

An integrated approach to assessing soil biological health

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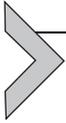
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Abstract

Soil health is an emerging field that holistically approaches key challenges within soil science through a chemical, physical, and biological lens. In recent years, soil health has become foundational to sustainability goals within the context of regenerative agriculture including climate mitigation and reversing biodiversity trends. However, given the vast array of indicators used to quantify soil health, there are still several unknowns regarding which indicators can most effectively indicate specific soil health outcomes and ecosystem functioning. Key linkages between indicators and soil health outcomes are especially lacking in the subfield of soil biological health. Moreover, the subfield of soil biological health, must expand its current portfolio of indicators to play an active role in soil biodiversity assessments. Here we propose an integrative approach to quantitatively assess soil biological health. We outline numerous key indicators that are important to consider because of their position in the soil food web and linkages to key soil processes. We recommend that future soil health

assessments directly measure soil organisms in conjunction with indicators that reflect key ecosystem functions. Exploratory factor analysis, a powerful quantitative method, for integrating soil biological health indicators is also discussed.



1. Introduction

The field of soil health has expanded due to a growing interest from farmers, extension educators, government agencies, and scientists (Karlen et al., 2019). Soil health resonates with these different groups because of its dual goals of enhancing agronomic performance and supporting environmental sustainability (O'Neill et al., 2021; Wade et al., 2020). Although disagreements remain surrounding the definition and meaning of soil health (Bünemann et al., 2018; Janzen et al., 2021), most agree that soil health is the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans (<https://www.nrcs.usda.gov/conservation-basics/natural-resource-concerns/soils/soil-health>). Soil health provides a holistic framework that considers soil chemical, physical, and biological processes equally (Doran, 2002). This is a strong departure from previous soil testing frameworks that tend to focus solely on soil nutrient status to inform external inputs such as fertilizers and amendments (Franzuebbers et al., 2022; O'Neill et al., 2021).

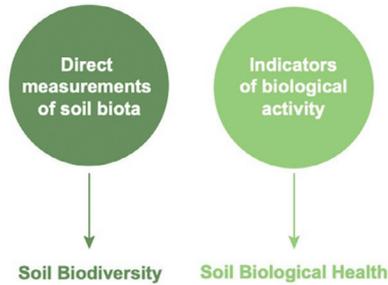
The primary goal of soil health is to connect a wide range of measurements to key ecosystem processes and functions to ensure that society reaches outcomes associated with crop production, biodiversity, climate regulation, and water quality (Kihara et al., 2020; Lehmann et al., 2020; Martin and Sprunger, 2022a). Given the vast array of chemical, physical, and biological processes that exist in any given system, a large range of indicators are needed for quantification and monitoring (Rinot et al., 2019). However, there is no silver bullet soil health indicator that can suitably reflect all soil health outcomes that practitioners seek to achieve (Wade et al., 2022). To compensate, existing soil health frameworks rely on dozens of indicators to quantify soil health (Moebius-Clune et al., 2016). Soil health as a field is still grappling with how to identify specific indicators that are most relevant and informative for a given situation (Wade et al., 2022). Moreover, there is an internal debate on whether soil health tends to focus too heavily on soil biology (Coyne et al., 2022) and not enough on physio-chemical properties, while others argue that soil biological health is vastly underdeveloped compared to other soil health components (Bünemann et al., 2018; Martin et al., 2022).

While there is plenty of evidence to demonstrate that soil biological health indicators are vastly underdeveloped, it is also true that there has been a resurgence of research on soil biology within the context of soil health in recent years (Fierer et al., 2021; Martin and Sprunger, 2022a; McDaniel, 2017). This new focus has partially come about because farmers and practitioners have directly asked researchers for indicators that reflect soil biota (Sprunger, 2015). Moreover, soil biological health indicators are often able to detect differences in soil conditions earlier than other types of indicators (O'Neill et al., 2021). As Pankhurst et al. (1997) explain, physical and chemical components are often fixed due to geographical constraints, while biological components are sensitive and responsive to recent changes in management.

This chapter seeks to introduce soil biological health as an integration between soil biology and ecosystem functioning. Specifically, our objectives are to (1) Identify key soil biological health indicators and link each indicator to a specific ecosystem function within the soil food web, (2) Assess how various measures of soil biology can be integrated into the soil health framework, and (3) Elaborate on future research needs and discuss key statistical approaches that can effectively integrate organismal data with biological activity for a more robust soil biological health framework.

Although scientists have struggled to quantify soil biological health over the years, there is overwhelming agreement that soil biological health should focus on the living component of soil (McDaniel, 2017; Pankhurst et al., 1997). Traditionally, there have been two approaches to measuring soil biological health (Fig. 1). One approach has been to directly measure soil biota, which is often key for biodiversity assessments but is often challenging to relate to ecological function. Second, has been to rely on indicators that reflect biological activity in the soil, which serve as strong proxies for ecological function. The latter is currently the approach that is best represented within the soil health framework to date (Moebius-Clune et al., 2016). These two approaches span multiple fields and until recently have been conducted in isolation, with very different goals. Although it is known that soil biota are regulators of key ecosystem services, scientists continue to struggle with linking the microbiome to ecosystem function and by extension soil health (Finlay et al., 1997; Widder et al., 2016). However, more recently scientists have worked to collect global datasets that link soil biodiversity to multifunctionality, which is critical as scientists continue to investigate linkages between soil biodiversity and broader sustainability goals (Delgado-Baquerizo et al., 2020).

A Traditional approach to studying soil biology



B Integrated approach to assessing soil biology

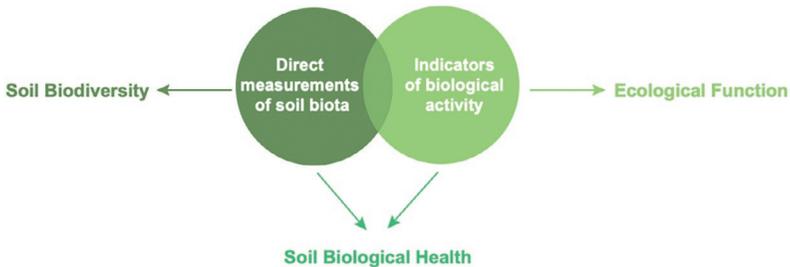


Fig. 1 (A) Conceptual framework depicting the traditional approach toward assessing soil biology where direct measures of soil biota are often conducted separately from indicators that reflect soil biological activity. (B) An integrated approach to assessing soil biology that accounts for direct measures of soil fauna (structure) and indicators that reflect biological activity and key ecosystem processes (function).

Given the challenges associated with directly measuring soil organisms, numerous indicators of soil biological health have been developed to reflect biological activity (Culman et al., 2012; Franzluebbers et al., 2000; Moebius-Clune et al., 2016). These indicators have been fundamental to Cornell University's Comprehensive Assessment of Soil health, the NRCS standard protocol, and various other soil health frameworks that have emerged over the past two decades (Moebius-Clune et al., 2016; NRCS, 2019). Furthermore, these biological indicators have been the basis for quantifying soil health largely within an agroecological context and integrating more organismal indicators could make soil health even more relevant for other ecosystems. We've also seen modest advances in connecting these indicators to soil health outcomes (Martin et al., 2022;

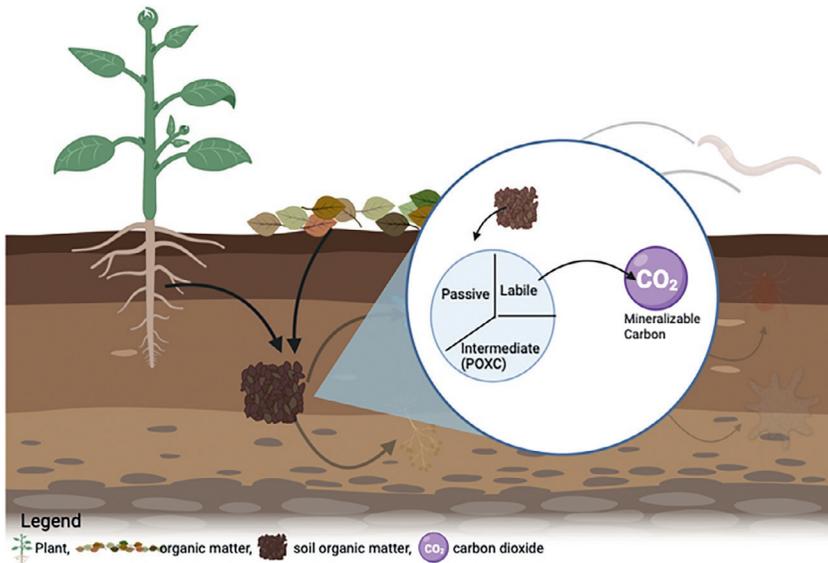


Fig. 3 Soil organic matter depicted as three key pools of soil C: passive, intermediate, and labile and the soil health indicators that can represent these key soil C pools. Created with *BioRender.com*.

