Identification of new microbial functional standards for soil quality assessment

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Abstract. The activity of microorganisms in soil is important for a robust functioning of soil and related ecosystem services. Hence, there is a necessity to identify the composition, diversity and function of the soil microbiome in order to determine its natural properties, functioning and operating range as well as to assess ecotoxicological effects due to anthropogenic activities. Numerous microbiological methods currently exist in the literature and new, more advanced methods continue to be developed; however, only a limited number of these methods are standardized. Consequently, there is a need to identify the most promising non-standardized methods for assessing soil quality and to transform them into standards. In agreement with the "Ecosystem Service Approach", new methods should focus more on soil microbial functions, including nutrient cycling and greenhouse gas emission, pest control and plant growth promotion, carbon cycling and sequestration, as well as soil structure development and filter function. The few existing standardized methods available that focus on the function of the soil microbiome mostly include measurements, like basal respiration, enzyme activities, and biodegradation of organic matter, under well-defined conditions in the lab. This paper sets out to summarize and expand on recent discussions within the International Organization for Standardization (ISO), Soil Quality - Biological Characterization sub-committee (ISO TC 190/SC 4) where a need was identified to develop scientifically sound methods, which would best fulfil the practical needs of future users for assessing soil quality, going beyond the existing test systems. Of particular note is the current evolution of molecular methods in microbial ecology that use quantitative real time PCR (qPCR) to produce a large number of new functional endpoints which are more sensitive as compared to 'classical' methods. Quantitative PCR assesses the abundance of microbes that catalyse major transformation steps in nitrogen and phosphorus cycling, greenhouse gas emissions, chemical transformations including pesticide degradation, and plant growth promotion pathways based on the assessment of marker gene sequences that drive the related processes. In the assessment of soil quality methods, it was found that most methods focus on bacteria and related endpoints. Techniques to describe fungal communities as well as their functional traits are far less represented. As such, techniques to analyse fungal enzyme activities are proposed. Additionally, methods for the determination of microbial growth rates and efficiencies, including the use of glomalin as a biochemical marker for soil aggregation, are discussed. Furthermore, field methods indicative of carbon turnover, including the litter bag test and a modification to the tea bag test, are presented. However, it is obvious, that with increasing developments in high throughput sequencing technologies and big data analyses, including metagenomics analysis, it will be possible to implement these technologies into the standardisation process for assessing the functions of the soil microbiome. Overall, it is suggested that endpoints should represent a potential function of soil

52 microorganisms rather than actual activity levels, as the latter can largely be dependent on short-term variable soil
53 properties such as pedoclimatic conditions, nutrient availability, and anthropogenic soil cultivation activities.

1 Introduction

Soils are one of the world's hotspots for biodiversity (Parker, 2010). Biota – both micro- and macro-organisms – in soil form strong networks and complex food webs, which determine the efficacy of the soil ecosystem functions (e.g. nutrient cycling, C storage and turnover, water retention, and modulation of soil structure) (Creamer et al., 2016). These functions support a range of ecosystem services that are indispensable for soil use in agri-, horti- or silviculture (Nannipieri et al., 2017). At the same time, soil biota are strongly impacted by various anthropogenic activities including ongoing global and climate change, pollution, as well as degradation and destruction of the terrestrial environment (Gomiero, 2016; Montgomery, 2008; Wagg et al., 2014). Consequently, investigations of the soil biome structure and function became an emerging topic in soil and environmental sciences (Griffiths and Philippot, 2013). As such, the number of studies describing the ecology of soils and ecosystem functioning has increased significantly over the past few decades and has resulted in the development of new methods for the assessment of microbial communities (e.g. Guillaume et al., 2016; Tian et al., 2018). In comparison, the ecotoxicological assessment of human impacts (e.g. chemical pollution and mechanical compaction) using single species tests, which are well-established methods, has remained constant (Brookes, 1995; Joergensen and Emmerling, 2006).

Characterizing the natural state of a soil's biome is a quite challenging task. In addition to its huge structural and functional diversity, soil biomes are influenced by strong temporal dynamics including seasonal weather conditions and the enormous spatial heterogeneity, which ranges from field scale to microscale (Kuffner et al., 2012; Regan et al., 2014; Suriyavirun et al., 2019). All of these intrinsic properties hinder the interpretation of data obtained from the analysis of soil biomes and the measurement of their functional traits.

Despite the fundamental methodological advances over the past years, which allow for an in-depth analysis of microbiomes and, to some extent, other soil-living organisms (e.g. Joergensen and Emmerling, 2006; Paul, 2015; Yates et al., 2016), only a limited number of soil biological methods have been standardized (for details see section 3). As a result, large and often significant deviations are observed in the results obtained when non-standardized methods are used (e.g. Strickland and Rousk, 2010). This is especially true for methods that are based on high throughput sequencing approaches, where variability and bias in data can occur from the "wet-lab" steps right through the various bioinformatics pipeline analysis steps (Quince et al., 2017). Therefore, the comparability between datasets generated by different laboratories using different methods or modified protocols of the same method is problematic. Consequently, the development of quality indices and threshold values, respectively, for

assessing soil quality is nearly impossible (Bastida et al., 2008). Presumably, this is why the number of meta-analyses in soil biology remains small.

Given that there is a lack of harmonization between existing methods and, at the same time, a proliferation of new methods, there is a need to identify the most promising methods described in the literature that can be standardized to produce reliable indicators for soil quality (e.g. Philippot et al., 2012). At the Annual International Organization for Standardization (ISO) meeting of TC 190 (Soil Quality) in Fukuoka, Japan in October 2013, a decision was made to compile a list of available methods and to identify those that would be suitable for assessing soil quality. Additionally, during a subsequent meeting of ISO TC 190/SC 4/WG 4 (Microbiological Methods) held in Paris, France in March 2014, further discussions focused the criteria for suitable methods to be comprised of microbial functional indicators. In this paper, we summarize the major outcomes of the discussions which took place over the past several years within ISO TC 190/SC 4. Therefore, besides collating a list of criteria for the selection of test methods for the future analysis of microbial functions in soil, the aim of this paper is to present our opinion, as members of the ISO TC 190 committee, to initiate further discussion on possible methods that should be standardized for future soil quality assessments.

