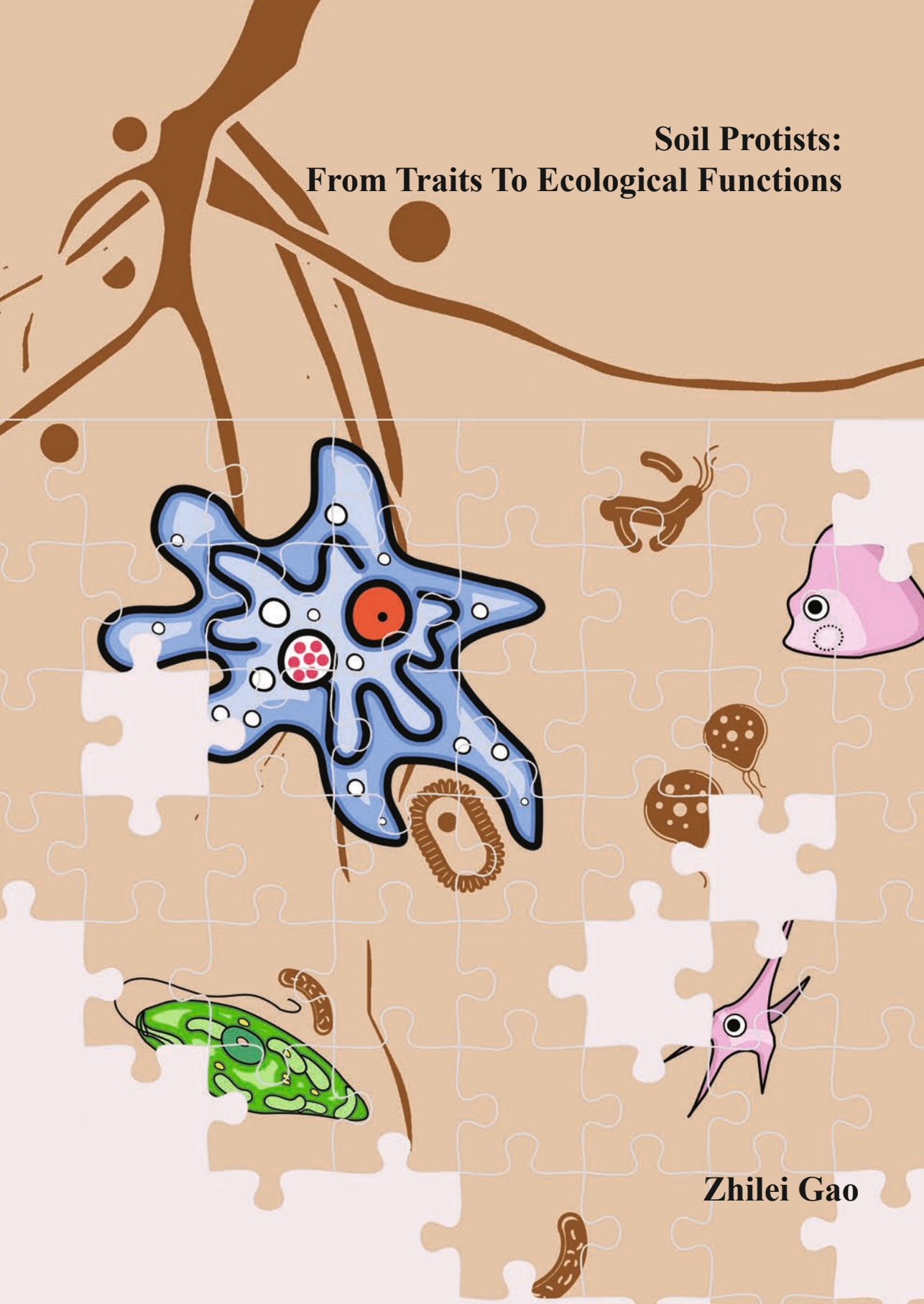
Soil Protists:
From Traits To Ecological Functions



Zhilei Gao

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ISBN 978-90-393-7274-6

Cover design by Yidong Wang

Illustraintions on page 12, 14, 17, 87, 91, 98 and 110 were desgined with BioRender.

Printed by Ridderprint BV, www.ridderprint.nl

Soil Protists: From Traits To Ecological Functions

Bodemprotisten: van Eigenschappen tot Ecologische Functies
(met een samenvatting in het Nederlands)

土壤原生生物：从性状到生态功能
(内附中文摘要)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

woensdag 21 oktober 2020 des ochtends te 11.00 uur

door

Zhilei Gao

geboren op 22 oktober 1990
te Beijing, China

Promotor: Prof. dr. G.A. Kowalchuk

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This thesis was accomplished with financial support from China Scholarship Council (No. 201506350026 to Zhilei Gao) and ECOstyle.

Contents

Chapter 1

General Introduction

Zhilei Gao

The living soil

Soil is not just the 'dirt', it is a living natural resource that provides the basis for plant growth. Soils are therefore essential in the provisioning of food, clothing and building materials. Many soil properties are due to the activities of soil-dwelling organisms, encompassing not only visible animals but also an enormous diversity of microorganisms (Fierer, 2017). Soil microorganisms play crucial roles in terrestrial ecosystem functioning, including improving soil fertility, sequestration of carbon, nitrogen mineralization, decomposition and supporting plant health and growth (Dilly et al., 2004; Heijden et al., 2008; Berendsen et al., 2012; Bardgett & Van Der Putten, 2014). Bacteria and fungi have traditionally been the most studied groups of soil-dwelling microorganisms, with numerous studies highlighting both their high abundance and biodiversity (Frey et al., 1999; Tedersoo et al., 2014; Delgado-Baquerizo et al., 2018). However, pure focus on bacteria and fungi only tells part of the story in soil; we still have relatively little insight into the forces that drive patterns of bacterial and fungal abundance and diversity. Many other soil organisms interact within the complex soil food web, and these interactions may be critical in determining soil microbiome structure and function (Thakur & Geisen, 2019). Soil protists represent one of the most important predators of soil microbes that can exercise top-down regulation of the soil microbiome.

What are protists?

Protists are a paraphyletic group encompassing most eukaryotic lineages, with the exception of fungi, animals and plants. This unusual classification is a legacy of the scientific discovery of protists, starting with the invention of the microscope by Antoni van Leeuwenhoek in the 17th century. Most people are still not familiar with the term 'protist'. Instead, the term 'protozoa' has typically received more public attention perhaps due to its association with rather rare, but particularly horrific, protozoan infections (Visvesvara et al., 2007). However, after modern classification based upon phylogenetic relatedness (Adl et al., 2005), protozoa is currently termed as heterotrophic protist to avoid confusion. The term 'protozoa' has gradually been discouraged in modern eukaryote taxonomy classification (Adl et al., 2013), although this term is occasionally still used in the medical sciences (Marcos & Gotuzzo, 2013).

Protists are reasonably well studied in aquatic systems, with heterotrophic protists displaying greater diversity than marine plants and animals based on the marine plankton size spectrum (de Vargas et al., 2015). Furthermore, protist predation results in major mortality of both heterotrophic and autotrophic bacteria. More recently, DNA-based studies have revealed an expectedly high diversity of soil protists (Bates

et al., 2013; Geisen, 2016a; Mahé et al., 2017; Xiong et al., 2018), sparking an increased research focus on these organisms. However, despite the growing interest, soil protists still receive far less attention than aquatic protists (Geisen et al., 2017) or other soil microorganisms such as bacteria and fungi.

Morphological diversity of protists

The vast morphological diversity of protists first started to come into view with the help of more sophisticated microscopes (Page, 1967; Foissner, 1999b; Smirnov & Brown, 2004). Soil protists display a remarkable diversity with respect to size, with body length ranging from micrometers up to 1 millimeter (Geisen et al., 2017). Other than the protist size, soil protists also display a range of distinct morphologies, even included plant-like morphologies of phototrophic protists that were formerly term as algae (Seppey et al., 2017). Additionally, some soil protists exhibit a fungi-like morphology, such as oomycete and species coined as 'slime molds', which take on a flexible amoeboid shape when food resources are abundant (see Fig.1 (l)) and congregate into a single body called a (pseudo-) plasmodium when food becomes scarce (scan Fig.1 (u), click P1-1 movement). Depending on the conditions, the pseudoplasmodium can also form a fruiting body called a sporocarp.

Protists encompass a range of morphotypes, such as flagellate (the cell with 2 - 4 flagella or cilia, see Fig.1 (d)); naked amoeba (amoeba with flexible cell shape, see Fig.1 (n)); testate amoeba (amoeba with a shell, see Fig.1 (r)); ciliate (the cell with a great numbers of flagella or cilia). These morphotypes are the result of convergent evolution and phylogenetically distinct groups can display very similar morphology (Fig.1 (f) and (j)).

Phylogenetic diversity of protists

Protists dominate the eukaryotic tree of life (See Fig.1 in Adl et al., 2012), and the advance of molecular studies has revealed an extremely high and unexpected species diversity that supersedes that predicted by morphological characterizations (Boenigk et al., 2005). In particular, the diversity of naked amoeba, such as in the class Heterolobosea, was underestimated due to their indistinguishable morphotypes under the microscope. The use of DNA sequence-based approaches targeting the small subunit (SSU) rRNA gene, as well as other gene makers, can help resolve phylogenetic affiliations at different levels of taxonomic resolution. For instance, sequence analyses targeting the highly variable Internal Transcribed Spacer (ITS) regions allow for detailed classification of some groups of morphologically similar protists to the genus, species and even potentially to the strain level (De Jonckheere, 2004).

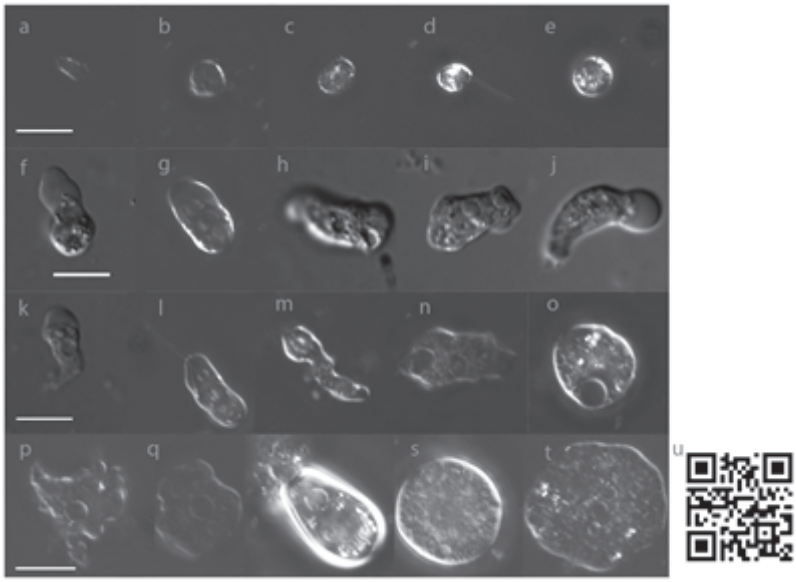


Figure.1 Morphotypes of 20 protists species used in this study, the order of each protist is visualized by the size (length). (a) *Rosculus* sp. C10D3; (b) *Mycamoeba* new sp.16; (c) *Cercomonas* sp. C7D2; (d) *Cercomonas lentalike* C5D3; (e) *Cercomonas* sp. S24D2; (f) *Allovahlkampfia* sp. NL10; (g) *Acanthamoeba* sp. C13D2; (h) *Naegleria clarki* P145-4; (i) *Naegleria clarki* NL81; (j) *Vahlkampfi soli* CN7; (k) *Heterolobosea* sp. S18D10; (l) *Didymium* sp. P1-1; (m) *Vermamoeba vermiformis* 7; (n) *Acanthamoeba* sp. C2D2; (o) *Vannella* sp. 33; (p) *Vannella* sp. P147; (q) *Vannella* sp. 45; (r) *Cryptodifflugia* sp. 75; (s) *Famella* new sp. 27; (t) *Cochliopodium minus* 76; (u) QR code of iamoeba.space (https://iamoeba.space/), in which all strains' movements were exhibited.

By aligning molecular data to the functional characteristics of cultivated protist strains, it is increasingly possible to putatively assign protist DNA sequences to a specific protist functional group. Thus, molecular approaches have not only been instrumental in revealing the enormous diversity of protists, they have also provided important insights into the ecological roles of protists (Mahé et al., 2017; Xiong et al., 2018). It should be noted that functional assignments are currently still according to our knowledge of a relatively limited range of well-characterized taxa. Further studies into revealing protist life history strategies and interactions with potential prey within the soil food-web are therefore necessary to improve our ability to link molecular data with inferences of protist ecology.

What do protists do in soil?

Protists are versatile microorganisms in soil and display a broad range of functional

roles. The best-known role of soil protists is as bacteria feeders. Nevertheless, soil protists can also be fungal feeders, other eukaryote feeders, primary producers or parasites (Bates et al., 2013; Jassey et al., 2015; Geisen et al., 2015c; Singer et al., 2016; Geisen, 2016b; Mahé et al., 2017). In this present work, I will zoom in on predation on bacteria as protists have long been regarded as the main consumers of soil bacteria. Protist consumption of bacteria can lead to the release of nutrients locked in bacterial biomass. These released nutrients, especially nitrogen in the form of NH_3, are related to the higher C:N ratio of protists as compared to bacteria (Sherr et al., 1983). However, protists do not feed on all prey bacteria equally, with selective feeding being based for instance on prey size, surface properties, motility and toxicity (Matz & Kjelleberg, 2005; Jousset, 2012). Furthermore, selective predation by protists can shift prey bacterial community structure, which may be linking with changes in soil community functioning (Bonkowski & Brandt, 2002; Rosenberg et al., 2009; Krome et al., 2009).

Protist-induced impacts on ecosystem functioning

Nutrient cycling

Soil protists increase the turnover of nutrients, such as nitrogen, phosphorus, and micronutrients. Most soil nutrients are usually locked within bacterial biomass. As protists consume bacterial prey, they make these nutrients available to other microbes, a phenomenon referred to as the microbial loop (Clarholm 1985). This nutrient availability induced by protists will increase microbial turnover and stimulate microbial activity, including microbial respiration (Trap et al. 2015) (Fig.2).

Such protist-related increases in nutrient turnover can benefit plants in two general ways. In the first place, the increased liberation of nutrients can become directly available to the plant to facilitate growth and plant nutrition (Ritz & Griffiths, 1987; Kuikman et al., 1990; Bonkowski et al., 2000). In addition to such direct effects, the associated increases in microbial activity can activate enzyme systems that lead to further increases in nutrient availability to the plant. In particular, protists can strongly facilitate mobilization of nitrogen from soil organic matter, thereby stimulating decomposition and promoting plant growth (Bonkowski et al., 2000; Koller et al., 2013a). The total effects of higher nutrient availability can lead both to an increase in plant biomass as well as improved nutrition in the plant, with positive effects of protists on plant carbon, nitrogen and phosphorous uptake (Bonkowski et al., 2000, 2001b; Somasundaram et al., 2008; Krome et al., 2009). Protists are also important for the efficient functioning of arbuscular mycorrhizal fungi, which have a limited ability to produce the enzymes required for soil organic matter breakdown. Protists increase nutrient mineralization by hyphae-associated microorganisms, which can

then be taken up by the mycorrhiza and transferred to the host plant (Herdler et al., 2007; Koller et al., 2013c) (Fig.2)

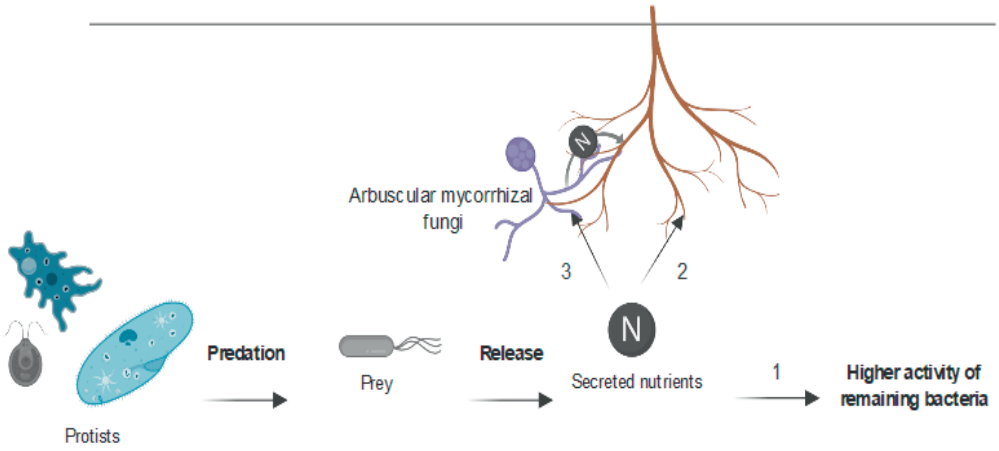


Figure.2 Predation by protists as a driver of nutrient cycling. Protists consume bacterial prey and release nutrients, those nutrients are available for 1: remaining bacteria; 2: plant uptake; 3: Arbuscular mycorrhizal fungi (AMF) that translocate nutrients to roots.

Shifts in microbiome and functionality

Non-specific effects of protists on bacterial community

Protist predation generally decreases total bacterial abundance and biomass (Ekelund et al., 2009; Rosenberg et al., 2009). However, predation stimulates total bacterial activity and species turnover by increasing the respiratory quotient and enhancing niche spaces for active species (Bonkowski, 2004).

Selective predation and its impacts on bacterial communities

Protists possess refined mechanisms for prey selection, allowing them to discriminate between potential prey based upon cell size, surface properties, motility and toxicity (Gasol et al., 1995; Matz & Jürgens, 2001, 2005; Jousset et al., 2006). For instance, marine protists exhibit size-selective predation strategies that has led to a preference for larger bacterial cells (Chrzanowski & Šimek, 1990; Baltar et al., 2016). Other structural features of potential bacterial prey, such as flagella and polysaccharides, also play a crucial role in prey recognition (Wildschutte et al., 2004). Moreover, chemical cues from prey bacteria may also influence predator selective feeding (Schulz-Bohm et al., 2017).

Selective feeding by protists can have important consequences for microbiome structure and function. Protist predation helps to maintain diversity within bacterial communities by feeding on the most dominant strains and enabling marginal bacteria to improve their competitiveness, thereby leading to increased bacterial evenness (Bell et al., 2010; Saleem et al., 2012). In addition, selective feeding by protists can result in shifts in rhizosphere microbiome composition and functionality. For instance, selective feeding can give a selective advantage to specific bacterial taxa that can avoid predation (Kreuzer et al., 2006; Rosenberg et al., 2009) (Fig.3), thereby promoting for instance Gram-positive bacteria in the rhizosphere that relatively well protected by their thick cell wall (Rønn et al., 2002; Murase et al., 2006). Moreover, selective feeding by protists favors rhizosphere microbes that can produce plant hormones. For instance, *Acanthamoeba castellanii* was shown to increase the relative abundance of auxin producers and increase plant-free auxin concentrations (Bonkowski & Brandt, 2002). However, in other cases, protist predation was not observed to affect the density of IAA-producing bacteria in soil (Vestergård et al., 2007).

Different protist species vary greatly in their effect on bacterial community composition. It can be hypothesized that impacts on soil microbial communities might be related to protist phylogeny. Any such relationship would clearly depend strongly on the taxonomic level investigated, and it has been observed that even closely related protist species can have highly distinct impacts on prey bacterial community structure (Glücksman et al., 2010). On the other hand, Pedersen et al (2011) showed that high-level taxonomic affiliations could be correlated with crucial characteristics linked to interactions with prey, such as sensitivity to bacterial defense compounds (Pedersen et al., 2011). Thus, while we do know that protist feeding differs between species, most studies to date have focused on only one or very few protist species, usually confined to a narrow phylogenetic range, thereby limiting our ability to predict how specific protists will impact soil-borne microbial communities.

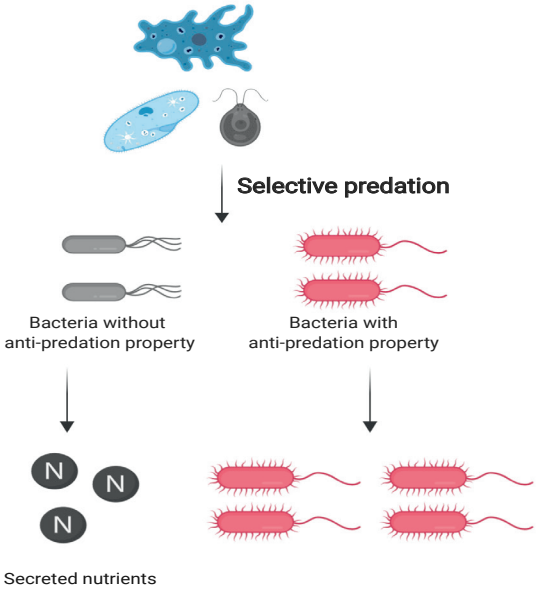


Figure.3 Impact of selective predation by protists on bacterial community. Bacteria that cannot express anti-predator strategies are consumed by protists and release nutrients. Conversely, bacteria that are able to express anti-predator strategies, for instance changing surface properties, increasing motility and release toxin, will gain a competitive advantage at the cost of sensitive bacterial populations.

Microevolution

Protists may play an important role in driving evolutionary changes in potential bacterial prey species. For instance, protist selective pressure may select for bacterial strains with a heightened ability to produce secondary metabolites that deter protist predation. Mutations that increase such secondary metabolite production would be positively selected, and this selection should come at a metabolic cost, thereby creating a selection tradeoff in the absence of protist predation. Thus, the selective pressures of protists can drive diversification in prey populations (Meyer & Kassen, 2007).

Protists can also support microbiome cooperation by consuming defectors that do not express public goods providing protection against protists such as toxic metabolites (Jousset et al., 2009; Friman et al., 2014). Such compounds benefit the entire population regardless of the individual investment, and defectors can still take advantage of public goods without investing their fair share. Although total population susceptibility may be a product of population-level secondary metabolite

production, protists may be able to distinguish between individual cells with high and low production of such compounds. In this way, protist selective feeding on defectors could serve to increase the cooperator population and thereby further stabilize microbial cooperation. Protist-imposed selection could also impact other co-evolutionary and multitrophic interactions. For instance, protists may weaken the selection strength of bacteria against bacteriophages and further phage infectivity (Friman & Buckling, 2013).

Missing gap: linking protist traits to their impacts on soil communities

To date, most studies of protist ecology have largely focused on ciliates and testate amoeba because of their distinct morphological features and the relative ease with which they can be cultivated (Foissner, 1999b). Despite their high abundance and diversity in soil communities, naked amoeba are still understudied, partially due to challenges related to their identification (Smirnov & Brown, 2004). Both cultivation and molecular-based approaches have shown naked amoeba to be dominant members in soil protist community (Finlay et al., 2000; Robinson et al., 2002; Geisen et al., 2015d), yet the majority of studies examining the functionality in soil protists has been restricted to *Acanthamoeba castellanii* or other single protist species. In order to more fully explore the ecology of protists in the soil ecosystems, it is imperative that we expand the number and diversity of protists stains used in functional characterizations.

As argued above, phylogenetic affiliation might not provide a solid framework for explaining or predicting the effects of protist predation on soil-borne bacterial communities. I therefore propose that a trait-based approach, as suggested by Dumack et al. (2019), might offer new insights into protist ecology and impacts of preferential feeding behaviors. In addition to growth rate, I principally focused on morphological traits, as these are generally straightforward to examine, and they have previously been shown to influence predator and prey interactions (Brose et al., 2019).

Objectives and outline of the thesis

To date, it remains unknown to what extent protist predation can be predicted because most studies have focused on only a single or a limited range of protist and prey species. To fill in this gap, the main objective of this thesis was to investigate if protists predation could be predicted using a trait-based approach. I focused on 20 well-characterized soil protist species, and further investigated how protist

morphological traits were linked to impacts of protist predation on soil-borne bacterial communities. Specifically, I examined the extent to which predation effects can be linked to either high-level phylogeny (**Chapter 2**) or morphological traits (**Chapter 3**). Trophic interactions between protists and bacteria can influence a range of ecological functions, including microbial cooperation. To examine this issue, I examined the stability of microbial cooperation in the presence of protist predation and competition (**Chapter 4**). Finally, I provide a combined synthesis of protists-driven effects on the rhizosphere microbiome, thereby providing a new perspective on trait-based approaches that could be used to improve rhizosphere functionality and plant growth (**Chapter 5**) (Fig.4).

In **Chapter 2** I start by clarifying the taxonomic affiliations of the protists isolates used in this thesis, with a special focus on Heterolobosea amoeba, which are widespread and diverse in soil. Given the lack of morphologically useful characteristics in Heterolobosea amoeba, I combined morphological identification and phylogeny analysis of Heterolobosean amoeba based upon sequencing the small subunit (SSU) rRNA gene and internal transcribed spacer (ITS) region.

In **Chapter 3**, I investigate the relationship between protist traits and their effects on prey bacterial community structure. I measured a range of protists traits, including growth rate, length, width, morphology and volume across my target 20 protist species covering the main phylogenetic lineages found in soil. I further used microcosm experiments to assess the effect of each species on the structure of a semi-natural soil bacterial community. This work revealed that protists traits, especially cell volume, could be linked to their predation effect on the bacterial community.

I then examine how protist predation affects the stability of cooperative interactions in the soil microbiome. To this end, in **Chapter 4** I tested the relative importance of predation and competition as drivers of cooperation. I combined a pair of focal cooperator and defector strains in the presence of competing soil bacterial community and protists. I then followed the benefit of cooperation and defector invasion when they are rare. This work revealed that multitrophic interactions strongly impacted microbial cooperation.

In **Chapter 5**, I synthesize existing knowledge on soil protists and demonstrate their importance as regulators of the rhizosphere microbiome. I first summarized the different reported interactions between predators and prey in the rhizosphere. I then addressed the known and hypothesized consequences of protists on microbiome functionality and plant performance. This chapter concludes with the presentation

of a framework to guide efforts to harness protists as a microbiome enhancer in sustainable agriculture.

In **Chapter 6**, I discuss the results in this thesis and further provide personal perspectives on the future directions and priorities related to research of soil protists.

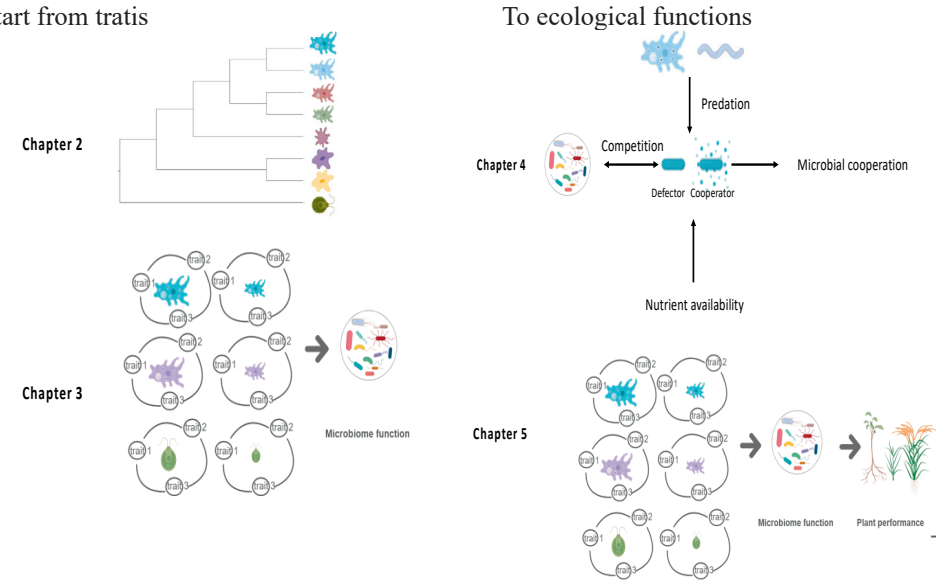
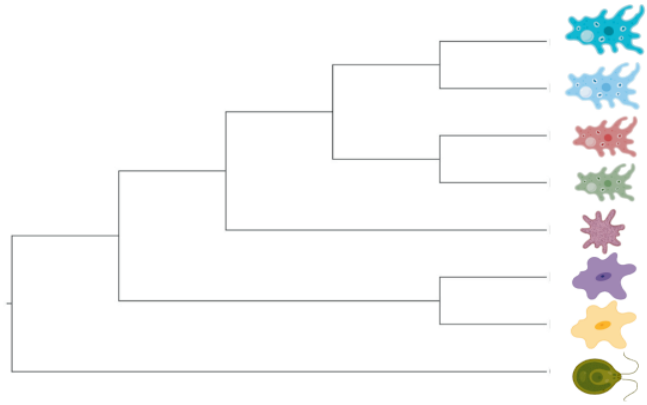


Figure.4 Cartoon of each chapter of this thesis. Chapter 2 shows the taxonomic affiliations of the protists isolates with a special focus on Heterolobosea amoeba; Chapter 3 presents protist traits and their effects on prey bacterial community structure; Chapter 4 examines the stability of microbial cooperation in the presence of protist predation and competition; Chapter 5 provides a combined synthesis of protist-driven effects on the rhizosphere microbiome, thereby providing a new perspective on trait-based approaches.



Chapter 2

Five clusters in the genus *Allovahlkampfia* and the description of the new species *Vahlkampfia soli*

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Abstract

The class Heterolobosea is one of the major groups of free-living protists in terrestrial ecosystems. The described diversity of soil heteroloboseans has rapidly increased in the last years, but we likely have only scratched the surface of the largely unknown diversity of heterolobosean amoebae in soils. Here, we provide an updated phylogeny of this group, including the report of a new species. We isolated four amoeba strains with morphological characteristics common for Heterolobosea from soils samples in the Netherlands and China. Two strains always moved in a limax locomotive form, one strain only occasionally showed a limax locomotive form and one strain mostly exhibited a flabellate form. Together with 18S rRNA gene and internal transcribed spacer (ITS) marker sequencing, one strain could be identified as *Naegleria clarki*, while the other three could only reliably be identified to the genus level: two strains as *Allovahlkampfia* spp. and one strain as *Vahlkampfia* sp. Among the allovahlkampfiids, one strain was closely related to *Allovahlkampfia* sp. Nl64, while the other one most closely resembled *'Solumitrus' palustris*, a species that has repeatedly been shown to belong to the genus *Allovahlkampfia*. As there are only two valid species described within *Allovahkampfia*, we combined all published sequences related to *Allovahlkampfia* and propose five new clusters within this genus. One strain was most closely related to *Vahlkampfia orchilla*, but sufficiently distinct based upon both morphology and DNA sequence data to propose this strain as a new species, *Vahlkampfia soli*, as it is the first described *Vahlkampfia* species from soil. Together, our study extends the described diversity of soil heteroloboseans through the description of a new *Vahlkampfia* species and by revising the morphologically and phylogenetically diverse genus *Allovahlkampfia*.

Introduction

Protists within the Heterolobosea have a global distribution ranging from tropical to polar regions, including extreme environments (Jacob & Patterson, 1990; Park & Simpson, 2011; Reeder et al., 2015; Tyml et al., 2016). Heteroloboseans are morphologically diverse, including amoeba, flagellates, amoeboflagellates and "slime molds" (Brugerolle & Simpson, 2004; Yubuki & Leander, 2008; Brown et al., 2012; Harding et al., 2013; Pánek et al., 2017). During locomotion, most heterolobosean amoeba adapt a cylindrical limax shape with eruptive bulges (Page, 1985). Some heterolobosean species, including several *Naegleria* species (the most well-studied heterolobosean genus due to particular attention for the human pathogenic *N. fowlerii*), can shift between an amoeba and a flagellate stage (De Jonckheere, 2002; Visvesvara et al., 2007; De Jonckheere, 2011, 2012).

