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Review Article

REVIEW ARTICLE

# Soil protists: a fertile frontier in soil biology research

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**One sentence summary:** We provide an overview of what soil protists are, their morphological and taxonomical diversity, their functional importance in soils and especially for plant performance, how they respond to human impact and how they may be applied as bioindicators and in agriculture.
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## ABSTRACT

Protists include all eukaryotes except plants, fungi and animals. They are an essential, yet often forgotten, component of the soil microbiome. Method developments have now furthered our understanding of the real taxonomic and functional diversity of soil protists. They occupy key roles in microbial foodwebs as consumers of bacteria, fungi and other small eukaryotes. As parasites of plants, animals and even of larger protists, they regulate populations and shape communities. Pathogenic forms play a major role in public health issues as human parasites, or act as agricultural pests. Predatory soil protists release nutrients enhancing plant growth. Soil protists are of key importance for our understanding of eukaryotic evolution and microbial biogeography. Soil protists are also useful in applied research as bioindicators of soil quality, as

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models in ecotoxicology and as potential biofertilizers and biocontrol agents. In this review, we provide an overview of the enormous morphological, taxonomical and functional diversity of soil protists, and discuss current challenges and opportunities in soil protistology. Research in soil biology would clearly benefit from incorporating more protistology alongside the study of bacteria, fungi and animals.

**Keywords:** Biogeography; Functional diversity; Plant performance; Soil food web; Soil microbiome; Taxonomic diversity

## INTRODUCTION

### Protist diversity and functional roles in ecosystems

Protists constitute the invisible majority of eukaryotes (Fig. 1). They include all eukaryotes outside land plants (Embryophyta), animals and arguably Fungi. They are predominantly unicellular, and span the entire eukaryotic tree of life (Adl *et al.* 2012; Fig. 1). Their mophological and lifestyle diversity is immense. Protists range from 'picoeukaryotic' taxa that are smaller than many bacteria (Moon-van der Staay, De Wachter and Vaulot 2001; Caron *et al.* 2009; Not *et al.* 2009) to plasmodium-forming slime mold taxa and marine green algae in the genus *Caulerpa* that form the largest single celled organisms on the planet and several meters large multicellular brown algae (kelps). Protists also include flexible-bodied 'naked amoeboid' or armoured forms (e.g. diatoms, testate amoebae). They can be both photoautotrophs ('algae'), heterotrophs ('protozoa')—or mixotrophic, obtaining carbon both photoautotrophically and heterotrophically (Geisen and Bonkowski 2017; Fig. 2). Many protists live as mutualistic or parasitic symbionts with animals, plants, fungi and other protists, or host ectosymbiotic and/or endosymbiotic prokaryotes (de Vargas *et al.* 2015; Fig. 2b).

Protists are present in all biomes on Earth including extreme environments such as those with low or high pH values, low or high temperature and salt stress (Petz 1997; Rodriguez-Zaragoza and Garcia 1997; Amaral Zettler *et al.* 2002; Rodriguez-Zaragoza, Mayzlish and Steinberger 2005; De Jonckheere 2006; Geisen *et al.* 2015a; Shmakova, Bondarenko and Smirnov 2016). Their numbers commonly reach tens of thousands of individuals per gram of bulk soil (Finlay 2002; Geisen *et al.* 2014) or per millilitre in aquatic systems (de Vargas *et al.* 2015). Their diversity and community structure vary among habitats, and, therefore, the community structure of protists particularly in soils provides a valuable indication of environmental conditions (Foissner 1997; Payne 2013).

Soil protist diversity has long been underestimated, but methodological advances, such as in environmental DNA isolation and ultra-deep high-throughput sequencing are revealing a diversity that has been qualified 'near imponderable' (Foissner 1999c; Mahé *et al.* 2017; Box 1). For example, the total plankton diversity in the euphotic zone of the world's oceans has been estimated up to about 150,000 operational taxonomic units (OTUs) based on 18S rRNA gene sequences (de Vargas *et al.* 2015). Soils host a different and, possibly, even higher protist diversity than aquatic ecosystems, but this diversity is still mostly unknown (Grossmann *et al.* 2016; Mahé *et al.* 2017).

Protists are also highly relevant for human health and economy (Box 2). Among them, we find several devastating human pathogens such as the malaria-causing agent *Plasmodium falciparum*, and intestinal parasites such as *Giardia duodenalis* and *Entamoeba histolytica*. There are, however, also symbiotic protists that are part of the human gut microbiome, such as *Blastocystis* or *Dientamoeba* (Parfrey *et al.* 2014; Scanlan *et al.* 2014). Protist pathogens are present in the soil environment as propagules, and other, non-pathogenic soil protists may act as vectors of human and animal pathogens (Box 2). Some protists can cause severe economic damage as plant pests. Notably, the oomycete *Phytophtora infestans* is held responsible for the Irish famine in the nineteenth century that lead to millions of deaths and provoked a massive exodus towards North- and South America, and Australia (Kinealy 1994). Other than oomycetes, plant-pathogenic protists include rhizarians such as plasmodiophorids and zoosporic Fungi, all of which occur in soil environments (Geisen *et al.* 2015c, Figs. 2b and 4e).

Less well-known than their pathogenic relatives, free-living protists are also of major importance for ecosystem stability and as providers of ecosystem services. Their predatory action releases nutrients from their prey, thereby playing a major role in soil fertility (Clarholm 1985; Bonkowski 2004; Fig. 4a); they also control microbial populations through predation (including bacteria, archaea, but also fungi and algae), thus influencing indirectly the functioning of those in ecosystems (Figs. 4b-f).

In this review, we provide an overview on the current state of knowledge on soil protists. We emphasize new methodological advances that will provide a better understanding of their taxonomic diversity in soils. We review the impact of (a)biotic factors on protist individuals and communities, and discuss how they respond or adapt to these drivers. We further describe the functional importance of soil protists, their role for ecosystem functioning and the services they provide. Finally, we highlight their potential for use in bio-indication and bio-engineering, and we point out research gaps and future needs for soil protistology research.

### Protist classification: a changing perspective

Until the end of the 20th century, protist taxonomy and broad classification were based primarily on their modes of nutrition and their overall morphology. The nutrition mode was used to distinguish photoautotrophs ('algae') from heterotrophs ('protozoa' and 'lower fungi,' *pro parte*) that primarily adopt phagocytosis to ingest and digest other microorganisms. Fungus-like protists included organisms that use absorptive nutrition. This may be active by secretion of extracellular digestive enzymes to hydrolyze organic macromolecules followed by absorbtion of the monomeric products (lysotrophy; Zuck 1953), or passive (osmotrophy) by absorbtion of already monomeric organic compounds from the environment (Spiegel 2016). Algae and 'lower fungi' were subjects of subdisciplines of botany, whereas protozoa (ciliates, flagellates and amoebae, depending on their locomotion type) were associated with zoology, and each grouping was classified according to its disciplinary affiliations (Lahr, Lara and Mitchell 2012).

Advances in light and electron microscopy, molecular phylogenetic/-genomic studies of cultured protists, protist sequences obtained through culture-independent approaches, and the development of newer paradigms for phylogenetic analyses over the last 50 years (Box 1) have allowed us to gain a much more accurate picture of protist evolution and diversity

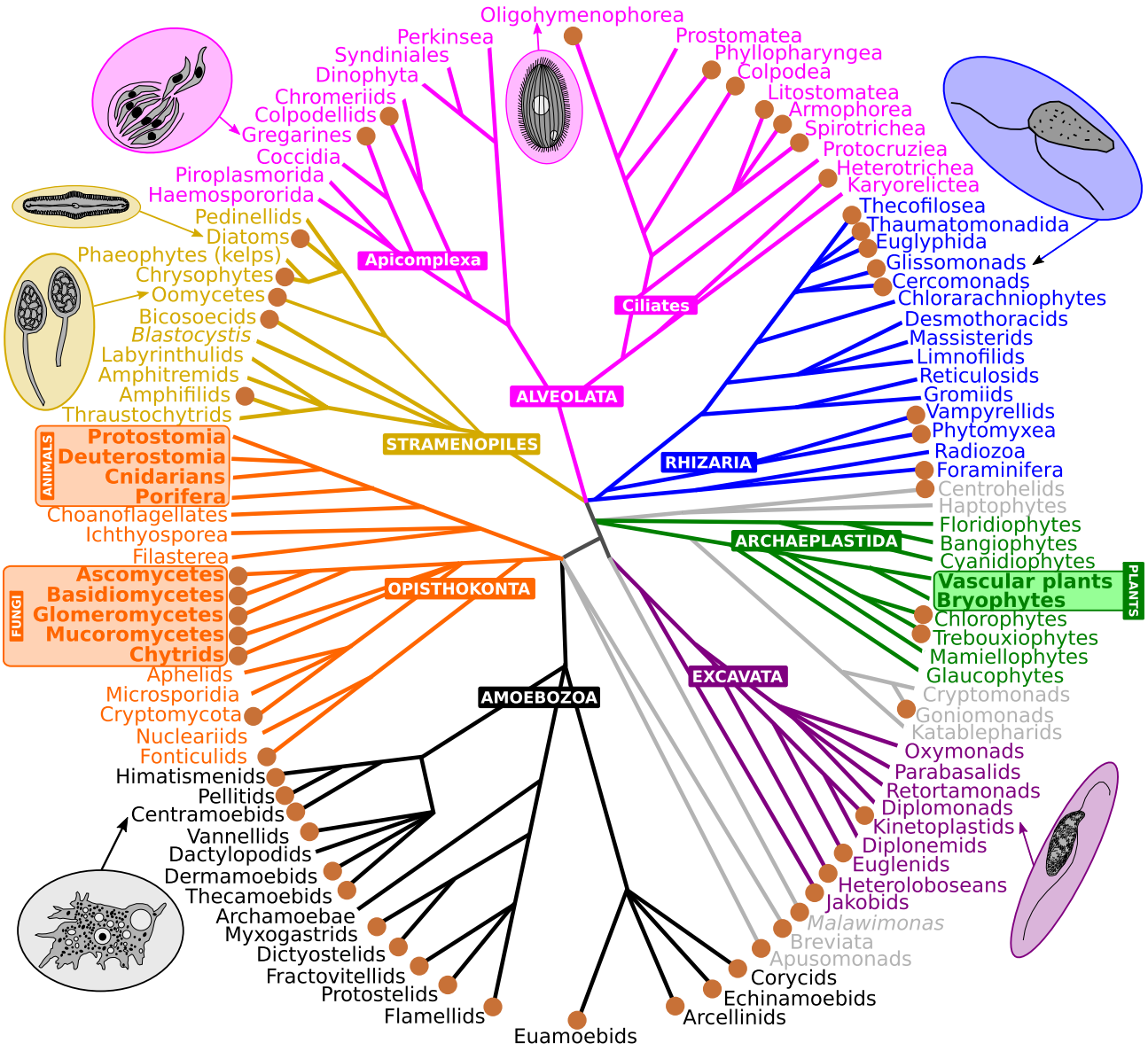


**Figure 1.** Schematic representation of the tree of eukaryotes. Brown dots represent groups that are particularly diverse and abundant in soils. Multicellular, non-protist taxa are highlighted in boxes. Groups whose affiliation is still subject to discussion are shown in light grey.

(Fig. 1). This increase in knowledge is reflected in the regular revision of protozoan, and more recently general eukaryote classification carried out by the International Society of Protistologists (née Society of Protozoologists). The evolution of protist classification can be followed through the publications Honigberg *et al.* (1964), Levine *et al.* (1980), and Adl *et al.* (2012), where a gradual change in protist classification from classical typological thinking to data-based modern systematics can be witnessed. The recent trends in modern systematics are supported by a more robust classification of protists (Fig. 1). This has suggested that the eukaryotic tree is composed of several supergroups, including Amoebozoa, Obazoa (which includes Opisthokonta), Archaeplastida (including Cryptista; Burki *et al.* 2016), SAR (which includes Stramenopila, Alveolata, and Rhizaria), and Excavata (Adl *et al.* 2012). In addition, a few species-poor lineages form deep nodes within the eukaryotic tree of life (Pawlowski 2013).

**Box 1:** METHODS USED TO STUDY SOIL PROTIST DIVERSITY

There are various methods available to study the diversity and abundance of soil protists (Geisen and Bonkowski 2017). Mainly in the past, direct observation and culture methods were applied (Ekelund 2002; Smirnov and Brown 2004; Adl, Coleman and Read 2006; Wilkinson and Mitchell 2010; Geisen and Bonkowski 2017). Although molecular methods are now preferred, the more traditional approaches are still useful to obtain quantitative estimates of soil protists (Acosta-Mercado and Lynn 2003), to help DNA barcoding of morphologically identified taxa (e.g. Kosakyan *et al.* 2013), or to obtain pure cultures of novel taxa (Blandenier *et al.* 2017).

