lists the different measurement errors, sample size and concentration ranges we used in our simulation of small and large $[CO_2]$ regimes. *OLS* Ordinary least squares *GMR* geometric mean regression

In Eq. 1, δ refers to the isotopic value of the component expressed in δ notation: $\delta = (R_{sample}/R_{standard} - 1) \times 1,000\text{‰}$, where R is the molar ratio of heavy to light isotopes. The C isotope ratio ($^{13}C/^{12}C$) is expressed relative to the standard Vienna Pee Dee belemnite. The Keeling linear mixing model equation that relates the observed $\delta^{13}C$ to the observed $[CO_2]$ is given in Eq. 2.

$$\delta_{obs} = \frac{[CO_2]_{bg}}{[CO_2]_{obs}} (\delta_{bg} - \delta_s) + \delta_s \quad (2)$$

The estimate of source $\delta^{13}C_R$ is obtained as the intercept of this linear model, regressing δ_{obs} versus $1/[CO_2]$.

Using Eq. 1, Miller and Tans (2003) derived a different linear mixing model equation where the source value of $\delta^{13}C$ is unknown:

$$\delta_{obs}[CO_2]_{obs} = \delta_{bg}[CO_2]_{bg} - [CO_2]_s(\delta_{obs} - \delta_{bg}) \quad (3)$$

From which an equation of the form $y = mx + b$ is derived:

$$\delta_{obs}[CO_2]_{obs} = \delta_s[CO_2]_{obs} - [CO_2]_{bg}(\delta_{bg} - \delta_s) \quad (4)$$

In this case, $\delta^{13}C_R$ is estimated as the slope of the regression line of $\delta_{obs} \times [CO_2]_{obs}$ versus $[CO_2]$ rather than the intercept of δ versus $1/[CO_2]$, as in Eq. 2. For our analysis, δ_s and δ_{obs} in the above equations are equivalent to the symbols $\delta^{13}C_R$ and $\delta^{13}C$, respectively, which we use to explain our methods.

The presentation of equations and discussion of the OLS and GMR approaches are discussed in online resource 1 (Electronic supplementary material; ESM). When OLS is used to estimate regression parameters, the uncertainty of the estimators relies on the assumption that the independent variable in the regression ($[CO_2]$ for Miller-Tans and $1/[CO_2]$ for Keeling) is measured without error. In contrast, GMR is used when there are errors associated with both the independent and dependent variables (Legendre and Legendre 1998).

Simulation variables ($[CO_2]_{range}$, $[CO_2]$ error, $\delta^{13}C$ error and sample size)

To examine the impacts of the two mixing models and the two regression approaches, we generated artificial data sets that were based on a known value of $\delta^{13}C_R$. This known value is the "true" value to which we compare simulation estimates. For each artificial dataset, paired values of $[CO_2]$ and $\delta^{13}C$ were generated with varying ranges of $[CO_2]$, error in $[CO_2]$ values, error in $\delta^{13}C$ values, and sample size, in a factorial design (Fig. 1). A separate set of artificial datasets were generated to be representative of conditions involving small $[CO_2]_{range}$ (i.e., those typical of applications involving ecosystem respiration) and large

$[\text{CO}_2]_{\text{range}}$ measurement regimes (i.e., those typical of applications involving tree stem and soil respiration). More details on the simulation procedure are given in online resource 2 (ESM).

We generated 10,000 x - y data pairs of size n . For each simulated dataset, we fit each of the four models and estimated the isotopic composition of respiration. We thus obtained four distributions with 10,000 estimates of $\delta^{13}\text{C}_R$ for each set of experimental conditions; for each distribution we calculated the mean value and the SD. We repeated this process for each of the 81 different combinations of experimental conditions. We refer to the difference between the mean value of the simulated distribution and the "true" value as the "mixing model bias". We refer to the SDs of the simulated distributions as "mixing model uncertainty".

