A methodological framework to embrace soil biodiversity

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# Abstract

Soils host the vast majority of life on Earth including microorganisms and animals, and supporting all terrestrial vegetation. While soil organisms are pivotal for ecosystem functioning, the assemblages of different biota from a taxonomic and functional perspective, as well as how these different organisms interact, remains poorly known. We provide a brief overview of the taxonomic and functional diversity of all major groups of soil biota across different scales and organism sizes, ranging from viruses to prokaryotes and eukaryotes. This reveals knowledge gaps in relation to all soil biodiversity groups, which are especially evident for viruses, protists, micro- and meso-fauna.  We review currently-available methods to study the taxonomic and functional diversity of soil organisms by grouping all commonly-used methods into morphological, biochemical and molecular approaches. We list potentials and limitations of the methods to reveal that there is, as yet, no single method to fully characterize the biodiversity even of a single group of soil biota. Yet, we stress that we now have the methods available to enable scientists to disentangle the taxonomic and functional diversity of virtually all soil organisms. We provide a user-friendly guide to help researchers address a wider variety of soil biodiversity in their studies by discussing and critically analysing the various potentials and limitations of diverse methods to study distinct groups of soil life. We highlight that integrative methodological approaches, ideally in collaborative interactions, are key to advancing our understanding of soil biodiversity, such as the combination of morphological and molecular approaches to overcome method-specific limitations. Together, integrative efforts can provide information on the abundance, biomass, diversity and function of several groups of soil biota simultaneously. This newly-obtained integrative information on soil biodiversity will help define the importance of soil biodiversity in ecosystem processes, functions, and services, and serve to refine food-web and earth system models.

Keywords: Microorganisms; Fauna; Molecular methods; Food-webs; Biodiversity; Soil functions and health

# 1. Introduction

Soils represents a complex habitat sustaining a huge diversity of organisms that are structured by and embedded within the physical matrix, together building the most diverse of all ecosystems (Curtis et al., 2002; Decaëns, 2010). Plants are part of this diversity and, as the dominant fraction of soil biomass (Bar-On et al., 2018), they have a major impact on the soil habitat and the rest of the biodiversity that resides within the belowground matrix. Plants structure soil physics, chemistry and other soil biota due to litter inputs and root growth (Berg and Smalla, 2009). In this perspectives paper, we focus on all such other organisms including microorganisms and soil animals. Billions of bacterial cells, tens of thousands of protist cells and kilometres of fungal hyphae typically inhabit a single gram of soil from anywhere on the planet, each represented by an enormous taxon-diversity (Orgiazzi et al., 2016; Fierer, 2017; Zhang et al., 2017; Geisen et al., 2018a; Table 1). Multi-cellular soil animals (Metazoa) are conventionally separated into microfauna (e.g., rotifers, tardigrades, nematodes), mesofauna (e.g., collembolans, mites, enchytraeids) and macrofauna (e.g., ants, termites, earthworms). This highlights a small proportion as numerous other groups (here we define *group* as a gross taxonomically-distinct assembly of soil organisms, e.g. bacteria or nematodes) are highly diverse, most of which contain tens of thousands or more species (Table 1; Orgiazzi et al., 2016). These groups interact across different temporal and spatial scales and also adapt and co-evolve (Fig. 1 and Fig. 2). Yet, we have a limited understanding of the diversity within, and the interactions among, these different groups of soil organisms.

This lack of knowledge is at least partly due to the complexity of the opaque soil matrix that necessitates the application of elaborate methodologies to unravel this hidden diversity. Recent advances in molecular high-throughput sequencing (HTS) technologies have mainly been applied to reveal the community structures of microbial prokaryotes (bacteria and archaea) and eukaryotic fungi across most soil biomes (Tedersoo et al., 2014; Maestre et al., 2015; Prosser, 2015; Zhou et al., 2015; Thompson et al., 2017; Delgado-Baquerizo et al., 2018c). Despite these promising advances, molecular approaches are less often applied for surveying the diversity, composition and spatial and temporal variability of higher trophic levels of soil fauna, viz. microbial protists and Metazoa (Table 1; Orgiazzi et al., 2015; Geisen et al., 2017).

Indeed, there are few causal studies linking biodiversity across different groups of soil organisms with soil ecosystem functioning (de Vries et al., 2013; Wagg et al., 2014; Delgado-Baquerizo et al., 2017; Morriën et al., 2017; Wang et al., 2019). This partly explains why the most widely applied food web models have essentially remained unchanged for the last 30 years (Hunt et al., 1987; Bradford, 2016). Ongoing research using gut content analyses, isotope probing and species-specific interaction assays is producing missing functional information, providing insights that should be implemented in more accurate food web models (Digel et al., 2014; Kramer et al., 2016; Morriën et al., 2017). However, the majority of these food web models are limited to certain components of the food web (Brose and Scheu, 2014; Heidemann et al., 2014). Non-trophic interactions, including entire groups such as viruses and parasites, are missing in soil food web models (Hawlena and Zaguri, 2016), despite their likely importance in structuring plant and animal communities (Fig. 1).

In this paper, we provide an overview of the methods to study the taxonomic and functional diversity of all major groups of soil organisms. We argue that in order to better understand the functioning of intimately connected soil biodiversity in soil ecosystems, we need to concurrently explore species abundances, distributions, and interactions across organism and group boundaries. To achieve this, we will need to use integrative methodologies that capture the taxonomic and functional diversity of soil biota and species interactions across trophic levels, space and time. We highlight that a methodological toolbox is now available and should be used to fill existing knowledge gaps on all soil organism groups, both individually and in combination, and argue that this will bring us closer to understanding the complexity of soil biodiversity, i.e. to disentangle the soil holobiome. This will help to continue advancing our understanding of soil biodiversity (Box 1).

# 2. Taxonomic and functional biodiversity of soils

Soil biodiversity is multifaceted in its taxonomic composition, with each major group consisting of a plethora of taxonomically and functionally dissimilar taxa (Table 1). Their global biomass is second only to that of trees, with at least viruses, prokaryotes, fungi, protists and earthworms each individually having higher biomass than all humans (Table 1; Bar-On et al., 2018). The diversity and complexity of soil biodiversity as well as training on individual groups of soil biota have resulted in a focus on single groups of soil life in the vast majority of soil biodiversity studies (Supplementary Table 3). Differences in methodologies to study distinct groups of soil biota further underlie this phenomenon. For example, bacteria and fungi can be studied simultaneously by phospholipid fatty-acid (PLFA) analyses and DNA/RNA-based sequencing, while mites and Collembola are co-extracted using heat-drying the soil followed by microscopic species determination. In comparison to those groups, studies focusing on other combinations of soil biodiversity, and those investigating more than two groups of soil life, are rare (Supplementary Table 3). Here we want to challenge the approach of analysing individual taxa in isolation and encourage the scientific community to include interactions and multiple organisms in studies (Table 1; Section 4.).