at various stages of decomposition. These different rates of decay lead to functionally different pools of C (Cotrufo and Lavelle, 2022; Wander, 2004). For example, the largest pool consists of C that has persisted in the soil for hundreds to thousands of years. This pool of C is physically protected from microbial activity and is considered passive due to its stability (Kravchenko et al., 2015). Next, there is an intermediate or slow pool of C that generally persists in the soil for a few decades. Lastly, there is a small active or labile pool of C that has a mean residence time that ranges from 24 h to a year (Fig. 3) (Paul et al., 1999). This labile C pool is sensitive to recent changes in management, drives nutrient mineralization, and is critical for crop productivity (Culman et al., 2013; Sprunger et al., 2020). Continued advances in methodology have led to rapid and affordable soil health metrics that reflect key mechanisms of soil C accrual and decay (Hurisso et al., 2016; Martin and Sprunger, 2022a). Moreover, these indicators have become central to a variety of soil health assessments including the Cornell Soil Health Test, the NRCS Soil Health Technical Note, and the Soil Health Institute (Liptzin et al., 2022; Moebius-Clune et al., 2016; NRCS, 2019).

2.1.1 Mineralizable carbon

Soil respiration, referred to here, as Mineralizable C is a soil biological health indicator that measures the amount of CO₂ mineralized from soil over a set period of time (Fig. 3). Mineralizable C is determined via aerobic incubations performed on re-wetted soils (Robertson and Paul, 2000). Mineralizable C can be determined over a 300+ day period to quantify active, slow, and passive pools of C in conjunction with acid hydrolysis (Paul et al., 1999; Sprunger and Robertson, 2018). However, within the soil health framework, mineralizable C tends to be assessed via short-term incubations ranging from 1 to 3 days (Franzluebbers et al., 2000; Haney et al., 2008).

Mineralizable C is prominent within the soil health framework because it is a rapid and inexpensive measure that reflects the biologically available pool of C and can serve as an early indicator of how the total soil organic matter pool may respond to management and land use change (Table 1, Hurisso et al., 2016; Sprunger et al., 2020). It reliably detects differences in soil C more effectively than other indicators and seems to align best with farmer perceptions of field-based conditions (O'Neill et al., 2021). Mineralizable C can also detect larger magnitude differences across a range of systems within relatively short periods of time (Sprunger and Robertson, 2018). For instance, Sprunger et al. (2020) found that mineralizable C within the perennial polyculture systems were twice the value of mineralizable C found within the annual row crops systems (Fig. 4A). However, while mineralizable C can reliably detect differences in soil C dynamics across management more readily than other indicators, mineralizable C is also extremely variable (Davidson et al., 2002). Spatial, temporal, and analytical differences can largely explain mineralizable C variability (Wade et al., 2018). At the field scale, mineralizable C is sensitive to recent weather patterns and within season management, which leads to extreme variability,

Table 1 The frequency of prominent soil health indicators cited in the literature using a “Web of Science” Search Functions between the years 2010 and 2022.

Soil health indicator	Count
ACE Protein	66
POXC	233
Soil Respiration or “Mineralizable Carbon”	5698

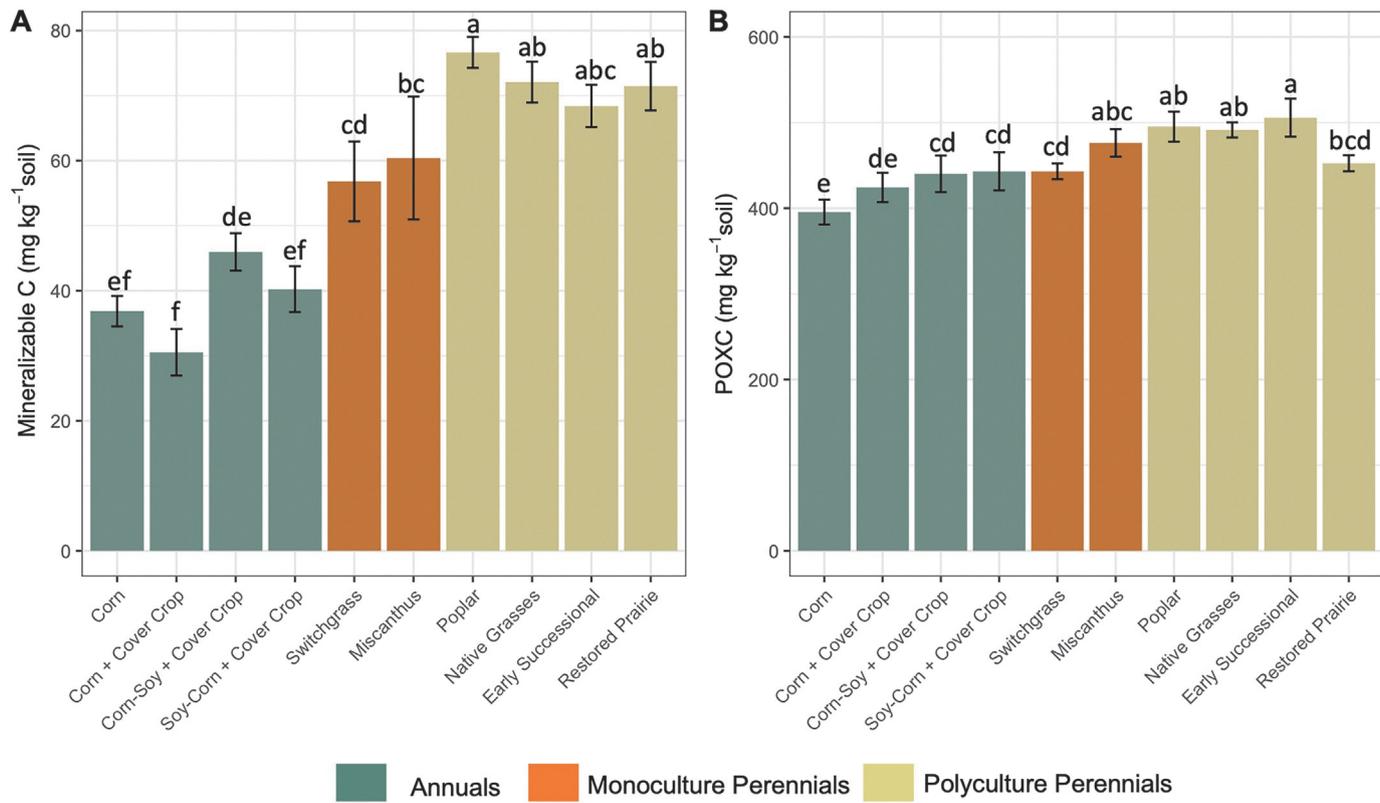


Fig. 4 (A) Mineralizable C and (B) Permanganate Oxidizable C (POXC) measured across 10 systems ranging in crop diversity and perennality. Bars with different letters are significant at $P < 0.05$. Data adapted from Sprunger, C.D., Martin, T., Mann, M., 2020. Systems with greater perennality and crop diversity enhance soil biological health. *Agric. Environ. Lett.* 5, e20030. with permission from authors.

making it difficult to consistently track through time (Martin and Sprunger, 2022a). Thus, there must be an element of caution when working to form management decisions based solely on mineralizable C due to the associated variability.

