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This Technical Note is a draft document provided for public comment via Federal Register Notice Docket No. NRCS-2018-0006, Notice of Recommended Standard Methods for use as Soil Health Indicator Measurements

United States Department of Agriculture
Natural Resources Conservation Service

August 2018

Soil Health
Technical Note No. SH-XX

Recommended Soil Health Indicators and Associated Laboratory Procedures

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Acknowledgments

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Catalyzed by the success of USDA NRCS' "Unlock the Secrets in the Soil" campaign (begun in 2011), we would like to acknowledge the significant organizational and financial contributions to this effort by the Samuel Roberts Noble Foundation and the Farm Foundation, who partnered to design and initiate the "Soil Renaissance" in 2013. The mission was to reawaken the public to the importance of soil health for enhancing healthy, profitable, and sustainable natural resource systems, with an overall goal of making soil health the cornerstone of land use management decisions.

Through the Soil Renaissance, several committee workshops were organized and sponsored by the Samuel Roberts Noble Foundation and Farm Foundation from November 2014 to February 2016, with leadership on technical content and focus provided by USDA NRCS and ARS. Each workshop was attended by a mix of university, government, nonprofit, and private industry scientists; field conservationists; and farmers. Over the course of these committee workshops, attendees addressed such topics as research and education needs, collaborations, communication plans, sampling protocols, leveraging existing programs and databases, archiving samples, soil health indicators, quality assurance and quality control, service laboratory adoption issues, regionalizing interpretations, and others.

From these efforts, USDA led teams of scientists from USDA ARS, USDA NRCS, Cornell University, University of Georgia, Kansas State University, University of Missouri, Ohio State University, Oregon State University, Texas A&M University, and Brookside Laboratories, in the writing of draft topical papers on the current best available methods available to measure specific soil health indicators. Topics addressed included such items as specific research supporting the indicator, recommended analytical methods, and identification of concerns and issues. These

papers were compiled and presented by the authors at the first annual meeting of the Soil Health Institute in July 2016 and served as a foundation for this technical note.

We acknowledge the many organizations and individuals that have contributed to such a fruitful collaboration since 2014. We greatly appreciate the commitments made to this important endeavor, and we look forward to continuing public-private partnerships to enhance soil health for the benefit of generations to come. We also acknowledge the time and effort from many individuals within the USDA ARS, NRCS, and National Institute of Food and Agriculture, as well as Soil Health Institute and a number of universities, who reviewed this document and provided many helpful suggestions.

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Recommended Soil Health Indicators and Associated Laboratory Procedures

Introduction

The modern soil health (SH) movement has its roots in the oil embargo of 1973 that spurred a renewed interest in investigating how the soil microbial population could be used to replenish nitrogen (N) available from soil, due to huge increases in the price of N fertilizers. Soon after, there was a push to implement minimum and no-till conservation practices on the land to reduce anthropogenic erosion. In the 1980s, a consortium of public and private entities provided information to land managers on the best ways to implement these new practices. Concurrently, leading experts in soil quality were developing definitions and recommended data to characterize soil quality as affected by human management (Doran et al. 1994, Doran and Jones 1996). While soil microbial methodologies were relatively primitive at the time, soil biology was always an integral part of the scientific effort to improve the understanding and measurement of soil characteristics. Eventually, as the capacity to study soil biology improved, discussion of soil quality was replaced by a discussion of soil health as a means of communicating the importance of understanding and managing the soil as a living, breathing ecosystem. NRCS has defined soil health as "the capacity of the soil to function as a vital living ecosystem that supports plants, animals, and humans."

Over the last four decades, laboratory methods have been developed and refined for studying, quantifying, and monitoring the biological and physical SH status of the soil. However, unlike the chemical methods made available to the public for nutrient status assessment and management recommendations, these biological and physical methods have remained largely within the research community.

To improve our understanding and ability to influence the soil's response to changes in human management, we need to move beyond the current soil chemical approach to a more complex view of soil physical, chemical, and biological constraints to function, and interactions between these. Today it is possible to identify SH constraints that impact a variety of soil, water, plant, and other resource concerns with a combination of field observations and laboratory tests. While qualitative or semi-quantitative field observations can be used for preliminary identification of these constraints, identifying the specific underlying causes and management practices to address them, often requires further quantitative laboratory analysis. Also, for accountability purposes, there is a need to quantify the current soil health status and eventual trends in condition after implementing soil health management systems that are sponsored and funded through public agencies. As a first step in this process, SH indicators must be selected that represent key dimensions of SH and reflect constraints to soil functioning, with the ultimate goal of providing useful information for interpretation and management recommendations.

Once a suite of SH indicators has been selected, there is a critical need for standardization of field and laboratory methods and protocols. Currently, sampling and handling procedures in the field also vary widely, leading to inconsistent results and interpretation. Recognizing this, there is a separate document being prepared to address sampling and handling issues. As with all soil measurements (e.g., pH, salinity, extractable N, phosphorus (P), and potassium (K), etc.), SH indicators vary spatially and temporally. Care needs to be taken with the sampling scheme (e.g.,

compositing from an adequate number of subsamples to make inferences about a sampled area), sampling methods (e.g., soil volume and depth), timing of sampling (e.g., seasonal, annual), and application of the appropriate statistical methods. On the analytical side, methods for laboratory measurement of SH indicators vary significantly. Within the NRCS, standardization of soil characterization methods has allowed for large-scale data integration and comparison. Without a similar approach involving rigorous standardization of SH methods, variation among laboratories hinders our ability to evaluate SH changes over time and space, and to interpret values appropriately given soil type and climate. This makes regional and national compilations of SH data difficult to interpret. Standardization of methods and protocols, along with appropriate proficiency testing, will facilitate production of high quality data with a high degree of interpretability. This will facilitate development and use of a national set of regionally appropriate interpretation functions (i.e., scoring algorithms) to transform raw data generated by multiple laboratories. This will in turn allow those interpretation functions to appropriately account for soil and environmental factors, and be used for on farm management decision making.

Specifically, private and public soil testing laboratories that choose to adhere to the standardized methods supported by a public-private partnership effort will be able to offer SH testing in conjunction with interpretation functions and recommendations based on a large dataset achieved through multiorganizational contributions. This output can then serve multiple public and private purposes, including 1) use by producers and their advisors in conservation planning and in NRCS technical and financial assistance activities, 2) use by consultants, technical service providers, and other agricultural service providers in planning and monitoring soil health management systems, 3) for leveraging partnerships and efforts using such assessments across multiple organizations and geographical scales and increasing the value of investments made publicly and privately in SH assessment.

Our current knowledge is incomplete, but is built on a strong framework covering decades of research and expertise. With further attention and investment from collaborating partners, the knowledge gaps will be addressed, and future improvements will continue to advance the science behind SH. A mechanism to maintain an up-to-date set of SH measurement standards will be part of the overall scope of NRCS SH activities.

This document covers indicators and methods for assessing the functioning of soil processes that were recommended as current best available for the above goals from work by a group of over 100 scientists that collaborated to meet identified goals in USDA-led subcommittee activities and in multiorganizational workshops. In addition, a section on currently available SH assessments and interpretation tools has been included.

Information derived from a typical soil fertility report (i.e., NPK, micronutrients, pH, salinity, sodicity, etc.) and several important soil physical indicators such as compaction and water holding capacity, are beyond the scope of this technical note, although they should be considered in a full SH assessment. Laboratory methods, interpretations, and recommendations for fertility indicators were established at the State level based on data that were calibrated to potential yields. However, for many of these indicators it is unclear how the various methods, often specific to a region, compare. To enhance the utility of fertility indicators, understanding how different methods compare is essential. Some information for this purpose already exists along with on-going efforts elsewhere to standardize fertility indicators, updating potential yields based

on more recent information (e.g., modern crop cultivars, divergence in soil health status), and include more advanced modeling techniques for nutrient recommendations, N in particular.

It should be understood that all soil measurements, including those of biological and biochemical activity presented here, depend on sample size and sample preparation (e.g., soil sampling depth and timing, storage duration and conditions, selection of sieve size, grinding, etc.).

What Makes a Good Soil Health Indicator and Method?

Four main criteria for selecting best available soil health indicators and associated methods were developed by the scientific SH community in the scientific literature and discussed and adopted during the 2014–2016 workshops. The indicators and methods selected could change and evolve according to the following three factors: 1) eventually, additional soil processes may be added to those discussed in this paper, and better or additional SH indicators and methods may be developed; 2) the indicators and methods presented in this document should be reviewed every 5 to 10 years, depending on the degree of advancements in methods; and 3) standardization of pre-analytical soil processing (e.g., degree of aggregation, sieving, grinding), is as important as the analytical methods themselves in determining analytical results, thus, standardization in soil preparation should also be periodically reviewed and updated. Analytical methods described herein include recommendations for standard pre-analysis processing.

The four main criteria for selecting reliable soil health indicators and methods are as follows.

I. Soil Health Indicator Effectiveness.
   A. Management-Sensitive.—The indicator is sensitive to changes in soil and crop management systems.
   B. Short-term Sensitivity.—The indicator is generally able to detect changes within 1 to 3 years in subhumid to humid climates with significant changes in management. Changes are likely to take longer in semiarid to arid climates, or with minor changes in management.
   C. Interpretable
      1. The indicator (by itself) represents specific physical, chemical, or biological soil processes or conditions relevant to agricultural production and environmental outcomes.
      2. Interpretation with other tests: If not by itself, then the indicator's representation of specific processes/conditions *can be interpreted* if measured in conjunction with one to two other tests.
   D. Useful.—The indicator provides useful information towards assessing the SH status of an area and towards addressing specific resource concerns.

II. Production Readiness.—Readiness for use in commercial production laboratories in terms of—
   A. Ease of Use:
      1. Sampling (for field conservation planners, consultants, other agricultural service providers, and producers).
      2. Sample submission
      3. Lab sample prep (for laboratories)
      4. Lab measurements (for laboratories)
   B. Cost effectiveness for producers on a per-sample basis:
      1. Labor and supply expenses

2. Specialized equipment cost
3. Laboratory space and time requirements/overhead

III. Measurement repeatability. The level of precision of the method is within acceptable limits.

IV. Interpretable for agricultural management decisions.
   A. Measured values are "directionally understood" (i.e., more is better, less is better, optimum).
   B. Some management practices that improve the measure are known.
   C. Regional potential ranges to define relative poor/good functioning are known.
   D. Outcome based (yield, resilience, risk, environmental) thresholds are known.

Recommended Indicators and Methods

Soil processes/conditions below have been recommended to be assessed using the following indicators and methods. Hyperlinks will navigate to the appropriate place within the paper. Additional runner-up methods are listed with the draw-backs of their use included in the notes section. Methods that are currently used by the USDA Soil Management Assessment Framework (SMAF) and the Cornell's publicly available Comprehensive Assessment of Soil Health (CASH) are noted.

Table 1. Recommended Indicators and Methods

Soil Process	Soil Health Indicators	Methods Considered	Notes
Organic Matter Cycling & C Sequestration	Soil organic C (SOC) content	Dry combustion	**Recommended Method.** Nelson and Sommers (1996). The standard operating procedure (SOP) is from Soil Survey Staff (2014), pp. 464–471. If the soil sample is above pH 7.2, then it must be corrected to inorganic carbon (Sherrod et al. 2002), See appendix 1. Used by SMAF.
		Wet oxidation	Gives numbers comparable to dry combustion, but has chemical wastes and is more labor intensive.
	Mass loss	Loss on ignition (LOI)	Most commonly used by commercial labs, but needs to be calibrated for each MLRA. Used by CASH.
Soil Structural Stability (Infiltration)	Aggregation	ARS wet macroaggregate stability	**Recommended Method.** Kemper & Rosenau (1986). Subsequently published by Nimmo and Perkins (2002). SOP from Mikha and Rice (2004). See appendix 2. Used by SMAF.
		NRCS wet aggregation	Based on Kemper and Rosenau (1986), this method pre-wets the samples (Soil Survey Staff 2014, pp. 213–216).
		Cornell sprinkle infiltrometer	Schindelbeck et al. (2016). Used by CASH. Values from this method have not yet been correlated with the wet-sieve method.
General Microbial Activity	Short-term C mineralization (STCM; a.k.a. respiration)	Carbon dioxide (CO_2) respired, 4-day incubation	**Recommended Method.** Schindelbeck et al. (2016), see appendix 3. A 4-day soil incubation (CO_2 measured by electrical conductivity, gas chromatography, or

Soil Process	Soil Health Indicators	Methods Considered	Notes
			titration). Used by CASH. Being added to SMAF.
		CO_2 respired, 24-hr incubation	Like the previous method, but with a shorter incubation time, e.g., Haney et al. 2017, Solvita®, or other 24-hr methods). Often has high variability amongst replicates.
General Microbial Activity	Enzyme activity A suite of enzymes is recommended	β-Glucosidase (BG)	**Recommended Method.** Eivazi and Tabatabai (1988) as presented by Deng and Popova (2011). See appendix 4. Also in Soil Survey Staff (2014), pp. 513–518. Involved in the C-cycle. Used in SMAF.
		N-acetyl-β-D-glucosaminidase (NAG)	**Recommended Method**. Parham and Deng (2000) as presented by Deng and Popova (2011). See appendix 4. Involved in the C- & N-cycle. It is being added to SMAF.
		Phosphomonoesterases (acid/alkaline phosphatase; Pase)	**Recommended Method.** Eivazi and Tabatabai (1977) as presented by Acosta-Martínez and Tabatabai (2011). See appendix 4. Involved in the P-cycle. Both present in all soils, with acid Pase dominating in soils ≤7.2 and alkaline Pase in soils >7.2. Being added to SMAF.
		Arylsulfatase (AS)	**Recommended Method.** Tabatabai (1970) presented by Klose et al. (2011). See appendix 4. Involved in the S-cycle. Being added to SMAF.
		Another 10 enzymes were considered, but for various reasons they were eliminated (couldn't be done on air-dried samples; not enough papers in the literature to ascertain trends and thresholds; too expensive).	
Carbon Food Source	Readily available C pool	Permanganate oxidizable C (POXC)	**Recommended Method.** Weil et al. 2003. SOP from Schindelbeck et al. 2016. See appendix 5. It is also in Soil Survey Staff (2014), pp. 505–509. Used by CASH. Being added to SMAF.
		Particulate organic matter	Good method. The fraction is operationally defined, with many methods in use. A method appropriate for soil test labs should soon be available. Being added to SMAF.
		28-day C mineralization	Too long (same method as the STCM method, but has a longer incubation).
		Cold/hot water extractable organic C (WEOC)	Cold WEOC (Haney et al. 2017). Hot WEOC (Ghani et al. 2003). Gives a snapshot of what is available in the soil solution at time of sampling. May not reflect total pool.
		Soluble carbohydrates	An older method no longer in wide use.
		Substrate-induced respiration	Research method; it is labor intensive.
		Microbial biomass C (fumigation-	A research method that is time/labor intensive. Used by SMAF.