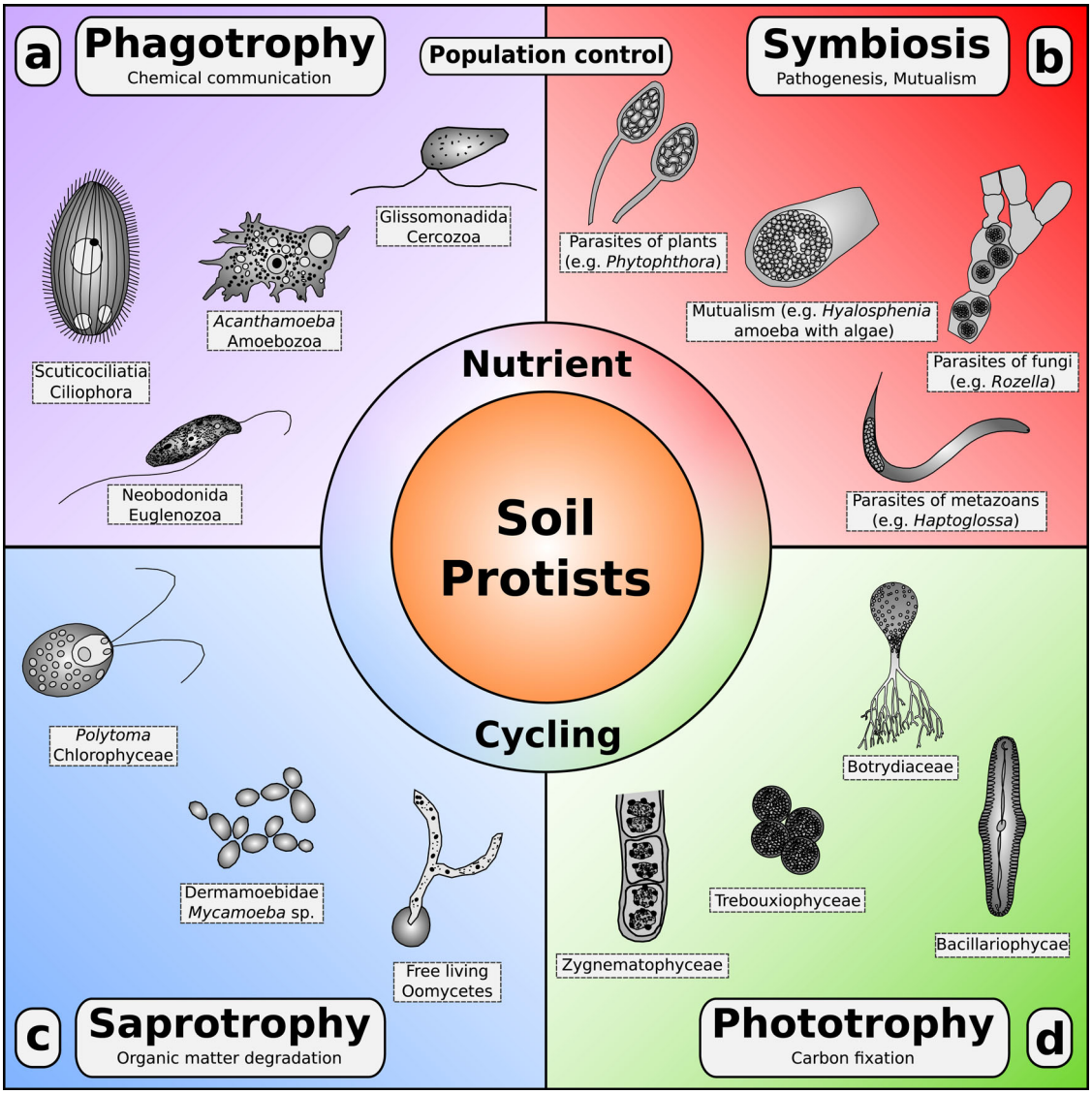


**Figure 2.** Overview of protist functional/ecological versatility. (**a**) Many soil protists are unicellular phagotrophs feeding on bacteria, whereas some feed on fungi, other protists and animals; thereby they affect soil biodiversity and chemically interact or communicate with other soil organisms; (**b**) some protists live in symbiosis, including parasitism, commensalism and mutualism, with fungi, other protists, plants and animals; this also affects soil biodiversity, but also plants and aboveground animals; (**c**) some soil protists, such as oomycetes, can participate as saprotrophs in organic matter degradation; (**d**) some soil protists contain chlorophyll (they are usually called algae), and can be phototrophic or mixotrophic. All functional groups of soil protists provide key roles for nutrient cycling in soils. Note: All illustrated protists are common soil inhabitants.

Sequencing of DNA isolated from environmental samples (often called *environmental* DNA), either of pre-amplified taxonomically conserved marker regions or of total DNA, has become the standard for soil microbial diversity assessments (Prosser 2015). Methods based on amplicon cloning and sequencing using the Sanger method were rarely directly applied to the study of soil protist diversity, primarily due to the over-dominance of fungal sequences, rendering it impossible to study protist communities at a reasonable cost (Lesaulnier *et al.* 2008). The first screenings focused on and revealed considerable protist diversity in aquatic systems including marine (López-García *et al.* 2001; Moon-van der Staay, De Wachter and Vaulot 2001), freshwater including extreme habitats (Amaral Zettler *et al.* 2002) and anoxic sediments (Dawson and Pace 2002). Soil eukaryotic molecular diversity remained virtually unknown. Group-specific approaches were later applied to soils and revealed a wealth of new forms and deep-branching clades such as in Kinetoplastida (Rasmussen *et al.* 2001), Cercozoa (Bass and Cavalier-Smith 2004; Bass *et al.* 2016), Ciliophora (Lara *et al.* 2007b), Myxomycetes (Fiore-Donno *et al.* 2016), Foraminifera (Lejzerowicz *et al.* 2010) and euglyphid testate amoebae (Lara *et al.* 2016).

High-throughput sequencing approaches now allow the retrieval of a much broader range of the soil protist diversity (Bates *et al.* 2013; Geisen *et al.* 2015c; Geisen 2016a; Mahé *et al.* 2017). Although these approaches are increasingly being used, they do have important shortcomings and unbiased methods capable of targeting the entire diversity of soil protists do not exist (yet). Considerable developments remain to be done with respect to sequencing technology (i.e. obtaining longer amplicons), the choice of appropriate primers for protist markers (the quest for the truly 'universal primers' if such a thing is possible at all). In general, the improvement of DNA-based methods to

study diversity should aim at avoiding or at least controlling biases during amplification, or perform sequencing without amplification at all (Wintzingerode, Göbel and Stackebrandt 1997; Geisen *et al.* 2015c; Geisen and Bonkowski 2017). Protist genetic markers that are most frequently targeted include parts of the gene for the 18S ribosomal RNA, such as the hyper-variable V4 or V9 regions (Adl, Habura and Eglit 2014; Hu *et al.* 2015; Mahé *et al.* 2017). The V4 region has the advantage of containing more phylogenetic information that can be used to infer phylogenetic trees (Pawlowski *et al.* 2012; Dunthorn *et al.* 2014), and there are more references in available databases such as the commonly used Silva (Pruesse *et al.* 2007) or the Protist Ribosomal Reference database (PR$^2$) (Guillou *et al.* 2013). By contrast, the V9 region is shorter and therefore more suitable for ultra-deep short-read sequencing approaches using, e.g. Illumina HiSeq. The V9 region has a less variable sequence length among protists than the V4. As longer sequences are less well-represented in ultra-high sequencing approaches, protist diversity might be more reliably represented when targeting the V9 compared with the V4 region. An added advantage of the V9 region is it being close to the Internal Transcribed Spacer (ITS), a gene region preferentially used for fungal molecular taxonomy (Schoch *et al.* 2012; Tedersoo *et al.* 2016).

An additional problem for 18S rRNA gene-based diversity approaches of protists is that introns or other insertions may be present both in the V4 and V9 regions. Examples include the testate amoeba *Difflugia bacillariarum*, which possesses an intron of 427 bp disrupting the V9 region (Gomaa *et al.* 2012); and many glissomonads, a common group of soil phagotrophs, that possess a ca. 600 bp class I intron in the V9 region (Ekelund, Daugbjerg and Fredslund 2004; Howe *et al.* 2009). Frequent insertions occur in the V4 region of the SSU rRNA, as well (Torres-Machorro *et al.* 2010). The diversity of protists is such that it is highly unlikely with a single primer pair to amplify the whole range of species (Pawlowski *et al.* 2012). In-silico experiments showed strong bias in what is amplified depending on the choice of primer pairs (Adl, Habura and Eglit 2014). Researchers are thus left with a trade-off: 1) a more complete coverage of most protist taxa simultaneously, but with some groups being missed, or 2) using several group-specific primers to target more specifically some groups (Lentendu *et al.* 2014), but at a higher cost per sample and an incomplete coverage of the overall protist diversity.

Besides these technical considerations—and we would argue even more so -, a major obstacle in the interpretation of high throughput based environmental DNA studies is the biological interpretation of the sequence data. Indeed, many sequences are currently impossible to assign with confidence to any known group, and are generally mentioned in the literature as 'other eukaryotes'. They can sometimes represent a significant proportion of all reads (Li *et al.* 2016; Mahé *et al.* 2017; Seppey *et al.* 2017). Consequently, our interpretation of the massively produced data is, at best, systematically incomplete (Geisen 2016a). There is therefore an important need for a massive effort in descriptive research, which would provide genetic, but also morphological and functional information on living protists (Heger *et al.* 2014; Mitchell 2015; Geisen 2016a; Geisen and Bonkowski 2017).

## SOIL PROTISTS: MORPHOLOGICAL DIVERSITY AND CLASSIFICATION

### Morphological and functional diversity of soil protists

The morphological and functional diversity of soil protists is immense. Soil protists span at least six orders of magnitude in length (Geisen *et al.* 2017), from a few micrometers, such as *Mycamoeba* (Blandenier *et al.* 2017), to cells reaching more than 1 millimeter, such as the extremely thin and ramified *Darbyshirella* (Berney *et al.* 2015). Both autotrophic and heterotrophic protists have a fundamental importance in food webs. Photosynthetic protists may provide an important carbon input in soils (Schmidt, Dyckmans and Schrader 2016; Seppey *et al.* 2017), although the magnitude of their carbon fixation has not been quantified. Heterotrophic phagotrophic protists release nutrients via microbial predation, which are then made available to plants, thus stimulating growth (Clarholm 1985b; Hunt *et al.* 1987; de Ruiter, Neutel and Moore 1995; Bonkowski and Clarholm 2012). Fungi-like and parasitic protists are abundant and diverse in soils, but their roles at the community and ecosystem levels have been less well studied (Geisen and Bonkowski 2017). This may change as a result of methodological advances (Box 1).

In the following we will first provide a concise overview of protist taxa common in soils as they have been classically defined based on morphology and then provide state-of-the-art phylogenetic placement according to the most recent classification (Adl *et al.* 2012) (Fig. 1).

### Photoautotrophic soil protists (traditionally termed algae)

Algae (in their classical terms), but now more correctly named *photoautotrophic* protists were divided into taxa based on their accessory photosynthetic pigments. Most photoautotrophic soil protists are found within the eukaryotic supergroups Stramenopiles (Diatoms, Eustigmatophyceae and Xanthophyceae (Flechtner, Johansen and Clark 1998; Zancan, Trevisan and Paoletti 2006)) and Archaeplastidae (Chlorophyceae and Trebouxiophyceae (Zancan, Trevisan and Paoletti 2006; Seppey *et al.* 2017)). Therefore, algae, as a whole, are highly polyphyletic. Recent sequencing of 18S rRNA gene amplicons from environmental DNA demonstrated the presence of dinoflagellates and even haptophytes in soils (Bates *et al.* 2013; Mahé *et al.* 2016). However, it remains to be determined if these are active forms—as was shown for foraminifera (Meisterfeld, Holzmann and Pawlowski 2001; Lejzerowicz *et al.* 2010; Geisen *et al.* 2015e)—or spores from aquatic organisms.

### Fungi-like protists

Most 'lower fungi' found in soils that are now considered protists have been classified as oomycetes (i.e. peronosporomycetes), a monophyletic group within the Stramenopiles. Oomycetes are osmo- and lysotrophic, and can be free-living, facultative or obligate parasitic of other oomycetes, fungi, plants and animals (Lara and Belbahri 2011). Oomycete marker sequences are abundantly present in environmental DNA-based diversity surveys (Geisen *et al.* 2015c; Singer *et al.* 2016). A large, culture-based survey of oomycetes across 64 plots used in soybean culturing (Canada and USA) provided no less than 216 OTUs based on ITS sequencing (Rojas *et al.* 2017). Each of these OTUs, however, can potentially represent several biological species (Schroeder *et al.* 2013). As obligate parasitic oomycetes are not likely to be recovered with cultivation-dependent approaches (Lara and Belbahri 2011), their diversity in this study may have been underestimated.

