Contemporary issues, current best practice and ways forward in soil protist ecology

Molecular Ecology Resources

Geisen, Stefan; Lara, Enrique; Mitchell, Edward

https://doi.org/10.1111/1755-0998.13819

This publication is made publicly available in the institutional repository of Wageningen University and Research, under the terms of article 25fa of the Dutch Copyright Act, also known as the Amendment Taverne. This has been done with explicit consent by the author.

Article 25fa states that the author of a short scientific work funded either wholly or partially by Dutch public funds is entitled to make that work publicly available for no consideration following a reasonable period of time after the work was first published, provided that clear reference is made to the source of the first publication of the work.

This publication is distributed under The Association of Universities in the Netherlands (VSNU) 'Article 25fa implementation' project. In this project research outputs of researchers employed by Dutch Universities that comply with the legal requirements of Article 25fa of the Dutch Copyright Act are distributed online and free of cost or other barriers in institutional repositories. Research outputs are distributed six months after their first online publication in the original published version and with proper attribution to the source of the original publication.

You are permitted to download and use the publication for personal purposes. All rights remain with the author(s) and / or copyright owner(s) of this work. Any use of the publication or parts of it other than authorised under article 25fa of the Dutch Copyright act is prohibited. Wageningen University & Research and the author(s) of this publication shall not be held responsible or liable for any damages resulting from your (re)use of this publication.

For questions regarding the public availability of this publication please contact openscience.library@wur.nl

Received: 31 October 2022 | Revised: 23 February 2023 | Accepted: 17 May 2023

DOI: 10.1111/1755-0998.13819

MOLECULAR ECOLOGY RESOURCES WILEY

OPINION

Contemporary issues, current best practice and ways forward in soil protist ecology

Stefan Geisen[1] | **Enrique Lara**[2] | **Edward Mitchell**[3]

[1]Laboratory of Nematology, Wageningen University, Wageningen, The Netherlands

[2]Real Jardín Botánico-CSIC, Madrid, Spain

[3]Laboratory of Soil Biodiversity, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

Correspondence
Stefan Geisen, Laboratory of Nematology, Wageningen University, Wageningen, The Netherlands.
Email: stefan.geisen@wur.nl

Handling Editor: Simon Creer

Abstract

Soil protists are increasingly studied due to a release from previous methodological constraints and the acknowledgement of their immense diversity and functional importance in ecosystems. However, these studies often lack sufficient depth in knowledge, which is visible in the form of falsely used terms and false- or over-interpreted data with conclusions that cannot be drawn from the data obtained. As we welcome that also non-experts include protists in their still mostly bacterial and/or fungal-focused studies, our aim here is to help avoid some common errors. We provide suggestions for current terms to use when working on soil protists, like protist instead of protozoa, predator instead of grazer, microorganisms rather than microflora and other terms to be used to describe the prey spectrum of protists. We then highlight some dos and don'ts in soil protist ecology including challenges related to interpreting 18S rRNA gene amplicon sequencing data. We caution against the use of standard bioinformatic settings optimized for bacteria and the uncritical reliance on incomplete and partly erroneous reference databases. We also show why causal inferences cannot be drawn from sequence-based correlation analyses or any sampling/monitoring, study in the field without thorough experimental confirmation and sound understanding of the biology of taxa. Together, we envision this work to help non-experts to more easily include protists in their soil ecology analyses and obtain more reliable interpretations from their protist data and other biodiversity data that, in the end, will contribute to a better understanding of soil ecology.

KEYWORDS
High-throughput sequencing, microbial ecology, predator–prey interactions, protists, soil biodiversity

1 | INTRODUCTION

Studying soil protist diversity is becoming a routine element in soil ecological research due to their importance as key microbiome predators (Thakur & Geisen, 2019). Protists influence the composition of microbial communities and their functioning (Gao et al., 2019; Jousset, 2017), and determine changes in plant performance (Jousset, 2017) and carbon cycling (Geisen et al., 2018, 2021; Jassey et al., 2015). Protists compose a major part of soil biodiversity, including potentially millions of species (Geisen et al., 2019), with soil protist species richness being more variable and higher than in freshwater or marine ecosystems (Singer et al., 2021). Beyond their interactions with bacteria, protists feed on other soil biotas like other protists, fungi and small Metazoans (Geisen et al., 2015), while being plant and animal symbionts that ranging from mutualists (e.g. lichens) to parasites (e.g. larger animals (Geisen et al., 2020)). Therefore, protists play key roles in all terrestrial systems as drivers of major ecosystem functions and services (Geisen et al., 2020).

The last decade has substantially increased insights on protists in soils and beyond, especially due to developments in molecular approaches. Molecular studies have confirmed the huge diversity of protists both in terms of deep phylogenetic diversity (protists represent the vast majority of eukaryotic lineages) and taxonomic richness allowing clarification of the classification (Adl et al., 2019; Keeling & Burki, 2019) and improvements in reference databases (Glockner et al., 2017; Guillou et al., 2013). Global community efforts such as UniEuk (Berney et al., 2017) are further contributing to increasing the knowledge of protists and its visibility in the scientific community. All these efforts were made possible to a large extent thanks to the development of high-throughput sequencing techniques that now allow easy exploration of protist communities in soils (Geisen et al., 2018). Biogeographic and macroecological studies up to the global scale have revealed protist diversity patterns at the level of communities (Aslani et al., 2022; Oliverio et al., 2020; Singer, Metz, et al., 2019), genus or species (Lara et al., 2016) and intraspecific taxa or cryptic species (Janik et al., 2020; Pinseel et al., 2020; Singer, Mitchell, et al., 2019). In addition, manipulative experiments and subsequent sequencing analyses have revealed potential protist keystone species and communities linked to changes in plant performance and ecosystem functions (Guo et al., 2022; Jiang et al., 2020). Also, many studies have appeared on responses of protists to changes in land management or other anthropogenic factors (Reczuga et al., 2020; Wu et al., 2022) and ecological gradients at the regional to local scale (Fernandez et al., 2022; Mazel et al., 2022; Singer et al., 2018).

We applaud the recent boost in soil protist research, especially studies that include protist community analyses in addition to those targeting bacteria and fungi. This development had indeed long been called for and we hope it continues. However, this fast development is not without risks as knowledge of protists lags behind that of plants, animals, bacteria and fungi. Indeed, many studies that include protists clearly lack the required depth in knowledge on protists leading to obvious errors in reported data and their interpretation. To avoid these caveats and help advance the field, we aim to reach out to soil ecologists starting or considering working on soil protists. We approach this by first giving an overview of the terms to be used when working on protists, followed by a guide on methods and approaches to allow addressing certain ecological questions involving protists and finally, outline some ideas for future studies that may help disentangle some poorly known aspects of soil protist diversity and ecology. We hope this will help us gain deeper insights into soil protists, their ecology and to better understand soil functioning by fully integrating soil protists in soil ecological research.

2 | TERMS TO BE USED IN PROTIST ECOLOGY

We here provide an overview of terms that we believe should be used in soil protist studies with reasons why. First of all, the term *protist** (including all eukaryotes that do not evolve multicellularity through embryonic development, therefore excluding animals, plants and fungi (sometimes also included as protists) (Adl et al., 2005; Taylor, 2003)) should consistently be used rather than *protozoa** (best referred to as heterotrophic protists). Arguably being a semantic discussion, the term *protist* is less problematic than *protozoa* as often discussed by experts, but still having missed the entire field of scientists who shifted towards or included protists in their research (Figure 1). In short, single-celled eukaryotes include also many other functional units like phototrophic taxa, which are highly common and abundant in soils (Jassey et al., 2022; Oliverio et al., 2020; Seppey et al., 2017). These could be called algae to split