2 Criteria for the selection of methods

Several papers addressing the task to identify suitable methods to be used as biotic indicators (usually including faunal indicators) were published in the last few years, mainly in the context of EU research projects (e.g. Bispo et al., 2009; Faber et al., 2013; Ritz et al., 2009; Römbke et al., 2010). Here, we propose to base the selection of soil quality methods more on the "Ecosystem Service Approach" (MEA 2005) which is increasingly recognized by both environmental scientists and regulatory agencies (Breure et al., 2012; Galic et al., 2012) and which takes into account that soils have been raised to the rank of a natural resource to be protected. As a consequence, and in addition to method development and application (including the assessment of biodiversity as a prerequisite for soil function), the focus of future activities should be the determination of soil microbial function as recommended endpoints (Kvas et al., 2017; Nienstedt et al., 2012; van der Putten et al., 2010; TEEB, 2010). Consequently, we propose to assess both existing and new methods for the selection of microbial functional tests that support various soil ecosystem services. This structures our approach and simplifies the identification of ecologically relevant methods, as well as, presumably increases their acceptance by users, including the regulatory and stakeholder community. The following **soil functions** and **ecosystem services** have been defined and are proposed to be used as a starting point for the development of future methods (MEA, 2005; Ockleford et al., 2017):

113 (1) Biodiversity, genetic resources, cultural services;
114 (2) Food web support;
115 (3) Biodegradation of pollutants;
116 (4) Nutrient cycling (for example N and P);
117 (5) Pest control and plant growth promotion;
118 (6) Carbon cycling and sequestration;
119 (7) Greenhouse gas emissions; and
120 (8) Soil structure affecting soil water, gas balance and filtration function.
121 A second major criterion for selecting methods for standardization is its usability. The method should be applicable
122 in regulations (e.g. European and National agencies registering chemicals or products) and for the evaluation of
123 soil ecology and functioning as fundamental aspects of soil quality (e.g. by stakeholders and researchers).
124 Moreover, the routine use of methods to inform farmers and site owners on soil quality as continuous assessments
125 of their land and land-use practises could be an additional condition that would require the choice of easy-to-use
126 methods or possibly encourage the simplification of existing methods. Overall frequently used methods generate
127 more data, which in turn is of high importance for the validation of threshold values. Therefore, the aim of this
128 process is to identify methods that are scientifically sound and best fulfil the practical needs of future users. The
129 most appropriate new methods, including those proposed in this article, need to be evaluated using the criteria
130 required for the standardization of ISO methods.
131 To assess possible methods, a list of criteria was used based on the 'logical sieve' approach (Ritz et al.,
132 2009). The list of criteria for the identification of functional indicators and associated methodologies (Table 1)
133 was an outcome of the EU FP7 EcoFINDERS project (Faber et al., 2013). The criteria were compiled after sending
134 a questionnaire to 25 partner institutions primarily working in the field of environmental science; mainly
135 representing academia but also regulators and subcontracting laboratories. These criteria are applicable for
136 different kinds of indicators and methods, including those addressing the functions of soil microbial communities.
137 In the following sections, we assume that existing ISO standardized methods partly already fulfil these criteria,
138 but not all relevant endpoints can be measured.
139
140 **3 Existing and new methods**
141 Current methods that have already been implemented as ISO standards are found in Table 2, whereas methods that
142 might be considered for future standardization are in Table 3. The compilation in Table 2 comprises methods to

quantify microbial biomass (e.g. through fumigation extraction of microbial biomass carbon (MBC) and DNA) (**Function 6**) as well as for (further) analysis of structural microbial diversity (e.g. determination of microbial fingerprints by phospholipid fatty acids (PLFA) analysis) (**Function 1**). Additionally, microbial biomass, measured as respiratory activity, has been included in Table 2, although not directly linked to one of the ecosystem services, as it provides important information on the activity of the complete microbiome (i.e. microflora and microfauna). Soil basal respiration normalized to MBC (ISO 14240-1 and ISO 14240-2, 1997, Table 2) yields the metabolic quotient qCO_2, which is a sensitive indicator for microbial carbon use efficiency (Anderson and Domsch, 1993). However, its use as an endpoint to assess anthropogenic and natural impacts on the soil microbiome has been controversially discussed in literature (Wardle and Ghani, 1995). The microbial quotient (MBC related to organic carbon content of a soil) is an indicator revealing changes in the microbial dynamic equilibrium of soils in response to exposure to natural or anthropogenic stressors (Pankhurst et al., 2001).

The biodiversity function (**Function 1**) addresses parameters related to the structural diversity of the soil microbiome. Here, respective ISO guidelines analysing PLFA, phospholipid ether lipids (PLEL) (ISO/TS 29843-1, 2010; ISO/TS 29843-2, 2011) and DNA (ISO 11063, 2012; ISO 17601, 2016), have already been well implemented into guidelines (Table 2). In addition, high throughput sequencing of barcodes of the ribosomal operon (16S rRNA gene for bacteria and archaea and ITS [internal transcribed spacer] region for fungi) have generated a large amount of data (Schöler et al., 2017). These approaches have been also used successfully for other microbial groups like protists (using the 18S rRNA gene as a target). As it is well accepted that the use of different primer pairs introduce different biases (Ramirez et al., 2018), standards have been recommended by international initiatives. For example, the Earth Microbiome project (www.earthmicrobiome.org) recommended a primer pair targeting the V4 region of the 16S rRNA gene and ITS2 region for bacterial and fungal barcoding, respectively. Bioinformatics pipelines used for barcoding approaches are already well standardized and shared worldwide among the scientists, which makes possible the cross comparison of various datasets from different labs. Unfortunately, this is not yet the case for the pipelines to analyse metagenomics datasets that are still under constant evolution making difficult cross-comparisons.

