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Sources of Variability that Compromise Mineralizable Carbon as a Soil Health Indicator

Jordon Wade*
Steven W. Culman
Tunsisa T. Hurisso
Ohio State Univ.
School of Environ. & Natural Resources
1680 Madison Avenue
Wooster, OH 44691

Robert O. Miller
Dep. of Soil and Crop Sciences
Colorado State Univ.
Fort Collins, CO 80523

Lucas Baker
Brookside Laboratories
New Bremen, OH 45869

William R. Horwath
Dep. of Land Air, and Water Resources
Univ. of California-Davis
One Shields Avenue
Davis, CA 95616

Mineralizable C, or C that is respired upon the rewetting of dried soil, is a common metric of soil health, but the metric still lacks a widely accepted and standardized protocol. A standardized protocol is an essential first step in quality control needed for a robust soil test. Here we examined numerous sources of laboratory variability associated with mineralizable C, with the overall goal of understanding the influence of each source on final values. Mineralizable C had twofold to 20-fold greater inter-laboratory variability than other commonly used soil tests, leading to a high degree of uncertainty associated with the interpretation of results. Procedural differences—such as sieve size and the method of rewetting—significantly influenced measurements of mineralizable C and underscore the need for the development of a standardized and universally adopted protocol. Capillary rewetting consistently suppressed mineralizable C relative to rewetting with a specific amount of water and is therefore not a recommended approach. However, the sensitivity of mineralizable C to changes in management did not differ among incubation intervals of 6, 24, and 72 h. While these procedural effects may influence inter-laboratory variability, there was also a considerable amount of analytical variability associated with mineralizable C measurements within a laboratory that is highly dependent on soil type.

Abbreviations: ALP, Agricultural Proficiency Laboratory; CV, coefficient of variation; WHC, water-holding capacity.

Core Ideas

- **Inter-laboratory variability for mineralizable C is greater than for other commercial soil tests.**
- **Water content and direction of rewetting both affect values of mineralizable C.**
- **As a soil health indicator, mineralizable C should have a standardized protocol.**
- **Analytical variability of mineralizable C is highly affected by soil type.**

The development of commercially viable soil health testing focused on biological properties is an essential step for improving the sustainability of our agricultural production systems (Kibblewhite et al., 2008). The burst of respiration on rewetting of air-dried soil, commonly referred to as "the Birch effect" (Birch, 1959) or the "flush of CO_2 on rewetting" (Franzluebbers et al., 2000), hereafter referred to as 'mineralizable C', is a potentially valuable tool in helping growers better understand the role that the microbial community plays in their soil (Franzluebbers, 2016). Mineralizable C has been widely accepted as an important metric of the overall health and quality of a soil (Karlen et al., 1997; Moebius-Clune et al., 2016) and has been used as an integrated measurement of soil microbial biomass (Anderson and Domsch, 1978), microbial activity (Wang et al., 2003), and soil carbon availability (Franzluebbers et al., 2000; Wang et al., 2003).

The strong response from growers for currently available commercial tests of mineralizable C (e.g., gel paddle field test, Solvita and Woods End Laboratories, Mt. Vernon, ME) illustrates the demand for a rapid measure of soil biological activity and health. Additionally, governmental institutions have also begun to support the use of mineralizable C to measure improvements in soil quality and have established incentive programs for growers to use respiration measurements to track changes in their fields after improvements in management (USDA–NRCS, 2015). However, integrating biology—a central component of the framework of soil

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*Corresponding author (jordonwade@gmail.com).

health (Doran and Zeiss, 2000)—results in increased complexity and, as with any new method, additional caution must be exercised on the interpretation and use of the results. Mineralizable C, has been used extensively in research trials and although it has been shown to consistently differentiate among imposed treatment effects on a given soil type, there is no recognized standard operating procedure that has been utilized across soil types. Standardization is one of many essential steps in the creation of a robust soil health indicator that can be translated across systems, soil type, and commercial test laboratories.

As with any laboratory metric, there are many potential sources of variation in mineralizable C measurements. One substantial source of variability is inter-laboratory variability, which is the basis for testing laboratory proficiency. However, proficiency testing assumes a standardization of methods among laboratories, which has not been the case thus far in multiple studies surrounding mineralizable C measurements. Variations in the methodology have included: sieve sizes ranging from 2 to 6 mm (Franzluebbers et al., 2000; Castro Bustamante and Hartz, 2016; Franzluebbers, 2016; Morrow et al., 2016), incubation intervals ranging from 6 to 72 h (Franzluebbers et al., 2000; Haney and Haney, 2010; Wade et al., 2016), and differences in the direction and final water content on rewetting (Haney and Haney, 2010; Sherrod et al., 2012; Wade et al., 2016). Therefore, it is also necessary to investigate how any procedural or methodological differences may contribute to the variability of mineralizable C. Given that mineralizable C is a biologically influenced metric, investigation of this variability is particularly salient when attempting to draw robust and accurate conclusions. Included in the consideration of methodology is the length of incubation for mineralizable C measurements, which has also differed among studies (Franzluebbers et al., 2000; Haney et al., 2008a; Wade et al., 2016).