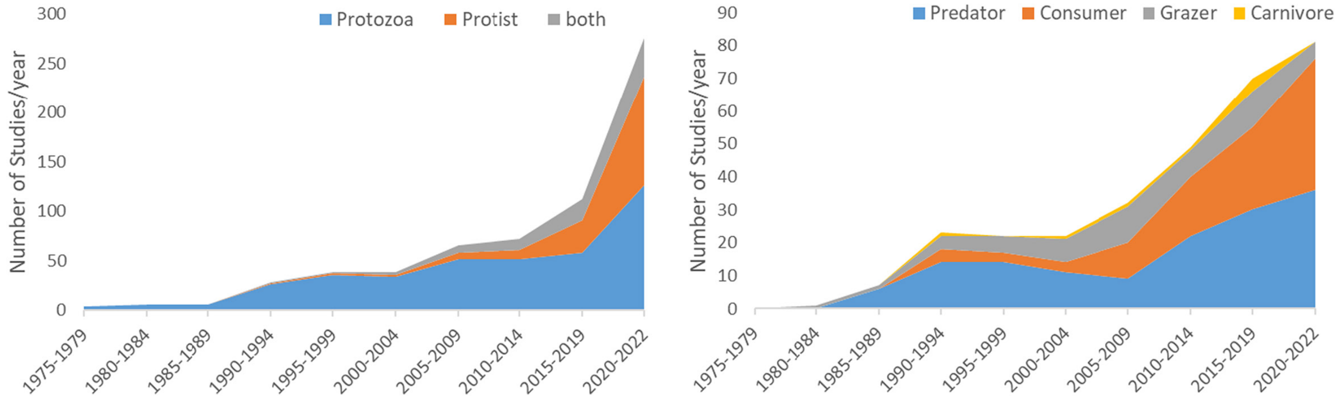

FIGURE 1 Overview of the terms used for soil protists as an average of yearly studies per 5 years. Left: use of the term protists, protozoa or both, showing an increase in the use of *protist* which should be continued (search terms used for the Web of Science topic search were (*soil* AND protist* NOT protozoa*) or (*soil* AND protozoa* NOT protist*) or (*soil* AND protozoa* AND protist*)). Right: Terms used to denote feeding of soil protist, showing not only increased use of the terms predator and consumer but also that the term grazer is still used. We propose to generally avoid calling protists grazers and use the terms predator or even general comment (search was similar as above, using the topic search that excluded other terms and also nematodes as created many false-positive results. For example, for the term predators we used the search 'soil* AND (protist* OR protozoa*) AND predat* NOT graz* NOT consum* NOT nematod*'. The search was limited to studies published by 31 December 2022 and was conducted on 21 February 2023.

the animal-like heterotrophic protozoans from the plant-like photosynthetic protists. Yet, many taxonomic clades contain both phototrophic and heterotrophic taxa that often cannot be differentiated based on amplicon sequencing data. In soil chrysophyceae, and especially in Ochromonadales, the photosynthetic function has been lost many times independently among closely related lineages (Bock et al., 2022). Furthermore, in soil, most phototrophic protists also consume other organisms or organic molecules. These organisms are coined constitutive mixotrophs when using vertically inherited plastids, or endosymbiotic non-constitutive mixotrophs when they rely on green photobionts for photosynthesis (Mitra et al., 2016). In both cases, these mixotrophs often favour one or the other strategy depending on the availability of nutrients (Selosse et al., 2017).

Microflora, a term still seen in soil ecological literature and mostly includes bacteria, archaea and fungi, as well as *Microfauna* which includes protists, should be abandoned. Microflora means 'minute plants' and, therefore, does not match the mostly heterotrophic bacteria, archaea and fungi. Microfauna means 'minute animals' that do not make sense for the phylogenetically diverse protists (and also protozoa) that cover the entire eukaryotic tree, but not a single one belonging to the group of animals (Metazoa). Even functionally, the term microfauna is misleading as many protists are often not (only) predators as they play many functional roles (see below for the definition and details on other functional groups). As such, protists are *microorganisms* (Caron et al., 2009), which also represent the fact that protists are mostly invisible to the naked eye (with some exceptions, such as macroscopic brown algae). Of course, it can be debated if this term is appropriate for organisms that are at one point of their life cycle visible with the naked eye like many myxomycetes, but this discussion would span many non-protists like fungi with their sometimes-massive fruiting body and multi-meter-long mycelia. Therefore, currently, all microbial groups should be called by their more precise taxonomic affiliation (e.g. bacteria, archaea, microbial eukaryotes or groups included within each domain), and the term microorganisms can often simply be avoided.

Protists should generally *not be called grazers*. The term grazer would imply that protists eat part of their prey that then can regrow, like a cow eating upper parts of grasses that can then re-grow from lower parts of shoots and roots. Most soil protists, in comparison, kill entire prey organisms after engulfment through phagocytosis (the feeding behaviour of some ciliates has even been referred to as 'raptorial' (Verni & Gualtieri, 1997)) and more rarely by sucking the cell content using specialized organs (e.g. 'suctorian' ciliates, Viridiraptoridae) (Hess & Melkonian, 2013). Only for some groups, the term grazer might still hold, such as for those protists that do not kill the entire organism including few specialized fungal feeders (see below) (Petz et al., 1986). Only when treating bacterial or algal colonies or biofilms as superorganisms, this definition could be extended to most phagotrophic protists. Then, however, nearly all predators in aboveground or marine systems that prey on swarms, herds or packs should be considered grazer. Therefore, the term *predator* should be used for free-living, heterotrophic protists, or *consumer* that equally captures grazers and predators, a proposal that also follows the trend seen in the use of feeding terminology (Figure 1). Finally, the rarely used term *'carnivore'* (Figure 1) should not be applied to protists, as it implies the action of eating meat (i.e. tissues from a vertebrate; in contrast with an insectivore, for instance). Likely very few protists feed on small metazoans, but even these cannot be assigned to meat. We rather recommend using terms like second-order predator or, again, conservatively integrating all as *consumers*.

3 | FUNCTIONAL ROLES OF SOIL PROTISTS: FREQUENT CONFUSIONS

Documenting the taxonomic composition of communities and diversity patterns at different scales is valuable for ecological studies. However, the functional role of organisms is often ecologically more relevant than the taxonomic composition. We, therefore, encourage including functional roles (inferred from taxonomic assignation) in any ecological study. Yet, some considerations need to be made when assigning protist communities to functional groups. Protist consumers are *not only general bacterivores*, but each taxon has its specific prey spectrum. Most protists phagocytose their prey (engulf prey items as a whole) and therefore size (or aperture size in the case of testate amoebae or the buccal apparatus of ciliates) can determine what protists can feed upon (Jassey et al., 2016; Meyer et al., 2013). Thus, smaller flagellated species can be expected to feed mostly on bacteria. However, ample examples exist that oppose this simple view. Many traditionally considered bacterivorous protists are, in fact, feeding on a diverse range of organisms including bacteria, fungi, algae and nematodes (Geisen et al., 2016; Seppey et al., 2017). As comprehensive feeding tests including multiple prey organisms are needed, but not done, the precise feeding range of distinct protist species cannot be inferred. Some soil species have been shown to have highly specialized food regimes, like grossglockneriid ciliates, whose specialized buccal apparatus can only suck fungal hyphae (Foissner, 1999a; Petz et al., 1986). Also, the testate amoeba *Phryganella paradoxa* needs the frustules of its diatom prey to build its test (Dumack et al., 2019). Many protists, especially larger ciliates and amoeboid protists prey partly or even exclusively on other eukaryotes like fungi and other protists (Foissner, 1998; Gilbert et al., 2000; Hess & Suthaus, 2022; Jassey et al., 2012, 2013). Fungivorous protists are even more diverse as only some larger protist species were previously tested for fungivory, while many protists that were considered exclusively bacterivorous have been shown to also feed on yeasts and fungal spores (Geisen et al., 2016). Other organisms have even been documented eating organisms larger than themselves; examples include protists being able to feed on much larger filamentous algae (Hess & Suthaus, 2022), suctorian ciliates preying on large protists that they do not need to engulf, or small testate amoebae that prey in groups (pack hunting) on larger nematodes (Geisen et al., 2015). Such findings are often made by chance, but typically require direct observation under the microscope, something that is typically not done in most contemporary molecular studies (Ekelund, 1998; Heger et al., 2014; Mitchell, 2015). Together, we propose to classify free-living heterotrophic protists as predators or consumers rather than aiming at more closely specifying their feeding range to, for example, bacterivorous or fungivorous, as we hardly have reliable data to draw these conclusions. When inferring more precise feeding modes, the obtained information should be treated with care.

While most soil protists tend to act as consumers in soil food webs (Singer et al., 2021), protists occupy many other functional levels. Parasites can be extremely abundant in certain ecosystems, especially Apicomplexa (Mahe et al., 2017). These organisms live associated with animals and particularly aberrantly infect arthropods and annelids (Del Campo et al., 2019). When referring to Apicomplexa, the first example that comes to mind is *Plasmodium*, the malaria agent, which causes 600,000 human deaths per year (WHO, 2021). However, while some apicomplexans do act as parasites (i.e. taking resources from the host and possibly killing it), there are many instances, where Apicomplexans behave as mere commensals (Rueckert et al., 2019), and sometimes even as mutualists (Bollatti & Ceballos, 2014). Although their effect on soil ecosystems still needs to be assessed, Apicomplexans currently cannot be considered regulators of metazoan populations. Another common group of protists generally tagged as parasitic is Peronosporomycetes (=oomycetes). While many of these fungal-like protists have the capacity to infect plants, animals, fungi and other protists, only a few are obligate biotrophs (Lara & Belbahri, 2011) and some are even probably not symbiotic at all (Bennett et al., 2018; Blackwell et al., 2015). In summary, *most symbiotic soil protists are not strictly speaking parasites*; we recommend using the word *'symbiont'*, which does not imply any negative interaction with host's fitness.