Soil Process	Soil Health Indicators	Methods Considered	Notes
		incubation, fumigation-extraction	
Bioavailable Nitrogen	Available organic N pool	Autoclaved citrate extractable (ACE) protein content	**Recommended Method**. Schindelbeck et al. (2016). See appendix 6. Modification, published by Hurisso et al. (2018), from Wright and Upadhyaya (1998).
		Cold water extractable organic N (WEON)	Used by the Soil Health Nutrient Tool (Haney et al. 2017). Not enough data available at this time.
		Correlation with short-term C mineralization	Picone et al. (2002). Has promise, but requires more evaluation with broader number of soils and management systems.
		7-day anaerobic potentially mineralizable N	Drinkwater et al. (1996). The 7-day incubation is too long for high-throughput labs.
		28-day aerobic PMN incubation	Used in the USDA/ARS Conservation Effects Assessment Projects (CEAP) soil health assessments (e.g., Stott et al. 2011). Too long.
		Illinois soil N test (ISNT)	Nitrogen available as amino-sugar (e.g., Sharifi et al. 2007). Measures a constant fraction of total soil N. Usually evaluated against yield rather than soil health.
		β-glucosaminidase activity (NAG)	See soil enzyme activity above.
		Protease	Must use fresh soil.
Microbial Diversity	Community structure	Phospholipid fatty acid (PLFA)	**Recommended Method.** PLFA (Buyer and Sasser 2012). See appendix 7. PLFA is an older method. It is offered by some commercial labs. It gives coarse community structural information. Rapid advances are being made in this area, however these methods are still in the research realm at this time.
		Ester-linked fatty acid methyl ester profile (EL-FAME)	EL-FAME is a newer method and less expensive, but is less suitable due to lack of fungal markers.
		"Sampling for Life"	**Recommended.** If appropriate storage is available, we recommend archiving samples until newer methods are available.

Discussion of Indicators and Methods by Soil Process

Soil Organic Matter Dynamics

There was an early consensus regarding the recommended indicator and method for this process, therefore no draft topical paper was prepared.

The soil organic matter (SOM) cycle begins with carbon dioxide (CO_2) from the air, which is continuously bound into organic substrates through photosynthesis and chemosynthesis. This bound carbon can be released as CO_2 for reuse by living plants and, subsequently, animals. This process is brought about primarily through biodegradation of organic residues within soil and water systems. During a 1-year period, degradation of most types of plant residues returns about 55 to 70 percent of the bound carbon to the atmosphere as CO_2, 5 to 15 percent is incorporated into soil biomass, and the remaining carbon is partially stabilized in the soil as new humus (Jenkinson 1971, Stott et al. 1983).

Soil organic matter generally constitutes less than 10 percent by mass of the surface horizon of most mineral soils. In arid soils, SOM may account for only one to two percent or less of the surface horizon (Stott and Martin 1989, 1990). Nonetheless, SOM is a very important matrix within soil, and its beneficial properties include—

- Improves soil physical structure (e.g., improved aggregation).
- Slow release of plant nutrient elements, especially N.
- Aids in trace element nutrition of plants through chelation reactions.
- Aids in solubilization of plant nutrients from insoluble minerals.
- Has a high adsorptive or exchange capacity for nutrient cations.
- Certain components may exert growth-promoting effects.
- Supports a greater and more varied soil biological population, which favors biological control of pests and pathogens.
- Reduces toxicity of both natural and anthropogenic toxic substances.
- Improves available water-holding capacity (especially in soils with a high sand content).

Management strategies influence not only the amount of SOM in the soil, but also how SOM is distributed in the various organic matter fractions (how readily available is the C and N associated with the SOM) and how other soil properties are modified. Management practices can also alter the soil microbial community, which drives 80 to 90 percent of soil processes in which the decomposition and transformation of organic residues into SOM occurs. The dynamic nature of soil microbial communities makes them a sensitive indicator for assessing soil health alterations related to SOM due to changing management practices.

Candidate Indicator

- Soil organic carbon (SOC) content.

Candidate Indicator Methods (methods considered for recommendation)

- Dry combustion (Nelson and Sommers 1996) (instrumentation: high temperature, infrared spectroscopy). See appendix 1. Also available in Soil Survey Staff (2014), (pp. 464–471). This is the recommended method.

  - If the soil sample pH ≥7.2, then the dry combustion value should be corrected for inorganic C (IC) as measured by the method of Sherrod et al. (2002).
- Wet oxidation (Walkley-Black as found in Nelson and Sommers (1996)).
- Loss on ignition (LOI), Schulte et al. (1991).

Discussion

Due to the influence of SOM, often measured as SOC, on so many processes in the soil, it is considered the most important baseline measurement of soil health (Doran and Parkin 1994, Larson and Pierce 1991). The primary, long-established indicator for this process is SOM content, usually measured as SOC content (Nelson and Sommers 1996, Sikora and Stott 1996). Changes in SOC content compared to previous measurements or knowledge of the potential levels attainable by similar soil types provide a snapshot of current health status of a soil. While invaluable as a baseline measurement, SOC content changes relatively slowly, often taking 3 to 5 years before significant shifts can be detected in humid or subhumid environments, or longer in arid and semiarid regions. Other indicators that change more readily in response to management systems are necessary in the short-term, and several are included in the following sections.

The established measurement (Nelson and Sommers 1996) of total C is by dry combustion, which gives values highly correlated with those obtained with Walkley-Black wet oxidation, an older method, in soils with pH < 7.2 (Tabatabai and Bremner 1970, Yeomans and Bremner 1991, and personal experience). Dry combustion consists of heating a small soil sample to 1200°C, which converts soil C to CO_2 and is measured using infrared spectroscopy. Often research papers will cite instrument guidelines rather than a published paper. For many soils, SOC is considered equal to the total C (TC) values. However, if the soil pH is greater than 7.0 with deposits of calcium, $(Ca)CO_3$, and magnesium, $(Mg)CO_3$, carbonates, then TC must be corrected for the inorganic C (IC) content (Sherrod et al. 2002). SOC determined by subtraction of IC from TC measured by dry combustion correlates well with SOC determined by the Walkley-Black wet oxidation method (Sherrod et al. 2002). Dry combustion requires an investment in equipment, but is the most accurate measurement, and the instrument can run 24 hours a day, 7 days a week. While the wet oxidation method is a less expensive alternative, it does result in hazardous waste, with the attendant disposal costs. For either method, care needs to be taken in soil preparation by the receiving laboratory to remove root and plant residue material (Conyers et al. 2011).

For both the dry combustion and wet oxidation methods, the SOC results are converted to SOM using a 1.74 conversion factor. It has been shown that the conversion factor is not constant across soils (Pribyl 2010). Thus, it would be more accurate to report findings as SOC rather that SOM.

The major issue with measurements for SOM is that many commercial labs still use the loss-on-ignition (LOI) method (Schulte et al. 1991, Sikora and Stott 1996), which is less expensive and does not generate chemical wastes, but can lead to significant inaccuracies. The LOI method involves heating a soil sample in a muffle furnace at various temperatures (Ghabbour et al. 2014), and the change in mass is determined. Most labs have developed a linear relationship between LOI and SOC for local soils, however, such correlations can differ across soils and regions. Several papers have highlighted variations from region to region, finding a LOI:TC ratio from 1.08 to 5.76 (Broadbent 1953, David 1988, Hoogsteen et al. 2015, Howard and

Howard 1990, Read and Ridgell 1922), to as much as 15.4 in a beech forest soil (Christensen and Malmros 1982). Values obtained by LOI also need to be corrected for IC (Tabatabai 1996).

Konen et al. (2002) collected 255 samples from soil horizons A, Ap, and AB from noncalcareous soils in selected major land resource areas (MLRAs) in the North Central United States. Within each MLRA, strong linear relationships were observed between LOI and TC measured by dry combustion, with coefficient of determination (R^2) values ranging from 0.94 to 0.98. Predictive equations developed were significantly different for individual MLRAs, reinforcing the need for development of unique predictive equations for individual soil-geographic regions.

Assessment and Interpretation

The consensus is that SOM follows a "more-is-better" trend. This indicator (method: dry combustion) is included in both SMAF and CASH, with scoring algorithms that shift with soil taxonomic classification (SMAF only), texture, and climate (SMAF only), with established end points. The current SMAF algorithms were developed using NRCS data and soil literature. The reason that CASH has used only texture is that it was developed for New York (and later used by neighboring States), so classification and climate were considered unnecessary. In contrast, SMAF was developed to be used nationally and internationally.

Soil Structural Stability

This section draws in part from a draft topical paper by Skye Wills (lead), Maysoon Mikha, Doug Wysocki, Leticia Sonon, Tony Provin, Harold van Es, Chuck Rice, and Willie Durham.

Soil health contributes to the soil's capacity to protect watersheds by regulating infiltration and partitioning of water and to prevent water and air pollution by buffering potential pollutants (National Research Council 1993, Stott et al. 1999). To regulate infiltration and partition water flow, a good quality agricultural soil must exhibit several characteristics. These include a structure that allows water to infiltrate, the capability of retaining beneficial amounts of water, a low tendency to crust or form a surface seal, and the ability to resist erosive forces.

Soil aggregates are recognized as an important soil property that mediates hydrologic and biological processes (Cambardella and Elliott 1993, Mikha and Rice 2004, Six et al. 2000a, b). Large (macro, >250 μm) aggregates have been shown to be sensitive to soil management and related to soil C and nutrient cycling (Angers and Chenu, 1998, Tisdall and Oades 1982, Blanco-Canqui et al. 2015). Microaggregation (53-250 μm), in general, is an inherent soil property influenced by mineralogy and texture, while the degree of macroaggregation is highly influenced by management (e.g., Moebius et al. 2007, Mitchell et al. 2017). Soils with low aggregate stability are more susceptible to crusting and surface sealing, as the initial step in this process is the breakdown of surface soil macroaggregates (LeBissonnais 1996). This has several impacts: increased erosion by water, reduced water infiltration and storage, reduced air exchange, poor seedling emergence, and increased stress to the plant population among others. Soils with low aggregate stability are more likely to have poor drainage and surface ponding may occur after heavy rains, delaying field operations. In soils with a high clay content, strong aggregation makes a soil less dense and easier to work with and improves water drainage. Good aggregation improves a soil's resilience to extreme weather events. Soil macroaggregate stability is related to soil biology, with microorganisms producing glues (soil carbohydrates) that along with fungal hyphae and fine roots bind the aggregates together. Since aggregation integrates soil biological, chemical and physical properties, it is an important indicator of SH.

Candidate Indicators

- Macroaggregate Stability.—Currently this is the most widely used and accepted method.
- While there are other measures of soil structure and strength that can be evaluated through visual (e.g., Guimarães 2011), mechanical or spectral assessment; there do not appear to be any candidates at this time that quantitatively measure soil biophysical components at this scale.

Candidate Indicator Methods

- Wet Sieve, with no pre-wetting and corrected for sand (Kemper and Rosenau 1986). The SOP for the recommended method is from Mikha and Rice (2004) (appendix 2). This is the recommended method.
- Wet Sieve, with pre-wetting (Soil Survey Staff 2014, pp 213).
- Mini-rainfall simulator (Moebius-Clune et al. 2016, Schindelbeck 2016).

Discussion

Macroaggregation is, after SOC content, one of the best integrators of physical, chemical and biological soil processes. The Kemper and Rosenau (1986) and the earlier Yoder (1936) method is widely used and scientifically robust. Nimmo and Perkins (2002) reiterated this method in their review of available methods. Over time, slight variations of the standard method used by various laboratories makes comparison of datasets challenging. The SOP presented in appendix 2 has been used by several USDA/ARS cross-location projects (Mikha et al. 2004, Stott et al. 2011), as well as used for the development of the SMAF macroaggregation algorithms. The theory behind the method is that agitation and quick wetting mimics slaking disruption by rainfall (rain on dry soil is considered the most disruptive event). In this procedure, 40 to 50 g (≈0.4 g per cm^2 sieve area) of 8-mm sieved air-dried soil is spread over a 2.00 mm sieve and nested with 250 and 53 µm sieves and a catch pan, deionized water is rapidly added and then the nest is oscillated mechanically with a given time (10 min), stroke length (4 cm) and frequency (30 cycles per minute). Multiple nests of sieves can be done at the same time, in separate cylinders, depending on the configuration of the machine. The aggregates remaining on the 250 µm sieve are considered the stable macroaggregates, while microaggregates are retained on the 53 µm. The soil mass is corrected for stone and sand content. The USDA-NRCS method (Soil Survey Lab 2014, pp 212–216) is robust, but presoaks the samples overnight before agitation, thus minimizing disruption through slaking.

The Cornell method (Moebius-Clune et al. 2016, Schindelbeck 2016, pp 44–46) uses a mini-rainfall simulator, in which a single layer of aggregates from 0.25–2.00 mm in size is spread on a 250 µm sieve and placed under the simulator which delivers 12.5 mm of water in drop form in 5 minutes. Anything remaining on the sieve is collected, dried, and weighed. The remaining mass is corrected for stone content (but not sand grains of < 0.25mm diameter). While the theory and physical processes evaluated are robust, this method is fundamentally different from most other aggregate stability metrics.

All methods can detect differences between treatments (soil health management systems, or SHMS vs. non-SHMS) within 1 to 3 years in humid or temperate annual crop systems. Significant changes will take longer in drier areas or with minor changes in management.

Assessment and Interpretation
Consensus is that aggregate stability follows the more-is-better trend, and both SMAF and CASH include this indicator in their SH assessments. However, the SMAF scoring algorithm is based on Kemper and Rosenau's (1986) method, while CASH developed texture-specific scoring functions based on values measured using the mini-rainfall simulator. In SMAF, the raw data for macroaggregation is interpreted and scores modified based on soil suborder, texture, and iron-oxide content.

General Microbial Activity: Short-term Carbon Mineralization

This section was derived, in part, from a draft topical paper by Alan Franzluebbers (lead) Veronica Acosta-Martínez, Steve Culman, Richard Dick, Willie Durham, Rick Haney, Michael Lehman, David Myrold, Diane Stott, and Skye Wills.

Soil is a living, breathing ecosystem. Biological activity occurs in response to food (energy) sources. Health of agricultural soils depends largely on conservation management practices that promote SOM accumulation. Total SOM changes slowly, but active fractions are more dynamic. A key indicator of healthy soil is potential biological activity, which can be measured rapidly with soil testing via short-term C mineralization following rewetting of dried soil, i.e., carbo mineralization (Franzluebbers et al. 1996). This early research outlined many of the hypotheses and issues that remain important today:

- Soil N supplying potential is related to soil microbial biomass and activity;
- Soil microbial biomass is sensitive to changes in amount and composition of active fractions of SOM.

Measuring C mineralization by incubation is a well-established technique (e.g., Martin et al. 1980, Paul et al. 1999, Stott et al. 1983), usually using longer incubation times (28-day, 6-mo, 1-yr, and longer). It is considered one of the best ways to determine C pools based on recalcitrance and physical availability of the carbon compounds. Soil is sampled, air-dried, and rewetted to a standard water content, and then incubated at a constant temperature for a given amount of time.