Classical identification of heterolobosean species has mostly relied upon morphological characters (Page, 1967). However, molecular tools have revealed that morphological characters can be ambiguous, with limited ability to distinguish between species due to presence of only a limited number of discernable heterolobosean phenotypic characters. Molecular sequencing using barcoding regions such as the 18S rRNA gene and the ITS (internal transcribed spacer) region has substantially increased the taxonomic resolution within Heterolobosea and corrected the often erroneous taxon relatedness previously proposed based on morphological characters (Brown & De Jonckheere, 1999; De Jonckheere & Brown, 2005). For example, the genus designation *Vahlkampfia* was shown to be genuinely paraphyletic, resulting in the erection of several new genera including *Tetramitus, Neovahlkampfia* and *Paravahlkampfia* (De Jonckheere et al., 1997; Brown & De Jonckheere, 1999; De Jonckheere & Brown, 2005).

To date, approximately 150 heterolobosean species, including ~26 soil heterolobosean species, have been described (Sandon, 1927; De Jonckheere, 2002; Brown & De Jonckheere, 2004; Murase et al., 2010; De Jonckheere et al., 2011; Anderson et al., 2011; Geisen et al., 2015a; De Obeso Fernadez Del Valle & Maciver, 2017). Despite the fact that heterolobosean species are ubiquitously found in soil habitats, these species have to date only been placed in seven out of total of 35 genera described within the Heterolobosea: *Naegleria, Allovahlkampfia, Fumarolamoeba, Parafumarolamoeba, Paravahlkampfia, Tetramitus and Vrihiamoeba* (Pánek et al., 2017).

As explained above, *Naegleria* spp. have received a disproportionate amount of

attention in research focused on (soil) Heterolobosea, which has led to a thorough molecular species definition within the genus *Naegleria* (De Jonckheere, 2004). However, much less is known about other heterolobosean genera. For instance, recent studies have demonstrated the ubiquity and high diversity of *Allovahlkampfia* in soils, but only two species, *A. spelaea* (Walochnik & Mulec, 2009) and *A. minuta* (De Obeso Fernadez Del Valle & Maciver, 2017) have been formally described. A third species, which was previously misidentified as *'Solumitrus' palustris* (Anderson et al., 2011), has repeatedly been shown to be placed within *Allovahlkampfia* (Brown et al., 2012; Geisen et al., 2015a).

The aim of the current study was to update the existing knowledge concerning the diversity and phylogeny of soil Heterolobosea. To this end, we isolated four distinct heterolobosean amoeba from soils of different origins, examined each strain's morphology and tested ranges of thermotolerance. We also sequenced the 18S rRNA gene and the ITS region, including the 5.8S rRNA gene, to allow for robust phylogenic analysis of each strain. We could show that one strain was closely affiliated with a known species within *Naegleria*, while two strains were identified as new species/strains of *Allovahlkampfia* and one strain represented a new species within the genus *Vahlkampfia*.

Methods

Isolation and cultivation

Four strains were isolated from two different soils; NL81 was isolated from a green house in Rotterdam, the Netherlands (51°55'32"; 4°29'39"); CN7 was isolated from the rhizosphere of tomato in Qilin town, Jiangsu province, China (32°03'09", 118°55'36"); NL28 was isolated from a grassland soil in the province Friesland, The Netherlands (51°56'60"; 6°10'59"); NL10 was isolated from rhizosphere soil of Centaurea stoebe in the Netherlands (51°51'60"; 5°53'34"). Isolations used one gram of soil sample suspended in 20 mL PAS (Page's Amoeba Saline). Soil suspensions were gently shaken for 30 min at Laboshake (Gerhardt GmbH & Co. KG, Königswinter, Germany), and one microliter of the mixed soil suspension was pipetted into each well of 96-well plates (Costar, Corning, New York, USA) containing *Escherichia coli* OP50 (*E.coli*) as food source. After several days of incubation at 15°C, we screened each well to select novel protists under an inverted microscope Nikon Eclipse TS100-F (NIKON, Tokyo, Japan). Wells containing potentially novel protists were further diluted several times in order to purify a single protist strain. All protists strains were maintained at 15°C and regularly transferred

to new medium with *E.coli*.

In order to examine thermotolerance, we grew each protist species with *E.coli* in 96-well plates and incubated them at 15, 20, 25, 28, 32, 37 ℃. We then tracked the number of protists cells each day until encystment or extinction.

Morphological analysis

All four isolates were identified under locomotion, stationary and cyst stages. Ten microliters of each active protist or protist cysts were deposited on a glass slide, immediately covered by the glass slip, and the edges were sealed using nail polish. The images of each protist trophozoite and cyst were acquired using a Nikon Eclipse Ti-E inverted microscope (NIKON, Tokyo, Japan) with Differential interference contrast (DIC) using a Plan Fluor 40x 1.30 N.A. oil objective (Nikon) and a CoolSNAP HQ2 camera (Photometrics). The 16-bit images were projected onto the CCD chip at a magnification of 107.5 nm/pixel with intermediate magnification 1.5X (Nikon). The images were captured with 50 ms exposure time using MicroManager (v.1.4.22. ImageJ 1.48v). Time-lapse movies were generated with a time interval 1 second to determine locomotive forms. Protist length and width were measured for at least 10 protist trophozoites or cysts per strain in ImageJ (1.48v). In short, the scale was set by the image scale (1 pixel=107.5 nm), and we further measured length or width by drawing a line from the top to bottom of the protist trophozoite. The formation of floating and flagellate forms of each protist were investigated.

DNA extraction and amplification

Protist DNA was extracted from 100 μL of protist culture using the E.Z.N.A Bacterial DNA extraction Kit (Omega, Bio-Tek Inc., Georgia, USA) and DNeasy Blood & Tissue Kit (QIAGEN N.V., Maryland, USA) following the manufacturer's instructions, with an additional 2 min bead-beating step at maximum speed to improve the DNA yield. Extracted DNA was stored at -20℃ before polymerase chain reaction (PCR) amplification.

The 18S rRNA gene was amplified using various pairs of general eukaryotic primers as shown in Supplementary Table 1. The PCR procedure utilized an initial 3 min 95℃ denaturation step followed by 38 cycles of 95 ℃ for 30s, a strain-specific annealing temperature (see Supplementary Table 1) for 30s, elongation at 72℃ for 90s, and a final extension step of 5 minutes.

The ITS region, including the 5.8S rRNA gene, was amplified using the primers JITS-F and JITS-F (De Jonckheere & Brown, 2005), PCR amplifications were run in a Veriti 96-well thermal cycler (Applied Biosystems, California, USA) with the following program: 95°C for 5 min, followed by 30 cycles with 95°C 30s, 50°Cfor 60s, and ending with 72°C for 120s.

PCR products (25 µL) were subjected to gel electrophoresis in 1% agarose dissolved in Tris-borate buffer (2.5 mM disodium EDTA, 89 mM Tris base, and 8.9 mM boric acid). The band containing the PCR product of interest was then excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN N.V., Maryland, USA). The cleaned PCR products were then sequenced (BaseClear B.V., Leiden, The Netherlands) using species-specific primers (Supplementary Table 1).

Phylogenetic analysis

The taxonomic affiliation of each strain was first determined by comparison against NCBI GenBank using a BLASTn search. To obtain resolved phylogenetic affiliations of our strains, the sequences of closely affiliated species were also downloaded for further analysis. In total, we generated the following four datasets, one including 30 18S rRNA gene sequences; one including the ITS regions for the same set of 30 species; one including the 18S rRNA gene and ITS region for 18 species in the genus *Allovahlkampfia*; and one including the same region for 9 species in the genus *Vahlkampfia*. All datasets were aligned with MAFFT (version 7) using the FFT-NS-2 method (Katoh et al., 2017) and further manually adjusted in SeaView (version 4.7) (Gouy et al., 2010) to curate potential sequencing errors.

Maximum likelihood phylogenetic trees of all datasets were constructed within SeaView. In order to assess the stability of the clades, phylogenetic analysis of all datasets was performed based on Bayesian analysis using MrBayes 3.2 (Huelsenbeck & Ronquist, 2001) and maximum likelihood using RAxML (v0.9.0) (Kozlov et al., 2019). Bayesian analysis was conducted under 6 General Time Reversible (GTR) substitution types with assumptions of rate variations across sites according to gamma + invariable distribution. Markov chain Monte Carlo simulations were performed for 10,000 generations, further sampled every 100 generations. The first 100 samples were discarded as burnin. Maximum likelihood analysis was also based on GTR + GAMMA + I model for all datasets. All phylogenetic trees were illustrated in FigTree (v1.4.4).

Results

Culture and morphological identification

All four heterolobosean isolates exhibited a typical heterolobosean eruptive locomotion and showed an irregular shape with eruptive pseudopods to various directions during the non-locomotive stage (Fig.1). All strains formed cysts (Fig.1). No specific floating forms, flagellate stages or formation of fruiting bodies could be observed for any of the strains examined.

Strain NL10 mostly adopted an elongated limax locomotive form, but occasionally showed an elongated flabellate shape (Fig.1a). A clear and large hyaline part was observed in the locomotive form. Trophozoites (length: 6.02-11.1 µm, width: 6.02-10.33 µm) of NL10 were uninucleate with one contractile vacuole being observed. One nucleus was observed (nucleus diameter: 4.70-2.62 µm, nucleolus diameter: 2.27-1.69 µm). Cysts were round but profoundly differed in size, ranging from 6.02 to 11.1 µm (Fig.1b). NL10 could tolerate temperatures of up to 37°C (Table 1).

Strain NL28 occasionally moved in a limax shape, but more often moved in an eclipse shape with an enlarged anterior with a small hyaline area (Fig.1c). At the posterior end, strain NL28 showed a bulbous uroidal filament (Fig.1c). One to three contractile vacuoles could be observed in trophozoites (length: 15.94-21.97 µm, width: 5.33-18.92 µm) of strain NL28. One nucleus (diameter: 3.02-6.42 µm) and one nucleolus (diameter: 1.69-3.39 µm) was present. Cysts were round with a clear and separated wall. Strain NL28 could tolerate temperatures up to 20 °C b, as even cysts were not formed above that temperature (Table 1).

Strain NL81 mostly showed a limax shape during locomotion. The locomotive trophozoite was 14.51-42.30 µm in length and 7.83-21.48 µm in width and formed a clear hyaline cap at the anterior end (Fig1.d, Table 1). One nucleus with a single nucleolus located in the center was observed in NL81 (nucleus diameter: 2.25-4.56 µm, nucleolus diameter: 1.66-3.52 µm). One large contractile vacuole was observed in the granular cytoplasm at the posterior end. Cysts of NL81 were round (diameter: 6.64-13.74 µm), with a clear nucleus and a perinuclear layer of granules, surrounded by a smooth and separated cyst wall (Fig1.d). The trophozoites and cysts of strain NL81 had a thermotolerance of 20 °C (Table 1).

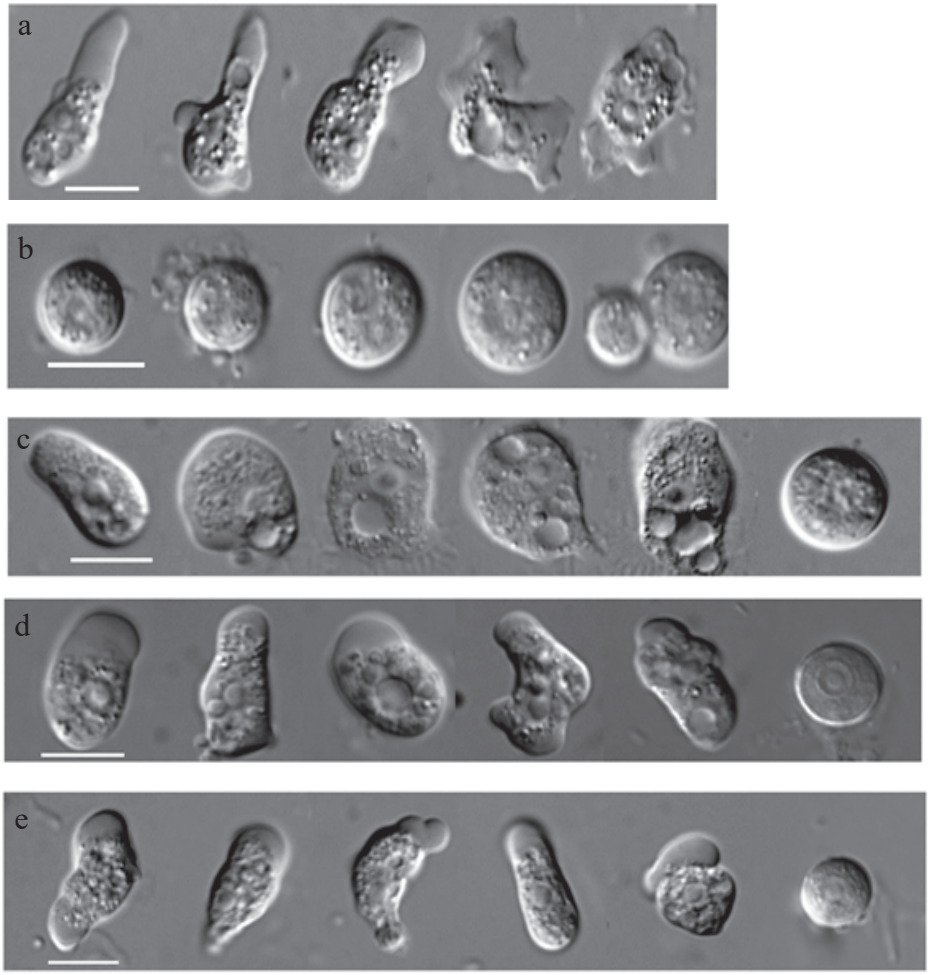


Figure.1 Differential interference contrast (DIC) images showing trophozoites and cysts of all strains described in this study. (a) NL10 (*Allovahlkampfia* sp.); (b) cysts of NL10, note that size difference in cysts; (c) NL28 (*Allovahlkampfia* sp.); (d) NL81 (*Naegleria clarki*); (e) CN7 (*Vahlkampfia* sp.); Scale bar: 10µm.

CN7 showed a limax shape during locomotion. However, the hyaline area became larger and formed asymmetrical bulges pointing towards different directions during when the amoebae stopped moving (Fig.1e). Amoebae were 13.65-24.17 µm long and 4.42-11.38 µm wide during locomotion. The anterior end of CN7 was usually broader than the posterior end, where sometimes a bulbous uroid was formed (Fig.1e). No contractile vacuole was found in CN7. Cysts (diameter: 7.24-12.23 µm) were round with a smooth wall and were uninucleate with a centered nucleolus (diameter: 2.05-3.11 µm). CN7 trophozoite had a thermotolerance of up to 37°C (Table 1)

Phylogenetic analysis

We sequenced nearly the complete 18S rRNA gene of all strains (Table 2) except NL81. Only half of the full-length 18S rRNA gene sequence could be obtained for NL81, possibly because of primer limitations. The lengths of the ITS region, including the 5.8S rRNA gene, were variable with especially NL81 showing a longer ITS region than the other strains (Table 2). The 5.8S rRNA gene was 161bp long in NL10 and strain NL28, while the 5.8S rRNA gene was longer (175bp) in NL81 and shorter (156bp) in CN7 (Table 2).

Blast searches of the 18S rRNA gene sequence revealed NL81 as *Naegleria clarki* based on best hits and phylogenetic analyses (Fig.2,3). NL28 and NL10 were placed within the genus *Allovahlkampfia* NL28 showed the closest affiliation with *Allovahlkampfia spelaea* (Walochnik & Mulec, 2009), and NL10 with *Allovahlkampfia* sp. Nl64 (Geisen et al., 2015a) (Fig.2). The BLAST search of the ITS regions also revealed the same affiliation for NL10 to *Allovahlkampfia* sp. Nl64. In contrast, the Blast search of the ITS region suggested that strain NL28 most closely resembled *'Solumitrus' palustris* (99% query cover but 89.9% identity) (Fig.3). CN7 most closely matched *Vahlkampfia inornate* when Blasting the 18S rRNA gene, while Blasting the ITS region suggested *Vahlkampfia orchilla* as the closest relative.

Table 1. List of strains with their origins and morphological characters

Strain	Origin	GPS	Max Temp (°C)	Locomotive from	Trophozoite length range/average (µm)	Trophozoite width range/average (µm)	Cyst diameter range/average (µm)	Nucleus diameter range/average (µm)	Nucleolus diameter range/average (µm)
CN7	China	32°03'09", 118°55'36"	37	limax	13.65-24.17/ 18.70±0.69	4.42-11.38/ 7.28±0.44	7.24-12.23/ 9.59±0.20	2.25-3.82/ 3.26±0.10	2.05-3.11/ 2.38±0.07
NL10	Netherlands	51°51'60"; 5°53'34"	37	Mostly limax / Occasionally flabellate	13.65-21.21/ 17.46±0.80	5.59-9.94/ 7.56±0.39	6.02-11.10/ 8.53±0.23	4.70-2.62/ 3.30±0.15	2.27-1.69/ 1.93±0.03
NL81	Netherlands	51°55'32"; 4°29'39"	20	limax	14.51-42.30/ 21.07±1.39	7.83-21.48/ 14.04±0.81	6.64-13.74/ 10.27±0.23	2.25-4.56/ 3.30±0.13	1.66-3.52/ 2.22±0.12
NL28	Netherlands	51°56'60"; 6°10'59"	20	Occasionally limax	15.94-21.97/ 22.79±1.11	5.33-18.92/ 9.82±0.79	8.35-13.34/ 10.21±0.21	3.02-6.42/ 4.14±0.17	1.69-3.39/ 2.60±0.10

Table 2. Sequence length (bp) of the 18S rRNA gene and ITS region of al strains

Strains	18S rRNA gene	ITS1	5.8S	ITS2
CN7	1883	167	156	456
NL10	2051	186	161	147
NL81	1318 (~half length)	441	175	481
NL28	2103	305	161	152

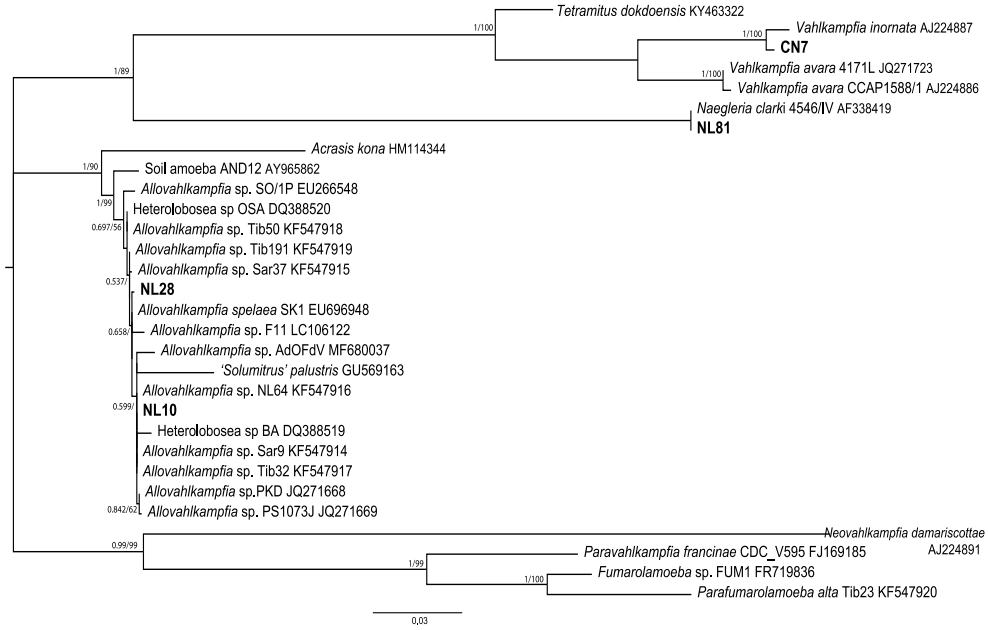


Figure.2. Maximum-likelihood tree based on 18S rRNA gene sequences. Support value at each node presented for BI/RA x ML. Support values <0.5 BI and 50% RA x ML are not shown. GenBank accession numbers of the 18S rRNA sequence used in the analysis are listed next to the taxon names.

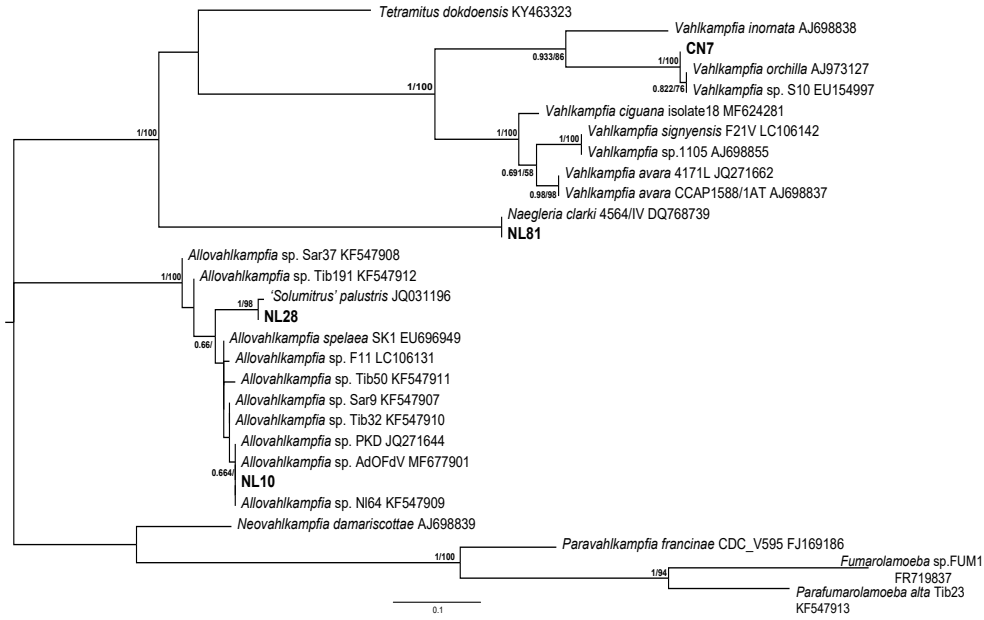


Figure.3. Maximum-likelihood tree based on 5.8S rRNA gene sequences. Support value at each node presented for BI/RA x ML. Support values <0.5 BI and 50% RA x ML are not shown. GenBank accession numbers of the 5.8S rRNA sequences used in the analysis are listed next to the taxon names.

As NL81 was clearly identified as *Naegleria clarki*, we focused our further analyses on the remaining three strains. For the two strains resembling *Allovahlkampfia* spp., we constructed phylogenetic trees containing all published sequences resembling *Allovahlkampfia* strains and further compared their sequence similarities (Fig.4 ITS region, Table 3). This revealed that NL10 most closely resembled *Allovahlkampfia*. sp. Nl64 (Fig.4 ITS region). Strain NL28 showed the highest similarity to *'Solumitrus' palustris* based on the 18S rRNA (99.3%) and 5.8S rRNA genes (99.4%) (Table 3).

To resolve the identity of CN7, we performed phylogenetic analyses based on the 5.8S rRNA gene sequences with published sequences resembling *Vahlkampfia* strains (Fig.5). CN7 formed a distinct, well-supported branch. Sequence similarity of all strains in the closely related branches showed that CN7 had 99.0 % similarity with closest related species *Vahlkampfia inornate* (18S rRNA gene), while showing 99.3 % similarity with *Vahlkampfia orchilla* (5.8S rRNA gene sequences; Table 4).

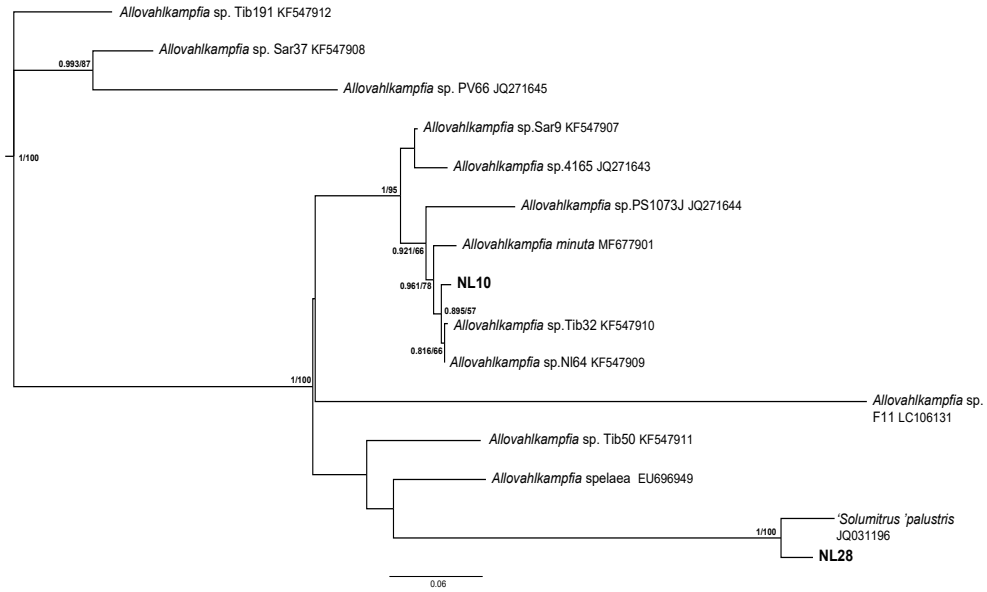


Figure.4. Maximum-likelihood tree based on the entire ITS region in *Allovahlkampfia* strains sequences. Support value at each node presented for BI/RA x ML. Support values <0.5 BI and 50% RA x ML are not shown. GenBank accession numbers of the ITS sequences used in the analysis are listed next to the taxon names.

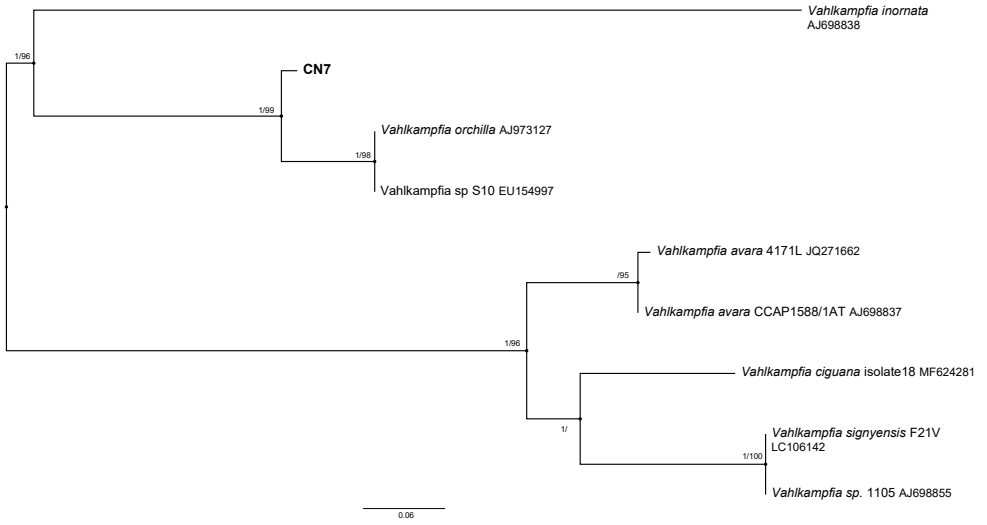


Figure.5. Maximum-likelihood tree based on the entire ITS region in *Vahlkampfia* strains sequences. Support value at each node presented for BI/RA x ML. Support values <0.5 BI and 50% RA x ML are not shown. GenBank accession numbers of the ITS sequences used in the analysis are listed next to the taxon names.

Table 3. Percentage identity matrix based on manually modified alignments of the 18S rRNA (below the diagonal, in bold) and 5.8S rDNA (above the diagonal, in bold) among *Allovahlkampfia* strains and Soil amoeba AND 12 as outgroup. NA, no value due to lack of a reference sequence.