The next functional group of protists ubiquitous in soil are phototrophs. These organisms play an important role as carbon sinks in the global carbon cycle (Jassey et al., 2022). While these organisms have been relatively well studied in arid soils as biocrust components (Maier et al., 2018), phototrophs are even more abundant in vegetated, wet and acidic soils (Jassey et al., 2022). Phototrophic protists have specific adaptations to soils, such as synthesizing photoprotectants and osmoprotectants (Gustavs et al., 2010, 2011); even protist predators of phototrophic protists exist (Seppey et al., 2017) showing that *phototrophic protists are genuine members of soil communities*.

4 | DOS AND DON'TS IN METHODS AND CLAIMS ON SOIL PROTISTS

Almost all current studies on soil protists are molecular, by far most applying *18S rRNA gene amplicon sequencing*. High-throughput sequencing of environmental DNA has clearly advanced soil protist ecology and microbiology in general by allowing the simultaneous analysis of numerous samples after a routine DNA extraction procedure. Such analyses typically reveal a high diversity (tens to hundreds of thousands) of protist taxa, including many previously unknown taxa (Geisen & Bonkowski, 2018; Santos et al., 2015). However, at the present state of methodological development, it is practically impossible to account for the full microeukaryotic diversity in a soil sample based on an environmental DNA survey, given the immense variability of sizes, genomes and cellular properties (Lara et al., 2022). For this reason, many claims that are commonly made are unfortunately not correct and cannot be concluded using solely sequencing data due to several caveats:

(1) Many studies rely on primer-based PCR. Yet, even the most broadly targeting ('universal') primer pair can never fully cover all protist taxa. Indeed, parts or entire groups of common soil protists like Amoebozoa and Heterolobosea (Geisen et al., 2014) are typically under-represented or altogether lacking due to mismatches and amplicon lengths variations (e.g. long introns are common in many protists) as shown in Vaulot et al. (2022).

(2) In addition to primer-related issues, DNA extraction is never complete as some protist taxa have hard cysts or shells, which reduce the efficiency of DNA extraction, while others have highly breakable cell membranes. Issues arise even before extraction, as rough treatments such as sample sieving disrupt fragile organisms, such as large-network-forming Amoebozoa (Berney et al., 2015). This makes nucleic acid extraction complicated as DNA from easy-to-break protists may be sheared when harsh procedures are performed, while DNA from those with hard cell walls might not be obtained at all. This extraction bias impacts diversity estimates and species abundance (i.e. taxa perceived as rare could be more common than believed) and thereby results in distorted protist community profiles (Santos et al., 2017).

(3) The use of different settings when applying bioinformatics pipelines may also artificially alter richness estimations, which precludes direct comparisons between studies (Caron & Hu, 2019; Xiong et al., 2019).

(4) A critical problem is related to non-specialists interpreting protist sequencing data (arguably impossible to do for all taxa, but even present for the most abundant or taxa mentioned in the paper) due to errors in automated sequence annotation they fail to identify.

(5) Finally, inference of causality frequently is not supported by the data due to an erroneous understanding of basic population ecology principles.

These different causes of errors need to be understood and are addressed hereafter.

Taxonomic richness is often the first detail reported in sequence-based studies, yet this information on the γ-diversity in a given study is often ecologically meaningless. A reliable richness of information would imply a rather complete investigation of the community present. Despite the fact that often thousands of protist taxa are covered in a sample, this number is far from complete. In fact, it is impossible to sample the total species richness of protists in a sample with current and likely near-future sequencing techniques due to above-mentioned DNA- and PCR-based issues. The sheer abundance and diversity of protists in a soil sample with co-amplification of fungi, plants and animals, as common for broadly targeted 18S rRNA gene sequencing, neglects the possibility of deciphering the entire diversity of protists in a sample. This bias is introduced by the fact that rarer ones, those with long amplicon reads and those with non-conserved primer-binding sites, will hardly ever be sequenced (Lara et al., 2022). Therefore, cross-study comparisons of γ-diversity should only be used to compare richness between sites or treatments with the same sampling, extraction and sequencing protocols, and not among studies where such factors usually differ to various degrees.

Furthermore, the true biological meaning of *rare sequences* still escapes understanding. It has been hypothesized that the rare biosphere corresponds to rare species or single propagules of inactive organisms awaiting the right conditions to build populations (Lynch & Neufeld, 2015), and it is assumed that rare species can drive important functions (Bahram et al., 2022; Jousset et al., 2017). However, currently, it is difficult to accurately depict which less abundant sequences represent true rare species as many errors prevail in bioinformatic analyses that inflate the real diversity and create artificial rare species (Reeder & Knight, 2009; Xiong et al., 2019) that often do not follow ecological expectable patterns of known dominant species (Schiaffino et al., 2016). We propose to carefully interpret rare sequences by conservative sequences curation and that can be assigned taxonomically in ecological studies. Disregarding this word of caution, larger ecological community patterns are mostly not affected by rare species (Xiong et al., 2019). Therefore, it should be possible to address the main ecological questions independently from including or excluding rare species.

The general importance and changes in abundance of distinct protist taxa should be reflected upon as sequencing data are *never quantitative*, but at best *semi-quantitative* and *qualitative*. As for the rare biosphere issue discussed, the real meaning of diversity can be questioned as a difference in diversity in the order of hundreds to thousands for soil protists might not be as relevant as other biodiversity facets such as biomass or activity (Schuldt et al., 2018; Shade, 2017). And here again, natural history approaches such as direct observation and experiments are essential to determine feeding habits as well as simply characterizing the biomass of taxa which can quite considerably vary within a group of closely related species (Kosakyan et al., 2015). This perception is hardly used in soil microbial ecology and not at all for protists due to the current lack of methods to reliably estimate biomass of entire protist communities. Therefore, claims based on metabarcoding data alone should be more cautious; alternatively, groups that can be quantified, such as ciliates and testate amoebae (Foissner, 1999b; Kosakyan et al., 2015), can be added as a proxy for whole-community biomass. Such approaches have proven useful to address current and past ecosystem functioning questions (Jassey et al., 2016; Koenig et al., 2018; Reczuga et al., 2018). We agree that these morphological quantification efforts cannot be done in high throughput, but the caveats of relative data should not be forgotten.

The obtained *community composition* is always a representation of the approach taken such as the PCR setup. Therefore, any claims on the true composition of protists in a sample should not be made. Of course, community comparison within a study should be made, as issues mentioned above are study specific if samples are treated equally. In contrast, in-depth comparisons among studies are difficult in nearly all cases when sampling and analysis protocols vary (Ramirez, Knight, et al., 2018). We currently miss any reliable information on the community composition of protists in soils, something that can only accurately be obtained with intense and thorough calibration studies that we would call for in some targeted studies that will make cross-study comparisons more reliable.

Sequence annotation is often problematic. Errors of wrongly barcoded organisms are largely reduced in curated reference databases like PR2 (Guillou et al., 2013), Silva (Pruesse et al., 2007) and soon UniEuk (Berney et al., 2017). However, other issues in sequence annotation prevail, many originating from the need to work with OTUs (Operational Taxonomic Units, or Molecular OTUs, MOTUs) or ASVs (Amplicon Sequence Variants) in amplicon sequence data that do not equate species (Callahan et al., 2017). OTUs that group amplicon reads into single OTUs with mostly a similarity of 97% were used almost since the onset of high-throughput sequencing studies to remove erroneous sequences produced by sequencing machines (Edgar, 2010). Consequently, OTUs are conglomerates of mostly many species which often might have different niches, functions and geographical distributions. Therefore, most amplicon studies now use ASVs that use a more stringent sequence curation to keep largely correct sequences independent of their similarity to different sequences. This increases the chance that closely related species with very similar sequences be differentiated, especially when dealing with standard short-read amplicon sequences. Alternatively, SWARMs are built by clustering together sequence reads that cluster together in distinct networks (Mahe et al., 2014, 2021). The issues with ASVs (and SWARMs), however, are that errors can remain after sequence curation and that species with intragenomic diversity in the barcoding region are treated as separate ASVs, and therefore species, inflating biodiversity estimates (Caron & Hu, 2019). These issues have implications not only for richness information and assessing the rarity of species but also for ecological questions, such as assessing true biogeographic patterns. In general, taxonomic assignments of OTUs/ASVs to fine taxonomic resolution (i.e. species level and below) should be avoided as 18S rRNA amplicon sequencing of short barcoding regions is almost always unable to differentiate between species, often even genera due to the slow evolutionary rate of this highly conserved gene (Lara et al., 2022). We also suggest manually investigating the most abundant sequences and those reported in the main text of the study for their correct assignment, such as with Blast searches or phylogenetic placements. Also increasing efforts need to be taken to fill databases with correctly annotated sequences to eventually enable identification of strain, species or genus information of a given (amplicon) sequence.