The ultimate goal of a universal protocol would be to minimize sources of unwanted variability so that the use of mineralizable C as a metric of soil health would be as robust as possible. Therefore, this study seeks to: (i) assess inter-laboratory and analytical agreement for current commercially available mineralizable C tests; (ii) evaluate the effects of methodological differences—such as soil sieve size, water content, and direction of rewetting—on mineralizable C values; and (iii) determine the length of incubation that is most sensitive for detecting treatment and/or management differences.

MATERIALS AND METHODS

Data Description

Our analysis included soil from eight studies on 72 agricultural cropland sites from across the United States (Table 1). In addition to traditional soil measurements (Table 2) mineralizable C was measured using permutations of soil processing and rewetting protocols (n = 1142 individual observations) to determine the sources of variation associated with these procedures (sieve size, water content, direction of rewetting). Additionally, selected studies were used to determine the analytical and inter-laboratory variability associated with measurements of mineralizable C. A description of methods and analyses performed on each study is given in Table 3.

Soil Analyses

Soil physiochemical characteristics, such as pH, textural characteristics, and soil C and N contents are listed in Table 2. To determine the effect of grinding or sieve size on mineralizable C, soils from the New York Grain study were air-dried and either hand-sieved to <8 mm, hand-sieved to <2 mm, or ground to <0.75 mm. Soils from the Agricultural Proficiency Laboratory (ALP) were either ground to <2 mm or to <0.8 mm with an Agvise flail mill. Soil water-holding capacity (WHC) was calculated as the difference in weight between a saturated soil that was allowed to drain for an hour and the weight after the soil was oven-dried for 24 h at 105°C.

Mineralizable Carbon

Mineralizable C measurements were taken during incubations ranging from 6 to 72 h of 10 to 40 g of air-dry soil. The amount of soil used for each incubation was consistent within each study. For all studies excepting the ALP study, gas samples were collected by extracting 1 to 5 mL from the headspace of a 0.4-L Mason jar capped with a metal lid and a butyl rubber septum, and these were run on an infrared gas analyzer (model S-151, Qubit Systems Inc., Kingston, Canada). Mineralizable C was calculated as the difference between a sample and a control, using the total headspace and the ideal gas law (Zibilske, 1994) at a constant temperature of 22°C. In the ALP study, mineralizable C was measured by gel paddles (Solvita and Woods End Laboratories, Mt. Vernon, ME) at a constant temperature of 23°C. Rewetting of the air-dried soil was done either through capillary rewetting from below using the methods described in Haney and Haney (2010) or by adding a percentage (25, 50, 75, or 100%) of the calculated WHC with deionized water dispensed directly on to the soil surface with a micropipette. In addition to rewetting from above, air-dried soil samples were also rewetted at 50% WHC from the bottom to assess the effect of direction of rewetting on mineralizable C. For rewetting from the bottom, 50-mL polypropylene beakers with four to five 6.5-mm diameter holes drilled in the bottom and glass microfiber filter were filled with soil and placed in the microcosm, which had been filled with the appropriate amount of deionized water. All measurement methods (i.e., incubation length and instrumentation) are presented in Table 3. Depending on the study, soils were air-dried and stored at room temperature before mineralizable C analyses were performed. Storage time ranged and generally clustered into three groups: <1 yr (California Grower Survey, West Side Research and Extension Center, and Russell Ranch Sustainable Agriculture Facility), 2 to 4 yr (California Tomato Survey, Ohio Urban Garden, and Windsor Organic Research Trial), or 9 to 11 yr (New York Grain and select soils from the ALP study). While the long-term storage of air-dried soil is known to increase the rewetting effect on mineralizable C measurements (De Nobili

Table 1. Description of study sites used for comparative analyses and their associated references.

Study and abbreviation	Description	State and location	No. of sites	No. of plots per site	Reference
Agricultural Laboratory Proficiency (ALP)	Soils from across the United States and Canada processed similarly to assess laboratory variability	Numerous†‡	27	1	None
California Grower Survey	Survey of grower fields across four growing regions of CA using mineral fertilizer with and without cover crops	California 38°37′ to 36°49′ N, 121°51′ to 19°49′ W	21	3–4	Wade et al., 2016
California Tomato Survey	Organically managed tomato fields using compost and manures as fertilizer sources	California 38°33′ to 38°51′ N, 121°48′ to 122°12′ W	13	1	Bowles et al., 2014, 2015
New York Grain	Grain farms across a management-induced soil fertility gradient	New York 42°36′ to 42°44′ N, 76°42′ to 77°03′ W	7	1–6	Schipanski et al., 2010; Schipanski and Drinkwater, 2011
Ohio Urban Garden	Urban garden using compost, compost + biochar, or compost + sudangrass cover crop	Ohio 41°04′49″ N, 80°40′35″ W	1	24	Beniston et al., 2016
Russell Ranch Sustainable Agriculture Facility)	Long-term research trial involving corn-tomato rotations with mineral fertilizer, mineral fertilizer + leguminous cover crops, or leguminous cover crops + compost and/or manure	California 38°32′ N, 121°52′ W	1	9	Wade et al., 2016
West Side Research and Extension Center	Research plots with 15 yr of minimal vs. conventional tillage, with and without cover crops	California 36°20′ N, 120°7′ W	1	21	Mitchell et al., 2015
Windsor Organic Research Trial	Organic conversion trial with cropland converted from perennial ley, vegetable crops, or row crops, with compost, manure, or cover crop organic additions	Illinois 40°06′ N, 88°16′ W	1	36	Ugarte and Wander, 2013

† ALP laboratory samples for inter-laboratory variability were from Arizona, British Columbia, Alabama, California, Connecticut, Florida, Idaho, Iowa, Kansas, Maine, Minnesota, Montana, Nebraska, Ontario, Quebec, South Carolina, South Dakota, Texas, and Wisconsin.