	NL10	NL28	*A.spelaea*	Nl64	*'S' palustris*	SO/1P	BA	Sar9	PS1073J	Tib32	OSA	Tib50	Tib191	Sar37	F11	*A.minuta*	AND12
NL10	**100**	91.9	98.7	100	92.9	NA	NA	99.4	100	99.4	NA	98.7	95.5	94.2	98.1	100	NA
NL28	96.1	**100**	94.8	93.5	99.4	NA	NA	94.2	93.5	94.2	NA	93.5	93.5	93.5	94.2	93.5	NA
Allovahlkampfia. spelaea	96.0	97.6	**100**	98.7	94.2	NA	NA	99.4	98.7	99.4	NA	98.7	96.8	95.5	99.4	98.7	NA
Allovahlkampfia sp. Nl64	99.4	96.1	96.1	**100**	92.9	NA	NA	99.4	100	99.4	NA	98.7	95.5	94.2	98.1	100	NA
'Solumitrus' palustris	92.5	99.3	94.2	92.4	**100**	NA	NA	93.5	92.9	93.5	NA	92.9	92.9	92.9	93.5	92.9	NA
Allovahlkampfia sp. SO/1P	95.5	96.6	96.4	95.2	93.7	**100**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Heterolobosea sp.BA	98.0	96.3	96.4	98.3	92.7	95.4	**100**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Allovahlkampfia sp. Sar9	98.2	96.7	96.7	98.6	93.9	95.6	99.1	**100**	99.4	100	NA	98.1	96.1	94.8	98.7	99.4	NA
Allovahlkampfia sp. PS1073J	98.8	96.0	95.9	99.3	92.4	95.2	98.3	98.5	**100**	99.4	NA	98.7	95.5	94.2	98.1	100	NA
Allovahlkampfia sp. Tib32	99.4	96.0	96.0	100	92.4	95.1	98.3	98.5	99.3	**100**	NA	98.1	96.1	94.8	98.7	99.4	NA
Heterolobosea sp.OSA	96.2	97.5	97.6	96.5	94.9	96.2	96.5	96.8	96.3	96.5	**100**	NA	NA	NA	NA	NA	NA
Allovahlkampfia sp. Tib50	96.3	97.5	97.6	96.4	95.0	96.2	96.5	96.8	96.3	96.5	99.9	**100**	95.5	94.2	98.1	98.7	NA
Allovahlkampfia sp. Tib191	95.8	97.3	97.4	95.9	94.9	97.2	96.0	96.4	95.7	95.8	96.7	96.7	**100**	98.7	96.1	95.5	NA
Allovahlkampfia sp. Sar37	95.4	97.6	97.1	95.6	95.0	97.3	95.7	95.9	95.4	95.8	96.5	96.6	98.3	**100**	94.8	94.2	NA
Allovahlkampfia sp . F11	95.8	98.0	97.2	96.0	96.2	96.4	96.2	96.5	95.9	95.9	97.0	97.0	96.7	96.7	**100**	98.1	NA
Allovahlkampfia. minuta	98.7	95.6	95.6	99.3	92.3	94.5	97.6	97.9	98.8	99.6	95.8	95.9	95.2	95.1	95.9	**100**	NA
Soil amoeba AND12	90.7	91.0	90.5	90.8	87.2	90.6	90.6	91.0	90.8	90.7	90.6	90.5	91.0	90.8	90.7	90.0	**100**

Table 4. Percentage identity matrix obtained with manually modified alignments of the 18S rRNA (below the diagonal, in bold) and 5.8S rRNA gene reads (above the diagonal, in bold) among *Vahlkampfia* strains. NA, no value due to lack of a reference sequence

	CN7	*V.orchilla*	S10	*V. avara* 4171L	*V. avara* CCAP1588/1AT	*V. ciguana* 18	F21V	1105	*V. inornata*
CN7	**100**	99.3	99.3	75.3	75.3	74.0	73.3	73.3	81.6
Vahlkampfia orchilla	NA	**100**	100	74.7	74.7	74.7	72.6	72.6	81.0
Vahlkampfia sp.S10	NA	NA	**100**	74.7	74.7	74.7	72.6	72.6	81.0
Vahlkampfia avara 4171L	93.9	NA	NA	**100**	100	94.0	92.7	92.7	74.8
Vahlkampfia avara CCAP1588/1AT	94.4	NA	NA	99.7	**100**	94.0	92.7	92.7	74.8
Vahlkampfia ciguana isolate18	NA	NA	NA	NA	NA	**100**	91.4	91.4	76.2
Vahlkampfia signyensis F21V	NA	NA	NA	NA	NA	NA	**100**	91.4	76.2
Vahlkampfia sp. 1105	NA	NA	NA	NA	NA	NA	NA	**100**	72.8
Vahlkampfia inornata	99.0	NA	NA	94.6	94.1	NA	NA	NA	**100**

Phylogenetic analysis supported the heterogeneity in the genus *Allovahlkampfia* as previously addressed by Geisen et al., 2015a (Fig.4). By closely comparing all strains in the genus *Allovahlkampfia* (Table 5, 6), we propose to divide the genus into five clusters (Fig. 4, Table 5,6). The first cluster contains Tib191, Sar37 (Geisen et al., 2015a) and PV66, as these species show a limax locomotive form and showed a similarity within the ITS region between 85.3-89.2%. All strains in this first cluster had a 162-163 bp long 5.8S rRNA gene, which was 1-2 bp longer than that of other groups (161 bp) (Table 5). The second cluster consists of seven strains (Sar9, 4165, Tib32, NL10, Nl64, *A. minuta*, PS1073J) (Geisen et al., 2015a; De Obeso Fernadez Del Valle & Maciver, 2017). These seven strains clustered as a branch in the genus *Allovahlkampfia* with 92.9-99.8% similarity across the entire ITS region (Fig. 4, Table 6). Within the second cluster, Tib32, NL10 and Nl64 showed the highest degree of similarity (99.0-99.8 %) with 1-5 bp differences. Despite the highest similarity, these three species show slight morphological differences, specifically with NL10 showing a mostly limax locomotive form compared to the other two strains that mostly moved in a flabellate form. The third group consists of *A. spelaea* (Walochnik & Mulec, 2009) and Tib50 (Geisen et al., 2015a) with higher ITS similarity (89.3%) with each other in comparison to other strains. The fourth cluster consists of the single strain F11, which has a low similarity (68.3-79.3%) with any other described strain in the genus *Allovahlkampfia*. The last cluster consists of NL28 and *'Solumitrus' palustris*, which show 95.0 % similarity across the entire ITS region (Table 6). Both strains show a unique bulbous uroid (Table 5, Fig.1c).

Table 5. Information of clusters in the genus *Allovahlkampfia*. NA, no available information.

	Strains	Location	Origins	Elevation	Max Temp (°C)	ITS1 (bp)	5.8S (bp)	ITS2 (bp)	Locomotive form
Cluster 1	Tib 191	Soil	Tibet, China	4149	<37	129	163	162	Limax
	Sar37	Soil	Sardinia, Italy	181	<37	117	163	130	Limax
	PV66	Beer bottle	Czech Republic	NA	NA	NA	162	NA	NA
Cluster 2	Sar 9	Soil	Sardinia, Italy	181	<37	163	161	128	Mostly limax
	4165	Liver	Czech Republic	NA	NA	NA	161	NA	NA
	Tib32	Soil	Tibet, China	4149	<37	154	161	127	Mostly flabellate
	NL10	Soil	Netherlands	NA	37	186	161	147	Mostly limax / Occasionally flabellate
	Nl64	Soil	Netherlands	57	<30	155	161	127	Mostly flabellate
	A. minuta	Lakeside	United Kingdom	NA	<28	NA	161	NA	flabellate
	PS1073J	Liver	NA	NA	NA	NA	161	NA	NA
Cluster 3	*A.spelaea*	Škocjanske jame	Austria	NA	<42	NA	161	NA	Mostly limax
	Tib50	Soil	Tibet, China	4149	<37	153	161	125	Limax or Flabellate
Cluster 4	F11	Freshwater lake	Vega Island, Antarctica	NA	NA	110	161	105	Limax
Cluster 5	NL28	Soil	Austria	NA	20	305	161	152	Occasionally limax / bulbous uroid
	'Solumitrus' palustris	Freshwater marsh	USA	NA	NA	NA	161	NA	Limax / bulbous uroid

Table 6. Percentage identity matrix obtained with the entire ITS region of all reported strains in clusters of genus *Allovahlkampfia* after modification. Note that below the diagonal (in blank) is percentage of similarity and above the diagonal (in blank) is the non- identical base pairs.

		Cluster 1			Cluster 2							Cluster 3		Cluster 4	Cluster 5	
		Tib191	Sar37	PV66	Sar9	4165	Tib32	NL10	Nl64	*A. minuta*	PS1073	*A. spelaea*	Tib50	F11	NL28	*'S' palustris*
Cluster 1	Tib191		45	56	83	89	82	82	83	81	97	83	78	103	104	109
	Sar37	89.2		43	85	91	82	83	83	82	96	82	75	100	98	98
	PV66	85.3	88.4		85	77	82	83	83	75	77	74	74	89	101	100
Cluster 2	Sar9	80.6	79.7	77.7		10	16	17	17	19	34	61	61	109	108	115
	4165	77.2	76.2	78.0	97.8		24	26	25	19	23	65	67	91	99	106
	Tib32	80.7	80.3	78.3	96.7	94.6		5	1	9	28	65	62	107	108	115
	NL10	80.7	80.0	78.0	96.5	94.2	99.0		4	11	29	67	60	108	110	117
	Nl64	80.4	80.0	78.0	96.5	94.4	99.8	99.2		8	27	66	61	108	109	116
	A. minuta	76.9	76.1	76.1	95.4	95.4	97.8	97.3	98.0		18	64	60	93	94	102
	PS1073J	77.0	76.8	79.5	92.9	94.8	94.1	93.9	94.3	95.6		75	70	105	116	125
Cluster 3	*A. spelaea*	79.9	79.8	79.8	87.1	85.1	86.2	85.8	86.0	84.0	83.8		48	121	96	104
	Tib50	81.1	81.4	79.8	86.8	84.3	86.5	87.0	86.7	85.4	84.5	89.3		102	98	100
Cluster 4	F11	73.3	73.5	74.1	75.2	77.6	75.5	75.3	75.2	76.8	75.7	72.7	75.9		130	139
Cluster 5	NL28	75.3	76.3	73.3	77.5	77.7	77.3	76.9	77.1	76.7	75.3	79.3	78.6	70.1		24
	'Solumitrus' palustris	74.4	76.5	73.6	76.2	76.3	76.0	75.7	75.8	75.0	73.6	77.8	78.4	68.3	95.0	

Discussion

In this study, we isolated four soil Heterolobosean strains, one of which we describe as the new species *Vahlkampfia soli*. By adding NL10 and NL28 to the genus *Allovahlkampfia*, we also amend the genus, proposing that it should contain (at least) five separate species.

For CN7, which we eventually named *V. soli*, we found that it was most closely related to *V. inorate* based on the 18S rRNA gene, but to *V. orchilla* (*V. orchilla* type strain and *V. orchilla* strain 10) based on the ITS region (De Jonckheere, 2006a; Yera et al., 2008). We attribute the lack of congruence to the absence of a published 18S rRNA gene sequence of *V. orchilla* in GenBank. Based on 5.8S rRNA gene read comparison between reported species in the genus *Vahlkampfia* (Table 4), we believe that *V. orchilla* is the sister species of *V. soli*. Future additions of sequences of *Vahlkampfia* spp. will help to better reveal the relatedness of this likely still poorly sampled genus. We justify the erection of a new species not only based on profound molecular differences but also on distinct morphological features. In particular, CN7 lacks pink pigments that are present in *V. orchilla* (De Jonckheere, 2006a). CN7 is smaller and has a bulbous compared with a filamentous uroid present in *V. inorate*.

So far, all reported species in the genus *Vahlkampfia* were isolated from aquatic ecosystem or inside humans (Supplementary Table 2). The only possible exception is *Vahlkampfia signyensis* strain 1105 that was found in soils from the South Orkney Islands on Antarctica, but indeed was suggested to potentially represent a marine species (Garstecki et al., 2005). *Vahlkampfia* species were reported from soil (Rahdar et al., 2016), but just based on potentially outdated morphological and therefore hardly reliable characteristics. As such, *V. soli* represents the first unequivocal soil *Vahlkampfia* species.

NL81 strongly resembled *Naegleria clarki* (Dyková et al., 2006). Interestingly, our strain showed a much lower thermotolerance than previously reported (De Jonckheere, 2014), especially considering that *Naegleria* species commonly have a high thermotolerance. This could be caused by the origin of the different strains, with the described *Naegleria clarki* strain originating form fish organs with potentially higher body temperature (Dyková et al., 2001) than the strain originating from temperate soil. No flagellate stages could be initiated in NL81, which is contrasting previous studies (De Jonckheere, 2014).

NL10 was affiliated with the genus *Allovahlkampfia* and was mostly related to Nl64

and Tib32 based on the 18S rRNA gene sequences - note that these two sequences were nearly identical except for the group I intron, which present in Nl64 but is absent in Tib32 (Geisen et al., 2015a). However, further analyses showed that NL10 was only closely related to Nl64 (Table 3), due to the slight 0.6 % dissimilarity between Nl64 and Tib32 in the 5.8 S rRNA gene reported by Geisen et al., 2015a. Our study also confirmed that NL10 has fewer bp difference with Nl64 than Tib32 based on the ITS region (Table 6). Considerable variabilities in the morphology within *Allovahlkampfia* species could also be found, which was consistent with previous studies (Walochnik & Mulec, 2009; Geisen et al., 2015a). NL10 adopted more of an elongated flabellate locomotive form occasionally, which is different from its closest relative Nl64 that exhibits mostly a flabellate locomotive form (Geisen et al., 2015a). The size of our strain was most strikingly different from that of Nl64, as trophozoite length of NL10 only reached half of the length of Nl64. Interestingly the mean cyst diameter of NL10 was larger than the Nl64, which might be due to profound variations between cyst sizes within NL10, resulting in a bigger value of mean cyst diameters (Fig.1b, Table 1). Thermotolerance also varied between Nl64 (below 30°C) and NL10 (37°C). Geisen et al., 2015a also reported similar levels of variability with respect to ability to grow at high temperatures when comparing the closely related species Tib32 and Nl64.

Strain NL28 showed different best BLAST matches, which is partly due to sequence errors present in *'S'. palustris* reported in previous studies (Brown et al., 2012; Harding et al., 2013; Geisen et al., 2015a). We further modified the alignments and found that NL28 was closely related with *'S'. palustris*, which was also supported by our phylogenetic analysis (Fig2, Table 3). Our study showed that NL28 and *'S'. palustris* were clustered in one group within *Allovahlkampfia*. This confirms previous reports suggesting that *'S'. palustris* should be included within *Allovahlkampfia* and that analyses should trim the end of the sequences of *'S'. palustris* to eliminate these errors (Brown et al., 2012; Geisen et al., 2015a). Morphological variabilities confirmed differences between strain NL28 and the closely related *'S'. palustris*, with NL28 being shorter and wider than the described *'S'. palustris* (Anderson et al., 2011).

Our results were in line with the observed heterogeneity within the genus *Allovahlkampfia* as addressed by Geisen et al, 2015a. By adding two *Allovahlkampfia* strains, we proposed five *Allovahlkampfia* clusters based on the entire ITS region of the described strains (Fig.4). Strains from the first cluster always had 1-2 bp longer 5.8S rRNA gene as compared to the clusters (Table 6), and both of the two described strains (Tib191 and Sar37) were morphologically similar and displayed a limax

locomotive form (Geisen et al., 2015a). The second cluster contained seven strains, in which Nl64, NL10 and Tib32 showed only 1-5 bp length differences within each other's ITS region sequences. However, these three strains had a slight difference in their locomotive form; Tib32 and Nl64 showed mostly a flabellate shape, while NL10 showed mostly a limaxand occasionally a flabellate shape (Fig.1a, Table 5). Variability in morphology could also be observed within the second cluster, with specifically Sar9 showing the mostly limax shape, which differed from Tib32 and Nl64. NL10 might possess the transitional morphotype between limax and flabellate, and there might be an additional group within the second cluster (Fig.4), which needs to be evaluated in future studies. The fourth cluster was generally distinct from other groups in the genus *Allovahlkampfia*. This might be because F11 was isolated from Antarctica and evolved differently in extreme conditions, akin to other differences observed for instance in the *Naegleria* polar cluster (De Jonckheere, 2006b). More polar *Allovahlkampfia* strains are needed in the future in order to confirm similarity within polar *Allovahlkampfia* strains. We included *'Solumitrus' palustris* and NL28 in the fifth cluster of the genus *Allovahlkampfia*, as these two strains showed the highest similarity with each other, and both of them showed a unique bulbous uroid (Table 5, 6). We suggest the five clusters mainly based on the entire ITS sequences, however, species lacking ITS sequences could not be included in the clusters. For example, our phylogenetic analysis also confirmed that Heterolobosea OSA had 94.9-97.6% similarity with other *Allovahlkampfia* species. Future studies utilizing new molecular data for identifying the clusters within the genus *Allovahlkampfia* are needed to confirm the cluster definition.

Soil Amoeba AND12 formed a new clade in between two closely related genera, *Allovahlkampfia* and *Acrasis* as found previously (De Jonckheere et al., 2011; Geisen et al., 2015a). The genus *Acrasis* is known for its sorocarp form, however, in the closely related genus *Allovahlkampfia*, only strain BA was so far reported to induce sorocarp formation (Brown et al., 2012). Little is known concerning the link between *Allovahlkampfia* and *Acrasis*, making soil amoeba strain AND12 an interesting target for further study (Lara et al., 2007).

Conclusions

We report strain CH7 as a new species, *Vahlkampfia soli*, the first described *Vahlkampfia* species isolated form soil. Furthermore, we identified two novel strains that allowed us to revise the genus into 5 clusters and propose that new species should be based on diverse clusters of sequenced and morphologically identified strains. Together, our study extends the knowledge on soil Heterolobosea, but suggests that

we are far from having captured a nearly complete inventory of this group of soil protists.

Diagnosis

New species description: *Vahlkampfia soli*

Morphology: Trophozoites 13.65-24.17 µm (average 18.70 µm) in lengths and 5.0-11.38 µm (average 7.28 µm) in widths; limax locomotion with highly eruptive pseudopodia; broader anterior than posterior end, where sometimes a bulbous uroid was formed; no contractile vacuole visible. Cysts round with a smooth wall and uninucleate with a centered nucleolus (nucleus diameter: 3.26 ± 0.1 µm, nucleolus diameter: 2.38 ± 0.07 µm); Thermotolerance of up to 37°C.

Phylogeny: 18S rRNA sequences of CN7 showed 99.0 % similarity with the closest related species *Vahlkampfia inornate*. 5.8S rRNA gene sequences of CN7 had 99.3 % similarity with closest related species *Vahlkampfia orchilla*.
Differences with closely related species: no pink pigment as present in *V. orchilla* (De Jonckheere, 2006a). Smaller than *V. inorate* and forming a bulbous instead of a filamentous uroid present in *V. inorate*.

Food: bacterivorous

Habitat: Soil

Origin: Qilin town, Jiangsu province, China (32°03'09", 118°55'36")

Etymology: CN7 is the first described *Vahlkampfia* species isolated from soil, the species name soli denotes its habitat in Latin (*soil – soli*)

Genus *Allovahlkampfia*, emended

Cluster 1 - *Allovahlkampfia iam*

Species: Tib191, Sar37 (Geisen et al., 2015a), PV66 (JQ271645)

Morphology: limax locomotive form

Habitat: soil, but also extracted from a beer bottle

Phylogeny: The 5.8S rRNA gene is 162 or 163bp in lengths; high within- cluster diversity of the entire ITS region (85.3 -89.2% similarities with each other), but more than to other clusters (73.3 -81.4%).

Etymology: Three species in this group have 1-2bp longer of 5.8S rRNA gene than species in other groups (161bp). The name iam denotes their longer 5.8S rRNA gene in Latin (*longer - iam*)

Cluster 2 - *Allovahlkampfia minuta* emended

Species: Sar9, Tib32, Nl64 (Geisen et al., 2015a), NL10, *A. minuta* (De Obeso Fernadez Del Valle & Maciver, 2017), 4165 (JQ271643), PS1073J (JQ271644)

Morphology: limax or flabellate locomotive form

Habitat: soil, liver, lakeside

Phylogeny: The 5.8S rRNA gene is 161bp in length; high within- cluster diversity of the entire ITS region (92.9 -99.8% similarities with each other), but more than to other clusters (77.9 -87.0%).

Etymology: This cluster name is consistent with the reported species *A. minuta* (De Obeso Fernadez Del Valle & Maciver, 2017)

Cluster 3 - *Allovahlkampfia spelaea* emended

Species: Tib50 (Geisen et al., 2015a), *A. spelaea* (Walochnik & Mulec, 2009)

Morphology: limax or flabellate locomotive form

Habitat: soil, stromatolitic stalagmites

Phylogeny: 5.8S rRNA gene is 161bp in length; high within- cluster diversity of the entire ITS region (89.3 % similarity with each other), but more than to other clusters (72.7-86.2%).

Etymology: This cluster name is in consistent with reported species *A. spelaea*

(Walochnik & Mulec, 2009)

Cluster 4 - *Allovahlkampfia antartica*

Species: F11 (Tyml et al., 2016)

Morphology: limax locomotive form

Habitat: Freshwater, Vega Island, Antarctica

Phylogeny: 5.8S rRNA gene is 161bp in length, this cluster species sequences of the entire ITS region had 68.3 -77.6% similarity with other clusters.

Etymology: F11 was isolated form Antarctica, cluster name antartica denotes its habitat

Cluster 5 – *Allovahlkampfia palustris* emended

Species: NL28, *'Solumitrus' palustris* (Anderson et al., 2011)

Morphology: limax locomotive form with bulbous uroid

Habitat: soil, freshwater

Phylogeny: 5.8S rRNA gene is 161bp in length; high within- cluster diversity of the entire ITS region (95.0 % similarity with each other), but more than to other clusters (68.3 -79.3%).

Etymology: This cluster name is consistent with the reported species *'Solumitrus' palustris* (Anderson et al., 2011) due to its similarity to the genus *Allovahlkampfia*, we suggest *Allovahlkampfia palustris* as the new cluster name.

Acknowledgements

Z.G. was supported by Chinese Scholarship Council (CSC), S.G. by a NWO-VENI grant from the Netherlands Organisation for Scientific Research (016.Veni.181.078). Moreover, we thank Xueyang Sun for the help of extracting DNA of protists strains. We appreciate Dr. Ilya Grigoriev and Biology Imaging Center (Utrecht University, the Netherlands) for the help of taking protist images and videos.

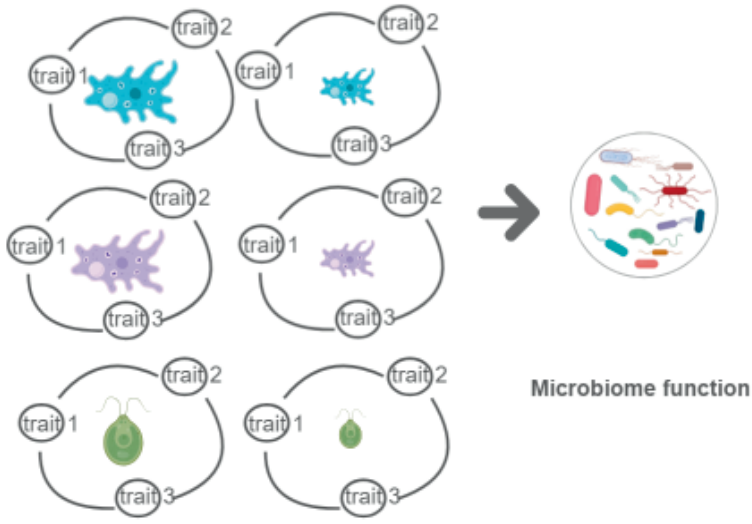
Supplementary material

Supplementary Table 1. Primers and different annealing temperature used for the 18S rRNA gene sequences

Strains	Forward Primer	Reverse Primer	Annealing temperature (°C)
NL28	Pre3NDf (Bass & Cavalier-Smith, 2004)	EukB (Medlin et al., 1988)	50
CN7	RibA (James et al., 1994)	RibB (James et al., 1994)	50
NL10	Pre3NDf	EukB	60
NL81	Pre3NDf	EukB	60

Supplementary Table 2. Origins of reported strains in genus *Vahlkampfia*.

Species	Accession number	Origins	Reference
Vahlkampfia orchilla	AJ973127	river	De Jonckheere, 2006a
Vahlkampfia orchilla strain 10	EU154997	contact lens	Yera et al., 2008
Vahlkampfia inornata	AJ698838	water	Page, 1967
Vahlkampfia signyensis	*LC106142*	freshwater lake	Tyml et al., 2016
Vahlkampfia sp. 1105	AJ698855	water	De Jonckheere & Brown, 2005
Vahlkampfia ciguana	MF624281	Nile water	Al-Herrawy & Gad, 2015
Vahlkampfia avara 4171L	JQ271662	kidney	Dyková & Kostka
Vahlkampfia avara CCAP1588/1AT	AJ698837	water	De Jonckheere & Brown, 2005
Vahlkampfia avara	LC191909	soil	Rahdar et al., 2016
Vahlkampfia avara	KC164242	compost	Conza et al., 2013


trait 1
trait 2
trait 3
Microbiome function

Chapter 3

Protist volume is linked to differential impacts on bacterial community structure

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Abstract

Protists are increasingly being recognized as important regulators of the soil microbiome via their selective feeding on bacterial prey and by exerting top-down control within the microbial community. However, microbiome predation has only been investigated for few protist species, rendering it impossible to obtain any general knowledge-insights on differences between predators and any traits potentially affecting these predator-prey interactions. Here, we identified traits and determined feeding impact of 20 phylogenetically and morphologically distinct protist species on microbiome composition to assess to what extent protist traits can predict their impact on microbiome function. Protist volume was the major trait that could be linked with changes in bacterial community structure, regardless of protist morphological similarity and phylogenetic relatedness. Especially large volume protist increased the abundance of bacteria reported to possess traits that may help avoid predation. Our study suggests that protist traits, especially protist volume, can predict predator and prey interactions and thereby impact soil microbiome composition and potentially functioning.

Introduction

Soil microorganisms are highly diverse and are known to provide multiple terrestrial ecosystem functions, such as enhancing soil fertility, driving nutrient cycling and impacting plant growth and health (Heijden et al., 2008; Berendsen et al., 2012). Advances in DNA sequencing methodologies have provided new windows of observation with respect to revealing the breadth of diversity within the soil microbiome (Fierer, 2017). In addition to providing cultivation-independent inventories of bacterial and fungal diversity, such nucleic acid (DNA and RNA) - based approaches have also led to a much greater appreciation of the distribution and importance of soil protists (Geisen et al., 2015d).

Protists represent a catch-all group that encompasses the vast majority of the phylogenetic diversity within the eukaryotic domain (Adl et al., 2012). Despite their large numbers and high diversity across various ecosystems, they have often received less attention than bacteria or fungi (Geisen et al., 2017). Within soil habitats, recent studies have revealed a large diversity of protists across various soil types, as well as patterns in protist community structure related to environmental conditions and experimental treatments (Fiore-Donno et al., 2016; Xiong et al., 2018). Soil protists also display a diverse range of morphological characters, such as in cell morphology as well as sizes ranging from a few micrometers up to the centimeter scale (Geisen et al., 2017).

Although protists can display diverse life-history strategies and positions within the soil food web (Geisen et al., 2018), arguably their most important impact on the soil microbiome comes through their predation on soil bacteria. Protists can selectively feed on the bacterial community leading to a shift in bacterial community structure (Rønn et al., 2002; Rosenberg et al., 2009). Such selection can lead to an increased relative abundance of bacterial populations that have developed various strategies to resist protist predation, for instance by changing their surface properties, increasing their size or producing secondary metabolites (Jürgens & Matz, 2002; Jousset et al., 2006). Thus, selective protist predation can lead to changes in soil microbiome functioning. Although some of the mechanisms by which protists impact bacterial communities have been studied using model protists species, such as *Acanthamoeba castellanii* or close relatives (Rosenberg et al., 2009; Glücksman et al., 2010), little is yet known about how protists traits dictate subsequent impacts on bacterial communities.

Given the broad phylogenetic and morphological diversity of protists as their ability to exert top down control on their bacterial prey, an important step to predict protist impacts on the soil microbiome lies in linking protist traits with their specific changes in soil microbial community structure. At the macroecological scale, size is

regarded as one of the most fundamental characteristics, because size can be linked to distribution, reproduction rate and interactions with potential prey (Peters, 1987; Brose et al., 2006). With respect to protists, size and morphology can also dictate to what extent they can gain access to small soil pores between soil (micro-) aggregates (Finlay & Fenchel, 2001). Similar patterns can also be found in aquatic ecosystems, where protist size determines the relationship between predator and prey, for instance, as exemplified by the increased bacterivorous activity in marine and freshwater in response to small-sized protists (<10μm) (Sherr & Sherr, 1991). Glücksman et al., 2010 showed for soil cercozoans that other traits, such as volume and plasticity, are responsible for driving changes in bacterial community structure. Moreover, morphotype can be an indicator of protist-induced bacterial community shifts, as the flexible body shape of amoeba species, with their elongated pseudopodia, allows them to reach and penetrate into small water-filled soil pores (Elliott et al., 1980; Darbyshire, 2005).

Soil protists are known to be potentially important drivers of soil microbiome structure and functions (Gao & Karlsson, et al., 2019), but we still lack the knowledge to relate protist identity and traits to subsequent impacts on microbial communities. This gap impedes our ability to predict the exact function of potentially protist species-specific predation on the soil microbiome. To fill in this gap, a systematic and broader approach to link protists traits with impacts on their prey is needed. We therefore collected 20 protist species across a broad taxonomic and morphological range. We then assessed the predation impact of each species on a microbial prey community composed of diverse bacteria. We tested whether protist effects on bacterial communities could be related to phylogenetic affiliation or to morphological or physiological features. Our study seeks to provide a better understanding of predator - prey interactions, thereby improving the prediction on the consequences of protist predation on soil microbiome functioning.

Material and Method

Preparation of protists cultures

Twenty protists were isolated from soil in China and the Netherlands, all protists were cultivated with *E.coli* OP50 (*Escherichia coli* OP50) as food resources at 15°C. Protists were washed two times with PAS (Page's Amoeba Saline) by centrifugation (800 g, 5 min) to remove *E.coli*. The numbers of protists were evaluated under an inverted microscope (Nikon Eclipse TS 100, Tokyo, Japan) and adjusted to 1000 individuals mL^{-1}.

Protists traits measurement

Fifty-microliters of the protist suspension were incubated with 50 µL *E.coli* (10^6 CFU ml^{-1}) and PAS in 96 - well plate (Corning Incorporated, Kennebunk, USA) with six replications. All traits measurement experiments were incubated at 15°C.

Length and width measurement

Images of each protist species were taken under the inverted microscope after 1-2 days of incubation. We measured the lengths and widths of trophozoites and cysts by ImageJ (1.48v) for at least 10 individuals of each protist.

Growth rate measurement

Numbers of protists were determined daily under the inverted microscope for a period of one week. Growth data of each protist species was fitted to a growth parametric model (*grofit :: gcFitModel*) to calculate the growth rate of each species.

Volume calculation

We first determined morphotypes of each protist species and categorized them into three groups based on their morphotypes (Smirnov & Brown, 2004; Smirnov et al., 2011)

Group A: Morphotypes were always cylindrical or sub-cylindrical; as such, we used a cylindrical volume as an approximation of protists volume:
$VA = h\pi r^2$
h=length, r =width/2

Group B: Morphotypes of this group were either cylindrical or flat, depending on environmental conditions; as such, we used a half cylindrical volume as an approximation of protists volume:
$VB = 1/2\ h\pi r^2$
h=length, r =width/2

Group C: Morphotypes were always flat, so we calculated the volume based on fan shape; as such, we used a fan shape area as an approximation of protists volume:
VC=lr/2
l=length, r=width

When protist volume was below 500 μm^3, we categorized these species as small volume protists, those above 500 μm^3 we categorized as large protists.

Protist phylogenetic relationship

DNA extraction and amplicon

Protist DNA was extracted from 100 μL protist sample using the E.Z.N.A Bacterial DNA extraction Kit (Omega, Bio-Tek Inc., Georgia, USA) and DNeasy Blood & Tissue Kit (QIAGEN N.V., Maryland, USA) following the manufacturer's instructions, with an additional 2 min bead-beating step to improve the yield of protists DNA extraction. The extracted DNA samples were stored at -20℃ prior to subsequent polymerase chain reaction (PCR).
The 18S rRNA gene was amplified using several pairs of general eukaryotic primers as shown in Supplementary Table 1. The general PCR procedure began with 3 min 95℃ denaturation and was followed by 38 cycles of 95 ℃ for 30s, a strain- specific annealing temperature for 30s, 72℃ for 90s, and 5 minutes of final extension.

Twenty-five microliters of PCR products were subjected to electrophoresis in 1% agarose dissolved in Tris-borate buffer (2.5 mM disodium EDTA, 89 mM Tris base, and 8.9 mM boric acid). The gel with targeted fragments was cut and purified using the QIAquick Gel Extraction Kit (QIAGEN N.V., Maryland, USA). Purified PCR products were sequenced (BaseClear B.V., Leiden, The Netherlands) addition of the appropriate primers (Supplementary Table 1).

Protist taxonomic affiliation

Resulting sequences were manually curated in BioEdit (version 4.0.6) and assembled to obtain near full-length 18S rRNA gene reads. These reads were subjected to BLASTn searches against NCBI GenBank. To obtain resolved phylogenetic affiliations of our strains, the sequences of the closest affiliated species (1-2 species) were downloaded for further analysis. All sequences, including 20 protists strain and 24 closely related species, were aligned in MAFFT (version 7) using the FFT-NS-2 method (Katoh et al., 2017) and further manually adjusted in SeaView (version 4.7) (Gouy et al., 2010) to check for correct sequences and an accurate alignment of all sequences.