A major problem in (soil) protistology and ecology in general – and perhaps the biggest problem that we highlight here – is to *infer causality* from environmental sampling studies or from any, often sequencing-based, correlative study (Addicott et al., 2022). More and more studies infer interactions among protists or between protists and their presumable prey based on amplicon sequencing data, despite the fact that inferring specific aspects such as predator–prey links is impossible for several reasons. The first is that predatory protists are never fully specialized in a single prey species. While it has been widely acknowledged that food regimes of protists vary between species, all predatory protists known so far eat diverse bacterial and fungal species (Amacker et al., 2022; Geisen et al., 2016). The genus/family-level resolution obtained by OTUs/ASVs adds another level of uncertainty in high-detail assignment of functional groups as distinct protist species can differ in their feeding patterns (Amacker et al., 2022; Glucksman et al., 2010). Species within the same genus can even differ in their coarse functional positions like *Pythium* species that can range from plant parasitic to animal parasitic (Rai et al., 2020). Last, predator–prey dynamics, such as known from Lotka–Volterra or functional response theories (Berryman, 1992), rule out the possibility that there is always a positive or negative correlation between predator and prey, both for single predator–prey links and even more if multiple overlapping predator–prey links co-exist (Figure 2). In fact, predator–prey correlations can range from positive to negative depending on the cycle the predator and the prey are in, as shown in the seminal paper that coined the soil microbial loop (Clarholm, 1981). The size of protists and bacteria also induces an important issue: interactions happen at the microscale possibly within aggregates, which is many orders of magnitude smaller than the volume of soil used for DNA extraction. As a result, interactions perceived based on sequencing data may correspond to organisms that do not even co-occur (Erktan et al., 2020; Vos et al., 2013). Together, these aspects render it difficult to uncover distinct protist taxa linked to single prey items as suggested by network links, and, therefore, predator–prey '*interactions*' should not be studied with correlative sequencing approaches. Of course, network or other correlative approaches *can* provide valuable information on *potential* links such as much more specialized host–parasite interactions (Singer et al., 2020) and symbioses like in lichens (Nazem-Bokaee et al., 2021), or information on key/hub taxa as well as inform about systems' structure (Ramirez, Geisen, et al., 2018). In fact, correlation-based approaches can be highly valuable as hypotheses generators that provide the basis for more targeted experiments that should include direct observation and experimental testing (Ramirez, Geisen, et al., 2018). Until such targeted experiments are done – which we strongly advocate to extend sequence-based work only – the information gained from correlation analyses should be treated with care.

Together, there are several biases that should be taken care of such as carefully dealing with diversity and compositional differences, as well as avoiding any larger claims on correlative data alone. Yet, metabarcoding is of major value within a given study to compare treatments, which should be the major point in most ecological studies. We argue that one simply has to know the biases inherent to specific approaches like those induced by distinct primers. In Table 1, we provide a short guide that we envision to help conduct

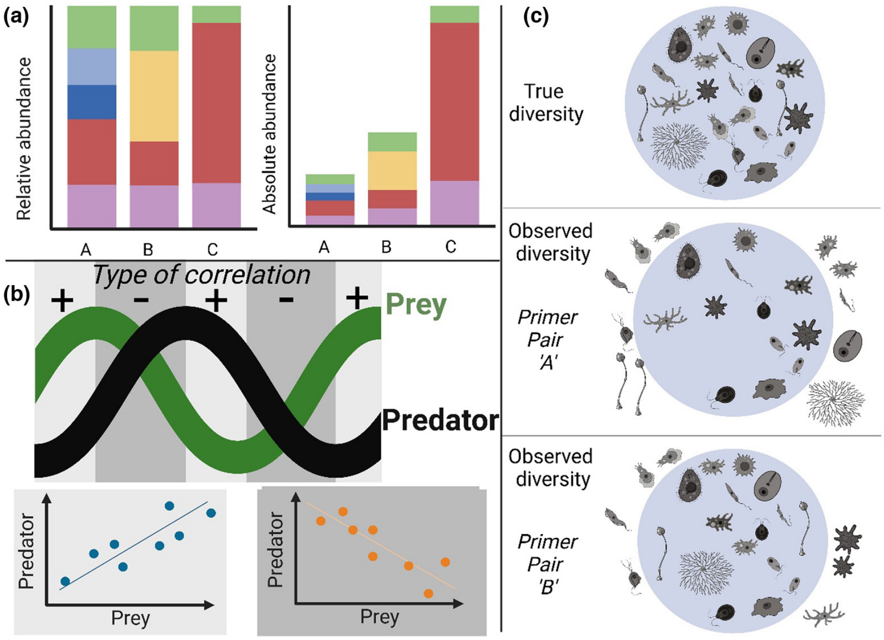

FIGURE 2 Common issues in protist and microbial sequence-based analyses. (a) Patterns obtained by sequencing efforts are of relative nature, which might lead to false conclusions. Richness is highest in A but as the absolute abundance of organisms is highest in C, the functional impact in the ecosystem might be highest; also, differences in taxon abundance appearing in relative terms might not be true when absolute abundance values were shown. (b) Typical textbook pattern of predator–prey dynamics with one predator and one prey. Even in this system correlations can be negative (dark grey) to positive (light grey) at a given point in time depending on the phase of the interaction–correlation in the above part illustrated in lower boxes with corresponding colour coding. Considering that predatory protists are never specialized towards one single prey item and that there are thousands of predators at the local scale in soil, correlation analyses can never reliably inform about predator–prey interactions. (c) The diversity and composition of protists recovered with any approach is never complete but is a representation determined by method used, such as primers.

TABLE 1 Guide for ecological studies on soil protists, which can be transferred to other fields of research. We acknowledge that this table is not complete but lists the major points that we believe should be considered before, during and after a study on soil protists.

	To do	Why
Pre-start	Get expertise - Read papers - Include experts	Information about state-of-the-art knowledge on protist biology and systematics
	Define - question(s) - aim(s) - hypotheses (testable and falsifiable)	To optimize the study setup
	Chose approach (methodology including appropriate primers (Vaulot et al., 2022) and experimental design)	To address question(s), aim(s) and hypotheses (QAHs)
Analyses	Perform thorough sequence analyses including bioinformatics (e.g. clustering, etc.), see (Lentendu et al., 2023) & (Lara et al., 2022)	To optimize obtained information, remove speculations on rare species and avoid inflating diversity estimates
	Richness information is only useful within the same study, remove cross-study comparisons of diversity information	All sampling/analyses steps impact outcome (Figure 2c)
	Avoid presence/absence analyses	Sequencing-related issues (e.g. primers and sequencing depth) make information on potential absence unreliable (Figure 2b)
Interpretation	Avoid claims on absolute abundance	Data are semi-quantitative and qualitative (Figure 2a)
	Correlations do not equal interactions (e.g. inform about predator–prey links) and avoid claims on causation	Organisms often never meet, omnivores distort any constant pattern and dynamics of prey are dyssynchronous with predator (Figure 2b)
	Link back to QAHs	Stick to those as this helps paper structure and avoids overstating
	Be open to issues (e.g. primers and gaps in methods)	No study is perfect – be honest about what you can claim. Rejecting a hypothesis can be a major breakthrough!

work on soil protist ecology to reduce issues inherent to most contemporary studies.