Although microbial diversity, per se, is not strongly correlated with a particular functional capacity, it is clear that the loss of diversity can have an impact on microbial function (Thiele-Bruhn et al., 2012); at least for relatively specific functions performed by narrow microbial guilds or taxa. This applies even more, when certain taxa are closely linked to very specific functions including nitrifiers, methanogens, arbuscular- and ecto-mycorrhizal fungi, and biocontrol microorganisms like *Trichoderma* (e.g. Hartmann et al., 2009; Hayat et al.,

2010; Lugtenberg and Kamilova, 2009; Peng et al., 2008; Singh et al., 2007; Xia et al., 2011). Therefore, the interpretation of the outcomes from microbial community-based testing tends to be straightforward and closely linked to **Function 4** and **Function 5**.

Food web support (**Function 2**) of higher trophic levels no doubt starts from soil microorganisms and propagates through the trophic levels (e.g. earthworms) that are consumed by birds and mammals (Haynes, 2014; Scheu et al., 2002; Scheu et al., 2005). However, the role of the microbiota in the soil food web is not fully understood, since many eukaryotic organisms can be considered as meta-organisms, which carry their "own microbiome" that itself is essential for life supporting functions. From this, it is unclear if environmental microbiomes and host specific microbiomes complement one another. So far there are no comprehensive methods (especially not those addressing microbial functions) or standards available to address this problem. The use of stable isotope labelling of select carbon or nutrient sources as a promising approach to follow food webs and degradation pathways might provide one possibility in the future to assess food webs in soil. This would be accomplished by combining carbon and nitrogen stable isotope fluxes determination with phylogenetic analysis of the microorganisms labelled with the stable isotopes (e.g. Coban et al., 2015; Traugott et al., 2013; Lueders et al., 2004).

Methods to assess the biodegradation of pollutants (**Function 3**), as described above, are already implemented into ISO guidelines (Table 2) and are part of legal frameworks including pesticide directives (EU Regulation1107/2009/EC; European Commission, 2009). A number of standard methods for the determination of the potential of soils to degrade organic chemicals (**Function 3**) under both aerobic (ISO 14239, 2017) and anaerobic (ISO 15473, 2002) conditions are available. However, in the past, the development of standard methods was mainly driven by the need to assess the ecotoxicological effects of anthropogenic activities, such as chemical contamination of soils, rather than to describe and understand the natural properties and functions of soils. Defining methods for the determination of adverse effects of contaminants on soil biota was not only done by ISO, but it was also a major task of other organizations such as the Organization for Economic Co-Operation and Development OECD). For example, there are OECD guidelines, tests No. 216 and 217, for testing the long-term effects of single exposure chemicals on soil microbial nitrogen and carbon transformation, respectively (OECD, 2000a; 2000b). As a result, it was decided early that the standardization of methods for toxicity testing should not be the primary aim of the ISO sub-committee (ISO TC 190/SC 4). Metagenomics lead to the rapid discovery of new genes, which catalyse degradation processes of xenobiotics and, consequently, offer new insight in the study of ecology of microbial degraders. The transfer of this knowledge into operational bioindicators for the estimation

of soil filtration capabilities (e.g. by using qPCR or RT-qPCR assays to quantify those genes and their expression in soils, respectively) will be one of the major tasks in soil science in the upcoming years. Jeffries et al. (2018) for example were able to successfully predict the degradation rates of organophosphorus based pesticides in a contaminated soil by using metagenomics based functional profiling.

Some of the existing standard methods that are listed in Table 2 focus on the estimation of enzyme activities useful for soil quality assessment, which mainly contribute to **Function 4**. Here, the potential dehydrogenase activity measurement is an indicator for general (potential) oxidoreductase activity in soil. Since this measurement has been frequently used, there are large amounts of baseline data available on the toxic effects of a range of pollutants in soil. Recently, additional potential enzyme activities related to the C, N, P and S cycle have been used and are either standardized or are in the process for standardization.

The current evolution of molecular methods in microbial ecology has resulted in a large number of new endpoints. It is well known that many of the new endpoints (e.g. using quantitative real-time PCR (qPCR)) are more sensitive than classical methods that had been standardized in the past (Ribbons et al., 2016; Schulz et al., 2016). This new metagenomics approach will be of high importance in the future, as it allows for the implementation of information on new functional traits that can be standardized into an analytical pipeline. Direct sequencing of soil DNA extracts, called metagenomics, is nowadays a method that can easily be implemented to obtain an overview of in-soil living organisms, including microorganisms. It is recognized that metagenomics is no longer limited by sequencing possibilities but more by the availabilities of large-scale computing clusters to analyse the amount of data generated. The future success of metagenomics in soil surveys will mainly depend on the possibilities to standardize bioinformatics pipelines as well as on the availability of tools for big data analysis and artificial intelligence. However, it must be mentioned that even a well-standardized and automated workflow will generate only data on the relative abundance of nucleotide sequences and not absolute values (Geisen et al., 2019). Thus at the moment for the assessment of new methods linked to **Functions 4 to 8**, qPCR from soil DNA extracts (ISO 17601, 2016) plays a very important role in determining the abundance of single marker gene sequences, which are indicative of specific transformation processes or soil functions. For example, the quantification of nitrogen fixing microbes, nitrifiers and denitrifiers has been successfully implemented using the *nifH*, *amoA* and *nirS/nirK* genes as markers, respectively (Henry et al., 2004; Hirsch et al., 2010; Ollivier et al., 2010; Sessitsch et al., 2006). Similarly, the quantification of microorganisms involved in the β-ketoadipate pathway has been implemented by targeting *pcaH* (El Azhari et al., 2008) and *catA* (El Azhari et al., 2010) gene