In order to assess the stability of clades, phylogenetic analysis of all sequences was

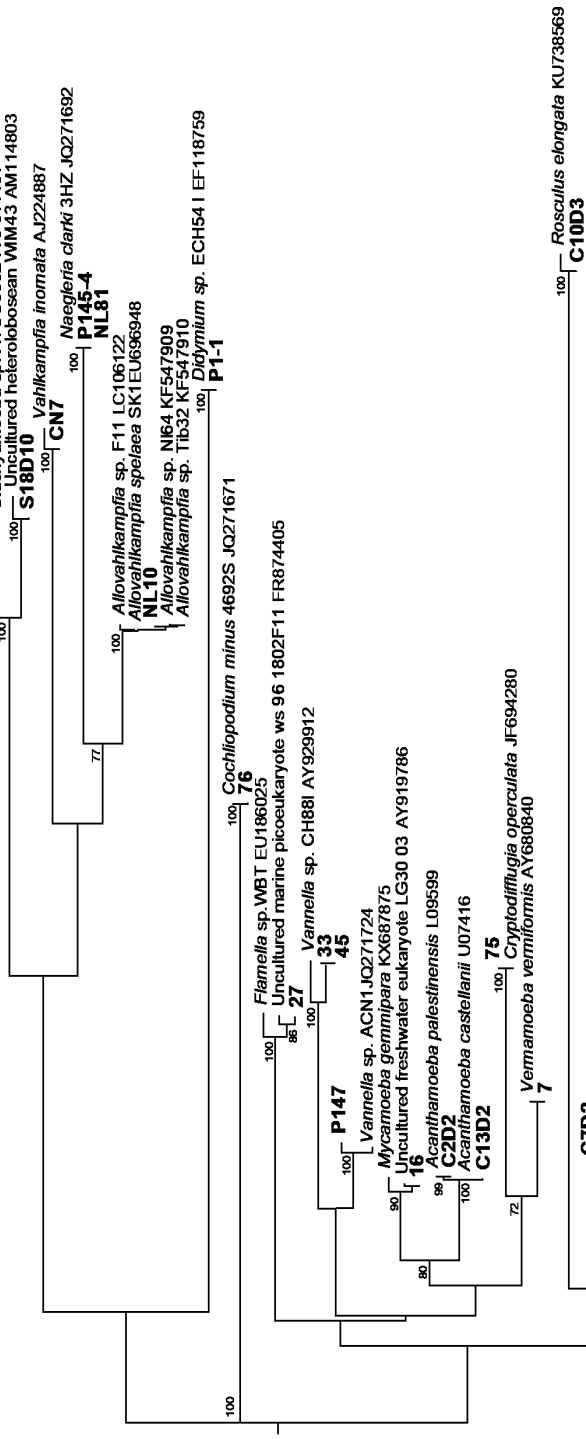


Figure.1 Maximum-likelihood tree based on 18S rRNA gene sequences of 20 protists species and their closest relatives. Support value at each node presented for RA x ML. Support values < 50% RA x ML are not shown. GenBank accession numbers of the 18S rRNA sequence used in the analysis are listed next to the taxon names. 20 protists species names are in bold.

performed based on maximum likelihood using RAxML (v0.9.0) (Kozlov et al., 2019). The phylogenetic tree was visually optimized in FigTree (v1.4.4).

Nearly full length 18S rRNA gene sequences were obtained for all protist strains used in this study, revealing a broad range of taxonomy (Fig.1). Eleven strains were affiliated with the supergroup of Amoebozoa, which is the most abundant supergroup of soil protists (Geisen et al., 2015d); Five strains were affiliated with the Excavate supergroup; Four species were affiliated with the Rhizaria supergroup (Supplementary Table 2).

Preparation of predator- free soil bacterial communities

Predator - free soil bacterial communities were extracted from a natural soil (from the Botanische Tuinen, de Uithof, Utrecht, the Netherlands) (52°05'16"; 5°10'14"). Fifty grams of soil were dried overnight and further blended in a kitchen blender with 200 mL 0.1% pyrophosphate buffer for 2 minutes. Bacteria were extracted from the soil suspension using Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), which is a density gradient centrifugation medium. Ten milliliters of Percoll and ten milliliters of soil suspension were added to a 50 mL Falcon tube and centrifuged using a fixed-angle rotor at 10000 x g for 20 min (Thermo Fisher Scientific, Langenselbold, Germany). To remove protists from natural soil, the top layer of soil suspension was transferred to a new falcon tube and further vacuum filtered using glass filters with pore sizes of 3 µm, 1.6 µm and 1.2 µm. The filtrate was diluted 10 times and incubated in 250 mL culture flasks in 1/300 TSB medium (Tryptic Soy Broth). Culture flasks were incubated at 15°C and checked under the microscope daily to test for contamination by protists (Adapted from (Rønn et al., 2002)).

Experimental set-up

One hundred microliters of predator- free soil bacterial suspension was used to inoculate 3 g of gamma-sterilized sandy soil in a 5 mL Eppendorf tube. After 2 hours of incubation, either 400 µL protist suspension (one of the 20 protist species) or PAS as control treatment was inoculated into the tube, with 5 replications for each protist treatment. In addition, we added 100 µL PAS into the tube to help the inoculant settle into the soil. The tubes were covered with parafilm to avoid contamination while allowing for air exchange. After 10 days, the soil was sampled for further analysis.

Soil DNA extraction and 16S rRNA gene tag sequencing

DNA from 0.5 g soil sample was extracted using the DNeasy PowerSoil Kit (QIAGEN, USA), according to the manufacturer's instructions. Bacterial 16S rRNA gene tag sequencing was carried out using a two-step PCR protocol. Soil DNA was diluted 5 times and amplified by modified primers (Caporaso et al., 2011). The first-step PCR was performed in a 96-well PCR plate with 12.5 µL of Dream Taq Green Master Mix (2X), 5 µL DNA template, 9.5 µL DNA- free water, and 2µL of 5µM combined forward and reverse primers (Supplementary Table 1). PCR amplification was conducted in a Veriti 96 well thermal cycler (Applied Biosystems, California, USA) using a program, consisting of 95℃ for 3 min, following by 26 cycles with 95℃ 20s, 55℃ for 30s, 72℃ for 30s.

PCR products were purified according to the 16S Metagenomic Sequencing Library Preparation protocol (PCR clean-up). Purified PCR products were further amplified using barcoded primers in a second round of PCR (Baym et al., 2015), with the following thermocycling scheme; 95℃ for 3 min, followed by 8 cycles of 95℃ for 30s, 55℃ for 30s, 72℃ for 30s. PCR products were purified as described above, resulting purified DNA quantified on a Qubit 3 Fluorometer (ThermoFisher, USA) and equal amounts of each product combined for 16S rRNA gene tag sequencing (Illumina Inc., San Diego, USA) using a 250-bp V2 paired-end protocol on a MiSeq sequencer (Utrecht Sequencing Facility).

DNA reads were processed (pair-ends merge, quality filter, trimming) using USEARCH , which is a part of the Uparse pipeline (Edgar, 2013). Sequences were identified and clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity level with the QUIIME1 (Caporaso et al., 2010) strategy using the UCLUST algorithm (Edgar, 2010). Subsequently, 16S OTUs were aligned against the SILVA 16S reference database, version 128 (Glöckner et al., 2017)

Rarefaction of the OTU table to 12000 reads per sample was performed using the rarefaction script of QIIME1. At this threshold, seven samples had to be removed from the dataset due to low read numbers. The resulting OTU table was used for further statistical analysis.

Data analysis

Analysis of bacterial community structure was conducted by Nonmetric Multidimensional Scaling (NMDS) based on Bray Curtis dissimilarity (*vegan :: metaMDS*) (Oksanen et al., 2007). The distance of bacterial community was

computed based on Bray Curtis dissimilarity (*vegan :: vegdist*) or UniFrac distance (*phyloseq :: distance*) (McMurdie & Holmes, 2013). Pair-wise protist traits distance was calculated by Euclidean distance (*vegan :: vegdist*). We used linear regression (*base :: lm*) and mantel tests (*vegan :: mantel*) to analyze the association between pair-wise prey bacterial community Bray Curtis dissimilarity and pair-wise protist trait distance. DEseq2 (Love et al., 2014) was used to determine which OTUs were significantly affected by protists with relatively small versus large volumes. All results were conducted in R version 3.6.

Results

Protists traits

The collection of 20 protists strains displayed a wide range of morphological and functional traits. The lengths of physically active protists (trophozoites) ranged from 5.68 to 33.77 µm and widths varied between 4.84 and 18.15 µm. Likewise, lengths and widths of protist cysts showed a large degree of variation between the strains examined (length: 4.58-17.62 µm, width: 4.39-12.21 µm). Similarly, large differences were observed in growth rates in cultures growing on *E.coli* (Supplementary Table 2). Protist volume ranged from 100.035 μm^3 to 4064.89 μm^3 (Supplementary Table 2).

Relationship between phylogenetic affiliation and the impact on bacterial community structure

Different protist strains in our study had disparate impacts on the bacterial community structure. We tested the degree to which phylogenic affiliation could explain the impacts of protist inoculation on bacterial community structure. We first calculated pair-wise phylogenetical distance of all strains, based upon 18S rRNA gene sequences. This matrix was matched against compositional dissimilarity of bacterial communities as determined by pair-wise Bray Curtis dissimilarity. We did not detect a correlation between pair-wise phylogenetic protist distance and Bray Curtis dissimilarity (Supplementary Fig.1). This lack of a correlation between protist phylogenetic affiliation and protist-induced shifts in bacteria community structure is illustrated by the fact that even closely related protist species, such as *Vannella* sp. strain 33 and *Vannella* sp. strain 45, had highly contrasting impacts on bacterial communities (Fig.2).

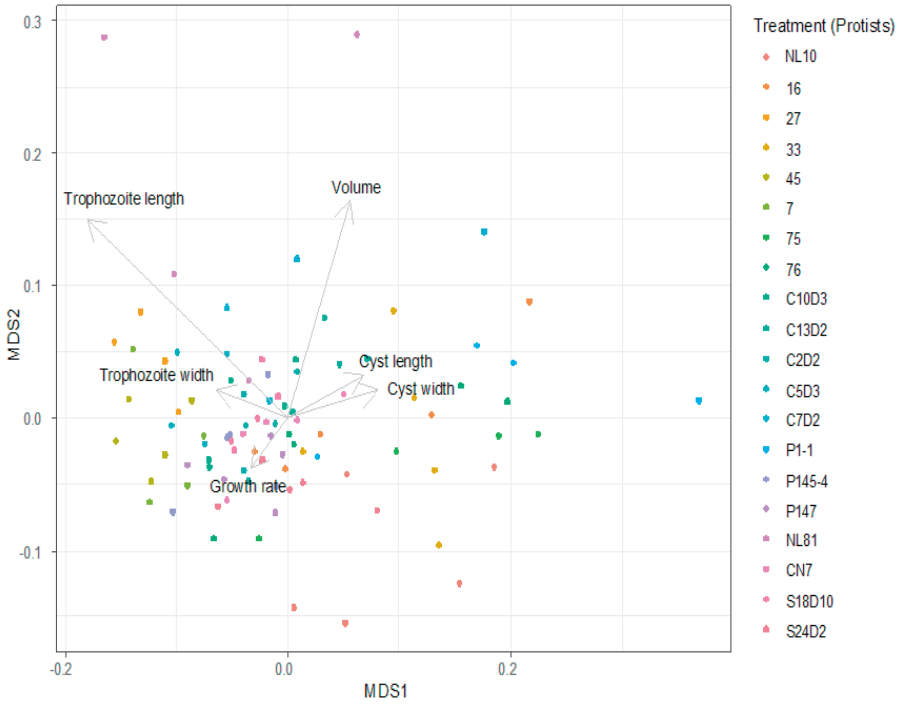


Figure.2 Effect of protist traits on prey bacterial communities, which was assessed by Nonmetric Multidimensional Scaling. Points with different colors represent different protists treatments; arrows represent different protist traits, note that the length of arrow means to what extent that specific protist trait influences prey bacterial communities

Relationship between protists traits and impacts on microbial community structure

We examined the degree to which several protist traits could explain protist-induced impacts on bacterial community structure (Fig.2). Variation in protist trophozoite length and protist volume exhibited the greatest explanatory power of the traits tested, as illustrated by the length of the vectors in Fig.2. To further examine these relationships, we calculated the pairs-wise Bray Curtis dissimilarity and pair-wise protist traits distance. This comparison showed that pair-wise Bray Curtis dissimilarity was significantly associated with pair-wise protist volume distance (Fig.3), which was further verified by linear regression and a Mantel test (Supplementary Table 3). This association between protist volume and protist-induced bacterial community was also found when taking prey bacteria phylogeny into consideration (Supplementary Fig.2). However, we could not find any correlation between protist length and shifts in bacterial community structure (Supplementary Table 3). As shown in the Fig.2, other traits such as growth rate, protist cyst size and trophozoite width had

little impact on the bacterial structure, and no correlations between those traits and bacterial community changes were found (Supplementary Table 3).

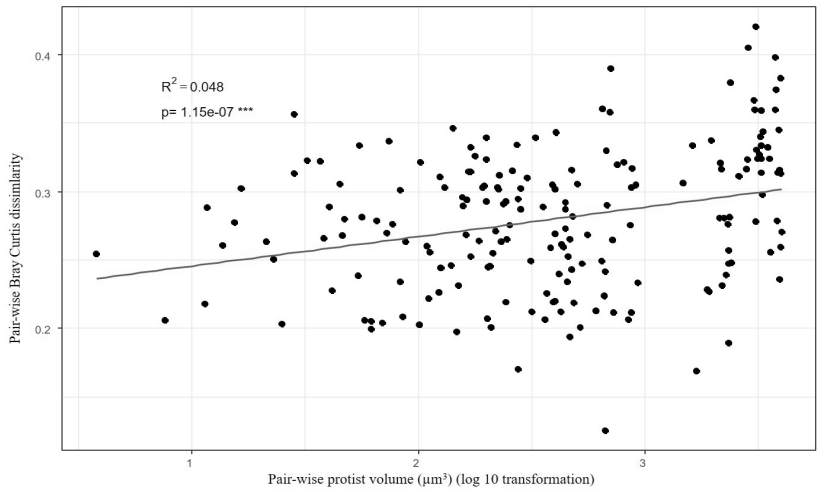
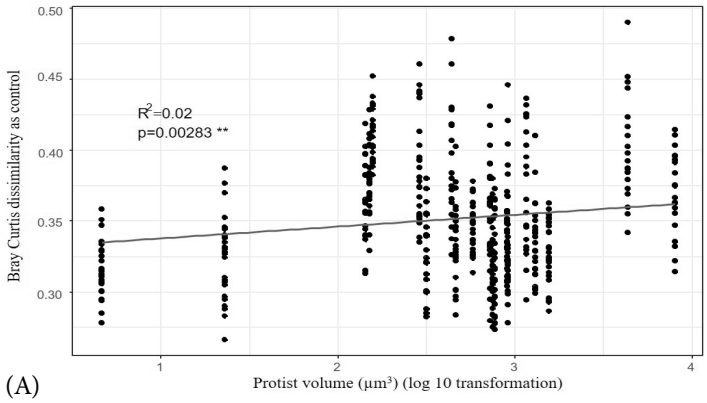
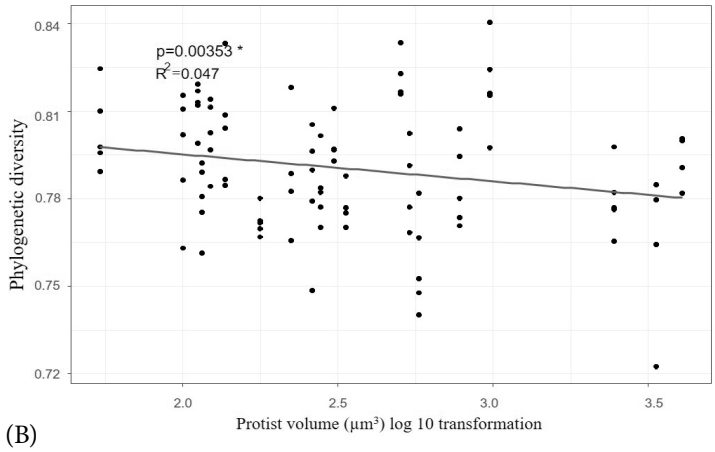


Figure.3 Correlation between pair-wise log 10 transformed protist volume distance and pair-wise Bray Curtis dissimilarity of prey bacterial communities.

We examined protist volume further by comparing the dissimilarity of bacterial community induced by protist treatments and control treatment without any protists. We found that protists with a larger volume tended to yield more dissimilar bacterial communities as compared to the control (Fig.4(A)), and this dissimilarity can be explained by the lower phylogenetic diversity of the prey bacterial community (Fig.4(B)). Larger volume protists tended to decrease the phylogenetic diversity within the bacteria community.



(A)



(B)

Figure.4 (A) Correlation between protists volume distance after log 10 transformation and Bray Curtis dissimilarity as control without protists; (B) Correlation between protists volume distance after log 10 transformation and phylogenetic diversity of prey bacterial communities.

To examine further how protist volume is related to impacts on bacterial community composition, we grouped our collection of 20 protists into groups comprised of small or large protists according to calculated volume; the small protists had volumes less than 500 µm^3; the remaining protists were assigned into the large protist group (Supplementary Table 2). In total, 12 OTUs showed significant relationships with respect to protist size classes. Five OTUs were significantly increased by smaller volume protists (Fig.5), while larger volume protists increased relative abundance of seven OTUs. The choice to divide protists strains using a cutoff of 500 µm^3 was based upon the fact that this gave a rather balanced analysis, while also adhering somewhat to a natural separation in the data (Supplementary Fig.3). We also examined a range

of different groupings of protists volumes, yielding very similar results (not shown).

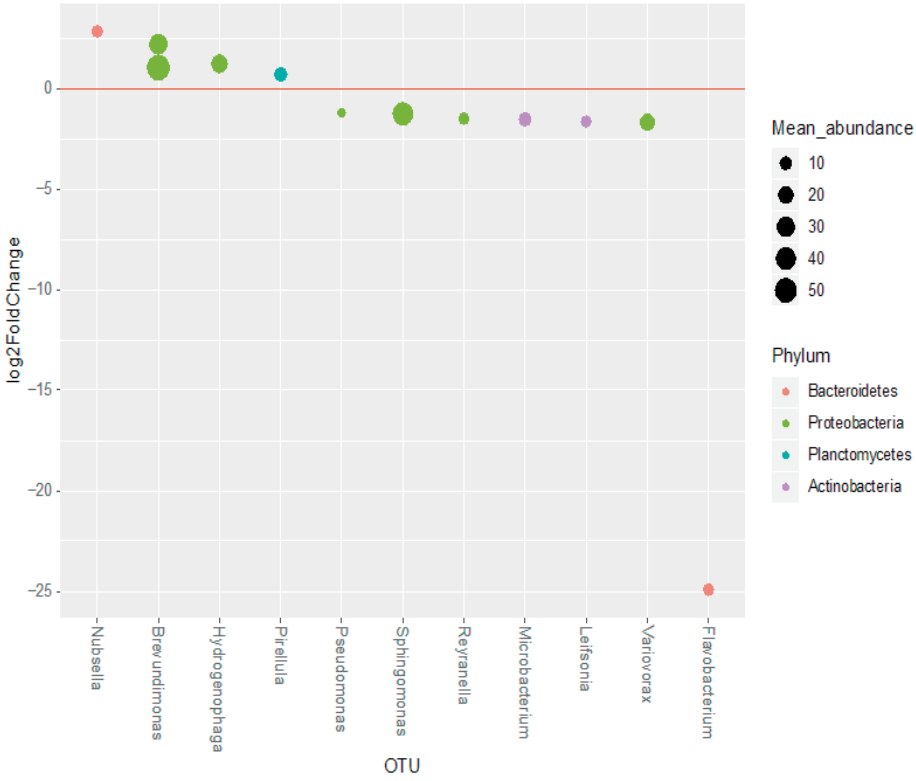


Figure.5 Significantly changed OTUs by predation of small or large volume protists. For each bacterial OTU, a log2 fold change was calculated to represent the extent of the change. Changes in log2 below 0 (read line) indicate that a particular OTU is overrepresented in communities subjected to protists with a large volume. Conversely, log2 fold changes above 0 (red line) indicate a relative overrepresentation of a given OTU in communities inoculated with smaller volume protists. Names of OTUs are represented by genus names; note that two OTUs fall under *Brevundimonas* that were overrepresented in communities subjected to protists with a small volume. Colors of each OTU indicate phylum information; sizes of each OTU indicate mean abundance.

Discussion

Protists are important predators of bacteria, a trophic interaction that it is important in soil food-webs and nutrient cycling (Clarholm, 1985). Protists represent the vast majority of eukaryotic phylogenetic and functional diversity (Adl et al., 2012; de Vargas et al., 2015; Geisen & Bonkowski, 2017), suggesting that different protists

species may have highly disparate impacts on soil-borne microbial communities and consequently soil functioning. However, studies to date that have tried to address the differential feeding behaviors of protists have been limited to simple one-on-one interactions, a limited range of protist taxa or low-resolution taxonomic identification of bacterial communities. We therefore sought to examine the extent to which soil protist traits can be linked to the impacts that they exert on bacterial communities via predation. We utilized a collection of 20 soil protist species representing a broad range of taxonomic groups (Fig.1). The majority of protist strains exerted a significant impact on bacterial community structure (Fig.2), suggesting that different protist strains had differential feeding behaviors when confronted with a diverse assemblage of potential bacterial prey species. We, however, did not detect any correlation between the phylogenetic relationship of protist strains and their impacts on bacterial communities (Supplementary Fig.1). We further examined if protists traits, such as growth rate, size and volume, could be linked to their predation impacts. We found that a significant portion of protist predation effects could be explained by protist cell volume (Fig.3).

Interestingly, we found no evidence for a link between protist phylogeny and their predation effects on bacterial community composition and diversity (Fig.2). Our results are in accordance with prior studies, which showed that even closely related species had differential predation effects on prey bacteria (Glücksman et al., 2010). Even though protist predation on specific bacterial strain can be predicted through high-level relatedness (Pedersen et al., 2011), our study could not find the link between predator high-level phylogenic relatedness and effects on the prey bacterial community (Fig.1,2), suggesting species-specific patterns of protist predation.

We adopted a more ecological approach, namely an examination based upon protists traits. We first measured traits of each protist strain, such as growth rate, size and volume, revealing a broad range of traits across the collection used in our study (Supplementary Table 2). We further attempted to link protist traits to their predation and found that protist traits are strongly associated with prey bacterial community structure (Fig.2).

Among the protist traits examined, we found cell volume was most strongly linked with protist predation effects (Fig.3,4, Supplementary Fig.2). However, other measured protists traits, such as length, width and growth rate did not have detectable effects. We expected that the protist trophozoite size would affect feeding impacts, since as smaller protists, in particular small trophozoite length, can access the soil pores in order to capture more prey bacteria (Finlay & Fenchel, 2001). It has to be note that we

only used one homogenized soil that might reduce the differences on natural settings. We did not detect a relationship between protist length and feeding-induced changes in the bacterial community. Thus, it appears that sheer ability to reach prey micro-habitats is not sufficient to predict predator -prey interactions besides experimental set-up limitation. Indeed, several steps are involved in the predator-prey interaction, for instance, including ingestion and digestion abilities (Matz & Kjelleberg, 2005; Jousset, 2012). With this in mind, we also expected high feeding and reproduction rates to be linked with stronger impacts on bacterial community structure, but no such relationship was found in our study (Supplementary Table 3).

Our results showed that protist volume was related to the impact of protist predation on the bacterial community (Fig.3,4, Supplementary Fig.2). This is in accordance to a prior study at microbial ecological scale by Glücksman et al., (2010), which showed that the differential volumes within Cercozoan species were correlated with the protist predation activity. Also at macroecological scale, predator size can also influence predation preference and serve as a proxy for feeding capacity (Schneider et al., 2012; Brose et al., 2019). It is important to know that inclusion of geometric body morphotypes (Hillebrand et al., 1999) was critical to our calculations of protist volumes. Use of such a combined volume calculation acts an effective wrapper incorporating several measures of size, thereby providing more information that for instance cell length, whose influence actually opposed that of cell volume (Fig.2).

We further examined how cell volume of protists related to specific changes in bacterial communities. For this we defined large and small protist size classes (Supplementary Table 2) and examined which bacterial taxa were over or underrepresented after incubation with either relatively small or large protists (Fig.5). We found that a range of OTUs that showed differential abundance according to protist size class, with five over in small volume treatment and seven over in big volume treatment. There was no relationship between protist size class and total bacteria density based upon QPCR data (not shown).

Interestingly, we found that large volume protists increased the relative abundance of some bacterial taxa that have been reported to have potential mechanisms to avoid predation by smaller volume protists (Supplementary Table 4). For instance, large volume protist treatments hosted relatively larger populations of *Pseudomonas, Variovorax,* and *Flavobacterium* (Rickard et al., 2003; Pehl et al., 2012; Rasamiravaka et al., 2015; Ríos-Castillo et al., 2018), which have been shown to form biofilms to defend against protist predation (Matz & Kjelleberg, 2005; Jousset, 2012). *Variovorax* spp. are also known to have the potential for a high level of, which may help in

the escape from protist predation (Matz & Jürgens, 2005). Secondary metabolite production by *Pseudomonas* sp. has been shown to not only prevent plant pathogen infection but also resist protist predation (Chin-A-Woeng et al., 2003; Jousset et al., 2006). Given the overlap between bacterial traits that help avoid protist predation and those involved in plant defense against pathogens (Iavicoli et al., 2003; Jousset et al., 2010), our study suggests that larger volume protists might therefore steer bacterial communities toward improved plant health promotion. However, this link would still have to be tested experimentally.

The specific changes of prey bacteria community can be caused by protist feeding strategy, specifically size selective feeding strategy. Studies on aquatic protists reported that heterotrophic nanoflagellates (HNF) and ciliates exerted strong predation pressure on bacterial cells ranging from 1-3 μm (Gonzalez et al., 1990; Monger & Landry, 1991). We therefore used bacterial cell length information available in the literature to examine if bacterial cell length of those bacterial OTUs differentially affected by protists size classes exhibited differences in cell length. We were unable to detect any relationship between predator volume and affected prey size (Supplementary Table 4), although it must be said that this analysis was limited to highly incomplete and somewhat speculative prey trait data.

Our study suggests that using protist morphological traits is more promising to predict predation impacts than using approaches related to protist phylogeny. However, we only were able to measure a limited range of morphological traits. Clearly, information concerning more functional traits linked to protist predation, such as nutrient requirement and tolerance to bacterial compounds, needs to also be taken into consideration (Jousset et al., 2006; Dumack et al., 2019). Moreover, protist functional traits can also interact with bacterial functional traits. Thus, studies of interactions between protists and prey would benefit from a combined approach, including both protist and bacterial traits. Such studies could also be extended to examine impacts of protist predation on ecosystem functioning, especially in the rhizosphere (Rosenberg et al, 2009; Krome et al., 2009), thereby potentially linking protist traits to plant protection, growth and health (Gao & Karlsson, et al., 2019).

To fully appreciate the consequences of protist predation on the soil microbiome, it appears to be important to take protist traits into consideration. Trait-based approaches have been demonstrated to be highly informative in other facets of microbial ecology, leading us to a better understanding on the relationship between community composition and ecosystem functioning that transcends taxonomy (McGill et al., 2006; Krause et al., 2014). Similar trait-based approaches have previously applied

for bacteria and fungi (Bouskill et al., 2012; Ho et al., 2013; Crowther et al., 2014). Our protist traits, such as cell volume which are linked to predation, can be a criterion of functional grouping in trait-based approaches of protists, thereby providing a predictive perspective of protist predation effects on soil microbiome functioning.

Conclusions

We observed species-specific effects of protist predation on soil microbial communities, and this effect was not linked to the protist phylogeny. Protist traits, especially cell volume, were found be more informative predictors of predation effects on the soil microbiome. For instance, larger volume protists lead to the resulting bacterial populations with potential for good predation defense strategies, traits that may overlap with those that support plant protection. Our study enhances the understanding in predator and prey interaction through the use of protists traits.

Acknowledgements

Z.G. was supported by Chinese Scholarship Council (CSC), S.G. by a NWO-VENI grant from the Netherlands Organisation for Scientific Research (016.Veni.181.078). Moreover, we would thank Xueyang Sun for the help in generating 18S rRNA gene sequences of our protists strains. We also acknowledge Nathalie Amacker, Jie Hu, Qiqi Lv and Peter Veenhuizen for their help with laboratory experiments. We thank Wu Xiong for suggestions on the analysis of next-generation sequence data.

Supplementary material

Supplementary Table 1. Sequences of primers used for protists phylogeny of 18S rRNA gene sequence and 16S rRNA gene tag sequencing preparation. NA means not available.

Sequence	Primers	Primers sequence	Reference
18S rRNA Sequence	Pre_3ndfor	CAGCAGGCGCGCAAATTACC	Bass & Cavalier-Smith, 2004
	V4_1for	CCAGCASCYGCGGTAATWCC	Hartikainen et al., 2016
	3NDfor	GGCAAGTCTGGTGCCAG	Cavalier-Smith et al., 2009
	12Nrev	AACGGCCATGCACCACC	NA
	RibA	ACCTGGTTGATCCTGCCAGT	James et al., 1994
	RibB	TGATCCATCTGCAGGTTCACCTAC	James et al., 1994
	Euk1A_18Sfor	CTGGTTGATCCTGCCAG	Sogin & Gunderson, 1987
	Euk1A_18Srev	TGATCCTTCTGCAGGTTCACCTAC	Medlin et al., 1988
16S rRNA gene tag sequencing	16SforA_NGS	TCGTCGGCAGCGTCAGATGTG-TATAAGAGACAGNNNNNaGTGC-CAGCMGCCGCGGTAA	Caporaso et al., 2010
	16SforB_NGS	TCGTCGGCAGCGTCAGATGTG-TATAAGAGACAGNNNNNNaGTGC-CAGCMGCCGCGGTAA	
	16SforC_NGS	TCGTCGGCAGCGTCAGATGTG-TATAAGAGACAGNNNNNNNaGTG-CCAGCMGCCGCGGTAA	
	16SrevA_NGS	GTCTCGTGGGCTCGGAGATGTG-TATAAGAGACAGNNNNNGGAC-TACHVGGGTWTCTAAT	
	16SrevB_NGS	GTCTCGTGGGCTCGGAGATGTG-TATAAGAGACAGNNNNNNGGAC-TACHVGGGTWTCTAAT	
	16SrevC_NGS	GTCTCGTGGGCTCGGAGATGT-GTATAAGAGACAGNNNNNNttG-GACTACHVGGGTWTCTAAT	

Supplementary Table 2. Overview of protists traits and phylogeny information. Note that trophozoite length and width of *Cryptodifflugia* sp.75 represents the length and width of *Cryptodifflugia* sp. 75 shell.