Cumulative C mineralization over several weeks should be considered a robust estimate of potential soil biological activity. The exact length of time for such incubation has not been well defined. Excessively long incubation of greater than a couple of month's leads to increasingly greater depletion of available C substrates and do not represent conditions present during a typical growing season. Several different research groups have generally converged upon 1 to 8 weeks as reasonable. Although the rate of C mineralization changes dramatically during soil incubation following rewetting of dried soil, the relative differences that occur among samples is consistent at any one point in time.

A target of estimating soil biological activity should relate to steady-state C mineralization. Soil depth matters, and this point needs to be emphasized when assessing soil biological activity. Sampling depth should be kept consistent through time and among fields being compared. The actual depth chosen needs to be carefully considered prior to initiation of a soil health assessment. Multiple depths of sampling are a valid approach.

Candidate Indicators

- Short-term C mineralization (STCM).

Candidate Indicator Methods

- A 4-day incubation, soil incubation with a base trap; CO_2 measured via titration, change in electrical conductivity, or gas chromatography (Schindelbeck et al. 2016) presented in appendix 3.
- A 3-day incubation (Franzluebbers et al. 2000).
- 24 hr incubation (e.g., Haney et al. 2017, Solvita®, or other 24-hr method).

Discussion

Respiration has been a standard for SH assessment for decades. Draeger tubes were used to measure respiration in the field with the NRCS soil quality test kits since the 1990s (https://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_050956.pdf). Laboratory measures using optimal temperature and water content and longer incubation times are used to separate different C pools in the SOM (e.g., Stott et al. 1983, 1990). A set of soils from Texas was initially tested using the flush of CO_2 under controlled lab conditions in 1 day compared with net N mineralization, soil microbial biomass C, and total organic C (Franzluebbers et al. 1996). Although a strong association existed between the flush of CO_2 in 1 day with that evolved in 3 days, the longer incubation time is considered to yield more reliable estimates of C mineralization (Franzluebbers et al. 2000). Cornell's CASH (Moebius-Clune et al. 2016, Schindelbeck et al. 2016) concluded that a 4-day incubation is required to obtain sufficient precision for a reliable assessment of the soil processes involved.

Assessment and Interpretation

Improvements in SH are positively correlated to increases in respiration in much of the literature, although there continues to be debate about interpretability. It is best to interpret this indicator in conjunction with SOC and time of sampling. This indicator, using the 4-day incubation and quantified using electrical conductivity, is included in CASH (Schindelbeck et al. 2016). It is being added to SMAF.

General Microbial Activity: Enzyme Activities

Soil metabolic activity, measured as enzyme activities (EAs) produced by microbes, drives the decomposition of plant, animal and microbial materials with ultimate impacts in C sequestration, nutrient availability, soil productivity, and the global C cycle. Among indicators of soil microbial activity (e.g., short-term C mineralization, N-mineralization, DNA sequencing), EAs are sensitive, early indicators of changes in SH related to soil biogeochemical cycling and SOM dynamics due to variations in land use, management, pollution, and climate.

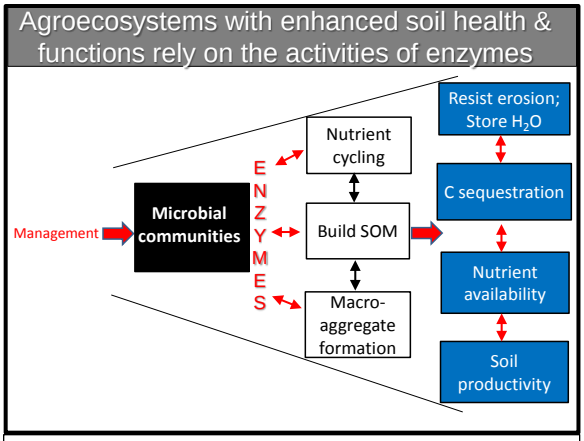

Figure 1. Microbial indicators of soil health are important drivers in a healthy soil leading to improved functions such as soil stability and resistance to erosion. Diagram courtesy of Dr. Veronica Acosta-Martínez.

The key ecological role of EAs in soil decomposition processes has been recognized since 1899 by Woods, and enzyme assays have been used since the 1960s (Dick and Burns 2011) (fig. 1). About 10 EAs have been generally used to represent C (β-glucosidase), C

and N (β-glucosaminidase), N (aspartase, asparaginase, urease), P (acid and alkaline phosphomonoesterase), and S (arylsulfatase) cycling (fig. 2). Many studies have shown that crop rotation, fertilization, tillage, and amendments can affect EAs by altering soil structure, bulk density, soil pH, and amounts and distribution of organic matter and nutrients in soil (Acosta-Martínez et al. 2011, Lehman et al. 2015). For example, soils under crop rotations generally show higher EAs compared to monocropping systems, owing to diversified organic inputs, improved soil structure, nearly year-round rhizosphere and plant cover, and higher root density (Bandick and Dick 1999, Deng et al. 2000, Klose et al. 1999). Inorganic fertilizer applications can affect EAs through higher plant yields, crop residue amounts, and changes in soil pH and soil solution chemistry. However, the addition of enzyme reaction products by inorganic fertilizers can also suppress enzyme synthesis (Dick 1997).

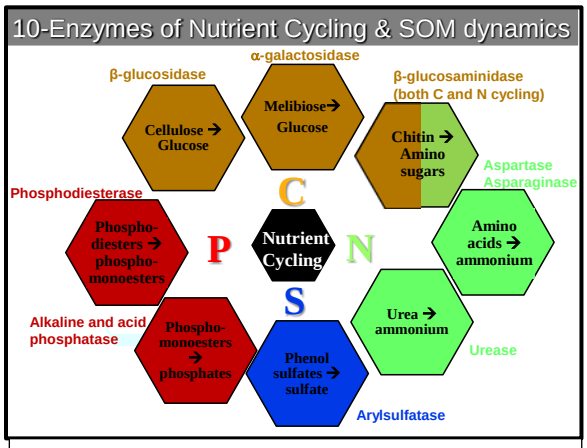

Figure 2. Ten enzyme activities involved in nutrient cycling) and the molecules involved in the reactions mediated by each enzyme. Diagram courtesy of Dr. Veronica Acosta-Martínez.

In addition to management practices, soil type has a strong influence on EAs. For instance, while changes in individual EAs due to tillage have been found in soils with high organic matter content (e.g., Deng and Tabatabai 1996a,b; Stott et al. 2014; Stott et al. 2013), no detectable effects on individual EAs were detected in a study of sandy soils. Instead, a combined effect of multiple EAs was observed (Acosta-Martínez et al. 2011). Although, the addition of enzyme reaction products by inorganic fertilizers can also suppress some enzyme synthesis (Dick 1997), careful selection of the EA has also allowed to distinguish among the effects of inorganic fertilizer and application rates due to the impacts on plant yields, crop residue amounts, and changes in soil pH and soil solution chemistry. Changes in EAs may even anticipate changes not quantifiable in SOM as affected by soil management and crop yields, allowing producers to redirect management. As it can be gathered from this overview, EAs can provide one of the most sensitive measures to assist in the selection and combination of management with enhanced soil health related to improvements in biogeochemical cycling and SOM dynamics.

The more common, validated enzyme assays are found in the "Methods of Soil Enzymology" (Dick 2011). Many EAs can be evaluated in air-dried soil conditions with simple protocols, results can be obtained in < 3hr that are cost effective and have been correlated to other biochemical analyses (Ndiaye et al. 2000). Significant correlations have been found between EAs and microbial biomass (Acosta-Martínez et al. 2011), arbuscular mycorrhizal fungi (AMF) (Cotton et al. 2013, Davinic et al. 2013), and soil aggregation (Stott et al. 2013, Wilson et al. 2009). All the selected enzymes are classified as hydrolases and can provide one of the most sensitive measures to assist in the selection and combination of management with enhanced soil health related to improvements in biogeochemical cycling and SOM dynamics.

Candidate Indicators and Methods

- C-cycling EA→ β-glucosidase (BG). Involved in cellulose degradation, most abundant polysaccharide in nature. Eivazi and Tabatabai (1988) as presented by Deng and Popova (2011).

- N- and C-cycling EA → N-acetyl-β-D-glucosaminidase (NAG). Involved in chitin degradation, second most abundant polysaccharide in nature. Parham and Deng (2000) as presented by Deng and Popova (2011).
- P-cycling EA → Phosphomonoesterase (Acid/Alkaline Phosphatase; Pase). Involved in organic P mineralization including lipids, DNA. Eivazi and Tabatabai (1977) as presented by Acosta-Martínez and Tabatabai (2011). Acid Pase used in soils with pH<7.1, while Alkaline Pase is used for higher pH soils. The method is the same except for the optimum pH.
- S-cycling EA → Arylsulfatase (AS). Involved in the mineralization of organic S sources, amino acids. Tabatabai (1970) as presented by Klose et al. (2011)
- SOPs (Dick 2011) are presented in appendix 4.

Discussion

Among 10–15 EAs used as indicators of soil health, four have been targeted (listed in the previous subsection) for their important roles in C, N, P, and S cycling. There are many published reviews covering the impact of management on the activities (e.g., Tabatabai 1994, Acosta-Martínez and Tabatabai 2011, Deng and Popova 2011, Dick and Burns 2011, Klose et al. 2011, Nannipieri et al. 2011, Wallenstein and Burns 2011). The enzymes selected are hydrolases, which catalyze the hydrolysis of various chemical bonds (e.g., ester, glucosyl) by reaction with water, leading to inorganic forms that can be taken up by plants (i.e., phosphates or sulfates for phosphatases and sulfatases, respectively) or monomers that are important energy sources for soil organisms (β-glucosidase or β-glucosaminidase). The enzymes selected are the most commonly assayed enzyme activities in soils and use similar approaches (*p*-nitrophenol released), facilitating comparisons across regions.

Assessment and Interpretation

It is generally agreed in the literature that higher EAs are present in healthier soils, as they are necessary for improved nutrient cycling in the soil (thus following the more-is-better model).

Among EAs, β-glucosidase has interpretive scoring algorithms within SMAF (Stott et al. 2010). The inclusion of this EA in soil health assessments will facilitate comparisons established over decades as this is one of the most commonly assayed enzymes in soil.

Evaluating more than one EA is needed to obtain a better overview of soil biogeochemical cycling and different reactions involved in SOM transformation. Calculation of a geometric mean (multiplying the values of all EAs then taking the root value of the number of EAs) for several EAs as defined by García-Ruiz et al. (2008) holds promise in providing an index of changes in biogeochemical cycling (Cotton et al. 2013, Acosta-Martínez and Cotton 2017).

Carbon Food Source

This section draws from the draft topical paper developed by Michael Robotham (lead), Steve Culman, Kristen Veum, Jennifer Moore-Kucera, Harold van Es.

The soil microbial population depends on access to soil C for food and energy. This pool of C is a small portion of the SOM, and the larger the pool, the larger the microbial population that can be supported. There are a variety of methods that measure some proxy of this C pool, often referred to as “active carbon”.

Candidate Indicators and Methods

- Permanganate oxidizable C (Weil et al. 2003); the SOP is provided by Schindelbeck et al. 2016 (see appendix 5), and is also available in Soil Survey Staff (2014) pp. 505–509.
- Particulate organic matter (Cambardella and Elliot 1992).
- 28-day C mineralization (base trap w/ CO_2 measured by GC, titration, or electrical conductivity).
- Water extractable organic C (WEOC) (Haney et al. 2017).
- Soluble carbohydrates.
- Substrate-induced respiration.
- Microbial biomass C (fumigation-incubation, fumigation-extraction).

Discussion

It is difficult to separate the indicator from the method because each method measures a slightly different pool of SOC, however, there is a high degree of correlation between them.

Permanganate oxidizable carbon (POXC) has been a widely used SH-related measure for many years. It serves as a proxy for SH functions and is generally thought to be a representation of the soil C pool that serves as a C food source for soil microbes. Weil et al. (2003) suggests that POXC represents a highly active fraction of SOC, but it may have a closer association to a more stabilized fraction of SOC (Culman et al. 2012, Morrow et al. 2016). Increasing POXC reflects practices that promote SOM accumulation and it is considered a useful indicator of long-term soil C sequestration (Hurisso et al. 2016).

A method using 0.02 M potassium permanganate was first published in 2003 (Weil et al. 2003) and has since been adopted as a standard methodology by many, including the USDA NRCS Kellogg Soil Survey Laboratory (Soil Survey Staff 2014). It has also been a component of the Cornell Assessment of Soil Health protocol since 2006 (Moebius-Clune et al. 2016). Multiple studies have found that differences in POXC are related to differences in management and that these differences are detectable in a relatively short (1 to 3 years) time frame. These values have also found a significant positive correlation with other soil properties that are commonly thought to be related to SH including: total SOC (Weil et al. 2003, Culman et al. 2012, Morrow et al. 2016); particulate organic C (Culman et al. 2012); soluble carbohydrates (Weil et al. 2003); substrate-induced respiration (Weil et al. 2013); and microbial biomass C (Weil et al. 2003, Culman et al. 2012). Research has shown that POXC is an early indicator of SH response to changes in crop and soil management, usually responding to management much sooner than SOC content.

Particulate organic matter (POM) is positively correlated to POXC, but POXC is generally thought to better represent the fraction of C available to soil microbes. While POM is highly sensitive to management changes (e.g., Cambardella and Elliott 1992), the method is not well suited to commercial labs and until recently had only been used in research labs. The Ohio State University Soil Health Lab is now offering variations of POM.

The 28-day C mineralization (incubation with base trap, CO_2 measured by GC or titration) is an old method that has long been the standard for measuring the readily available C pool (see the previous section on short-term C mineralization), but the down side is the length of time, taking up valuable shelf space that is at a premium in a high-throughput lab. There is good evidence that the 3- to 4-day incubation is highly correlated with the 28-day incubation.

Hot water extractable C is positively correlated to POXC, but POXC is thought to better represent the fraction of C available to soil microbes. Hot water extractable C is currently being used as an indicator in the ongoing New Zealand soil quality monitoring project (Stevenson, personal communication). This may be an alternative to POXC, but lacks widespread use within the United States. Cold-water extractable organic C (WEOC) is used by the Soil Health Nutrient Tool (aka Haney test), but the tool has not been published in a peer-reviewed journal. Mitchell et al. (2017) found mixed results with WEOC in arid, irrigated cropping systems in California. Older literature includes cold- and hot-water extractable C, but the methods were abandoned due to lack of correlations with changes in management. Newer instrumentation may allow better measurement of this C pool.

Soluble carbohydrate and substrate-induced respiration are research methods that have been used in the past, but seldom used now, and are not viable candidates for a high throughput lab.

Microbial biomass (MBC) is a widely used method based on Jenkinson et al. (2004). It is the method used by the SMAF and was initially considered for use in CASH but POXC was chosen instead, as a faster method for a production lab. MBC was included in SMAF based on its role as a readily available pool of C and N and an association with improved soil structural functioning (Elliott and Coleman 1988, Hendrix et al. 1990). Research has shown that indeed soil microbial biomass and activity are often highly related (Culman et al. 2012, Wardle 1992), and that both are generally related to soil N-supplying potential (Bonde et al. 1988, Stanford and Smith 1976). Although modifications of soil microbial biomass methodology have eliminated the 10-day incubation needed for chloroform fumigation-incubation (Jenkinson and Powlson 1976) by simply extracting soluble C following a single day of fumigation (Vance et al. 1987), field-moist soil is still recommended. Soil microbial biomass C estimation was shown to be possible by drying soil and pre-incubating soil for approximately10 days before fumigation and further incubation for 10 days, thus requiring at least 21 days.