‡ ALP laboratory samples for sieve size, water content, direction of water addition, and analytical variability were from Iowa, Montana, Nebraska, North Dakota, Ohio, Saskatchewan, and Texas.

et al., 2006; Kaiser et al., 2015), for this study we assumed that any artifacts due to sample storage would equally influence all treatments within a study. We did not find any evidence that storage increased mineralizable C amounts ($p = 0.47$; data not shown) or variability. To further ensure that differences in storage time did not impact our results, all statistical analyses were constrained to individual studies; when comparing data across multiple studies, we used 'study' as a covariate in the analysis.

Statistical Analyses

All statistical analyses were performed in RStudio (RStudio Team, 2016). Linear regressions were run using the *lm()* command. To obtain *F*-values and p-values for associated differences, *anova()* in the *car* package (Fox and Weisberg, 2011) was used to perform a Type II ANOVA. This type of ANOVA only tests each effect after the other effects are accounted for, and this results in a more conservative attribution of significance than

Table 2. Soil physical and chemical characteristics for each study. Values are mean (minimum, maximum).

Study and abbreviation	Soil organic C	Total N	C/N	pH (1:1 soil/water)	Clay	Sand
	g kg^{-1} soil				g kg^{-1} soil	
Agricultural Laboratory Proficiency	18.1 (4.2, 55.7)	1.6 (0.3, 4.1)	11.1 (7.1, 13.5)	6.3 (4.6, 8.1)	195.1 (39.0, 320.0)	485.5 (128.7, 919.3)
California Grower Survey	9.4 (3.7, 19.7)	1.0 (0.4, 1.7)	9.0 (6.7, 13.5)	7.1 (5.1, 8.4)	329.6 (76.8, 608.0)	508.8 (244.8, 910.4)
California Tomato Survey	13.3 (5.9, 22.1)	1.5 (0.7, 2.2)	8.9 (7.6, 10.5)	6.7 (6.1, 7.3)	158.1 (88.7, 222.1)	264.4 (133.4, 461.4)
New York Grain	19.0 (12.9, 26.8)	1.7 (1.2, 2.7)	11.1 (9.7, 12.9)	7.0 (6.2, 7.8)	273.9 (169.0, 369.5)	436.1 (336.5, 543.0)
Ohio Urban Garden	56.0 (10.4, 112.6)	4.1 (0.8, 8.3)	13.6 (12.2, 15.5)	7.7 (7.4, 8.0)	168.0	36.8
Russell Ranch Sustainable Agriculture Facility	10.7 (5.6, 15.4)	1.2 (0.5, 2.0)	9.4 (7.3, 11.9)	7.2 (6.5, 8.2)	323.2 (147.2, 390.4)	404.9 (132.8, 651.2)
West Side Researchand Extension Center	6.2 (4.7, 8.0)	0.7 (0.5, 0.9)	8.9 (7.6, 10.5)	7.4 (6.7, 7.8)	358.8 (259.2, 531.2)	444.4 (356.8, 558.4)
Windsor Organic Research Trial	23.1 (13.4, 33.1)	1.8 (1.1, 2.3)	12.9 (11.4, 15.7)	nd†	nd	nd

† nd, not determined.

Table 3. Studies and methods used to investigate sources of variability in measurements of mineralizable C.

Source of variability	Table or figure in present article	Study used†	Sample size	Measurement method‡	Length of incubation
			n		h
Inter-laboratory variability	Figure 1	CGS	28	IRGA (analytical laboratory§), Gel paddles (commercial laboratories)	24
Inter-laboratory variability	Table 4	ALP	480	Gel paddles	24
Sieve size	Table 5	ALP, NYG	585	IRGA (NYG), Gel paddles (ALP)	24
Water content	Table 6	RRSAF, WSREC	30	IRGA	6, 24, 72
Direction of rewetting	Table 7	ALP	126	Gel paddles	24
Incubation length/ sensitivity analysis	Table 8	CGS¶, CTS¶, OUG¶, RRSAF¶, WSREC¶, WORT¶	452	IRGA	6, 24, 72
Analytical variability	Table 9	ALP, NYG, OUG	219	IRGA (NYG, OUG), Gel paddles (ALP)	24

† Study abbreviations: ALP, Agricultural Laboratory Proficiency; CGS, California Grower Survey; CTS, California Tomato Survey; NYG, New York Grain; OUG, Ohio Urban Garden; RRSAF, Russell Ranch Sustainable Agriculture Facility; WSREC, West Side Research and Extension Center; WORT, Windsor Organic Research Trial.

‡ IRGA, infrared gas analyzer; Gel paddles, field test product of Solvita and Woods End Laboratories, Mt. Vernon, ME.

§ Analytical laboratory at Univ. of California-Davis.