Despite the high variability, mineralizable C strongly correlates with total soil organic matter and at times serves as a strong predictor of agronomic performance (Culman et al., 2013; Fine et al., 2017; Sprunger et al., 2019a). Mineralizable C is also strongly associated with nutrient mineralization and nitrogen availability. Aside from crop productivity and nutrient cycling, researchers have been slow to link mineralizable C to other ecosystem services, including biodiversity. A recent cross-site analysis found modest linkages between microbial community structure and mineralizable C and found that under reduced tillage systems key taxa inform mineralizable C models (Rieke et al., 2022). Moreover, when using the microbiome to predict measures of soil health using 900+ samples, mineralizable C was among the least predicted soil biological health indicators (Wilhelm et al., 2022). This demonstrates that we are not yet at the stage where the soil microbiome as determined by 16S rRNA gene amplicon sequencing can replace soil biological health indicators that effectively reflect function. That said, given the heightened interest in soil health there is more pressure from stakeholders and policymakers to demonstrate key linkages between soil biological health indicators and ecosystem services (Ball et al., 2018). Mineralizable C has been touted as an indicator that reflects overall soil health. Yet few studies have demonstrated the linkages between mineralizable C and soil health outcomes, aside from soil C accumulation. Future studies should continue to assess the ecological importance of increasing mineralizable C within an agricultural context.

2.1.2 Permanganate oxidizable carbon

Permanganate oxidizable carbon (POXC) is measured via a chemical oxidation of organic matter by a potassium permanganate solution and is thought to reflect readily available C (Fig. 3) (Culman et al., 2012; Weil et al., 2003). While POXC is based on a chemical reaction, it is considered a soil biological health indicator because it appears to represent a biologically available pool of C (Wade et al., 2021). However, researchers have found that POXC is more closely associated with heavier particulate organic C fractions and seems to reflect soil C stabilization processes in comparison

to other common indicators including microbial biomass (Culman et al., 2012; Hurisso et al., 2016). While more empirical studies are needed, there is also early evidence to suggest that potassium permanganate oxidizes multiple elements and compounds, including carbonaceous compounds (Kleber et al., 2021; Wade et al., 2021). This is a departure from the initial view of POXC and could help explain why POXC behaves differently from other labile C fractions and reflects a more processed pool of C. Nevertheless, this serves as early evidence that referring to POXC as “Active C” may indeed be a misnomer. A chief example how POXC behaves differently from Mineralizable C is presented in Fig. 4B, which shows that the magnitude difference between systems is much reduced for POXC relative to mineralizable C (Fig. 4A), demonstrating that these two indicators are reflecting two functionally different pools of C. Taken together, POXC appears to serve as an early indicator of soil C sequestration (Culman et al., 2012; Hurisso et al., 2016; Sprunger et al., 2020). Permanganate oxidizable C is an attractive soil biological health indicator because it is rapid, inexpensive, and can consistently detect differences across a range of ecosystems and management intensities (Hurisso et al., 2016, 2018; Martin and Sprunger, 2022a). However, more work is needed to fully understand its functionally operational role in the context of soil C dynamics.

2.1.3 Exploring soil C trajectories using mineralizable C and POXC

Coupled together, mineralizable C and POXC can provide powerful insight into soil C trajectories because they reflect functionally different pools of C. Hurisso et al. (2016) developed a framework that comparatively analyzes mineralizable C and POXC using a linear regression model. The framework assesses the average residuals from a linear model in a given treatment or system. Positive residuals indicate systems that are more influenced by POXC or stabilization processes, whereas negative residuals indicate a system influenced more by mineralization processes (Hurisso et al., 2016). Since the development of this framework, numerous publications have adopted this framework to identify soil C carbon trajectories of a given system (Sprunger et al., 2019a, 2020; Wade et al., 2019). For instance, Sprunger et al. (2020) calculated average residuals across a biodiversity gradient and generally found that systems with greater perenniality and biodiversity tended to be influenced more by POXC or stabilization forces relative to annual systems that were influenced more so by mineralization processes. This demonstrates that perennial polyculture systems are accumulating C

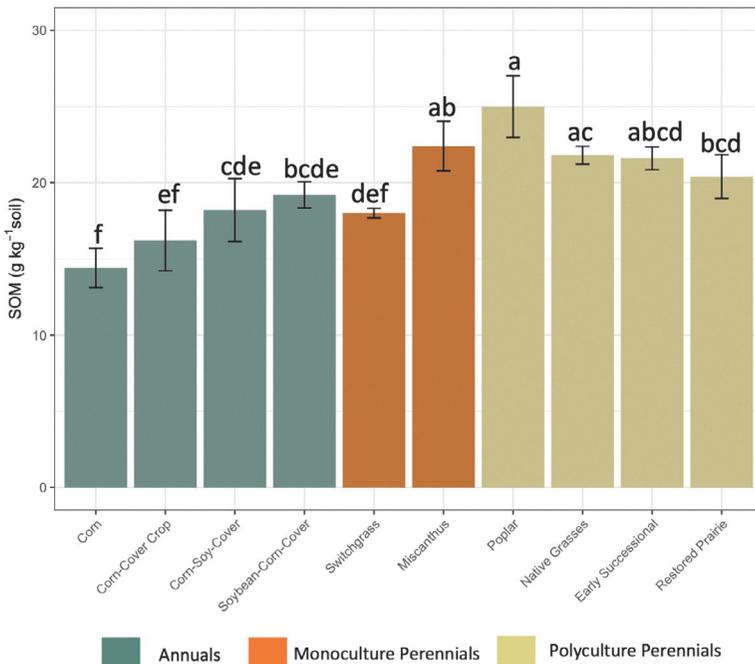


Fig. 5 Soil organic matter determined via loss on ignition measured across 10 systems ranging in crop diversity and perennality. Bars with different letters are significant at $P < 0.05$. Data adapted from Sprunger, C.D., Martin, T., Mann, M., 2020. Systems with greater perennality and crop diversity enhance soil biological health. *Agric. Environ. Lett.* 5, e20030. with permission from authors.

more rapidly relative to annual row crop systems. Understanding, where a system falls along a soil C trajectory will become more critical as the soil C credit market advances and companies continue to track soil C sequestration in real-time (Oldfield et al., 2022a,b). Moreover, both POXC and Mineralizable C are more sensitive and can likely detect changes in soil C earlier than SOM (Figs. 4 and 5).

2.2 Soil nitrogen

Soil nitrogen (N) status is a major driver of optimal crop growth in agroecosystems and is also the most consequential nutrient given its rapid transformations and subsequent impacts on the environment (Gardner and Drinkwater, 2009; Robertson and Vitousek, 2009). Despite its centrality within agroecosystems, it has received less attention within the context of soil biological health (Grandy et al., 2022). Until recently, most soil tests

have relied heavily on soil inorganic N in the form of ammonium and nitrate. Basing N management solely on inorganic N is challenging because these forms of N change frequently over the course of a single growing season (Culman et al., 2013). Alternatively, measuring total soil N is also an option but raises several challenges. First, because it is assessing the entire soil N pool, it may not be as sensitive to recent changes in management, which is an important criterion for a soil biological health indicator (Hurisso and Culman, 2021). Second, total nitrogen can be costly and time consuming, which makes it an undesirable soil biological health indicator. Scientists have worked to develop indicators that better reflect biologically available pools of N, soil N status, and overall soil health.

2.2.1 Autoclaved citrate-extractable protein

The intricate link between soil organic matter and N cycling can be measured via soil proteins (Fig. 6). Proteins account for the largest pool of organically bound nitrogen found in the soil (Gillespie et al., 2011). For this reason, researchers have developed a soil biological health indicator called, autoclaved citrate-extractable protein (soil protein) to target the organically bound pool of N (Hurisso and Culman, 2021; Hurisso et al., 2018; Moebius-Clune et al., 2016). Soil protein is attractive for several

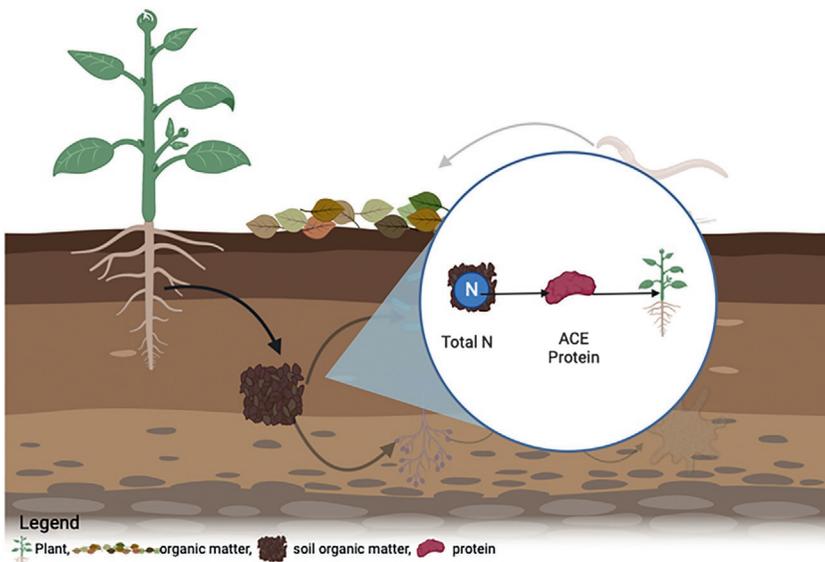


Fig. 6 The transformation of available nitrogen for plant uptake as indicated by Autoclaved-Citrate Extractable Soil Protein. Created with [BioRender.com](https://www.biorender.com).