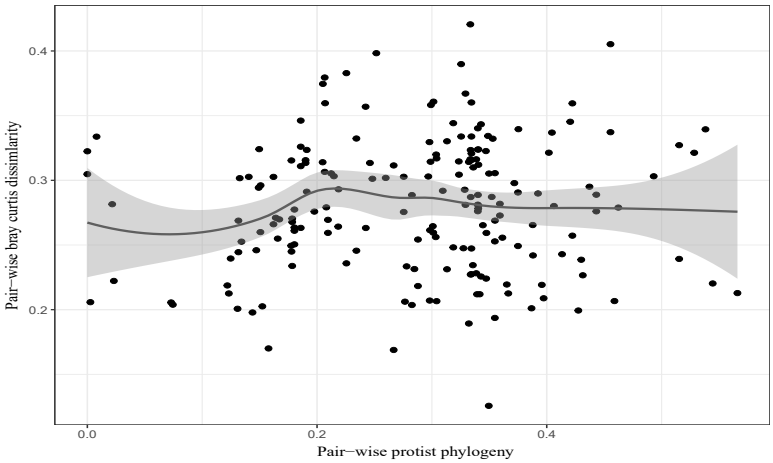
Protists	Phylogeny	Supergroup	Growth rate (individuals/ day)	Trophozoite length (µm)	Trophozoite width(µm)	Cyst length(µm)	Cyst width(µm)	Morphotype	Morphotype group	Volume ($µm^3$)	Volume group
C5D3	*Cercomonas lentalike*	Rhizaria	158.40	11.01	6.22	10.25	9.23	Flagellate	A	334.548	Small
C7D2	*Cercomonas lentalike*	Rhizaria	138.20	10.82	5.13	7.71	7.40	Flagellate	A	223.641	Small
C13D2	*Acanthamoeba* sp.	Amoebozoa	24.50	17.23	14.28	13.5	12.21	Acanthopodial	C	123.022	Small
S18D10	*Heterolobosea* sp.	Excavata	43.40	19.98	6.07	7.94	6.76	Eruptive	A	578.18	Large
S24D2	*Cercomonas* sp.	Rhizaria	165.80	11.14	5.64	8.10	7.57	Flagellate	A	278.313	Small
P145-4	*Naegleria clar*ki	Excavata	13.75	22.60	11.75	10.62	9.62	Eruptive	A	2450.61	Large
CN7	*Vahlkampfi soli*	Excavata	7.73	18.73	7.28	10.46	9.31	Eruptive	A	779.633	Large
C2D2	*Acanthamoeba* sp.	Amoebozoa	42.54	18.92	12.20	13.5	12.21	Acanthopodial	C	115.412	Small
76	*Cochliopodium minus*	Amoebozoa	435.81	18.53	12.05	7.87	7.09	Lens-like	C	111.643	Small
75	*Cryptodifflugia* sp.	Amoebozoa	4.67	18.58	16.69	17.62	16.10	Testated	A	4064.89	Large
33	*Vannella* sp.	Amoebozoa	77.00	20.10	13.59	11.07	10.03	Fan-shape	C	136.58	Small
NL10	*Allovahlkampfia* sp.	Excavata	234.39	13.67	9.55	8.53	7.90	Eruptive	A	979.186	Large
P1-1	*Didymium* sp.	Amoebozoa	9.00	15.69	9.05	10.54	9.06	Branched	B	504.638	Large
16	*Mycamoeba* new sp.	Amoebozoa	2.88	5.87	4.84	5.02	4.39	Flabellate	B	53.9994	Small
C10D3	*Rosculus* sp.	Rhizaria	124.00	7.60	7.70	4.58	3.73	Flabellate	B	176.952	Small
P147	*Vannella* new sp.	Amoebozoa	21.30	23.97	21.85	11.07	10.03	Fan-shape	C	261.872	Small
7	*Vermamoeba vermiformis*	Amoebozoa	447.90	14.11	6.96	8.01	7.04	Monotatic	A	536.828	Large
27	*Famella* new sp.	Amoebozoa	7.90	33.77	18.15	10.55	9.00	Flamellian	C	306.463	Small
NL81	*Naegleria clarki*	Excavata	11.65	21.70	14.04	11.08	10.06	Eruptive	A	3359.57	Large
45	*Vannella* newsp.	Amoebozoa	0.12	15.98	12.52	11.07	10.03	Fan-shape	C	100.035	Small

Supplementary Table 3.Tests (linear regression and Mantel statistics) for a significant association between pair-wise Bray Curtis dissimilarity of prey bacterial communities and pair- wise protist traits distance.

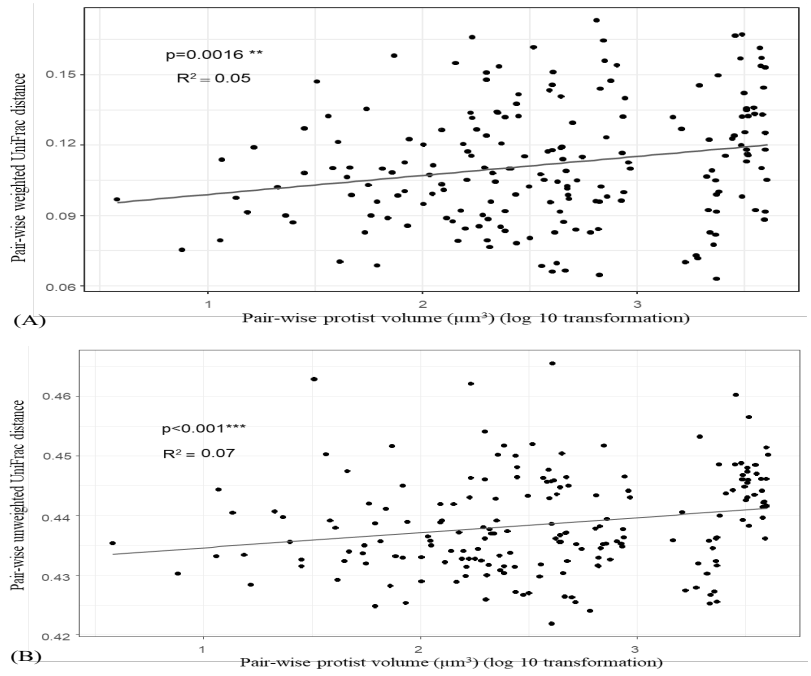
Predictor	Linear regression		Mantel test	
	F - statistic	**P value**	**Mantel r**	**Significance**
Phylogeny distance	0.796	0.3734	0.06493	0.32
Traits distance				
Trophozoite length	1.814	0.1796	0.09776	0.251
Trophozoite width	0.05618	0.813	-0.01728	0.497
Cyst length	0.4265	0.514	-0.04758	0.592
Cyst width	0.4939	0.483	-0.05119	0.651
Growth rate	0.1449	0.704	0.02775	0.403
Volume	16.96	<0.001 ***	0.3731	0.025

Supplementary Table 4. Potential functions of significantly changed OTUs by small and large volume protists.

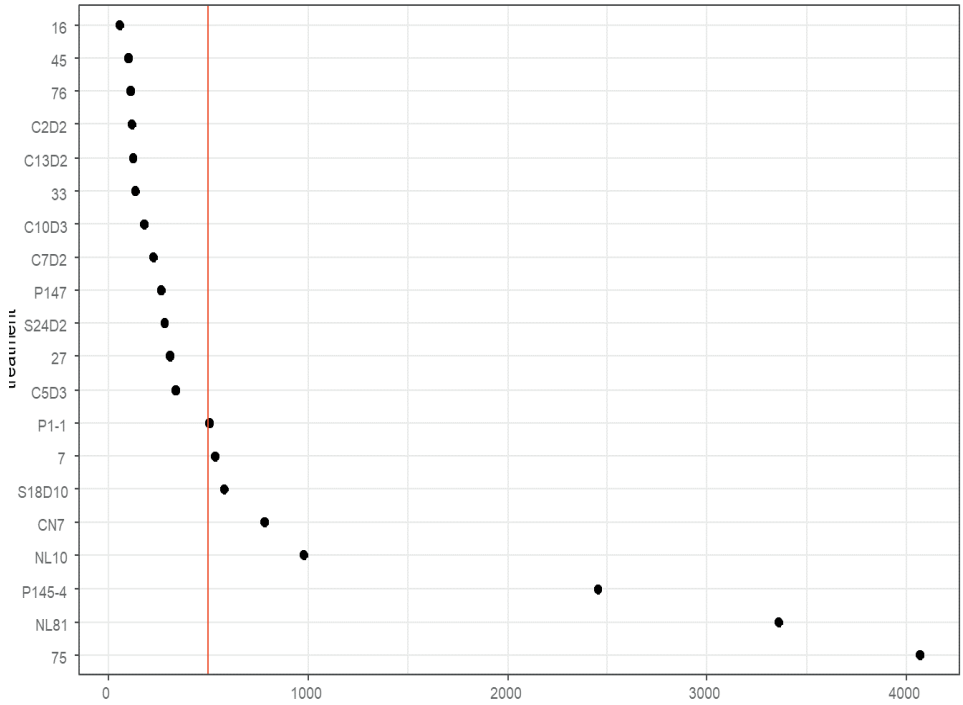
OTUs	length (μm)	Increased in	Functions
Nubsella	1.0-5.0	Small volume protists	*Nubsella zeaxanthinifaciens* produces zeaxanthin, which is carotenoid alcohols (Ryan & Pembroke, 2018)
Brevundimonas	1.0-4.0	Small volume protists	Emerging global opportunistic pathogens Survival on Mars (Dartnell et al., 2010)
Hydrogenophaga	0.6-5.5	Small volume protists	Hydrogen-Oxidizing bacteria (Willems et al., 1989)
Pirellula	1.12	Small volume protists	Plant pathogen (Glöckner et al., 2003)
Pseudomonas	1.5-5.0	Large volume protists	Biofilm formation, Antibiotic resistance (Rasamiravaka et al., 2015)
*Sphingomon*as	1.4	Large volume protists	Yellow-pigmented colonies Biodegradative and biosynthetic capabilities (Balkwill et al., 2006)
Reyranella	1.59	Large volume protists	Bacteria associated with Amoeba (Pagnier et al., 2012)
Microbacterium	1.0-4.0	Large volume protists	Antibiotic and heavy metal tolerance (Learman et al., 2019)
Leifsonia	1.2-2.5	Large volume protists	Ratoon stunting pathogen (Davis et al., 1980)
Variovorax	0.7-3.0	Large volume protists	Motility and biofilm formation (Jamieson et al., 2009; Pehl et al., 2012)
Flavobacterium	3.0-5.0	Large volume protists	Biofilm (Ríos-Castillo et al., 2018)



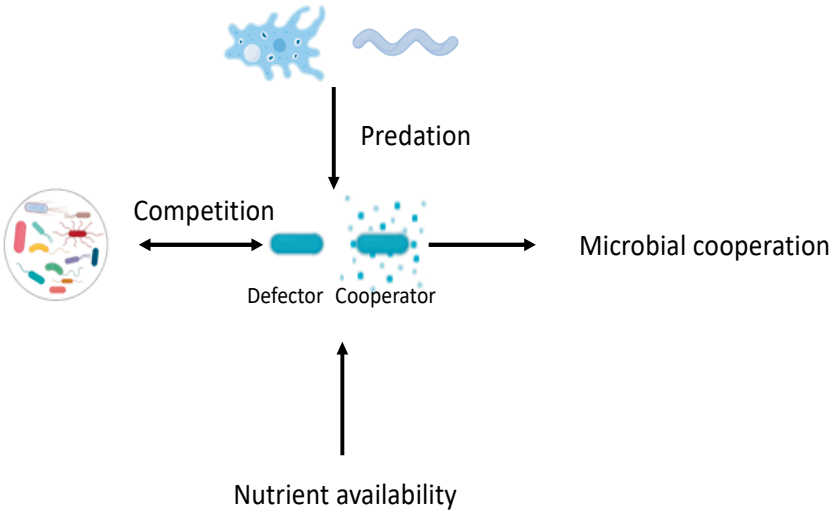
Supplementary Figure.1 Relationship between pair-wise protist phylogenetic distance and pair-wise bray curtis dissimilarity of prey bacterial communities



Supplementary Figure.2 Correlation between pair-wise protist volume distance and prey bacterial communities dissimilarity based on (A) weighted UniFrac distance and (B) un-weighted UniFrac distance.



Supplementary Figure.3 Protists volumes were ordered by size; red line indicates the cut-off (500 um^3) of small and large volume protists, protists' volume below 500 um^3 are small volume protists (left side of red line), protists' volume above 500 um^3 are large volume protists (right side of red line).


Predation
Competition
Microbial cooperation
Defector
Cooperator
Nutrient availability

Chapter 4

Multitrophic interactions determine the stability of bacterial cooperation

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Abstract

Microorganisms often cooperate and jointly invest into public goods that increase their fitness. Evolutionary theory predicts however that cooperation should rapidly break down under the pressure of defectors, raising the question about why cooperative behavior is so widespread in nature. Here we address to which extent resource availability and multitrophic interactions including competition and predation jointly stabilize cooperation by shifting the costs and benefits of public good production. We grew the model cooperator *Pseudomonas protegens* CHA0, producing several bioactive secondary metabolites, and a signal-blind isogenic mutant as defector, as focal species along three orthogonal gradients of resource availability, competition with a background semi-natural bacterial community and predation by protists. We measured under each condition the benefit of cooperation when grown alone and the defector invasion when defectors are rare. We observed strong interactions between the different interactions, competition increased the net benefit of cooperation at low and intermediate resource availabilities, however, predation followed the opposite pattern, with increasing the net benefit of cooperation at intermediate and high resource availabilities. Our results further showed the multitrophic interactions constrained the defector invasion and promote cooperation, in particular, competition prevented the spread of defectors, but this effect strongly depended on resource availability. Our study suggests that cooperation may be more stable than expected when placed in a multitrophic context.

Introduction

The widespread occurrence of cooperation in microorganisms remains puzzling from an evolutionary perspective (Crespi, 2001; West et al., 2006). Several bacteria, despite of being single-cell organisms, engage in cooperative behaviors with their neighbors. They produce for instance extracellular public goods such as siderophores, enzymes or antimicrobial compounds (Griffin et al., 2004; Diggle et al., 2007). As these compounds benefit both the producer and neighboring organisms, their production is susceptible to defectors taking advantages of public goods without contributing to their production (Travisano & Velicer, 2004; West et al., 2007; Rankin et al., 2007). Consequently, in absence of control mechanisms, cooperation is predicted to be unstable, a prediction in direct contradiction with the high prevalence of cooperative behaviors in natural communities. Cooperation can be stabilized by a range of environmental parameters including resource availability, policing, scale of competition or predation (Duffy & Défago, 2000; Brockhurst et al., 2008; Jousset et al., 2009; Manhes & Velicer, 2011; Inglis et al., 2011). To date, most studies on the stability of cooperation have been based on model systems including few or no species outside of the focal species. However, in natural systems, microorganisms typically grow within multispecies communities, where their individual fitness is constrained, in addition to kin competition, by the presence of diverse competitors and predators (Fierer, 2017). In this work we aim to understand better the dynamics of cooperation in a multitrophic context and disentangle which trophic interactions are the most determinant as a driver of cooperative behaviors. In particular, we focus on the fitness gain confered by cooperation and on the defector invasion when rare.

We therefore grew a model focal pair of cooperator and defector, grown alone or with a rare initial defector frequency. We used *Pseudomonas protegens* CHA0 as a model cooperator, a plant-associated bacteria producing a range of secondary metabolites functioning as public goods (Voisard et al., 1994; Schnider-Keel et al., 2000; Jousset et al., 2008). We used an isogenic *gacS*$^{-}$ mutant as defector, a signal-blind mutant lacking most secondary metabolites produced by the wild type (Zuber et al., 2003; Jousset et al., 2006). We measured the benefits of cooperation in two ways. First, we compared the fitness of the cooperator and defectors when placed as a focal species along three fully orthogonal gradients of nutrient availability, competition with a semi-natural bacterial community and predation by two free-living amoebae. We then measured the ability of a rare defector to invade a focal population of cooperators under the same range of biotic interactions.

We hypothesized that multitrophic interactions increase the benefits of cooperation,

as several of the produced public goods are directly involved in antagonizing competitors and predators (Jousset et al., 2006, 2009; Bruce et al., 2017). We also expected that resource availability increase cooperation because increasing resource supply could reduce costs of cooperative behaviors (Brockhurst et al., 2008). In terms of invasion of a cooperator by a rare defector, we expected that high resource availability would reduce the probability of invasion by limiting the metabolic burden of cooperation, while selective predators may in the opposite drive defectors extinct by overconsuming them (Jousset et al., 2009; Friman et al., 2013).

Material and methods

Strain selection

We used *Pseudomonas protegens* CHA0 (Voisard et al., 1994; Jousset et al., 2006) as cooperator and its *gacS* isogenic mutant CHA19 (Zuber et al., 2003; Jousset et al., 2006) as signal-blind defector that do not produce most of secondary metabolites functioning as public goods. Both strains were with chromosomally tagged with green fluorescent protein and a kanamycin resistance cassette (Jousset et al., 2006). Bacteria were kept as frozen glycerol stocks. Prior to experiments, single colonies were picked and grown overnight 1/3 Tryptic Soy Broth (TSB, Tryptone 17g L^{-1}, Soy Peptone 3g L^{-1}, Glucose 2.5 g L^{-1}, NaCl 5.0 g L^{-1}, K_2HPO_4 2.5g L^{-1}). Tryptone and Soy Peptone were purchased from BD Diagnostic systems (Heidelberg, Germany) and washed three times using the diluted phosphate buffer Page's Amoeba Saline (PAS) (Page, 1988) to remove nutrients and reduce the salt concentrations to a level allowing further predation experiments. Both strains were adjusted to an initial concentration of 10^6 CFU mL^{-1}.

Predator and competitors

We used two protists as predator, the Heterolobosea amoeba S18D10, which was isolated form sandy soil in the Netherlands and *Acanthamoeba* sp. C2D2, which was isolated form clay soil in the Netherlands as predators. Both strains were identified based on 18S rRNA gene using eukaryotic 18S primers sets Euk1A (Sogin & Gunderson, 1987) and EukB (Medlin et al., 1988). Protists were grown on *Escherichia coli* OP50 at 15°C and washed twice by centrifugation (800 g, 5 min) to remove *E.coli*. Due to technical limitations we could only reduce *E.coli* density to 1000 CFU mL^{-1} in the protist culture. However, this bacteria grows very poorly at the temperature used for further experiments, limiting its impact on the results. Protist populations were measured under inverted microscope Nikon Eclipse TS 100

(NIKON, Tokyo, Japan) and adjusted to at 1000 individuals mL^{-1}.

We used as competitors a semi-natural soil bacteria community extracted from a sandy soil (Botanical garden at de Uithof, Utrecht, the Netherlands) (52°05'16"; 5°10'14"), using a standardized protocol (Rønn et al., 2002). Briefly, 50 g soil was air-dried overnight and blended in a kitchen blender with 200 mL 0.1% pyrophosphate buffer for 2 minutes. After decantation, bacteria were extracted from the soil slurry by density gradient centrifugation: 10 mL Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and 10 mL soil suspension were gently added to 50 mL falcon tube on the top of Percoll and centrifuged using fixed-angle rotor 10000 x g for 20 min with acceleration and deceleration 1 (Thermo Fisher Scientific, Langenselbold, Germany). The top layer was transferred to a new falcon tube (Greiner Bio-one, Frickenhausen, Germany) and successively vacuum-filtered on glass microfiber filters with pore sizes 3 µm, 1.6 µm and 1.2 µm (Whatman GE Healthcare, Chicago, USA). The filtrate was diluted 1:10 and incubated in in 250 mL culture flasks (Greiner Bio-one, Frickenhausen, Germany) in 1/300 TSB. Culture flasks were incubated at 15°C and checked under the microscope for contamination with protists. Bacterial communities without predator contamination were pooled together and centrifuged (4500 rpm, 10min) to remove access nutrients before use.

Experimental set-up

Benefit of cooperation

The cooperator or defector separately were subjected to three environmental variables: resource availability gradients (0.2, 0.5, 1.0 g L^{-1} TSB), predators (Heterolobosea sp. or *Acanthamoeba* sp., 5000 individuals mL^{-1}) , competitor (predator - free soil bacteria community, 10^6 CFU mL^{-1}) and combined variables with competitor and predation.

Invasion by rare defectors

The cooperator and defector are mixed at a cooperator to defector ratio of 99:1. This ratio was selected to keep defectors as rare as possible at the beginning while avoiding too much bottleneck effects. The combination was also subjected to above-mentioned three environmental variables.

Each treatment had six replications, which were randomly placed in 96 well plates (Corning Incorporated, Kennebunk, USA). All replications were grown statically at

20°C in obscurity.

Bacteria enumeration

After seven days, twenty microliters of each well were sampled and diluted 10000 times. Ten microliters of all samples were plated out on skim milk agar (Tryptic soy broth 3g L^{-1}, agar 15g L^{-1} and skim milk 150 mL L^{-1}) supplemented with antibiotics (Kanamycin 50µg mL^{-1}, Chloramphenicol 10µg mL^{-1}, Ampicillin 40µg mL^{-1}, Cycloheximide 75µg mL^{-1}). All plates were incubated at 28°C for two days. We identified cooperators on the base of a halo on the silk milk agar and color change on tryptophan side-chain oxidase (TSO) overlay (Oberhansli et al., 1991), conversely, defectors were identified on the base of the lack of a halo and no color change.

Data analysis

All statistics analysis was performed under R version 3.6.

Benefit of cooperation for the focal species

We calculated the strength of each interaction (predation, competition and mix of competition and predation) at each resource concentration as the log10-transformed ratio of focal species density when grown alone in the target treatment. The net benefit of cooperation for each condition was defined as the difference between the interaction strengths on the cooperator and the defector.

Invasion of cooperators by rare defectors

We calculated the ability of defectors to invade a cooperator focal population. We categorized invasion at the end of the experiment as failed (defector density dropped below detection) or successful. We used Generalized Linear Models (*MASS::glm*) with a binomial distribution to analyze the multitrophic effects on the invasion success (Venables & Ripley, 2013).

Result

Benefits of cooperation

We first assessed the impact of trophic interactions on the population of the

cooperators and defectors growing separately as focal species. Both cooperators and defector population density were negatively affected by biotic interactions in most of the treatments (Fig.1 A, B). However, cooperator population was consistently higher than the defector, indicating a large decrease in defector density in comparison to the cooperator density. At the lowest resource availability treatment, the presence of a background community increased cooperator absolute density, pointing to potential facilitation rather than competition. When the focal species was composed fully of defectors, all multitrophic interactions strongly decreased the defector density in comparison to the control treatment (Fig.1 B). Interestingly, when competition and predation were combined, they showed an additive effect on cooperator or defector populations (Fig.1 A, B).

The net benefit of cooperation was defined here as difference of each trophic interaction on the cooperator and defector. The benefit of cooperation further varied with resource availability (Fig.1 C): Cooperation conferred a competitive advantage in the presence of competing bacteria especially at low resources availability, but this advantage vanished when resource availability increased. In contrast, the benefit of cooperation in terms of predation resistance followed the opposite pattern and only was apparent at intermediate and high resource concentration. This highlights that cooperation can help cope with both competition and predation, yet in a resource-dependent way.

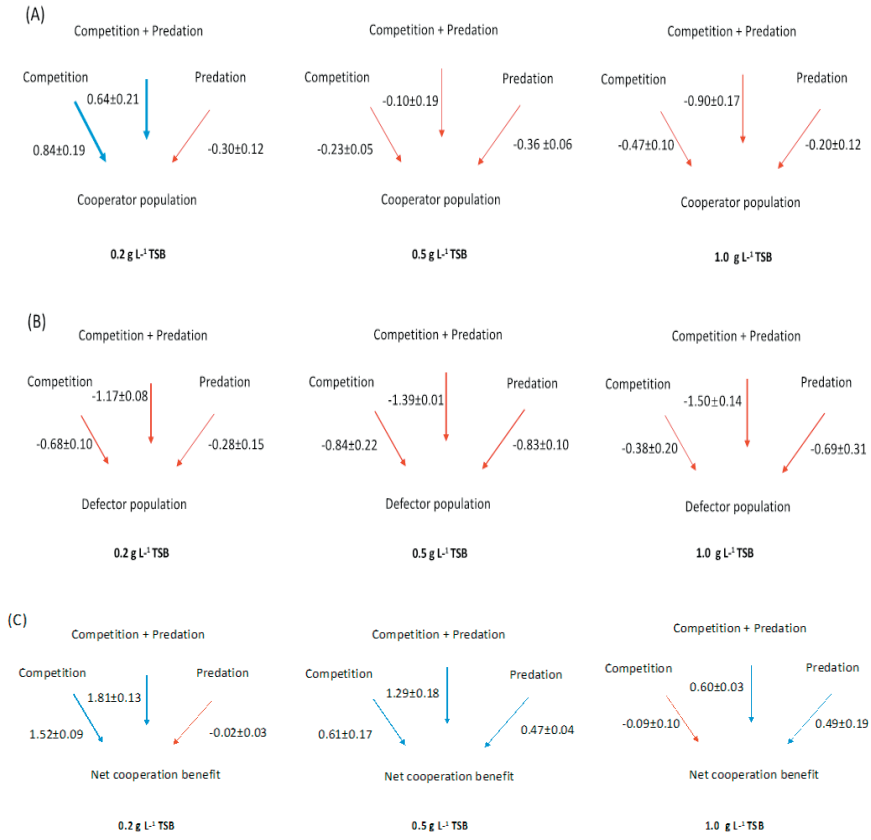


Figure.1 Interactive effects of resource availability, predation and competition on the growth of cooperator focal species *Pseudomonas protegens* CHA0 (A) and the defector *gacS*- mutant CHA19 (B). Interaction strength was defined as the ratio between focal species density when grown alone or in presence of the specific natural enemies. Red and blue arrows facilitation or antagonism, respectively. Note each interaction strength was log10 transformed. (C) Net benefit of cooperation in presence of different resource availabilities, competitors and predators. The net benefit is defined as the difference of each trophic interaction on the cooperator and defector. Red and blue arrows indicate a net benefit or disadvantage of cooperation, respectively.

Effect of multitrophic interactions on the defector invasion

We next investigated the stability of cooperation by assessing invasion of a cooperative population by rare defectors present at an original relative abundance of 1% (Fig.2).

We found that multitrophic interactions strongly constrained defector invasion at the end of the experiment (Fig.2). In absence of natural enemies, defectors could invade between 50% and 83% of the communities (Fig.2). Predation drove defectors to extinction (below detection) in all replicate communities, yet this effect was only apparent at intermediate and high resource availability (Fig.2: Predation; Table 1), matching well the benefits of cooperation under these conditions.

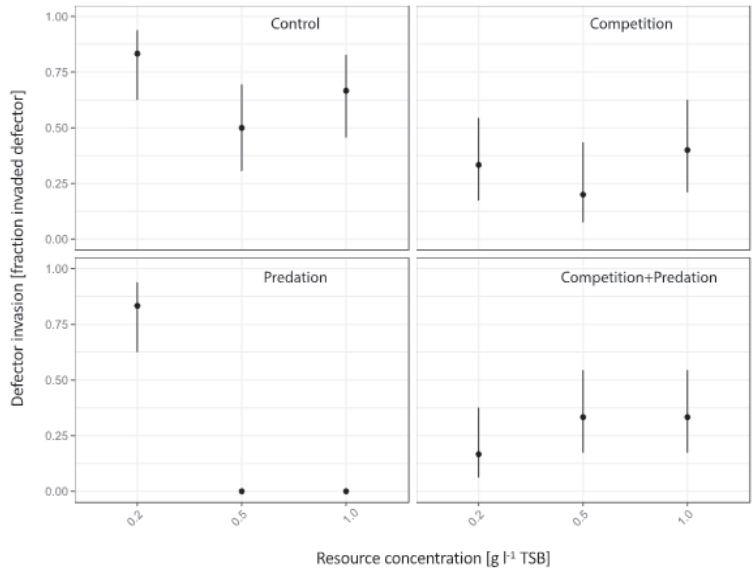


Figure.2 Probability of invasion by the signal-blind defector *Pseudomonas protegens* CHA0 ΔgacS under predation and competition when rare (1% initial relative abundance), defined as the proportion of the defectors could successfully persist above detection (6 replications in each treatment). Control: cooperators and defectors were grown alone. Competition: defectors and cooperators were grown in presence of a semi-natural bacterial community; Predation: defectors and cooperators were grown in presence of the bacterivorous amoeba Heterolobosea sp.; Competition + Predation: defectors and cooperators were grown in presence of both a semi-natural bacterial community and the predator Heterolobosea sp..

Competition with a background microbial community also constrained defector invasion and there was a significant interactive effect with resource availability (Fig.2: Competition; Table 1). Competition and predation had interactive effects, with invasion being intermediate in predation + competition treatments (Fig.2: Competition + Predation; Table 1).

Table 1. ANOVA table summarizing the interactive effects of Resource availability (RA), Competition (CO) and Predation (PR) on the probability of invasion of cooperators by rare defectors.

	Df	$\chi 2$	Pr (>χ)
Null			
CO	1	2.3613	0.12438
PR	1	3.6273	*0.05684* .
RA	2	4.5053	0.10512
CO X PR	1	2.3449	0.12570
CO X RA	2	8.4405	**0.01469** *
PR X RA	2	0.6386	0.72665
CO: PR: RA	2	4.6082	*0.09985* .
Residuals	69	93.51	

Discussion

Benefits of cooperation

Microbial cooperation is an essential but yet hard to manage component of a range of microbiome-mediated services including crop protection, nitrogen fixation, or bioremediation of organic pollutants (Simms & Lee Taylor, 2002; Abraham et al., 2002; Denison et al., 2003; Megharaj et al., 2011; Besset-Manzoni et al., 2018). There is a growing awareness that the dynamics and stability of cooperation is highly dependent on the environmental context, with for instance the presence of natural enemies repeatedly reported to promote cooperation (Jousset et al., 2009; Morgan et al., 2012; Mumford & Friman, 2017). However, these measures are all made in a disparate set of experimental conditions, making it hard to stitch them together into an overarching predictive framework. Here we sought to address how different interactions such as competition and predation interactively shape the fitness of cooperators and defectors, allowing to predict better the fate of cooperation in a range of environmental conditions.

In line with previous studies, multitrophic interactions increased the benefits of cooperation for the focal species. In almost all treatments, the presence of an intact GacS gene (Heeb & Haas, 2001), which is essential for cell-to-cell communication and secondary metabolite production helped the focal species *Pseudomonas protegens* CHA0 survive competition and predation. This pattern is in line with previous studies showing that secondary metabolites of this strain can help resist

predation (Jousset et al., 2006) and competition (Bruce et al., 2017).

Resource availability further modulated the importance of both competition and cooperation. For instance, cooperator population was increased at low nutrient availability but not at high resource availability in presence of competition. This matches well previous studies showing that resource availability can affect the costs and benefits of cooperation, with for instance high resource availability being likely to reduce costs of public goods production (Brockhurst et al., 2008). In a multitrophic context, competition increased the net benefit of cooperation at low and intermediate resource availabilities. In contrast, predation effects on the net benefit of cooperation had the opposite pattern along resource availability, in particular predation increased the net benefit of cooperation at intermediate and high resource availabilities. Consistence to previous studies we show that predators are major factors shaping cooperation at higher nutrient level, because defensive traits of prey is costly (Abrams, 2000) and high nutrients enable prey to develop more anti-predator traits (Jürgens & Matz, 2002; Friman et al., 2008; Corno & Jürgens, 2008). Moreover, due to these differential impacts, when competition and predation were combined, net benefit of cooperation was always positive regardless of resource availability (Fig.1 C).