Another important group of fungi-like protists are the 'slime moulds', amoeboid organisms that sporulate in a manner that is reminiscent of Fungi. 'Slime moulds' are found primarily in the supergroup Amoebozoa (Shadwick *et al.* 2009), though some are

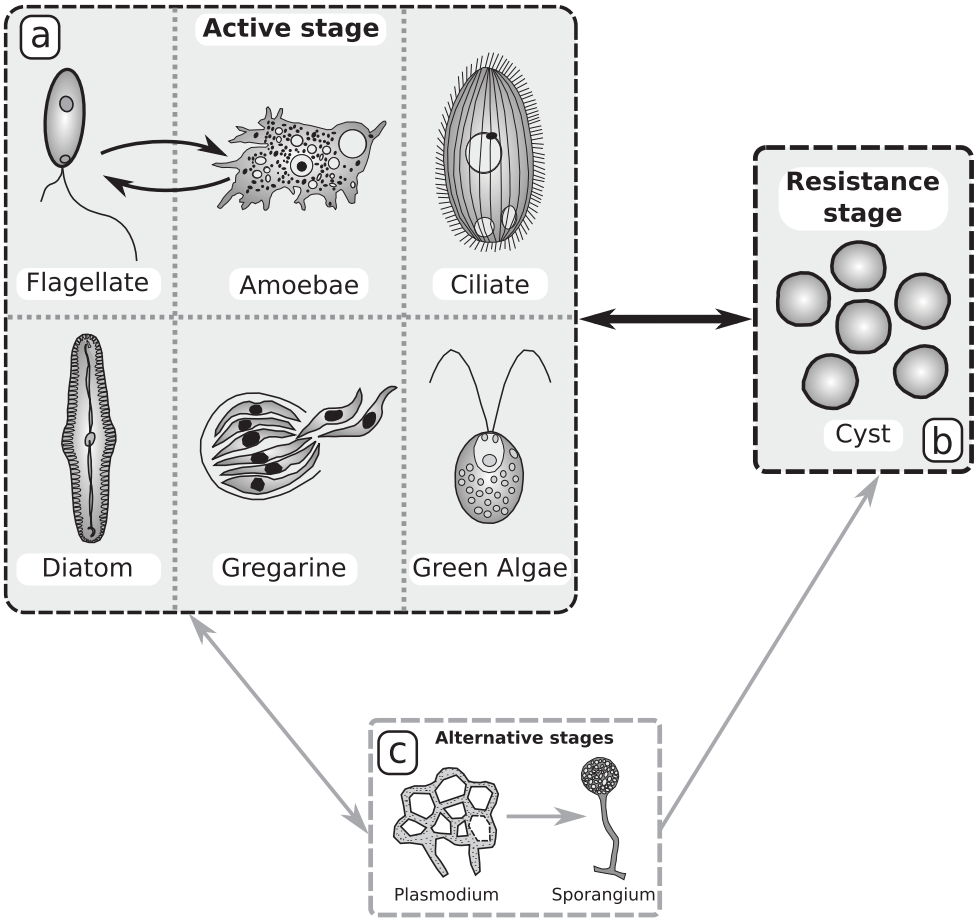


**Figure 3.** Typical life stages of soil protists. Most soil protists are either (**a**) active or in a resting stage as cysts (**b**), in which they can survive unfavorable conditions (in particular drought), which are common in soils. Certain soil protists from various taxonomic groups may fuse cells to become plasmodial and form common reproductive structures, such as sorocarpic 'slime moulds'; (**c**) the classical distinction between amoebae and flagellates is phylogenetically and ecologically problematic as protist from different taxa can switch between being an amoeba or a flagellate (a), other common soil protists can display both cell stages at the same time.

found within Opisthokonta, SAR (Stramenopila, Alveolata and Rhizaria), and Excavata. Their sporulating structures, or fruiting bodies, may develop from just a single precursor cell, a process called sporocarpy, or they may develop from aggregations of cells into a multicellular mass, named sorocarpy (Fig. 3). All sporocarpic and some sorocarpic species are found within the Amoebozoa (Fiore-Donno *et al.* 2010; Kang *et al.* 2017; Spiegel *et al.* 2017), whereas the remainder of the sorocarpic forms are found throughout the whole eukaryotic tree (Brown and Silberman 2013).

## Heterotrophic soil protists (traditionally termed protozoa)

Formerly, heterotrophic soil protists were combined under the term protozoa, as a component of the microfauna. They were classified based on their morphology into four broad categories: flagellates, ciliates, naked and testate amoebae, plus the parasitic Sporozoa (Foissner 1999a). Although these categories are gradually being abandoned in modern literature, they are still widely used in ecological studies that use morphological tools to characterize protists. In order to link the former with molecular approches, we provide below an updated summary of protozoan taxa classically defined morphologically.

Flagellates have cells that move using long motility organelles called either flagella or cilia, terms that are essentially synonymous as they refer to homologous structures. There are usually one to four flagella or cilia on the cell (mostly two), and more rarely many more. Flagellate cells range from relatively rigid to fairly flexible, but they tend to maintain a more or less constant shape. Flagella are not only used for motility, but as sensory organelles as well, and often help to direct food particles to the cell body for ingestion (Mitchell 2007). Flagellates are a paraphyletic group and are found in all eukaryotic supergroups. In soils, they include Excavata, as well as Bicosoecids and Chrysophytes within the Stramenopiles (Lentendu *et al.* 2014; Geisen *et al.* 2015e; Mahé *et al.* 2017; Seppey *et al.* 2017; Fig. 1).

Amoebae are organisms with flexible cell shape, which changes in order to move and to ingest food particles. Amoebae form transient cell extensions called pseudopodia. Most members of the supergroup Amoebozoa, and a great percentage of members of Rhizaria (SAR), are amoeboid (Smirnov and Brown 2004; Smirnov *et al.* 2011; Bass *et al.* 2016). The amoeboid lifestyle is probably used as well by soil foraminiferans (SAR), a primarily marine group of protozoa that were also recently found to be widespread in soils (Lejzerowicz *et al.* 2010; Geisen *et al.* 2015e). Some Opisthokonts and one group of Excavata, the Heterolobosea, contain amoeboid members as well, for example, the genera *Naegleria* and *Allovahlkampfia* (De

Jonckheere 2014; Geisen *et al.* 2015a); Fig. 1). Many soil Rhizaria possess flagella but also have the ability to produce pseudopodia, and can therefore be associated to both amoebae and flagellates. This combined morphological variability appears particularly well suitable for foraging between soil aggregates. This group within Rhizaria includes the Glissomonads and Cercomonads (Fig. 1), which are among the most abundant protists in soils (Geisen *et al.* 2014, 2015e).

Certain amoeboid taxa have developed a test or shell, which is used as a protection against desiccation and, probably, against predation. Shell-containing amoebae are found in three eukaryotic supergroups, the Amoebozoa (Nikolaev 2005), Rhizaria (Bhattacharya, Helmchen and Melkonian 1995; Dumack, Baumann and Bonkowski 2016) and Stramenopiles (Gomaa, Mitchell and Lara 2013). They are encountered mostly in the litter soil horizon (Geisen *et al.* 2015e), as many larger species are prevented from reaching deeper horizons due to their large test. The largest cells of this group of organisms can reach 150 $\mu$m, like some *Bullinularia*, *Centropyxis* or *Distomatopyxis* (Arcellinida; Amoebozoa) (Meisterfeld 2002a). Rhizarian testate amoebae such as *Euglypha* (Silicofilosea; Rhizaria) use their thin pseudopodia (called filopodia) to forage within aggregates (Meisterfeld 2002b). The shells or tests are typically composed of proteins, reinforced by self-secreted mineral elements, most often silica (e.g. for *Euglypha*, *Quadrulella*, and *Trinema*), but also calcium carbonate (for *Paraquadrula*) (Meisterfeld 2002b). Other genera use materials collected in the surroundings, or use mineral scales recycled from their prey (often smaller testate amoebae—see section below on nutrient cycling, Lahr *et al.* 2015).

Ciliates are a taxon-rich group of the Alveolata in the supergroup SAR (Foissner 1998). They are the only classical group of protozoans that has proven to be monophyletic. Ciliate cells are characterized by having two types of nuclei. A diploid germ nucleus has the purpose to provide the genome to gamete nuclei following meiosis, and a polyploid nucleus, where gene expression takes place (Foissner 1998; Dunthorn *et al.* 2015). Ciliates typically have hundreds of short flagella/cilia arranged in rows along their cell bodies. Ciliate cells may be fairly rigid or flexible, but they usually maintain a recognizable shape. Amongst all ciliates classes, Colpodea and Haptoria are notably more diverse and well represented in terrestrial systems than in aquatic ones (Foissner 1987; Foissner and Oertel 2009; Fig. 1).

### Parasitic soil protists

Sporozoa was a name given to protists that spent large portions of their metabolically active lives as intracellular parasites of other eukaryotes. In absence of a host cell, sporozoa are dormant, encased in a cell wall, the spore stage. Sporozoans are polyphyletic, encompassing the taxon-rich Apicomplexa within Alveolata (Fig. 1), and the Microsporidia, a subclade within the Nucletmycea, that branch of Opisthokonta (Fig. 1). The importance of the Apicomplexa in soils has been discovered only very recently. Sporozoa were reported in the earlier literature (Foissner 1987 and references therein), but high throughput sequencing approaches (Box 1) are now revealing their true diversity and ubiquity in soils (Bates *et al.* 2013; Geisen *et al.* 2015e; Mahé *et al.* 2017), and their likely major role as parasites of soil invertebrates (Geisen *et al.* 2015c; Mahé *et al.* 2017). A study of soil eukaryotic diversity in three Neotropical forests based on environmental DNA showed that apicomplexan Gregarines (Fig. 1) dominated the protist diversity in both relative abundance and OTU richness (Mahé *et al.* 2017).

### Implications of changing classification

The simplistic earlier view of protist classification ignored some important functional aspects of soil protistology. The above-mentioned Cercomonads and Glissomonads can be seen as intermediates between flagellated and amoeboid organisms. Some algae, especially most autotrophic members of the dinoflagellates (Alveolata), and many unicellular and colonial Ochrophyta (Stramenopiles) are mixotrophic, because they combine the ability to photosynthesize with the capacity to phagocytize (Fig. 2). Most autotrophs are probably capable of some osmotrophy, and many different algae can survive without light if provided appropriate organic compounds in their environment (Amblard 1991). These examples illustrate that the old classification actually blurs relevant ecological information and a phylogenetically relevant classification be applied. Bridging molecular data with knowledge on individual species ecology is certainly one of the timeliest challenges in soil protist ecology. Achieving this goal with an acceptable level of detail requires a collaborative effort between experts in protist systematics, classical taxonomy, molecular techniques and ecology. Soil protistology is therefore in itself a multi-disciplinary research field that needs to be connected with research on other groups of soil organisms (Geisen 2016a; Geisen *et al.* 2017; Xiong *et al.* 2017). This is especially crucial if we are to take full benefit from the tremendous potential of high throughput sequencing data. Optimal exploitation of these data requires sound databases and the ability to critically interpret the output of taxonomic assignations. Failure to do so will inevitably lead to erroneous ecological interpretations (Geisen and Bonkowski 2017).

## FUNCTIONAL ROLES OF PROTISTS IN FOOD WEBS

Soil protists assume a broad range of functional roles; beyond their best-known role as consumers of bacteria they also act as primary producers, fungal feeders, predators of other protists and micro-metazoa, and as parasites of plants, protists and metazoa (Adl 2003; Adl and Gupta 2006; Geisen 2016b; Geisen *et al.* 2017). The main ecological functions of soil protists are summarized in Fig. 2.

### Primary production

Photosynthetic protists occur in soils (Fig. 2d) and are most abundant in the sunlit uppermost soil layers where they contribute to the formation of biological crusts (Bamforth 2008). Although environmental DNA surveys suggest that photosynthetic protists represent a small part of all soil protists, their contribution to the soil organic carbon input is non-negligible, even under temperate climates (Seppey *et al.* 2017). Photosynthetic mixotrophic protists are well documented as an important functional group in aquatic systems (Ward and Follows 2016). In carbon-rich peatland soils, mixotrophic protists contribute significantly to carbon sequestration (Jassey *et al.* 2015), but their overall importance for carbon sequestration across other soil ecosystems remains unknown.

### Element cycling

The essential role of soil protists in nutrient cycling is inferred from their predation on bacteria and other soil organisms (Fig. 2a), which we detail in the following sections. Soil protists, especially soil diatoms (Kidder and Gierlowski-Kordesch 2005)

and euglyphid testate amoebae, take up silicon (Si) from the environment to build Si structures (Aoki, Hoshino and Matsubara 2007; Sommer *et al.* 2013; Puppe *et al.* 2014). Their contribution to Si cycling has been estimated to be approximately equal to the forest trees (Aoki, Hoshino and Matsubara 2007). Hyalospheniid testate amoebae that prey on euglyphids are also believed to play a major role in the Si cycle (Lahr *et al.* 2015). Evolutionary radiations in these groups coincide with major changes in the composition of terrestrial vegetation as well as climate, suggesting a possible causal relationship (Wilkinson 2008; Lahr *et al.* 2015).

## Bacterivory (and phagotrophy in general)

Bacterivory by heterotrophic protists leads to the release of nutrients (Fig. 2a). In marine systems, bacterivory is mainly attributed to small protists, collectively termed heterotrophic micro- and nanoflagellates (Azam *et al.* 1983). In soils, a wide variety of protists is bacterivorous, constituting a major cause of bacterial mortality (Hunt *et al.* 1987; de Ruiter, Neutel and Moore 1995; Clarholm 2005). Due to the higher C:N ratio of protists than their bacterial prey, nitrogen is excreted as a waste product, mainly in form of $NH_3$ (Sherr, Sherr and Berman 1983). Liberated nitrogen (and other nutrients) become available to all organisms, including microorganisms and plants (see plant-protist interaction section). The importance of heterotrophic protists is linked to their inherent characteristics: First, protist turnover can be extremely rapid with division times of often only a few hours (Fenchel 1982). Second, soils contain an enormous seedbank of dormant protists ranging between $10^4$ and $10^7$ individuals per gram of soil (Adl and Coleman 2005). Under suitable environmental conditions, these organisms become active and their density follows closely the increase of their bacterial prey (Clarholm 1981; Adl and Coleman 2005).

Protists do not prey on all bacteria equally (Singh 1941, 1942). Small bacterivorous species are often morphologically quite similar, but differ in their ecological optima and their food regimes (Koch and Ekelund 2005; Howe *et al.* 2011). Indeed, contrasted food preferences have been documented even for phylogenetically closely related protist species (Glücksman *et al.* 2010; Pedersen *et al.* 2011). Although the underlying mechanisms that determine soil protist feeding preferences still need to be assessed more systematically (Flues, Bass and Bonkowski 2017), food preferences of individual strains of bacterivorous protists appear highly reproducible (Rosenberg *et al.* 2009).