¶ Data have been previously published in part or in full in Hurisso et al. (2016).

other methods of calculation (Langsrud, 2003). For all lettered differences, Tukey's HSD test was performed using the *HSD.test* command in the *agricolae* package (de Mendiburu, 2016). The sensitivity analysis for mineralizable C incubation length was performed using the *aov()* and the conservative Type II *Anova()* command in the *car* package, with the experimental factors (e.g., tillage, management practices) modeled as predictor variables. Three separate analyses were run with the incubation length (6, 24, or 72 h) as the response variable. The corresponding *F*-values were then representative of the magnitude of the effect exhibited by the predictor variable (experimental factors) on the response variable (incubation length).

To assess analytical and inter-laboratory variability, each soil was run in triplicate for each laboratory × treatment combination. The analytical variability among these replicates, represented as the coefficient of variation (CV), was calculated using the standard deviation normalized by the mean. To investigate the effect of these methodological differences on analytical variability (e.g., if sieving alters the analytical variability of mineralizable C), the CV values were used as a response variable and the treatments—study, site and/or field within a study, sieving, water content, and direction of water addition—were used as predictor variables. Analytical variability from the treatments was determined in two separate laboratories (Table 3) and the effects were nested within these laboratories to isolate from any inter-laboratory variation in these measurements. To calculate inter-laboratory variability, three replicates were averaged to obtain the mean mineralizable C values for a given soil from that laboratory. This value was then combined with the means obtained in other laboratories to calculate the inter-laboratory variability (expressed as a CV-value) for that soil. The CV for inter-laboratory variability was calculated using the median in place of the mean due to the high degree of skewness in the distribution. Similar to prior analyses, *F*-values and p-values were obtained using the *aov()* command and the conservative Type II ANOVA using the *Anova()* command in the *car* package.

RESULTS AND DISCUSSION

Inter-laboratory Variability

Low inter-laboratory variability is a primary criterion for a robust soil health metric. If different laboratories return different values for the same soil sample, the efficacy of that data is greatly diminished. Although numerous studies have shown mineralizable C to be sensitive to management practices and outcomes (Fraser et al., 1988; Franzluebbers et al., 2000; Haney et al., 2001; Schomberg et al., 2009; Culman et al., 2013; Castro Bustamante and Hartz, 2016; Wade et al., 2016), these relative differences, expressed via linear correlations, are not enough to meet the criteria of repeatability necessary for a soil health metric. Therefore, assessing absolute differences among laboratories is essential. To study this difference, seven independent soil samples were analyzed for mineralizable C at three certified (Solvita Partner Plus, Woods End Laboratories, Mt. Vernon, ME) commercial laboratories and one analytical laboratory (Univ. of California-Davis) (Table 3). Comparison of these results show that although there were moderately strong linear relationships, there were significant absolute differences among laboratories (Fig. 1). In four of the six comparisons, there were considerable absolute differences ($p < 0.01$) among the values obtained by different laboratories. However, there was no clear relationship between the strength of linear relationship (R^2 values) and mean absolute differences among laboratories, with the greatest R^2 value ($R^2 = 0.77$) corresponding to a highly significant absolute difference ($p < 0.0001$) and the lowest R^2 value ($R^2 = 0.50$) corresponding to the statistically insignificant absolute difference ($p < 0.05$). Some differences in absolute mineralizable C values may be attributable to the equipment differences between the infrared gas analyzer (IRGA) used in the Univ. of California-Davis laboratory and the gel paddles used in commercial laboratories (Table 3). This is supported by the similar slopes in the relationships between the laboratory at Univ. of California-Davis and the commercial laboratories.

However, the differences between regression lines and 1:1 lines that persist among commercial laboratories suggest that

there is also significant amount of uncontrolled error associated with the use of gel paddles (Fig. 1). Previous work has shown strong linear relationships (R^2 > 0.90) among traditional methods of measuring mineralizable C, such as IRGA, gas chromatography, and a NaOH base trap (Haney et al., 2008b; Sherrod et al., 2012). However, these strong relationships are not shared in the relationships between the gel paddle and the traditional methods of measuring respiration, such as NaOH base trap (R^2 = 0.82) and IRGA (R^2 = 0.79) from Haney et al. (2008b) and with NaOH base traps (R^2 = 0.84) in Haney et al. (2008a). Since the slopes and intercepts of the methods of NaOH and IRGA were comparable across studies (Haney et al., 2008a, 2008b; Sherrod et al., 2012), the IRGA-measured mineralizable C measurements should be considered the more consistent of the two methods (Table 3). In addition to the vetting and adoption of consistency in instrumentation, the focus of future study should be on establishing agreement in absolute rather than relative terms, which will be crucial to begin translating mineralizable C values from one laboratory to values obtained in another laboratory. Accordingly, the remainder of this paper will focus on variations and aberrations in absolute values of respiration rather than correlative values.