Results and discussion

Small $[\text{CO}_2]_{\text{range}}$ regime

When used with the OLS regression approach, the Keeling and Miller-Tans models produced similarly biased estimates of $\delta^{13}\text{C}_R$ (Table 1) which is consistent with previous studies (Ohlsson et al. 2005; Zobitz et al. 2006). However, when used with the GMR regression approach, there is a distinct difference in the pattern of bias between the mixing models, a relationship that has not previously been discussed in the literature (Fig. 2). The largest difference in $\delta^{13}\text{C}_R$ estimates between the Keeling and Miller-Tans models was 3.4‰ and the Keeling model consistently produced more negatively biased estimates of $\delta^{13}\text{C}_R$ with respect to the Miller-Tans model. As Fig. 2 exemplifies, if we assume that the $\delta^{13}\text{C}$ error is small then the Keeling-GMR estimate will provide a more accurate estimate of $\delta^{13}\text{C}_R$ and conversely, if the error in $\delta^{13}\text{C}$ is large, on the

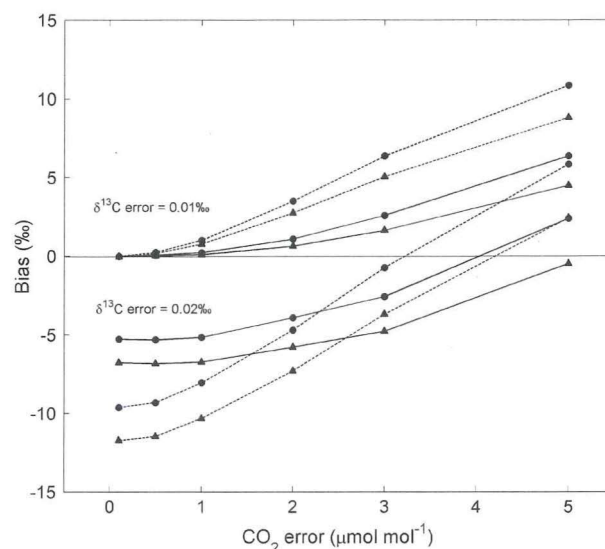


Fig. 2 Patterns of stable C isotope composition of respiration ($\delta^{13}\text{C}_R$) estimate bias simulated from Miller-Tans (circle) and Keeling (triangle) models used with geometric mean regression (GMR). Bias as a function of $[\text{CO}_2]$ measurement error (x -axis) and varying levels of ^{13}C error (0.01 and 0.2‰), sample size [$n = 5$ (solid line) and 21 (dashed line)], with a $[\text{CO}_2]_{\text{range}}$ of $10 \mu\text{mol mol}^{-1}$. Bias is calculated as the difference between $\delta^{13}\text{C}_R$ defined by the truth model and the average of the mixing-model simulation distributions

order of 0.2‰, then the Miller-Tans model is more accurate at low levels of CO_2 error.

The bias of small $[\text{CO}_2]$ range regimes is highly influenced by the relative levels of $\delta^{13}\text{C}$ error and $[\text{CO}_2]$ error, levels of error that are addressed differently by each regression approach due to their underlying assumptions. GMR assumes that error is present in both the x and y variables of the regression. If the variance of the error distribution is similar for both x and y , which is usually assumed in the use of GMR (Legendre and Legendre 1998; McArdle 2003), then the GMR estimate has a small bias. This relationship is seen in online resource.3a (ESM),

Table 1 Bias of estimates of the stable C isotopic composition of respiration ($\delta^{13}\text{C}_R$; ‰) for difference ranges of small $[\text{CO}_2]$ ($[\text{CO}_2]_{\text{range}}$) and large $[\text{CO}_2]$ ($\mu\text{mol mol}^{-1}$) regimes