Differences in feeding habits of protists on their bacterial prey can at least partly be attributed to a wide arsenal of strategies against protist attack evolved by distinct bacteria. This includes changes in bacterial cell size, colony formation, escape by movement and production of toxic compounds (Matz and Kjelleberg 2005; Jousset *et al.* 2006; Brüssow 2007; Jousset 2012). Indeed, it is not unlikely that co-evolution between bacterial prey and their predators has been a major driver for protists' diversification; for instance, differentially sized protist species can feed on differentially sized bacteria, while different protist species vary in their sensitivities to bacterial chemical defences (Jousset *et al.* 2006; Pedersen *et al.*, 2010, 2011). In addition, several bacterial species evolved to infect their protist predators. Some of those became symbionts, playing mutualistic and even parasitic roles (Brüssow 2007; Jousset 2012, Box 2). The eco-evolutionary arms race has likely enabled protist species to sense suitable prey and avoid potentially deleterious organisms via bacterial produced volatiles (Schulz-Bohm *et al.* 2017).

Differential feeding of protists has direct consequences on population dynamics and community assembly of their prey (Hünninghaus *et al.* 2017). In experimental studies, bacterial diversity increased with an increasing diversity of up to three protist species (Saleem *et al.* 2012), suggesting that the diversity of thousands of protist species in soils is not only a consequence of differential feeding, but is also maintaining a higher bacterial community diversity.

It has long been assumed that the main prey of protists in soils are bacteria. This paradigm in food web models still widely persists (Hunt *et al.* 1987; de Ruiter, Neutel and Moore 1995; Holtkamp *et al.* 2008, 2011; Tiunov *et al.* 2015; Trap *et al.* 2016). The paradigm of soil protists being merely bacterivores is due to (1) media to cultivate and enumerate heterotrophic protists that preferentially enrich bacteria and therefore bacterivorous protists and (2) the focus of ecological studies on very few bacterivorous species (e.g. Bonkowski 2004; Huws *et al.* 2008; Jousset *et al.* 2009; Rosenberg *et al.* 2009; Bjørnlund *et al.* 2012). More and more evidence, however, suggests that other feeding and functional roles of protists are equally important including feeding on fungi (Geisen *et al.* 2016) and even predation on other eukaryotes such as animals (Geisen 2016b). Newer studies thus challenge the still prevalent view of soil protists being mostly bacterivores (Figs. 2a, 4a and 4b).

## Predation on small eukaryotes

Protists from many different taxa take up prey that is in the right size range to be engulfed (Boenigk *et al.* 2002), but not necessarily indiscriminately (see above). Many previously assumed bacterivorous soil protists are likely facultative omnivores as they are also capable of ingesting and growing on yeasts, or conidia and spores of fungi (Geisen *et al.* 2016; Geisen 2016b; Figs. 2 and 4). Other taxa are purely fungivores. The Grossglockneriidae, a distinct family of ciliates, obligatorily depend on fungi as their sole food source as their highly specialized cytostome does not allow the ingestion of a prokaryotic cell (Foissner 1999b). The Viridiraptoridae (Glissomonadida; Rhizaria) have also highly specialized cytoskeletal structures to perforate algal cell walls (Busch and Hess 2017). These organisms, originally observed as algal feeders in freshwater, also seem to be common in soils (Seppey *et al.* 2017).

Several larger-sized protists prey exclusively on a wide range of other eukaryotes, including predatory ciliates, vampyrellid amoebae, *Thecamoeba* spp. (Page 1977; Berger 1979; Hess, Sausen and Melkonian 2012; Berney *et al.* 2013; Geisen *et al* 2016) and most testate amoebae from family Hyalospheniidae that need euglyphid testate amoebae to build their own tests (Lahr *et al.* 2015). Even soil animals can serve as prey for protists; nematodes and rotifers are consumed by large testate amoebae (Yeates and Foissner 1995; Gilbert *et al.* 2000). Protists' abilities to consume alternate prey even challenges several conceptions of predator-prey interactions. For instance, some smaller testate amoebae that can live on bacteria and fungi as a sole food source have developed a pack-hunting strategy to cooperatively prey and benefit from consuming much larger sized nematodes (Geisen *et al.* 2015d). Altogether, feeding on eukaryotic (micro)organisms is common and widespread among different phylogenetically distinct groups of protists and should therefore be incorporated into more realistic soil food web models.

## Absorptive nutrition

Eukaryotic microbes, such as fungi, are involved in decomposition processes (Fig. 2c). Many oomycetes are free-living and contribute to the decomposition of organic matter through

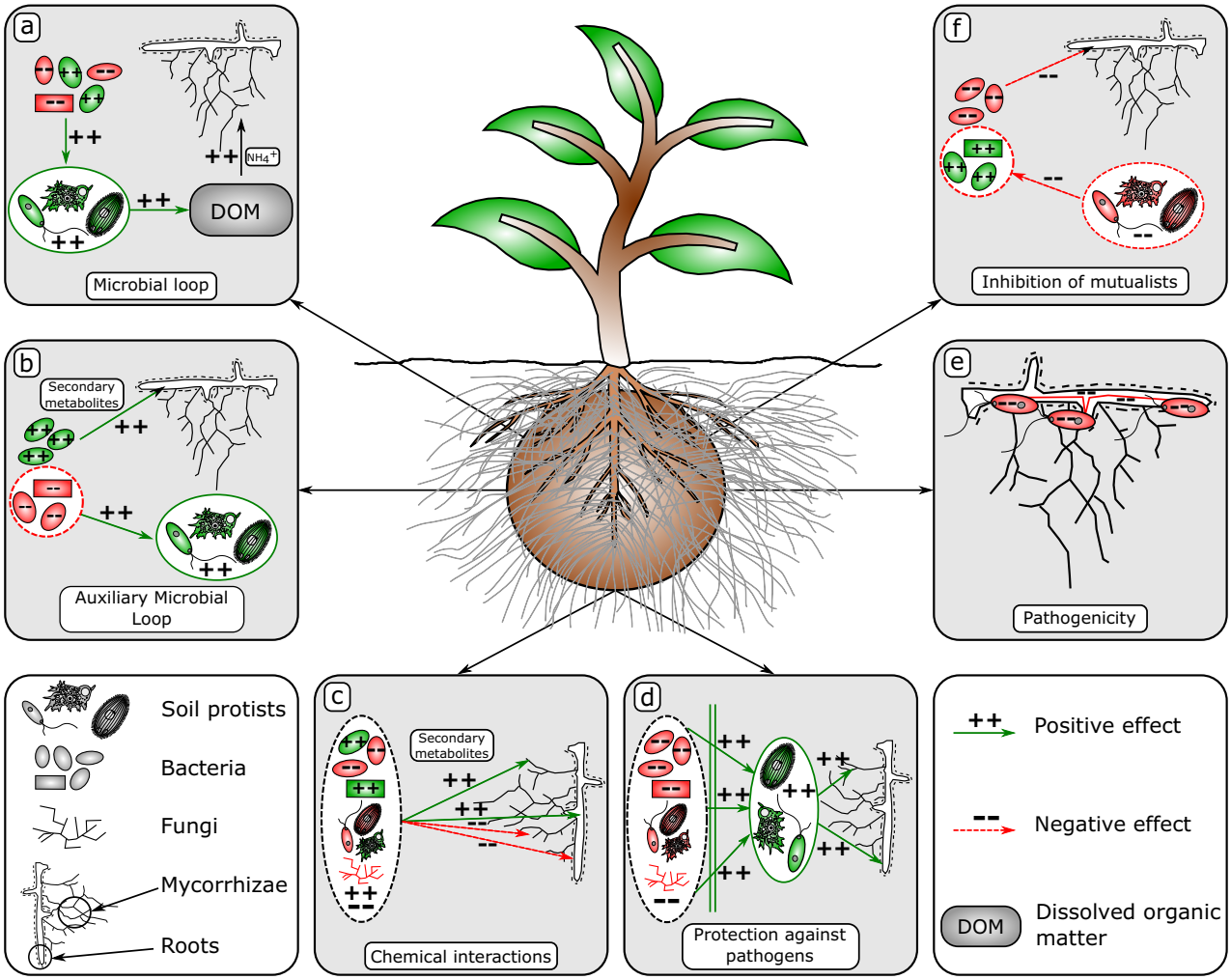


**Figure 4.** Overview of various interactions between plants and protists. (**a**) Protists can positively stimulate plant performance (green, '++') due to nutrient release as a result of consuming other microorganisms (the microbial loop); (**b**) they may further stimulate bacteria to produce secondary metabolites (the auxiliary microbial loop); (**c**,**d**) chemical interactions of protists directly or of protist prey with plants, and protection of plants against pathogens via competition or predation; (**e**) some protists are directly plant parasitic; (**f**) protists can also negatively impact plant performance (red, '–') due to direct release of harmful substances or stimulation of bacteria producing deleterious secondary metabolites for plants, or by inhibition of plant mutualists.

lysotrophy (Kramer *et al.* 2016). Osmotrophy is also frequent, and the fact that many protists such as *Acanthamoeba castellani* (Amoebozoa) can grow in axenic conditions purely feeding on nutrient-rich media without any additional prey demonstrates that they can take in nutrients directly from the environment (Neff 1957). Soil protists from a variety of taxa have this ability, such as *Andalucia godoyi* (Excavata) (Lara, Chatzinotas and Simpson 2006), ciliates (Alveolata; SAR), cercomonads (Rhizaria; SAR) and amoebozoans. The presence of structures related to osmotrophy i.e. coated pits and vesicles; it can be supposed that many soil protists are capable of actively participating in decomposition processes just like prokaryotes and Fungi do. The range of substrates that different species can exploit would be relevant for models on soil nutrient cycling.

## Parasitism

Soil protists do not only ingest their prey, they can also parasitize other organisms (Fig. 2b), including humans (Box 2). Sequence-based studies have revealed high (relative) abundances and diversities of parasitic protist OTUs in soils (Bates *et al.* 2013; Geisen *et al.* 2015c; Dupont *et al.* 2016; Grossmann *et al.* 2016; Mahé *et al.* 2017). Parasitic protists are spread across the eukaryotic tree of life (Fig. 1). In soils, however, the most notorious group of parasites are apicomplexans (supergroups: SAR, Alveolata), which most familiarly contain the prominent examples *Plasmodium falciparum* (causative agent of malaria) and *Toxoplasma gondii* (causative agent of toxoplasmosis). In soils, apicomplexan Gregarines (Gregarinasina, Fig. 1) are very diverse and abundant. In particular, in tropical rainforests they can contribute to more than half of total protist diversity (Mahe *et al.* 2017). Apicomplexans are obligate parasites of invertebrates and vertebrates, and likely play an important role in controlling animal populations and even local animal diversity (Mahé *et al.* 2017). By parasitizing larger, macroscopic organisms (and in certain cases eventually killing them), soil protists release nutrients into the soil. Parasites of other groups of organisms are also common, such as Cryptomycota, belonging to the sister group of Fungi, the Opisthosporidia (Karpov *et al.* 2014). These protists can infect a large range of microbial eukaryotes, including chytrids, Blastocladiomycota, oomycetes and some green algae (Held 1981), diatoms (Jones *et al.* 2011) and Amoebozoa

(Corsaro *et al.* 2014). Finally, parasitic protists can also infect plants, and some of them are important plant pathogens. We will detail their specificities in a following section.

**Box 2:** Soil-borne protists as human pathogens

SOIL PROTISTS AND PUBLIC HEALTH

Altogether, almost 15% of all known protists show symbiotic (mutualistic or parasitic) life styles and around 100 protist species can infect humans (Walochnik and Aspöck 2012). The majority of these are not significantly harmful to their hosts, with several species even being commensals (Parfrey *et al.* 2014). However, some protists can cause fatal diseases. Among these are not only the important vector-borne parasites such as the malaria causing agents *Plasmodium* spp. that infect hundreds of millions of people and are a main human mortality cause (Snow *et al.* 2005), and food-borne diseases such as *Toxoplasma gondii* that affect millions and kill hundred thousands (Torgerson *et al.* 2015; Burgess *et al.* 2017). However, also water and soil-borne protists, including *Naegleria fowleri* and *Entamoeba histolytica* infect humans. Many infections with protists show a more severe progression in immunocompromised hosts (e.g. toxoplasmosis, cryptosporidiosis, granulomatous amoebic encephalitis).

Several soil-borne protists are opportunistic human pathogens, and soil can therefore be an important source of infection (Santamaría and Toranzos 2003). While the majority of protist pathogens need a host for replication, few can conclude their entire life cycle without a host. Examples are the facultative (or potentially) pathogenic amoebae, including mainly amoebozoan taxa (e.g. *Acanthamoeba*, *Balamuthia*, *Sappinia*), but also the excavate *Naegleria fowleri*. Most of the soil-borne, medically relevant protists seem to be cosmopolitan, but since many rely on faecal–oral transmission they are generally more common in low-income countries with poor sanitation. An overview of the most important protist pathogens that can be transmitted through soil is given in Table 1.

The importance of soil as a 'vehicle' for pathogens should not be underestimated. The main route of infection for soil-borne protist pathogens is by ingestion, either of food contaminated with soil (e.g. salad, vegetables), or contaminated water, or via contaminated hands. This route is of primary importance for e.g. *Giardia* spp., *Cryptosporidium* spp., *Cystoisospora belli*, *Cyclospora cayetanensis*, *Toxoplasma gondii*, *Balantidium coli* and *E. histolytica* (Curry, Turner and Lucas 1991; Marcos and Gotuzzo 2013; Pérez *et al.* 2016). Other body openings and skin lesions also offer the possibility for protists to infect humans, for example, *Naegleria fowleri* (nasal route), *Acanthamoeba* spp. (eye/inhalation/skin) and *Balamuthia mandrillaris* (inhalation/skin) (Schuster and Visvesvara 2004a; Visvesvara, Moura and Schuster 2007).