sequences. Various methods for the assessment of soil microbial **Function 4** (nutrient cycling), **Function 5** (pest control and plant growth promotion) and **Function 7** (greenhouse gas emissions) are proposed based on the qPCR analysis of gene sequences coding for enzymes which trigger the respective function (e.g. Fish et al., 2013; Ribbons et al., 2016; Smith and Osborn, 2009). Additionally, it should be noted that molecular methods based on the assessment of specific marker genes for estimating the degradation potential in soil have already been proposed both for PAHs (e.g. Cebron et al., 2008) and individual pesticides (e.g. Martin-Laurent et al., 2004). These could be interesting for future standardization; however, if a method is very compound-specific and targeted, this could limit its application range. Thus, these specific approaches will not be discussed further in this article.

Major advantages of qPCR assays to quantify gene sequence numbers, which can be used as proxies for a given microbial process, are that they are: (i) highly standardized, sensitive, selective and reproducible, (ii) designed for high throughput analysis, (iii) available for a wide range of targets, and (iv) methods that are relatively cheap once the necessary analytical devices are on hand. Some training on the method is required, however, once trained the assays are easy to perform. For example, numerous studies have already used the microbial functional genes involved in nitrogen cycling to determine the status and to assess induced changes in the soil microbial community (Levy-Booth et al., 2014; Nannipieri and Eldor, 2009; Wallenstein et al., 2006). Consequently, the number of functional genes that are suited for use as specific indicators of soil function are continuing to grow in the literature as researcher gain experience in this field and data becomes more prevalent. Also, evidence is increasing that functional gene abundance and community structure are closely linked to related microbial activities and their increase or decrease, e.g. through agricultural fertilizer regime or soil contamination (Levy-Booth et al., 2014; Ouyang et al., 2018; Xue et al., 2018). However, also contrasting findings have been reported, pointing to the fact that functional gene abundance and diversity is less affected by short-term changes, e.g. due to soil moisture changes (Zhang et al., 2019). A critical meta-analysis of existing data and reports, respectively, would be timely to better identify and generalize the linkage of functional gene abundance and ecosystem services.

Disadvantages, on the other hand, are that: (i) the quality of qPCR data depends on soil DNA extracts (PCR inhibition), (ii) primer pairs, even degenerated ones, might not successfully amplify all microbes of the functional group of interest, (iii) only genetic potential is resolved, and (iv) there is no differentiation between active, dormant or dead microorganisms, when working with DNA as a template for the qPCR reaction. The analysis of total RNA and of mRNA, which could help to overcome the latter problem, is currently not a suitable alternative as it is highly dynamic in time and space and needs special care to stabilize the RNA extracted from

complex environmental matrices to avoid its degradation. Another problem of DNA analysis is the biological representativeness of the results is solely based on a relatively small amount of soil (from few hundred mg to ten g of soil) from which the DNA extracted. The use of small soil samples (< 1 g) simplifies the sample preparation process for molecular biologists; however, it provides a poor representation of the indigenous soil microbial community in the naturally inhomogeneous soil. Typically, the α-biodiversity declines with sample size while that of ß-biodiversity increases (Nicol et al., 2003; Penton et al., 2016). Lastly, it must be noted that the high repeatability and reproducibility of molecular biology methods, including qPCR assays, depends on extraction, purification and amplification of DNA or RNA. This is typically performed using commercial extraction kits; however, by simply changing the commercial supplier of a kit can substantially change the results (Brooks et al., 2015; Feinstein et al., 2009). This clearly challenges standardization since standard methods must not hinge on a specific supplier.

Recently, molecular tools for the assessment of the microbial phosphorous turnover (**Function 4)** have been published (Bergkemper et al., 2016) where metagenomics data have been used for the construction of primers for P mineralization, transport and uptake. As another example, the relevance of anaerobic ammonium oxidation (anammox) for N cycling in soils has increased (Levy-Booth et al., 2014) along with the development of analytical methods for high throughput analysis. Among the microorganisms in soil that substantially govern pest control and plant growth promotion (**Function 5**), arbuscular mycorrhizae and ectomycorrhizal fungi are of high relevance. These microorganisms are especially abundant in the rhizosphere (Hartmann et al., 2009; Hayat et al., 2010; Lugtenberg and Kamilova, 2009). Methods related to **Function 5** are listed in Table 3.

Several options exist for (additional) standardized methods to test **Function 6** (carbon cycling and sequestration) (Table 3). For **Function 6**, there is a need to implement more fungal activity analysis as most tests described mostly assess bacterial activities. Thus, the integration of more fungal enzyme activities into the suite of standardized methods for soil quality assessment is essential (for example determining the turnover of complex natural compounds such as lignin) (Baldrian, 2006). The ligninolytic enzymes laccase and Mn-peroxidase, as well as the chitin degrading 1,4-α-*N*-acetylglucosaminidase, are typical fungal enzymes of interest for ecosystem services (Jiang et al., 2014; Šnajdr et al., 2008). However, since other organisms also produce these enzymes, including bacteria and plants (Bollag, 1992; de Gonzalo et al., 2016), current methods do not specifically target fungal enzyme activities. As a result, the implementation of molecular methods for assessing fungal communities are far less developed than those for bacterial communities (Table 3). First approaches to close this gap have been made in recent years. For example, molecular systems to detect genes coding for laccases and other ligninolytic

enzymes as well as other fungal activities in carbon cycling have been published and applied (Edwards et al., 2011; Chen et al., 2013; Hannula and van Veen, 2016). Also for genes involved in the fungal nitrogen turnover, primer pairs have been successfully developed (Gorfer et al., 2011). However here, even more than for bacteria, it is critical to link copy numbers of genes directly to the size of the respective functional population, as in many cases one organism can harbour multiple operons coding for the same genes involved in a given function.