Assessment and Interpretation

The consensus in the literature is that higher values of POXC represent a more highly functioning soil than do lower values and when SH management systems are implemented, improvements in SH are positively correlated to increases in POXC. Currently POXC is only included in CASH, with SMAF using the more labor-intensive MBC. With enough data from other regions, the algorithms used by CASH could be used for developing scoring functions in other parts of the country, as it has proven quite robust within the Northeast.

Bioavailable Nitrogen

This section draws from the draft topical paper authored by Brandon Smith (lead), Alan Franzluebbers, Daniel Moebius-Clune, Steve Culman, Chuck Rice, Willie Durham, Jude Maul, and Maysoon Mikha.

Nitrogen is critical to the growth and maintenance of all living organisms. The microbial population requires N for building proteins required to sustain life. Understanding the nitrogen supply stored in the SOM is basic to understanding how well the soil can support the microbial population necessary for sustaining proper soil functioning, (e.g., nutrient cycling, structural stability, water infiltration and storage, residue breakdown, among others).

Candidate Indicators and Methods

- Autoclaved citrate extractable (ACE) protein content (Schindelbeck et al. 2016) as modified (Hurisso et al. 2018) from Wright and Upadhyaya (1998). See appendix 6.
- Water extractable organic N (Haney et al. 2017).
- Correlation with short-term C mineralization (Franzluebbers et al. 1996).
- 7-day potentially mineralizable N (e.g., Drinkwater et al. 1996).
- 28-day aerobic incubation at field capacity as used in Conservation Effects Assessment Projects (see the SOC section in this document).
- Beta-glucosaminidase activity (NAG, see the soil enzyme activity section in this document).
- Protease.
- Illinois soil nitrogen test (estimates soil amino sugar N).
- Solvita® Labile Amino Nitrogen (SLAN) test.

Discussion

ACE protein is an indicator of high molecular weight organic N storage, and the coupling of the C and N cycles. Soil organic matter is primarily derived from plant and microbial material. Within this material, a majority of the N is found in proteins, and as soil microbes decompose proteins and other organic materials (e.g., manures), the proteins are used to increase the microbial biomass or may be reconfigured into other N containing compounds. As the biomass turns over, proteins may become incorporated, in some form, into SOM. Soil proteins represent the largest pool of organically bound N in the SOM, which soil microbes can mineralize, and make available for plant uptake (Nannipieri and Eldor 2009, Kleber et al. 2007, Rillig et al. 2007). Furthermore, the extracellular enzyme-mediated depolymerization of proteins to amino acids, rather than breakdown of amino acids to ammonium (NH_4^+), has been identified as a major rate-limiting step in soil N cycling (Jan et al. 2009, Mooshammer et al. 2012, Schimel and Bennett 2004). Proteins can therefore influence the functionality of soil by storing (immobilization) and subsequently releasing N through mineralization processes. There are many research papers in the literature that support the ACE protein method as a sensitive indicator for distinguishing among treatments, i.e., among differing levels of SH. In the literature, it has commonly been referred to as "glomalin" or "glomalin-like" substances (e.g., Balota et al. 2016, Lozano et al. 2016, Luna et al. 2016, Nogueira et al. 2016, Sandeep et al. 2016, Singh et al. 2016, Turgay et al. 2015). Although the early literature attributed the extracted proteins to the fungal taxonomic group *Glomales* (hence the name glomalin), there is clear evidence that the extracted proteins represent a wide range of organic sources, and that they reflect key soil ecosystem functions or processes likely including N supply (Rosier et al. 2006, Hurisso et al. 2018).

Correlation with CO_2 flush may be an alternative to measuring ACE proteins (Picone et al. 2002, Franzluebbers and Stuedemann 2015), but would be reflective of the microbial activity, not of the quantity and quality of the organic matter source (specifically its N-containing fraction). A combination of source and activity (ACE proteins and respiration) would conceptually lead to better information about N availability (Hurisso et al. 2018).

The inclusion of potentially mineralizable N (PMN) in SMAF is based on its relation to nutrient availability and a theorized relationship between microbial activity and plant productivity (Hendrix et al. 1990, Sparling 1997). Unfortunately, this method is time consuming, between 7

and 28 days, dependent on the method used. In addition, some of the colorimetric methods generate hazardous chemical waste that must be disposed of properly. This makes it impractical for a high throughput lab.

The cold water-soluble organic nitrogen (WEON) test, as used in the Soil Health Nutrient Tool (Haney et al. 2017) may have potential. Mitchell et al. (2017) found a positive trend between WEON and implementation of conservation practices (no-till, cover crops).

The Illinois Soil Nitrogen Test (ISNT), or SLAN or direct steam distillation, estimates the amount of readily mineralizable soil organic N in the form of amino-sugars. Reportedly, the ISNT analysis will reflect soil organic N mineralization for the next 2–3 years. It has been tested primarily in corn systems and used to predict how much N fertilizer to use. Osterhaus et al. (2008) found that ISNT values were not related to observed economic optimum N rates in the corn N response experiments. They also noted that ISNT had no ability to separate N-responsive from nonresponsive sites. ISNT was highly correlated with SOM (R^2=0.88).

For possible use of N-acetyl-β-D-glucosaminidase (NAG) or proteases, please see the Metabolic Activity section. NAG is involved in the decomposition of amino-sugars, a subgroup of proteins. The thought is that NAG activity is correlated with the presence of its substrate, the amino-sugars. The use of protease activity follows a similar concept. Proteases are not recommended methods due to the need to use fresh, field-moist soil.

Assessment and Interpretation
It is generally agreed that the more N in the SOM, the healthier the soil, and the more that can be mineralized to support plant growth (more-is-better). ACE protein is in CASH; PMN is in SMAF, but PMN takes too long for a production laboratory. ACE protein is being added to SMAF.

Microbial Diversity

Soil health has been defined as “the continued capacity to function as a vital living ecosystem” that sustains plants, animals, and humans while maintaining or enhancing water and air quality. At the core of this definition is the “living ecosystem” that includes soil biota representing an array of trophic levels present in astounding quantities (Lehman et al. 2015). Among the life in the soil, the microorganisms represent the most numerous and metabolically complex. Essentially every aspect of plant biology is affected by interactions with microbes (Reid and Greene 2012); many of these interactions have been known for more than one hundred years. For example, rhizobia were first described by Martinus Beijerinck in 1888, whereas other relationships are just being discovered now due to the rapid advancement of molecular techniques. Some key functions performed by soil microbes relevant to agriculture and the environment include—

- Decomposition and formation of SOM.
- Nutrient provision and cycling.
- Formation and stabilization of soil aggregates.
- Protection from plant pathogens and pests.
- Production of plant growth promoting chemicals.
- Enhancement of water availability for plants.
- Neutralization of toxic compounds.

- Maintenance of vast genetic information of unknown and untapped potential.

Key challenges to identification and adoption of methods for microbial community composition and structure include a shift of how soil is sampled, transported, and stored along with a paucity of interpretable or actionable information and high cost of analysis. Thresholds or ranges to define relative poor/good functioning are currently not known yet could be established if standard procedures are followed. Despite the perceived challenges facing development of interpretation criteria for microbial methods, ignoring the microbial community in a large SH sampling or monitoring network would be a lost opportunity and a grave omission.

Candidate Indicator and Methods

- Phospholipid fatty acid profile (PLFA) (e.g., Buyer and Sasser 2012), see appendix 7.
- Ester-linked fatty acid methyl ester profile (EL-FAME).
- Simply sample soil properly and archive for future microbial assessments.

Discussion

In comparison to other SH assessment categories, microbial population methods are relatively novel and do not have a long-established history with known trends and thresholds. Common methods for coarse-level microbial characterization through lipid profiling include PFLA and EL-FAME, which provide an estimate of the microbial biomass and similar information regarding shifts in the microbial community. This information can then be translated into functional attributes important for SH including enhanced nutrient cycling or soil stabilization that can be supported by other SH indicators such as enzyme assays, N mineralization potential, aggregate stability, etc. For instance, arbuscular mycorrhizal fungi (AMF) and certain groups of bacteria (usually Gram negative), have been shown to play important roles in the formation and stabilization of soil aggregates.

Thus far, certain directional generalizations have been established by the research community and published works. For example, increases in total and AMF biomass (estimated from total fatty acid content) and the fungal-to-bacterial ratio have been associated with enhanced SH and have served as sensitive indicators to reflect short-term changes in management (1 to 3 years). Additional ratios obtained from lipid profiles include “stress ratios” and the ratio of Gram-positive-to-Gram-negative bacteria which tend to decline with implementation of SH management practices. PLFA is currently being offered by some commercial labs. However, EL-FAME provides essentially the same information and is less expensive per sample to conduct. Miura et al. (2017) compared the two methods and found that for fungi, the PLFA method is more suitable than EL-FAME

Due to the lack of a widely accepted, direct measure of community structure, one additional option is to sample soil properly and archive it appropriately for future microbial assessments. This would involve “sampling for life” whereby soil samples are kept cool and shipped rapidly (1 to 2 days) on ice for sample storage. Samples should then be maintained at −80°C. The archived sample set, in conjunction with the other soil health tests and metadata from the field, would create an unparalleled opportunity that many soil microbial ecologists would be very interested in pursuing. Furthermore, this option should be considered regardless if PLFA or EL-FAME methods are employed so that novel technologies can be leveraged in the future. This

option would require a storage facility and represents an opportunity for public and private partnerships to create and maintain the archive.

Assessment and Interpretation
Neither PLFA nor EL-FAME has an interpretation at this time, however, as samples are collected, eventually there should be enough to develop a robust interpretation (scoring curves).

Soil Health Assessments (Interpretations)

Soil Management Assessment Framework (SMAF)

SMAF, developed by the USDA ARS and NRCS, provides site-specific interpretations for soil health indicator results. The SMAF uses measured soil indicator data to assess management effects on soil functions using a three-step process that includes indicator selection, indicator interpretation, and integration into an index. Indicators used in the SMAF include soil physical, chemical, and biological characteristics that are management sensitive and therefore dynamic. Currently, the SMAF includes 11 indicators with scoring curves consisting of interpretation algorithms (some including logic functions). They are: wet macroaggregate stability, bulk density, electrical conductivity (salinity), pH, Na-adsorption ratio (used only in naturally high Na soils, western irrigated lands, and specialized situations such as high-tunnels), extractable P and K, SOC, microbial biomass C (MBC), potentially mineralizable N (PMN), and B-glucosidase activity (BGA) (Andrews et al. 2004, Stott et al. 2010, Wienhold et al. 2009).

SMAF uses broad soil taxonomic groups (soil suborders) as a foundation for assessment, allowing for the modification of many of the scoring indicator values based on soil suborder characteristics, and providing a contextual basis for indicator interpretation. Soil health and its assessment is soil and site specific and depends on a variety of factors, including inherent soil characteristics, environmental influences such as climate, and human values such as intended land use, management goals, and environmental protection, all of which are considered (and can be manipulated by the user) in the SMAF tool.

Currently, the SMAF includes four microbial or biochemical indicators: SOC, PMN, MBC, and β-glucosidase activity, all represented by more-is-better curves (Andrews et al. 2004). SOC is considered the leading baseline indicator, and while it is a chemical measurement, it is a product of organic matter formation and degradation that is primarily microbially mediated.

To increase the sensitivity of the SMAF to management impacts, the development of additional indicator scoring curves has been encouraged. Scoring curve development is a multistep process starting with the identification of an indicator, determining the type of relationship between the indicator and a specific soil function, identifying an appropriate mathematical equation(s) describing that relationship, and validating the scoring curve (Andrews et al. 2004, Stott et al. 2010, Wienhold et al. 2009). There are basically three types of relationships between scoring curves and soil function: (i) more is better (upper asymptotic sigmoid curve), (ii) less is better (lower asymptotic sigmoid curve), and (iii) having a midpoint optimum (Gaussian function) (Andrews et al. 2004, Karlen and Stott 1994).

Cornell's Comprehensive Assessment of Soil Health

There is also a farmer-oriented assessment tool: the Cornell CASH (Idowu et al. 2008, Moebius-Clune et al. 2016). This assessment evaluates relative soil functioning with respect to crop

production and environmental impact. Most of the scores are effectively percentile ratings, comparing a measured value to the known population distribution in a textural group. Future work needs to associate thresholds with agronomic and environmental outcomes appropriate to soil, climate, and production system. CASH was initially based on SMAF, but as CASH moved into a high-throughput lab setting, it shifted to indicators with faster procedures (e.g., ACE proteins substituting for PMN, POXC for microbial biomass C). Since it was originally developed for New York, the decision was made to vary scoring functions by texture, but to drop the adjustments due to soil inherent characteristics represented by the soil taxonomic classification, as well as climatic conditions. This approach appears to work well within the region, however CASH scoring functions for outside of the Northeast are still in development.

Other Soil Health Assessments

The USDA NRCS Soil Conditioning Index (SCI) estimates the effects of crop management on SOC levels (NRCS 2002). The SCI was designed to determine if SOC levels would increase, decrease, or remain stable under a given management system. When the SCI was compared with the SMAF SOC indicator (a more direct comparison than using the full suite of SMAF indictors), the SMAF SOC was more successful in separating the tested cropping systems (Zobeck et al. 2007, Zobeck et al. 2008, Zobeck et al. 2015).

The AgroEcosystem Performance Assessment Tool (AEPAT) is a research-oriented index methodology that ranks agroecosystem performance among management practices for chosen functions and indicators (Liebig et al. 2004). There is general agreement between the AEPAT and the SMAF (Wienhold et al. 2006), however, the input requirements and intended uses of the two tools are different, making a direct comparison difficult.

Karlen et al. (2008) compared the SMAF with the SCI, the soil tillage intensity rating tool (STIR), and the N-leaching index that have been incorporated in the Revised Universal Soil Loss Equation, Version 2 (RUSLE2) (USDA-ARS et al. 2014). The RUSLE2 estimates soil loss due to rill and interrill erosion caused by rainfall on cropland (USDA-ARS 2005, Lightle 2007). STIR was developed to replace the soil disturbance rating used in the original SCI and can function as a stand-alone rating to evaluate tillage and planting effects on factors other than ground cover and surface residue distribution. The N-leaching index is computed based on the soil hydrologic group and annual and winter rainfall (Pierce et al. 1991) and can be used to compare the potential for N leaching among various management systems. The SMAF soil quality index was significantly negatively correlated with soil loss as calculated by RUSLE2 and the N-leaching index, significantly positively correlated with the SCI, and not correlated with the STIR rating (0.08). SMAF appeared to provide more information about the effects of management practices within the watershed examined (Karlen et al. 2008).