Water is the primary medium for protist infection by the oral route, and both air and water are modes of protist dispersal. However, cysts and oocysts can withstand desiccation and can survive in soil for very long periods of time, sometimes many years (Shmakova, Bondarenko and Smirnov 2016). Among the amoebozoan taxa there are several genera with particularly resilient cysts, an example being *Acanthamoeba* (de Jonckheere 1991; Mergeryan 1991). This genus often hosts bacteria, including pathogens, which are not digested but live endocytobiotically (Barker and Brown 1994; Molmeret *et al.* 2005; Van der Henst *et al.* 2016). A prominent (or well-documented) example is *Legionella pneumophila* that can cause serious lung infection and that has been shown to be more invasive for human cells after passage through amoebae (Cirillo, Falkow and Tompkins 1994; Hwang, Katayama and Ohgaki 2006). Bacteria are not the only pathogens hosted by *Acanthamoeba* sp., which can contain a whole array of viruses and yeasts like *Cryptococcus neoformans* (Guimaraes *et al.* 2016). Table 2 gives an overview of the soil protists known to function as 'Trojan horses'.

## ADAPTIVE STRATEGIES OF SOIL PROTISTS

Life in soils presents several challenges that protists have to face. Unlike in aquatic systems that have a higher buffering capacity against sudden changes in environmental conditions, soils are highly variable. For instance, (1) soils are subject to desiccation, which is for protists as organisms that inhabit the aqueous part in soil a main challenge. (2) With the exception of low elevation equatorial, tropical and hyper oceanic regions, the topsoil is subject to freeze-thaw cycles that poses a challenge for the vast majority of protists inhabiting this layer. (3) Active dispersal over long distances of minute protists as well as passive mass flow as in aquatic systems is limited in soils. (4) Sunlight only reaches the uppermost part of soil, while the main part of soil remains dark. (5) Soil pore space provides distinct niches for protists but also for their prey, which can hinder successful predation. We provide examples for adaptations to these obstacles for protists to live in soils but acknowledge that other factors, such as oxygen availability, or patchiness of nutrient resources are likewise constraints that protists need to deal with.

### Resistant structures

The ability to build resistant structures (e.g. cysts) can be considered as a prerequisite for a protist to be able to live in soils (Fig. 3). The only exceptions are those that inhabit permanently wet soils; indeed, it has been shown that air-drying rainforest soils reduced considerably the diversity of ciliate communities. The species retrieved after drying consisted only of generalists, which has led to an underestimation of the true diversity and the specificity of ciliate communities (Foissner 1997). Cysts consist of coccoid structures that are formed by organisms from very different phylogenetic backgrounds, and are regulated by different mechanisms. For instance, cystein proteases and corresponding inhibitors control cyst formation/excystment in the soil amoeba *Acanthamoeba castellani* (Moon *et al.* 2011; Lee *et al.* 2013). Phospholipase D controls cyst formation in the (parasitic) amoebozoan *Entamoeba invadens* (Ehrenkaufer *et al.* 2013). The cyst wall composition is lineage specific, but has been studied so far only in pathogenic species. *Entamoeba invadans* has chitin as a main component of cyst wall, but *Giardia* (Excavata) cysts are mainly composed of an N-acetylgalactosamine polymer (Samuelson and Robbins 2011).

The cysts of free-living, non-parasitic protist species have been less studied. These structures may be very efficient in preserving protists for weeks and even for years against environmental stresses such as drought. Cysts of *Protosiphon botryoides* (Chlorophyceae) have survived 50 years in dry soil before becoming active again (Lewis and Trainor 2012). Particularly resistant cysts of *Acanthamoeba* and *Flamella* cysts in the permafrost from the late Pleistocene (i.e. 30–60,000 years) have been revived (Mazur, Hadas and Iwanicka 1995; Shmakova and Rivkina 2015; Shmakova, Bondarenko and Smirnov 2016).

**Table 1.** Soil-borne protists as human pathogens (including also protists with only permanent stages in the soil).

| Protist pathogen | Occurrence | Stage in soil | Reservoirs | Disease | Selected References |
|---|---|---|---|---|---|
| **Excavates** | | | | | |
| *Giardia duodenalis* | worldwide | cysts | animals (including humans) | diarrhea | Olson *et al.* 1999; Santamaría and Toranzos 2003; Dado *et al.* 2012; Balderrama-Carmona *et al.* 2014; Minetti *et al.* 2016 |
| *Naegleria fowleri* | worldwide | trophzoites and cysts | soil | PAME | Das 1970; Schuster and Visvesvara 2004b; Moussa *et al.* 2015 |
| **Alveolates** | | | | | |
| *Toxoplasma gondii* | worldwide | oocysts | cats | cerebral and ocular toxoplasmosis (opportunistic) | Hill and Dubey 2002; Elmore *et al.* 2010 |
| *Cryptosporidium* spp. | worldwide | oocysts | animals (including humans) | diarrhea (mainly opportunistic) | Olson *et al.* 1999; Santamaría and Toranzos 2003; Dado *et al.* 2012; Balderrama-Carmona *et al.* 2014 |
| *Cystoisospora belli* | mainly tropics and subtropics | oocysts | humans | diarrhea | Özkayhan 2006; Tiyo *et al.* 2008; Ros Die and Nogueira Coito 2017 |
| *Cyclospora cayetanensis* | mainly tropics | oocysts | humans | diarrhea | Chacín-Bonilla 2008; Giangaspero *et al.* 2015 |
| *Balantidium coli* | worldwide (particularly in regions with intensive pig farming) | cysts | pigs | hemorrhagic diarrhea | Schuster and Visvesvara 2004b; Schuster and Ramirez-Avila 2008 |
| **Stramenopiles** | | | | | |
| *Blastocystis hominis* | worldwide | cysts | humans, other animals? | diarrhea (mainly opportunistic) | Stensvold and Clark 2016 |
| **Amoebozoans** | | | | | |
| *Entamoeba histolytica* | mainly tropics and subtropics | cysts | humans | diarrhea, liver abscess | Santamaría and Toranzos 2003; Dado *et al.* 2012; da Silva *et al.* 2016 |
| *Acanthamoeba* spp. | worldwide | trophzoites and cysts | soil | keratitis; GAE (opportunistic) | Nagington *et al.* 1974; Tsvetkova *et al.* 2004; Schuster and Visvesvara 2004b |
| *Balamuthia mandrillaris* | worldwide | trophzoites and cysts | soil | GAE (mainly opportunistic) | Visvesvara, Schuster and Martinez 1993; Schuster and Visvesvara 2004b |
| *Sappinia* spp. | worldwide | trophzoites and cysts | soil | GAE (opportunistic) | Gelman *et al.* 2001; Qvarnstrom *et al.* 2009 |
| *Vermamoeba vermiformis* | worldwide | trophzoites and cysts | soil | eye infections | Lorenzo-Morales *et al.* 2007; Reyes-Batlle *et al.* 2016 |
| **Fungi-related** | | | | | |
| Microsporidia | worldwide | spores | animals (including humans) | intestinal and systemic infections (opportunistic) | Graczyk *et al.* 2007; Dado *et al.* 2012 |
| *Pneumocystis jirovecii* | worldwide | spores | animals (including humans) | pneumonia (opportunistic) | Hughes, Bartley and Smith 1983 |

GAE: granulomatous amoebic encephalitis; PAME: primary amoebic meningoencephalitis

## Dispersal structure and mechanisms

While the majority of soil protists lack specific structures or mechanisms for active or passive long-distance dispersal (see biogeography section), some of them developed special structures, such as spores (Fig. 3). With few exceptions, spores and cysts are morphologically distinct (Fig. 3). While it may seem just semantics to distinguish between cysts (which are sessile and less easily dispersed) and spores, there is a long tradition of using these terms to refer to these two functionally distinct cell types (Spiegel 2016). Dispersal structures are often macroscopic (like in mushrooms and 'myxomycetes'), varying in structure and composition but generally composed by a stalk and a sporangium/fruiting body. Sporocarps, where a single cell develops into a stalked spore-bearing structure, are unique to Amoebozoa (Kang *et al.* 2017; Spiegel *et al.* 2017). Sorocarps where the spore-bearing structure is a result of the aggregation of many cells into multicellular mass can be encountered very commonly across almost all eukaryotic supergroups (Spiegel *et al.* 2004).

The myxogastrids and the protosteloid amoebae are sporocarpic members within Amoebozoa (Shadwick *et al.* 2009). Protosteloid amoebae occur separately in several clades of Amoebozoa suggesting that spore formation might be a synapomorphic character for the entire supergroup Amoebozoa (Kang *et al.* 2017; Spiegel *et al.* 2017). Sorocarps exists in amoebozoan

**Table 2.** Protists with known 'Trojan horse' function.

| Protist host | Transported pathogen | Disease | References |
|---|---|---|---|
| **Amoebozoans** | **Viruses** | | |
| *Acanthamoeba* | Enteroviruses | diarrhea | Greub and Raoult 2004 |
| | Mimi viruses | (possibly pneumonia) | La Scola *et al.* 2005 |
| | **Bacteria** | | |
| *Acanthamoeba*, *Balamuthia*, *Dictyostelium*, *Sappinia*, *Vermamoeba*, etc. | *Acinetobacter* spp. | nosocomial infections | Cateau *et al.* 2011 |
| | *Burkholderia cepacia* | opportunistic infections | Lamothe, Thyssen and Valvano 2004 |
| | *Burkholderia pseudomallei* | melioidosis | Inglis *et al.* 2000 |
| | *Chlamydia pneumoniae* | pneumonia | Essig *et al.* 1997 |
| | *Coxiella burnetii* | Q fever | La Scola and Raoult 2001 |
| | *Escherichia coli* O157:H7 | (hemorrhagic) diarrhea | Barker *et al.* 1999 |
| | *Francisella tularensis* | tularemia | Gustafsson 1989, Abd *et al.* 2003 |
| | *Helicobacter pylori* | gastritis | Winiecka-Krusnell *et al.* 2002 |
| | *Legionella* spp. | legionnaires' disease, opportunistic infections | Rowbotham 1980 |
| | *Listeria monocytogenes* | diarrhea, opportunistic infections | Ly & Mueller 1990 |
| | *Mycobacterium bovis* | tuberculosis | Taylor *et al.* 2003 |
| | *Mycobacterium leprae* | leprosy | Jadin 1975 Lahiri & Krahenbuhl 2008, |
| | Nontuberculous mycobacteria (NTM) | opportunistic infections | Krishna-Prasad & Gupta 1978 Steinert *et al.* 1998 Mura *et al.* 2006, |
| | *Pseudomonas aeruginosa* | keratitis, opportunistic infections | Michel *et al.* 1995 |
| | *Ralstonia pickettii* | nosocomial infections | Michel & Hauröder 1997 |
| | *Salmonella* spp. | diarrhea | King *et al.* 1988 |
| | *Shigella sonnei* | diarrhea | King *et al.* 1988 |
| | *Simkania negevensis* | (possibly pneumonia) | Kahane *et al.* 1998 Fasoli *et al.* 2008, |
| | *Vibrio cholerae* | cholera | Thom *et al.* 1992 Abd, Weintraub and Sandström 2005, Van der Henst *et al.* 2016 |
| | *Yersinia enterocolitica* | diarrhea | King *et al.* 1988, Nikul'shin *et al.* 1992 |
| | *Yersinia pestis* | plague | |
| | **Fungi** | | |
| ***Acanthamoeba*** | *Cryptococcus neoformans* | opportunistic infections | Abd *et al.* 2003 |
| | *Histoplasma capsulatum* | opportunistic infections | Steenbergen *et al.* 2004 |
| **Ciliates** | **Bacteria** | | |
| ***Tetrahymena*** | *Campylobacter jejuni* | (hemorrhagic) diarrhea | Snelling *et al.* 2005 |
| | *Francisella tularensis* | tularemia | Kormilitsyna *et al.* 1993 |
| | *Shigella sonnei* | diarrhea | King *et al.* 1988 |
| | *Yersinia enterocolitica* | diarrhea | King *et al.* 1988 |
| **Heteroloboseans** | **Bacteria** | | |
| ***Naegleria*** | *Legionella* spp. | legionnaires' disease, opportunistic infections | Rowbotham 1980 |
| | *Vibrio cholerae* | cholera | Thom *et al.* 1992 |

Dictyostelia and *Copromyxa* (Kang *et al.* 2017), but also in several taxa in diverse supergroups such as in one ciliate (Olive and Blanton 1980), Heterolobosea (Brown, Silberman and Spiegel 2010), Opisthokonts (Brown, Spiegel and Silberman 2009), Rhizaria (Brown *et al.* 2012), and Stramenopiles (Tice *et al.* 2016). Therefore, these structures seem to have been developed independently as a winning strategy for dispersal in terrestrial environments.

## Adaptations to low light conditions

The absence of light in deeper soil layers prevents the growth of phototrophic organisms. When deprived of light, photosynthetic protists must rely on dissolved organic nutrients in the pore water, thus becoming temporarily heterotrophic and feeding by osmotrophy. As for many photosynthetic protists, freshwater and soil diatoms are known to grow in the dark if the right amount of nutrients is available (Lewin 1953; Amblard 1991). Some groups that evolved towards a mixotrophic or phagotrophic lifestyle, such as some of the Chrysophyceae, can use their modified plastids (leucoplasts) as storage structures for starch or oil (Preisig and Hibberd 1982; Cavalier-Smith and Hourihane 1996; Mylnikov, Mylnikova and Tikhonenkov 2008). Some groups of photosynthetic protists (Figueroa-Martínez *et al.* 2015; Gentil *et al.* 2017; López-García *et al.* 2017) lost the ability for photosynthesis in the course of evolution and became strictly heterotrophic, such as common *Spumella*-like chrysophytes (Boenigk *et al.* 2005; Findenig, Chatzinotas and Boenigk 2010).