Abundance and diversity of soil organisms are unquestionably important, yet the functioning of certain groups and soil biodiversity as a whole seems ecologically more meaningful (Kardol et al., 2016; Shade, 2017). Functional roles are commonly differentiated between bacteria and fungi, and higher trophic level groups (protists and fauna). Bacteria and fungi are functionally often defined based on their bottom-up roles in ecosystem processes, including nutrient cycling and litter decomposition, which support soil ecosystem services such as crop production and climate regulation (Baveye et al., 2016). Functional units studied are, for example, methanotrophic, denitrifying and nutrient mineralizing microbial taxa (Fierer, 2017; Table 1). In contrast, functioning of protists and fauna is predominantly defined by their trophic position in the food web and is often considered to affect ecosystem services provided by prokaryotes and fungi via top-down regulation (Table 1). Invertebrates such as earthworms and other ecosystem engineers including fungi represent notable exceptions (Lavelle et al., 2006; Rillig and Mummey, 2006) as these are involved in other functions directly, such as soil formation, water infiltration and biological regulation. Subsequently, we will focus mostly on the functioning of organisms in food webs as these govern microbial-controlled ecosystem processes, and have been studied less frequently over recent decades (Geisen et al., 2017).

Functional assignments are dependant on our knowledge on specific taxa. Functioning of well-known groups is commonly derived from taxonomically identified organisms. Thus, taxonomic information can inform about the likely gross ecological function of many members of fungi (Nguyen et al., 2016), protists (Xiong et al., 2018), nematodes (Sieriebriennikov et al., 2014), mites (Birkhofer et al., 2016; de Groot et al., 2016) and overall soil fauna (Ehnes et al., 2011). However, functional knowledge for most soil organisms remains limited, extrapolated or oversimplified (Fig. 1), and studying simple trophic interactions between soil organisms, as well as non-trophic interactions (Kéfi et al., 2012) is essential to understand feeding links and functional roles of specific taxa.

Traditionally, most groups of soil organisms are placed in one or a few trophic positions in the soil food web (Hunt et al., 1987; Briones, 2014). However, all taxonomic groups of soil organisms are functionally diverse and indeed can be grouped in various positions in the food web as shown for protists (Geisen et al., 2018a), nematodes (Yeates et al., 1993b), collembolans (Potapov et al., 2016) and mites (Walter and Proctor, 2013; Birkhofer et al., 2016) (Fig. 1). Functional overlap between taxonomically distinct groups of soil organisms is commonplace, such as between bacteria and fungi that compete for the same nutrient sources (Bahram et al., 2018), bacterivorous protists, nematodes and microfauna that prey on bacteria (de Ruiter et al., 1995; Morriën et al., 2017) or nematodes, collembolans, earthworms and insect larvae competing for protist prey (Crotty et al., 2012; Fig. 1). Therefore, groupings into functional rather than taxonomic groups are likely to be more ecologically meaningful (Fig. 1). While discrepancies in traditional food webs are now increasingly being acknowledged (Bradford (2016) and associated articles), these approaches still need to be included in redefined soil food webs. For that, careful consideration of available methods and method integrations are needed, which is the focus of this article.

# 3. Potentials and limitations of available methodologies to study soil biodiversity

We will now provide an overview on the most generally used and informative methods to study distinct groups of soil biodiversity. The multiple approaches available to study abundance, composition and diversity of groups of soil organisms can be divided into three main categories: (1) morphological, (2) biochemical and (3) molecular methods (Fig. 3; Table 2).

### 3.1. Morphological methods

Soils impose one major obstacle in comparison to aquatic and above-ground environments: while there is promise to potentially study soil organisms non-destructively *in-situ* in soils (Koebernick et al., 2017), most biota need to be isolated from the soil matrix. Thus, soil invertebrates need to be extracted and microorganisms cultivated before morphological identification can be conducted. However, isolation based methods for microorganisms only select for easily cultivable and fast growing taxa, which can represent as little as 1 % of the entire diversity as illustrated for bacteria (Amann et al., 1995). However, almost all bacterial diversity has been cultivated if the commonly used standard similarity threshold of 97% in the 16S rRNA gene is adopted (Martiny, 2019). This suggests that using the right culturing conditions and approaches – a painstaking and hardly feasible effort – all taxa are theoretically cultivable. Cultivable microorganisms can be difficult to identify morphologically. For bacteria, identification can be performed using a pattern of physiological and morphological characteristics (Whitman et al., 2012). The same holds for protists, which can be identified and enumerated by microscopy using cultivation-based techniques or soil dilution techniques (Foissner, 1999; Geisen and Bonkowski, 2018). Yet, the vast majority of protist diversity present in soil samples, particularly omnipresent parasites, are difficult to culture (Mahé et al., 2017). Host-dependency and therefore difficulties in cultivation are most prevalent for viruses including bacteriophages, because they cannot be cultured without their specific host bacteria. When cultured with their host bacteria, phage morphology can be studied using electron microscopy and their abundances quantified using indirect plaque assays (Williamson et al., 2017). Around 17% of described fungal taxa are cultivable, although it is estimated that >95% of fungal diversity remains unknown (Bridge and Spooner, 2001; Hawksworth and Lucking, 2017).

While fast-growing fungi can be identified morphologically (Watanabe, 2010), culturing methods are mainly used to calculate growth rates and substrate utilization, and infrequently for fungal identification. Sporocarp forming soil fungi can be identified using visible parts of the fungus (Andrew et al., 2019), and spores of arbuscular mycorrhizal fungi (AMF) can be isolated from soils and used to identify AMF species present (Jansa et al., 2002). In addition, many fungi exhibit distinct morphological structures between the sexual and asexual stages (teleomorph vs. anamorph), adding to the confusion of morphology-based fungal taxonomy (Geiser et al., 2007).

Soil animals can be identified and enumerated directly after extraction. Indeed, extraction of soil animals commonly depends on active movement and therefore provides valuable information on the activity of targeted organisms (Bartlett et al., 2010; Gibb et al., 2010; Geisen et al., 2018b). For instance, wet extraction methods are applied to extract nematodes using Oostenbrink elutriators or Baermann funnels, and enchytraeids, rotifers and tardigrades using Baermann funnels (Schmelz and Collado, 2010). Active microarthropods (including mites, some arachnids and collembolans), insects, myriapods, and other macrofauna are extracted from soils by heat-drying the soil in Berlese funnels and similar extractors (i.e. Tullgren funnels, MacFadyen extractors) (Tullgren, 1918), while chemical (e.g. 4% formalin and either mustard or allyl isothiocyanate (AITC) solutions) or electrical expulsions are applied to extract earthworms (Römbke et al., 2006). Pitfall trapping is widely used for estimating the diversity of agile surface-living predators like spiders and ground beetles; emergence traps allow collecting of imago insects and there is potential for automatized determination of abundances and activity of caught organisms (Dombos et al., 2017). A variety of extraction methods (e.g. Winkler apparatus, Tullgren funnels, MacFadyen extractors, Berlese funnels) are available for collecting larger surface-dwelling arthropods, including ants and beetles (Krell et al., 2005; Longino et al., 2014)). However, these animal extraction techniques that target active stages can underrepresent the total diversity by more than 30% (André et al., 2002). Alternative techniques that also cover inactive stages and dead organisms include centrifugation based or flotation methods that use solvents (e.g. hexane) or dense solutions (e.g., sugar) to extract nematodes and microarthropods (Jenkins, 1964; Ducarme et al., 1998; Arribas et al., 2016), but work best in low organic and mainly mineral soils. Larger soil animals including earthworms and immobile stages of insects (such as cocoons, lethargic individuals and pupae) as well as animals that avoid escaping heat and dryness such as molluscs can be collected by hand-sorting, but hand-sorting negatively selects for deep burrowers, small taxa and juveniles that often dominate assemblages (Smith et al., 2008; Bartlett et al., 2010). Various techniques are used for inventorying social insects such as ants and termites, but, as yet, none of them is fully effective and universally applicable (e.g. Jones and Eggleton, 2000; Dawes-Gromadzki, 2003). Importantly, none of these techniques recover all taxa equally (McSorley and Walter, 1991).