Mixing model		K.GMR			K.OLS			MT.GMR			MT.OLS		
Regime	Range	Min.	Average	Max.	Min.	Average	Max.	Min.	Average	Max.	Min.	Average	Max.
Small $[\text{CO}_2]_{\text{range}}$	10	-11.73	-0.80	8.81	0.00	4.40	13.97	-9.61	0.48	10.85	0.00	4.40	13.97
	40	-1.04	-0.07	1.16	-0.03	0.49	2.44	-0.76	0.08	1.51	-0.03	0.49	2.43
	100	-0.21	-0.03	0.19	-0.02	0.08	0.42	-0.14	0.01	0.26	-0.02	0.07	0.41
Large $[\text{CO}_2]_{\text{range}}$	1,000	-0.56	0.02	0.90	-0.39	0.17	1.75	-0.22	0.05	0.57	-0.11	0.12	0.92
	5,000	-0.03	0.00	0.04	-0.02	0.00	0.09	-0.01	0.00	0.01	-0.01	0.00	0.02
	10,000	-0.01	0.00	0.01	-0.01	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.01

Estimates were made by using simulated data with different combinations of mixing models [Keeling (K) and Miller-Tans (MT)] and regression approaches [ordinary least squares (OLS) and geometric mean regression (GMR)], *Min.* Minimum, *Max.* maximum

where the $[\text{CO}_2]$ error and $\delta^{13}\text{C}$ error change at an optimal rate, resulting in a bias close to zero. When we simulated estimates using the largest level of $[\text{CO}_2]$ error and smallest level of $\delta^{13}\text{C}$ error, the bias was consistently positive and conversely, the bias was negative when the isotopic error was large and the CO_2 error was small.

A positive bias also occurred with the OLS estimator. In general the OLS regression (with either the Keeling or Miller-Tans mixing models) produced a systematic positive bias that increased with increasing $[\text{CO}_2]$ error. A bias in excess of 1‰ is possible at $[\text{CO}_2]$ accuracies on the order of $1 \mu\text{mol mol}^{-1}$ and a small concentration range. For example, when $[\text{CO}_2]_{\text{range}}$ is small we show the bias can be as great as 14‰; however, the bias is improved to within 2.5‰ with a moderate increase in the concentration range ($40 \mu\text{mol mol}^{-1}$ in this study). This positive bias in the OLS regression estimates can be partly understood by the assumptions behind the regression approach. For OLS we assume that measurements of x are made without error. This assumption works well when $[\text{CO}_2]$ measurement error is low; however, when random error is introduced, variation in x causes the estimated covariance between x and y to be less than the expected covariance value, causing the estimated slope to be too small and ultimately resulting in a regression slope closer to zero (McArdle 2003). In the case of small $[\text{CO}_2]_{\text{range}}$ regimes, a mixing model with a regression slope closer to zero translates to a positively biased estimate of its isotopic signal.

Similar to the model bias results, the OLS approach used with either the Keeling or Miller-Tans mixing model produced the most precise estimates of $\delta^{13}\text{C}_R$ (Table 2). For all models, the SD of the model estimates improved with an increase in the concentration range and sample size (online resource.3c-d, ESM). We found a dynamic interaction between $[\text{CO}_2]$ error and $\delta^{13}\text{C}$ error toward explaining model precision. Precision decreased linearly with an increase in $\delta^{13}\text{C}$ error when the level of CO_2 error was low. Conversely, when $\delta^{13}\text{C}$ error was low the SDs increased logarithmically with CO_2 error and appeared to

reach a maximum within the range of CO_2 error levels we used. Intermediate values of $\delta^{13}\text{C}$ and CO_2 error led to greater SDs that were primarily driven by the level of $\delta^{13}\text{C}$ error. The precision of OLS $\delta^{13}\text{C}_R$ estimates will therefore improve with large $[\text{CO}_2]_{\text{range}}$ and accurate measures of $\delta^{13}\text{C}$.