### Adaptations to soil pore size

Soils are characterized by a range of pores of different diameters and connectivity depending on the soil type and water content (Havlicek and Mitchell 2014). Many soil protists present morphological adaptations allowing them to hide or hunt between or within soil aggregates. For example, some small euglyphid testate amoebae produce elongated sub-cylindrical shells (e.g. *Trinema lineare*) (Meisterfeld 2002b), whereas ciliates of the class Stichotrichia have an elongated shape that is well adapted to edaphic conditions (Foissner 1998). Euglyphid testate amoebae, on the other hand, produce very slender pseudopodia (called filopodia) that might allow them to capture prey otherwise inaccessible within too small pores. Generally small sizes and a variable body shape of amoebae and cercomonads, the most abundant and diverse members of soil protists, are likely adaptations to access soil pores that would be impossible to access for larger taxa and those with rigid body shapes.

## PLANT PROTIST-INTERACTIONS

### From the soil microbial loop to the plant microbiome

Antedating the concept of microbial stoichiometry and postulating an active role of plants in plant-microbial interactions, Clarholm's seminal paper on the microbial loop in soil highlighted the perception of protist roles in terrestrial ecosystems (Clarholm 1985b). More than 30 years later, the progress in molecular methods (Box 1) has profoundly changed our understanding of microbial, and in particular, of protist diversity in soils. At the same time, it has become appreciated that all living plants are colonized internally and externally by a diversity of microorganisms (Rodriguez *et al.* 2009; Porras-Alfaro and Bayman 2011; van der Heijden and Schlaeppi 2015), including protists (i.e. the plant 'microbiome').

While most research on the plant's 'microbiome' is focused on rhizosphere interactions, other microbes including protists also occur in the phyllosphere. Protists are still absent in reviews of the phyllosphere microbiome (Vorholt 2012; Peñuelas and Terradas 2014). Bacterivorous phyllosphere protists are diverse and have peculiar life cycles (Bamforth 1973; Spiegel *et al.* 2004, 2017; Ploch *et al.* 2016); they differ morphologically and in their life cycle from rhizosphere taxa (Mueller and Mueller 1970; Dumack *et al.* 2017). Phyllosphere bacterivorous protists shape the bacterial community assembly, such as by favouring pseudomonads, and change more than a dozen metabolic core functions in bacteria (Flues, Bass and Bonkowski 2017). In line, plant pathogenic oomycetes change the structure of prokaryotic communities (Agler *et al.* 2016). These examples show that also aboveground protists represent an integral component of the plant's microbiome. In the following, however, we focus on plant-protist interactions in the rhizosphere and ask how future research on plant-protist interactions can and should contribute to our knowledge gain (Fig. 4).

### The microbial loop in soil and its connection to fungal root symbionts

Clarholm's (1985b) concept of the 'microbial loop in soil' in response to the microbial loop in aquatic systems (Azam *et al.* 1983) remains a cornerstone of our perception on plant-protist interactions. As consumers of a quickly re-growing bacterial biomass, protists would constantly remobilize about one third of the consumed nitrogen as ammonia especially in the plant rhizosphere, where the continuous release of root exudates prevents carbon limitation of microbial growth (Griffiths 1994; Bonkowski and Clarholm 2012). Clarholm's microbial loop in soil differed from views of the microbial loop in aquatic systems (Azam *et al.* 1983) by postulating an active role of plant roots in plant-protist interactions.

The significance of nutrient transfer through protist consumption of microbial biomass to plants has been clearly confirmed (Kuikman *et al.* 1990; Ekelund and Rønn 1994), and is particularly strong when fresh and nitrogen-rich organic material provides hotspots of microbial mineralization and for root foraging (Griffiths 1994; Bonkowski, Griffiths and Scrimgeour 2000; Ekelund *et al.* 2009; Koller *et al.* 2013a). Integrating the microbial loop and ecological stoichiometry provides a new framework to predict more accurately protist effects on plant nutrition (Trap *et al.* 2016). This becomes particularly important when interactions with other root symbionts are taken into account. Plant nutrient uptake is intimately linked to symbioses with Fungi (Zuccaro, Lahrmann and Langen 2014), and we will not gain a full understanding of protists' roles in plant nutrition unless taking their contributions into account (Fig. 4).

It seems that protists are the key trophic link facilitating nitrogen-uptake of arbuscular mycorrhizal fungi, while bacteria alone may in some cases even suppress root mycorrhization by nutrient competition (Leigh, Fitter and Hodge 2011; Hodge and Storer 2015). Arbuscular mycorrhizal (AM) Fungi are well known to improve uptake of phosphor by plants (Smith and Smith 2011), but they completely lack the enzymatic machinery for nitrogen mineralization (Veresoglou, Chen and Rillig 2012) and rely on other microorganisms for the supply of mineral nitrogen. AM hyphae were shown to be extremely efficient in the uptake of nitrogen released from bacteria by protist predators (Herdler *et al.* 2008; Koller *et al.* 2013a; Koller *et al.* 2013b), providing compelling evidence for a tight functional coupling of the microbial loop and nutrient foraging by AM Fungi (Koller *et al.* 2013a; Bukovská *et al.* 2016). Further experiments separating the effects of fungal symbionts, bacteria and protists are needed to gain a mechanistic understanding of the interplay of the rhizosphere microbiome. Interactions of protists with ecto-mycorrhizal (EM) Fungi were shown to be even more complex and included changes in root architecture as well as trade-offs between EM symbionts and protists (Jentschke *et al.* 1995; Bonkowski, Jentschke and Scheu 2001). As explained above, many protists are able to prey on both fungi and bacteria, suggesting that nutrients originating from both bacteria and fungi can be channelled together already at the next trophic level in soil food webs (Geisen *et al.* 2016; Geisen 2016b; Fig. 2a).

It has to be noted, however, that other organisms than protists participate in the microbial loop in soil. Myxobacteria are preying on a broad range of other soil prokaryotes, and recent studies claimed that their impact on bacterial mortality could be higher than protist predation in certain cases (Lueders *et al.* 2006; Morgan *et al.* 2010; Lloyd and Whitworth 2017). Studies from marine systems suggest that 20–50% of bacterial production falls victim to viruses, the 'viral shunt' (Suttle 2007; Jiao *et al.* 2010). Bacteriophages can be extremely abundant in soil systems (Buée *et al.* 2009), where bacterial mortality is determined by rapid cycles of coevolution with the phages (Gómez and Buckling 2011). Conflicting selection pressures may prevent a stable adaptation of bacteria to either one of their predators, resulting in a constant coevolutionary arms race between bacteria, phages and protists (Friman and Buckling 2013, 2014). Protists may further shape these interactions by direct consumption of viruses (Deng *et al.* 2014). Accordingly, it could well be that protists in the

microbial loop interact with both a 'viral shunt' and a 'prokaryote loop'.

### Beyond the microbial loop

Not all plant growth promotion by protists can be attributed to improved nutrient uptake when calculating total nutrient contents of plants from published data (Kuikman *et al.* 1990; Jentschke *et al.* 1995; Alphei, Bonkowski and Scheu 1996; Krome *et al.* 2009; Koller *et al.* 2013b). Other plant traits, such as lateral root growth and photosynthesis were commonly enhanced in these studies, suggesting alternative mechanisms (Bonkowski and Clarholm 2012).

In recent years, it has become clear that each plant species assembles a specific subset of the soil microbial community in its rhizosphere, endosphere and phyllosphere (Bulgarelli *et al.* 2013; Berg *et al.* 2014). Protists are both an integral part of the microbiome, as well as an external force shaping its assembly, but targeted research on the protist microbiome and how that feeds back on plant performance remains surprisingly scarce.

### Shifts of microbiome assembly and function

Selective predation on bacteria by soil protists was demonstrated for some species based on morphological and chemical traits, creating positive selective pressures for consumption-resistant taxa and resulting in predictable patterns of bacterial community assembly (see section 'Functional roles of protists in food webs'). Network analyses can show how well the results gained with few protist model organisms can be scaled up to the community level (Flues, Bass and Bonkowski 2017).

Both the degree of protist prey selection and bacterial defence mechanisms shape the resulting bacterial community composition. This in turn has a significant influence on the function and microevolution of the plant microbiome. Complementarity of protist feeding modes, resource availability to prey, and growth-defence trade-offs were all shown to influence microbial community assembly (Corno and Jürgens 2008; Friman *et al.* 2008; Glücksman *et al.* 2010; Saleem *et al.* 2013). Since protists differ in their feeding modes and preferences, high species richness of protists should reduce bacterial biomass drastically through feeding on a wide variety of bacterial prey (Saleem *et al.* 2012). In view of the diversity of soil protist taxa uncovered by high-throughput sequencing studies (Geisen *et al.* 2015e; Fiore-Donno *et al.* 2016; Grossmann *et al.* 2016; Harder *et al.* 2016; Mahé *et al.* 2017), the degree to which diversity of predation will induce random or predictable functional changes of plant-associated microorganisms is a decisive question.

The interactions of protists with other microbes as studied most commonly with the model species *Acanthamoeba castellanii* and their bacterial prey has direct effects on the root architecture and exudation. For instance, lateral root branching is induced in a range of gymno- and angiosperms (Jentschke *et al.* 1995; Bonkowski, Jentschke and Scheu 2001; Kreuzer *et al.* 2006), suggesting a common response of terrestrial plants to protists. Root architecture is under hormonal control, where synthesis of active free auxins and auxin deactivation by conjugation regulates a critical balance for lateral root formation. Krome *et al.* (2010) confirmed the relationship between root architecture and the presence of protists in cress plants grown in presence of *A. castellanii*. Indeed, while bacteria alone increased conjugated auxin in shoots of *Lepidium sativum* grown on agar, the addition of *A. castellani* initiated lateral root growth concomitant with an increase of the active free auxins. Bonkowski and Brandt (2002) found indications that predation by *A. castellanii* may favour bacteria producing auxins like indole-3-acetic acid (IAA), but other protists failed to influence root branching and or IAA production (Vestergård *et al.* 2007).

The non-uniform effects of protists to shape plant growth architecture through their predation on microbes highlights the need to identify protist traits that determine their interactions with other microbes and eventually plants in order to better predict protist-microbe-plant interactions (Martiny *et al.* 2015; Schleuning, Fründ and García 2015).

### Plant parasitic protists

Plants are intimately connected to their microbiome, especially in the rhizosphere. Therefore, coevolution of plants and members of the microbiome is common (Lundberg *et al.* 2012; Yeoh *et al.* 2017). Some plants growing in low-nutrient conditions have even developed specific feeding structures to acquire additional nutrients from trapped and digested rhizosphere protists (Barthlott *et al.* 1998). However, the interactions between plants and plant parasitic protists seem much more common.

The most important plant parasitic protists are Oomycetes (Peronosporomycetes; Stramenopiles) (Ben Ali *et al.* 2002; Ruggiero *et al.* 2015). Another group of plant pathogens are the phytomyxid Plasmodiophorida (Rhizaria; SAR) (Neuhauser, Bulman and Kirchmair 2010; Neuhauser *et al.* 2014), showing that plant parasites independently evolved in different protist supergroups. These plant pathogenic protist groups actively reprogram root development and the immune system of plants (Schulze-Lefert and Panstruga 2011). Well-known oomycetes are the downy mildews, *Phytophthora* spp. (root rot), *Pythium* spp. (damping off disease), *Albugo* spp., and *Plasmopara viticola* (Dick 2001; Savory, Leonard and Richards 2015).

The phytomyxea evolved in the Cercozoa (Bulman *et al.* 2001) and include *Plasmodiophora brassicae* the agent of clubroot disease in Brassicaceae (Dixon 2009), or of powdery scab in potatoes (*Spongospora* spp.), as well as carriers of viruses in beets and cereals (*Polymyxa* spp.) (Kanyuka, Ward and Adams 2003). Oomycetes and phytomyxids can be very numerous and diverse in natural habitats (Geisen *et al.* 2015c; Singer *et al.* 2016; Mahé *et al.* 2017), but only a minority is causing visible disease symptoms. Oomycetes form a functional continuum from obligate saprotrophs to facultative pathogens to obligate biotrophic plant parasites (Savory, Leonard and Richards 2015) and many phytomyxids spend part of their life cycle as plant endophytes, while others evolved to hyperparasites of oomycetes or endoparasites of algae (Neuhauser *et al.* 2014).

## COMMUNITY ECOLOGY AND BIOINDICATION

### Fundamental bases for using soil protists as bioindicators

Soil protist community ecology aims at understanding the patterns and drivers of their community structure, trophic interactions with the rest of the soil food web and symbiosis with other organisms at different spatial and temporal scales. Understanding the factors structuring soil protist communities is a prerequisite for using soil protists to infer environmental quality and for forecasting future changes in ecosystem functioning. Soil protists are useful bioindicators in natural and agroecosystems (Bonnet 1984; Foissner 1987, 1997) and are ideal model organisms to address many fundamental and applied questions in ecology (Payne 2013; Altermatt *et al.* 2015). Soil

protists are well suited to develop various monitoring tools for soil fertility, the impact of natural or anthropogenic environmental changes, such as pollution. Protists also indicate ecosystem recovery (e.g. following restoration or conversion to organic farming) because they: 1) have short generation times, their response to external changes is faster than macroscopic organisms; 2) are distributed across all soil types and environmental conditions, including places where few multicellular eukaryotes survive; 3) are functionally diverse and play key roles in soil food webs, biogeochemical cycling and for plant growth; and 4) are abundant and diverse and thus small samples suffice to obtain valuable data (Foissner 1999a; Adl and Gupta 2006; Payne 2013).