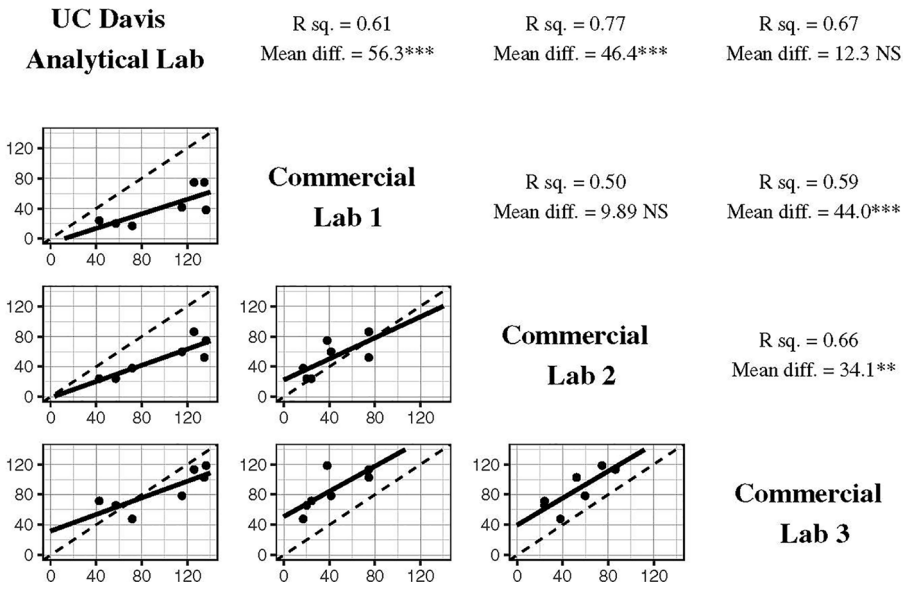

Fig. 1. Differences in 24-h mineralizable C values (mg CO_2–C kg^{-1} air-dried soil) obtained in three commercial laboratories and one analytical laboratory. Dashed line represents a 1:1 relationship, indicating perfect agreement of values. Mean diff. is the average difference among laboratories for a sample, with associated significance obtained by *t* test. Asterisks (*, **, and *) indicate significance at the $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively. NS, no significant difference.**

To determine the expected variation in mineralizable C values among laboratories and compare this variability to other traditional soil metrics (total C, total N, pH, clay, etc.), a set of 20 soils was sent to commercial laboratories, where each measurement was run in triplicate for each soil. The mean value of these three analytical reps was then compiled across laboratories for each soil to determine the inter-laboratory CV for each soil. A wide range of variability was shown among laboratories measuring 24-h mineralizable C, with inter-laboratory CV values ranging from 4.21 to 53.17% across 20 soils. The mean inter-laboratory CV was greater than the median (Table 4), with a significant skewness value of 1.19 (p < 0.05; data not shown) for the sample size (Pearson and Hartley, 1970), both of which indicate a long right tail on the distribution of CV values. This suggests that inter-laboratory variability is not evenly distributed across all of the 20 soils, but that most of the variability was actually less than the mean value in Table 4 and that median values may be a more appropriate measure. The median inter-laboratory CV value for mineralizable C (16.0%) is 2.8- to 19.3-fold greater than the median inter-laboratory CV values for other commonly utilized soil test metrics, such as total C and total N on combustion (2.93% and 5.63%, respectively), pH (0.83%), or clay content (5.69%). Additionally, mineralizable C inter-laboratory CV was highly variable by soil, with CV values ranging from 4.2 to 53.2%. Taken together, mineralizable C measurements were much more variable among laboratories than other commercially available soil measurements, and this variability is likely soil-specific.

Table 4. Comparison of the inter-laboratory coefficient of variability (CV%) for *n* = 20 soils for 24-h mineralizable C and other commonly used laboratory procedures.

Metric and method†	No. of laboratories	Mean‡	Standard error	Median	Maximum	Minimum
Mineralizable C (gel paddles)	8	19.8	3.1	16.0	53.17	4.21
Total C (combustion)	13	10.3	7.3	2.9	149.19	1.06
Total N (combustion)	15	11.8	4.5	5.6	86.36	2.00
pH (1:1 soil/water)	59	0.9	0.1	0.8	1.59	0.54
Sand	26	4.4	0.8	3.6	16.48	0.80
Silt	26	5.7	0.8	4.9	17.10	2.28
Clay	26	7.6	1.1	5.7	20.33	2.26
Nitrate	42	5.0	0.4	4.4	10.19	2.93

† Each metric was run in triplicate in each laboratory, the mean of which was averaged with other laboratories to establish the inter-laboratory CV for each soil.

‡ Mean, the mean inter-laboratory CV for all 20 soil types.

Table 5. Mean values ± standard error of 24-h mineralizable C for the Agricultural Laboratory Proficiency (ALP) and New York Grain studies (NYG).

	n	Ground	2 mm	8 mm
		——— mg CO_2–C kg^{-1} soil ———		
ALP	63	51.1 ± 3.7 a†	50.6 ± 3.1 a	nd‡
NYG	151	75.4 ± 2.3 a	76.5 ± 2.4 a	64.7 ± 2.3 b

† Different lowercase letters within rows indicate significant differences ($p < 0.05$) by Tukey's HSD test.

‡ nd, not determined.