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Appendix 1

Soil Organic Carbon

This SOP is based on the one found in the USDA NRCS Kellogg Soil Survey Laboratory Methods Manual (Soil Survey Staff 2014, pp. 464–471) with a few minor additions for clarification (LECO 2014). Directions specific to a specific instrument were eliminated. Please refer to methods provided for your specific instrument. It is based on the method outlined by Nelson and Sommers (1996). For soils with a pH ≥ 7.2, inorganic C needs to be measured (Sherrod 2002).

Summary of Method for Total C
An air-dry (80 mesh, <180 μm) sample is packed in a tin foil or placed on a ceramic boat, weighed, and analyzed for total C by an elemental analyzer. Depending on the instrument used, total N and S may also be measured.

The elemental analyzer works according to the principle of catalytic tube combustion in an oxygenated CO_2 atmosphere and high temperature. The combustion gases should be free from foreign gases. The desired measured component, CO_2, is separated from the bulk sample with the help of specific adsorption columns and are determined in succession with a thermal conductivity detector. Helium is the flushing and carrier gas.

If the soil sample has a pH < 7.2, total C is considered to be equal to soil organic carbon. If pH ≥ 7.2, then inorganic C should be measured by the method of Sherrod et al. (2002) and subtracted from the total C value.

Interferences
Contamination through body grease or perspiration must be avoided in sample packing. Insufficient O_2 dosing reduces the catalysts, decreasing their effectiveness and durability.

Safety
Exhaust gas pipes should lead into a ventilated fume hood. Aggressive combustible products should not be analyzed. Before working on electrical connections (adsorption columns) or before changing reaction tubes, the instrument must be cooled down and cooled off. Gloves and safety glasses should be worn at all times during operation and maintenance of instrument.

Equipment
A C or elemental analyzer equipped with an automatic sample feeder and an online electronic balance (±0.1 mg sensitivity). Parts will depend on the make and model of the analyzer chosen. Many instruments will include—

- Combustion tube.
- Reduction tube.
- Gas purification (u-tube).
- O_2 lance.
- Tin foil cups and/or ceramic boats.
- Computer with software for the analyzer and a printer.

Reagents
The reagents needed will vary depending on the analyzer, use the reagents specified for total C analysis for your analyzer.

Procedure
Refer to the manufacturer's manual for operation and maintenance of the elemental analyzer. Conditioning of the elemental analyzer and determination of factor and blank value limit are part of the daily measuring routine. The analyzer furnace temperature should be 1350°C to achieve full combustion (e.g., Leco Corp. 2014). Soil used for the analysis should be finely ground. The soil sample size used will be between 0.200 to 0.05g oven-dried weight, depending on the instrument.

A calibration that covers the desired working range of the C samples should be performed periodically. The final calculation is:

$$Total\ C\ (\%) = \frac{[g\ CO_2 sample] - [g\ CO_2, blank]}{g\ oven - dried\ soil} \times 0.2727 \times 100$$

Soil Inorganic Carbon

Total C for a soil sample with pH ≥ 7.2 needs to be corrected for inorganic C content (Sherrod et al. 2002). The SOP was prepared by David DenHaan and approved by Amy Morrow, USDA ARS National Laboratory for Agriculture and the Environment.

Instrumentation
Transducer with voltmeter

Scope and Application
- This method measures the increase in pressure when CO_2 gas is liberated upon addition of 50percent concentrated hydrochloric acid (HCl) with 3 percent iron chloride ($FeCl_3$) in a sealed container.
- This method is applicable to finely ground soil samples that have been pre-screened for effervescence upon acid addition.
- The analytical range is 0.01 percent inorganic C on a weight/weight basis.

Summary
- This SOP describes the analysis of finely ground soil samples for C in carbonate form by measuring the pressure change in a sealed container upon addition of 50 percent HCl with 3 percent $FeCl_3$. One gram of sample ± 0.005 g is weighed into 20 mL serum bottles and sealed with 20 mm butyl stopper and 20 mm aluminum seal. The samples are injected with 2 mL of the acid and allowed to react for 6 hours. When CO_2 is released, the pressure in the serum bottle increases. This pressure is measured with a transducer in millivolts. The pressure measurements are compared against standards made with sand and calcium carbonate.

Safety
All laboratory personnel should wear lab coats, protective safety glasses, and protective gloves while handling samples and reagents.

Equipment
- 20 mL serum bottles
- 20mm butyl stoppers
- 20mm aluminum seals
- A balance capable of measuring 0.005 g
- Pressure transducer connected to a voltmeter as described by Sherrod et al. (2002)
- 2ml luer-lock syringe
- 25 gauge, 1.5" luer-lock needle

Reagents and Consumables
- Concentrated hydrochloric acid (HCl), A.C.S. certified at about 12.1 N
- Deionized water with at least 14 mega ohms-cm resistance at 25 °C.
- 50 percent HCl with 3 percent $FeCl_3$: In a 250 mL volumetric flask add 7.5 g $FeCl_3$ and dissolve with less than 125 mL deionized water, add 125 mL concentrated HCl, dilute to volume.
- Sand
- Calcium carbonate ($CaCO_3$)
- Inorganic carbon standards (table A1.1)

Calibration and Standardization
- Twelve standards are used and a blank that consists of sand.
- The standard readings are into a spreadsheet the slope, intercept and regression coefficient are calculated.
- The regression coefficient (r) should be 0.995 or greater.
- Several controls are run to validate the curve and the samples – Two duplicates of North American Proficiency Testing samples providing a low, medium, and high inorganic carbon range.

Quality Control
Ten percent of samples are run in duplicate.

Table A-1.1. Composition of Inorganic C Standards.

	$CaCO_3$ (g)	C from $CaCO_3$ (g)	g sand	Inorganic C (%)
Std 1	0.02	0.002	19.98	0.012
Std 2	0.04	0.005	19.96	0.024
Std 3	0.05	0.006	19.95	0.030
Std 4	0.07	0.008	19.93	0.042
Std 5	0.10	0.012	19.90	0.060
Std 6	0.20	0.024	19.80	0.120

	$CaCO_3$ (g)	C from $CaCO_3$ (g)	g sand	Inorganic C (%)
Std7	0.40	0.048	19.60	0.240
Std 8	0.70	0.084	19.30	0.420
Std 9	1.00	0.120	19.00	0.600
Std 10	2.00	0.240	18.00	1.200
Std 11	3.00	0.360	17.00	1.800
Std 12	4.00	0.480	16.00	2.400

Procedure

1) 1 gram ± 0.005 g of sample and standards are weighed into 20 mL serum bottles.
2) The bottles are sealed with a butyl stopper and an aluminum seal and crimped.
3) 2 mL 50 percent HCl with 3 percent FeCl3 is drawn up into a luer-lock syringe with a 25 gauge 1.5” needle and injected through the butyl stopper.
4) Allow samples to react for 6 hours with the reagent before analyzed with the transducer. The voltage is recorded.
5) Results are entered into a spreadsheet to convert millivolts into Inorganic Carbon (%), using the standard curve that was developed (table A1.1).
6) Organic C (%) = % total C (%) value obtained through dry combustion (above) minus the Inorganic C (%) value.

Precision and Accuracy

The inorganic C (%) values between 0.01 and 0.03 are between the MDL and LDL standards.

Appendix 2

Aggregate Stability

The purpose of the aggregate analysis is to determine the size distribution of the water-stable aggregates and the amount of aggregation. A soil sample is placed on a nest of screens under water. The screens are moved up and down for a specified amount of time. The mass of the soil remaining on the individual screens is determined, corrected for sand/gravel and the percent macro and microaggregate stability is calculated.

The SOP presented here is from Mikha and Rice (2004). The number of sieves used were reduced so that there are only three fractions: macroaggregates (>250 μm), microaggregates (250-53 μm), and nonaggregated material (>53 μm).

Materials and Equipment

- Electronic balance with a link to a computer so that weights can be recorded directly into a spreadsheet.
- Labeled weigh boats (two per sample) A and B.
- Labeled aluminum pans (e.g., 1 lb bread loaf pans), three per subsample—example:
  1A – 10, 1A – 60, 1A – 270
  1B – 10, 1B – 60, 1B – 270.
- Yoder-style wet sieving apparatus (set for 30 cycles per min) and water tubes (4 per machine).
- Sieves: #10 (2.00 mm), #60 (250 μm), #270 (53 μm).
- Spreadsheet to record weights with appropriate reference numbers.
- Source of DI water for filling sieve tubes and rinsing bottles.
- Rubber policemen for aggregate breakdown.
- Forced air drying ovens.
- DI water source.
- Optional: a tall bread rack on wheels to store samples during drying process.

Procedure

A 25 g sub-sample of air-dried, 8 mm air-dried soil is analyzed for water stable aggregation. A modified Yoder sieving machine (see Kemper and Rosenau 1986), set to 30 strokes per minute (min) for 5 min, is used to determine the percent of water stable aggregates present in the soil sample. Samples are not pre-wetted (Kemper and Chepil 1965), to represent the soil in its most vulnerable state. Deionized (DI) water should be used during the procedure so as not to introduce chemical dispersion of the aggregates.

Safety

- No food or drink in area.
- Gloves and lab coat recommended.

Sample collection

Samples should be collected at least 2 to 3 days before analysis. While the soil is moist and friable, gently break it up (don't crush or compact) and passed through an 8-mm sieve. Then air-dry the soil and store until time to analyze.

1) Labeling and sample preparation
   a) Label (pan ID#) and record all pan weights.
   b) Label large weigh boats with lab ID#.
   c) Homogenize soil samples by dumping them onto butcher paper and grabbing corners to mix.
   d) Weigh and record 25.0 g (± 0.25 g) of soil into weigh boats.
2) Sieving machine set up
   a) Check that machine runs for 30 cycles per minute.
   b) Check bracket and tube conditions, overall condition of equipment, deal with any problems.
   c) Note that there are 4 tubes of one size and 4 tubes of another size; be sure to use similar tube sizes for sample reps A and B.
   d) Put sieves in order (2 mm on top; 1 mm, 0.5 mm, 0.25 mm on bottom).
   e) Hang sieves in the sieving bracket.
   f) Fill tube with DI water, water level should be just touching the screen on the top sieve.
3) Procedure
   a) Pour soil sample in the top sieve, start machine and timer (5 min).
   b) Sieve each sample for 5 min.
   c) When finished sieving, remove the sieve set and position to drain excess water for a few minutes (set the sieves angled on the top of the tube).
      Using DI water from the sink or from wash bottles, rinse soil from each sieve into its corresponding pre-weighed pan. NOTE: Water from the tubes will need to be passed through the #270 (53 µm) sieve and treated the same as the other sieve fractions.
   d) If the pan becomes too full to transport easily, use a second (pre-weighed) pan. Be sure to note on the pan that it is a second pan and for which sample ID#.
   e) Place filled pans in the soil drying oven at 70 °C, until all water has evaporated, and the soil is dry (usually overnight).
   f) Remove pans from oven when dry and record weight (1^{st} weight = pan + soil + [sand, gravel]).
      - Put pans in numerical order before entering data weights—this will help to eliminate data entry errors.
      - Watch the balance and the file to be sure you are capturing the weight—the balance will sometimes take a few seconds to stabilize—use a balance cover if there is too much air movement in the lab and the balance seems unstable.
   g) Save these pan + soil combinations for the next procedure (correction for sand and gravel).
4) Correction for sand and gravel
   a) Remaining soil is now rinsed into the same size sieve.
      By means of a gentle stream of water and a rubber policeman the fine particles are washed through the screen. Use the rubber policeman to crush aggregates on the side walls of the sieves, avoid crushing aggregates on the screens themselves.
   b) Sand and gravel remaining on the screen are washed into the same labeled pan and placed in the soil drying oven at 70 °C, until all water has evaporated, and soil is dry (usually overnight).
      Remove pans from the oven when dry and record weight (2^{nd} weight = pan + [sand, gravel]).

5) Cleanup
   a) Make sure any remaining soil samples are safely put away where they won't be inadvertently dumped and labeled.
   b) Rinse and invert to dry. Sieve machine tubes, sieves, pans, and weigh boats used for the day.

Calculations

Soil + Sand	Pan + Soil + [Sand,Gravel] (1st weight (wt)) less the pan wt
Sand	Pan + [Sand,Gravel] (2nd wt) less the pan wt
Corrected Soil Aggregate wt	Soil + Sand – Sand
Total C Soil Aggregate wt	Per Sample = Sum of all sieve size classes
Total Sand, Gravel	Per Sample = Sum of all sieve size classes
Total Corrected Sample wt	Sample wt less [Sand,Gravel] sum
Total Water Stable Soil Aggregate Fraction	(Total Corrected Soil Aggregate wt) / (Total Corrected Sample wt)
Sieve Mean Diameter	Average of the lower limit and the upper limit of sieve (Opening in screen passed + opening in screen retained) / 2
Fraction Soil Aggregates in each fraction class	Corrected Soil Aggregate wt / Corrected Sample wt
Fraction data for Mean Weight Diameter (MWD) calculation	For each size class: Sieve Mean Diameter X Fraction Soil Aggregate
Mean Weight Diameter (MWD)	Sum of above product for each sample (mm)

Appendix 3

Short Term Carbon Mineralization

This SOP is from Schindelbeck et al. (2016) under "Soil Respiration". It is a measure of the amount of C that is readily available to the microbial population as food and energy. It is based on the amount of CO_2 released from a soil by microbial activity during a specific incubation period and water content. The CO_2 concentration in this method is by electrical conductivity. Alternatively, the CO_2 concentration can also be measured by titration or by gas chromatography.

Materials and Equipment

Pre-Setup

- Soil samples (sieved to 8 mm, air dried to constant mass).
- Jars with lids (wide mouth, 1-pint canning jars, with standard 2-part lids.
- Labeling tape and Sharpie® or similar marker.
- Filter papers (55 mm).
- Weighing paper (3" x 3" or 4" x 4").
- Pre-perforated aluminum weigh boats (with 9 holes in bottom, using a needle).
- Trap assembly (beaker on a pizza stool, attached using foam tape).
- Jar rack that holds at least 22 jars.
- Record keeping notebook or spreadsheet.

Incubation Initiation

- Jar or beaker with distilled, deionized H_2O (ddH_2O).
- Jar or beaker with 0.5 M KOH (keep covered).
- Large beaker to cover the KOH jar or beaker.
- Paper towels 10 mL pipettor 10 mL pipette tips.
- Marker.
- Gloves.