5 | CONCLUSIONS

Insights into soil ecology, microbiology and protists are rapidly accumulating, however, at the risk of cutting corners due to overconfidence in methods. We highlight contemporary issues in the scientific literature on protists. We here show that some of the claims made in currently widely accepted scientific practices would benefit from a deeper knowledge of a topic and a more critical view of methods and their outcomes. We do not vote against molecular methods, as we strongly believe in their benefits. But we strongly recommend to thoroughly consider the conclusions that can be drawn from those tools. We think that partly a misunderstanding of tools and a potential need for overstatements in the publishing landscape have led to the imprint of many contemporary issues, such as here shown for work on soil protists. As such, and because protists are an essential part of soil biology and functioning, we provide a roadmap for ecological studies including soil protists. We show how to correctly use soil protist (and in the end microbiological and ecological) data and standardize terms used when working on soil protists, which should help authors and reviewers in analysing, writing and evaluating literature. Most of the issues we highlight can be translated to other biodiversity groups in soil and other environments. As such, we hope to provide a matrix to bring more natural history into soil protist and microbial ecology in general, in addition to enhancing nomenclature and terminology for the benefit of overall scientific clarity.

AUTHOR CONTRIBUTIONS

SG initiated the work and conceived the first draft which was substantially modified with input from EL and EM.

ACKNOWLEDGEMENTS

EL was supported by a grant from the Spanish Government PID2021-128499NB-I00 10.13039/501100011033/ (MCIU/AEI/FEDER, UE) and EM by grant no. 182531 from the Swiss National Science Foundation (SNSF).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

No data are linked to this paper.

ORCID

Stefan Geisen https://orcid.org/0000-0003-0734-727X

Enrique Lara https://orcid.org/0000-0001-8500-522X
Edward Mitchell https://orcid.org/0000-0003-0358-506X

REFERENCES

Addicott, E. T., Fenichel, E. P., Bradford, M. A., Pinsky, M. L., & Wood, S. A. (2022). Toward an improved understanding of causation in the ecological sciences. *Frontiers in Ecology and the Environment*, *20*(8), 474–480. https://doi.org/10.1002/fee.2530

Adl, S. M., Bass, D., Lane, C. E., Lukes, J., Schoch, C. L., Smirnov, A., Agatha, S., Berney, C., Brown, M. W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., Del Campo, J., Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., … Zhang, Q. (2019). Revisions to the classification, nomenclature, and diversity of eukaryotes. *The Journal of Eukaryotic Microbiology*, *66*(1), 4–119. https://doi.org/10.1111/jeu.12691

Adl, S. M., Simpson, A. G., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A., Fredericq, S., James, T. Y., Karpov, S., Kugrens, P., Krug, J., Lane, C. E., Lewis, L. A., Lodge, J., Lynn, D. H., Mann, D. G., … Taylor, M. F. (2005). The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *The Journal of Eukaryotic Microbiology*, *52*(5), 399–451. https://doi.org/10.1111/j.1550-7408.2005.00053.x

Amacker, N., Gao, Z., Hu, J., Jousset, A. L. C., Kowalchuk, G. A., & Geisen, S. (2022). Protist feeding patterns and growth rate are related to their predatory impacts on soil bacterial communities. *FEMS Microbiology Ecology*, *98*(6), fiac057. https://doi.org/10.1093/femsec/fiac057

Aslani, F., Geisen, S., Ning, D., Tedersoo, L., & Bahram, M. (2022). Towards revealing the global diversity and community assembly of soil eukaryotes. *Ecology Letters*, *25*(1), 65–76. https://doi.org/10.1111/ele.13904

Bahram, M., Espenberg, M., Parn, J., Lehtovirta-Morley, L., Anslan, S., Kasak, K., Kõljalg, U., Liira, J., Maddison, M., Moora, M., Niinemets, Ü., Öpik, M., Pärtel, M., Soosaar, K., Zobel, M., Hildebrand, F., Tedersoo, L., & Mander, U. (2022). Structure and function of the soil microbiome underlying N(2)O emissions from global wetlands. *Nature Communications*, *13*(1), 1430. https://doi.org/10.1038/s41467-022-29161-3

Bennett, R. M., Devanadera, M. K., & Dedeles, G. R. (2018). A revision of *Salispina*, its placement in a new family, Salispinaceae (Rhipidiales), and description of a fourth species. *S. hoi Sp. Nov. IMA Fungus*, *9*(2), 259–269. https://doi.org/10.5598/imafungus.2018.09.02.03

Berney, C., Ciuprina, A., Bender, S., Brodie, J., Edgcomb, V., Kim, E., Rajan, J., Parfrey, L. W., Adl, S., Audic, S., Bass, D., Caron, D. A., Cochrane, G., Czech, L., Dunthorn, M., Geisen, S., Glöckner, F. O., Mahé, F., Quast, C., … de Vargas, C. (2017). UniEuk: Time to speak a common language in Protistology! *The Journal of Eukaryotic Microbiology*, *64*(3), 407–411. https://doi.org/10.1111/jeu.12414

Berney, C., Geisen, S., Van Wichelen, J., Nitsche, F., Vanormelingen, P., Bonkowski, M., & Bass, D. (2015). Expansion of the 'Reticulosphere': Diversity of novel branching and network-forming amoebae helps to define Variosea (Amoebozoa). *Protist*, *166*(2), 271–295. https://doi.org/10.1016/j.protis.2015.04.001

Berryman, A. A. (1992). The Orgins and evolution of predator-prey theory. *Ecology*, *73*(5), 1530–1535. https://doi.org/10.2307/1940005

Blackwell, W. H., Letcher, P. M., & Powell, M. J. (2015). A review and update of the genus *Sapromyces* (Straminipila: Oomycota). *Phytologia*, *97*(2), 82–93.

Bock, C., Olefeld, J. L., Vogt, J. C., Albach, D. C., & Boenigk, J. (2022). Phylogenetic and functional diversity of Chrysophyceae in inland waters. *Organisms, Diversity and Evolution*, *22*(2), 327–341. https://doi.org/10.1007/s13127-022-00554-y

Bollatti, F., & Ceballos, A. (2014). Effect of gregarines (Apicomplexa: Sporozoa) on survival and weight loss of Victorwithius similis (Arachnida: Pseudoscorpiones). *Journal of Invertebrate Pathology*, *117*, 13–18. https://doi.org/10.1016/j.jip.2014.01.002

Callahan, B. J., McMurdie, P. J., & Holmes, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME Journal*, *11*(12), 2639–2643. https://doi.org/10.1038/ismej.2017.119

Caron, D. A., & Hu, S. K. (2019). Are we overestimating Protistan diversity in nature? *Trends in Microbiology*, *27*(3), 197–205. https://doi.org/10.1016/j.tim.2018.10.009

Caron, D. A., Worden, A. Z., Countway, P. D., Demir, E., & Heidelberg, K. B. (2009). Protists are microbes too: A perspective. *The ISME Journal*, *3*(1), 4–12. https://doi.org/10.1038/ismej.2008.101

Clarholm, M. (1981). Protozoan grazing of bacteria in soil – impact and importance. *Microbial Ecology*, *7*, 343–350.

Del Campo, J., Heger, T. J., Rodriguez-Martinez, R., Worden, A. Z., Richards, T. A., Massana, R., & Keeling, P. J. (2019). Assessing the diversity and distribution of apicomplexans in host and free-living environments using high-throughput amplicon data and a phylogenetically informed reference framework. *Frontiers in Microbiology*, *10*, 2373. https://doi.org/10.3389/fmicb.2019.02373

Dumack, K., Kahlich, C., Lahr, D. J. G., & Bonkowski, M. (2019). Reinvestigation of *Phryganella paradoxa* (Arcellinida, Amoebozoa) Penard 1902. *The Journal of Eukaryotic Microbiology*, *66*(2), 232–243. https://doi.org/10.1111/jeu.12665

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Ekelund, F. (1998). Enumeration and abundance of mycophagous protozoa in soil, with special emphasis on heterotrophic flagellates. *Soil Biology and Biochemistry*, *30*(10–11), 1343–1347. https://doi.org/10.1016/s0038-0717(97)00266-6

Erktan, A., Or, D., & Scheu, S. (2020). The physical structure of soil: Determinant and consequence of trophic interactions. *Soil Biology and Biochemistry*, *148*, 107876. https://doi.org/10.1016/j.soilbio.2020.107876

Fernandez, L. D., Seppey, C. V. W., Singer, D., Fournier, B., Tatti, D., Mitchell, E. A. D., & Lara, E. (2022). Niche conservatism drives the Elevational diversity gradient in major groups of free-living soil unicellular eukaryotes. *Microbial Ecology*, *83*(2), 459–469. https://doi.org/10.1007/s00248-021-01771-2

Foissner, W. (1998). An updated compilation of world soil ciliates (protozoa, Ciliophora), with ecological notes, new records, and descriptions of new species. *European Journal of Protistology*, *34*(2), 195–235. https://doi.org/10.1016/s0932-4739(98)80028-x

Foissner, W. (1999a). Description of two new, Mycophagous soil ciliates (Ciliophora, Colpodea): Fungiphrya strobli n. g., n. sp. and Grossglockneria ovata n. sp. *The Journal of Eukaryotic Microbiology*, *46*(1), 34–42. https://doi.org/10.1111/j.1550-7408.1999.tb04581.x

Foissner, W. (1999b). Soil protozoa as bioindicators: Pros and cons, methods, diversity, representative examples. *Agriculture, Ecosystems & Environment*, *74*(1–3), 95–112.