Sieve Size

Soil processing (e.g., sieving of air-dried soils) can alter the results obtained in analyses. Given that the susceptibility of soil C to mineralization is largely controlled by physical protection rather than chemical recalcitrance (Kleber et al., 2011; Dungait et al., 2012), we hypothesized that sieving soil to smaller size fractions would reduce the physical protection of soil C and result in higher values of mineralizable C. Finely ground soil (<0.75 and <0.8 mm in the New York Grain and ALP studies, respectively) and soil of <2 mm had similar mineralizable C values in both New York Grain and ALP studies, but soil that was sieved to <8 mm had a significantly ($p < 0.05$) lower values of mineralizable C relative to the other sieve sizes (Table 5). These results agree with previous findings by Franzluebbers (1999b) that the physical protection of mineralizable C reaches a threshold at 2 mm, and below this threshold, additional disturbances do not increase C mineralization. Additionally, our results show that the effect of sieve size on mineralizable C observed at 72-h by Franzluebbers (1999b) is also evident at 24 h (Table 5). Thus, sieving or grinding soil to different sizes can influence mineralizable C values, and standardization for soil processing will be required for a better comparison of treatment effects on soil health across studies.

Water Content

The water content of incubated soil had a significant effect on soil mineralizable C (Table 6). We observed a bell-shaped response curve of mineralizable C to water content, similar to previous studies (Linn and Doran, 1984; Franzluebbers, 2016), with a maximum response typically occurring between 50 and 75% WHC. The greatest mineralizable C value in the Russell Ranch Sustainable Agriculture Facility study at 72 h was at 100%WHC, but this was not significantly different from 50 or 75% WHC ($p < 0.05$; Table 6). The capillary method of rewetting bringing soil to 100% WHC had a distinct inhibitory effect on mineralizable C at both locations and all intervals, with the effect being more pronounced at the shorter intervals of 6 and 24 h (Table 6). The glistening soil surface observed when using capillary rewetting and the further inhibition of mineralizable C measurements over 100% WHC suggest that the capillary rewetting method proposed by Haney and Haney (2010) can result in supersaturated (>100% WHC) soils that do not optimize heterotrophic respiration incubations. This is in agreement with previous studies that have shown that 50 to 60% of saturation simultaneously optimizes substrate transport and gas diffusivity across C contents (Linn and Doran, 1984; Hashimoto and Komatsu, 2006; Moyano et al., 2013)

When directly comparing the two water contents that are currently utilized in commercial soil laboratories and in previous studies (50% WHC and the capillary method of rewetting), the 50% WHC had significantly greater mineralizable C values across all combinations of site and time interval, except at the 72-h interval at Russell Ranch Sustainable Agriculture Facility, which were statistically similar (Table 6). These trends show that the greater mineralizable C values measured at 50% WHC could allow for greater sensitivity of analysis (Castro Bustamante and Hartz, 2016; Wade et al., 2016) and therefore an increased ability to detect statistical differences due to management (Ladoni et al., 2015). Together, these results show that although the capillary method of rewetting represents a significant decrease in labor and analysis time, the decrease in sensitivity of response likely offsets the benefits. Both gravimetric (Culman et al., 2013; Castro Bustamante and Hartz, 2016; Wade et al., 2016) and volumetric measurements (Franzluebbers, 1999a; Franzluebbers et al., 2000; Haney et al., 2001) have been used in previous stud-

Table 6. Interactive effects of water content and incubation interval on mineralizable C for two long-term research trials in California (*n* = 3 fields for each value).

		Mineralizable C		
Location	Water content†	6 h	24 h	72 h
		——— mg CO2–C kg^{-1} soil ———		
Russell Ranch Sustainable Agriculture Facility	25% WHC	79.7 a‡	119.1 bc	144.0 c
	50% WHC	103.8 a	168.4 ab	267.2 ab
	75% WHC	105.8 a	197.1 a	349.6 a
	100% WHC	91.4 a	169.3 ab	353.4 a
	Capillary method	24.7 b	61.1 c	170.8 bc
West Side Research and Extension Center	25% WHC	39.0 a	88.7 bc	284.0 ab
	50% WHC	51.2 a	141.7 a	444.2 a
	75% WHC	34.8 ab	126.2 ab	396.4 ab
	100% WHC	18.6 bc	90.2 abc	341.5 ab
	Capillary method	14.1 c	68.0 c	263.1 b

† WHC, water holding capacity, calculated as the difference in weight between a saturated soil that was allowed to drain for an hour and the weight after the soil was oven-dried for 24 h at 105°C; Capillary method, soil rewetted from below by the methods of Haney and Haney (2010).

‡ Different lowercase letters indicate significant differences ($p < 0.05$) within Location × Mineralizable C interval by Tukey's HSD Test.

ies and, while they have been found to be related to one another (Haney and Haney, 2010), the two approaches have not been comparatively evaluated.

Method of Rewetting

We assessed the effect of method of rewetting on mineralizable C by adding water (50% WHC) to air-dried soil (1) from the top as well as (2) from the bottom and then compared with (3) capillary rewetting from below. The absolute differences in 24-h mineralizable C were greater between the directions of rewetting at 50% than between differing water contents when rewetted from below (Table 7). Thus, similar to the results found in Table 6, capillary rewetting inhibited respiration, relative to the 50% WHC, even when accounting for differences in direction of rewetting (Table 7). The difference between top- and bottom-wetted soils at 50% WHC is likely due to differences in water flow: wetting from above would fill all pores, followed by the draining of water from the macropores over a short time interval, whereas wetting from below is primarily driven by capillary action, which would result in slower and more unequal distribution of moisture toward the top of the soil column (McCoy et al., 1994). This effect may be mitigated with incubation intervals longer than the 24-h period investigated here, although currently, no studies have been conducted on the topic. Therefore, these results suggest that rewetting from above will optimize the sensitivity of the measurement for 24-h incubations.