Post-Incubation Reading

- Incubated samples in jars.
- Electrical conductivity (EC) meter.
- KimWipes® or similar laboratory tissues.
- Cut strips of filter papers.
- Gloves.
- Large (1/2 gal) jar for waste KOH.
- Wash tub with ddH_2O for trap assemblies.
- Trash receptacle for other jar contents.
- HCl to neutralize KOH.
- pH test strips.
- Stir plate.
- Stir bar

Method

- Start with sieved, air-dried soil. Gather clean, dry mason jars with lids, filter papers, labeling tape, and marker, pre-perforated aluminum weigh boats, and weighing papers.
- Have a jar rack ready to place jars with soil samples in after weighing.
- Label a set of jars for the range of samples you will be weighing out.
- Include 2 replicates (2 jars) per soil sample, appending "a" and "b" to the sample number (e.g., L-123a and L-123b).
- Use labeling tape with marker rather than writing on jar, to facilitate jar cleanup.
- Add 2 filter papers to the bottom of each jar, offset from each other. Use long forceps if necessary for adjusting placement of filter papers.
- Weigh 20.00 g dry soil into a pre-perforated aluminum weigh boat. Use a weigh paper below the weigh boat on the balance pan to catch soil that falls through the perforations in the bottom of the boat. Be sure to re-tare the balance when placing a new weighing paper on it.
- Place the weigh boat into the jar. Use long large forceps to pick up the weigh boat by the back tab, holding with the tips far enough down to pick up the boat without it flexing. Gently place weigh boat onto the weighing papers in the jar. Tap soil remaining on the weighing paper on the balance pan into pile of soil in weigh boat while transferring the weigh boat to the jar.
- Jars can be stored with soil pre-weighed a few days ahead of time, cover with individual lids or with a sheet of Kraft paper.

<u>Prepare Respirometer Jars for Incubation Set Up</u>

- Start with a set of jars with soil samples pre-weighed in them.
- Gather trap assemblies (10 mL beakers stuck to plastic tripod "pizza stools," using foam tape).
- Place one trap assembly into each respirometer jar, pressing the legs of the stool down into the soil sample to allow it to firmly stand, and to better conform the shape of the flat-bottomed weigh boat to the slightly domed jar bottom.
- Set up a rack at a time (or a couple racks) of trap assemblies with soil samples to save time in advance.

<u>Set up Incubations</u>

- Place fresh paper towels on a clean space on the bench. Label one "KOH – 9 mL," and the other "H_2O – 7.5 mL".
- Place a fresh 10 mL pipette tip on each of the labelled paper towel.
- Place stock KOH into a clean, dry 500 mL beaker—label the beaker "KOH 0.5 M." Place ddH_2O into another clean, dry beaker. Label this beaker "ddH_2O". Cover these beakers until use to excessive air exposure.
- To a few jars at a time (a set of 11 at a time is convenient, 10 samples plus a blank):
    - Add 9 mL 0.5 M KOH to trap beakers in jars. Take extra care to avoid dripping any KOH onto the soil sample or other jar contents.
    - Add 7.5 mL ddH_2O into each jar via the inside wall of the jar, as far down as you can safely and effectively hold the pipette tip without it contacting anything else in the jar (the amount of water may need to be adjusted for high sand or high clay content

soils—the ideal water content is 60 to 70 percent field capacity (Linn and Doran 1984).

- Immediately place a lid flat onto each jar, minimizing the amount of time that the jar is open. Minimize the difference in amount of time that different jars are open, and keep blanks in sequence with sample jars. Close the jars securely with the screw top rings. Screw on tight enough to make an airtight seal. Keep in mind that opening the jars at the end of the incubation without spilling the traps.
- Carefully move closed jars to a holding rack, avoiding tipping the trap assemblies inside.
- Set rack on shelf, with a note indicating sample number range, date and time set up (note beginning time for batch, when traps began to be dispensed) and date and time to take down (4 days incubation time, start reading at the same time of day as the batch incubations were started—timing from when traps are dispensed).
- Discard used tips, paper towels, etc. Discard extra KOH after neutralizing. Do not set aside and use for further traps, other than those set up in an immediate tandem run. CO_2 absorption from the air, and evaporative concentration of the solution make it inadvisable to reuse.
- Incubate 4 days at room temperature. Avoid major temperature fluctuations.

Measure After Incubation

- Calibrate EC meter according to manufacturer's directions. Make sure to blot probe dry with a KimWipe and use a filter paper strip to blot KOH off the metal temperature probe stud in the probe opening. Blot, don't wipe.
- Probe can be set dry on a paper towel in between measurements if it is already blotted dry. Do not let KOH dry onto the probe.
- Carefully retrieve jar rack from shelf, and move it to the bench.
- Gently remove a run of jars, in order, as when setting up the incubation.
- Carefully remove the rings from the run of jars, leaving the flat lids in place—they should stay sealed on the tops of the jars.
- Pop the lid off the first jar and place the probe into the trap beaker, giving a brief gentle stir with the probe in the process, then letting it rest at the bottom of the trap beaker. Let the reading stabilize and then record it.
- After taking the reading, immediately blot the probe. In the case of the first jar, this should be some used KOH rather than a sample proper.
- If doing stretches of 11 or 12 jars, try to match the timing of reading with how long it takes to set up the set of jars initially, and avoid having jars sit open for very long.
- When the rack is finished, rinse the probe well in the tap water beaker, and then again in the ddH_2O beaker. Set onto paper towel to dry.

Cleanup

- Neutralize KOH before disposal. Place jar on stir plate in fume hood with sash drawn low, and place a stir bar in the jar, turn stir function on at low to moderate speed. Wear gloves and eye protection. Neutralize with HCl while stirring, and test with litmus paper strip. When neutral, dispose of it according to local requirements.
- Separate trap beakers from pizza stools. This can be easier after they have soaked for some time in a tub of water, as small amounts of residual KOH will make the water basic and this

will loosen the adhesive. Set beakers into a tub of distilled water to soak overnight before cleaning. Set pizza stools aside to be cleaned and reused.

- Empty jars into waste bin, discarding weigh boat and filter papers with the soil.
- Wash and rinse all glassware well, rinsing several times at the end with distilled water and then a final rinse in ddH_2O. Air-dry. If any residues become apparent after drying, wash again and rise well. Residues may interfere with further usage.

Calculations

Nine milliliters of the 0.5 M KOH can theoretically accommodate 0.009 L * 0.25 mol/L * 44.01 g/mol * 1000 mg/g = 99.025 mg CO_2. Of this total trap capacity of 99.025 mg CO_2, some fraction is actually absorbed. The difference between the measured EC for a sample (or blank) and the EC of the "raw" KOH is a quantity referred to as the "observed EC drop." This quantity is some fraction of the total possible drop, which we can call "full capacity EC drop." Dividing the observed EC drop by the full capacity EC drop gives a fraction that is equivalent to the fraction of the total trap capacity for CO_2 absorption that is used. So, it is key that all measurements are made at the same temperature. If EC_{raw} is the electrical conductivity of pure 0.5 M KOH and EC_{sat} is the electrical conductivity of 0.25 M K_2CO_3 and EC_{sample} is the electrical conductivity of the trap associated with a particular sample, and P is the proportion of the trap capacity for CO_2 absorption that is actually used, then

$$((EC_{raw} - EC_{sample})/(EC_{raw} - EC_{sat}))=P$$

and

$$P*(\text{trap capacity in mg}) = CO_2 \text{ mg absorbed by the trap in question.}$$

Multiple replicates of each sample should be run, and the values averaged. If these differ from each other by more than a set threshold (suggest an average deviation of 5 percent from the common mean), then the sample should be rerun.

Appendix 4

Enzyme Assays

The four enzymes proposed for use are—

- β-glucosidase (BG) that is involved in the C-cycle (Eivazi and Tabatabai 1988, Deng and Popova 2011),
- *N*-acetyl-β-D-glucosaminidase (NAG) that is involved in both the C- and N-Cycle (Parham and Deng 2000, Deng and Popova 2011),
- Phosphomonoesterases (acid/alkaline phosphatase; Pase) is involved in the P-cycle (Eivazi and Tabatabai 1977, Acosta-Martínez and Tabatabai 2011), and
- Arylsulfatase (AS) that is involved in the S-cycle (Tabatabai 1970, Klose et al. 2011).

This SOP is from the lab of Veronica Acosta-Martinez, USDA ARS, for methods based on *p*-nitrophenol release, and Soil Survey Staff (2014, pp. 513–519)

Preparation for Assay

A general assay protocol is described below as it applies for the four enzyme assays suggested. The activities of enzymes can be measured in air-dried soil conditions to facilitate sample handling/ space-storage required and comparison across regions. Reagents and their preparation is given in table 4.1. The original solution amounts and distribution of organic matter and nutrients in soil (Deng and Popova 2011, Acosta-Martinez and Tabatabai 2011, Klose et al. 2011) have been reduced in half of the original assays to reduce overall cost with lower reagents needed and waste generated. Make sure there is sufficient substrate for your soil type to ensure the procedure is not substrate limited. Healthy soils may require the original amounts, so double the values in table 4.1. Additionally, toluene is not part of these assay protocols due to environmental, safety and cost considerations.

Before starting an assay, ensure that enough buffer is available at room temperature (about 6 mL per sample), and prepare appropriate substrate with buffer for the total amount of samples to be analyzed (assume 1.5 mL per sample). It is not necessary to run a control for each soil sample if the same soil type has been evaluated in the lab and control absorbance readings have been consistent. Thus, certain samples can be selected to have controls for the same soil type under different management practices, which can reduce the total number of samples to be analyzed for each assay.

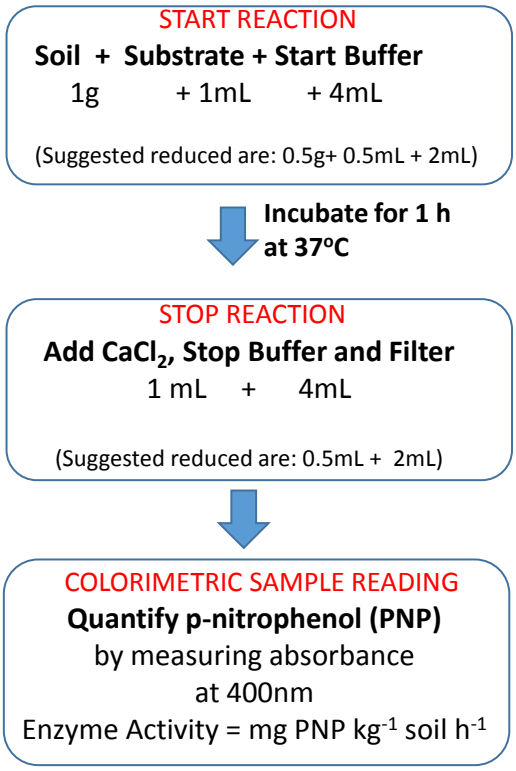

Figure 4.1. Flow sheet for the determination of enzyme activities that have *p*-nitrophenol as a reaction product.

Equipment

- Electronic balance, ±1.0-mg sensitivity.
- Magnetic stir plate, with stir bars.
- Incubator set to 37 ºC.

- Funnel stand to accommodate several glass funnels.
- Colorimeter or spectrophotometer, set at 410 nm.

Materials

- Volumetric flasks, acid washed, 100 mL, 1000 mL.
- Incubation flasks, Erlenmeyer flasks, acid washed, 25 mL, fitted with No. 1 stoppers.
- Tray to hold Erlenmeyer flasks for easy transfer to and from incubator.
- Funnels, long stem, about 50-mm diameter.
- Filter paper to fit funnel, Whatman® 2V.
- Test tubes to capture filtrates.
- Pipettes, 10 mL, with tips.
- Cuvettes to fit colorimeter or spectrometer.

Reagents

- Deionized water (dH_2O).
- Modified universal buffer (MUB) stock solution: Dissolve 12.1 g of tris(hydroxymethyl)aminomethane (THAM), 11.6 g of maleic acid, 14.0 g of citric acid, and 6.3 g of boric acid (H_3BO_3) in 488 mL of 1 N sodium hydroxide (NaOH) and dilute the solution to 1 L with dH_2O. Store it in a refrigerator.
- Calcium chloride ($CaC1_2$), 0.5 M: Dissolve 73.5 g of $CaC1_2 \cdot 2H_2O$ in about 700 mL of dH_2O, and dilute the volume to 1 L with dH_2O.
- Sodium hydroxide (NaOH), 0.5 M: Dissolve 20 g of NaOH in about 700 mL of dH_2O, and dilute the volume to 1 L with dH_2O.
- For buffers and start and stop reagents, see table 4.1, for ones that pertain to each enzyme of interest.
- Standard *p*-nitrophenol solution: Dissolve 1.0 g of *p*-nitrophenol in about 700 mL of dH_2O and dilute the solution to 1 L with dH_2O. Store the solution in a refrigerator.

Procedure

Start reaction

- Add 0.5 g of soil to each 25 mL Erlenmeyer flask.
- Label the replicates with A and B, and use C for the control.
- Add 2 mL of *Start Buffer* to the soil in each Erlenmeyer flask (A, B, and C).
- Add 0.5 mL of *Substrate* to A and B ONLY! Place a stopper in each flask (A, B, and C).
- Swirl each flask gently and place in an incubator at 37 °C for 1 hour.

Stop reaction

- Remove flasks from incubator and remove stoppers.
- Add 0.5 mL of $CaCl_2$ to the soil in each flask (A, B, and C).
- Add 2 mL of *Stop Buffer or Solution* to A and B first, then add to C. Swirl gently after each addition.
- Add 0.5 mL of *Substrate* to C ONLY! Then swirl.
- Pour into funnel lined with filter paper, capture solution in test tubes and let stand for ~30 min until fully filtered.

Colorimetric Sample Reading

- Remove filter from funnel and place into filter hazardous waste bottle. Remove the test tube and place into a rack.
- If necessary, dilute each sample to get an absorbance ≤1.3 (dilute all samples the same if possible). Use lowest dilution possible (i.e., values closest to but below 1.3). DO NOT dilute the controls. Suggested dilution approach: Add 2–3 mL of solution to the cuvettes with a pipette, washing the pipette after each set of 2 reps (i.e., transfer 1a and 1b, wash, etc.).
- Read samples in a spectrophotometer. Note any dilution factors for all samples.
- All *p*-nitrophenol waste is considered hazardous waste (includes all filtrate, any sample that was diluted and read in the spectrophotometer).

Standards

- Prepare calibration curve plotting absorbance at 410 nm versus amount of *p*-nitrophenol using the appropriate buffer using standard *p*-nitrophenol calibration solutions so that the final concentrations are 50, 40, 30, 20, 10, 0 µg *p*-nitrophenol.
- To prepare this graph, dilute 1 mL of the standard *p*-nitrophenol solution to 100 mL in a volumetric flask and mix the solution thoroughly.
- Pipette 0-, 1-, 2-, 3-, 4-, and 5-mL aliquots of this diluted standard solution into Erlenmeyer flasks), adjust the volume to 5 mL by addition of water (i.e., 5, 4, 3, 2, 1, and 0 mL, respectively), and proceed as described in the enzyme assay protocol after incubation of the soil sample (i.e., add the stop solutions). Measure the yellow color intensity of the filtrate with a spectrophotometer set at 410 nm.

Table App-4.1: Description of the enzyme assay procedure and reagents needed for determining enzyme activities in soils.