reasons. First, soil protein reflects a bioavailable pool of N, which has direct impacts on several ecosystem functions, including plant N uptake, N storage, and N synchrony, all of which are key for reflecting the N status of soil (Hurisso and Culman, 2021). Second, soil protein is considered a more rapid method relative to other N status indicators including potentially mineralizable nitrogen. This means that commercial laboratories could scale up protein analyzes quite effectively (Hurisso et al., 2018).

Although soil protein is a relatively new soil health indicator, field-based experiments have demonstrated its close association with other soil health indicators and responsiveness to management (Geisseler et al., 2019; Sprunger et al., 2019a; Williams et al., 2020). For instance, when analyzing over 2000 soil samples from the Midwest and Northeast, Fine et al. (2017) found that protein was most highly correlated with Organic Matter, POXC, and Respiration. These findings reaffirm the notion that the soil protein procedure extracts proteins from a wide range of sources within the soil organic matter pool and demonstrates its close association with both soil carbon and nitrogen cycling in the soil (Hurisso and Culman, 2021; Hurisso et al., 2018). Additionally, Martin and Sprunger (2021a), demonstrate that soil protein and fine root N are significantly correlated. This indicates that the soil protein pool is serving as source of bioavailable N in row-crop agriculture. Future research should monitor N exchange between plant and soil using stable isotopes to fully assess when soil protein is serving as a source or sink for N and in what contexts. Beyond having close associations with key soil biological health indicators, soil protein is sensitive to management, which is an important attribute for any soil health indicator. For example, Roper et al. (2017) found that no-till management increased soil protein relative to conventional tillage. Sprunger et al. (2020) also demonstrate that perennials and perennial polycultures enhance soil protein relative to annual row-crops after 9 years of establishment (Fig. 7).

The ability to predict agronomic performance is a major criteria of soil health indicators (Culman et al., 2013; Wade et al., 2020). This is particularly true for nitrogen centric soil biological health indicators that could help farmers manage for crop productivity, while balancing N additions. For instance, Wade et al. (2020) found that soil biological health, including soil protein, served as a strong predictor of a soil's responsiveness to N fertilization and substantially contributed to improved yields across the Midwest. In small holder farming systems in Kenya, Sprunger et al. (2019a) found that soil protein was the top predictor of maize yields across four sites with contrasting soil types. While soil protein is a promising soil health indicator

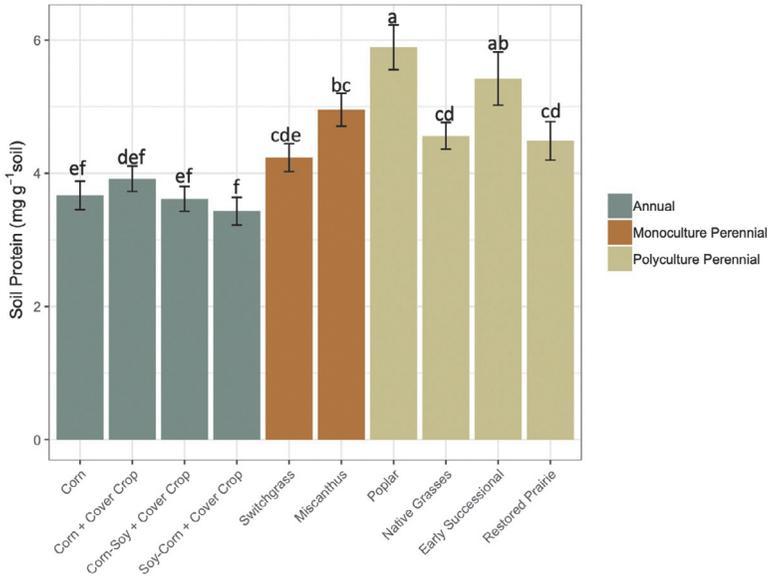


Fig. 7 Autoclave-Citrate Extractable Soil Protein measured across 10 systems ranging in crop diversity and perennality. Bars with different letters are significant at $P < 0.05$. Data adapted from Sprunger, C.D., Martin, T., Mann, M., 2020. Systems with greater perennality and crop diversity enhance soil biological health. *Agric. Environ. Lett.* 5, e20030. with permission from authors.

Table 2 Nematode food web indices and the ecological function they reflect.

Index	Inferences into function	Reference
Structure Index	Trophic complexity and diversity	Ferris et al. (2001), Ferris and Bongers (2006)
Enrichment Index	Organic inputs and nutrient cycling	Ferris et al. (2001), Ferris and Bongers (2006)
Channel Index	Fungal: bacterial decomposition pathways	Ferris et al. (2001), Ferris and Bongers (2006)
Basal Index	Amount of disturbance	Bongers and Bongers (1998)

that reflects N status in soils, to date, it is much less used relative to mineralizable C and POXC (Table 2). This is likely the result of soil protein being a newer indicator, that people are less familiar with. Moreover, scientists are still working to assess the mechanisms that soil protein reflects and how sensitive it is to recent changes in management (Geisseler et al., 2019; Sprunger et al., 2021).

2.3 Enzymes

Soil enzymes are valuable soil health indicators and provide key information on biogeochemical reactions within the soil (Dick et al., 1997). Enzymes measure the potential of a particular protein to catalyze its substrate (Fig. 8). Enzyme activity can be interpreted as a composite of the activity from various sources such as cell membranes, the soil solution microbial debris, or stabilization within the soil matrix (Nannipieri et al., 2012, 2018). Specifically, enzymes depolymerize organic compounds and generate soluble monomer and oligomers that can be recognized by microbes and taken into the cell (Wallenstein and Burns, 2011). Enzymes in the soil that break down organic matter can function as either hydrolytic or oxidative (Dick and Burns, 2011). Hydrolytic enzymes are substrate-specific and cleave specific bonds that turn polymers into monomers. Whereas oxidative enzymes catalyze an oxidation reaction.

These specific enzymes have the potential to be important indicators within the soil health framework because of their ability to reflect key processes in the soil. That said, enzymes exist tangentially and have not been fully incorporated as part of major soil health assessments (Moebius-Clune et al., 2016). Enzymes could prove to be an ideal soil health indicator

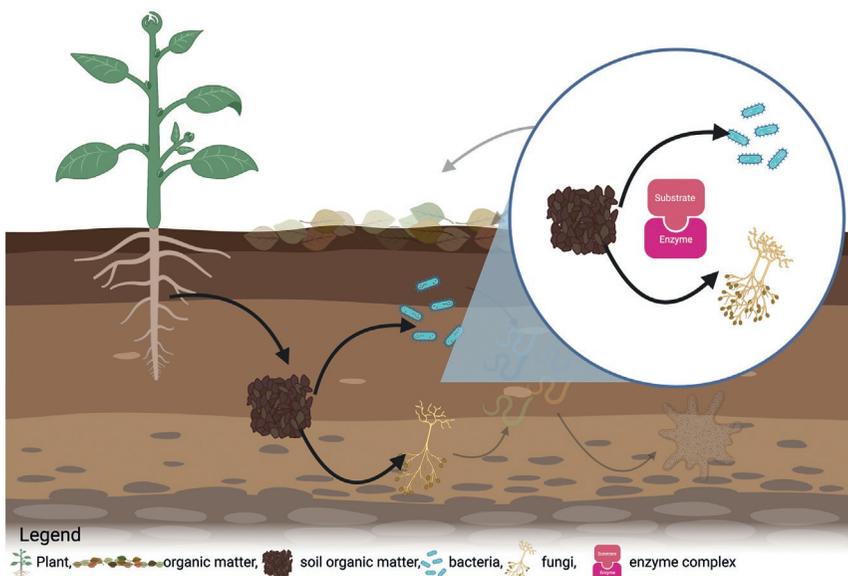


Fig. 8 Enzymes can indicate the breakdown of organic matter for microbial consumption. Created with *BioRender.com*.