Length of Incubation

Several commercial implementations of mineralizable C have different lengths of incubations ranging from 24 to 96 h.

Table 7. Difference in 24-h mineralizable C by water content and direction of water addition.

Water content†	Direction of water addition	24-h mineralizable C
		mg CO_2–C kg^{-1}
50% WHC	Top	70.66 a‡
50% WHC	Bottom	49.90 b
Capillary wetting	Bottom	31.95 c

† WHC, water holding capacity, calculated as the difference in weight between a saturated soil that was allowed to drain for an hour and the weight after the soil was oven-dried for 24 h at 105°C; Capillary method, soil rewetted from below by the methods described in Haney and Haney (2010).

‡ Different lowercase letters indicate significant differences ($p < 0.05$) by Tukey's HSD Test, $n = 42$ for each mean value.

Although it is well documented that short-term mineralizable C measurements correspond well to longer incubation intervals (Franzluebbers et al., 2000; Haney et al., 2008b), it is unclear if there is an incubation duration that is more sensitive to treatment differences within a trial. To assess the sensitivity of mineralizable C measurements at different incubation intervals to experimental factors, we used *F*-statistics generated from analysis of variance models in which incubation length (6, 24, or 72 h) was used to compare the degree of treatment effect across incubation times. Incubation duration served as a response variable with experimental factors (e.g., site, fertilizer source, tillage) as predictor variables. The sensitivity of the incubation interval to differences in experimental factors was mixed (Table 8). The 6- and 72-h intervals had the greatest sensitivity to experimental factors, and these were selected as the most sensitive indicator 33 and 58% of the time, respectively (Table 8). However, both the 6- and 72-h incubation intervals were able to detect statistically

Table 8. Comparison of the relative sensitivity of mineralizable C measurement interval (6, 24, and 72 h incubation) to detecting experimental factors associated with each study. F-values were generated using the mineralizable C interval as a response variable for each of the experimental factors (including significant interactions). Bolded *F*-values represent the interval that yielded the greatest sensitivity for a given experimental factor within a study.

		Mineralizable C *F*-value		
Study	Experimental factor	6 h	24 h	72 h
California Grower Survey	Site	2.49 ***	4.91 ***	**5.49** ***
	Growing region	**7.24** ***	6.06 ***	7.01 ***
	Cover crop use	0.84	0.83	**1.74**
California Tomato Survey	Site	**5.53** ***	1.71 †	1.39
	Fertilizer source	**4.69** *	1.97	0.56
Ohio Urban Garden	Management	1.53	1.25	**2.40** †
Russell Ranch Sustainable Agriculture Facility	Management	7.10 **	**12.67** ***	6.89 **
West Side Research and Extension Center	Cover crop use	7.67 **	8.89 **	**13.59** ***
	Tillage	1.50	1.81	**2.87**
	Cover crop × tillage	0.87	1.08	**3.06** †
Windsor Organic Research Trial	Management	0.18	0.43	**0.62**
	Fertilizer source	**2.17**	1.56	1.50
Total instances where F-value was greatest		4	1	7
Total instances where F-value was statistically significant ($p < 0.10$)		6	5	6
Percentage where interval was most sensitive		33%	8%	58%
Percentage where interval was statistically significant ($p < 0.10$)		50%	42%	50%

* Significant at $p < 0.05$.

** Significant at $p < 0.01$.

*** Significant at $p < 0.001$.

† Significant at $p < 0.10$.

significant differences in 50% of the studied factors. Similarly, the 24-h incubation interval was able to detect significant differences in 40% of our experimental factors.

There were also no distinct trends in terms of management practices that were better detected by mineralizable C measurements. In general, mineralizable C was sensitive to inputs of labile carbon and to differences between sites (Table 8). While many differences in labile C inputs were detected, such as cover crops in the West Side Research and Extension Center study, management in both the Russell Ranch Sustainable Agriculture Facility and Ohio Urban Garden, and fertilizer source in the California Tomato Survey, there were also many differences that respiration was not sensitive to, such as fertilizer source in the Windsor Organic Research Trial study and cover crop use in the California Grower Survey. Most of the effect of the management differences were shown across all three incubation intervals, with the exceptions of fertilizer source in the California Tomato Survey and management in the Ohio Urban Garden, which were only detected by 6- and 72-h mineralizable C, respectively. The ability to differentiate between sites was found in both of the multi-site studies, wherein the 6-h incubation data were the most sensitive in the California Tomato Survey and the 72-h data were the most sensitive in the California Grower Survey. However, given that both of these were found within the Central Valley of California, it is unclear if these trends would be consistent in other climates and edaphic conditions. The current sensitivity analysis showed that no single mineralizable C interval was consistently more effective at detecting treatment differences, although relaxing the threshold of statistical significance may increase the efficacy of these metrics in an applied setting (Morrow et al., 2016).