Large $[\text{CO}_2]_{\text{range}}$ regimes

Overall, the bias of the estimates for the simulated large $[\text{CO}_2]_{\text{range}}$ regimes was small with a range of -0.004 to 0.173‰ on average (Table 1). When the results are classified by concentration regime, sample size, CO_2 error, $\delta^{13}\text{C}$ error or and mixing model combination then patterns in bias are more apparent (online resource.3b, ESM). The greatest absolute bias occurred with the Keeling mixing-model used with either regression approach while the minimum absolute bias occurred with the Miller-Tans mixing model implemented with the GMR regression approach. Importantly, for large $[\text{CO}_2]_{\text{range}}$ regimes the mixing model-regression combination does not behave similarly to that for small $[\text{CO}_2]_{\text{range}}$ regimes.

In contrast to the small $[\text{CO}_2]_{\text{range}}$ regimes, the Keeling and Miller-Tans models used with the OLS regression did not yield equivalent results and the Keeling estimate, in general, had the greatest bias. In Fig. 3, the increase in Keeling bias used with both regression models is shown in relation to the different levels of CO_2 and $\delta^{13}\text{C}$ error. The GMR approach was consistently the least biased when the sample size was greater than three. When we compare the Miller-Tans and the Keeling model applied with the GMR approach (Fig. 4) we can see that the Keeling model is both the most positively and negatively biased estimator when sample size is equal to three or ten. The level of $[\text{CO}_2]$ error was the primary driver of bias and precision in the simulations in contrast to the small $[\text{CO}_2]_{\text{range}}$ regime where the interaction of both CO_2 and ^{13}C error influenced model bias.

The precision of the model estimate was greatly influenced by the $[\text{CO}_2]_{\text{range}}$ (Table 2). For a $[\text{CO}_2]_{\text{range}}$

Table 2 SD of $\delta^{13}\text{C}_R$ (‰) for different concentration ranges of small $[\text{CO}_2]_{\text{range}}$ and large $[\text{CO}_2]_{\text{range}}$ ($\mu\text{mol mol}^{-1}$) regimes

Mixing model	Range	K.GMR			K.OLS			MT.GMR			MT.OLS		
		Min.	Average	Max.	Min.	Average	Max.	Min.	Average	Max.	Min.	Average	Max.
Small $[\text{CO}_2]_{\text{range}}$	10	0.31	4.15	13.37	0.31	4.01	11.43	0.31	4.13	13.35	0.31	4.01	11.43
	40	0.08	1.35	4.01	0.08	1.32	3.84	0.08	1.35	3.98	0.08	1.32	3.84
	100	0.03	0.56	1.57	0.03	0.56	1.56	0.03	0.56	1.55	0.03	0.56	1.54
Large $[\text{CO}_2]_{\text{range}}$	1,000	0.01	1.50	4.17	0.01	1.44	3.95	0.01	1.01	3.15	0.01	0.99	3.08
	5,000	0.00	0.43	1.14	0.00	0.42	1.11	0.01	0.22	0.61	0.01	0.22	0.61
	10,000	0.00	0.24	0.69	0.00	0.24	0.67	0.01	0.13	0.35	0.01	0.13	0.35

Estimates were made by using simulated data with different combinations of mixing models (K and MT) and regression approaches (OLS and GMR). For abbreviations, see Table 1