because of their high throughput potential and capacity to reflect key soil health outcomes. Specifically, enzyme activities have been found to be rapid, reproducible, and sensitive measurements of soil nutrient cycling (Dick et al., 1997). For example, sulfur cycling is essential for protein and enzymes synthesis for all organisms (Klose et al., 2011). A suite of enzymes can be utilized to assess the sulfur cycle in its transformation between organic, inorganic, and gaseous form. Thus far, the most intensively investigated enzyme that reflects sulfur cycling is arylsulfatase, which is essential for the degradation of ester sulfates (Fitzgerald et al., 1978; Germida, 2005; Haneklaus et al., 2007). Nitrogen cycling is essential for the maintenance of plant health and growth and can be assessed through a suite of enzyme activities. The most popular enzyme activities that are used are amino-hydases (Urease, L-Asparagine, L-Glutaminase, and Amidase), these enzymes particularly focus on N-mineralization within the nitrogen cycle (Kandeler et al., 2011). Phosphorus is essential for plant growth and is the second most limiting nutrient. Phosphatase enzymes are essential for mineralizing soil organic P into available P for plant uptake. Phosphomonoesters, phosphor-diester, inorganic pyrophosphatase, and trimetaphosphatase enzymes can be measured to understand phosphorus transformation in the soil (Acosta-Martínez and Tabatabai, 2011). However, phosphomono-esterase activity is the most commonly measured enzyme. Enzymes are also used to break down carbon (cellulose and lignin). For instance, β -glucosidase is often used to indicate carbon cycling (Lazcano et al., 2013).

Enzyme activities have thus far been used in a suite of experiments to assess differences between agricultural management, changes in climate change, as well as assessments of soil remediation (Bogati and Walczak, 2022; Bowles et al., 2014; Brockett et al., 2012; Burns et al., 2013; Stone et al., 2016; Xiao et al., 2018; Zuber and Villamil, 2016). Although enzymes appear to be an effective soil health indicator due to their sensitivities and capacities to reflect nutrient cycling more research is needed on protocol standardization (Wade et al., 2021). There are still large discrepancies regarding optimal pH, incubation time, and temperature (Nannipieri et al., 2018). Additional experiments that mechanistically show what each enzyme is directly measuring and reassurance of how to interpretate these results, especially in field based conditions are needed (Fierer et al., 2021; Margenot and Daughtridge, 2022).



3. Directly measuring soil organisms as a means of assessing soil health

A chief goal of soil health is to improve soil biodiversity for enhanced ecological function (Maroun and Atkins, 2021; Pankhurst et al., 1997). Yet, direct measurements of soil fauna are generally left out of soil health assessments. Instead, soil scientists rely on soil health assessments that reflect soil biological activity. One major reason for this is that directly measuring soil organisms can be informative for assessing abundance and biodiversity metrics but less inferences can be made toward ecological function, which many believe are directly linked to soil health outcomes (Fierer et al., 2021). However, given major threats of soil biodiversity loss within terrestrial landscapes, soil health assessments should directly work to measure soil organisms while simultaneously working to link soil microbes and fauna to function (Martin and Sprunger, 2022b; Nielsen et al., 2011). There is a resurgence of research focused on incorporating microbiology into the soil health framework (Rieke et al., 2022; Wilhelm et al., 2022). This is partially because molecular methods have advanced and become more affordable. Moreover, there is a growing demand from stakeholders, who would like direct measurements of soil organisms to be included in soil health assessments (Sprunger, 2015).

For instance, Fierer et al. (2021) review a wide range of microbial indices and outline their strengths and weaknesses when it comes to assessing soil health. While the majority of metrics reviewed (i.e., microbial community composition, fungal:bacterial ratios, mycorrhizae) are elegantly used in scientific experiments, providing inferences in how they relate to function in a practical setting is a limited. For instance, how might a farmer use such data to make more informed management decisions? Here we propose direct measures of soil biota that we believe can more easily be linked to function and therefore more useful in a soil health and soil management context.

3.1 Fatty acid methyl ester

Fatty acid methyl ester (FAME) profiling is used to characterize microbial community composition (Fig. 9). Specifically, certain types of fatty acids can be found in the phospholipid membrane and are specific to different

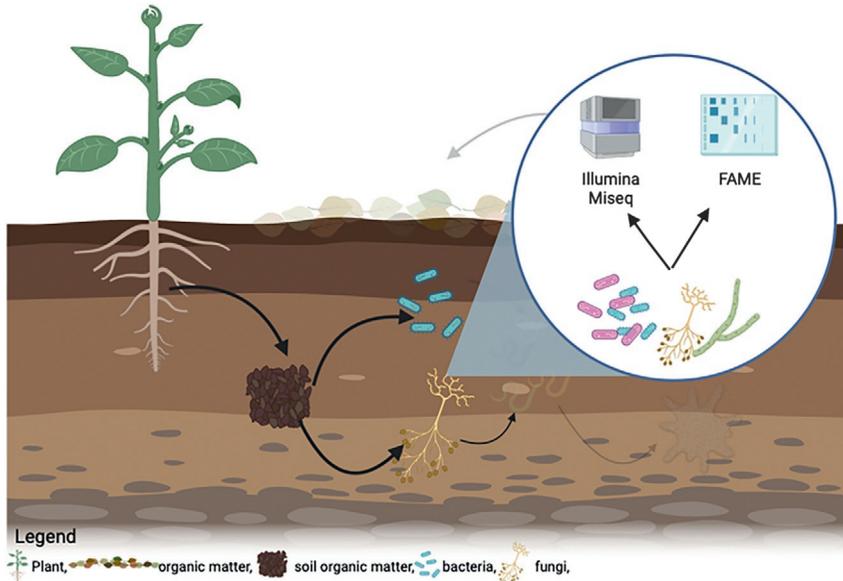


Fig. 9 Both Fatty Acid Methyl Ester (FAME) and high-throughput metabarcoding techniques can indicate microbial community diversity and composition. *Created with BioRender.com.*

microbial groups (Zelles, 1999). This allows membrane biomarkers to serve as identifiers for key microbial groups. Characterizations of FAME allows scientists to assess the proportion of microbial groups such as arbuscular mycorrhizal fungi, Gram-positive bacteria, Gram-negative bacteria, actinobacteria, and saprophytic fungi (Bardgett and McAlister, 1999; Fanin et al., 2019; Moore-Kucera and Dick, 2008). In addition, fatty acids in the phospholipid membranes change in response to disturbance making FAME a good indicator of detecting disturbance and differences among microbial communities, especially in response to environmental perturbations (Li et al., 2020; Muhammad et al., 2021).

The use of FAME has led to knowledge on the microbial community structure and function in a wide range of ecosystems and under varying land use management. The identification of certain microbial groups is directly related to function as these ratios have been found to be linked to essential ecosystem processes. For example, fungi:bacteria ratios have been used to indicate the response to environmental stressors (Bardgett and McAlister, 1999; Moore-Kucera and Dick, 2008). In addition, the comparison between Gram positive and Gram-negative bacteria has been used as an indicator of energy limitations and carbon availability because of the greater association

of Gram-positive bacteria on labile plant derived C than compared to Gram-negative bacteria (Fanin et al., 2019). FAME can also indicate the proportion of arbuscular mycorrhizal, which is vital for nutrient cycling and maintained plant health (Fr ac et al., 2018; Li et al., 2018).