Gao, Z., Karlsson, I., Geisen, S., Kowalchuk, G., & Jousset, A. (2019). Protists: Puppet masters of the rhizosphere microbiome. *Trends in Plant Science*, *24*(2), 165–176. https://doi.org/10.1016/j.tplants.2018.10.011

Geisen, S., Bandow, C., Römbke, J., & Bonkowski, M. (2014). Soil water availability strongly alters the community composition of soil protists. *Pedobiologia*, *57*(4–6), 205–213. https://doi.org/10.1016/j.pedobi.2014.10.001

Geisen, S., & Bonkowski, M. (2018). Methodological advances to study the diversity of soil protists and their functioning in soil food webs. *Applied Soil Ecology*, *123*, 328–333. https://doi.org/10.1016/j.apsoil.2017.05.021

Geisen, S., Briones, M. J. I., Gan, H., Behan-Pelletier, V. M., Friman, V.-P., de Groot, G. A., Hannula, S. E., Lindo, Z., Philippot, L., Tiunov, A. V.,

& Wall, D. H. (2019). A methodological framework to embrace soil biodiversity. *Soil Biology and Biochemistry*, *136*, 107536. https://doi.org/10.1016/j.soilbio.2019.107536

Geisen, S., Hu, S., Dela Cruz, T. E. E., & Veen, G. F. C. (2021). Protists as catalyzers of microbial litter breakdown and carbon cycling at different temperature regimes. *The ISME Journal*, *15*(2), 618–621. https://doi.org/10.1038/s41396-020-00792-y

Geisen, S., Koller, R., Hünninghaus, M., Dumack, K., Urich, T., & Bonkowski, M. (2016). The soil food web revisited: Diverse and widespread mycophagous soil protists. *Soil Biology and Biochemistry*, *94*, 10–18. https://doi.org/10.1016/j.soilbio.2015.11.010

Geisen, S., Lara, E., Mitchell, E. A. D., Völcker, E., & Krashevska, V. (2020). Soil protist life matters! *Soil Organisms*, *92*(3), 189–196. https://doi.org/10.25674/so92iss3pp189

Geisen, S., Mitchell, E. A. D., Adl, S., Bonkowski, M., Dunthorn, M., Ekelund, F., Fernández, L. D., Jousset, A., Krashevska, V., Singer, D., Spiegel, F. W., Walochnik, J., & Lara, E. (2018). Soil protists: A fertile frontier in soil biology research. *FEMS Microbiology Reviews*, *42*(3), 293–323. https://doi.org/10.1093/femsre/fuy006

Geisen, S., Rosengarten, J., Koller, R., Mulder, C., Urich, T., & Bonkowski, M. (2015). Pack hunting by a common soil amoeba on nematodes. *Environmental Microbiology*, *17*(11), 4538–4546. https://doi.org/10.1111/1462-2920.12949

Gilbert, D., Amblard, C., Bourdier, G., Francez, A. J., & Mitchell, E. A. D. (2000). Feeding habits of testate amoeba. *A Review. Annee Biologique*, *39*(2), 57–68.

Glockner, F. O., Yilmaz, P., Quast, C., Gerken, J., Beccati, A., Ciuprina, A., … Ludwig, W. (2017). 25 years of serving the community with ribosomal RNA gene reference databases and tools. *Journal of Biotechnology*, *261*, 169–176. https://doi.org/10.1016/j.jbiotec.2017.06.1198

Glucksman, E., Bell, T., Griffiths, R. I., & Bass, D. (2010). Closely related protist strains have different grazing impacts on natural bacterial communities. *Environmental Microbiology*, *12*(12), 3105–3113. https://doi.org/10.1111/j.1462-2920.2010.02283.x

Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., Boutte, C., Burgaud, G., de Vargas, C., Decelle, J., del Campo, J., Dolan, J. R., Dunthorn, M., Edvardsen, B., Holzmann, M., Kooistra, W. H., Lara, E., le Bescot, N., Logares, R., … Christen, R. (2013). The Protist ribosomal reference database (PR2): A catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. *Nucleic Acids Research*, *41*(Database issue), D597–D604. https://doi.org/10.1093/nar/gks1160

Guo, S., Tao, C., Jousset, A., Xiong, W., Wang, Z., Shen, Z., Wang, B., Xu, Z., Gao, Z., Liu, S., Li, R., Ruan, Y., Shen, Q., Kowalchuk, G. A., & Geisen, S. (2022). Trophic interactions between predatory protists and pathogen-suppressive bacteria impact plant health. *The ISME Journal*, *16*(8), 1932–1943. https://doi.org/10.1038/s41396-022-01244-5

Gustavs, L., Eggert, A., Michalik, D., & Karsten, U. (2010). Physiological and biochemical responses of green microalgae from different habitats to osmotic and matric stress. *Protoplasma*, *243*(1–4), 3–14. https://doi.org/10.1007/s00709-009-0060-9

Gustavs, L., Gors, M., & Karsten, U. (2011). Polyol patterns in biofilm-forming Aeroterrestrial green algae (Trebouxiophyceae, Chlorophyta)(1). *Journal of Phycology*, *47*(3), 533–537. https://doi.org/10.1111/j.1529-8817.2011.00979.x

Heger, T. J., Edgcomb, V. P., Kim, E., Lukes, J., Leander, B. S., & Yubuki, N. (2014). A resurgence in field research is essential to better understand the diversity, ecology, and evolution of microbial eukaryotes. *Journal of Eukaryotic Microbiology*, *61*(2), 214–223. https://doi.org/10.1111/jeu.12095

Hess, S., & Melkonian, M. (2013). The mystery of clade X: *Orciraptor* gen. Nov. and *Viridiraptor* gen. Nov. are highly specialised, algivorous amoeboflagellates (Glissomonadida, Cercozoa). *Protist*, *164*(5), 706–747. https://doi.org/10.1016/j.protis.2013.07.003

Hess, S., & Suthaus, A. (2022). The Vampyrellid Amoebae (Vampyrellida, Rhizaria). *Protist*, *173*(1), 125854. https://doi.org/10.1016/j.protis.2021.125854

Janik, P., Lado, C., & Ronikier, A. (2020). Range-wide Phylogeography of a Nivicolous Protist Didymium nivicola Meyl. (Myxomycetes, Amoebozoa): Striking contrasts between the northern and the southern hemisphere. *Protist*, *171*(6), 125771. https://doi.org/10.1016/j.protis.2020.125771

Jassey, V. E., Signarbieux, C., Hattenschwiler, S., Bragazza, L., Buttler, A., Delarue, F., … Robroek, B. J. (2015). An unexpected role for mixotrophs in the response of peatland carbon cycling to climate warming. *Scientific Reports*, *5*, 16931. https://doi.org/10.1038/srep16931

Jassey, V. E. J., Lamentowicz, M., Bragazza, L., Hofsommer, M. L., Mills, R. T. E., Buttler, A., Signarbieux, C., & Robroek, B. J. M. (2016). Loss of testate amoeba functional diversity with increasing frost intensity across a continental gradient reduces microbial activity in peatlands. *European Journal of Protistology*, *55*, 190–202. https://doi.org/10.1016/j.ejop.2016.04.007

Jassey, V. E. J., Meyer, C., Dupuy, C., Bernard, N., Mitchell, E. A. D., Toussaint, M.-L., Metian, M., Chatelain, A. P., & Gilbert, D. (2013). To what extent do food preferences explain the trophic position of heterotrophic and mixotrophic microbial consumers in a *sphagnum* peatland? *Microbial Ecology*, *66*(3), 571–580. https://doi.org/10.1007/s00248-013-0262-8

Jassey, V. E. J., Shimano, S., Dupuy, C., Toussaint, M.-L., & Gilbert, D. (2012). Characterizing the feeding habits of the testate amoebae *Hyalosphenia papilio* and *Nebela tincta* along a narrow "fen-bog" gradient using digestive vacuole content and 13C and 15N isotopic analyses. *Protist*, *163*(3), 451–464. https://doi.org/10.1016/j.protis.2011.07.006

Jassey, V. E. J., Walcker, R., Kardol, P., Geisen, S., Heger, T., Lamentowicz, M., Hamard, S., & Lara, E. (2022). Contribution of soil algae to the global carbon cycle. *The New Phytologist*, *234*(1), 64–76. https://doi.org/10.1111/nph.17950

Jiang, Y., Luan, L., Hu, K., Liu, M., Chen, Z., Geisen, S., Chen, X., Li, H., Xu, Q., Bonkowski, M., & Sun, B. (2020). Trophic interactions as determinants of the arbuscular mycorrhizal fungal community with cascading plant-promoting consequences. *Microbiome*, *8*(1), 142. https://doi.org/10.1186/s40168-020-00918-6

Jousset, A. (2017). Application of Protists to improve plant growth in sustainable agriculture. In S. Mehnaz (Ed.), *Rhizotrophs: Plant growth promotion to bioremediation* (pp. 263–273). Springer Singapore.