Subsequent to isolation, soil animals are typically identified morphologically. The long history of studies on different soil animal groups has resulted in profound knowledge on species differentiation and experts can identify most soil animals to family or genus. However, morphological identification to the species level is limited to a handful of taxonomists globally, is time-consuming and laborious, and therefore impractical for most ecological work that includes several dozens of samples (Orgiazzi et al., 2016; Nielsen, 2019). Therefore, taxa are often identified at lower taxonomic resolution (family or genus), particularly nematodes and mesofauna, for which microscopic analyses are essential for identification. For example, nematode taxa in ecological studies are commonly divided into feeding groups based on differences in the morphology of mouth parts and digestive organs, and sometimes further classified to family or genus (Yeates et al., 1993b). Nematodes and microarthropods are often killed before identification to avoid community changes over time before sample processing. While reducing temporal variability, organism identification can be more challenging as taxa can change in shape and colour, and species-characteristic movement patterns are lost. Similarly, enchytraeids become opaque when fixed making certain internal structures, such as spermathecae and salivary glands, impossible to see and therefore they need to be identified alive (Schmelz and Collado, 2010). This limits the ability to process a high number of samples due to constraints on storage time (Schmelz and Collado, 2010). Microarthropods are identified to family, genus, sometimes species level using a range of morphological characters (Walter and Proctor, 2013), but problems arise when trying to identify juvenile stages, with some mites undergoing metamorphosis and hence, exhibiting several development stages (instars), some of them being inactive.

### 3.2. Biochemical methods

Several biochemical methods are used to estimate microbial biomass. These can be differentiated into static approaches that determine cellular components such as ATP, DNA and RNA (Blagodatskaya and Kuzyakov, 2013). These compounds are needed to keep cellular processes ongoing and therefore provide information on the total biomass including active and quiescent cells. Yet, concentration of these compounds varies substantially over time, and depends on variation in abiotic parameters, making estimates on the activity and biomass of microorganisms less accurate (Blagodatskaya and Kuzyakov, 2013; Blazewicz et al., 2013). In contrast, dynamic approaches can address the proportion of active organisms and measure metabolic processes such as enzyme activity, respiration and substrate utilization (Bailey et al., 2002; Chapman et al., 2007; Blagodatskaya and Kuzyakov, 2013). While enzyme activity is suggested to inform about active soil biota, many enzymes are active extracellularly for prolonged periods of time (Schimel and Schaeffer, 2012; Burns et al., 2013). Therefore, quantifying enzyme activity informs on both active and inactive soil organisms at a given point in time. Enzyme activity and microbial respiration are widely used proxies for soil functioning and due to their fairly high throughput, used, for example, to reveal responses of soil microorganisms to climate change (Frey et al., 2008; Bradford et al., 2019). However, these methods only measure process rates and have limited taxonomic resolution, providing only crude information on total contents of those compounds present in soils that are produced mainly by the most abundant organisms: bacteria and fungi (Chapman et al., 2007; Blagodatskaya and Kuzyakov, 2013).

Other approaches to study microbial biomass in soils are fumigation-extraction methods ((Joergensen, 1996; Powlson et al., 2017). Analysis of soil samples for PLFAs and neutral lipid fatty acids (NLFAs) originating from cell membranes provide microbial biomass estimates including that of specific bacterial groups, such as Gram-positive (many terminally branched fatty acids such as i15:0 or a15:0) and Gram-negative bacteria (many monounsaturated fatty acids such as 16:1ω9c or 18:1ω5c) and the most abundant soil fungal groups, including ascomycetes and basidiomycetes (18:2ω6c) (Frostegård and Bååth, 1996; Ruess and Chamberlain, 2010; Willers et al., 2015). In turn, some PLFA markers are non-specific and can be used to study different groups of organisms, such as the 16:1ω7c used to study Gram-negative bacteria as well as cyanobacteria and diatoms and even all “fungal specific markers” including 18:1ω9c and 18:2ω6c that also are used to investigate cyanobacteria, green algae and diatoms (Willers et al., 2015). When surface soil is removed, which commonly contains these photosynthetic microorganisms, these markers have been found to measure mainly fungi. The fungal specific membrane compound ergosterol can be measured to estimate fungal abundance and seems to be both robust and among the most accurate to estimate fungal biomass (Grant and West, 1986; Baldrian et al., 2013). Mucoromycota, including AMF, are studied using the specific NLFA marker 16:1ω5 (Olsson et al., 1995). Fatty acid composition might also provide information on community composition and abundances of soil animals (Kühn et al., 2018). Biochemical markers to investigate the taxonomic soil diversity are mostly crude; they can only differentiate between coarse microbial and animal groups and none of these markers is entirely specific for individual taxa (Ruess and Chamberlain, 2010). However, they can be useful in revealing large shifts in soil food-web structure from fungal dominated to bacterial dominated in response to, for example, land use change (van der Wal et al., 2006; de Vries et al., 2013; Sechi et al., 2014; Fanin et al., 2019), while the Gram-positive to Gram-negative ratio has been recently shown to be a good indicator of soil carbon use efficiency (CUE) (Fanin et al., 2019).

More recently, “omics” approaches have been implemented for proteins (metaproteomics) and metabolites (metabolomics) in soils. Despite the great promise in providing functional information for soil biodiversity (Marchesi and Ravel, 2015; Fig. 3), the presence of a given protein or metabolite, even in the case of enzymes, only informs about potential rather than actual functions present (Becher et al., 2013). In addition, these approaches are rarely performed in soils because calibrations to adjust for incomplete and biased protein and metabolite extraction have to be conducted (Greenfield et al., 2018), while reliable protein and metabolite annotations remain complicated as the identity of many soil biota and their expressed proteins and metabolites often remains unknown (Becher et al., 2013; Heyer et al., 2017).

Stable isotope analysis of soil animals and fungi does not provide information on the taxonomic composition of soil communities as such, but helps to elaborate the structure of soil food webs (Potapov et al., 2019). Application of stable isotope markers, such as 15N and 13C, allows detailed tracing of the flow of nutrients and carbon through the soil food-web primarily through active consumption (Crotty et al., 2011). Radiocarbon (14C) dating has allowed estimations of the age of the carbon assimilated and mobilized by soil invertebrates (Briones et al., 2010). These approaches, however, are rather expensive, require expertise for accurate dating interpretations and a rather high minimum mass for analyses which makes it impractical for smaller sized soil organisms. Gut-content analysis combined with stable isotopes can elucidate actual feeding relationships in soils, but this individual-based analysis is in its infancy for larger-scaled ecological studies (Potapov et al., 2019). Yet, the determined food-source taken up can sometimes originate from cross-feeding of consumers that have taken up the determined food-source. As such data obtained has to be interpreted carefully to avoid false-interpretation of direct feeding interactions.