The method of community level physiological profiling (CLPP) using the Biolog™ system (Biolog, Hayward CA, USA) was first developed in the late 1980s to identify bacteria of clinical importance by assessing the consumption of 95 different carbon sources in a microtiter plate. The technique was then extended to identify bacterial strains from environmental mixed microbial communities samples using select carbon sources (Garland, 1997). Currently, the technique is frequently used to assess the effects of contaminants on soil microbial activity (Bloem and Breure, 2003; Schmitt et al., 2004). As such, the CLPP method has become a measure of microbial functional diversity in soil (e.g. Gomez et al., 2006) and was used to distinguish the biodiversity of soil microbial communities in monitoring programs (Rutgers et al., 2016). Even though the method is easy to use, it does have some drawbacks (Winding and Hendriksen, 2007). The technique is based on the utilisation of select carbon sources, which when consumed result in reduction, and thus colour change, of a tetrazolium indicator dye (Garland and Mills, 1991). This reaction is based on the dehydrogenase enzyme activity of cultivable, fast growing, aerobic, eutrophic microorganisms (largely bacteria). Consequently, this technique does not reflect the full spectrum of microbial species within a mixed soil community. Additionally, due to the artificial growth conditions required in the test, it is argued that the method does not reflect the microbial community diversity and its function of a given soil (Glimm et al., 1997). On the other hand, however, standardized conditions allows for direct comparisons between microbial communities in different sites, for example, independent of the abiotic conditions, thus making CLPP a popular method for toxicology testing (Preston-Mafham et al., 2002).

Isothermal micro-calorimetry is another technique that involves the direct measurement of energetics in soil and provides a functional link between energy flow and the composition of belowground microbial communities at a high taxonomic level (Herrmann et al., 2014). With this method, an integrative determination of the metabolic activity of soil bacteria and fungi is achieved. The integrated assessment of substances' and energy turnover has high potential to elucidate the regulation of soil ecological functions. However, the substantial costs for the acquisition of this very specific instrumentation is considered as a major drawback. Furthermore, the

measurement requires water saturation of the soil and, thus, the samples are modified. Since calorimetry has been rarely used and data and publications are few, this method is considered not ready for standardization.

The methods targeting thymidine or leucine incorporation into microbial biomass can be used to determine microbial growth rates and efficiencies (Bååth et al., 2001; Rousk, 2016). Growth rate is a fundamental reference for numerous other microbial properties and functions. For example, it is required to calculate microbial carbon use efficiency (CUE) as a key-parameter describing C-substrate turnover and storage in soil (Liu et al., 2018; Spohn et al., 2016; Takriti et al., 2018). Furthermore, the method can be used to assess the adverse effects of toxic chemicals on the microbial community (Modrzyński et al., 2016; Rousk et al., 2009a). The drawbacks of these two methods are: (i) specific training is required, (ii) laboratories must have a permit to manipulate radioactive isotopes, and (iii) there are higher costs for proper handling and disposal of ^{3}H-labelled radioactive material. As an alternative, the incorporation of the stable isotope ^{18}O from labelled water into soil microbial DNA can be used to distinguish growing and non-growing microorganisms based on the gradient-separation of [^{18}O]DNA and [^{16}O]DNA (Schwartz, 2007). The ^{18}O stable isotope method has been improved by sequencing a marker gene from fractions retrieved from ultracentrifugation to produce taxon density curves; thus enabling researchers to estimate the percent isotope composition of each microbial taxon's genome (Schwartz et al., 2016). This method continues to be advanced and, although not used often, could have a high potential for future standardization.

There are simplistic methods available to determine organic matter decomposition, which are indicative of C cycling (**Function 6**). The tests listed in Table 3 are based on measuring the weight loss of introduced organic materials of different complexity in soil over time. The tests are relatively easy to perform and inexpensive, however, degradation activity is not exclusive to microorganisms but can also include invertebrates. The OECD litter bag test (OECD, 2006) for site specific assessment of organic matter decomposition uses wheat straw as the substrate and provides clear evidence of cellulose degradation. In general, the litter bag tests provides evidence for the degradation of naturally occurring plant material in soil. Results do, however, depend on the mesh size of the litter bags (increasing exclusion of soil animals with decreasing mesh size). On the other hand, plant material or litter is hard to standardise with the results largely depending on the composition of the plant material. As such, artificial cellulose has been successfully used for a laboratory procedure to assess organic matter decomposition (Kvas et al., 2017). Another alternative to the litter bag test is the use of tea bags (Keuskamp et al., 2013). Tea bags can be purchased to contain a consistent quality of material, and so this method is preferred by citizen science (e.g. farmers to assess the soil quality of their land). In order to better distinguish the degrading abilities of different

soil microbiomes, the test could be modified to use different types of tea that contain recalcitrant material to a different extent. Another test for future method development is the Bait Lamina test (ISO 18311, 2016) used to assess the degradation of organic matter in field soil by grazing invertebrates (Jänsch et al., 2013; Kvas et al., 2017). It is a simple test that can easily be adapted for use under controlled laboratory conditions (Jänsch et al., 2017).