Jousset, A., Bienhold, C., Chatzinotas, A., Gallien, L., Gobet, A., Kurm, V., Küsel, K., Rillig, M. C., Rivett, D. W., Salles, J. F., van der Heijden, M. G. A., Youssef, N. H., Zhang, X., Wei, Z., & Hol, W. H. (2017). Where less may be more: How the rare biosphere pulls ecosystems strings. *The ISME Journal*, *11*(4), 853–862. https://doi.org/10.1038/ismej.2016.174

Keeling, P. J., & Burki, F. (2019). Progress towards the tree of eukaryotes. *Current Biology*, *29*(16), R808–R817. https://doi.org/10.1016/j.cub.2019.07.031

Koenig, I., Mulot, M., & Mitchell, E. A. D. (2018). Taxonomic and functional traits responses of sphagnum peatland testate amoebae to experimentally manipulated water table. *Ecological Indicators*, *85*, 342–351. https://doi.org/10.1016/j.ecolind.2017.10.017

Kosakyan, A., Mulot, M., Mitchell, E. A., & Lara, E. (2015). Environmental DNA COI barcoding for quantitative analysis of protists communities: A test using the Nebela collaris complex (Amoebozoa; Arcellinida; Hyalospheniidae). *European Journal of Protistology*, *51*(4), 311–320. https://doi.org/10.1016/j.ejop.2015.06.005

Lara, E., & Belbahri, L. (2011). SSU rRNA reveals major trends in oomycete evolution. *Fungal Diversity*, *49*(1), 93–100. https://doi.org/10.1007/s13225-011-0098-9

Lara, E., Roussel-Delif, L., Fournier, B., Wilkinson, D. M., & Mitchell, E. A. D. (2016). Soil microorganisms behave like macroscopic organisms: Patterns in the global distribution of soil euglyphid testate amoebae. *Journal of Biogeography*, *43*(3), 520–532. https://doi.org/10.1111/jbi.12660

Lara, E., Singer, D., & Geisen, S. (2022). Discrepancies between prokaryotes and eukaryotes need to be considered in soil DNA-based studies. *Environmental Microbiology*, *24*(9), 3829–3839.

Lentendu, G., Lara, E., & Geisen, S. (2023). Metabarcoding approaches for soil eukaryotes, protists, and microfauna. In F. Martin & S. Uroz (Eds.), *Microbial environmental genomics (MEG)* (pp. 1–16). Springer US.

Lynch, M. D., & Neufeld, J. D. (2015). Ecology and exploration of the rare biosphere. *Nature Reviews. Microbiology*, *13*(4), 217–229. https://doi.org/10.1038/nrmicro3400

Mahe, F., Czech, L., Stamatakis, A., Quince, C., de Vargas, C., Dunthorn, M., & Rognes, T. (2021). Swarm v3: Towards tera-scale amplicon clustering. *Bioinformatics*, *38*(1), 267–269. https://doi.org/10.1093/bioinformatics/btab493

Mahe, F., de Vargas, C., Bass, D., Czech, L., Stamatakis, A., Lara, E., … Dunthorn, M. (2017). Parasites dominate hyperdiverse soil protist communities in Neotropical rainforests. *Nat Ecol Evol*, *1*(4), 91. https://doi.org/10.1038/s41559-017-0091

Mahe, F., Rognes, T., Quince, C., de Vargas, C., & Dunthorn, M. (2014). Swarm: Robust and fast clustering method for amplicon-based studies. *PeerJ*, *2*, e593. https://doi.org/10.7717/peerj.593

Maier, S., Tamm, A., Wu, D., Caesar, J., Grube, M., & Weber, B. (2018). Photoautotrophic organisms control microbial abundance, diversity, and physiology in different types of biological soil crusts. *The ISME Journal*, *12*(4), 1032–1046. https://doi.org/10.1038/s41396-018-0062-8

Mazel, F., Malard, L., Niculita-Hirzel, H., Yashiro, E., Mod, H. K., Mitchell, E. A. D., Singer, D., Buri, A., Pinto, E., Guex, N., Lara, E., & Guisan, A. (2022). Soil protist function varies with elevation in the Swiss Alps. *Environmental Microbiology*, *24*(4), 1689–1702. https://doi.org/10.1111/1462-2920.15686

Meyer, C., Desalme, D., Bernard, N., Binet, P., Toussaint, M.-L., & Gilbert, D. (2013). Using testate amoeba as potential biointegrators of atmospheric deposition of phenanthrene (polycyclic aromatic hydrocarbon) on "moss/soil interface-testate amoeba community" microecosystems. *Ecotoxicology*, *22*(2), 287–294. https://doi.org/10.1007/s10646-012-1025-x

Mitchell, E. A. D. (2015). Pack hunting by minute soil testate amoebae: Nematode hell is a naturalist's paradise. *Environmental Microbiology*, *17*(11), 4145–4147. https://doi.org/10.1111/1462-2920.13025

Mitra, A., Flynn, K. J., Tillmann, U., Raven, J. A., Caron, D., Stoecker, D. K., Not, F., Hansen, P. J., Hallegraeff, G., Sanders, R., Wilken, S., McManus, G., Johnson, M., Pitta, P., Våge, S., Berge, T., Calbet, A., Thingstad, F., Jeong, H. J., … Lundgren, V. (2016). Defining planktonic Protist functional groups on mechanisms for energy and nutrient acquisition: Incorporation of diverse Mixotrophic strategies. *Protist*, *167*(2), 106–120. https://doi.org/10.1016/j.protis.2016.01.003

Nazem-Bokaee, H., Hom, E. F. Y., Warden, A. C., Mathews, S., & Gueidan, C. (2021). Towards a systems biology approach to understanding the lichen Symbiosis: Opportunities and challenges of implementing network modelling. *Frontiers in Microbiology*, *12*, 667864. https://doi.org/10.3389/fmicb.2021.667864

Oliverio, A. M., Geisen, S., Delgado-Baquerizo, M., Maestre, F. T., Turner, B. L., & Fierer, N. (2020). The global-scale distributions of soil protists and their contributions to belowground systems. *Science Advances*, *6*(4), eaax8787. https://doi.org/10.1126/sciadv.aax8787

Petz, W., Foissner, W., Wirnsberger, E., Krautgartner, W. D., & Adam, H. (1986). Mycophagy, a new feeding strategy in autochthonous soil ciliates. *Naturwissenschaften*, *73*(9), 560–562. https://doi.org/10.1007/bf00368169

Pinseel, E., Janssens, S. B., Verleyen, E., Vanormelingen, P., Kohler, T. J., Biersma, E. M., Sabbe, K., van de Vijver, B., & Vyverman, W. (2020). Global radiation in a rare biosphere soil diatom. *Nature Communications*, *11*(1), 2382. https://doi.org/10.1038/s41467-020-16181-0

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., & Glockner, F. O. (2007). SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research*, *35*(21), 7188–7196. https://doi.org/10.1093/nar/gkm864

Rai, M., Abd-Elsalam, K. A., & Ingle, A. P. (2020). *Pythium: Diagnosis, diseases and management*. CRC Press.