### 3.3 Molecular methods

Molecular approaches have facilitated ease and detail of identification as well as taxon delimitation, up to the genus level or below, and have shed light on taxonomic relationships based on phylogenetic similarities among and within groups of organisms (Cristescu, 2014; Orgiazzi et al., 2015; Csuzdi et al., 2017). In barcoding approaches slowly evolving conservative genes such as those encoding for ribosomes (bacteria, protists, nematodes, collembolan, earthworms), cytochrome oxidase (mites, collembola, enchytraeids, earthworms) and histones (earthworms), or non-coding internal transcribed spacer region (fungi, but also animal groups) are used to differentiate species within most taxa (Hamilton et al., 2009; Iglesias Briones et al., 2009; Pawlowski et al., 2012; Schoch et al., 2012; Young et al., 2012; Porco et al., 2013; Anslan and Tedersoo, 2015). Viruses lack universally conserved phylogenetic marker genes, but some gene regions, such as the g23 gene encoding the major capsid protein of the T4-like phages, can be targeted to study distinct viral groups (Williamson et al., 2017). While molecular barcoding of individual specimens has been used to compare soil animal communities (Wu et al., 2011), this approach becomes rather inefficient for studying the vast diversity of soil organisms across systems (Cristescu, 2014).

For community profiling of soil biodiversity, nucleic acids have to be extracted first from soils or from extracted organisms. While this approach is fast and easy, it provides an artificial distorted representation of the community structure of taxa due to differential extraction efficiencies and PCR inhibition (Martin-Laurent et al., 2001). Nowadays, the obtained mixed nucleic acids are processed mainly by high-throughput sequencing (HTS), an approach that has transformed and facilitated soil biodiversity analyses especially of microorganisms (Prosser, 2015; Fierer, 2017). Different methods are classified as HTS. Among those, amplicon sequencing, also called *meta*barcoding, is currently being the most often applied approach to target different groups of soil biodiversity. In this method, molecular barcoding regions are amplified in parallel with a potential to simultaneously analyse hundreds of samples containing thousands of taxa (van Dijk et al., 2014). High-throughput sequencing now represents the standard to assess the community structures of prokaryotes (Thompson et al., 2017), fungi (Tedersoo et al., 2014) and protists (Geisen and Bonkowski, 2018). However, HTS has only recently been applied to study soil animals (Arribas et al., 2016; Delgado-Baquerizo et al., 2017; Geisen et al., 2018b; Oliverio et al., 2018) and viruses (Williamson et al., 2017; Emerson 2019). For mesofauna and larger aboveground animals, amplicon sequencing or metagenomics after enrichment of mitochondria has successfully enabled high-taxonomic resolution of soil animal communities (Andújar et al., 2015; Arribas et al., 2016; Liu et al., 2016). Ongoing developments in sequencing technologies, such as long read sequencing using Nanopore or PacBio sequencers, can enable higher taxonomic resolution of community analyses (Tedersoo et al., 2017), and can be employed without a PCR step.

A major limiting step in processing and therefore taxonomic identification of sequences obtained in these HTS efforts, is the lack of cumulative reference sequences that are needed to taxonomically assign obtained reads (Cristescu, 2014). Curated and exhaustive reference databases are present for bacteria, such as RDP, Greengenes and SILVA (Pruesse et al., 2007; Balvočiūtė and Huson, 2017), and to a lesser extent for fungi (UNITE (Abarenkov et al., 2010)) and protists (Silva and PR2 (Pruesse et al., 2007; Guillou et al., 2013)). This already shows a bias towards bacterial-dominated soil biodiversity inventories (Box 2). Yet, continuous efforts are needed to assemble taxonomic and functional information from pure cultures (Geisen, 2016) to more comprehensively understand soil biodiversity and enable reliable sequence annotation as well as interpretation. Genomic reconstructions from individual but also complex environmental species assemblies using metagenomics will help in linking taxonomy to the functional potential of individual taxa, with the focus on bacteria ideally being expanded to other groups of soil biodiversity (Box 2; Parks et al., 2017).

Quantification of microbial taxa has advanced the field and is increasingly being performed using qPCR (or similar alternatives such as digital PCR), with total or specific groups of bacteria and fungi commonly enumerated (Fierer et al., 2005; Rousk et al., 2010). A major issue, however, is that bacteria can have up to 15 copies of 16S rRNA gene copies per genome (Klappenbach et al., 2001; Větrovský and Baldrian, 2013), while fungal taxa are often multinucleate and thereby differ in their copy-numbers per organism (Baldrian et al., 2013; Song et al., 2014). Therefore, variation in 16S rRNA gene abundances quantified by qPCR can reflect both genomic copy number variation and variation in the abundance of microorganisms. This issue seems even more pronounced for protists, nematodes and larger soil animals, as copy numbers of barcoding genes commonly differ by orders of magnitude (Gong et al., 2013). However, targeting house-keeping or functional genes, present in one or a few copies per genome, as well as specific taxa with known gene copy numbers per genome can alleviate such bias for some organisms (Schaad and Frederick, 2002; Madani et al., 2005; Campos-Herrera et al., 2012).

Functional gene information can also be obtained by linking taxa to function in HTS approaches (Langille et al., 2013) or directly when studying functional gene diversity (Torsvik and Ovreas, 2002) and by omics HTS approaches (Dinsdale et al., 2008; Fierer et al., 2012; Prosser, 2015). Omics HTS approaches provide information not only on the composition of the soil biota but also on functional genes (metagenomics) and actively transcribed genes (metatranscriptomics) present in a sample (Urich et al., 2008; Prosser, 2015). Omics HTS techniques enable targeting all organisms from viruses to earthworms as well their functional genes in a single approach (see 4.), and reduce the accumulation of biases from applying different techniques. In the future, long-read sequencing will enable linking of taxonomic assignments with functional annotations in complex soil systems as entire genomes can be assembled from environmental samples (Luo et al., 2011; Howe et al., 2014). Clearly, continuous development in HTS methods will soon enable cost-efficient analyses of the community structure and potential functions of the entire soil biodiversity using one or few techniques.

Limitations inherent to all molecular approaches need to be considered; molecular validation of morphologically-based identification is still lacking (Beaulieu et al., 2019), leading to yet unresolved conflicted issues regarding species complexes and cryptic species (e.g. (Trontelj and Fišer, 2009; Emerson et al., 2011; Orgiazzi et al., 2015; Baulieu et al., 2019; Schäffer et al., 2019). DNA-based methods amplify DNA of organisms that are inactive (spores or cysts) or dead, thereby affecting diversity estimates (Carini et al., 2016). An alternative is to target rRNA after reverse transcription into cDNA. Praised as a tool to determine and quantify active organisms (or the ratio of rRNA to rDNA), the paradigm of RNA representing active organisms is likely flawed (Blazewicz et al., 2013; Dlott et al., 2015; Steven et al., 2017) and we have yet to determine what rRNA really identifies. Independent of which type of nucleic acid is being targeted, differential PCR amplification efficiency adds further biases, including PCR biases and lengths of the amplified barcoding region (von Wintzingerode et al., 1997). As such, both real-time PCR and metabarcoding approaches are strongly dependent on the coverage and specificity of the primers used (Tremblay et al., 2015).