Analytical Variability

To determine the source and magnitude of analytical variability associated with mineralizable C measurements, samples that had been treated with procedural variations (e.g., sieve size and water additions) were run in triplicate to obtain a CV for a given procedure. These CV values were then used as response variables in a linear model to determine their effect, as well as study-specific treatment and edaphic effects, on analytical variability. The magnitude of the effects associated with these variables, that is, *F*-values, were then evaluated to determine their statistical effect on analytical variability (Table 9). A range of CV values from 0.5 to 84.4%, with a mean of 18.4% and a median of 12.4% were found (data not shown), and these agree with the range of analytical variability found in the literature (Ahn et al., 2009; Zagal et al., 2009; Morrow et al., 2016). The examined soil processing sources of variability—sieve size, water content, and direction of rewetting—all showed that they did not significantly increase the analytical variability (Table 9) of 24-h mineralizable C. This is not to say that these sources do not contribute to variation in the method, but rather how they are standardized (i.e., ground vs. sieved soil) has little influence on the repeatability of the measurement for a given field or soil type.

Table 9. The effect of each factor on the precision of 24-h mineralizable C, as measured by coefficients of variation among triplicate replications. *F*-values are based on a type II ANOVA with all factors included.

Factor	*F*-value
Study	10.82 **
Field	13.54 ***
Sieve size	0.54 ns†
Water content	2.25 ns
Direction of water addition	1.45 ns

** Significant at $p < 0.01$.

*** Significant at $p < 0.001$.

† ns, no significance.

There were significant differences in the analytical variability between studies and between soils or fields within a study (Table 9). Some soils within a study had higher intrinsic analytical variability than others and some studies had higher analytical variability than others. The analytical variability associated with these respective sources of variability was highly significant, although the soil- or field-level variability was slightly greater than the difference between studies (F_{Study} = 10.82 and F_{Field} = 13.54; Table 9).

The between-study variability can be attributed to differences in instrumentation (Table 3) and climatic differences, although the relative importance of these factors is unclear. However, between-site variability is in agreement with the results obtained from the inter-laboratory variability tests (data not shown), in which some soils were more variable across laboratories than others.

This soil-specific variability is attributable many edaphic characteristics, such as carbon or O_2 availability, soil aggregation, and soil texture (Linn and Doran, 1984; Mikutta and Kaiser, 2011; Moyano et al., 2013; Angert et al., 2015; Yan et al., 2016), many of which would not be addressed simply by sieving (Table 9). Additionally, the soil mineral composition can significantly alter the potential for hysteresis effects on drying (Kaiser et al., 2015) and on the wettability on rewetting (Woche et al., 2017), which has been previously shown to alter the mineralizable C measurements (Goebel et al., 2007). The relative importance of these potential confounding factors would likely be especially salient at lower concentrations of mineralizable C (Paterson and Sim, 2013) that are thought to indicate less "healthy" soils.

This study has examined several common method variations in mineralizable C procedures, but these variations are by no means exhaustive. Additional factors not examined here, such as soil column height, drying time, drying temperature, and incubation temperature (Creamer et al., 2014), have yet to be optimized, but may also contribute significantly to variability and should be investigated.

Broader Implications for Variability of Mineralizable C

Similar to other soil measurements, mineralizable C has multiple sources of variability: spatial, temporal and analytical. However, our findings that these sources of variability are soil-specific may be a substantial hurdle to a repeatable measurement

of mineralizable C and to its utility as a robust soil health metric. Here we have used a conservative Type II ANOVA to determine effect sizes, suggesting that the potentially confounding effects are even greater when more liberal analyses are performed. Several in situ studies of respiration have found that samples sizes of up to 75 separate samples are needed to achieve 95% confidence in values ±10% of a population mean (Davidson et al., 2002; Adachi et al., 2005) to account for these multiple sources of variability. In the current study, the analytical variability is exemplified in the lack of statistical differences at the 95% confidence level between the means of 25% WHC (284.0 mg CO_2–C kg^{-1} soil) and 50% WHC (444.2 mg CO_2–C kg^{-1} soil) in the West Side Research and Extension Center study (Table 6), despite a 56% increase in mean mineralizable C measured. In a commercial setting, this analytical variability can result in unreliable and/or inconsistent recommendations when using a single measurement. If additional analytical replicates were to be suggested, this would increase the cost of analysis and may serve as a financial barrier for growers (Carlisle, 2016).

CONCLUSIONS

Mineralizable C is currently being used in multiple commercial tests as an indicator of soil health. Previous studies have often focused on a narrow range of soils in a given study or have examined linear relationships of mineralizable C with other variables, obscuring the potential discrepancies in absolute values that can be obtained using this metric. However, there are many sources of variation that contribute to differences in absolute mineralizable C measurements. Sieve size, water content, and direction of rewetting were all found to be significant sources of variability, underscoring the need for standardization of soil handling procedures to help minimize experimental error across locations. In particular, the capillary method of rewetting inhibited mineralizable C measurements, which would likely result in decreased analytical sensitivity and hence we recommend not using this method to rewet soils in mineralizable C analyses. Calculating water content to be added on a soil-by-soil basis will undoubtedly increase the analysis time and cost, but will improve the overall accuracy of the measurement. We found no evidence that flail grinding to pass through a 2-mm sieve (as is commonly practiced in commercial laboratories) negatively impacts the measurement, nor that 6, 24, or 72 h yielded results that were more sensitive to management differences. Therefore, we see no justification in modifying the most common approach of a 24-h incubation on soil ground to <2 mm. Even after controlling for procedural variations, the repeatability of the metric varied widely across soils and studies. Until the sources of analytical variability are better understood, we recommend that mineralizable C measurements be run with analytical replication.

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