Ramirez, K. S., Geisen, S., Morrien, E., Snoek, B. L., & van der Putten, W. H. (2018). Network analyses can advance above-belowground ecology. *Trends in Plant Science*, *23*(9), 759–768. https://doi.org/10.1016/j.tplants.2018.06.009

Ramirez, K. S., Knight, C. G., de Hollander, M., Brearley, F. Q., Constantinides, B., Cotton, A., Creer, S., Crowther, T. W., Davison, J., Delgado-Baquerizo, M., Dorrepaal, E., Elliott, D. R., Fox, G., Griffiths, R. I., Hale, C., Hartman, K., Houlden, A., Jones, D. L., Krab, E. J., … de Vries, F. T. (2018). Detecting macroecological patterns in bacterial communities across independent studies of global soils. *Nature Microbiology*, *3*(2), 189–196. https://doi.org/10.1038/s41564-017-0062-x

Reczuga, M. K., Lamentowicz, M., Mulot, M., Mitchell, E. A. D., Buttler, A., Chojnicki, B., Słowiński, M., Binet, P., Chiapusio, G., Gilbert, D., Słowińska, S., & Jassey, V. E. J. (2018). Predator–prey mass ratio drives microbial activity under dry conditions in sphagnum peatlands. *Ecology and Evolution*, *8*(11), 5752–5764. https://doi.org/10.1002/ece3.4114

Reczuga, M. K., Seppey, C. V. W., Mulot, M., Jassey, V. E. J., Buttler, A., Słowińska, S., Słowiński, M., Lara, E., Lamentowicz, M., & Mitchell, E. A. D. (2020). Assessing the responses of Sphagnum micro-eukaryotes to climate changes using high throughput sequencing. *PeerJ*, *8*, e9821. https://doi.org/10.7717/peerj.9821

Reeder, J., & Knight, R. (2009). The 'rare biosphere': A reality check. *Nature Methods*, *6*(9), 636–637. https://doi.org/10.1038/nmeth0909-636

Rueckert, S., Betts, E. L., & Tsaousis, A. D. (2019). The symbiotic Spectrum: Where do the gregarines fit? *Trends in Parasitology*, *35*(9), 687–694. https://doi.org/10.1016/j.pt.2019.06.013

Santos, S. S., Nielsen, T. K., Hansen, L. H., & Winding, A. (2015). Comparison of three DNA extraction methods for recovery of soil protist DNA. *Journal of Microbiological Methods*, *115*, 13–19. https://doi.org/10.1016/j.mimet.2015.05.011

Santos, S. S., Nunes, I., Nielsen, T. K., Jacquiod, S., Hansen, L. H., & Winding, A. (2017). Soil DNA extraction procedure influences Protist 18S rRNA Gene Community profiling outcome. *Protist*, *168*(3), 283–293. https://doi.org/10.1016/j.protis.2017.03.002

Schiaffino, M. R., Lara, E., Fernandez, L. D., Balague, V., Singer, D., Seppey, C. C., Massana, R., Izaguirre, I., & Izaguirre, I. (2016). Microbial eukaryote communities exhibit robust biogeographical patterns along a gradient of Patagonian and Antarctic lakes. *Environmental Microbiology*, *18*(12), 5249–5264. https://doi.org/10.1111/1462-2920.13566

Schuldt, A., Assmann, T., Brezzi, M., Buscot, F., Eichenberg, D., Gutknecht, J., Härdtle, W., He, J. S., Klein, A. M., Kühn, P., Liu, X., Ma, K., Niklaus, P. A., Pietsch, K. A., Purahong, W., Scherer-Lorenzen, M., Schmid, B., Scholten, T., Staab, M., … Bruelheide, H. (2018). Biodiversity across trophic levels drives multifunctionality in highly diverse forests. *Nature Communications*, *9*(1), 2989. https://doi.org/10.1038/s41467-018-05421-z

Selosse, M. A., Charpin, M., & Not, F. (2017). Mixotrophy everywhere on land and in water: The grand ecart hypothesis. *Ecology Letters*, *20*(2), 246–263. https://doi.org/10.1111/ele.12714

Seppey, C. V. W., Singer, D., Dumack, K., Fournier, B., Belbahri, L., Mitchell, E. A. D., & Lara, E. (2017). Distribution patterns of soil microbial eukaryotes suggests widespread algivory by phagotrophic protists as an alternative pathway for nutrient cycling. *Soil Biology and Biochemistry*, *112*(Supplement C), 68–76. https://doi.org/10.1016/j.soilbio.2017.05.002

Shade, A. (2017). Diversity is the question, not the answer. *The ISME Journal*, *11*(1), 1–6. https://doi.org/10.1038/ismej.2016.118

Singer, D., Duckert, C., Heděnec, P., Lara, E., Hiltbrunner, E., & Mitchell, E. A. D. (2020). High-throughput sequencing of litter and moss eDNA reveals a positive correlation between the diversity of Apicomplexa and their invertebrate hosts across alpine habitats. *Soil Biology and Biochemistry*, *147*, 107837. https://doi.org/10.1016/j.soilbio.2020.107837

Singer, D., Kosakyan, A., Seppey, C. V. W., Pillonel, A., Fernandez, L. D., Fontaneto, D., … Lara, E. (2018). Environmental filtering and phylogenetic clustering correlate with the distribution patterns of cryptic protist species. *Ecology*, *99*(4), 904–914. https://doi.org/10.1002/ecy.2161

Singer, D., Metz, S., Unrein, F., Shimano, S., Mazei, Y., Mitchell, E. A. D., & Lara, E. (2019). Contrasted micro-eukaryotic diversity associated with sphagnum mosses in tropical, subtropical and temperate climatic zones. *Microbial Ecology*, *78*(3), 714–724. https://doi.org/10.1007/s00248-019-01325-7

Singer, D., Mitchell, E. A. D., Payne, R. J., Blandenier, Q., Duckert, C., Fernández, L. D., Fournier, B., Hernández, C. E., Granath, G., Rydin, H., Bragazza, L., Koronatova, N. G., Goia, I., Harris, L. I., Kajukało, K., Kosakyan, A., Lamentowicz, M., Kosykh, N. P., Vellak, K., & Lara, E. (2019). Dispersal limitations and historical factors determine the biogeography of specialized terrestrial protists. *Molecular Ecology*, *28*(12), 3089–3100. https://doi.org/10.1111/mec.15117

Singer, D., Seppey, C. V. W., Lentendu, G., Dunthorn, M., Bass, D., Belbahri, L., Blandenier, Q., Debroas, D., de Groot, G. A., de Vargas, C., Domaizon, I., Duckert, C., Izaguirre, I., Koenig, I., Mataloni, G., Schiaffino, M. R., Mitchell, E. A. D., Geisen, S., & Lara, E. (2021). Protist taxonomic and functional diversity in soil, freshwater and marine ecosystems. *Environment International*, *146*, 106262. https://doi.org/10.1016/j.envint.2020.106262

Taylor, F. J. (2003). The collapse of the two-kingdom system, the rise of protistology and the founding of the International Society for Evolutionary Protistology (ISEP). *International Journal of Systematic and Evolutionary Microbiology*, *53*(Pt 6), 1707–1714. https://doi.org/10.1099/ijs.0.02587-0

Thakur, M. P., & Geisen, S. (2019). Trophic regulations of the soil microbiome. *Trends in Microbiology*, *27*(9), 771–780. https://doi.org/10.1016/j.tim.2019.04.008

Vaulot, D., Geisen, S., Mahe, F., & Bass, D. (2022). pr2-primers: An 18S rRNA primer database for protists. *Molecular Ecology Resources*, *22*(1), 168–179. https://doi.org/10.1111/1755-0998.13465

Verni, F., & Gualtieri, P. (1997). Feeding behaviour in ciliated protists. *Micron*, *28*(6), 487–504. https://doi.org/10.1016/s0968-4328(97)00028-0

Vos, M., Wolf, A. B., Jennings, S. J., & Kowalchuk, G. A. (2013). Micro-scale determinants of bacterial diversity in soil. *FEMS Microbiology Reviews*, *37*(6), 936–954. https://doi.org/10.1111/1574-6976.12023

WHO. (2021). World Malaria Report 2021. Retrieved from. Switzerland:

Wu, C., Chao, Y., Shu, L., & Qiu, R. (2022). Interactions between soil protists and pollutants: An unsolved puzzle. *Journal of Hazardous Materials*, *429*, 128297. https://doi.org/10.1016/j.jhazmat.2022.128297

Xiong, W., Li, R., Guo, S., Karlsson, I., Jiao, Z., Xun, W., Kowalchuk, G. A., Shen, Q., & Geisen, S. (2019). Microbial amendments alter protist communities within the soil microbiome. *Soil Biology and Biochemistry*, *135*, 379–382. https://doi.org/10.1016/j.soilbio.2019.05.025

How to cite this article: Geisen, S., Lara, E., & Mitchell, E. (2023). Contemporary issues, current best practice and ways forward in soil protist ecology. *Molecular Ecology Resources*, *00*, 1–11. https://doi.org/10.1111/1755-0998.13819