To date there are two options to conduct fatty acid profiling: the use of phospholipid fatty acids (PLFA) or EI-FAME analyses. These analyses are different because of the lipid extraction techniques. Specifically, in PLFA phospholipids are fractionated into glycolipids and neutral lipids (Zelles, 1999). However, in EI-FAME the phospholipids are extracted collectively. Due to the separate extraction of the phospholipids by PLFA, this method is advantageous as these phospholipids have short turnover times and can therefore provide a snapshot in time (Zelles, 1999). Thus, PLFA methods are more so derived from living organisms than compared to EI-FAME which measures the fatty acids that have been ester linked to complex lipids, which persist longer in the soil (Fernandes et al., 2013; Zelles, 1999). Both analyses have been found to convey similar information. However, EI-FAME has been found to be cheaper as well as more rapid (Drijber et al., 2000). When comparing PLFA to EI-FAME across 172 soil samples from 14 states that varied in soil properties, results showed that that biomarkers were comparable between the two methods (Li et al., 2020). However fungal and actinobacteria biomarkers and were more abundant in EI-FAME, whereas bacteria biomarkers were more dominant and responsive to soil properties in PLFA's (Li et al., 2020). Moreover, numerous studies have concluded that the use of EI-FAME is useful for initial screening due to low cost and the method being faster. However, PLFA is recommended for use due to it being more sensitive to shifts in soil properties (Buyer and Sasser, 2012).

While FAME measurements are useful for gauging the overall structure and function of microbial groups, caution should be used. When interpreting the biomass of certain microbial subgroups, many of the fatty acid markers are not specific to the fungi or bacteria they are indicating. For example, the PLFA's cy17:0 and cy 19:0 are commonly used as an indicator of Gram-negative bacteria however, they can also be found in Gram-positive bacteria (Schoug et al., 2008). In addition, when using PLFA's as an estimate for rapid changes in the environment, variables such as temperature can slow turnover rates and results may not be an accurate depiction of microbial community shifts of a changing environment (Ranneklev and B  ath, 2003). Lastly, the results of Shannon's Diversity may not account for the complete fungal or bacterial diversity, given that

each fatty acid is not a representation of species (Frostegård et al., 2011). For a more granular assessment of microbial structure, more advanced molecular methods are needed.

3.2 High-throughput sequencing

High-throughput sequencing of microbial community DNA is a soil biological indicator of fungal and bacterial diversity and composition (Fig. 9). High-throughput sequencing utilizes the ITS1 and ITS2 markers to quantify fungal diversity, and the 16S rRNA gene marker to quantify bacterial diversity (Baldwin-Kordick et al., 2022; Vos et al., 2013). The amplification of these marker genes can yield relative abundance pertaining to the targeted microbial community. In addition, the use of high-throughput sequencing can allude to taxonomic shifts between systems. For example, Ling et al. (2016) found that microbial functional groups that involved carbon, nitrogen, and phosphorus-based metabolism were present more so at a significantly high abundance in organic systems than when compared to conventional agricultural systems.

Given that microbial communities are rapidly altered by disturbances, the use of high-throughput sequencing could serve as a rapid soil biological health indicator. Although still challenging to link to ecological function, advances have been made to demonstrate how key taxa are associated with key processes including decomposition and soil nutrient cycling (Leff et al., 2015; Zhang et al., 2016). For example, Zhang et al. (2019) found that soil N processes were significantly related to bacterial/archaeal community composition and the bacterial and fungal richness diversity. Additionally, the structure of the microbial community was found to have altered the soil environment and have indirect control on soil C and N processes (Zhang et al., 2019). This demonstrates that there is strong potential to integrate microbial taxa into the soil biological health framework.

Although high-throughput sequencing can give rapid assessments of the microbial community, this measure has its own pitfalls. Specifically, there are often tradeoffs between the quality of the data, the cost of sequencing, and the length of the reads (Pollock et al., 2018). In addition, the scale of observation can be heterogenous given that samples are taken from a smaller area where there are only a few dominant taxa (Lindahl et al., 2013). Biases can also be introduced when choosing the sequencing platform. The most common platform that is used is the Illumina Miseq (Illumina Inc., San Diego, CA, USA). However, Kozich et al. (2013) reported that this platform

can have an error rate of up to 10%. This error rate has been greatly reduced with the PacBio platform. However, tradeoffs arise as the use of this machine is more expensive and time consuming (Castaño et al., 2020; Tedersoo et al., 2018, 2020). Lastly, there is a current disconnect between linking microbial community composition and diversity to ecological functioning. Moreover, scientists have yet to discover the most effective way to translate high-throughput sequencing data to farmers, which is needed to comprehensively inform soil health goals.

3.3 Free-living nematodes

Nematodes were first introduced as important indicators of soil health in the early 2000s (Neher, 2001), however, the field of soil health has drastically changed in the past 20 years, thus the role of nematodes as a soil biological health indicator must be revisited. Nematodes represent the most abundant metazoa across terrestrial ecosystems and play a key role within the soil food web (Ferris et al., 2001). As specialists, nematodes, have important downstream impacts on microbial communities that subsequently influence key nutrient cycles (Ferris, 2010; Gebremikael et al., 2016). For instance, there are nematodes that solely feed on bacteria, nematodes that solely feed on fungi, herbivorous nematodes that feed on plant roots, carnivorous nematodes that prey on other nematodes, and lastly omnivorous nematodes that change feeding preferences based on environmental conditions (Fig. 10). The presence and absence of these various types of nematodes have been linked to soil food web function and can demonstrate key ecosystem processes and overall environmental health (Ferris et al., 2001). Moreover, nematodes are very sensitive to chemical and physical perturbations, which makes them an ideal indicator for soil health within agroecosystems (Fiscus and Neher, 2002). That said, while free-living nematodes are mentioned in the Comprehensive Assessment of Soil Health Framework (Moebius-Clune et al., 2016), soil health assessments in the United States, rarely include free-living nematodes.

With a renewed focus on soil biological health, reassessing how nematodes can be integrated into the soil health framework is critical for linking soil food web structure to soil health (Martin and Sprunger, 2022b; Martin et al., 2022). One reason why nematodes could serve as critical indicators of soil health is their direct influence on nitrogen availability and plant biomass production (DuPont et al., 2009; Ferris et al., 1998; Gebremikael et al., 2016). For instance, when bacterivorous nematodes feed on bacteria,

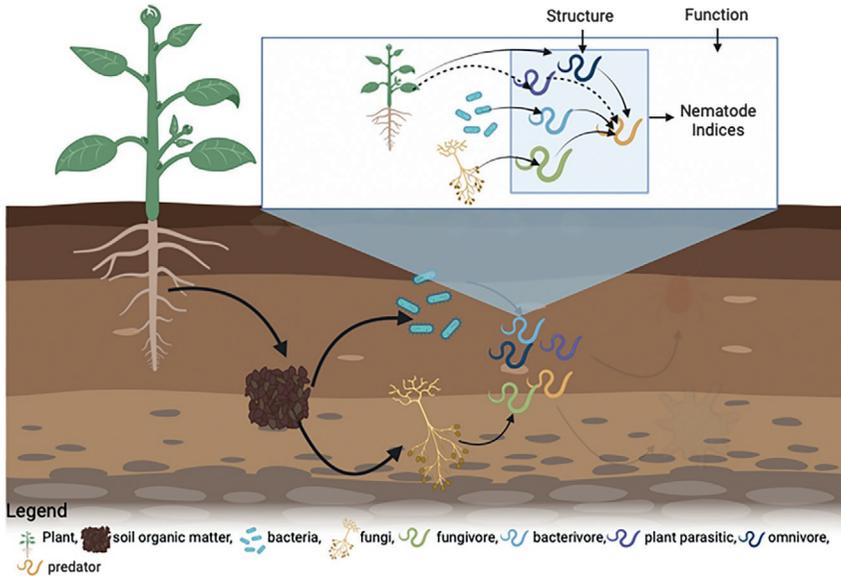


Fig. 10 Nematode feeding preference can be used to indicate soil food web structure and function. *Created with BioRender.com.*

they acquire N and then excrete the excess nutrients not required for their metabolic needs. Moreover, nematode traits can alter the amount of plant available N present in a system (Ferris et al., 1998), demonstrating that monitoring nematode community composition could provide key insight into N cycling and plant production, especially in systems that are N limited. Taken together, the trophic level position of nematodes can provide key inferences to soil food web structure, while also having direct implications for soil ecosystem functioning. This link to ecological function makes nematodes an especially promising indicator of soil biological health (Fig. 10).