Methods for the determination and assessment of greenhouse gas emissions from soil (**Function 7**) have already been standardized or are well advanced in the standardization process (Table 2). They are mostly focused on measuring concentrations of greenhouse gases, like CO_2, CH_4 and N_2O, as well as their fluxes as endpoints. In addition, molecular biology methods that estimate the relative abundance of functional microbial guilds or taxa gives new insight into the ecology of microorganisms involved in the formation of greenhouse gases. For example, the qPCR measurement of key N_2O functional genes has allowed researchers to link N_2O reduction capacity to reduced greenhouse gas emissions in soil amended with organic matter (Xu et al., 2018). Additionally, the quantification of functional gene sequences related to methane generation and methane oxidation, respectively, yields detailed insights into the functional potential of climate change-affected permafrost soils (Yergeau et al., 2010).

For **Function 8** (soil structure affecting soil water, gas balance and filtration function), there is clear evidence that microbial activity and biomolecules substantially contribute to the formation and stability of micro-aggregates, and thus to the structure, pore system and pre-consolidation stress of soils (Six et al., 2004). While existing parameters, such as enzyme activities, are not clearly indicative in this regard (Beck and Beck, 2000), glomalin can be considered as a biochemical marker of soil aggregation. This glycoprotein is produced by microorganisms, especially arbuscular mycorrhiza fungi, and significantly increases aggregate formation and stability (Rillig, 2004; Rillig and Mummey, 2006). The existing protocols for extraction (chemical extraction combined with autoclaving) and determination of glomalin, either by using the Bradford protein assay, enzyme-linked immunosorbent assay (ELISA), or LC-MS method (Bolliger et al., 2008; Janos et al., 2008), open the possibility for its standardization in the near future. It should be noted, however, that a well-equipped and experienced laboratory is required to perform this method.

4 Transforming standardized methods into indicators of soil quality

As recently underlined by the European Food Safety Agency (EFSA) in a scientific opinion ‘addressing the state of the science on risk assessment of plant protection products for in-soil organisms’, there is an urgent need to

modernize pesticide risk assessment by implementing specific protection goals for in-soil organisms which are key drivers of a wide range of functions supporting ecosystem services (Ockleford et al., 2017). There currently exists a multitude of methods that can potentially be used for this task. Here, we have identified in the body of this paper a number of methods that are presumably suitable for further evaluation and standardization with regard to their scientific value and practical applicability. These prospective standardized methods will not only be useful to identify adverse effects on the soil microbiome, but also to conduct comparable studies in laboratories all over the world to define normal operating ranges of microbial activity in soil and respective quality indices and threshold values.

It is clear that all parameters taken together reflect the potential of a microbial community to perform a certain function and not solely a specific (actual) activity. This is important to understand in order to interpret the values of a given endpoint in relation to both energy fluxes and compound transformation rates, which can largely depend on intrinsic properties such as pedoclimatic conditions, and nutrient availability as well as extrinsic properties such as anthropogenic effects, and soil cultivation measures. To make use of these methods as indicators for soil quality, there are several requirements that need to be included. These involves the assessment of the normal operating range of soil that include natural and dynamic fluctuations of a given endpoint. The methods need to be implemented into a framework, which takes into account site-specific conditions including soil type, pedoclimate and land-use. Undoubtedly, this requires further joint efforts in order to generate comprehensive databases from which normal operating ranges of values for a given proxy can be read. Such a task calls for standardized methods to obtain comparable data. Additionally, there is a requirement for the assessment of resistance and resilience of a given microbial endpoint to see how much it is affected by a soil disturbance and whether or not it can recover (e.g. return to its original state) after the disturbance has disappeared. Here the use of DNA based methods, which provide a measure of a microbial community's potential to perform a given process, might be more useful than using RNA. The RNA rather indicates actual activities, which may highly fluctuate in time and space, and thus are of less significance as an indicator. However, free DNA released from dead microbes is often highly resistant in soil, which might result in an over estimation of a potential function. This needs to be taken into account when interpreting the data. Recently, methods that extract DNA only from living cells have been described, but their use has not been yet introduced into recent standardization activities.

Also, the use of a test battery to measure a range of interconnected endpoints is recommended (Ockleford et al., 2017) to integrate different biological and other parameters (e.g. soil pH, organic carbon content) into multiparametric indices (Bastida et al., 2008; Kvas et al., 2017). At present, it appears to be favourable to use a

suite of different methods, i.e. functional gene analysis and microbial enzyme and/or degradation activities, for soil quality testing. Finally, to fully understand soil microbial functioning, a task was envisioned to investigate the linkage between the genetic functional potential and the available resources, termed the soil metaphenome (Jansson and Hofmockel, 2018). This will require even further integration and assessment of multiple parameters and test methods. Reaching that goal will surely promote soil ecological research but, at the current stage, may clearly go beyond the applied aim of standardization to release easy-to-use targeted methods.

The critical evaluation of existing and non-standardized methods is required to further select and standardize new methods to assess soil quality. For methods linked to the molecular analysis of soil microbiomes, there is a need to ensure that worldwide activities are synchronized to propose important standards that are well accepted by the scientific community. To improve the reproducibility of data it has been agreed that a complex mixture of microorganisms (MOCK) must be implemented as a control in every experiment, but so far no common agreement on the composition of a MOCK community has been reached. However, it is clear that if further developments of microbial bar coding and/or metagenomics methods are to be implemented into ISO guidelines, an MOCK is required.

ISO standardization committees are open circles and the presented selection and valuation of methods may not be complete. Environmental scientists are solicited to propose new work items enlarging the current catalogue of biological methods for future standardization. Accordingly, this opinion paper aims at initiating a broader discussion intended to improve the measurement of microbial functions for soil quality assessment. Lastly, it must be noted that standardization of methods is inevitably a balancing act. On one hand, standardization provides defined methods that are essential to obtain comparable data, e.g. for integration in large, joint databases. On the other hand, it requires setting a specific method for several years. Consequently, scientific progress cannot be easily adopted, or at least with a delay, considering that standards are revised every five years, which may be a barrier to the introduction of new approaches resulting from technological evolution, especially in the fast developing field of molecular biology methods. Hence, it is also the aim of this paper to have an open discussion to identify the best suitable methods with an assumed longer period of validity.