Protists are, however, not yet as widely used as bioindicators as this potential suggests, for several reasons: 1) they are less studied than prokaryotes, fungi and soil invertebrates, 2) there are too few protist specialists (taxonomists and ecologists), 3) not all taxonomic groups and ecosystems are equally well understood, and 4) while there is increasing recognition of the huge diversity of soil protists (Mahé *et al.* 2017), most species are still not described (Adl *et al.* 2007; Mora *et al.* 2011; Pawlowski *et al.* 2012) making their bioindication potential unknown for most groups. Despite these limitations, existing studies suffice to give an overview of key ecological factors driving protist diversity and function.

Various factors shape protist community structure: current and past geography and climate (see soil protist biogeography), abiotic and biotic characteristics of the environment—at the mm to cm scale, and the frequency and level of disturbance. Thus, natural and/or anthropogenic factors may drive protist species richness, abundance and composition. Assessing the relative role of these different factors is one of the goals of protist community ecology, which has been addressed by observational and experimental studies (Geisen *et al.* 2017). We highlight the most important natural and anthropogenic structuring factors of protist communities, and the established and potential applications of protists in bioindication.

## Key environmental factors affecting soil protist communities

Soil protists need water to be active. Consequently, all their functions are strictly limited by water availability in the soil pore space (Clarholm 1981; Geisen *et al.* 2014). Soil moisture is a key factor regulating soil protist diversity, density and community composition from arctic to tropical regions (Kennedy 1993; Anderson 2000; Krashevska *et al.* 2012; Tsyganov *et al.* 2013). Protist taxa with contrasted life styles and body sizes exhibit a broad range of tolerance to soil water availability, large taxa being typically more strongly affected by drought than small organisms (Geisen *et al.* 2014). Heterotrophic protist diversity generally peaks in continuously moist soils (Geisen *et al.* 2014), but some groups (e.g. dictyostelid cellular slime molds) become more diverse with alternating wet and dry seasons (Cavender *et al.* 2016). Water availability also controls the development of soil phototrophs (Shields and Durrell 1964; Holzinger and Karsten 2013), plant pathogenic oomycetes (Chadfield and Pautasso 2012; Cohen and Ben-Naim 2016) and apicomplexan animal parasites (Higgs and Nowell 2000; Kolman, Clopton and Clopton 2015). At the other end of the scale, excess of water leads to anoxia in soils. In general, growth rates of protists in anoxic conditions are less than 25% of those in oxic conditions (Fenchel and Finlay 1990). Tolerance to anoxia is variable among protist groups (Fenchel 2014). Certain species require oxygen but can withstand temporary anoxia by encysting (e.g. Schwarz and Frenzel 2003). On the other hand, many parasitic protists are adapted to anoxic conditions, a prerequisite to their lifestyle (e.g. Box 2). Nevertheless, there is also a large diversity of free-living protists that require at least partially anoxic conditions (microaerophiles) or that do not tolerate oxygen at all (strict anaerobes). Several groups have entirely lost their mitochondria to be specialised to anoxic conditions, especially within Excavata and Amoebozoa (Makiuchi & Nozaki 2014; Fig. 1). An entire order of ciliates (the Armophorea) is exclusively composed of anaerobic species, which occur in damp soils (Foissner 1998). These organisms form symbiotic associations with methanogenic Archaea (Finlay and Fenchel 1992) and thus take part in the methanogenesis process in rice fields (Schwarz and Frenzel 2003). Furthermore, protists prey on methanogenic bacteria in these environments, resulting in a release of methane carbon to non-methanogenic microorganisms (Murase, Noll and Frenzel 2006; Murase and Frenzel 2007).

Temperature affects soil protists, primarily by regulating moisture in warm regions through drought and in cold regions through freezing (Bamforth 1973). Accordingly, protists show a broad range of temperature optima and tolerances depending on the environment or life cycles studied (Liu, Yan and Chen 2015). Some protists tolerate frost and/or desiccation (Müller, Achilles-Day and Day 2010; Anderson 2016; Bischoff and Connington 2016), while others do not (Šabacká and Elster 2006). Subtle differences in temperature also influence protist communities as shown experimentally with a dominance of heterotrophic flagellates at 5°C and amoeboid protists at 23°C (Opperman, Wood & Harris 1989). High temperatures above 60°C prevent eukaryotic life (Tansey and Brock 1972; Clarke 2014), but temperatures above 35°C select for thermophilic eukaryotes, also in soils (De Jonckheere, Murase and Opperdoes 2011). Ciliates and kinetoplastids have been found in the Antarctic Dry Valleys, arguably one of the coldest and dryest environments on Earth (Niederberger *et al.* 2015).

In addition to soil moisture and temperature, local soil pH and conductivity frequently affect the density, diversity, species composition, distribution or activity of protists (Ekelund & Rønn 1994; Opravilová & Hájek 2006; Ehrmann *et al.* 2012; Mitchell *et al.* 2013; Dupont *et al.* 2016; Lara *et al.* 2016). Diversity often decreases under low pH, as shown for photosynthetic protists (Shields and Durrell 1964; Lukešová and Hoffmann 1996; Zancan, Trevisan and Paoletti 2006; Fránková *et al.* 2009; Antonelli *et al.* 2017). Nevertheless, acidic soils such as *Sphagnum* peatlands harbour diverse and often highly specific protist communities (Gilbert and Mitchell 2006; Lara *et al.* 2011b; Dupont *et al.* 2016). Soil protists are even present in acid mine drainage precipitates, with pH values as low as 2.5 (Rojas, Gutierrez and Bruns 2016), and with high iron (Fe) concentrations acting as an additional selective parameter.

Light intensity affects the abundance of photosynthetic protists (Shields and Durrell 1964; Lukešová and Hoffmann 1996) and the protist predators of these photosynthetic protists (Seppey *et al.* 2017). UV and red light have direct physiological effects on heterotrophic protists, and influence their dispersal. The multicellular slug stage of phagotrophic heterotrophic *Dictyostelium* expresses light-induced migration (positive phototaxis), and *Dictyostelium* responds by differential sporulation depending on light conditions, which can be explained by a dispersal optimization in more open environments (Häder and Poff 1979; Miura and Siegert 2000). UV-B intensity impacts (possibly indirectly through effects on prey organisms) the diversity of some phagotrophic protist species (e.g. Amoebozoa and

Rhizaria) (Robson *et al.* 2005). Exposure to UV can damage DNA and kill cysts of parasitic protists like *Eimeria* (Apicomplexa) (Thomas, Stanton and Seville 1995). Red light inhibits the sporulation by the plant parasitic *Peronospora* spp. (Cohen *et al.* 2013), which may again help avoid producing spores where they are less likely to be dispersed.

Carbon (C), nitrogen (N) and other nutrients shape also soil protist diversity. Ciliate, testate amoebae and algae diversity and density varied strongly along soil N gradients (Shields and Durrell 1964; Clarholm 2002; Acosta-Mercado and Lynn 2004; Bernasconi *et al.* 2011), while the diversity and density of testate amoebae were reduced by experimental C and phosphorus (P) addition but benefited from addition of N (Krashevska *et al.* 2010, 2014). This effect is often marked in nutrient-depleted environments such as peatlands (Gilbert *et al.* 1998a; Mitchell 2004). Albatross nests in sub-Antarctic islands represent also hotspots of diversity (Vincke *et al.* 2007). However, the experimental addition of nitrogen to tundra soil yielded no significant change in soil protist numbers (Stapleton *et al.* 2005), suggesting that community responses are complex and depend on the type of soil, and also on the protist species present.

Plants and vegetation type can affect soil protist communities in a variety of ways (Acosta-Mercado and Lynn 2004), through differences in the quality of litter or root exudates, changes in microclimate (e.g. open vs. forested land), and effects on bacterial or fungal communities. Rhizosphere differences between two different tropical plants significantly affected their ciliate communities (Acosta-Mercado and Lynn, 2006). Invasive plants such as *Reynoutria* (*Fallopia*) *japonica* or *Rhododendron ponticum*, which drastically modify the vegetation structure by forming monospecific stands, were shown to influence soil protist communities (Sutton and Wilkinson 2007; Vohník, Burdíková and Wilkinson 2012; Bischoff and Connington 2016). In peatlands, testate amoeba communities differ clearly between *Sphagnum* and brown moss rhizospheres (Heal 1961), with larger species observed in *Sphagnum* (Jassey *et al.* 2014). Higher plant functional diversity was shown to increase abundances of amoeboid protists (Ledeganck, Nijs and Beyens 2003; Scherber *et al.* 2010). Testate amoebae species richness increased along chronosequences in parallel to vegetation development (Smith 1985; Carlson *et al.* 2010; Bernasconi *et al.* 2011). However, the increase in amoeba species richness is not necessary correlated to vascular plant richness, since other factors such as soil organic C or N content also contribute to protist diversity (Carlson *et al.* 2010; Dassen *et al.* 2017). Protist communities respond to a complex combination of biotic and abiotic drivers whose interactions are still far from being understood.

Soil protist communities respond to anthropogenic perturbations such as tillage, pollution, land use intensification, pesticides, fertilizers and elevated $CO_2$ (Brussaard *et al.* 2016; Foissner 1997, 1999a; Li *et al.* 2005; Adl, Coleman and Read 2006; Zancan, Trevisan and Paoletti 2006; Lara *et al.* 2007a; Lentendu *et al.* 2014; Gabilondo *et al.* 2015; Imparato *et al.* 2016; Antonelli *et al.* 2017). Pesticides and pollution may also modify the impact of parasites, for example the relative abundance of Apicomplexa (gregarines and coccidids) in soil. Furthermore, the infection level of soil invertebrates increased with the application of herbicides, heavy metals and in regions with high $SO_2$ deposits (Foissner 1999a), possibly due to weakened hosts. The resistance to fungicides and insecticides varies among groups of heterotrophic protists (Petz and Foissner 1989). Addition of fertilizers to naturally nutrient-poor peatlands soils highly increases the growth of phototrophs (Gilbert *et al.* 1998b).

### Using soil protists as bioindicators

Organisms sensitive to environmental changes can be used as bioindicators. By measuring differences in their species identity, morphology or community structure, the strength and quality of impact on the environment over time can be inferred. Based on the application three categories of bioindicators have been proposed: environmental indicators, ecological indicators and biodiversity indicators (McGeogh 1998). Further, we consider that protists are: 1) key components of ecological diversity and ecosystem function and, therefore, pertinent for conservation actions, and 2) likely relevant indicators of diversity that may or may not be correlated to that of other groups (e.g. plants, insects, etc.).

Testate amoebae produce decay-resistant shells and are routinely used in palaeoenvironment studies for reconstruction of water table (in peatlands). They have a also a strong potential to infer other environmental changes, e.g. climate change, pollution, land use changes, fire history, nutrient status, as forensic trace evidence and dating cadavers (Wanner and Dunger 2001; Mitchell, Charman and Warner 2008; Payne and Babeshko 2016; Swindles and Ruffell 2009; Lamentowicz *et al.* 2013, 2015; Seppey 2013; Szelecz *et al.* 2014;). Parasitic protists have so far rarely been used as bioindicators in soils, but their increase could be used as indication of environmental perturbation (Dupont *et al.* 2016) (see above).

Ecotoxicological studies testing the effects of pollutants on the survival and reproduction of protists might be particularly informative as several protist taxa are quickly responding to fluctuations in their abiotic environment (Foissner 1997). Examples include the effects of oil spills on marine protists (Brussaard *et al.* 2016). Freshwater ciliates such as *Tetrahymena pyriformis* and other species are classical tools used for evaluating the toxicity of various chemicals (Sauvant, Pepin and Piccinni 1999). Likewise, several green algal species are also used (Franklin, Stauber and Apte 2002), amongst others. However, soil protists are surprisingly little included in ecotoxicoloical investigations. Soil ciliates have been tested against heavy metals (Díaz, Martín-González and Gutiérrez 2006), and *Dictyostelium discoideum* against pesticides (Amaroli 2015). The spectrum of protists tested (both functionally and taxonomically) is nevertheless narrow as compared with their immense diversity and should be expanded in order to better predict the effect of pollutants on ecosystem functioning.

## SOIL PROTIST BIOGEOGRAPHY

### Challenges and knowledge gaps in soil protist biogeography

Since more than two centuries (e.g. von Humboldt and Bonpland 1805; Wallace 1876; MacArthur and Wilson 1967) biogeography attempts to document and understand the patterns and causes of biodiversity along broad spatial gradients and time (Brown and Lomolino 1998; Gaston and Blackburn 2000). Species richness often varies in a regular fashion over space, giving rise to broad-scale diversity gradients known as biogeographical patterns. Two of the most remarkable biogeographical patterns are the latitudinal and the elevational diversity gradients. The existence of biogeographical patterns produced and maintained by ecological and long-term processes has been repeatedly demonstrated in multicellular organisms (i.e. plants and animals) (Wiens and Donoghue 2004; Hortal *et al.* 2011; Rivadeneira *et al.* 2011; Sanders and Rahbek 2012; Qian *et al.* 2013).