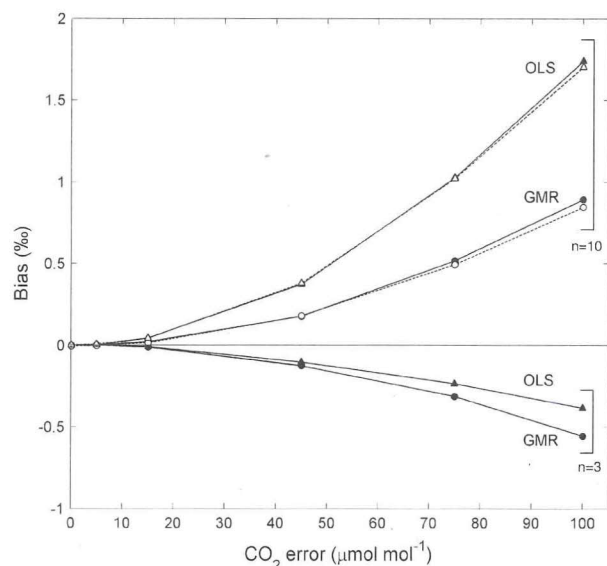


Fig. 3 Large $[\text{CO}_2]_{\text{range}}$ regime patterns of bias in simulated estimates of $\delta^{13}\text{C}_R$ from the Keeling mixing model used with the OLS and GMR regression approach. CO_2 error levels are listed along the x-axis, bias along the y-axis and varying levels of $\delta^{13}\text{C}$ error [0.01 (solid line and symbols) and 0.2‰ (dashed line and open symbols)] and sample size ($n = 3$ and 10), for a $[\text{CO}_2]_{\text{range}}$ of $1,000 \mu\text{mol} \cdot \text{mol}^{-1}$. Bias is calculated as the difference between $\delta^{13}\text{C}_R$ defined by the truth model and the average of the mixing-model simulation distributions. For abbreviations, see Fig. 1

$> 5,000 \mu\text{mol} \cdot \text{mol}^{-1}$, the maximum SD was 1.13‰ or less and for a $[\text{CO}_2]_{\text{range}}$ of $1,000 \mu\text{mol} \cdot \text{mol}^{-1}$ the maximum SD was 4.17‰ . Similar to the patterns in bias, precision was primarily driven by $[\text{CO}_2]$ error (online resource.3d, ESM). Overall, the Miller-Tans mixing model implemented with the GMR regression approach consistently produced the most precise estimate of $\delta^{13}\text{C}_R$.

Sample size effect

Pataki et al. (2003) reported that simply increasing sample size does not improve the estimate of $\delta^{13}\text{C}_R$. We found for all simulations the $\delta^{13}\text{C}_R$ bias actually increased with the number of samples used in the mixing model. We hypothesized this phenomenon was an artifact from generating datasets of size n equidistantly across the determined $[\text{CO}_2]_{\text{range}}$ in CO_2 space rather than $1/[\text{CO}_2]$ space; the dimension used for Keeling plots. We present results from a simulation addressing this point in online resource.4 (ESM).

The increase in bias with sample size is not explained by transformations in x (i.e., $[\text{CO}_2]$) but is rather a function of variance in the x variable in the regression as described in the statistical literature. This phenomenon is known to occur in OLS regression (Mandansky 1959; Stefanski 1985) and we suspect a similar phenomenon for GMR. The