Nematodes also have important controls over soil carbon cycling (Gebremikael et al., 2016; Margenot and Hodson, 2016; Martin and Sprunger, 2021b). Plant feeding nematodes have direct impacts on root decomposition that triggers the release of root exudates, which subsequently increases microbial growth and leads to a loss of CO₂ (Bardgett et al., 1997). In contrast, systems where root herbivory is minimal, C assimilation in roots increases (Yeates et al., 1998). Bacterivorous nematodes can also stimulate soil C accrual by suppressing the metabolic activity of bacteria (Neher, 2010). A recent meta-analysis also found that bacterivorous nematodes influenced soil C accumulation in micro-aggregates (Martin and

Sprunger, 2021b). A novel finding from Mielke et al. (2022) indicates that grazing stimulates bacteria that preferentially live in the rhizosphere. This in turn enhances the necromass of these same bacteria, which substantially contributes to soil organic matter formation. Exploring soil fauna–microbe interactions can provide key insights into soil organic matter cycling and should be a routine component of soil health assessments.

Nematologists have also worked to go beyond nematode community structure by creating nematode indices that reflect key ecosystem functions (Ferris et al., 2001) and that could be used as relevant indicators of soil health (Martin and Sprunger, 2022b; Melakeberhan et al., 2021). Nematodes fall along a 1–5 linear colonizer–persister (cp) scale, where nematodes are assigned values based on their r and k characteristics, i.e., generation times, fecundity, resistance to stressors (Du Preez et al., 2022; Ferris et al., 2001). For instance, bacterial feeders and some fungal feeders are assigned lower cp values, whereas omnivores and predators are assigned higher values (Ferris et al., 2001). Based on the weighted abundance of these functional guilds or cp values nematode indices can be calculated (Table 2). The structure index reflects trophic complexity and overall diversity of the nematode community but does not consider plant parasitic nematodes (Ferris and Bongers, 2006; Ferris et al., 2001). Thus, the sigma maturity index was developed to account for a measure of overall trophic complexity (Yeates, 1994). The enrichment index indicates organic matter inputs and nutrient cycling (Ferris and Bongers, 2006; Ferris et al., 2001). The channel index portrays key fungal:bacterial decomposition pathways and the basal index reflects ecosystem disturbance (Bongers et al., 1991; Ferris et al., 2001). The plant parasitic index was developed to identify the complexity of solely the plant parasitic nematode community (Bongers et al., 1997). Soil faunal profiles can be constructed from these nematode indices and have the potential to reflect ecosystem function and soil health status (Du Preez et al., 2018; Ferris et al., 2001; Sprunger et al., 2019a,b). For example, the relationship between the enrichment index and the structure index is often used to track ecological succession of the soil food web and overall ecosystem health. This framework demonstrates how soil food webs shift by management and could be a nice integration into the soil health framework (Fig. 11) (Melakeberhan et al., 2021).

Although indices provide a nice framework for assessing soil food web function, recent studies have found that nematode community structure data are more sensitive and can be more easily integrated into the soil health framework relative to nematode indices (Martin and Sprunger, 2022b;

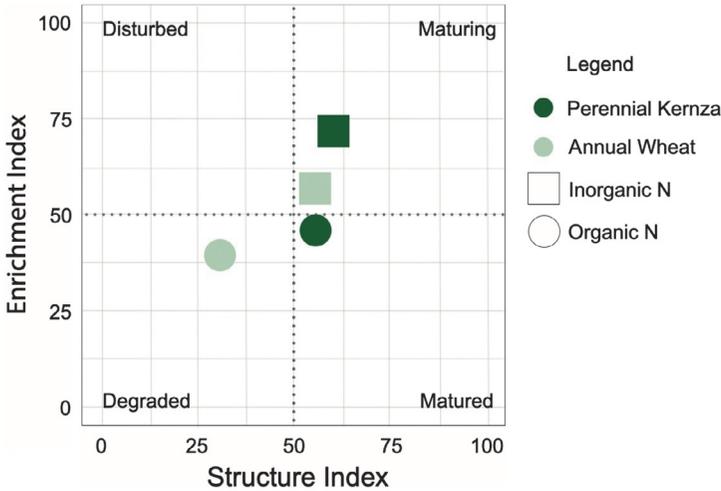
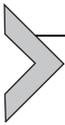


Fig. 11 Soil faunal profiles created by comparing structure and enrichment indices (Fierer et al., 2021). Nematode communities in a perennial grain system (squares) compared to annual wheat (circles) were more mature relative to annuals fertilized by mineral N. Data adapted from Sprunger, C.D., Culman, S.W., Peralta, A.L. DuPont, S.T., Lennon, J.T., Snapp, S.S., 2019b. Perennial grain crop roots and nitrogen management shape soil food webs and soil carbon dynamics. *Soil Biol. Biochem.* 137, 107573. with permission from authors.

Martin et al., 2022). For instance, Martin and Sprunger (2022b) found that nematode community structure shifted over the course of a single growing season, while indices largely remained the same. Moreover, when conducting an exploratory factor analysis, nematode community structure data significantly loaded on to the same factors as other soil health indicators, while nematode indices were isolated from other indicators (Martin et al., 2022). That said, there are numerous studies that demonstrate important shifts in nematode indices, especially in multi-year studies and in cases where contrasting systems are examined (i.e., annual vs perennials) (DuPont et al., 2014; Sprunger et al., 2019a,b).

Due to their position in the soil food web and linkages to ecological function, free living nematodes are a strong candidate for soil biological health indicators that should be further integrated into future assessments (Du Preez et al., 2022; Melakeberhan et al., 2021). Furthermore, given that nematodes are critical drivers of both carbon and nitrogen, indices and feeding groups should be incorporated into more holistic soil management plans. That said, while there are several upsides associated with nematode indicators, there are a few negatives as well. First and foremost, nematodes are

typically measured via microscopy and extensive training is needed for accurate nematode identification to family and genera (Neher, 2001, 2010). Until molecular methods for nematode quantification and identification advance (see section below), microscopy will be the gold standard, which requires a substantial amount of time and labor. In a recent review, Du Preez et al. (2022) outline the various ways in which nematode indices can be used to assess soil ecosystem health and functioning but also highlight the need for more refined cp and trophic group classifications. This refinement could help nematode indices become more sensitive and appropriate on a regional basis (Martin et al., 2022).

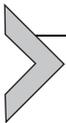


4. Molecular approaches needed to integrate nematology into the soil health framework

Free-living nematode communities have historically been identified via microscopy, which relies on the morphological identification of each individual organism (Bongers, 1990). However, this process is laborious and requires intensive training to carry out morphological identifications on individual nematodes. Thus, molecular approaches via DNA barcoding efforts have been implemented to make nematode quantification and identification more efficient and accessible (Schenk et al., 2019). That said, while progress has been made in recent years, there are several methodological and procedural advances that must be made for such approaches to yield accurate results. Currently, nematodes have been amplified using the 18S small subunit ribosomal RNA (Kenmotsu et al., 2020). However, debate lies as to which regions may serve as the best primer set for nematode identification. Particularly, the 18S rRNA, V6-V8 region primers have been found to be 74% accurate in identifying nematodes to genus (Sikder et al., 2020). However, Kenmotsu et al. (2020) reports that the DNA barcoding using the V4 is best for soil nematode taxonomic analysis. Results thus far have shown that the amplification and use of the 18S subunit is a potential method for the future of identifying free-living nematodes. Eyualem and Blaxter (2003) have found that the use of 18S was able to separate five cultured nematode isolates whereas morphological methods such as scanning electron microscopy were not able to differentiate between these five groups.

Although the use of metabarcoding is capable of distinguishing various nematodes the identification of the barcodes using reference databases and pipelines yield contrasting results. For example, the Basic Local

Alignment Search Tool (BLAST) reference data base has reported limitations when identifying nematode species (Kenmotsu et al., 2020), and can only identify nematodes to genus. Others have found that the use of the DADA2 R package pipeline is capable of assigning genus level assignments with high accuracy and low error rate, whereas species level assignments still had high error rates (Callahan et al., 2016). Additionally, Treonis et al. (2018) found that the use of the Quantitative Insights Into Microbial Ecology (QIIME) pipeline failed to assess the prevalence of nematode families and also detected some families that were not identified morphologically. Likewise, Schenk et al. (2019) found that when comparing between morphology, barcoding, and metabarcoding only three nematode species were shared, thus indicating that further work needs to be done in forming a more accurate database. Future research must continue to advance metabarcoding efforts for the accurate characterization of nematode to family and genus. In addition, there needs to be rigorous assessment of which 18S subunit region to target, as there is conflicting evidence of which primer is most effective. Given that many scientists view morphological identification of nematodes as a limitation to scaling up nematode research (Kekelis et al., 2022; Neher, 2001), advancing molecular methods for nematode characterizations could significantly amplify the use of nematode structure and function within the soil health framework.