Enzyme EC number	Substrate (disolved in start buffer)	Buffer used to start reaction Do as below & adjust to 1L with H_2O	Solution to stop reaction Do as below & adjust to 1L with H_2O	$CaCl_2$ *	Page in Soil Enzymology Book SSSA Book series, R.P.Dick (Ed)
β-Glucosidase **3.2.1.21**	*p*-Nitrophenyl-β-D glucopyranoside (0.05M) 1.506 g/ 100 mL buffer Sigma N-7006	MUB pH 6.0 [Take 200 ml stock MUB bring to pH with 0.1 N HCl]	0.1 M THAM pH 12.0 [12.2 g of THAM , adjust pH with 0.1 N NaOH]	yes	195-198
β-Glucosaminidase **3.2.1.52**	*p*-Nitrophenyl-N-acetyl-β-D-glucosaminide (10.0mM) 0.342 g/100 mL buffer Sigma N-9376	100 mM acetate buffer pH 5.5 [13.6 sodium acetate trihydrate adjust to pH with aceti acid 99%]	0.5 N NaOH [20g of sodium hydroxide]	yes	195-198
Alkaline phosphatase	*p*-Nitrophenyl phosphate (0.05M) 1.68 g /100 mL buffer 104-0	MUB pH 11.0 [Take 200 ml of stock MUB bring to pH with 0.1 N NaOH]	0.5 N NaOH [20g of sodium hydroxide]	yes	163-168
Acid phosphatase	*p*-Nitrophenyl phosphate (0.05M) 1.68 g /100 mL buffer	MUB 6.5 [Take 200 ml stock MUB bring to pH with 0.1 N HCl]	0.5 N NaOH [20g of sodium hydroxide]	yes	163-168
Phosphodiesterase	*Bis*-*p*-Nitrophenyl phosphate (0.05M) 1.82 g/ 100 mL buffer N-3002	0.05 M THAM pH 8.0 [Add 6.1 g THAM adjust pH with 0.1 M H_2SO_4]	0.1 M THAM pH 12.0 [12.2 g of THAM , adjust pH with 0.1 N NaOH]	yes	168-171
Arylsulfatase	*p*-Nitrophenyl sulfate (0.05M) 1.228 g/ 100 mL buffer N-3877	0.5 M acetate buffer pH 5.8 [68g sodium acetate trihydrate adjust pH with 1.7 mL acetic acid 99%]	0.5 N NaOH [20g of sodium hydroxide]	yes	133-137

***0.5 M $CaCl_2$ is prepared by dissolving 73.5g of $CaCl_2 \cdot 2H_2O$ in DI water with final volume of 1L.**

Appendix 5

Active Carbon

Active C (also known as permanganate-oxidizable C or POXC) measures the portion of soil organic matter that can serve as a readily available food and energy source for the soil microbial community, thus helping to maintain a healthy soil food web. To measure active C, soil is reacted with a potassium permanganate solution, which has a deep purple color. As the solution oxidizes, it loses some of its color. This loss of color upon reaction is directly proportional to the amount of active C in the soil sample, which is determined by using a spectrophotometer and calibrated against standards of known concentration. Duplicate soil samples are air dried to constant weight, shaken with 0.02 KMnO4 solution, allowed to settle, diluted and absorbance measured at 550 nm. The SOP is from Schindelbeck (2016) and is based on the method of Weil et al. (2003).

Materials and Equipment

- 50 mL centrifuge tubes with caps (e.g., Falcon® tubes) and racks.
- Bottle-top solution dispenser.
- pH meter and buffered calibration solutions.
- Analytical balance (3 significant figures).
- Colorimeter (with 550 nm setting).
- Kimwipes® or other laboratory tissue.
- 100–1000 µl pipettor and disposable tips.
- Platform shaker.
- Stop watch.
- Stir plate and stir bar.
- 1000 mL volumetric flasks, beakers, and graduated cylinder.
- Amber bottle.

Reagents

- Distilled H_2O (dH_2O).
- $KMnO_4$.
- $CaCl_2$.
- KOH.
- HCl.

Procedure

<u>Preparation of 0.2 M $KMnO_4$ stock solution:</u>

- Dissolve 11.09 g $CaCl_2$ in ~750mL dH_2O in a beaker. Dissolve completely, using stir plate with stir bar (final concentration, 0.1 M).
- Add 31.61 g $KMnO_4$ to the solution and a further 200 mL of dH_2O. Allow to dissolve completely (about1 hour), covering solution and stir plate with an opaque box or paper bag.
- Ensure that the pH meter is properly calibrated.
- Measure solution pH (final pH should be 7.2).

- Depending on pH measure, make a dilute (~0.1 M) acid or base solution using HCl or KOH. Using a pipettor, slowly add acid or base, while monitoring pH, until constant at 7.2.
- Pour solution into a 1 L volumetric flask and bring to 1000 mL with dH_2O. The solution is light sensitive—transfer to an opaque bottle, label, and date. Solution should remain stable for 3–6 months.

Standard curve

- Ensure that the colorimeter is set to 550 nm and zero with dH_2O.
- Dispense 45 mL dH_2O into each of three centrifuge tubes.
- Add additional dH_2O to the tubes in the following volumes:
  - tube 1: 3.75 mL.
  - tube 2: 2.50 mL.
  - tube 3, 0.00 mL.
- Add 0.2 M $KMnO_4$ to the tubes in the following volumes:
  - tube 1: 1.25 mL.
  - tube 2: 2.50 mL.
  - tube 3, 5.00 mL.
- Final concentrations of 50 mL $KMnO_4$ solutions are now 0.005 M, 0.01 M, 0.02 M. Cap and shake for 10 seconds.
- Dispense 20 mL distilled water into nine Falcon tubes – three for each standard solution.
- Add 0.2 mL of each standard to each respective triplicate set. Cap and shake for 10 seconds.
- Read and record the absorbance of each triplicate standard, filling the cuvette with one volume of standard and cleaning the outside with a Kimwipe to remove any liquid or smudges before each reading.
- Concentration = a + b * (absorbance). Determine the slope (b) and y-intercept (a) of a linear regression equation with concentration as the dependent variable (y) and absorbance as the independent variable (x).

Measuring Active Carbon in Soil Samples

- Each soil sample is run in duplicate, requiring 2 centrifuge tubes with 18 mL dH_2O and 2 centrifuge tubes with 20 mL dH2O.
- Samples are generally run in groups of 20 per rack (10 duplicate soil samples).
- Dispense enough distilled water into Falcon tubes for as many as ten soil samples and set aside.
- Into the centrifuge tubes, measure two 2.5 g replicates for each soil sample. (±0.005 g).
- Dispense 0.2 M $KMnO_4$ solution into a beaker in small amounts as needed (about 50 mL each) and cover with an opaque container to block light.
- In sequence, add 18 mL dH_2O to each tube containing soil. Then, in same sequence, begin redox reaction by adding 2 mL of 0.2 M $KMnO_4$ to each tube. Cap tightly.
- Place tubes and rack on the shaker at 120 rpm, start stopwatch and allow to shake for 2 min.
- After 2 min (do not stop stopwatch), remove samples from the shaker and ‘slosh’ solution in tubes to ensure that soil is not stuck to the cap or top of the tube. Uncap tubes. On bench-top, allow settling and reaction to continue for a further 8 min.

- After 10 min of total reaction time, remove 0.2 mL from each reaction tube and transfer to a centrifuge tube with 20 mL distilled water. Dispensing this 0.2 mL aliquot from the reaction tube into 20 mL distilled water is a 100× dilution; this ends the reaction.
- After all reactions have been stopped, cap the diluted sample tubes and shake by hand for 10 seconds.
- Read and record absorbance of each sample or control.
- Repeat duplicates with a difference in absorbance greater than 5 percent.
- Clean all materials, particularly colorimeter cuvette, using dH_2O.

Calculations

The bleaching (loss of purple color; reduction in absorbance) of the $KMnO_4$ is proportional to the amount of oxidizable C in the soil sample. It is assumed that 1 mole (mol) MnO_4 is consumed (reduced from Mn^{7+} to Mn^{2+}) in the oxidation of 0.75 mol (9000 mg) of C.

Active C (mg/kg) = [0.02 mol/L - (a + b * absorbance)] * (9000 mg C/mol) * (0.02 L solution/0.0025 kg soil).

Where:

0.02 mol/L is the initial solution concentration, (a + b * absorbance) is the post-reaction concentration, 9000 mg of C (0.75 mol) is assumed to be oxidized by 1 mol of MnO_4 changing from Mn^{7+} to Mn^{2+}, 0.02 L is the volume of $KMnO_4$ solution reacted, and 0.0025 kg is the weight of soil used.

Appendix 6

Bioavailable Nitrogen – ACE Protein

Bioavailable nitrogen as measured by extracting protein from the organic matter in soil samples using a neutral sodium citrate buffer to disaggregate soil and dissolve soil protein with high heat and pressure in an autoclave, and to quantify the protein content of such an extract using a bicinchoninic acid protein assay.

The extraction procedure used is a modification of an approach used to extract proteins from fungi and from soil (Keen & Legrand 1980, Wright & Upadhyaya 1996), which has been shown to extract proteins of numerous sources (Hurisso et al. 2018). The quantification assay used is a well-established procedure and chemistry, run at high temperature for an extended time to increase protein sensitivity and decrease variation by protein type (Walker 2002). The SOP is from Schindelbeck et al. (2016).

Materials and Equipment

- Soil (air-dried, sieved to 8 mm)
- 96-well spectrophotometric plate reader
- Glass extraction (centrifuge) tubes with caps
- Microcentrifuge tubes.
- Storage tubes in racks.
- Pipettors and Tips.
  - 1000 µl pipettor.
  - Large and small volume 8-channel Pipettors with 1000, 200, 20 µL tips.
- Pipetting reservoir.
- 96-well clear flat-bottom chimney well polystyrene plate.
- Tape seal for plate.
- 50 mL tube for mixing working reagent.
- Pipetting reservoir 96-well clear flat-bottom chimney well polystyrene plate.
- Tape seal for plate.
- Pierce bicinchoninic acid (BCA) reagents A and B.
- Standards set.
- 50 mL tube for mixing working reagent.

Reagents

- BCA reagents A and B (purchased).
- Protein standards set (purchased).
- 20 mM sodium citrate.
  - Prepare 20-L at a time. Use a 20-L carboy, with a cap and spigot, which has been rinsed thoroughly, using dH_2O as the final rinse.
  - In a 1-L beaker, with a stir bar, add 115.19 g Tribasic sodium citrate dihydrate (m.w. 294.10), and 1.603 g citric acid (or sufficient citric acid to yield a final pH of 7.0 – your water source may require slightly different amounts). Gently add about 500 mL dH_2O, stirring at moderate speed.

- When fully dissolved, pour solution into carboy, being sure to retain the stir bar with the beaker.
- Rinse the beaker three times, with dH_2O to full, with the stir bar in it, into the carboy, to ensure complete transfer of the citrate salts.
- Add about 10-L more dH_2O, then cover and agitate the carboy to mix contents. Fill the rest of the way to the graduated line (to 20-L total), using dH_2O, cover again and agitate to mix.
- Draw some solution through the spigot and discard so that the fresh solution is ready to be dispensed.

Procedure

Extraction

- Weigh soil into tubes.
- Label tubes in advance. Weigh out two replicates for each sample. Label these by appending 'a' and 'b' after the sample designation. Use labeling tape rather than marking straight on the glass tube.
- Weigh 3.00 g air-dried soil onto clean weigh paper. Transfer to glass extraction tube.
- Curling the paper into a funnel, and tapping on the back of it with a fingernail several times should leave no measurable mass of soil behind on the paper. You can use the same paper to weigh out a second rep of the same soil sample, which gives an opportunity to check to make sure that the paper weighs 0.00 g when put back on the balance. Use a clean weighing paper for each new sample.
- Cap tube gently.
- Add 24.00 mL extractant (20 mM sodium citrate, pH 7.0), using a bottle top dispenser. Dispense two or more times into a waste beaker to prime the dispenser. Make sure no bubbles are in the dispensing tube, as these will impact volume dispensed. Dispense additional aliquots into the waste beaker if there are bubbles or other volume-impacting issues with the dispenser. Always have the next tube under the spout when drawing the dispenser plunger up, as a small part of the volume comes out when you hit the top. This is factored into the volume setting for the dispenser. Make the draw up and the dispensing push down smooth and not stuttered as this will affect the volume dispensed.
- Cap tubes tightly after adding extractant.
- Shake at 180 rpm for 5 min.
- Remove from shaker, and swirl mixture to consolidate solids. Extractant and soil may be left on the sides of the tubes following shaking. With the caps on the tubes, swirl once or twice rapidly. This should wash these trailing amounts of soil back down into the extractant in the bottom of the tube, consolidating the contents.
- Loosen caps so they are not airtight, but still on the tube to protect contents. This is needed to avoid any pressure differential inside and outside of the tubes in the following, autoclave, step). Leave disengaged caps on top of tubes.
- Autoclave 30 min at full temperature. Follow the directions for the autoclave. Preheat autoclave while samples are shaking.
- Set aside to cool to room temperature before clarification.

Clarification

- Label, ahead of time, a set of 2.2 mL microcentrifuge tubes, and one or more racks of sample storage tubes (1.1 mL open top tubes, in strips of 8, racked in 96-place format) to accommodate the sample range to be clarified.
- Close caps on glass extraction tubes again, and resuspend solids by shaking for 1 min, then swirl to consolidate as above. Loosen or remove caps.
- Withdraw approximately 1.75 mL of mixture using a disposable plastic transfer pipet, and place this in a clean, labeled 2 mL microcentrifuge tube. Close microcentrifuge tube cap.
- Use fully labeled tubes because of high likelihood of sample order mix-up in these steps, moving from one rack format to another.
- Centrifuge at 10,000 × g for 3 min. Make sure that the settings are for 10 k gravities, not 10 k rpm—these are quite different
- Gently remove tubes one at a time, and transfer 1 mL of the cleared extract liquid layer to a storage tube (microtiter tube) in a 96-well format, using a 1000 µL pipettor with a new, clean tip. Avoid dislodging the pellet of solids at the bottom of the tube.
- Set aside rack with tubes in refrigerator overnight if not quantifying on the same day.

Quantification

Note: This method uses microtiter plates and reader, but could be converted to use of a standard spectrophotometer, although that would increase the amount of chemicals used.

- Remove sample tubes in 96-well format microtiter tube racks from refrigerator, as well as rack with standards, also in microtiter tube strips.
- Allow tubes to equilibrate with room temperature before quantifying.
- Ready a reaction plate, inspecting bottom to avoid scratches, and avoiding getting plate dusty.
- Preheat the heat block to 61.5 °C. Check to be sure there is no debris in the heating block that could scratch the bottom of the plate or prevent it seating well.
- Make ready a plate sealing tape sheet pad, and the plate sealing roller, before filling the plate.
- Prepare the BCA working reagent in a 50-mL centrifuge tube.
- Make enough for 200 µL per well, plus enough extra for a reservoir so that pipetting bubbles can be avoided.
- The working reagent is a 50:1 mixture of two parts: Reagent A (clear) and Reagent B (blue-green copper sulfate solution). For 25.5 mL put 0.5 mL (500 µl) of Reagent B into a centrifuge tube or clean small beaker, and then add 25 mL of reagent A to it. Stir or swirl to mix. A cloudiness that appears initially and then dissipates is normal. Set this aside, covered, while preparing the plate with samples and standards.
- Remove strip caps from tubes with standards (0, 125, 250, 500, 750, 1000, 1500, and 2000 µg per mL BCA), being careful not to splatter any. A small droplet of carryover would have a very large effect on these standards.
- Using the 8-channel, small-volume, multichannel pipettor and tips, pipette 10 µl of the standards into the first column of the reaction plate. Using a new set of tips, draw another 10 µl and place in the 7th column (just past the middle) of the plate. Dispense this droplet slowly onto the bottom of the wells, at the edge of the sidewall, maintaining contact with the side of the plate.