Invasion of cooperating population by rare defectors

Multitrophic interactions prevent the spreads of defectors when rare and even drove them to extinction in a majority of the communities. In absence of natural enemies, defectors could invade in 50-83% population (Fig.2 Control), this proportion can be reduced to 0 by predation (Fig.2 Predation). This is in line with prior studies showing that protists can selectively consume defectors that do not produce secondary metabolites (Jousset et al., 2009). It has to be noted that predation has a strong trend on driving the survival of defectors to extinction, especially at intermediate and high resource availabilities (Fig.2). We tested effects by predation on cooperation through two protists respectively, the result only showed a predation effect by Heterolobosea sp. and the same pattern of preventing defector invasion was found in presence of predation by *Acanthamoeba* sp. (Supplementary Fig.1). Our results showed that predators decreased the defector the most at intermediate and high resource availabilities, which is line with prior studies showing that prey defense is stronger at high resource environment (Friman et al., 2008).

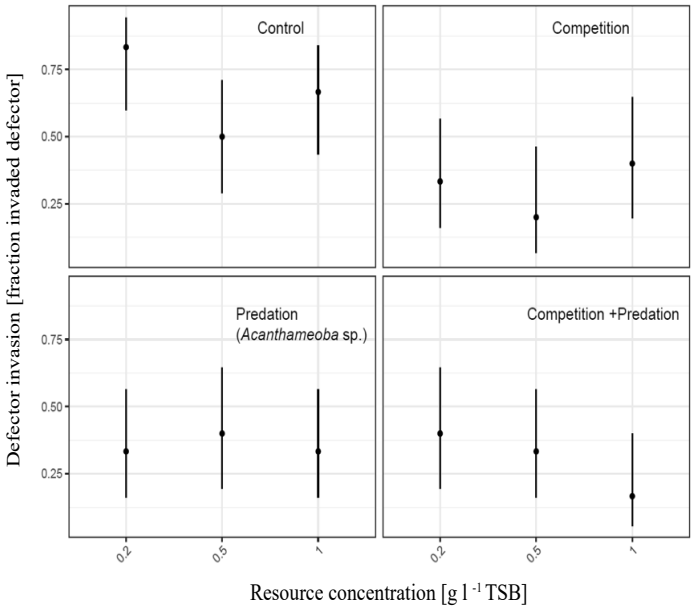
Our results showed that competition had no effect on the defector invasion, however, interaction between competition and resource availabilities significantly impacted

defector invasion, especially at low nutrient availability competition decreased the defector the most. This is contrast to our expectation because low resource availability would increase the selective benefit of cheating, thereby increase defector invasion (Harrison et al., 2008; Brockhurst et al., 2008). Our study suggests that competition with natural soil bacterial communities could prevent defector invasion but that strongly depended on resource availability.

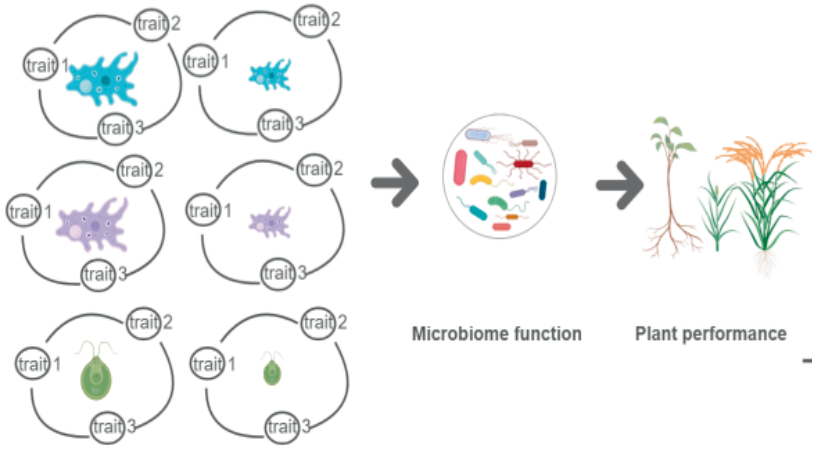
Conclusion

Multitrophic interactions promote the cooperation stability from an ecological and evolutionary perspective by increasing net benefits of cooperation and decreasing defector invasion. Predation increased the net benefit of cooperation at intermediate and high resource availabilities, however, cooperation followed the opposite pattern, increasing the net benefit of cooperation at low and intermediate resource availabilities. Predation constrained the spread of defectors when rare and enforced cooperation. Competition also prevented the defector invasion, but this effect significantly depended on resource availabilities. Our study suggests that cooperation may be more stable than expected when in the context of multitrophic interactions.

Supplementary material



Supplementary Figure.1: Probability of invasion by the signal-blind defector *Pseudomonas protegens* CHA0ΔgacS under predation and competition when rare (1% initial relative abundance), defined as the proportion the defectors could successfully persist above detection (6 replications in each treatment). Control: cooperators and defectors were grown alone. Competition: defectors and cooperators were grown in presence of a semi-natural bacterial community; Predation: defectors and cooperators were grown in presence of the bacterivorous amoeba *Acanthamoeba* sp; Competition + Predation: defectors and cooperators were grown in presence of both a semi-natural bacterial community and the predator *Acanthamoeba* sp..


trait 1
trait 2
trait 3
Microbiome function
Plant performance

Chapter 5

Protists – puppet masters of the rhizosphere microbiome

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Published as:

Gao Z, Karlsson I, Geisen S, Kowalchuk G, Jousset A. 2019.
Protists: Puppet Masters of the Rhizosphere Microbiome.
Trends in Plant Science 24: 165–176.

Highlights

- The rhizosphere microbiome is a major determinant of plant fitness
- The mechanisms driving microbiome assembly are however insufficiently known, impeding efficient microbiome management
- Here we present free-living protists as an overlooked yet core component of the microbiome that may offer a powerful leverage to improve microbiome function
- Protists shape microbiome structure by consuming bacteria and fungi and can select for plant-beneficial functional traits
- Protist predation increases microbiome provisioning of services required for improving plant growth and health

Outstanding Questions

- What is the relative importance of protist predation and resource competition for rhizosphere microbiome dynamics?
- What are the direct effects of protists on plant physiology?
- What are general and species-specific effects of protists on the rhizosphere microbiome and plant performance?
- What is the power of phylogeny and functional traits as predictors of the impact of protist on microbiome functioning and plant performance?
- What are the ecological functions of unknown protist taxa recently discovered via sequencing-based surveys that have not yet been isolated?
- How do free-living protists interact with microorganisms in the mycosphere?
- How can protists be used as a leverage to enhance microbiome function?
- How can application methods for protists as biostimulants be optimized?

Abstract

The rhizosphere microbiome is a central determinant of plant performance. Microbiome assembly has traditionally been investigated from a bottom-up perspective, assessing how resources such as root exudates drive microbiome assembly. However, the importance of predation as a driver of microbiome structure has to date largely remained overlooked. Here we review the importance of protists, a paraphyletic group of unicellular eukaryotes, as a key regulator of microbiome assembly. Protists can promote plant-beneficial functions within the microbiome, accelerate nutrient cycling and remove pathogens. We conclude that protists form an essential component of the rhizosphere microbiome and that accounting for predator-prey interactions would greatly improve our ability to predict and manage microbiome function at the service of plant growth and health.

Key words: protists, amoeba, rhizosphere microbiome, predation, plant-microbe interactions

A multi-trophic perspective to the rhizosphere microbiome

Plant growth, nutrition and health are to a large extent determined by the activity of associated microorganisms (Berg et al., 2016). In particular, plant roots are associated with an active multispecies community, the **rhizosphere microbiome** (Glossary), providing several important services to the plant. Root-associated microbes for instance mineralise nutrients, manipulate plant hormonal balance and suppress potential pathogens (Berendsen et al., 2012). The species composition of the rhizosphere microbiome is now recognized to have direct effects on host plant traits (Panke-Buisse et al., 2015). However, our understanding of the determinants of microbiome community assembly and composition is still lacunar, restricting our ability to predict and harness microbiome dynamics and functionality. To date, most studies seeking to address the mechanisms underlying microbiome composition, species turnover and function have focused on bottom-up drivers of microbial community composition, such as plant developmental stage, soil type and host genotype (Chaparro et al., 2014; Edwards et al., 2015; Wagner et al., 2016). While **bottom-up control** are certainly crucial, they represent only half of the story (Figure 1). Microorganisms in the rhizosphere are subjected to **top-down control** by a range of bacterial and eukaryotic consumers. Among them, free-living protists (Box 1), a highly diverse group of mostly unicellular eukaryotes (Geisen et al., 2018), in our opinion deserve a special attention. Protists are highly abundant and active consumers of bacteria and arguably fungi, impact community structure, and play a key role for nutrient cycling in the rhizosphere (Clarholm, 1985; de Ruiter et al., 1995; Rønn et al., 2002; Crotty et al., 2013; Kramer et al., 2016; Zhang & Lueders, 2017). This review primarily addresses the importance of free-living heterotrophic **protists**, feeding on other organisms. For the sake of simplicity, we hereafter refer to them as 'protists', deliberately omitting mutualistic or parasitic taxa, including animal parasites or plant pathogens.

Despite their ubiquity and ecological importance for soil functioning, protists are still a relatively misunderstood component of the soil and rhizosphere microbiome (Caron et al., 2008; Geisen et al., 2017). This knowledge gap is especially striking given that protists are comparably well investigated in aquatic ecosystems, where they are recognised as an integral part of the microbial food web. However, when it comes to soil, research has long focused on taxonomic species descriptions, with only a handful of scientists assessing interactions with other microorganisms and plants. This can partly be attributed to methodological constraints in studying protists as they can be difficult to extract and cultivate, and reliable molecular methods have only recently been developed (Geisen & Bonkowski, 2017). Better coverage of protist

databases (Berney et al., 2017) and the emergence of high-throughput sequencing approaches allowing in-depth interrogation of soil protist communities (Bates et al., 2013) provide new opportunities to explore the diversity and ecological importance of soil-borne protists. The time is ripe to shift the perception of plant-microbiome interactions beyond bacteria and fungi and integrate protistology more solidly into microbiome research.

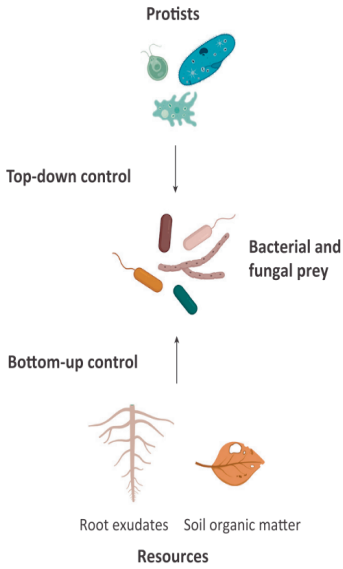

Figure.1 Bottom-up vs top-down drivers of the rhizosphere microbiome assembly

The microbiome is affected by a range of factors. Plants invest carbon into root exudates, which together with soil organic matter fuel microbial activity in the rhizosphere (bottom-up control). However, top-down control by protist consumers is also influencing microbiome dynamics and functionality.

We aim here to place the rhizosphere microbiome into a multitrophic perspective. We highlight the importance of free-living protists as an often overlooked but central group of rhizosphere organisms that drive both microbiome structure and interaction with the host plant. Our main goal is to combine the recent advances in protistology, microbiology and general ecology, fostering exchanges between different disciplines that often address the same topic but have long been disjointed. We synthesize the current knowledge on the roles of protists within the rhizosphere microbiome and propose new roads for future research. We demonstrate that protists pull the strings of several processes in the rhizosphere and steer community structure, function and evolution. Thanks to their versatility and central role as a major selective pressure on

rhizosphere microbes, we argue that protists function as "puppet masters" steering beneficial plant-microbe interactions that might be exploited to manipulate the rhizosphere microbiome functionality.

Box.1 What are protists?
Protists represent a paraphyletic, extremely diverse group of unicellular eukaryotes, encompassing by far the majority of eukaryotic phylogenetic diversity (Adl et al., 2012; Burki, 2014). Soil protists can be found in all eukaryotic supergroups: Amoebozoa, Obazoa, Archaeplastida, SAR and Excavata. Protists come in a wide range of morphological shapes and locomotion modes. Soil protistology has formerly focused entirely on heterotrophic taxa (previously 'protozoa') and have been grouped based on coarse morphological features into naked amoebae, testate amoebae, flagellates and ciliates. Yet, in-between forms are the rule and phylogenetic work has shown that all these groups with the exception of ciliates are paraphyletic, rendering ecological interpretations based on this morphological classification less meaningful. Many protists have a complex life cycle, most consisting of an active and a resting stage, mostly in the form of a cyst, but some intermediate forms are also common (Geisen et al., 2018). The inactive and persistent cyst stage is formed in response to unfavourable environmental conditions. These protist cysts form an important fraction of the soil microbial seed bank that can readily turn active in response more favourable conditions such as the increased moisture along with presence of suitable prey (Clarholm, 1981).

Protists are abundant members of the soil microbiome, typically present at densities of 10^4-10^8 per gram of soil (Adl & Coleman, 2005). Protists, like their bacterial and fungal prey, are especially enriched in the rhizosphere, the region directly surrounding, and influenced by, plant roots. The protist community composition is shaped by a number of factors in addition to the presence and composition of prey, including a range of biotic and abiotic factors such as plant species and soil properties such as pH and humidity (Bates et al., 2013; Dupont et al., 2016; Leff et al., 2018). The functional role of protists in soils is diversely linked to nutrient cycling and includes phagotrophy [consumption of other (micro)organisms such as bacteria], phototrophy, symbiosis, saprotrophy or a mix of these strategies (Geisen et al., 2018)

Predator-prey interactions in the rhizosphere microbiome

Protists interact with their preys in a variety of ways, including trophic interactions and chemical communication (Figure 2A). These different interactions can in turn result in important changes in microbiome structure and activity. In this section, we highlight different types of interactions between protists, bacteria and fungi. We will address the consequences for microbiome functioning and plant growth in the next section.

First, consumption of microorganisms by protists increase nutrient turnover. A reason for this is that protists have a higher C:N ratio than bacteria or fungi they are

consuming. They will therefore excrete the excess N, making it available for other microorganisms or the host plant (Clarholm, 1985; Cleveland & Liptzin, 2007). Further, by consuming dormant cells, protists release limiting micronutrients (that would otherwise remain locked in the microbial seed bank) that do not contribute to microbiome function (Bonkowski, 2004). This increased nutrient turnover can happen regardless of the traits from protists and their prey. In addition, microbial consumption may have a range trait-dependent effects on community structure and function when predation correlates with specific prey traits.

Most protists show strong prey selection patterns based on species-specific sets of traits. For instance, the size ratio between predator and prey restricts which preys can be ingested. Protist feeding mode and motility is also important. Amoeba can for example reach for tiny pores in the soil matrix thanks to their extremely plastic body shape and even digest biofilms thanks to the production of extracellular enzymes. Filtrate-feeding ciliates can eat single bacteria or microcolonies. They show a comparatively low selectivity but can have a high per-capita consumption rate (Clarholm et al., 2007). Different feeding types are associated with a given level of specialization. For instance, mycophagous Grossglockneriidae, a group of ciliates, have a specialized needle-like feeding structures only permitting feeding on fungi (Foissner, 1999a). Bacterivorous protists show refined patterns of prey selection and can discriminate bacteria on the base of their size (Baltar et al., 2016), surface properties (Wootton et al., 2007) or the presence of diffusible secondary metabolites (Jousset et al., 2006). They are further attracted or repelled by volatile compounds, such as terpenes, secreted by microorganisms (Schulz-Bohm et al., 2017). Protists respond in a species-specific manner to these volatiles (Schulz-Bohm et al., 2017).

Bacteria have evolved a range of defense mechanisms to prevent detection, ingestion or digestion by protists. These mechanisms can be either constitutive or be triggered by the presence of protists and this variation in palatability is a fundamental driver of selective feeding by protists. Bacteria sense chemical cues from protists and specifically respond to predation pressure by adaptations such as changes in cell size and shape (Pfandl et al., 2004), increased motility (Matz & Jürgens, 2005), surface properties and secretion of defensive secondary metabolites (Jousset et al., 2006). Secondary metabolites known to confer predation resistance against protists include: pigments like violacein (Matz et al., 2004), polyketide antibiotics, hydrogen cyanide, the *exoprotease AprA* (Jousset et al., 2006), cyclic lipopeptides (Mazzola et al., 2009; Götze et al., 2017). Several of the bacterial responses to predation are expressed on the population level, such as formation of biofilms or filaments which are less accessible for predators than single cells (Corno & Jürgens, 2006; Queck

et al., 2006). Likewise, several of the secondary metabolites conferring predator resistance are regulated by quorum-sensing, such as the pigment violacein produced by *Chromobacterium violaceum* (Matz et al., 2004). In *Pseudomonas* bacteria coordinate production of several antibiotics with anti-predator activity is induced in a density-dependent manner (Haas & Défago, 2005; Jousset et al., 2006).

Impact of protists on the microbiome

From biomass to function

Protist-prey interactions lead to a range of effects on several characteristics of the microbiome (Figure 2B). Predation typically decreases total bacterial biomass (Ekelund et al., 2009; Rosenberg et al., 2009). By increasing nutrient turnover, protist predation stimulates microbial activity; this is evidenced by increased microbial respiration and nutrient mineralization (Zahn et al., 2016; Hünninghaus et al., 2017). Simultaneously, selective feeding shifts rhizosphere microbiome composition and gives a selective advantage to microbial groups that can avoid predation (Jousset et al., 2008; Rosenberg et al., 2009). For instance, predation may promote Gram-positive bacteria, which thanks to their thick cell wall are harder to digest (Rønn et al., 2002; Murase et al., 2006). Moreover, protist predation can help maintain diversity within bacterial communities by feeding on the dominant taxa and thereby increase the relative abundance of formerly rare bacteria, leading to increased bacterial evenness and complementarity (Bell et al., 2010; Saleem et al., 2012).

Protist selective predation can further affect functional trait composition of the microbiome. Predation results in an increased abundance of organisms harbouring traits conferring resistance to protists (Jousset et al., 2009). Furthermore, predation can stimulate expression of several traits linked to defense (Jousset & Bonkowski, 2010). These anti-predator traits can be highly relevant for the delivery of microbiome function relevant for plant health. For instance, several secondary metabolites conferring resistance against consumption by protists are also involved in the suppression of plant pathogens and immunity (Jousset et al., 2006; Song et al., 2015). We later discuss in detail how these different effects on microbiome taxonomic and functional composition impact plant performance.

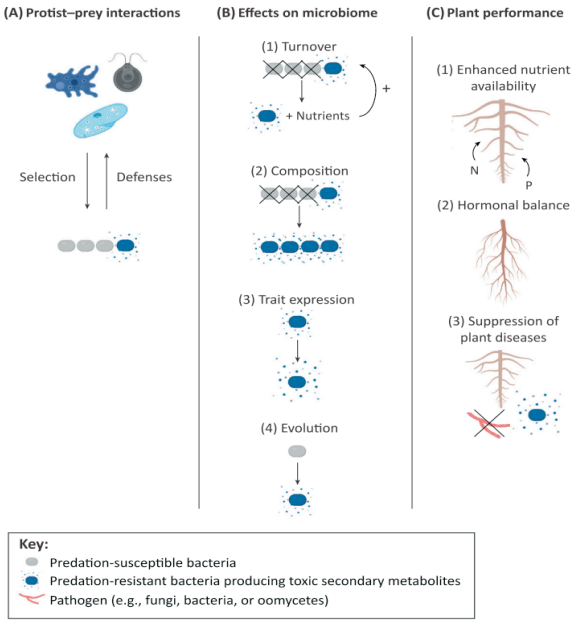

Figure.2 Protist-prey interactions drive rhizosphere microbiome function and plant performance

Protist-prey interactions, specifically the interplay between protist selective feeding and prey defence traits (A), affect several characteristics of the microbiome (B). Here we use production of secondary metabolites as an example of a bacterial defence trait. Protist consumption increases species turnover and subsequent nutrient release that fuels further microbial activity (1). Moreover, protist feeding is selective and shifts the taxonomic composition of the microbiome as well as the frequency of functional traits (2). Bacteria respond to protist consumption by increased expression of defence traits (3). Finally, protist consumption acts as an evolutionary force on microbial populations (4). These effects on and responses of the microbiome result in a range of changes in plant physiology, with various implications for plant performance (C). Importantly, protist consumption unlocks nutrients bound in rhizosphere microbes, which can be taken up by plant roots and stimulate growth (1). Moreover, protists can increase lateral root branching by promoting auxin-producing microbes (2). A last example is the production of certain antibiotics, which both confer resistance against protists and inhibit plant pathogens and may thus protect plants against diseases (3).

Protist species-specific effects

Recent studies have revealed unexpected diversity of soil protists (Dupont et al., 2016; Mahé et al., 2017). This diversity is also reflected in diverse interactions with their prey. Protists with different feeding modes have distinct effects on biofilm

morphology (Weitere et al., 2005; Böhme et al., 2009). For instance, *Acanthamoeba polyphaga*, which requires attachment to a firm surface to be able to feed, was most efficient in reducing the biomass of biofilms. In contrast, the flagellate *Bodo saltans* stimulated microcolony formation in biofilms, which conferred resistance against this protist (Weitere et al., 2005). Moreover, protists representing different feeding modes and motility types have species-specific effects on bacterial community structure and diversity (Saleem et al., 2012; Hünninghaus et al., 2017).

In addition to morphology, the phylogenetic affiliation of protists is an important predictor of their effect on microbial communities. Such phylogenetic patterns can emerge at different scales. For instance, when comparing the effects of nine Cercozoa species on model preys communities, protist phylogenetic distance could not explain variation in bacterial community structure (Glücksman et al., 2010). In another example, including protist species spanning several eukaryotic supergroups, broad–scale taxonomic affiliation could be correlated with sensitivity to bacterial defense compounds, a crucial characteristics linked to interactions with their prey (Pedersen et al., 2011). Still, most studies investigating the effects of protists on microbial community structure have been conducted with only one or very few model species. We advocate that further studies should include more protist species to unravel the links between protist taxonomy and traits and their impact on microbiome structure and function.

Microbiome evolution

Beyond ecological interactions, protists can also drive the evolutionary dynamics of the rhizosphere microbiome. Predation by protists creates a selection pressure that impacts the evolution of microbial traits that are relevant for interactions between microorganisms and with the host plant (Hiltunen & Becks, 2014). Predation can trigger diversification (Meyer & Kassen, 2007), thereby increasing the phenotypic pool available to the plant. Further, they may also guide the evolution of specific traits. A range of bacterial traits have likely evolved at least partially as an adaptation to protists, including size, surface properties or the secretion of defensive secondary metabolites (Pfandl et al., 2004; Jousset et al., 2006; Wootton et al., 2007). Alterations in these traits may impact bacterial growth, interactions with competitors and ultimately the host plant. For instance, surface molecules such as lipopolysaccharides play a central role in adhesion to roots and recognition by the plant immune system. Their alteration to avoid recognition by protists may thus change the way they interact with the plant (Wildschutte et al., 2004; Lugtenberg & Kamilova, 2009).

Protists also affect intraspecific interactions and can for instance enforce cooperation by consuming defectors that use plant- derived resources but do not provide plant-beneficial functions in return (Jousset et al., 2009). Therefore, they ensure the evolutionary stability of social behaviours required for plant growth and health, such as secondary metabolite production (Jousset et al., 2009; Friman et al., 2013). Evolution of protist resistance may also have impacts in a multitrophic context, with protists selecting for instance for bacteria susceptible to bacteriophages (Friman & Buckling, 2013).

Evolution of microorganisms within protists may also affect microbiome function. Protists carry several intracellular bacteria, ranging from pathogens to symbionts (Gast et al., 2009). Some of these bacteria are also opportunistic pathogens of humans and plants, and it has been proposed that virulence traits such as secretion systems and elicitors have evolved originally as an adaptation to survive within vacuoles (Erken et al., 2013). Protists may therefore function as a hotspot of pathogen evolution for both human - and plant-pathogenic bacteria (Brüssow, 2007). Vacuoles are also a hotspot for horizontal gene transfer between microorganisms (Schlimme et al., 1997), further exacerbated as predation by protists maintains conjunctive plasmids, another central mechanism of microbial evolution (Cairns et al., 2016). The different eco-evolutionary feedbacks of bacteria and protists still need to be investigated in more detail in the rhizosphere and integrated into the community level in order to understand protist effects on rhizosphere microbiome evolutionary dynamics.

Impact of protists on microbiome functionality and plant performance

The rhizosphere microbiome is increasingly recognized as an essential component shaping plant physiology, nutrition and health (Friesen et al., 2011; Berendsen et al., 2012). In the previous sections, we highlighted how protists can affect the functional and taxonomic composition of the rhizosphere microbiome (Figure 2B). Here, we will show that many of these changes can have concrete impacts on plant performance (Figure 2C).

Plant nutrition

Soil microorganisms play essential roles in plant nutrition by fixing nitrogen, mineralizing soil organic matter or solubilizing organically-bound nutrients that would otherwise remain inaccessible to the plant (Falkowski et al., 2008). Protists impact these activities in several ways. One central hypothesis, the **microbial loop**, postulates that most biomass turnover occurs at a microscopic level. Protist

consumption releases nutrients from bacterial biomass and make them available to plants (Clarholm, 1985), resulting in increased plant nutrition and growth (Krome et al., 2009). While much attention has been directed to the role of protists in nitrogen cycling, recent work has emphasized their importance for phosphorous mineralization in soil (Trap et al., 2015). The effects of protists are not restricted to plant biomass, but can also influence nutrition and biomass allocation, increasing for instance resource allocation to reproductive organs (Bonkowski et al., 2001a; Krome et al., 2009). The effect of protists on plant nutrition is more pronounced in presence of arbuscular mycorrhizal fungi, which on their own have a limited ability to produce the enzymes required for soil organic matter breakdown. Protists increase nutrient mineralization by hyphae-associated microorganisms, which can then be taken up by the mycorrhiza and transferred to the host plant (Koller et al., 2013b).

Protists may also affect nutrition by selecting for or against specific bacterial groups that are important for nutrient cycling and plant nutrition, such as nitrifiers (Rosenberg et al., 2009) or phosphate solubilizers (Gómez et al., 2010). Protist predation can moreover induce bacterial traits important for nutrient cycling, such as siderophores, which chelate iron and thus modulate iron availability in soil (Levrat et al., 1992). Finally, some protists such as testate amoebae are involved in the cycling of silica, an element required for plant stress tolerance (Wilkinson & Mitchell, 2010; Creevy et al., 2016) (Table 1).

Plant hormonal balance

Plants use various hormones to regulate their life history, including flowering time, root morphology and stress resistance (Davies, 2010). Each of these traits is linked with specific costs and benefits and a tight regulation is necessary to match the plant's phenotype to the specific environmental conditions it is facing. Root-associated microorganisms can influence plant hormonal balance in several ways. Several rhizosphere microorganisms can produce or degrade hormones such as ethylene, auxin, cytokinin or gibberellin, with broad repercussions on plant phenotype and fitness (Dodd et al., 2010; Ravanbakhsh et al., 2018). Protists can impact the effect of microorganisms on the plant hormonal balance by altering both the abundance and activity of the involved microorganisms: Protists promote for instance auxin-producing bacteria (Bonkowski & Brandt, 2002), thereby stimulating lateral root branching (Krome et al., 2010). Protists also increase cytokinin concentrations in plants, possibly as a result of the increased nitrate concentration that occurs when excess nitrogen is secreted (Krome et al., 2010). Finally, protists could alter the plant's

hormonal balance indirectly by affecting microbiome functions, for instance by increasing the production of bacterial metabolites such as 2,4-diacetylphloroglucinol (DAPG) (Jousset & Bonkowski, 2010), an antimicrobial compound that also interferes with auxin signaling (Brazelton et al., 2008). The strong effect of protists on the plant metabolome can most likely be linked to these multiple hormonal changes (Kuppardt et al., 2018).

Table 1 Effect of protists on rhizosphere functions.

Function	Impact of protists	Reference
Nutrient turnover	Increased plant carbon uptake	Bonkowski et al., 2000, 2001a; Krome et al., 2009
	Increased nitrogen release	Clarholm, 1985; Bonkowski et al., 2000
	Increased nitrogen mineralization	Ekelund et al., 2009; Koller et al., 2013b
	Increased plant nitrogen uptake	Bonkowski et al., 2000; Somasundaram et al., 2008; Ekelund et al., 2009; Krome et al., 2009; Koller et al., 2013a,c
	Increased plant phosphorus uptake	Bonkowski et al., 2001b
	Increased plant magnesium and calcium	Herdler et al., 2007
	Increased silica mineralization	Wilkinson & Mitchell, 2010; Creevy et al., 2016
Plant hormones	Increased plant free auxin	Krome et al., 2010
	Increased plant cytokinin levels	Krome et al., 2010
Disease suppression	Higher abundance of pathogen-suppressing bacteria	Jousset & Bonkowski, 2010

Plant health

Plants are confronted with a broad range of pathogens. Plant-associated microbes are unanimously recognized to be a central determinant of plant health by inhibiting pathogens and stimulating plant immunity, with a power equaling the defense traits encoded in the plant genome (Berendsen et al., 2012). However, not all microbiomes suppress disease equally, with impacts ranging from full disease suppression to disease promotion (Pieterse et al., 2016). Further, even if great strides have recently been made in correlating patterns of community structure to the presence of specific taxa or particular functional genes to disease suppression (Mendes et al., 2011; Wei et al., 2015), the mechanisms underlying the presence or absence of these microbiome characteristics remain elusive. As a result, disease suppression by microbial communities is still unpredictable and hard to manage as we only partially understand why a plant-protective microbiome configuration can emerge

and be preserved. Predation by protists may be a missing link to understand soil suppressiveness.

There are several potential means by which protists can influence the ability of the microbiome to suppress diseases. Predation by protists can select for bacteria producing compounds linked to disease suppression, such as cyclic lipopeptides, polyketides, alkaloids or hydrogen cyanide (Jousset et al., 2006; Mazzola et al., 2009; Klapper et al., 2016) and stimulate the expression of these traits (Jousset & Bonkowski, 2010; Song et al., 2015). The overlap between the suite of bacterial traits linked with predation resistance and pathogen suppression can be used as a tool to promote specific functions throughout the microbiome. It may also provide a stepping stone to enhance the performance of introduced biocontrol bacteria, which are implemented as a sustainable alternative to pesticides but often fail to establish under field conditions. The establishment of such **biocontrol agents** may be facilitated by protists. Selective feeding by protists may favor introduced, secondary metabolite-producing biocontrol *Pseudomonas protegens* (Jousset et al., 2008) by preferentially consuming less defended resident species that compete with the biocontrol agents. In addition, protists may even affect plant immunity: Some secondary metabolites overproduced by bacteria in their defense response to predators (Jousset et al., 2010) can prime plant immunity via the jasmonic acid pathway (Iavicoli et al., 2003), thereby enhancing resistance to a range of belowground and aboveground pathogens.

Protists may also contribute to disease suppression by directly consuming pathogens, reducing their survival in soil and potentially protecting plants (Chakraborty et al., 1983). Mycophagous protist groups such as vampyrellid amoebae and grossglockneriid ciliates are more widespread and abundant in different soils than previously thought (Geisen et al., 2015b). Moreover, several species previously considered bacterivorous were recently discovered to feed on a range of fungal spores and yeast cells, including plant pathogenic fungi (Geisen et al., 2015b). This widespread mycophagy suggests that mycophagous protists constitute a reservoir of biocontrol agents that could directly consume fungal pathogens. In addition to direct consumption, protists secrete several extracellular compounds, some of which show bactericidal effects that may prevent the growth of bacterial pathogens (Long et al., 2018). In conclusion, evidence from experiments in controlled environments suggest that protists can influence the disease suppressive ability of microbial communities directly or via changes in the microbiome composition. The next step is to investigate whether and how these promising findings can be translated into applications to control plant diseases.