5. Integrating soil biodiversity metrics into the soil health framework

In the sections outlined above, we demonstrate how key soil biological health indicators can be linked within the soil food web to reflect key ecosystem processes. Taken together, merging a diverse range of indicators can provide key insights into ecosystem functioning (Fig. 1). That said, to quantitatively merge these key indicators and measures, researchers must turn to quantitative means. To date, this has mainly been conducted via structural equation modeling. However, understanding the relationships between soil biological health indicators goes beyond relatedness. Distinguishing which soil biological health indicators are most important and reflective of ecosystem functioning in any given context may require quantitatively identifying specific soil health traits (Shukla et al., 2006; Wade et al., 2022). One method that has recently gained momentum is the use of exploratory factor analysis (EFA), which is a latent variable

analysis that identifies the underlying traits that measured variables share (Fabrigar and Wegener, 2011). Specifically, an EFA can quantitatively determine which soil biological health indicators share underlying traits and how these clusters can inform key soil health outcomes (Fig. 12). This is depicted in Fig. 12; whereby different indicators are loading on to a given latent variable based on shared underlying soil health traits. These shared traits can then inform important soil health outcomes. Moreover, EFA can be combined with confirmatory factor analysis and structural equation modeling to test the robustness of these underlying soil health traits (Wade et al., 2022).

Recent studies have applied EFA using soil health data. For instance, Wade et al. (2022) proposed EFA as a rigorous approach that can successfully link soil health assessments to soil health outcomes (i.e., ecosystem function). Similarly, Martin et al. (2022) were the first to integrate nematode community composition with other soil biological health indicators such as soil respiration and enzyme activities using EFA. Results demonstrated that certain nematode feeding groups shared key underlying soil health traits with POXC, Mineralizable C, and Protein, providing a mechanistic understanding of how nematodes influence mineralization and stabilization processes. Future assessments should incorporate EFAs to understand shared underlying traits between various soil biological indicators. For example, it is still unclear which aspects of the microbiome influence soil health outcomes (Fierer et al., 2021). Soil biological indicators, such as FAME and high-throughput sequencing could be integrated with other prominent soil biological indicators using EFA (Fig. 12). This would lead to a more quantitative understanding of the linkages between soil biodiversity and soil health.

Exploratory Factor Analysis should also be used to merge all aspects of soil health (biological, physical, and chemical) and would be an elegant way to integrate a larger number of indicators. Moreover, the field of soil biological health should make a more concerted effort to integrate indicators of pests and pathogens, as plant pathology metrics are acutely missing from current soil health frameworks (Larkin, 2015). This linkage could be conducted using EFAs to merge both beneficial and harmful pests to see how soil health traits inform disease suppressiveness. Lastly, the use of EFA will lead to stronger understandings of how soil type, agricultural management, and other context dependent factors have on underlying soil health traits and inform soil health outcomes (Martin et al., 2022; Wade et al., 2022).

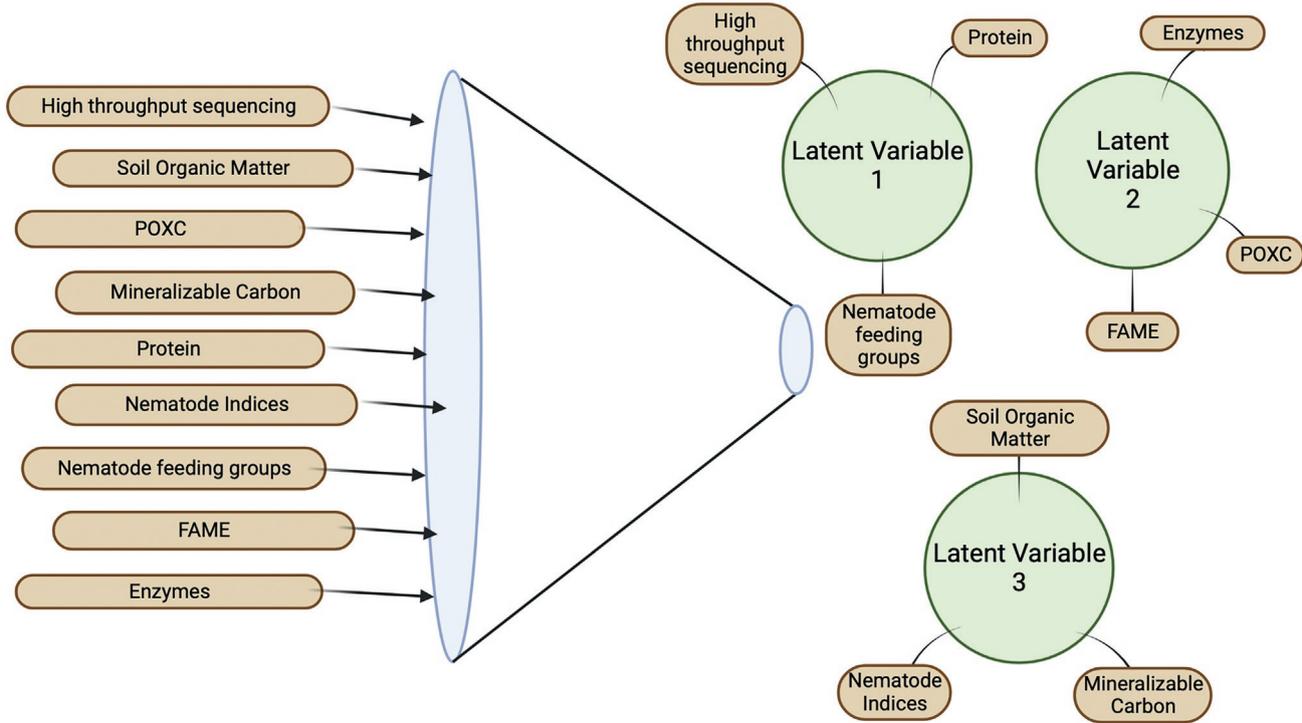
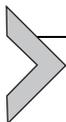


Fig. 12 Conceptual graphic of how soil biological health indicators can be integrated to identify shared underlying traits using exploratory factor analysis. Individual soil biological health indicators “load onto” latent variables based on shared soil health traits. *Figure created with BioRender.com.*



6. Soil biological health is fundamental to regenerative agriculture

Regenerative agriculture has gained renewed momentum as society grapples with continued declines in biodiversity, uncertain crop markets, and the ongoing climate crisis (Schreefel et al., 2020). Although, regenerative agriculture has numerous definitions, most agree that it goes beyond sustainable agriculture, and encompasses a more aggressive approach toward self-sustaining agricultural systems that deliver key ecosystem services including carbon sequestration, nutrient retention, greater crop productivity, and enhanced biodiversity (Giller et al., 2021). Perhaps most foundational to regenerative agriculture is the rejuvenation of soil, specifically soil biology. As Rodale (1983) states, regenerative agriculture must increase the soil biological production and that biological relationships in the system should be maximized. Unfortunately, due to land conversion, we have seen unprecedented losses of top-soil, which has led to reductions in soil biodiversity as well as specific taxa that provide key ecosystem functions (Nielsen et al., 2015; Tibbett et al., 2020). The implementation of regenerative agricultural practices is needed more than ever to tackle multiple environmental issues. The soil biological health indicators outlined in this chapter are sensitive to changes in management and should be used to closely monitor ecosystems responses to regenerative agricultural practices.

In this chapter, we have demonstrated how various measures of soil biology should be integrated for a more robust soil biological health framework, especially within an agricultural context. Through fostering relationships that maximize soil food web structure and function, key soil health outcomes including carbon sequestration and greater agronomic performance can be realized (Sprunger et al., 2019b; Stefan et al., 2021). It is also clear that management practices that feature perenniality and crop diversification should be pillars of regenerative agriculture, as such practices are extremely effective at enhancing soil biological health (Sprunger et al., 2020). Future research must continue to assess the underlying relationships that maximize soil biological health, so that key sustainability goals can be achieved. Lastly, the field of soil science should continue to prioritize research that seeks to integrate measures of soil biology into the soil health framework via molecular and quantitative based methods.

Acknowledgment

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