Code availability. Does not apply.

Data availability. Does not apply.

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Disclaimer.

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Table 1. List of criteria for the selection of indicators for microbial functional indicators, based on Faber et al. (2013) and Pulleman et al. (2012), with slight modifications by the authors.

	Criteria	Measured by	Low Score	High Score
a)	Practicability	Lab equipment	Very few labs have the equipment needed	All labs would be able to carry out the work
		Skills	Specialist skills are needed	General skills would suffice
b)	Cost efficiency	Capital start-up	More than €100 000	Less than €2000
		Cost per sample	More than €100	Less than €2
		Labour needed in the lab	High labour demand	Low labour demand
		Labour needed in the field	High labour demand	Low labour demand
c)	Policy relevance	Focus on ecosystem processes and services	Weak links with existing or planned legislation	Strong links with existing or planned legislation
d)	Sensitivity	Effect of soil properties	No response or idiosyncratic response	The indicator responds characteristically to change
		Effect of land use	No response or idiosyncratic response	The indicator responds characteristically to change
		Effect of disturbance	No response or idiosyncratic response	The indicator responds characteristically to change
e)	Selectivity		Endpoint affected by numerous variables	Endpoint only affected by parameter under investigation
f)	Reproducibi-lity		Low or largely varying reproducibility among replicates	Highly reproducible
g)	Use as an indicator	Status quo	Not in use already	In use already
h)	Handling and availability of organisms[1]		Rare and/or difficult to obtain Difficult to keep Largely varying quality/fitness Seasonal availability	Easy to obtain Easy to keep Easy to provide with constant quality/fitness Year-round availability
i)	Fit for use as an indicator	Significance / explanatory power	Weak relationship to ecological function	Strong relationship to ecological function
		Standardized	Methods are not ready for general use or standardization (i.e. low experience, no SOPs [2])	Methods are already in general use, preferably as standard (e.g. OECD)
		Spatio-temporally relevant	Spatio-temporally only relevant for a small plot at one point in time	Representative for more than one site and/or more than one point in time
		Understandable	Difficult to explain in a policy situation	Easily understood in a policy situation
j)	Experience	Literature data	Low amount of information on performance and outcome, e.g. <10 publications	High amount of information on the performance and outcome, e.g. >10 publications, existing ring test(s)
k)	Data evaluation	Database	No or hardly any existing data available or not freely available	Freely available and sound database for data evaluation

[1] Only relevant for faunal species. Does not apply to soil microorganisms that are tested with their natural abundance in mixed communities.

[2] Standard operating procedures

911 **Table 2.** Methods already validated and published as ISO standards for determining potential microbial biomass
912 and activities for soil quality.

Microbial biomass and respiration (some relations to Functions 1 and 6)	
ISO 14240-1	Determination of soil microbial biomass – Part 1: Substrate induced respiration method
ISO 12240-2	Determination of soil microbial biomass – Part 2: Fumigation – extraction method
ISO 16072	Laboratory method for determination of microbial soil respiration
ISO 17155	Determination of the activity of the soil microflora using respiration curves
ISO 11063	Direct soil DNA extraction
ISO 17601	Quantification of the abundance of microbial groups in soil DNA extract
ISO/TS 29843-1	Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis (
ISO/TS 29843-2:	Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method
(Potential) microbial enzymatic activities: C, N and P turnover (Functions 4 and 6)	
ISO/TS 22939 [1]	Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates
ISO 20130 [2]	Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates
ISO/TS 23753-1	Determination of dehydrogenase activity in soils — Part 1: Method using triphenyltetrazolium chloride (TTC)
ISO/TS 23753-2	Determination of dehydrogenase activity in soils — Part 2: Method using iodotetrazolium chloride (INT)
ISO 14238	Biological methods – Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes
ISO 15685	Determination of potential nitrification and inhibition of nitrification — Rapid test by ammonium oxidation
Potential microbial activities: biodegradation of pollutants (Function 3)	
ISO 11266	Guidance on laboratory testing for biodegradation of organic chemicals in soil under aerobic conditions
ISO 14239	Laboratory incubation systems for measuring the mineralization of organic chemicals in soil under aerobic conditions
ISO 15473	Guidance on laboratory testing for biodegradation of organic chemicals in soil under anaerobic conditions
Potential microbial activities: turnover greenhouse gases (Function 7)	
ISO 20951	Guidance on methods for measuring greenhouse gases (CO_2, N_2O, CH_4) and ammonia (NH_3) fluxes between soils and the atmosphere
ISO/TS 20131-1	Easy laboratory assessments of soil denitrification, a process source of N_2O emissions -- Part 1: Soil denitrifying enzymes activities
ISO/TS 20131-2	Easy laboratory assessments of soil denitrification, a process source of N_2O emissions -- Part 2: Assessment of the capacity of soils to reduce N_2O
Potential microbial activities: organic matter decomposition (Function 6)	
ISO/CD 23265 [3]	Test for measuring organic matter decomposition in contaminated soil

913 [1] Measured enzyme activities: Arylsulfatase E.C. 3.1.6.1; α-glucosidase E.C. 3.2.1.20; β-glucosidase E.C. 3.2.1.21;
914 β-xylosidase E.C. 3.2.1.37; cellobiosidase E.C. 3.2.1.91; N-acetylglucosaminidase E.C. 3.2.1.52;
915 phosphodiesterase E.C. 3.1.4.1; phosphomonoesterase E.C. 3.1.3.2; leucine-aminopeptidase E.C. 3.4.11.1;
916 alanine-aminopeptidase E.C. 3.4.11.12.
917 [2] Measured enzyme activities: Arylamidase E.C. 3.4.11.2; arylsulfatase E.C. 3.1.6.1; α-glucosidase E.C. 3.2.1.20;
918 β-glucosidase E.C. 3.2.1.21; β-galactosidase E.C. 3.2.1.22; N-acetylglucosaminidase E.C. 3.2.1.52; phosphatase
919 E.C. 3.1.4.1; acid phosphatase E.C. 3.1.4.1; alkaline phosphatase E.C. 3.1.4.1; urease E.C. 3.5.1.5.
920 [3] Degradation of cellulose under laboratory conditions.
921

Table 3. Potential new methods for the ISO standardization process and assessment according to the "logical sieve" selection criteria (described in Table 1).