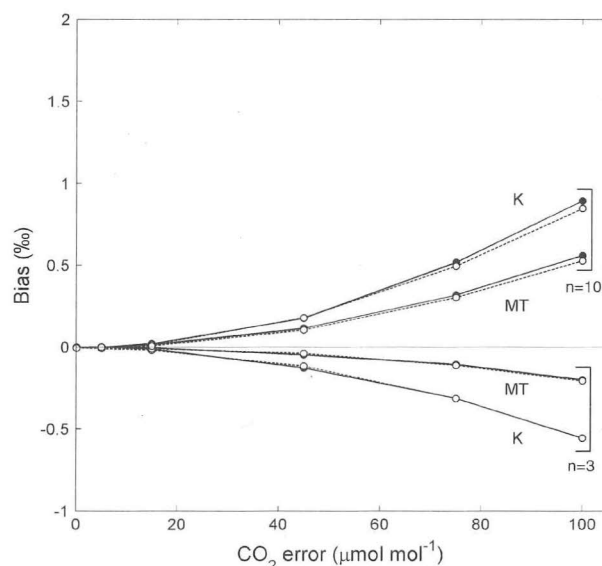


Fig. 4 Large $[\text{CO}_2]_{\text{range}}$ regime patterns of bias in simulated estimates of $\delta^{13}\text{C}_R$ from the Keeling (K) and Miller-Tans (MT) mixing models used with the GMR regression approach. $[\text{CO}_2]$ measurement error levels are listed along the x-axis, bias along the y-axis and varying levels of $\delta^{13}\text{C}$ [0.01 (solid line and symbols) and 0.2‰ (dashed line and open symbols)], and sample size ($n = 3$ and 10), for a $[\text{CO}_2]_{\text{range}}$ of $1,000 \mu\text{mol} \cdot \text{mol}^{-1}$. Bias is calculated as the difference between $\delta^{13}\text{C}_R$ defined by the truth model and the average of the mixing-model simulation distributions

explanation for the case of OLS is as follows. In the presence of measurement error in the covariate, the OLS slope estimator is biased for the true slope B , but it is a consistent estimator for:

$$\beta^+ = \beta^* \left(\frac{\sigma_x^2}{\sigma_x^2 + \sigma_e^2} \right) \quad (5)$$

where σ_x^2 is the marginal variance of x and σ_e^2 is the measurement error variance of the covariate (Mandansky 1959; Stefanski 1985). By "marginal variance" we refer to the variance that is present in the values of x ignoring the values of y . A negatively biased estimate of the slope parameter leads to a positively biased estimate of the intercept parameter or, in the case of the Keeling model, the estimate of $\delta^{13}\text{C}_R$.

In our simulation we chose to select either evenly spaced values of $[\text{CO}_2]$ along the $[\text{CO}_2]_{\text{range}}$ or evenly spaced values of $1/[\text{CO}_2]$ along $1/[\text{CO}_2]_{\text{range}}$ and we used one observation of $\delta^{13}\text{C}$ per value of $[\text{CO}_2]$ (i.e., we did not have replicate values). Therefore, the marginal variance of $1/[\text{CO}_2]$ is as large as it can be when the sample size is 2 and this marginal variance decreases as the sample size increases. As the sample size increases, the marginal variance decreases resulting in an estimate of β^+ less than B and an intercept greater than x ; ultimately, leading to an increase in bias. The results of a simulation where

Table 3 Simulation results for Eq. 5 in which uncertainty in $[\text{CO}_2]$ was $5 \mu\text{mol mol}^{-1}$ and the $[\text{CO}_2]_{\text{range}}$ was $40 \mu\text{mol mol}^{-1}$

Sample size	Estimated ^a marginal variance	Estimated ^b measurement error	$\left(\frac{\hat{\sigma}_x^2}{\hat{\sigma}_x^2 + \hat{\sigma}_e^2} \right)$	$\hat{\beta}^+$	Estimated ^c bias
3	1.57E-08	3.96E-11	0.9974878	7,481.158	0.05
13	6.61E-09	3.95E-11	0.9940549	7,455.411	0.11
21	6.04E-09	3.94E-11	0.9935109	7,451.332	0.12
50	5.55E-09	3.96E-11	0.9929155	7,446.866	0.13
100	5.39E-09	3.94E-11	0.9927433	7,445.575	0.14
1,000	5.24E-09	3.94E-11	0.9925374	7,444.031	0.14

^a Marginal variance estimated by the sample variance of $1/[\text{CO}_2]$

^b Estimated measurement error in $1/[\text{CO}_2]$ is based on the uncertainty in CO_2 of $5 \mu\text{mol mol}^{-1}$

^c Bias in the estimate of $\delta^{13}\text{C}_R$

$[\text{CO}_2]_{\text{range}}$ is $40 \mu\text{mol mol}^{-1}$, and the uncertainty in $[\text{CO}_2]$ is $5 \mu\text{mol mol}^{-1}$ are summarized in Table 3.