As most soil protists have not yet been formally described, determining their biogeographical distributions is challenging (Foissner 1999c; Chao *et al.* 2006; Geisen and Bonkowski 2017). Early views on protist biogeography favoured the idea of a cosmopolitan distribution (Penard 1902). It, however, gradually became obvious that at least some soil protist species were not cosmopolitan (Foissner 2006; Smith and Wilkinson 2007). Protist and microbial biogeography in general became very controversially discussed until about a decade ago (Finlay, Esteban and Fenchel 1996; Finlay 2002b; Fenchel and Finlay 2004; Foissner 2006, 2008; Martiny *et al.* 2006). This debate was largely tied to the question of taxonomic resolution (Mitchell and Meisterfeld 2005; Heger *et al.* 2009). As a consequence, the underlying processes responsible for generating theses pattern, other than in paleogeography (Foissner 1998; Smith and Wilkinson 2007), have not been investigated much (Aguilar *et al.* 2014; Fernández *et al.* 2016).

With new methodological developments including high-throughput sequencing (Box 1), research on protist biogeography has now clearly entered a new, much more positive era (Lepère *et al.* 2013; Logares *et al.* 2013; Lanzén *et al.* 2016; Lara *et al.* 2016). Initial studies have focused on archaea, bacteria, aquatic protists, fungi, and more recently, on soil protists (Foissner 1999c; Chao *et al.* 2006; Fontaneto *et al.* 2006; Bates *et al.* 2013; Fernández *et al.* 2016; Fournier *et al.* 2016; Lara *et al.* 2016). In the following, we summarise the state of knowledge and recent developments in soil protist biogeography.

## The latitudinal gradient in soil protist diversity

Research on protist biogeography has traditionally focused on studying the latitudinal diversity gradient over regional ( $>$ 1000 km) or global (from pole to pole) geographical scales (e.g. Hillebrand and Azovsky 2001; Azovsky and Mazei 2013; Bates *et al.* 2013; Fernández *et al.* 2016; Lara *et al.* 2016). In general, soil protist diversity follows predictable trends in latitudinal gradients, mirroring those traditionally found in plants and animals. One of the major challenges that faces the study of latitudinal diversity gradients is sampling bias. Latitudinal gradients stretch along hundreds to thousands of kilometres, and therefore, the screening of a representative fraction of soil protist diversity and distribution can be very challenging at such spatial scales. Indeed, a well-designed study to investigate regional or global latitudinal gradients in soil protist diversity would require the collection of samples at regular and narrow intervals to capture the true spatial heterogeneity of their communities. Even high-throughput sequencing studies have up to now examined only a limited number of samples along regional or global latitudinal gradients, often concentrating the sampling effort in the northern hemisphere or in historically well-studied regions (e.g. the Holarctic). As a result, such sampling biases still limit the conclusions of these studies (Belasky 1992; Yang, Ma and Kreft 2013).

## The elevational gradient in soil protist diversity

Elevation gradients are suitable for studying biogeographical patterns (Grytnes and McCain 2007; McCain and Grytnes 2010) and the logistics of such studies is easier than for latitudinal studies. Furthermore, many of the potential underlying causes that covary along other geographical gradients (history, climate, area, etc.) do not covary along altitudinal gradients. Finally, altitudinal gradients represent globally replicated gradients—essentially any altitudinal gradient contained between sea level and a mountaintop is a replicate, so they offer many opportunities to test patterns and potential causes for the spatial distribution of microbial diversity. By contrast, there are essentially only two latitudinal gradients either northwards and southwards from the equator towards the respective poles.

Soil protist diversity often follows the same elevational trends reported for multicellular organisms, including decreasing diversity with elevation (e.g. Todorov 1998; Heger *et al.* 2016) and unimodal diversity gradients (diversity peaks at mid-elevations, e.g. (Krashevska *et al.* 2007)). Research on plant and animal biogeography suggests that decreasing diversity gradients are common in wet altitudinal gradients because they often exhibit an increment of environmental adversity with elevation (Grytnes and McCain 2007; McCain and Grytnes 2010). Unimodal diversity gradients are common in dry altitudinal gradients because they often exhibit adverse environmental conditions at lower and higher elevations (Grytnes and McCain 2007; McCain and Grytnes 2010). Recent research suggests that environmental adversity might also be driving decreasing and unimodal gradients in soil protist diversity (Krashevska *et al.* 2007; Heger *et al.* 2016; Lanzén *et al.* 2016).

These findings suggest that the diversity of soil protists and multicellular organisms exhibits similar broad-scale diversity patterns, but the generality of these results still needs to be tested. For example, the elevational diversity gradient has mainly been investigated in soil testate amoebae (e.g. (Todorov 1998; Mitchell, Bragazza and Gerdol 2004; Krashevska *et al.* 2007; Heger *et al.* 2016)), while a considerable fraction of the unicellular terrestrial lineages that compose domain Eukaryota remains little studied (Shen *et al.* 2014; Lanzén *et al.* 2016). In addition, the elevational diversity gradient has only been investigated at the top of the altitudinal gradients (i.e. typically in mountainsides distributed between 1000 and 3000 m.a.s.l.). Altogether, this causes sampling biases that are likely to distort the results (McCain and Grytnes 2010). These biases possibly explain why some studies (e.g. Mitchell, Bragazza and Gerdol 2004; Tsyganov, Milbau and Beyens 2013; Shen *et al.* 2014; Lanzén *et al.* 2016) failed in finding any trend in protist alpha diversity along altitudinal gradients. Therefore, the elevational gradient in microbial diversity still needs to be investigated along full altitudinal gradients (i.e. from sea level to mountaintops). Finally, the elevational diversity gradient has been mostly investigated in altitudinal gradients located in similar ecoregions (i.e. in ecological and geographical regions with similar vegetation types, climate, history, etc.).

## Passive dispersal in soil protists

Another critical issue in protist biogeography is the dispersal capacity of these organisms. It is generally agreed that protists disperse passively, and soil organisms are transported by the wind or, incidentally, by animals such as ants (Villani *et al.* 2008), birds, other insects, and human activity (Wuthrich and Matthey 1980; Wilkinson 2010). Dispersal is potentially easier for those protists that are able to produce dormant forms (cysts) which can be extremely resistant and remain viable over long periods (Shmakova and Rivkina 2015). Such cyst-producing protists can thus be transported over large distances before reaching an environment where they can build a population. However, not all soil protists are able to form cysts and analyses of dried soil may thus lead to underestimation of the diversity, especially when studying soils that never dry out naturally (Foissner 1997). The capacity of being transported is also largely dependent on propagule size, as shown by modelling (Wilkinson *et al.* 2012) and population genetics (Lara *et al.* 2011a) studies. Dispersal capacities of soil protists therefore likely varies considerably among taxa and habitats.

### Processes driving biogeographical patterns in soil protists

The broad-scale patterns of soil protist diversity often correlate with one or more environmental variables. In particular, there is an emerging trend to find strong relationships among soil protist species richness and environmental variables related to water content (Bates *et al.* 2013; Serna-Chavez, Fierer and Van Bodegom 2013; Geisen *et al.* 2014), energy (i.e. temperature and biochemical turnover (Yang *et al.* 2006; Heger *et al.* 2016; Lara *et al.* 2016)) or both (i.e. a water-energy balance (Fernández *et al.* 2016)). The evaluation of these hypotheses has shown that ecological processes contribute to the maintenance of current biogeographical patterns of soil protists. However, future research should also incorporate the explicit evaluation of historical and evolutionary hypotheses (see for example (Fernández *et al.* 2016)). The assessment of these hypotheses will allow determining whether long-term processes also contribute to shaping soil protist biogeography as is commonly observed for macroscopic organisms.

Finally, the debate on protist ecology is no longer on whether all species or only some of them have a cosmopolitan distribution (Caron 2009; Hanson *et al.* 2012), but rather on the true magnitude and geographical patterns of their diversity. Of particular interests are the rules that underlie the distribution of these patterns, whether these patterns and rules are the same as for macroscopic organisms, and if not, why.

## CHALLENGES AND FUTURE PROSPECTS IN SOIL PROTISTOLOGY

In this paper, we provide a cumulative state-of-the-art overview of soil protist ecology, including methods to study protists, the taxononomic and functional diversity of soil protists soil protists as human pathogens, specific adaptations of protists to live in soils, the role of protists for plant performance, drivers that shape soil protist communities locally and biogeographic patterns of soil protist. While research on soil protists is clearly gaining momentum (Geisen *et al.* 2017), and we are rapidly advancing our understanding on soil protistology, there are important remaining research gaps, which we will highlight in the concluding paragraph.

### Describing the huge taxonomic and functional diversity

Even before the advent of high throughput sequencing (Box 1) it was clear that describing the full diversity of soil protists was a huge challenge. One advantage of the wealth of data that is being generated by high throughput sequencing studies is that we now have the possibility to prioritise further detailed descriptive taxonomical and ecological work. This can be achieved by selecting taxa that are of special interest either because of their phylogenetic position (i.e. under-studied clades or novel clade known only from molecular data), that they are potentially useful as bioindicators (e.g. forensic indicators, see Seppey *et al.* (2016)), or that are suspected to play a key ecological role, e.g. as identified by co-occurrence network analyses (Flues *et al.* 2017; Xiong *et al.* 2017). All this will require a tremendous effort of basic taxonomic description and basic ecological work. Soil protistology may indeed be entering a new 'golden age', but this will only come true with the necessary level of support for this field (Mitchell 2015). International, community based efforts, such as UniEuk, have now started (Berney *et al.* 2017) that will help at filling this knowledge gap.

### Protists as a soil health diagnostic tool

The idea of using protists for soil monitoring is not new, but practical applications have been limited by the difficulties associated with time-consuming morphological identification methods, which restricted their use to a few skilled specialists and prevented the parallel survey of numerous soil samples. We expect that the development of new molecular screening tools (Lara and Acosta-Mercado 2012) as well as simplified direct observation (Koenig, Feldmeyer-Christe and Mitchell 2015), and approaches based on functional traits (Fournier *et al.* 2012) will allow soil protists to become more important for applications as indicators of environmental health. With the increasing amount of eukaryotic genomes sequenced and the future development of novel, more performant sequencing technologies, the search for eukaryotic functional genes of interest in 'omics' approaches (including metagenomics and metatranscriptomics as it is currently done for bacteria; Prosser 2015) can be envisaged in the near future.

### Linking phylogeny to function for biotechnological applications

The enormous taxonomic and functional diversity of protists may be applied in biotechnology and agriculture. In order to exploit the appropriate protist species, however, there is a need to predict better their main functional characteristics, either from detailed genome information or from their phylogenetic affiliation. As an example, protist feeding patterns on bacteria and fungi are partly conserved in their phylogenetic affiliations (Glücksman *et al.* 2010; Geisen *et al.* 2016). Further, protists from different phyla show very distinct patterns of sensitivity to bacterial secondary metabolites, including, for instance, the sensitivity and ability to avoid volatile and soluble compounds produced by their prey (Pedersen *et al.* 2010; Schulz-Bohm *et al.* 2017). Different studies thus point to a phylogenetic conservation of protist predator-prey interactions, in which case, the taxonomic affiliation may provide a first rough prediction of the protist's functionality (Parry 2004). The distinct and consistent impacts of different protist phyla on ecosystem functioning may be exploitable in agricultural applications.

Based on the current state of research, one should, however, interpret protist functionality from taxonomy with caution, given that closely related species may have different lifestyles. For example, closely related oomycete species may be either pathogens or saprotrophs (Lara and Belbahri 2011; Savory, Leonard and Richards 2015), which makes a difference for potential applications. Similarly, chrysophytes have lost the photosynthetic ability several times in their evolutionary history and evolved into phagotrophs (Beisser *et al.* 2017). Some green algae have lost photosynthetic capacity and evolved into saprotrophs and pathogens (Figueroa-Martínez *et al.*, 2015). Further more explicit functional studies thus need to be conducted, especially for poorly-known clades, and coupled with genome sequencing to find more appropriate functional markers (Box 1; Geisen 2016a).

### Applications, e.g. in agriculture

Although to date very few applications are based on soil protists, they bear a great potential as a soil health improvement technology. Protists consuming bacteria can speed up nutrient turnover. This may come in particularly handy to enhance nu-

trient availability from organic fertilizers especially under low temperature. By increasing the availability of otherwise limiting nutrients, protists may also enhance bacterial metabolism, which may stimulate biodegradation rates of organic pollutants by soil microbial communities (Kota, Borden and Barlaz 1999).

Protists may be applied for biological control of plant diseases (Fig. 4d). Soil-borne pathogens are a major threat to food security. Conventional pesticide-based control strategies are losing their efficiency and causing negative side effects on human and environmental health. There is thus an urgent need for sustainable disease control strategies to replace pesticides. Root-associated microbiota can suppress diseases, and therefore hold a potential for agricultural applications. Protists can confer a competitive advantage to a range of native bacteria with the potential to protect plants against diseases, by selectively removing competitors and acting like 'bacterial bodyguards'. They can further stimulate antibiotics production by plant-growth promoting bacterial species (Mazzola *et al.* 2009; Jousset and Bonkowski 2010; Jousset *et al.* 2010; Song *et al.* 2015). Protists may thus help to enhance natural innate suppression in agricultural soils, by improving the ability of soil microbial communities to ward off pathogens, furthering the goals of more sustainable agriculture. In addition, parasites such as apicomplexans may also serve to control nematode and arthropod plant pests, while photoautotrophic protists may enhance soil oxygen levels that activates other microbes.

The first industrial companies are now developing protist-based strategies and the first industrially-produced biofertilizers are now on the market. As the range of possible applications of protists is indeed very broad, we are confident that protists will find more specific applications in the near future.

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