Method	Source	Function addressed	Assessment [1]									
			a	b	c	d	e	f	g	h	i	j	k
Function 4. Nutrient cycling (N and P)													
Functional genes assessed by real time qPCR													
Ammonium monoxygenase gene (*amoA*)	Levy-Booth et al., 2014	quantify the abundance of nitrifying microbes	1-2	3	5	5	5	5	5	na[2]	4	4	3
Ammonium monoxygenase gene (*amoB*)	Norton et al., 2002	quantify the abundance of nitrifying microbes	1-2	3	5	5	5	5	5	na	4	4	3
Nitrogenase gene (*nifH*)	Gaby and Buckley, 2012	quantify the abundance of N fixing microbes	1-2	3	5	5	5	5	5	na	4	4	3
Various genes driving P turnover	Bergkemper et al, 2016	quantify the abundance of microbes driving P transformation	1-2	3	5	5	5	5	5	na	4	4	3
Function 5. Pest control and plant growth promotion													
Specific mtDNA sequences assessed by real time qPCR	Voříšková et al., 2017	quantify the abundance of arbuscular mycorrhiza	1-2	3	5	5	5	5	5	na	4	3	2
Specific ITS sequences assessed by real time qPCR	Sakakibara et al., 2002	quantify the abundance of ectomycorrhizal fungi	1-2	3	5	5	5	5	5	na	4	3	2
Specific ITS sequences assessed by real time qPCR	Savazzini et al., 2008	quantify the abundance of biocontrol active Trichoderma fungi	1-2	3	5	5	5	5	5	na	4	3	2
Function 6. Carbon cycling and sequestration													
Enzyme activity of fungi	Eichlerová et al., 2012	determine activity of laccases	4	4	5	5	3	5	5	na	4	5	4
	Bach et al., 2013	determine activity of phenoloxidases	4	4	5	5	3	5	5	na	4	5	4
Community level physiological profiling (CLPP, "Biolog")	Garland and Mills, 1991	determine degradation of a set of carbon sources	3	4	3	1	1	5	3	na	1	5	3
Microcalorimetry	Prado and Airoldi, 2001; 2003	quantify microbial energy turnover	1	2	1	3	3	5	1	na	3	3	1
[^{3}H]-leucine or [^{3}H]-thymidine incorporation	Bååth, 1998; Bååth et al., 2001; Rousk et al., 2009b	quantify microbial growth rate and efficiency	1	2	5	5	4	5	5	na	4	4	2
[^{18}O] incorporation into DNA from labelled water	Schwartz, 2007; Schwartz et al., 2016	quantify microbial growth rate and efficiency	2	3	5	5	4	5	5	na	3	2	2
Organic matter decomposition	OECD, 2006; Knacker et al., 2003	assess organic matter degradation and therefore C cycling assess the	5	5	4	4	5	4	5	na	5	5	5
Litter bag technique	Bockhorst and Wardle, 2013	degradation of plant litter material											
Tea bag technique	Keuskamp et al., 2013	assess the degradation of tea leaves	5	5	4	4	5	4	5	na	5	5	5
Funct. genes within C cycle assessed by real time qPCR	El Azhari et al., 2008	quantify the abundance of microbes able to degrade protocatechuate (*pcaH*) a key intermedi-ary metabolite of the β-ketoadipate pathway	1-2	3	5	5	5	5	5	na	4	4	3
	El Azhari et al., 2010	quantify the abundance of microbes able to degrade catechol (*cat A*) a key intermediary metabolite of the β-ketoadipate pathway											

925 **Table 3.** Continued

Method	Source	Function addressed	Assessment [1]									
Function 7. Greenhouse gas emissions												
Methyl coenzyme M reductase (*mcrA*) assessed by real time qPCR	Steinberg and Regan, 2009	quantify the abundance of methane producing microbes	1-2	3	5	5	5	5	5	na	4	4	3
N_2O reductase gene (*nosZ*) assessed by real time qPCR	Jung et al., 2013	quantify the abundance of N_20 reducing microbes	1-2	3	5	5	5	5	5	na	4	4	3
Methane reductase gene *pmoA*)	Kolb et al., 2003	quantify the abundance of methane reducing microbes	1-2	3	5	5	5	5	5	na	4	4	3
Nitric oxide reductase gene *cnorA*) assessed by real time qPCR	Dandie et al., 2007	quantify the abundance of methane reducing microbes	1-2	3	5	5	5	5	5	na	4	4	3
Function 8. Soil structure affecting soil water, gas balance and filtration capacity												
Determination of glomalin	Bolliger et al., 2008; Janos et al., 2008; Wright et al., 1998	determine the content of glomalin in soil as a proxy of soil aggregation	3	3	4	4	4	5	3	na	4	4	2

926 [1] Overall scoring in case of several measures for one criterion. Fulfilment of criterion described by numbering
927 (colour code): 1 (red) very low; 2 (orange) low; 3 (yellow) medium; 4 (light green) good; 5 (dark green) very good.
928 [2] na = not applicable
929