Protists as a microbiome optimisation tool for sustainable agriculture

Protists hold promise for future strategies to enhance microbiome function and contribute to sustainable, high yield agricultural practices. One challenge of applying beneficial microbes is achieving stable formulations, which is easier for organisms forming resistant structures, such as spores (Schisler et al., 2004). Similarly, the ability of protists to form cysts can facilitate efficient large-scale production efforts for industrial applications such as seed coatings or soil amendments. The ability of protists to enhance nutrient cycling and promote plant growth make them interesting as **biostimulants**. Protists may for instance be used to speed up the mineralization of organic fertilizer and increase the survival and activity of beneficial microbes (Jousset et al., 2008; Weidner et al., 2017). The first protist-based biostimulants and plant protection products have already hit the market [Ecostyle (https://www.ecostyle.nl/groensector/protoplusr)] or are under development [amoéba (http://www.amoeba-biocide.com/en/news/w-magna-90-efficacy-mildew)]. We suggest that a targeted approach focusing on determining which protist traits are linked to enhanced plant performance may prove more fruitful in identifying beneficial protist taxa than the traditional screening of a large number of species (Figure 3). We foresee that, thanks to the overlap between predator defense and pathogen suppression, protists may be a promising soil health improvement technology, alone or in combination with introduced biocontrol microorganisms. Further, thanks to their key function as a regulator of the rhizosphere microbiome, protists may be an excellent target for soil enhancement practices. Protists readily respond to agricultural practices such as soil tillage (Zhang et al., 2015), fertilization (Lentendu et al., 2014) or pesticide application (Imparato et al., 2016) as well as sown plant species (Turner et al., 2013; Leff et al., 2018). Protists may thus form an important leverage between management practices and microbiome, helping managing microbiome function in a more targeted and efficient way. For instance, addition of organic fertilizer was shown to increase the relative abundance of heterotrophic protist taxa at the expense of parasites and pathogens (Xiong et al., 2018). Protists could also be the target of conservation biocontrol strategies where management practices, for example different cultivars, are applied to promote indigenous taxa with biocontrol activity. Future research should focus on identifying management practices that increase the abundance and positive effects of specific protists that in turn foster desired traits in the rhizosphere microbiome.

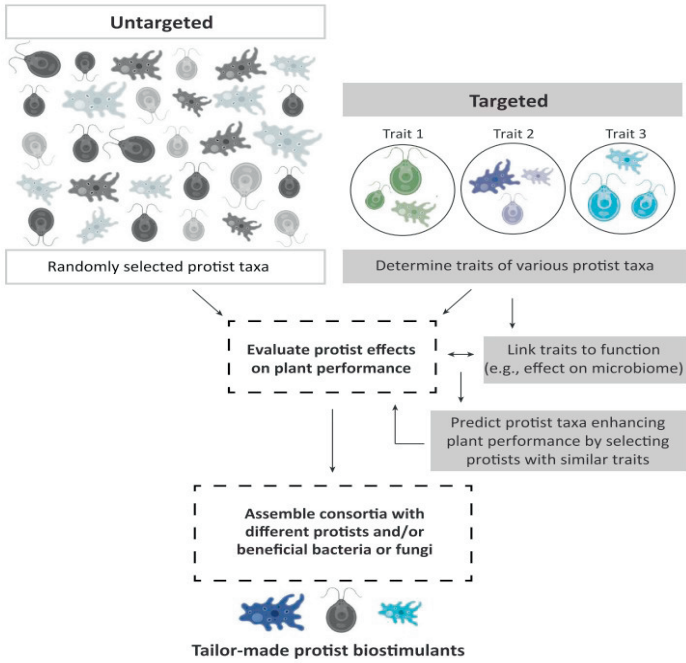

Figure.3 Strategies to identify beneficial protist taxa

In order to identify protist taxa suitable as biostimulants a targeted approach based on, for example, traits can be applied. In such an approach, protist traits are characterized and linked to functional information. Based on that information, a guided taxon choice can be made for future tests based on traits associated with beneficial effects on plants. In comparison, untargeted approaches randomly test the effect of a wide variety of protists on plant performance. We suggest the targeted approach to be more efficient in finding taxa with the desired effects over time, as it reduces the number of taxa that need to be screened and as it guides targeted screening for additional cultures. The aim of both approaches is to identify beneficial protist taxa that can be applied alone or in combination to enhance plant performance. Protists may also be combined with other beneficial microbes in order to reach synergistic effects.

Concluding Remarks and Future Perspectives

In this review, we have summarized recent developments on soil protists, pointing to their role as long-overlooked "puppet masters" of the rhizosphere, with broad implications for microbiome function and services to plants. Protists may be a missing link that helps us predict and enhance microbiome function. We advocate that future efforts targeting the rhizosphere microbiome should include free-living protists as top-down regulators of microbiome composition, balancing and completing the

current prevailing research focus on bottom-up drivers such as root exudates and plant genotype (see Outstanding Questions). Such a multitrophic approach could combine species distribution patterns retrieved from metabarcoding surveys with food-web modelling, providing testable predictions on the impact of given protists on microbiome structure (Wang & Brose, 2018). By better deciphering the rules underlying soil microbiome assembly and function, this approach will allow for designing improved strategies harnessing the beneficial functions of the rhizosphere microbiome. The time has come for protists to get out of their scientific niche and become the next biotechnological tool to engineer microbiomes to promote the functions that are needed to guarantee sustainable and resilient food production.

Glossary

Biostimulants: (microbial) organisms that promote plant performance such as by serving as biofertilizers

Biocontrol agent: organism that can be applied to reduce pests or diseases

Bottom-up control: the population size is determined by the availability of nutrients for growth or the productivity of primary producers.

Microbiome: the entity of interacting microbial taxa including bacteria, archaea, fungi, viruses, protists and other microbial eukaryotes.

Microbial loop: nutrient release through consumption of bacteria or fungi by higher trophic levels such as protists.

Protists: paraphyletic group comprising all eukaryotes with the exception of plants, fungi and animals. In the soil, encompasses photoautotrophic (algae), heterotrophic taxa (protozoa) and mixotrophs.

Rhizosphere: the zone in soils directly influenced by the presence of roots.

Top-down control: mortality due to consumption by organisms at higher trophic levels determine the size of the population.

Acknowledgements

ZG was supported by Chinese Scholarship Council (CSC), IK by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), and SG by a NWO-VENI grant from the Netherlands Organisation for Scientific Research (016.Veni.181.078).

5

Chapter 6

General discussion and future perspectives

Zhilei Gao

Protists are widely distributed and important members of the soil community. Soil-borne protist diversity and the importance of specific protist taxa are increasingly being recognized (Bates et al., 2013; Geisen et al., 2015d; Mahé et al., 2017). However, our understanding of protist ecology in soils remains highly rudimentary. This conspicuous knowledge on functions of protists is scare, because most studies to date have only focused on one or a few closely related protists taxa. This thesis seeks to fill this gap by considering a broad range of protist taxa in order to understand predator and prey interactions.

Within this thesis, I focused on 20 protist species, representing a broad phylogenetic and morphological diversity (**Chapter 1, 2, 3**). The central question of this thesis is whether the effects of protist predation can be predicted. I proposed to use protist traits as a means to address this question. I first investigated the phylogenetic affiliations of the protist species used in this thesis (**Chapter 2**). I then went on to characterize the growth rates and morphological traits within my protist strain collection (**Chapter 3**) and examined how these traits are related to the impacts of protist predation on soil-borne bacterial communities. Results showed that the effects of protists predation could be related to differences in protist cell volume. To understand the functionality of protists, I tested how predation affects microbial cooperation in soil and found that predation can favor cooperation, leading to lower defector density; an effect that was dependent on nutrient availability (**Chapter 4**). In **Chapter 5**, a range of approaches is reviewed to provide new perspectives and approaches to understand and utilize protist activities, especially as related to improving plant growth and health.

Integrating morphological and phylogenetic diversity

Determinations of cell morphology lie at the heart of traditional approaches of examining protist diversity, with particular attention to for instance the characterization of easily isolated ciliates with unique morphological features (Foissner, 1999b). However, other morphological groups, such as flagellates and naked amoeba, have remained highly understudied, partially due to their smaller sizes and the fact that they often have indistinguishable morphotypes under the light microscope (Smirnov & Brown, 2004). The development of molecular tools, such as 18S rRNA gene sequencing together with markers offering finer level phylogenetic resolution, such as internal transcribed spacer (ITS) regions, has enabled cultivation-independent interrogation of soil protists, revealing high levels of diversity and allowing us to distinguish between morphologically similar protist species. In **Chapter 2**, I isolated several new Heterolobosean amoeba from soil. I further combined morphological characterization and molecular tools to identify these species and proposed a new

species within the genus *Vahlkampfia*. This first soil *Vahlkampfia* species highlights the need for additional efforts in the future to cultivate previously unknown soil protists. The combination of morphological and molecular identification approaches can clearly contribute to advance our knowledge of protist diversity in soil (**Chapter 2**).

Thanks to improved high-throughput sequencing techniques, such as DNA barcoding, we are starting to recognize the enormous diversity of soil protists (Bates et al., 2013, Geisen et al., 2015d; Mahé et al., 2017). Despite this quantum leap in available sequence data, we still often lack sufficient information to interpret such data from a functional perspective. Although advances have been made in the assignment of protist marker sequences to functional groups (Geisen et al., 2016; Xiong et al., 2018), there is still an urgent need for additional, well-characterized reference strains, especially from taxonomic groups currently underrepresented or absent from current culture collections. As demonstrated in **Chapter 2**, such efforts not only inform the functional interpretation of sequence data, they also can provide much-needed insight into protist ecology.

Appreciating the ecological roles of soil protists

Protists are far more than bacterivores

Throughout this thesis, I have principally focused on the ability of protists to feed on bacterial prey (**Chapter 3, 4**). This attention to the bacterivorous activities of protists stems mostly from the fact that protists are commonly regarded as important drivers of bacterial mortality in soils (de Ruiter et al., 1995; Trap et al., 2015). However, it is important to recognize that soil protists can occupy a broad range of positions within the soil food-web. Recent studies have highlighted diverse interactions between protists and other soil organisms, suggesting that protists also play important roles in the consumption of archaea (Seppey et al., 2017), fungi (Ekelund, 1998; Adl & Gupta, 2006; Geisen, 2016b), nematodes (Geisen et al., 2015c) and even other protists (Jassey et al., 2012). In addition, parasitic and phototrophic protists should also not be overlooked (Zancan et al., 2006; Geisen et al., 2016; Mahé et al., 2017). We are still far away from understanding the full extent of tropic interactions involving protists, with most studies to date focusing on very specific interactions between a single protist and one particular target organism of interest. As suggested by this thesis (**Chapter 3**), studies examining a large collection of protists and involving complex microbial communities will help to advance our understanding of the ecological niches occupied by protists and help better define their roles within the

soil food web (Hunt et al., 1987; de Ruiter et al., 1995). It is expected that emerging isotope tracer methods, combined with metagenomics, will help such studies advance our knowledge of soil protist ecology.

Eco-evolution of protists and prey interactions

Interactions between predators and their prey represent classic examples for the study of coevolution. From the protist's perspective, perception of, for instance, volatile compounds produced by bacteria may result in differential feeding habits (Schulz-Bohm et al., 2017). From the bacterial prey's perspective, multiple pre-ingestion strategies impeding predation have been developed, such as changes in size, colonies formation, motility and production of effective toxic compounds (Matz & Kjelleberg, 2005; Jousset et al., 2006; Brüssow, 2007). In addition to pose a threat, the presence of protists may also offer a suitable habitat, with some bacteria evolving endosymbiotic relationships with specific protist species (Horn & Wagner, 2004).

It has been previously suggested that protists can increase the stability of microbial cooperation by selectively feeding on bacterial defectors (Jousset et al., 2009). The results presented in **Chapter 4** demonstrated that bacterial defectors that fail to produce certain secondary metabolites can be driven to extinction. As a result, bacterial cooperators that do produce these secondary metabolites benefited by the presence of the protist predator, with this positive effect of predation being strongest under conditions of intermediate and high resource availabilities (**Chapter 4**). These results are also in line with previous studies that reported a link between the positive effects of predation on cooperation depends and resource availability (Friman et al., 2008). It should be noted that I only tested such effects of predation for two protists strains out of a collection of 20 protist species. In order to demonstrate the general impacts of protist predation on bacterial cooperation, it will be necessary to examine such effects across a broader spectrum of protist species.

Moving toward a predictive framework to understand predator and prey interactions

Predator and prey interactions can result in shifts of microbial assembly and functions (Rønn et al., 2002; Rosenberg et al, 2009). Such shifts in microbial assembly may be due to differential abilities of potential prey bacteria to adapt to the presence of the protist predator. However, we are still lacking a more comprehensive framework to describe such effects, and this knowledge gap is principally caused by the narrow range of protist species for which selective feeding has been studied. To fill in this

knowledge gap, I investigated 20 protist species covering a broad morphological and phylogenetic diversity (**Chapter 1, 3**). One of the central questions in this thesis was whether protist predation can be predicted, and, if so, which protists characteristics are most informative to explain the impacts of protist predation on bacterial prey communities. I proposed that protists traits may help to explain such predation effects.

Protists have previously been shown to have some level of selective feeding on bacterial prey, and such selective feeding differs between protists species. In general, more phylogenetically related organisms might share a higher level of functionality. I therefore first examined if protist phylogeny could explain their predation effects. Previous results suggested that phylogeny may to some extent determine protist impacts on bacterial prey, depending on the phylogenetic level examined (Pedersen et al., 2011), and such patterns may be related to the production of bacterial defense compounds. The results presented in **Chapter 3**, show that protist phylogeny could not be linked to their predation effects on bacterial communities. This result was in agreements with previous studies that have shown that even closely related protist species can have highly contrasting effects on bacterial communities (Glücksman et al., 2010).

I further investigated if protist morphological traits could explain their predation impacts. To this end, I characterized a range of protist traits including growth rate, size, morphology and volume. I found that protist volume was significantly linked to their predation impacts. This result is in line with a prior study that examined closely related protist species (Glücksman et al., 2010). The work presented in this thesis demonstrated that protist volume can be linked to their predation effects even across a broad range of phylogenetically distinct protist taxa. Given that our protist collection had a high level of morphological diversity (**Chapter 1**), it was critical that cell volume calculations were not only based upon cell size, but that they also took geometric morphotype into consideration (**Chapter 3**). Future studies seeking to detangle predation and prey interactions as related to protist cell volume should therefore consider both cell size and morphotype.

In this thesis, mostly morphological traits of protists were investigated. However, protist predation might also be highly dependent on other traits, such as motility, nutrient requirements and habitat preferences (Dumack et al., 2019). It is therefore important to expand trait-based studies of protists to include other characteristics that are related to feeding impacts of soil-borne protists. As mentioned previously, protist predation on bacteria involves the interaction and coevolution of both interacting

parties. Bacterial traits clearly affect how they respond to protist predation. With this in mind, it could be highly informative to adopt an approach that includes both protist and bacterial traits. This would require a collection of protists with known traits and a collection of bacteria with known traits.

It should be noted that the soil bacteria community examined in **Chapter 3** only targeted a single soil type. Differences in soil bacteria community are affected by a range of factors that differ between different soils, including differences in soil texture, organic matter content, plant communities, land-use, pH and moisture (Kowalchuk et al., 2002; Fierer & Jackson, 2006; Singh et al., 2009; Lauber et al., 2013). It is therefore important that predator/prey interactions between protists and bacterial communities could be examined across a range of different soil types to determine if, for instance, protist cell volume is a general explanatory factor of protist predation impacts.

Moving toward to applications of soil protists

Plants recruit microbes via root exudates, those microbes recruited to the rhizosphere in turn help to support plant growth and resistance to pathogen attack (Mendes et al., 2011; Berendsen et al., 2012). In order to improve soil fertility, bacterial strains with desired traits have often been utilized to improve rhizosphere functionality. However, such introductions often have only a limited impact on plant performance, mainly because a single introduced species will only reach a limited population size in the highly diverse rhizosphere microbiome. In contrast, protists may despite of a small total abundance have cascading effect on the whole bacterial community. They therefore may offer a powerful tool to steer soil microbiome functionality at the service of plant performance (**Chapter 5**).

Trait-based strategies to identify beneficial protist taxa

This thesis proposed a framework in which protist traits are used to identify plant-beneficial protist taxa (**Chapter 5**). Morphological traits and taxonomic affiliation of 20 protists species were characterized and examined (**Chapter 2, 3**), showing that predation effects on the prey bacteria community could be linked to protist cell volume. Although it was shown that cell volume can help explain changes in bacteria communities, we still lack knowledge of how these shifts in bacterial community structure impact plant performance. Follow-up steps could involve evaluation of plant performance after transplantation of protist-affected bacteria communities in the presence and absence of specific plant pathogens. This would facilitate the

identification of protist taxa and traits associated with plant growth promotion and defense. Such a framework could also be expanded to include plant traits as well, such as shoot growth or root architecture to be linked with specific protist species or traits. Thus, such a framework could potentially catalog heterotrophic protists into broadly defined groups that share similar functional capabilities and ecological strategies: for instance, 'plant shoot promoting' taxa that increase plant shoot biomass, 'nutrition facilitating' taxa that can increase plant nutrient or 'defense promoting' taxa that can improve plant defense against pathogens. Note that these specific functional taxa are also related to the plant genotype, with cultivar-dependent effects (Fig.1). By adopting this framework, specific protists may be linked to the promotion of specific plant traits to allow for a tailor-made approach to improve plant performance.

Plant health

There is a growing body of evidence suggesting that protists are essential to maintain disease suppression. Predation by protists can select for bacteria producing compounds linked to disease suppression, such as cyclic lipopeptides and 2,4-DAPG (Jousset et al., 2006; Song et al., 2015). I also found that large volume protist could increase bacteria that putatively possess anti-predator strategies that overlap with functions involved in disease suppression (**Chapter 3**). It should, however, be stressed that this conclusion remains highly speculate due to its reliance on literature related to a limited range of isolated bacterial strains. Further efforts to verify this intriguing link are clearly necessary. For instance, large protists could be applied in the rhizosphere with subsequent examination of plant performance and quantification of the potential plant beneficial bacteria or bacterial genes mentioned in **Chapter 3** using qPCR.

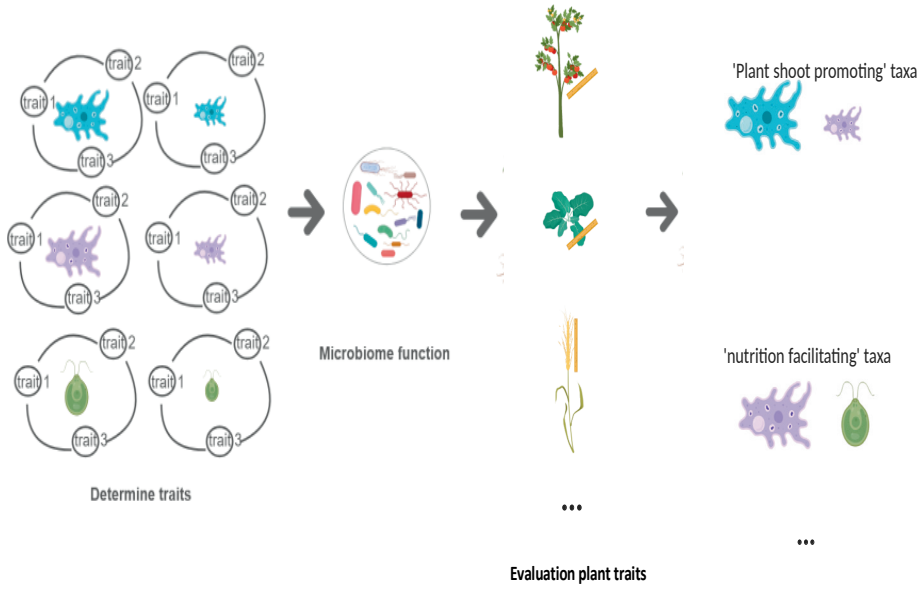

Figure 1. Cartoon of a targeted approach to improve plant performance by identifying plant-beneficial protist taxa.

Moreover, predation has been shown to stimulate beneficial bacterial activities *in vitro* by upregulating the production of secondary metabolites, siderophores and volatile compounds, which are also responsible for the disease suppressive ability of many biocontrol bacteria (Andersen & Winding, 2004; Jousset & Bonkowski, 2010; Weidner et al., 2017). The overlap between the suite of bacterial traits for antipredator and disease suppression abilities can be used to support the establishment of introduced biocontrol bacteria in soil systems through protist selective feeding on non-toxic bacteria, thereby increasing the abundance of toxic bacteria (Jousset et al., 2009). However, this negative effect of predation on non-toxic bacteria is also dependent on resource availability (**Chapter 4**). Taken together, protist predation therefore confers a selective advantage to bacteria that also carry genes responsible for biocontrol, indicating that they can improve the selective value of functional genes otherwise present at a too low density to efficiently protect the plant (Müller et al., 2013).

Protists may also be relevant for the plant immune response to pathogens. The selection of bacteria producing bioactive compounds may further serve to stimulate plant immunity. Protists were shown to increase *Pseudomonas* spp. abundance

(**Chapter 3, 4**) and some antibiotics of which can both confer resistance to predation and induce jasmonic acid-mediated plant immunity (Iavicoli et al., 2003). These links to plant immunity need to be tested in more detail and across different level of resource availabilities (**Chapter 4**).

Protists may finally contribute to disease suppression by directly consuming pathogens, reducing their survival in soil and potentially protecting plants (Chakraborty & Old, 1982; Chakraborty et al., 1983). The newly discovered high diversity of mycophagous protists in soil could also be a reservoir for protists with biocontrol effects on fungal pathogens (Geisen, 2016b).

Taken together, this thesis suggests that protists could be a potential biostimulant to boost the abundance of plant beneficial bacteria (**Chapter 3, 4**), however, the functional impacts of protist predation on bacterial communities still requires further research to prove this link and exploit protist activities to the benefit of plant health.

Concluding remarks

Within this thesis, I proposed the use of protist traits as a means to understand predator/prey interactions. I found that protist predation was not related to protist phylogeny, but protists traits, namely, protist cell volume was informative with respect to protist impacts on bacterial prey communities. Understanding predator/prey interactions could help us better predict resulting changes in microbiome functionality. Furthermore, this work has shown that predation can reinforce cooperation in soil, thereby leading to higher numbers of cooperators, and such cooperation may be linked to positive effects on plant disease suppression. Taken together, this thesis investigated the effects of protist traits on predation and ecological interactions, with the goal of providing a more systematic and ecological framework toward the utilization of potentially beneficial protists for improved soil functioning.

References

Abraham WR, Nogales B, Golyshin PN, Pieper DH, Timmis KN. 2002. Polychlorinated biphenyl-degrading microbial communities in soils and sediments. Current opinion in microbiology 5: 246–53.

Abrams PA. 2000. The Evolution of Predator-Prey Interactions: Theory and Evidence. Annual Review of Ecology and Systematics 31: 79–105.

Adl SM, Coleman DC. 2005. Dynamics of soil protozoa using a direct count method. Biology and Fertility of Soils 42: 168–171.

Adl MS, Gupta VS. 2006. Protists in soil ecology and forest nutrient cycling. Canadian Journal of Forest Research 36: 1805–1817.

Adl SM, Habura A, Eglit Y. 2013. Amplification primers of SSU rDNA for soil protists. Soil Biology and Biochemistry 69: 328–342.

Adl SM, Simpson AGB, Lane CE, Lukeš J, Bass D, Bowser SS, Brown MW, Burki F, Dunthorn M, Hampl V, et al. 2012. The revised classification of eukaryotes. Journal of Eukaryotic Microbiology 59: 429–493.

Al-Herrawy AZ, Gad MA. 2015. Isolation and molecular identification of naegleria fowleri from nile river, Egypt. Journal of the Egyptian Public Health Association 90: 161–165.

Andersen KS, Winding A. 2004. Non-target effects of bacterial biological control agents on soil Protozoa. Biology and Fertility of Soils 40: 230–236.

Anderson OR, Wand W, Faucher SP, Bi K, Shuman HA. 2011. A New Heterolobosean Amoeba *Solumitrus palustris* n. g., n. sp. Isolated from Freshwater Marsh Soil. Journal of Eukaryotic Microbiology 58: 60–67.

Balkwill DL, Fredrickson JK, Romine MF. 2006. Sphingomonas and Related Genera. In: The Prokaryotes. New York, NY: Springer New York, 605–629.

Baltar F, Palovaara J, Unrein F, Catala P, Horňák K, Šimek K, Vaqué D, Massana R, Gasol JM, Pinhassi J. 2016. Marine bacterial community structure resilience to changes in protist predation under phytoplankton bloom conditions. The ISME Journal 10: 568–581.

Bardgett RD, Van Der Putten WH. 2014. Belowground biodiversity and ecosystem functioning. Nature 515 505: 505–511.

Bass D, Cavalier-Smith T. 2004. Phylum-specific environmental DNA analysis reveals remarkably high global biodiversity of Cercozoa (Protozoa). International Journal Of Systematic And Evolutionary Microbiology 54: 2393–2404.

Bates ST, Clemente JC, Flores GE, Walters WA, Parfrey LW, Knight R, Fierer N. 2013. Global biogeography of highly diverse protistan communities in soil. The

ISME Journal 7: 652–659.

Baym M, Kryazhimskiy S, Lieberman TD, Chung H, Desai MM, Kishony RK. 2015. Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS ONE 10.

Bell T, Bonsall MB, Buckling A, Whiteley AS, Goodall T, Griffiths RI. 2010. Protists have divergent effects on bacterial diversity along a productivity gradient. Biology letters 6: 639–42.

Berendsen RL, Pieterse CMJ, Bakker PAHM. 2012. The rhizosphere microbiome and plant health. Trends in Plant Science 17: 478–486.

Berg G, Rybakova D, Grube M, Köberl M. 2016. The plant microbiome explored: Implications for experimental botany. Journal of Experimental Botany 67: 995–1002.

Berney C, Ciuprina A, Bender S, Brodie J, Edgcomb V, Kim E, Rajan J, Parfrey LW, Adl S, Audic S, et al. 2017. UniEuk : Time to Speak a Common Language in Protistology! Journal of Eukaryotic Microbiology 64: 407–411.

Besset-Manzoni Y, Rieusset L, Joly P, Comte G, Prigent-Combaret C. 2018. Exploiting rhizosphere microbial cooperation for developing sustainable agriculture strategies. Environmental Science and Pollution Research 25: 29953–29970.

Boenigk J, Pfandl K, Stadler P, Chatzinotas A. 2005. High diversity of the 'Spumella-like' flagellates: an investigation based on the SSU rRNA gene sequences of isolates from habitats located in six different geographic regions. Environmental Microbiology 7: 685–697.

Böhme A, Risse-Buhl U, Küsel K. 2009. Protists with different feeding modes change biofilm morphology. FEMS Microbiology Ecology 69: 158–169.

Bonkowski M. 2004. Protozoa and plant growth: The microbial loop in soil revisited. New Phytologist 162: 617–631.

Bonkowski M, Brandt F. 2002. Do soil protozoa enhance plant growth by hormonal effects? Soil Biology and Biochemistry 34: 1709–1715.

Bonkowski M, Geoghegan IE, Birch AN, Griffiths BS. 2001a. Effects of soil decomposer invertebrates (protozoa and earthworms) on an above-ground phytophagous insect (cereal aphid) mediated through changes in the host plant. Oikos 95: 441–450.

Bonkowski M, Griffiths B, Scrimgeour C. 2000. Substrate heterogeneity and microfauna in soil organic 'hotspots' as determinants of nitrogen capture and growth of ryegrass. Applied Soil Ecology 14: 37–53.

Bonkowski M, Jentschke G, Scheu S. 2001b. Contrasting effects of microbial partners in the rhizosphere: Interactions between Norway Spruce seedlings (*Picea abies Karst.*), mycorrhiza (*Paxillus involutus (Batsch)* Fr.) and naked amoebae

(protozoa). Applied Soil Ecology 18: 193–204.

Bouskill NJ, Tang J, Riley WJ, Brodie EL. 2012. Trait-based representation of biological nitrification: Model development, testing, and predicted community composition. Frontiers in Microbiology 3: 1–17.

Brazelton JN, Pfeufer EE, Sweat TA, Gardener BBM, Coenen C. 2008. 2,4-Diacetylphloroglucinol Alters Plant Root Development. Molecular Plant-Microbe Interactions 21: 1349–1358.

Brockhurst MA, Buckling A, Racey D, Gardner A. 2008. Resource supply and the evolution of public-goods cooperation in bacteria. BMC Biology 6: 20.

Brose U, Archambault P, Barnes AD, Bersier LF, Boy T, Canning-Clode J, Conti E, Dias M, Digel C, Dissanayake A, et al. 2019. Predator traits determine food-web architecture across ecosystems. Nature Ecology and Evolution 3: 919–927.

Brose U, Jonsson T, Berlow EL, Warren P, Banasek-Richter C, Bersier L-F, Blanchard JL, Brey T, Carpenter SR, Blandenier M-FC, et al. 2006. Consumer–Resource body-size relationship in natural food webs. Ecology 87: 2411–2417.

Brown S, De Jonckheere JF. 1999. A reevaluation of the amoeba genus *Vahlkampfia* based on SSUrDNA sequences. European Journal of Protistology 35: 49–54.

Brown S, De Jonckheere JF. 2004. Isolation of a new vahlkampfiid amoeba from soil: *Paravahlkampfia lenta* n. sp. European Journal of Protistology 40: 289–294.

Brown MW, Silberman JD, Spiegel FW. 2012. A contemporary evaluation of the acrasids (Acrasidae, Heterolobosea, Excavata). European Journal of Protistology 48: 103–123.

Bruce JB, West SA, Griffin AS. 2017. Bacteriocins and the assembly of natural *Pseudomonas fluorescens* populations. Journal of Evolutionary Biology 30: 352–360.

Brugerolle G, Simpson AGB. 2004. The Flagellar Apparatus of Heteroloboseans. Journal of Eukaryotic Microbiology 51: 96–107.

Brüssow H. 2007. Bacteria between protists and phages: from antipredation strategies to the evolution of pathogenicity. Molecular Microbiology 65: 583–589.

Burki F. 2014. The Eukaryotic Tree of Life from a Global Phylogenomic Perspective. Cold Spring Harbor Perspectives in Biology 6: a016147–a016147.

Cairns J, Jalasvuori M, Ojala V, Brockhurst M, Hiltunen T. 2016. Conjugation is necessary for a bacterial plasmid to survive under protozoan predation. Biology letters 12: 20150953.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pẽa AG, Goodrich JK, Gordon JI, et al. 2010. QIIME allows analysis of

high-throughput community sequencing data. Nature Methods 7: 335–336.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences of the United States of America 108 Suppl 1: 4516–22.

Caron DA, Worden AZ, Countway PD, Demir E, Heidelberg KB. 2008. Protists are microbes too: a perspective. The ISME Journal 3: 4–12.

Cavalier-Smith T, Lewis R, Chao EE, Oates B, Bass D. 2009. *Helkesimastix marina* n. sp. (Cercozoa: Sainouroidea superfam. n.) a Gliding Zooflagellate of Novel Ultrastructure and Unusual Ciliary Behaviour. Protist 160: 452–479.