While HTS based omics approaches (metagenomics and metatranscriptomics) are less prone to PCR-based issues, similar to metabarcoding approaches they are semi-quantitative and fail at providing absolute abundances of soil organisms. This can mask ecologically relevant information if not supplemented with absolute quantitative data (Vandeputte et al., 2017; Geisen et al., 2018b). Adding known quantities of organisms, or DNA of taxa not expected in a sample, or synthetic DNA, has been suggested to extrapolate total taxon abundance from sequencing data (Smets et al., 2016; Tkacz et al., 2018). Yet, this approach has still to be evaluated in complex soil communities, especially when considering the above-mentioned differences, such as in copy numbers. Another issue of omics approaches is that they are still costly, exceeding hundreds of dollars per sample. Therefore, ultra-deep sequencing is currently needed to cover a wide diversity of soil biodiversity in metagenomics approaches as metagenomic information especially contains less than 1 % of taxonomic barcoding genes, the vast majority being of bacterial origin (Tedersoo et al., 2015). While taxonomic barcoding information obtained in metatranscriptomic approaches is higher, the vast majority of barcoding information is of bacterial origin (>85 %) (Urich et al., 2008), and sample processing including RNA extraction and processing is more expensive than in comparable metagenomic approaches. As a result, omics approaches are as yet barely applicable to investigate a wider soil biodiversity in larger-scale ecological studies, and those omics approaches often focus only on bacteria (Box 2). Therefore, further method developments are needed to reduce sequencing costs. Functional omics data face several other limitations such as (1) the function of many genes remains unknown, (2) the presence of genes in metagenomes does not necessarily imply RNA transcription, and (3) transcribed genes detected in metatranscriptomes might not be translated into proteins, or alternatively, the synthesized proteins might not be active (Prosser, 2015).

# 4. Size and scale influence applicable methodologies

Sizes of soil biota span at least eight orders of magnitude ranging from few hundred nanometres for viruses and bacteria, to several centimetres and decimetres long for insects, earthworms and megafauna, such as moles (Fig. 2). Even taxa that are defined as microorganisms can reach macroscopic dimensions as some fungal networks can span several hectares in size (Ferguson et al., 2003). These differentially sized organisms inhabit specific soil compartments (Fig. 2). While earthworms, ants and termites act as ecosystem engineers and virtually transform entire soils, smaller organisms are bound to the soil matrix and move (microarthropods) or grow through air-filled soil pores (fungi), or are active in water-filled spaces around soil particles (viruses, bacteria, protists, nematodes; Nielsen, (2019); Fig. 2). This has implications for the ecological connectedness of different soil organism groups and the methodologies used to assess them: e.g., many viruses and bacteria inhabit soil pores that are inaccessible to other soil biota (Vos et al., 2013). Microbial taxa within the observed community structure might never encounter each other, as they inhabit spatially separated soil pores or microsites (Fig. 2). Commonly used extraction techniques targeting microorganisms normally utilize <1 g of soil and cannot resolve the spatial distribution of isolated soil taxa (Vos et al., 2013). Sampling size is also relevant for soil animals: samples exceeding 100 g might be needed to reliably estimate the diversity of even the smallest animals, e.g., nematodes, in soils (Wiesel et al., 2015). However, alternatives exist to at least get a representative overview of the diversity of larger, more freely moving organisms. These can be done using analyses of small soil volumes after thorough combination of individually taken, replicated soil samples after homogenization of larger amounts of soils such as by freeze-drying (Oliverio et al., 2018). Extraction protocols also exist that allow DNA extraction from up to 500 g of soil by targeting extracellular DNA, which can be subsequently studied using qPCR or HTS-based approaches (Taberlet et al., 2012). Thorough homogenization such as by mixing after freeze thawing also seems to enable the molecular (and presumably several biochemical) analyses of both microbial and animal communities in soils (Oliverio et al., 2018). Taken together, soil sampling (sampling size, scale, pooling of individual samples, timing, etc.) and post-sampling processing has to be considered when investigating different groups of soil biota (Gundale et al., 2017).

# 5. Consensus on integrated methodologies

The profound taxonomic and morphological differences between groups of soil biodiversity, as highlighted in Sections 3 and 4, and limitations inherent to all available methods, prevent the application of a single unbiased method to simultaneously study of all soil biodiversity (McSorley and Walter, 1991). Yet, a thorough understanding of methods, including their limitations, will help in optimizing sampling efforts and data interpretation. Fundamentally, the experts involved are the ones who can best judge which method is appropriate to answer a given scientific question. We provide a summary (Table 2) as well as a guide of the most common and informative methods to obtain both quantitative and qualitative information on the taxonomic and functional profile of soil biodiversity (Table 3, Fig. 3).

We especially emphasize the power of integrative approaches, to combine different methods to target different soil organisms as well as to provide as complete information as possible on the abundance, community structure and function performed by different groups of soil biota, and soil biodiversity as a whole. For instance, the isolated and morphologically identified organisms can be supplemented with molecular identification (Fig. 3). Cultured organisms also offer the possibility for in-depth biochemical and molecular examination of fatty acid profiles (Ruess and Chamberlain, 2010), molecular gut content analyses (Wallinger et al., 2017), bulk and compound-specific stable (Potapov et al., 2019) and radioactive isotope analyses (Briones et al., 2010), all provide detailed information on species-specific feeding and position in soil food-webs (Fig. 3). Cultures can also be subjected to targeted experimental trait analyses for examination of other functions, such as trophic interactions and the role on plant performance (Geisen, 2016; Fierer, 2017; Gao et al., 2019; Fig. 3).

Similar to the combination of morphological with biochemical and molecular methods, the information obtained by biochemical analyses can be strengthened when complemented with molecular approaches. For instance, the use of isotopes is especially powerful when stable isotope probing (SIP) is combined with HTS sequencing (DNA- and RNA-SIP; Morriën et al., (2017); Section 3.3; Fig. 3).

# 6. Beyond taxonomic profiling of soil biodiversity

Most methodological approaches highlighted so far focus on analysing soil biodiversity present in a sample. However, to fully understand the enormous complexity of the soil biodiversity including their interactions, we need to go beyond a stamp collection-like approach by linking taxonomic with functional information. Biodiversity manipulations are particularly useful to help decipher the ecological functioning of soil biodiversity. For example, communities of soil biota can be assembled *de novo* and inoculated into sterile soils. These efforts have shown, for instance, that an increased diversity (Wagg et al., 2011) and trophic food-web interactions (Bonkowski, 2004) enhance plant diversity and performance, respectively. However, due to the vast diversity and large number of interactions within soil (Figs. 1 & 2), these approaches are only applied to study a small fraction of soil biodiversity.