Recommendations

For relatively small $[\text{CO}_2]_{\text{range}}$ regimes, OLS is consistently the least biased estimator when $[\text{CO}_2]$ error is low, conditions for which GMR will generally have a negative bias. Zobitz et al. (2006) reported that measurement with a small $\delta^{13}\text{C}$ error is essential to achieve estimates of $\delta^{13}\text{C}_R$ with acceptable bias and uncertainty. Their analyses were restricted to conditions similar to those we used for simulations of small $[\text{CO}_2]_{\text{range}}$ regimes. In contrast to their results we found that estimates of $\delta^{13}\text{C}_R$ for large $[\text{CO}_2]_{\text{range}}$ regimes were improved by minimizing $[\text{CO}_2]$ error rather than minimizing $\delta^{13}\text{C}$ error. We consider the error levels used in our simulations to be conservative. The $[\text{CO}_2]$ error was on the order of 1–2% for the large $[\text{CO}_2]_{\text{range}}$ regimes. Our study illustrates that uncertainty in calibration gas could potentially have a significant impact on $\delta^{13}\text{C}_R$ estimates. Thus, researchers conducting studies involving large $[\text{CO}_2]_{\text{range}}$ regimes should strive to use standard gases with small $[\text{CO}_2]$ uncertainty. For instance, gases from the National Oceanic and Atmospheric Administration (NOAA) are accurate to within $0.07 \mu\text{mol mol}^{-1}$ for gas concentrations representative of atmospheric ambient conditions (Zhao and Tans 2006) while gas concentrations above $500 \mu\text{mol mol}^{-1}$ are expected to decrease in uncertainty at a rate of 0.01% $[\text{CO}_2]$ (Duane Kitizis, personal communication). Not all standards are equal, for instance gases available through industrial distributors are commonly accurate to $\pm 2\%$ of the target value and even secondary or field standards have been shown to drift up to $5 \mu\text{mol mol}^{-1}$ over time (Griffis et al. 2004). In large and small $[\text{CO}_2]_{\text{range}}$ applications, the actual error encountered in measuring the $[\text{CO}_2]$ may be larger than the reported precision of the

instrument due to the uncertainty in the standard gasses or additional measurement error incurred during sampling. Therefore, it is prudent to expect a relatively high $[\text{CO}_2]$ error in large $[\text{CO}_2]_{\text{range}}$ regimes, in which case the Miller-Tans-GMR mixing model had the smallest range in bias for the range of simulated conditions.

Bias and uncertainty were relatively insensitive to $\delta^{13}\text{C}$ error in large $[\text{CO}_2]_{\text{range}}$ regimes, most likely due to the overwhelming influence of $[\text{CO}_2]$ error on $\delta^{13}\text{C}_R$. However, the estimates of $\delta^{13}\text{C}_R$ did vary with n . With low n ($n = 3$) $\delta^{13}\text{C}_R$ is negatively biased, although the absolute range of the bias is small. For $n > 3$, the range of the bias increased with increasing n , indicating that priority should be placed on increasing $[\text{CO}_2]_{\text{range}}$ and reducing $[\text{CO}_2]$ error rather than increasing n to improve the bias and uncertainty of $\delta^{13}\text{C}_R$.

Conclusion

There are inherent differences between small and large $[\text{CO}_2]_{\text{range}}$ regimes regarding measurement assumptions, the interaction between CO_2 and ^{13}C error, and mixing model performance, differences that warrant separate recommendations for making estimates of $\delta^{13}\text{C}_R$. Our simulation results are consistent with those of Zobitz et al. (2006), which recommend the use of the Keeling-OLS combination for ecosystem respiration. For systems with large $[\text{CO}_2]_{\text{range}}$ (e.g., soil, tree stem, foliar respiration), many of the model combinations are functionally unbiased; however, the Miller-Tans-GMR mixing model is the least biased and most precise at moderate concentration ranges for situations when error is present in either $\delta^{13}\text{C}$ or $[\text{CO}_2]$ values.

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