- Recap standards with new cap strips and set aside.
- Change to new gloves before opening samples.
- Uncap samples with care, and pipet samples into the available columns of the plate. Keep careful track of which samples are where on the plate, and go in order.
- Pipet two replicate columns of each strip of eight sample tubes into the plate wells. Use two reaction replicates per extraction, with two extractions per soil sample. So, 4 wells on the plate will represent each soil sample.
- When all samples and standards have been placed in the appropriate wells of the reaction plate, recap the samples and set aside.
- Retrieve the premixed working reagent, and transfer it to a clean, dry, multichannel pipettor reservoir.
- Using the larger volume multichannel pipette and the 200 µl tips, add 200 µl of working reagent to each well of the reaction plate. Add the liquid slowly, the swirling action should mix the liquids in the wells, but if pipetted too vigorously, it will splash, making the plate unusable. Further mixing will happen naturally while the reaction is heating.
- When the plate is filled, seal with a tape seal, using the roller to press the sealing tape to the well tops as well as upper surface of the plate. Make sure the seal is sound. Don't let the plate skid around on the surface of the bench, as this could scratch the bottom, interfering with the optical clarity.
- When plate is sealed, place gently in heat block and cover.
- Start timer for 60 min.

To Read

- After the 60-min incubation, gently remove from heat block and place on benchtop to cool for at least 10 min undisturbed.
- When the plate has cooled, ensure that the sealing tape is well in place still. Invert and re-right the plate to incorporate the droplets, which will have collected on the tape seal. Be gentle.
- Carefully remove the tape seal without letting the plate be jarred by the motion.
- Follow the manufacturer instructions for your model of the plate reader, read the plate. Record the measurement readings.
- Place plate in tray of plate reader.

Calculations

Average absorbance values for multiple reaction replicates of the same extract, before calculating the protein concentration, and average concentration values across replicate extracts of the same soil sample. If the relative average deviation of replicates from their mean exceeds 5 percent, then the sample is flagged for re-running. Use the standard curve developed from the measurements of the standards.

Appendix 7

High-Throughput Neutral Lipid Fatty Acids (NLFA) and Phospholipid Fatty Acids (PLFA) Analysis of Soil

This SOP is from the lab of Jeff Buyer, USDA-ARS (Buyer and Sasser 2012), with interpretations from the University of Missouri Soil Testing lab courtesy of Donna Brandt.

Major Equipment

- Lyophilizer
- High-speed concentrator
- Ultrasonic cleaning bath
- Multichannel pipettor reagents

Bligh-Dyer Extractant

- 200 mL 50 mM PO4 buffer pH 7.4 (8.7 g K2HPO4 per liter)
- 500 mL methanol
- 250 mL chloroform
- Mix fresh daily, or at least weekly if many runs are anticipated

Transesterification Reagent

- 0.561 grams KOH
- 75 mL methanol
- 25 mL toluene
- Dissolve KOH in methanol and then add toluene
- Prepare weekly

Internal Standard

- Phospholipid: 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids Catalog # 850367P (white powder).
- Neutral lipid: Trinonadecanoin glyceride, Nu-Check-Prep Catalog #T-165.
- Dissolve 40.9 mg of phospholipid and 31.1 mg of neutral lipid in 10 mL of chloroform and bring to 20 mL with methanol (2.5 mM solution of phospholipid and 1.67 mM neutral lipid).
- Store at −20 °C.
- Just before extracting, warm to room temperature and add to an appropriate volume of extractant at a rate of 0.5 µL internal standard per mL of extractant and mix. This is equivalent to adding 10 nmoles of 19:0 phospholipid and 10 nmoles of 19:0 neutral lipid.

Soil Drying

- Weigh 95 13−100 screw-cap glass test tubes (without caps on).
- Add 1.5–2.0 grams soil to each test tube. If using a lyophilizer, freeze test tubes.
- Run overnight in a high-speed concentrator (e.g., SpeedVac®) at room temperature or use a freeze-dryer or lyophilizer. Lyophilization is preferred as tubes may occasionally break in the high-speed concentrator.
- Weigh tubes + dry soil to calculate dry weight of soil.
    - If storing samples before extraction, cap each tube with Teflon-lined screw cap and store in −20 °C freezer.

- If working with previously dried soils, simply weigh 1–2 grams into test tube.

Extraction

- Use one additional test tube as a blank.
- Add 4 mL of Bligh-Dyer extractant containing internal standard.
- Sonicate 10 min in an Ultrasonic Cleaning Bath* at room temperature.
- Incubate at room temperature with end-over-end shaking 2 hours.
- Centrifuge 10 min in SpeedVac* without vacuum.
- Transfer liquid phase to 13×100 test tube with polytetrafluoroethylene (PTFE)-lined screw cap.

Separation

- Add 1 mL each of chloroform and deionized water.
- Vortex 10 seconds and centrifuge 10 min in high-speed concentrator without vacuum.
- Aspirate top (aqueous) phase.
- Concentrate to dryness in test tubes at 30 °C (about 1 hr).
- Dissolve in 1 mL chloroform for chromatography.

Lipid Separation

- 50 mg silica gel SPE 96-well plate.
- Wash each well 3× with 1 mL methanol then 3×1 mL chloroform.
- Place clean 1.5 mL multi-tier microplate in the bottom of 96-well plate.
- Add extract to wells.
- Let sample drain into column, collecting the eluate (NLFA fraction 1). Seal with a Teflon/silicon cap mat.
- Change microplate for another clean 1.5 mL multitier microplate and repeat transfer with another 1 mL chloroform, collecting the eluate (NLFA fraction 2). Seal with Teflon/silicon cap mat.
- Wash with 1 mL chloroform and 1 mL acetone, discarding the eluate.
- Place clean 1.5 mL multitier in bottom of 96-well plate manifold.
- Elute phospholipids with 0.5 mL of 5:5:1 methanol:chloroform:H_2O. Seal with Teflon/silicon cap mat and store at 4 °C while processing the NLFA.
- Concentrate both NLFA fractions at 37 °C until down to approximately half the original volume (about 30 min).
- Transfer remaining volume in fraction 2 to fraction 1 using multichannel pipettor and use the high-speed concentrator take the sample to dryness (37 °C, 1 hr). Seal with Teflon/silicon cap mats and store at −20 °C.
- Use the high-speed concentrator to take the 5:5:1 fraction (PLFA) to dryness (70 °C, 30 min, then 37 °C until dry, about 2 hrs total). Seal with Teflon/silicon cap and store at −20 °C.

Transesterification and Transfer to GC Vials

- Carry out this whole procedure on either NLFA or PLFA first. Once complete, carry out procedure on the other.
- Let samples warm up to room temperature. Add 0.2 mL transesterification reagent and mix.
- 37 °C 15 min.

- Add 0.4 mL of 0.075 M acetic acid and 0.4 mL chloroform.
- Seal with Teflon/silicon cap mat, shake vigorously, let separate.
- Transfer bottom 0.3 mL to 1 mL multi-tier plate (E & K Scientific # EK-99234) using multichannel pipettor. If 1 mL tips displace too much volume or don't fit in wells, use two 150 µl transfers with 250 µl pipet tips.
- Repeat with another 0.4 mL chloroform, transferring bottom 0.4 mL this time. If 1 mL tips displace too much volume or don't fit in wells, use two 200 µl transfers with 250 µl pipet tips.
- If any aqueous phase is seen on top of the transferred chloroform, remove with clean disposable Pasteur pipets. Evaporate the chloroform in the high-speed concentrator at room temperature-remove as soon as dry (~ 45 min).
- Redissolve extract in 75 µL hexane.
- Transfer to limited-volume insert placed in GC vial and screw cap with PTFE/Silicone/PTFE Septa.

Gas Chromatography

Use a gas chromatograph (GC) equipped with an autosampler, split–splitless inlet, and flame ionization detector. Consult the analytical methods for your GC for details regarding the separation of FAMEs (fatty acid methyl esters). As an example, Buyer and Sasser (2012), using an appropriate column, used a split ratio of 30:1 with the hydrogen carrier gas at 1.2 mL/min constant flow rate. Initial oven temperature: 190 °C, ramping to 285 °C at 10 °C /min and then to 310 °C at 60 °C/min, followed by a hold at 310 °C for 2 min. Injector temperature: 250 °C. Detector temperature: 300 °C. The GC should have appropriate software for identifying microbial peaks.

Glassware Cleaning

- All glassware scrubbed carefully with detergent and thoroughly rinsed while wearing gloves.
- An ultrasonic cleaning bath is helpful. Any lipids will form a monolayer and spread over the entire surface of the wet glass, so gloves are absolutely necessary.
- If possible, bake glassware at 400–500 °C at least 2 hrs.
- Use a muffle furnace dedicated to clean glassware—no samples ever in this furnace.
- All screw caps shaken with hexane in test tube. Make sure Teflon liner is in place before using.
- Cap mats are cleaned by gentle scrubbing with soap and water, rinsed sequentially with DI water, ethanol, and chloroform, and then dried in a laminar flow hood.

Notes

- All organic solvents should be HPLC grade or better.
- Contamination is a major problem.
  - Run at least 1 blank with every batch of samples.
  - Always wear gloves. Nitrile gloves may be better than latex gloves.
  - GC caps must have PTFE/Silicone/PTFE Septa.
  - Test tube caps must be PTFE-lined.
  - Limited volume inserts must not have polyspring feet.

Guide to Understanding Phospholipid Fatty Acid (PLFA) Data and Initial Exploratory Analyses

Step-By-Step

- First, refer to biology texts, scientific literature, and other sources readily available online or at the library for basic information on PLFAs, PLFA nomenclature, or PLFA extraction A good paper describing PLFA vs. neutral fatty acids, non-ester-linked fatty acids, etc., is Zelles (1999).
- Use this guide to understand and explore your PLFA output files from the Soil Health Lab.
- Consult the scientific literature to find articles pertinent to your study (e.g., from similar management or ecosystems) for further information and ideas on interpretation of PLFA data.
- The exploratory techniques listed in this guide are a starting point for that process. Many ideas can be found in the PLFA literature. Ultimately, the final data/statistical analyses depend on the objectives of your study, the study design, the quality/quantity of the data, and the skillset of the analyst.
- Many criticisms of PLFA analyses, interpretations, and conclusions exist in the literature (e.g., Kaur et al. 2005; Frostegård et al. 2011).
- This guide is specific to the Buyer/Sasser extraction method Peak assignments, microbial groupings, etc., vary quite a bit in the literature and are dependent on the specific analyzer and software package.

General PLFA Nomenclature

PLFA are an essential structural component of all microbial cellular membranes. The PLFA nomenclature follows A:BωC pattern, where the "A" position identifies the number of C atoms in the fatty acid, position ''B'' is the number of double bonds, and "C" designates the C atom from the aliphatic end before the double bond. This is followed by a "c" for *cis* or a "t" for *trans* configuration of monoenoics (i.e., having only one double bond). The abbreviation "br" is used to designate branching. The prefixes "i" and "a" stand for *iso* and *anteiso*, respectively. Midchain branching is noted by "me," and cyclopropyl fatty acids are designated as "cy" or "cyclo." The position of hydroxy groups is noted. Saturated fatty acids can be straight or branched chains and have no double bonds. That is, the chain of C atoms is fully "saturated" with hydrogen atoms. Saturated straight chains are designated as 12:0, 13:0, 18:0, etc., and are ubiquitous. Monounsaturated fatty acids (MUFA) have only one double bond (e.g., 16:1ω7c), whereas polyunsaturated fatty acids (PUFA) have more than one double bond (e.g., 18:2ω6,9c or 18: 3ω3,6,9c).

Interpretations of Microbial Categories Assigned to Individual and/or Collective PLFA Biomarkers

Microbial Groups

These values do not reflect absolute biomass values.

Gram-negative (G-) bacteria.—A major component of the plant rhizosphere and improve plant growth by increasing solubility of many nutrients. Generally, G- bacteria dominate surface soils (versus G+) in the rooting zone and breakdown newly added organic matter. G- bacteria produce monounsaturated fats (MUFA) and cyclopropane PLFAs.

Gram-positive (G+) bacteria.—Common in the bulk soil and concentrated in the rhizosphere, but are not as closely tied to the rooting zone as are G- bacteria. The PLFA profiles of G+ species have high percentages of saturated branched-chain PLFAs such as 15:0iso and 15:0anteiso. Thus, the sum of iso and anteiso PLFAs provides an estimate of the abundance of the G+ bacteria (other than actinobacteria) in the sample.

Anaerobic bacteria.—Important under low oxygen conditions such as wet soils, deep soils, sediments, or the interior of soil macroaggregates. Anaerobic bacteria can be either G+ or G- bacteria. Dimethyl acetals (DMA), such as 16:1ω9c DMA (1,1-Dimethoxy-7-Hexadecene), are considered PLFA biomarkers for anaerobic bacteria.

Actinobacteria (formerly Actinomycetes).—G+ bacteria that are active in decomposition of organic matter and produce geosmin, a compound that generates the "earthy" smell of freshly tilled soils. They have distinctive PLFAs with a methyl branch at the 10^{th} C, such as 10Me16:0 (10-Methylhexadecanoic acid / 10-Methylpalmitic acid) and 10Me18:0, and others.

Methanotrophs**,** sometimes called methanophiles or methane-oxidizers.—Prokaryotes that can metabolize methane for C and energy. They are primarily G- bacteria. The primary MUFA marker for this group is 16:1ω8c (8-Hexadecenoic acid / cis-8-Palmitoleic acid).

Archaea (single-celled prokaryotes).—Universally distributed in soils and are important contributors to nitrification and NH_4 oxidation in agricultural and forest soils. However, PLFAs from archaea are ether-linked, not ester-linked, so they are ***not*** present in the Buyer-Sasser extraction /analysis.

Eukaryotes.—Include fungi, algae, nematodes, earthworms, insects, arthropods, and protozoa that are important in soil ecology. Eukaryotes have more complex cell structures than prokaryotes like bacteria. General eukaryotic markers include PUFAs.

Fungi.—Important in decomposition, especially recalcitrant organic compounds like lignin. A wide variety of fungi occur in soil. They range from single cell yeasts to some of the largest organisms in the world. The 18:2 w6c (9,12-Octadecadienoic acid / Linoleic acid) is assigned to fungi.

Arbuscular mycorrhizae fungi (AMF).—Grow in long, thin strands called hyphae and form mutually beneficial relationships with most plants. AMF have lipid storage organs called vesicles that contain the fatty acids 18:2ω6c as well as 16:1ω5c**,** which has been recommended as a biomarker for AMF, but is also found in bacteria.