An alternative approach is to reduce soil biodiversity through application of physical (e.g., gamma sterilization, heat, drought or freezing) or chemical stressors (e.g., chloroform fumigation or copper), or mechanically removing part of soil biodiversity (e.g., through sieving, dilution or inhibiting access by differentially sized mesh bags). Physical or chemical stressors remove less resistant taxa in a rather untargeted way; studies applying these stressors have revealed that biodiversity losses result in functional changes in soil communities (Griffiths et al., 2000; McNamara et al., 2003). Sieving and dilution techniques or constructing selective environments using mesh bags are applied primarily to remove rare taxa or reduce taxa of different sizes that often correspond with different trophic levels (as roughly illustrated in Figs. 1-3). These approaches have shown the importance of rare microbial taxa for plant performance (Hol et al., 2010), bacterial importance for ecosystem functioning (Bell et al., 2005; Philippot et al., 2013), the influence of differentially sized soil faunal groups on decomposition rates (Bradford et al., 2002) and the importance of biodiversity across groups for ecosystem multifunctionality (Wagg et al., 2014). We suggest that both simplistic and more complex biodiversity experiments are needed, and when integrated with a diverse set of state-of-the-art methods (Fig. 3) could provide a better and more mechanistic understanding of the vast biodiversity in soils.

Newly emerging analytical tools will help identify the groups and interactions of soil biodiversity that are functionally important in a given study. For instance, structural equation modelling can help in providing causal links of taxonomic and functional groups to determine the main functional drivers in a distinct study (Eisenhauer et al., 2015), such as by determining the importance of abiotic variables in structuring soil biodiversity (Delgado-Baquerizo et al., 2018a; Delgado-Baquerizo et al., 2018b). Network analyses can integrate nearly infinite data including taxonomic and functional data to explore co-occurrence and potential interactions, while network structure, such as network complexity, might inform about the state of a system. For instance, natural and stable systems have been associated with a more complex soil biodiversity network compared with agricultural and less stable systems (Morriën et al., 2017; de Araujo et al., 2018; Banerjee et al., 2019).

Moreover, novel experimental approaches or the combination of those experimental approaches that act at different scales will increase our understanding of soil systems in general. Mesocosm and microcosm experiments allow disentangling correlations observed in larger-scale field experiments from causality, as they allow highly-replicated, controlled manipulation of different abiotic and biotic factors. These often static systems are more and more being replaced by other systems such as the ‘rhizobox’ systems. Rhizoboxes allow repeated destructive or non-destructive sampling of soil biodiversity in planted systems, potentially allowing controlled study of community assembly and ecological succession in semi-natural environments (Wenzel et al., 2001; Au - Schmidt et al., 2018). These approaches will also allow the study of evolution at species level, changes in species and functional assemblages of soil biodiversity, especially when combined with mark-recapture methods of focal species of interest, such as pathogenic bacteria that can be re-isolated using selective media (Gomez and Buckling, 2011), or when methods are applied to study the dispersal of focal species such as by visual tagging of earthworms (Mathieu et al., 2018). Furthermore, isolation chip methods that allow culturing of bacteria *in situ* in soils using semipermeable devices might prove useful in studying interactions between species that cannot be easily cultured in lab conditions (Alessi et al., 2018). Yet, all these approaches are based on small-scaled systems that are more suitable to study microorganisms associated to plant growth and cannot replace classical mesocosm systems, such as in larger controlled model systems, to study most soil animals.

# 7. Challenges and steps forward

We now have the powerful tools and methods to broaden our understanding of the taxonomic and functional biodiversity in soils, and to provide a comprehensive understanding of connections among the many soil components.

We propose the following steps to accelerate the process of understanding soil biodiversity and its functional significance:

* Standardizing methodologies to assess biodiversity and its role in ecosystem functioning (Philippot et al., 2012), including sampling, laboratory work and analytical methods. Herein, we provide some guides that should help in studying different groups of soil biodiversity. However, we believe that a consensus of expert-driven standardization proposals is needed to convince the vast majority of researchers in a specific field to adopt standardized methods in order to make biodiversity analyses more consistent.
* Integrating quantitative techniques and qualitative approaches in large-scale environmental surveys, but also in simplified controlled experiments, to understand the mechanisms linking phylogeny, species-functions and community composition and to help in interpreting potential interactions with complex environmental data and the resulting effects on ecosystem services (Barnes et al., 2018).
* Adding taxonomic and functional information obtained experimentally to public databases (e.g. Edaphobase, DriloBASE Taxo, GBIF, BOLD). However, these data storage facilities should implement detailed data policies and provide DOIs to the data uploaded, making sure that the intellectual property of the data providers is safeguarded. Obtaining this data is tedious, and therefore, to encourage data sharing, data providers should benefit from these efforts, such as through appropriate citations of their work when data is used or by including them in joint collaborations.
* Bridging scientists working on individual groups of organisms with those studying the soil environment to get a better ecological understanding of soil biodiversity. This also refers to omics data that should be fully exploited rather than only focused on a few groups of organisms. Ongoing international initiatives, such as the Global Soil Biodiversity Initiative (GSBI; Box 1), can help bridge existing gaps. Integrated knowledge on soil biodiversity can only emerge from scientists with different expertise collaborating: interactions are crucial! This will necessitate ample funding initiatives that support basic and descriptive research (e.g., taxonomy, life-cycle studies).

Understanding soil biodiversity more holistically will be pivotal to address multiple ecological questions related to conservation and climate change mitigation (Creer et al., 2016), assessing ecosystem functions and services performed by soil biodiversity(Reiss et al., 2009) and enhancing ecological intensification in sustainable agriculture (Bender et al., 2016) that can promote human health (Wall et al., 2015). The importance of belowground organisms in influencing aboveground organisms (but also vice-versa) needs to be considered in future work (Bardgett and van der Putten, 2014). This growing knowledge on soil biodiversity also provides promising options for protecting and sustaining soils. More complete understanding of soil biodiversity enlarges the portfolio of management options for threatened soils. The application of integrative approaches and collaborative efforts will allow in-depth and holistic soil biodiversity analyses across taxonomic and functional groups. This will enhance our understanding of this cryptic and often overlooked ecosystem that is vital to support all terrestrial life.

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# Author Contributions

# SG initialized the study. MJIB and HG designed the figures. All authors contributed in gathering soil biodiversity data. SG wrote the first draft with substantial input and final approval from all authors.

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# Tables

Table 1: Overview of major morphological, taxonomic and functional characteristics of the dominant groups of biodiversity in soils (excluding deep subsurface soils due to lack of knowledge for most groups) including current knowledge on these organisms. Gt C: Gigaton carbon; Study #: number of studies using the term ‘soil’ and corresponding terms for soil biota groups in title, abstract or keywords. The search has been performed at the end of 2018. For additional details, see Supplementary Information.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Soil biota** | **Size of most taxa** | **Total global biomass (Gt C) in topsoil** | **Described species diversity in soils** | **Estimated species diversity in soils** | **Classical grouping** | **Taxonomic grouping** | **Main (additional) functional units** | **Soil functions;** services and disservices | **Study #** |
| Viruses | <0.2 m | 0.1 | Unknown | Unknown, likely >bacteria | NA | Viruses | Microbial, animal and plant controllers | Nutrient cycling; horizontal gene transfer; plant and animal pathogens | 4203 |
| Bacteria/ Archaea | 0.2-1.5 m | 7.5 | 15,000 | >1,000,000 | Microflora | Microorganisms Prokaryotes | Saprotrophs; photoautotrophs; (Animal and plant parasites/ mutualists) | Primary producers; plant growth promotion; nutrient cycling; contamination reduction; sources for biochemicals/pharmaceuticals; pest control; plant and animal pathogens | 55383 |
| Fungi | yeasts/spores: 3-50 μm; hyphae: >1cm | 12 | 73,000 | >1,000,000 | Microflora | Microorganisms Eukaryotes | Saprotrophs, plant mutualists; (animal and plant parasites) | Plant growth promotion; nutrient cycling; contamination reduction; sources for biochemicals/pharmaceuticals; pest control; plant and animal pathogens | 41366 |
| Protists | 5-50 m | 1.5 | 21,000 | >1,000,000 | Microfauna | Microorganisms Eukaryotes | Consumers of other microorganisms, photoautotrophs, Animal parasites; (omnivores) | Primary producers; nutrient cycling; plant and animal pathogens | 1896 |
| Nematodes | 0.1-5 mm | 0.01 | 25,000 | 1,000,000 | Microfauna | Metazoa Eukaryotes | Consumers of microorganisms, animal parasites, omnivores, predators (parasites) | Nutrient cycling; pest control; damaging plants; animal disease agents | 10308 |
| Collembola | 0.12 - 17 mm | 0.01 | 8,700 | 50,000 | Mesofauna | Metazoa Eukaryotes | Consumers of microorganisms, saprotrophs, predators | Nutrient cycling | 2626 |
| Acari | 60 m to 2-5 mm | 0.01 | 40,000 | 500,000 | Mesofauna | Metazoa Eukaryotes | Consumers of microorganisms, predators; saprotrophs | Nutrient cycling; pest control; damaging plants; disease vectors | 2634 |
| Enchytraeids | 1-30 mm | 0.05 | 700 | 7,000 | Mesofauna | Metazoa Eukaryotes | Saprotrophs | Nutrient cycling;  Ecosystem engineers | 682 |
| Earthworms | 3-15 cm | 0.15 | 7,000 | 30,000 | Macrofauna | Metazoa Eukaryotes | Saprotrophs | Nutrient cycling; ecosystem engineers | 7028 |
| Macro Arthropods | >1 mm | 0.05 | several 100,000 | up to 5,000,000 | Macrofauna | Metazoa Eukaryotes | Saprotrophs, predators, plant feeders | Nutrient cycling; human food; ecosystem engineers; pest control; damaging plants | 67855 |

Table 2: Overview of commonly used methods to study the diversity, abundance, biomass and functioning of soil organisms.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Method** | **Information provided** | **Main groups targeted** | **Advantages** | **Disadvantages** | **References** |
| **Morphological** | Direct microscopy (light, epifluorescent or electron microscopy) | Abundance | Bacteria, Fungi, Viruses | Materials cheap | Difficult to visualize organisms in soil samples, highly expert dependent | (Bloem, 1995; Li et al., 2004) |
| Flow cytometry | Abundance | Bacteria | Potentially high sample throughput | Requires cell detachment and purification | (Frossard et al., 2016) |
| Cultivation | (Abundance, diversity) | Bacteria, Fungi, Protist, Viruses | Materials cheap; organisms in culture for additional analyses or experiments | Dependant on cultivability; limited taxonomic resolution without including molecular approaches; favours fast growing taxa | (Geisen and Bonkowski, 2018) |
| Centrifugation followed by morphological identification | Abundance, diversity | Nematodes | Materials cheap; Well-established; Organisms available for additional analyses or experiments | Identification time consuming; expert dependent | (Hooper et al., 2005) |
| Wet extraction followed by morphological identification  (e.g. Oostenbrink or Berlese) | Abundance, diversity & biomass | Enchytraeids, Nematodes,  Rotifers,  Tardigrades | Low cost; targets only active taxa; Organisms available for additional analyses or experiments | Identification time consuming; expert dependent. | (Schmelz and Collado, 2010) |
| Heat extraction followed by morphological identification  (e.g. Tullgren) | Abundance, diversity & biomass | Mites, Collembola, mesofauna | Low cost; targets only active taxa; Organisms available for additional analyses | Identification time consuming; expert dependent. | (Tullgren, 1918) |
| Hand sorting, heat extraction, chemical expulsion, pitfall trapping followed by morphological identification | Abundance, diversity & biomass | Macrofauna including arachnids and Earthworms | Low cost; all methods except for hand sorting target only active taxa; Organisms available for additional analyses or experiments | Identification time-consuming; expert dependent | (Smith et al., 2008; Bartlett et al., 2010) |
| **Biochemical** | Substrate-induced respiration | Biomass | Microorganisms | Cheap; provides information on active microorganisms | Cannot distinguish between microbial groups | (Joergensen, 1996; Bailey et al., 2002) |
| Fumigation-extraction | Biomass | Microorganisms | Cheap and fast; information on the extractable part of microbial biomass C | Cannot distinguish between microbial groups | (Bailey et al., 2002; Powlson et al., 2017) |
| PLFA | Biomass, (Diversity) | Bacteria, Fungi | Enables biomass estimation of both bacteria and fungi, including bacterial subgroups (Gram +/-, Actinobacteria) | Little diversity information; time consuming; costly; dependent on hazardous chemicals | (Frostegård and Bååth, 1996; Bailey et al., 2002; Powlson et al., 2017) |
| NLFA | Biomass | Arbuscular mycorrhizal fungi | Most reliable method to estimate biomass of Glomeromycota | Glomeromycotan diversity or biomass of other soil groups not obtained; time consuming; costly; dependent on hazardous chemicals | (Olsson, 1999) |
| Ergosterol | Biomass | Ascomycete and basidiomycete fungi | Most reliable method to estimate fungal biomass | Not all fungal groups targeted such as Glomeromycota | (Frostegård and Bååth, 1996) |
| Metaproteomics | Functioning | Microbial-derived proteins | High-resolution functional information based on translated proteins | Databases incomplete, most proteins poorly annotated; work intensive and costly | (Schneider et al., 2012) |
| Metabolomics | Functioning | Microbial-derived proteins | High-resolution functional information based on metabolites | Databases incomplete, thus most metabolites poorly annotated; work intensive and costly | (Swenson et al., 2015) |
| Isotopic analysis (bulk and compound-specific) | Functioning | Various target groups | Depending on the methodology, can provide crude or more detailed information on trophic structure | Needs to be combined with other approaches; target groups should be analysed separately | (Potapov et al., 2019) |
| **Molecular** | qPCR | Abundance/ Biomass/ Functioning | All or specific groups of bacteria, fungi, nematodes; functional genes | Easy, cost-efficient; high-throughput; can target nearly any taxa or functional gene | For each taxon/function targeted, another primer pair is needed | (Fierer et al., 2005) |
| Metabarcoding/ Amplicon Sequencing | Diversity | All soil biodiversity /functional genes | Ultra-high throughput; most cost-efficient high-resolution diversity method | Amplification efficiency primer-dependent, distorting observed community structure | (Knight et al., 2018b) |
| Metagenomics | Diversity, Functioning | All organisms and functional genes simultaneously | High-resolution; potentially applicable to study all organisms and genes simultaneously | Costly: diversity large; most obtained information uninformative and unassignable | (Delmont et al., 2011) |
| Metatranscriptomics | Diversity, Functioning | All organisms and expressed functional genes simultaneously | High-resolution; applicable to all organisms and transcribed genes simultaneously | Costly: diversity large, RNA processing expensive; limited database coverage for functional genes; RNA does not only target active taxa | (Urich et al., 2008; Blagodatskaya and Kuzyakov, 2013) |

Table 3: Recommended method to capture the diversity and abundance of distinct groups of the soil biodiversity. For further details on advantages and disadvantages of respective methods, as well as references, see Table 2.

|  |  |  |  |
| --- | --- | --- | --- |
| **Soil biota** | **Information provided** | Recommended method | Best alternative method |
| Viruses | Abundance | Cultivation based techniques using specific bacterial hosts | Direct counting using microscopy |
| Diversity | Metagenomics | Comparison of morphological characteristics using microscopy |
| Bacteria/ Archaea | Biomass | PLFA | substrate-induced respiration, fumigation-extraction |
| Diversity | Amplicon sequencing of 16S rRNA gene | None |
| Fungi | Biomass | Ergosterol | PLFA |
| Diversity | Amplicon sequencing of ITS region | Amplicon sequencing of 18S or 28S rRNA gene or when phylogenetic analyses are envisioned |
| Protists | Abundance | Cultivation based techniques | None |
| Diversity | Amplicon sequencing of 18S rRNA gene | None |
| Nematodes | Abundance & Diversity | Extraction of active taxa followed by morphological quantification and amplicon sequencing for high-resolution taxonomic identification | Extraction followed by morphological quantification and taxonomic identification; much more time consuming, expert dependent and lower in resolution- but more standardized |
| Collembola | Abundance & Diversity | Heat-extraction followed by morphological quantification and morphological and molecular identification | Extraction followed by molecular identification of animals (yet, often insufficient database coverage); quantification of all animals by counting |
| Acari | Abundance & Diversity | Heat-extraction followed by morphological quantification and morphological and molecular identification | Wet extraction/flotation followed by morphological quantification and taxonomic identification |
| Enchytraeids | Abundance & Diversity | Extraction followed by morphological quantification and morphological and molecular identification | Extraction followed by molecular identification of animals; quantification of all animals by counting |
| Earthworms | Abundance & Diversity | Extraction/excavation followed by morphological quantification and morphological and molecular identification | Extraction followed by molecular identification of animals; quantification of all animals by counting |
| Macro Arthropods | Abundance & Diversity | Heat extraction followed by quantification of all animals by counting and molecular identification | Hand-sorting or pitfall trapping followed by quantification of all animals by counting and morphological identification |

# Figure legends

Fig. 1. The soil food web based on functional groups of soil organisms.

Conceptual scheme of a soil food web based on (trophic) functional rather than taxonomic groupings including previously neglected groups such as viruses and pathogens. This scheme illustrates that food web reconstructions that place organisms in single functional units solely according to their taxonomic identity and that use unidirectional nutrient flows are likely over-simplistic’

This simplified scheme focusing only on the dominant functional roles of different groups of soil organisms illustrates that organisms placed in different functional units and unidirectional nutrient flows (brown arrows) are likely to be over-simplistic. Figure legend as in Fig. 3.

Fig. 2. Soil biodiversity across different spatial scales in the soil matrix.

The diversity of interacting soil organisms is embedded within the soil matrix at different spatial scales. A) megafauna and macrofauna can be seen by eye when on the soil surface or after excavation (Fig. 3); B) mesofauna are hardly visible by eye and need to be extracted from soils before examination; C) microfauna and microorganisms, including D) the smallest bacteria and viruses need to be studied using specialized techniques. Circles indicate areas that are magnified in the following graph panels.

Fig. 3. Methodology to study soil biodiversity.

Various techniques are available to study each group of soil biota including morphological, biochemical and molecular methods. Sampling sizes differ between groups of organisms (brown filled squares) that need to be considered (Fig. 2). Bottom part in grey shows subsequent quantitative and qualitative methods to study taxonomic and functional biodiversity in soils. Circle size indicates the size of the targeted organisms. Extraction approaches are split into those that depend on dynamic (D) movement of organisms and passive (P) methods that capture both active and inactive organisms. Extraction SIA: Stable isotope analyses; FA: Fatty acid analyses; SIP: Stable isotope probing; SIR: substrate induced respiration, chloroform-fumigation).

# Box 1. Soil biodiversity initiatives

There has been a rapidly growing interest in soils and soil biodiversity over the last decade. Several local and global scale projects and initiatives have launched, which together have spread the awareness of the importance of soil and its biodiversity for human well-being. Here we list some of these examples:

* The World Soil day, celebrated on December 5th, was implemented to raise awareness of soil as a resource in 2002.
* In line with the World Soil day, the [Global Soil Week](https://globalsoilweek.org/) aims to link scientists with policy makers and end-users to discuss and implement options to manage land more sustainably.
* The [Global Soil Biodiversity Initiative](https://www.globalsoilbiodiversity.org/) launched in 2011 links scientists to the public at a global scale.
* The FAO announced 2015 as the [International Year of Soils](http://www.fao.org/soils-2015/en/) to highlight soils as a limiting and important resource for humanity.
* The [UN Global Forum on Food Security and Nutrition](http://www.fao.org/fsnforum/activities/discussions/biodiversity-mainstreaming) took place in 2018 to illustrate the importance of (soil) biodiversity to secure and optimize human nutrition
* A new emphasis is taking place on soil biodiversity assessments such as illustrated in global-biodiversity inventories (Tedersoo et al., 2014; Thompson et al., 2017; Bahram et al., 2018; Delgado-Baquerizo et al., 2018c) and large-scale projects ([LUCAS – EU project](https://esdac.jrc.ec.europa.eu/projects/lucas) and the [UN Convention of Biological Diversity](https://www.cbd.int/gbo4/)).

# Box 2. Benefits of expanding soil biodiversity studies beyond bacteria

Research methodology on bacteria is relatively standardized, with a limited set of techniques that allow a user-friendly and cumulative analysis of the entire taxonomic and functional diversity (Knight et al., 2018a). Yet, even those techniques are not entirely standardized, rendering fine-tuning and method-adjustments pivotal for correct ecological data interpretation: a step which is rarely taken by most researchers, who often bluntly use default setting taken from published studies. Biases inherent to all methods often are ignored, despite having the potential to artificially alter ecological results. Therefore, despite bacteria-centric studies far outnumbering studies on all other organism groups in soils (Geisen et al., 2017), this should not be equated to general knowledge of soil biodiversity. Expertise, especially on soil viruses, protists and animals is limited to few specialists, but their data interpretation is possibly more accurate and ecologically meaningful. Furthermore, methods to study soil animals have been developed and thoroughly tested for decades, simultaneously providing qualitative and quantitative data. The century-long history of functional experiments on many soil fauna groups provides unique insights into their functional composition and their community structure (Yeates et al., 1993a; Eissfeller et al., 2013; Geisen et al., 2018a), whereas the function of most bacterial taxa remains unassignable. Expanding traditional approaches to study soil animals with modern sequencing tools and other approaches, such as automated image analyses, opens previously unimaginable potentials (Geisen et al., 2018c). Thus integrated system-based approaches that simultaneously study diverse taxonomic groups of soil biota and their functioning might be the next frontier in soil biodiversity analyses (Zinger et al., 2016).

# SUPPORTING INFORMATION

Additional Supporting Information is provided online.