Chakraborty S, Old KM. 1982. Mycophagous soil amoeba: Interactions with three plant pathogenic fungi. Soil Biology and Biochemistry 14: 247–255.

Chakraborty S, Old KM, Warcup JH. 1983. Amoebae from a take-all suppressive soil which feed on *Gaeumannomyces graminis tritici* and other soil fungi. Soil Biology and Biochemistry 15: 17–24.

Chaparro JM, Badri D V, Vivanco JM. 2014. Rhizosphere microbiome assemblage is affected by plant development. The ISME journal 8: 790–803.

Chin-A-Woeng TFC, Bloemberg G V., Lugtenberg BJJ. 2003. Phenazines and their role in biocontrol by *Pseudomonas* bacteria. New Phytologist 157: 503–523.

Chrzanowski TH, Šimek K. 1990. Prey-size selection by freshwater flagellated protozoa. Limnology and Oceanography 35: 1429–1436.

Clarholm M. 1981. Protozoan grazing of bacteria in soil—impact and importance. Microbial Ecology 7: 343–350.

Clarholm M. 1985. Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. Soil Biology and Biochemistry 17: 181–187.

Clarholm M, Bonkowski M, Griffiths B. 2007. Protozoa and Other Protista in Soil. In: Van Elsas JD, Jansson JK, Trevors JT, eds. Modern Soil Microbiology. CRC Press, 147–175.

Cleveland CC, Liptzin D. 2007. C:N:P stoichiometry in soil: Is there a 'Redfield ratio' for the microbial biomass? Biogeochemistry 85: 235–252.

Conza L, Pagani SC, Gaia V. 2013. Presence of Legionella and Free-Living Amoebae in Composts and Bioaerosols from Composting Facilities. PLoS ONE 8.

Corno G, Jürgens K. 2006. Direct and Indirect Effects of Protist Predation on Population Size Structure of a Bacterial Strain with High Phenotypic Plasticity. Applied and Environmental Microbiology 72: 78–86.

Corno G, Jürgens K. 2008. Structural and functional patterns of bacterial communities in response to protist predation along an experimental productivity gradient. Environmental microbiology 10: 2857–71.

Creevy AL, Fisher J, Puppe D, Wilkinson DM. 2016. Protist diversity on a nature reserve in NW England—With particular reference to their role in soil biogenic silicon pools. Pedobiologia 59: 51–59.

Crespi BJ. 2001. The evolution of social behavior in microorganisms. Trends in Ecology & Evolution 16: 178–183.

Crotty F V., Adl SM, Blackshaw RP, Murray PJ. 2013. Measuring soil protist respiration and ingestion rates using stable isotopes. Soil Biology and Biochemistry 57: 919–921.

Crowther TW, Maynard DS, Crowther TR, Peccia J, Smith JR, Bradford MA. 2014. Untangling the fungal niche: The trait-based approach. Frontiers in Microbiology 5: 1–12.

Darbyshire JF. 2005. The use of soil biofilms for observing protozoan movement and feeding. FEMS Microbiology Letters 244: 329–333.

Dartnell LR, Hunter SJ, Lovell K V., Coates AJ, Ward JM. 2010. Low-temperature ionizing radiation resistance of deinococcus radiodurans and antarctic dry valley bacteria. Astrobiology 10: 717–732.

Davies PJ. 2010. The Plant Hormones: Their Nature, Occurrence, and Functions. In: Davies PJ, ed. Plant Hormones. Dordrecht: Springer Netherlands, 1–15.

Davis MJ, Gillaspie AG, Harris RW, Lawson RH. 1980. Ratoon stunting disease of sugarcane: Isolation of the causal bacterium. Science 210: 1365–1367.

Delgado-Baquerizo M, Oliverio AM, Brewer TE, Benavent-González A, Eldridge DJ, Bardgett RD, Maestre FT, Singh BK, Fierer N. 2018. A global atlas of the dominant bacteria found in soil. Science 359: 320–325.

Delwiche CF. 1999. Tracing the Thread of Plastid Diversity through the Tapestry of Life. The American Naturalist 154: S164–S177.

Denison RF, Bledsoe C, Kahn M, O'Gara F, Simms EL, Thomashow LS. 2003. Cooperation in the rhizosphere and the 'free rider' problem. Ecology 84: 838–845.

Diggle SP, Griffin AS, Campbell GS, West SA. 2007. Cooperation and conflict in quorum-sensing bacterial populations. Nature 450: 411–414.

Dilly O, Bloem J, Vos A, Munch JC. 2004. Bacterial diversity in agricultural soils during litter decomposition. Applied and environmental microbiology 70: 468–74.

Dodd IC, Zinovkina NY, Safronova VI, Belimov AA. 2010. Rhizobacterial mediation of plant hormone status. Annals of Applied Biology 157: 361–379.

Duffy BK, Défago G. 2000. Controlling instability in gacS-gacA regulatory genes during inoculant production of Pseudomonas fluorescens biocontrol strains. Applied and environmental microbiology 66: 3142–50.

Dumack K, Fiore-Donno AM, Bass D, Bonkowski M. 2019. Making sense of environmental sequencing data: ecologically important functional traits of the protistan groups Cercozoa and Endomyxa (Rhizaria). Molecular ecology resources: 0–1.

Dupont AÖC, Griffiths RI, Bell T, Bass D. 2016. Differences in soil micro-eukaryotic communities over soil pH gradients are strongly driven by parasites and saprotrophs. Environmental Microbiology 18: 2010–2024.

Dyková I, Kostka M. Illustrated guide to culture collection of free-living amoebae. Praha: Academia, 2013. 363 pp. ISBN 978-80-200-2176-2.

Dyková I, Kyselová I, Pecková H, Oborník M, Lukeš J. 2001. Identity of Naegleria strains isolated from organs of freshwater fishes. Diseases of Aquatic Organisms 46: 115–121.

Dyková I, Pecková H, Fiala I, Dvořáková H. 2006. Fish-isolated Naegleria strains and their phylogeny inferred from ITS and SSU rDNA sequences. Folia Parasitologica 53: 172–180.

Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26: 2460–2461.

Edgar RC. 2013. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10: 996–998.

Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, Eisen JA, Sundaresan V. 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. Proceedings of the National Academy of Sciences of the United States of America 112: E911-20.

Ekelund F. 1998. Enumeration and abundance of mycophagous protozoa in soil, with special emphasis on heterotrophic flagellates. Soil Biology and Biochemistry 30: 1343–1347.

Ekelund F, Saj S, Vestergård M, Bertaux J, Mikola J. 2009. The ‘soil microbial loop’ is not always needed to explain protozoan stimulation of plants. Soil Biology and Biochemistry 41: 2336–2342.

Elliott ET, Anderson R V., Coleman DC, Cole C V. 1980. Habitable Pore Space and Microbial Trophic Interactions. Oikos 35: 327.

Erken M, Lutz C, McDougald D. 2013. The rise of pathogens: predation as a factor driving the evolution of human pathogens in the environment. Microbial ecology 65: 860–8.

Falkowski PG, Fenchel T, Delong EF. 2008. The Microbial Engines That Drive Earth's Biogeochemical Cycles. Science 320: 1034–1039.

Fierer N. 2017. Embracing the unknown: disentangling the complexities of the soil microbiome. Nature Reviews Microbiology 15: 579–590.

Fierer N, Jackson RB. 2006. The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 103: 626–631.

Finlay BJ, Black HIJ, Brown S, Clarke KJ, Esteban GF, Hindle RM, Olmo JL, Rollett A, Vickerman K. 2000. Estimating the Growth Potential of the Soil Protozoan Community. Protist 151: 69–80.

Finlay BJ, Fenchel TOM. 2001. Protozoan community structure in a fractal soil environment. Protist 152: 203–218.

Fiore-Donno AM, Weinert J, Wubet T, Bonkowski M. 2016. Metacommunity analysis of amoeboid protists in grassland soils. Scientific Reports 6: 19068.

Foissner W. 1999a. Description of Two New, Mycophagous Soil Ciliates (Ciliophora, Colpodea): *Fungiphrya strobli* n. g., n. sp. and *Grossglockneria ovata* n. sp. The Journal of Eukaryotic Microbiology 46: 34–42.

Foissner W. 1999b. Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples. Agriculture, Ecosystems & Environment 74: 95–112.

Frey SD, Elliott ET, Paustian K. 1999. Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. Soil Biology and Biochemistry 31: 573–585.

Friesen ML, Porter SS, Stark SC, von Wettberg EJ, Sachs JL, Martinez-Romero E. 2011. Microbially mediated plant functional traits. Annual Review of Ecology, Evolution, and Systematics 42: 23–46.

Friman V-P, Buckling A. 2013. Effects of predation on real-time host-parasite coevolutionary dynamics. Ecology letters 16: 39–46.

Friman V-P, Diggle SP, Buckling A. 2013. Protist predation can favour cooperation within bacterial species. Biology Letters 9.

Friman V-P, Hiltunen T, Laakso J, Kaitala V. 2008. Availability of prey resources drives evolution of predator–prey interaction. Proceedings of the Royal Society of London B: Biological Sciences 275.

Friman V-P, Jousset A, Buckling A. 2014. Rapid prey evolution can alter the structure of predator-prey communities. Journal of Evolutionary Biology 27: 374–380.

Gao Z, Karlsson I, Geisen S, Kowalchuk G, Jousset A. 2019. Protists: Puppet Masters

of the Rhizosphere Microbiome. Trends in Plant Science 24: 165–176.

Garstecki T, Brown S, De Jonckheere JF. 2005. Description of *Vahlkampfia signyensis* n. sp. (Heterolobosea), based on morphological, ultrastructural and molecular characteristics. European Journal of Protistology 41: 119–127.

Gasol JM, Del Giorgio PA, Massana R, Duarte CM. 1995. Active versus inactive bacteria: size-dependence in a coastal marine plankton community. Marine Ecology Progress Series 128: 91–97.

Gast RJ, Sanders RW, Caron DA. 2009. Ecological strategies of protists and their symbiotic relationships with prokaryotic microbes. Trends in Microbiology 17: 563–569.

Geisen S. 2016a. Thorough high-throughput sequencing analyses unravels huge diversities of soil parasitic protists. Environmental Microbiology 18: 1669–1672.

Geisen S. 2016b. The bacterial-fungal energy channel concept challenged by enormous functional versatility of soil protists. Soil Biology and Biochemistry 102: 22–25.

Geisen S, Bonkowski M. 2017. Methodological advances to study the diversity of soil protists and their functioning in soil food webs. Applied Soil Ecology.

Geisen S, Bonkowski M, Zhang J, De Jonckheere JF. 2015a. Heterogeneity in the genus *Allovahlkampfia* and the description of the new genus *Parafumarolamoeba* (Vahlkampfiidae; Heterolobosea). European Journal of Protistology 51: 335–349.

Geisen S, Koller R, Hünninghaus M, Dumack K, Urich T, Bonkowski M. 2015b. The soil food web revisited: Diverse and widespread mycophagous soil protists. Soil Biology and Biochemistry 94: 10–18.

Geisen S, Laros I, Vizcaíno A, Bonkowski M, De Groot GA. 2016. Not all are free-living: High-throughput DNA metabarcoding reveals a diverse community of protists parasitizing soil metazoa. Molecular Ecology 24.

Geisen S, Mitchell EAD, Adl S, Bonkowski M, Dunthorn M, Ekelund F, Fernández LD, Jousset A, Krashevska V, Singer D, et al. 2018. Soil protists: a fertile frontier in soil biology research. FEMS Microbiology Reviews.

Geisen S, Mitchell EAD, Wilkinson DM, Adl S, Bonkowski M, Brown MW, Fiore-Donno AM, Heger TJ, Jassey VEJ, Krashevska V, et al. 2017. Soil protistology rebooted: 30 fundamental questions to start with. Soil Biology and Biochemistry 111: 94–103.

Geisen S, Rosengarten J, Koller R, Mulder C, Urich T, Bonkowski M. 2015c. Pack hunting by a common soil amoeba on nematodes. Environmental Microbiology 17: 4538–4546.

Geisen S, Tveit AT, Clark IM, Richter A, Svenning MM, Bonkowski M, Urich T.

2015d. Metatranscriptomic census of active protists in soils. The ISME Journal: 1–13.

Glöckner FO, Kube M, Bauer M, Teeling H, Lombardot T, Ludwig W, Gade D, Beck A, Borzym K, Heitmann K, et al. 2003. Complete genome sequence of the marine planctomycete Pirellula sp. strain 1. Proceedings of the National Academy of Sciences of the United States of America 100: 8298–8303.

Glöckner FO, Yilmaz P, Quast C, Gerken J, Beccati A, Ciuprina A, Bruns G, Yarza P, Peplies J, Westram R, et al. 2017. 25 years of serving the community with ribosomal RNA gene reference databases and tools. Journal of Biotechnology 261: 169–176.

Glücksman E, Bell T, Griffiths RI, Bass D. 2010. Closely related protist strains have different grazing impacts on natural bacterial communities. Environmental Microbiology 12: 3105–3113.

Gómez W, Buela L, Castro LT, Chaparro V, Ball MM, Yarzábal LA. 2010. Evidence for gluconic acid production by Enterobacter intermedium as an efficient strategy to avoid protozoan grazing. Soil Biology and Biochemistry 42: 822–830.

Gonzalez JM, Sherr EB, Sherr BF. 1990. Size-selective grazing on bacteria by natural assemblages of estuarine flagellates and ciliates. Applied and Environmental Microbiology 56: 583.

Götze S, Herbst-Irmer R, Klapper M, Görls H, Schneider KRA, Barnett R, Burks T, Neu U, Stallforth P. 2017. Structure, Biosynthesis, and Biological Activity of the Cyclic Lipopeptide Anikasin. ACS Chemical Biology 12: 2498–2502.

Gouy M, Guindon S, Gascuel O. 2010. SeaView Version 4: A Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree Building. Molecular Biology and Evolution 27: 221–224.

Griffin AS, West SA, Buckling A. 2004. Cooperation and competition in pathogenic bacteria. Nature 430: 1024–1027.

Haas D, Défago G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Reviews Microbiology 3: 307–319.

Harding T, Brown MW, Plotnikov A, Selivanova E, Park JS, Gunderson JH, Baumgartner M, Silberman JD, Roger AJ, Simpson AGB. 2013. Amoeba stages in the deepest branching heteroloboseans, including pharyngomonas: Evolutionary and systematic implications. Protist 164: 272–286.

Harrison F, Paul J, Massey RC, Buckling A. 2008. Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. ISME Journal 2: 49–55.

Hartikainen H, Bass D, Briscoe AG, Knipe H, Green AJ, Okamura B. 2016. Assessing myxozoan presence and diversity using environmental DNA. International Journal for Parasitology 46: 781–792.

Heeb S, Haas D. 2001. Regulatory Roles of the GacS/GacA Two-Component System in Plant-Associated and Other Gram-Negative Bacteria. Molecular plant-microbe interactions 14.12: 1351-1363.

Heijden MGA Van Der, Bardgett RD, Straalen NM Van. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11: 296–310.

Herdler S, Kreuzer K, Scheu S, Bonkowski M. 2007. Interactions between arbuscular mycorrhizal fungi (Glomus intraradices, Glomeromycota) and amoebae (Acanthamoeba castellanii, Protozoa) in the rhizosphere of rice (Oryza sativa). 40: 660–668.

Hiltunen T, Becks L. 2014. Consumer co-evolution as an important component of the eco-evolutionary feedback. Nature Communications 5: 5226.

Ho A, Kerckhof FM, Luke C, Reim A, Krause S, Boon N, Bodelier PLE. 2013. Conceptualizing functional traits and ecological characteristics of methane-oxidizing bacteria as life strategies. Environmental Microbiology Reports 5: 335–345.

Horn M, Wagner Mi. 2004. Bacterial Endosymbionts of Free-living Amoebae1. The Journal of Eukaryotic Microbiology 51: 509–514.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755.

Hünninghaus M, Koller R, Kramer S, Marhan S, Kandeler E, Bonkowski M. 2017. Changes in bacterial community composition and soil respiration indicate rapid successions of protist grazers during mineralization of maize crop residues. Pedobiologia 62: 1–8.

Hunt HW, Coleman DC, Ingham ER, Ingham RE, Elliott ET, Moore JC, Rose SL, Reid CPP, Morley CR. 1987. The detrital food web in a shortgrass prairie. Biology and Fertility of Soils 3–3.

Iavicoli A, Boutet E, Buchala A, Métraux J-P. 2003. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Molecular plant-microbe interactions : MPMI 16: 851–8.

Imparato V, Santos SS, Johansen A, Geisen S, Winding A. 2016. Stimulation of bacteria and protists in rhizosphere of glyphosate-treated barley. Applied Soil Ecology 98: 47–55.

Inglis RF, Roberts PG, Gardner A, Buckling A. 2011. Spite and the scale of competition in Pseudomonas aeruginosa. The American naturalist 178: 276–85.

Jacob L, Patterson DJ. 1990. Some flagellates (Protista) from tropical marine sediments. Journal of Natural History 24: 801–937.

James SA, Collins MD, Roberts IN. 1994. Genetic interrelationship among species of the genusZygosaccharomyces as revealed by small-subunit rRNA gene sequences. Yeast 10: 871–881.

Jamieson WD, Pehl MJ, Gregory GA, Orwin PM. 2009. Coordinated surface activities in *Variovorax paradoxus* EPS. BMC Microbiology 9: 124.

Jassey VEJ, Shimano S, Dupuy C, Toussaint ML, Gilbert D. 2012. Characterizing the Feeding Habits of the Testate Amoebae Hyalosphenia papilio and Nebela tincta along a Narrow ‘ Fen-Bog’ Gradient Using Digestive Vacuole Content and ^{13}C and ^{15}N Isotopic Analyses. Protist 163: 451–464.

Jassey VEJ, Signarbieux C, Hättenschwiler S, Bragazza L, Buttler A, Delarue F, Fournier B, Gilbert D, Laggoun-Défarge F, Lara E, et al. 2015. An unexpected role for mixotrophs in the response of peatland carbon cycling to climate warming. Scientific Reports 5: 16931.

De Jonckheere JF. 2002. A Century of Research on the Amoeboflagellate Genus *Naegleria* A Century of Research on the Amoeboflagellate Genus Naegleria. Acta protozoologica 41: 309–342.

De Jonckheere JF. 2004. Molecular Definition and the Ubiquity of Species in the Genus *Naegleria*. Protist 155: 89–103.

De Jonckheere JF. 2006a. Isolation and Molecular Identification of Vahlkampfiid Amoebae from an Island (Tenerife, Spain).

De Jonckheere JF. 2006b. Isolation and molecular identification of free-living amoebae of the genus *Naegleria* from Arctic and sub-Antarctic regions. European Journal of Protistology 42: 115–123.

De Jonckheere JF. 2011. Origin and evolution of the worldwide distributed pathogenic amoeboflagellate *Naegleria fowleri*. Infection, Genetics and Evolution 11: 1520–1528.

De Jonckheere JF. 2012. The impact of man on the occurrence of the pathogenic free-living amoeboflagellate *Naegleria fowleri*. Future Microbiology 7: 5–7.

De Jonckheere JF. 2014. What do we know by now about the genus *Naegleria*? Experimental Parasitology 145: S2–S9.

De Jonckheere JF, Brown S. 2005. The Identification of Vahlkampfiid Amoebae by ITS Sequencing. Protist 156: 89–96.

De Jonckheere JF, Brown S, Robinson BS. 1997. On the Identity of the Amoeboflagllates Didascalus throntoni and Adelphamoeba galeacystis. The Journal of Eukaryotic Microbiology 44: 52–54.

De Jonckheere JF, Murase J, Opperdoes FR. 2011. A new thermophilic heterolobosean amoeba, *Fumarolamoeba ceborucoi*, gen. nov., sp. nov., isolated near a fumarole at a

Volcano in Mexico. Acta Protozoologica 50: 41–48.

Jousset A. 2012. Ecological and evolutive implications of bacterial defences against predators. Environmental Microbiology 14: 1830–1843.

Jousset A, Bonkowski M. 2010a. The model predator *Acanthamoeba castellanii* induces the production of 2,4, DAPG by the biocontrol strain Pseudomonas fluorescens Q2-87. Soil Biology and Biochemistry 42: 1647–1649.

Jousset A, Lara E, Wall LG, Valverde C. 2006. Secondary metabolites help biocontrol strain *Pseudomonas fluorescens* CHA0 to escape protozoan grazing. Applied and environmental microbiology 72: 7083–90.

Jousset A, Rochat L, Péchy-Tarr M, Keel C, Scheu S, Bonkowski M. 2009. Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters. The ISME journal 3: 666–674.

Jousset A, Rochat L, Scheu S, Bonkowski M, Keel C. 2010. Predator-prey chemical warfare determines the expression of biocontrol genes by rhizosphere-associated Pseudomonas fluorescens. Applied and environmental microbiology 76: 5263–8.

Jousset A, Scheu S, Bonkowski M. 2008. Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria. Functional Ecology 22: 714–719.

Jürgens K, Matz C. 2002. Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. Antonie van Leeuwenhoek 81: 413–434.

Katoh K, Rozewicki J, Yamada KD. 2017. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics.

Klapper M, Götze S, Barnett R, Willing K, Stallforth P. 2016. Bacterial Alkaloids Prevent Amoebal Predation. Angewandte Chemie International Edition 55: 8944–8947.

Koller R, Robin C, Bonkowski M, Ruess L, Scheu S. 2013a. Litter quality as driving factor for plant nutrition via grazing of protozoa on soil microorganisms. FEMS Microbiology Ecology 85: 241–250.

Koller R, Rodriguez A, Robin C, Scheu S, Bonkowski M. 2013b. Protozoa enhance foraging efficiency of arbuscular mycorrhizal fungi for mineral nitrogen from organic matter in soil to the benefit of host plants. New Phytologist 199: 203–211.

Koller R, Scheu S, Bonkowski M, Robin C. 2013c. Protozoa stimulate N uptake and growth of arbuscular mycorrhizal plants. Soil Biology and Biochemistry 65: 204–210.

Kowalchuk GA, Buma DS, De Boer W, Klinkhamer PGL, Van Veen JA. 2002. Effects of above-ground plant species composition and diversity on the diversity of

soil-borne microorganisms.

Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. 2019. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference (J Wren, Ed.). Bioinformatics.

Kramer S, Dibbern D, Moll J, Huenninghaus M, Koller R, Krueger D, Marhan S, Urich T, Wubet T, Bonkowski M, et al. 2016. Resource Partitioning between Bacteria, Fungi, and Protists in the Detritusphere of an Agricultural Soil. Frontiers in microbiology 7: 1524.

Krause S, Le Roux X, Niklaus PA, van Bodegom PM, Lennon T. JT, Bertilsson S, Grossart HP, Philippot L, Bodelier PLE. 2014. Trait-based approaches for understanding microbial biodiversity and ecosystem functioning. Frontiers in Microbiology 5: 1–10.

Kreuzer K, Adamczyk J, Iijima M, Wagner M, Scheu S, Bonkowski M. 2006. Grazing of a common species of soil protozoa (*Acanthamoeba castellanii*) affects rhizosphere bacterial community composition and root architecture of rice (*Oryza sativa L.*). Soil Biology and Biochemistry 38: 1665–1672.

Krome K, Rosenberg K, Bonkowski M, Scheu S. 2009. Grazing of protozoa on rhizosphere bacteria alters growth and reproduction of Arabidopsis thaliana. Soil Biology and Biochemistry 41: 1866–1873.

Krome K, Rosenberg K, Dickler C, Kreuzer K, Ludwig-Müller J, Ullrich-Eberius C, Scheu S, Bonkowski M. 2010. Soil bacteria and protozoa affect root branching via effects on the auxin and cytokinin balance in plants. Plant and Soil 328: 191–201.

Kuikman PJ, Jansen AG, Van Veen JA, Zehnder AJB. 1990. Protozoan predation and the turnover of soil organic carbon and nitrogen in the presence of plants. Biol Fertil Soils 10: 22–28.

Kuppardt A, Fester T, Härtig C, Chatzinotas A. 2018. Rhizosphere Protists Change Metabolite Profiles in Zea mays. Frontiers in Microbiology 9: 1–11.

Lara E, Berney C, Ekelund F, Harms H, Chatzinotas A. 2007. Molecular comparison of cultivable protozoa from a pristine and a polycyclic aromatic hydrocarbon polluted site. Soil Biology and Biochemistry 39: 139–148.

Lauber CL, Ramirez KS, Aanderud Z, Lennon J, Fierer N. 2013. Temporal variability in soil microbial communities across land-use types. ISME Journal 7: 1641–1650.

Learman DR, Ahmad Z, Brookshier A, Henson MW, Hewitt V, Lis A, Morrison C, Robinson A, Todaro E, Wologo E, et al. 2019. Comparative genomics of 16 *Microbacterium* spp. that tolerate multiple heavy metals and antibiotics. PeerJ 6: e6258.

Leff JW, Bardgett RD, Wilkinson A, Jackson BG, Pritchard WJ, De Long JR, Oakley S, Mason KE, Ostle NJ, Johnson D, et al. 2018. Predicting the structure of

soil communities from plant community taxonomy, phylogeny, and traits. The ISME Journal 1

Lentendu G, Wubet T, Chatzinotas A, Wilhelm C, Buscot F, Schlegel M. 2014. Effects of long-term differential fertilization on eukaryotic microbial communities in an arable soil: a multiple barcoding approach. Molecular Ecology 23: 3341–3355.

Levrat P, Pussard M, Alabouvette C. 1992. Enhanced bacterial metabolism of a Pseudomonas strain in response to the addition of culture filtrate of a bacteriophagous amoeba. European Journal of Protistology 28: 79–84.

Long JJ, Jahn CE, Sánchez-Hidalgo A, Wheat W, Jackson M, Gonzalez-Juarrero M, Leach JE. 2018. Interactions of free-living amoebae with rice bacterial pathogens *Xanthomonas oryzae* pathovars *oryzae* and *oryzicola* (Z Wang, Ed.). PLOS ONE 13: e0202941.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15: 550.

Lugtenberg B, Kamilova F. 2009. Plant-Growth-Promoting Rhizobacteria. Annual Review of Microbiology 63: 541–556.

Mahé F, de Vargas C, Bass D, Czech L, Stamatakis A, Lara E, Singer D, Mayor J, Bunge J, Sernaker S, et al. 2017. Parasites dominate hyperdiverse soil protist communities in Neotropical rainforests. Nature Ecology & Evolution 1: 0091.

Manhes P, Velicer GJ. 2011. Experimental evolution of selfish policing in social bacteria. Proceedings of the National Academy of Sciences 108: 8357–8362.

Marcos LA, Gotuzzo E. 2013. Intestinal protozoan infections in the immunocompromised host. Current Opinion in Infectious Diseases: 1.

Matz C, Deines P, Boenigk J, Arndt H, Eberl L, Kjelleberg S, Jürgens K. 2004. Impact of violacein-producing bacteria on survival and feeding of bacterivorous nanoflagellates. Applied and environmental microbiology 70: 1593–9.

Matz C, Jürgens K. 2001. Effects of hydrophobic and electrostatic cell surface properties of bacteria on feeding rates of heterotrophic nanoflagellates. Applied and Environmental Microbiology 67: 814–820.

Matz C, Jürgens K. 2005. High motility reduces grazing mortality of planktonic bacteria. Applied and environmental microbiology 71: 921–9.

Matz C, Kjelleberg S. 2005. Off the hook-how bacteria survive protozoan grazing. Trends in microbiology 13: 302–7.

Mazzola M, de Bruijn I, Cohen MF, Raaijmakers JM. 2009. Protozoan-induced regulation of cyclic lipopeptide biosynthesis is an effective predation defense mechanism for *Pseudomonas fluorescens*. Applied and environmental microbiology 75: 6804–11.

McGill B, Enquist B, Weiher E, M W. 2006. Rebuilding community ecology from functional traits. Trends in Ecology & Evolution 21: 178–185.

McMurdie PJ, Holmes S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data (M Watson, Ed.). PLoS ONE 8: e61217.

Medlin L, Elwood HJ, Stickel S, Sogin ML. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71: 491–499.

Megharaj M, Ramakrishnan B, Venkateswarlu K, Sethunathan N, Naidu R. 2011. Bioremediation approaches for organic pollutants: A critical perspective. Environment International 37: 1362–1375.

Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JHM, Piceno YM, DeSantis TZ, Andersen GL, Bakker PAHM, et al. 2011. Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria. Science 332: 1097–1100.

Meyer JR, Kassen R. 2007. The effects of competition and predation on diversification in a model adaptive radiation. Nature 446: 432–435.

Monger BC, Landry MR. 1991. Prey-size dependency of grazing by free-living marine flagellates. Marine Ecology Progress Series 74: 239–248.

Morgan AD, Quigley BJZ, Brown SP, Buckling A. 2012. Selection on non-social traits limits the invasion of social cheats (M van Baalen, Ed.). Ecology Letters 15: 841–846.

Mumford R, Friman VP. 2017. Bacterial competition and quorum-sensing signalling shape the eco-evolutionary outcomes of model in vitro phage therapy. Evolutionary Applications 10: 161–169.

Murase J, Kawasaki M, De Jonckheere JF. 2010. Isolation of a new heterolobosean amoeba from a rice field soil: *Vrihiamoeba italica* gen. nov., sp. nov. European Journal of Protistology 46: 164–170.

Murase J, Noll M, Frenzel P. 2006. Impact of Protists on the Activity and Structure of the Bacterial Community in a Rice Field Soil. Applied and Environmental Microbiology 72: 5436–5444.

Oberhansli T, Defago G, Haas D. 1991. Indole-3-acetic acid (IAA) synthesis in the biocontrol strain CHA0 of *Pseudomonas fluorescens*: Role of tryptophan side chain oxidase. Journal of General Microbiology 137: 2273–2279.

De Obeso Fernadez Del Valle A, Maciver SK. 2017. *Allovahlkampfia minuta* nov. Sp., (acrasidae, heterolobosea, excavata) a new soil amoeba at the boundary of the acrasid cellular slime moulds. Acta Protozoologica 56: 181–189.

Oksanen J, Kindt R, Legendre P, O'Hara B, Stevens MHH, Oksanen MJ, Suggests M. 2007. The vegan package. Community ecology package 10: 631–637.

Page FC. and Blanton RL. 1985. The Heterolobosea (Sarcodina: Rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). Protistologica 21.1: 121–132.

Page FC. 1967. Taxonomic Criteria for Limax Amoebae, with Descriptions of 3 New Species of *Hartmannella* and 3 of *Vahlkampfia*. 14: 499–521.

Page FC. 1988. A New Key to Freshwater and Soil Gymnamoebae, with Instructions for Culture, Freshwater Biological Association, Ambleside (1988), 122 S., 55 Abb., z.T. als Tafeln. Brosch. Archiv für Protistenkunde 137: 55–56.

Pagnier I, Croce O, Robert C, Raoult D, La Scola B. 2012. Genome sequence of Reyranella massiliensis, a bacterium associated with amoebae. Journal of Bacteriology 194: 5698–5698.

Pánek T, Simpson AGB, Brown MW, Dyer BD. 2017. Heterolobosea. Handbook of the Protists:1-42.

Panke-Buisse K, Poole AC, Goodrich JK, Ley RE, Kao-Kniffin J. 2015. Selection on soil microbiomes reveals reproducible impacts on plant function. The ISME journal 9: 980–9.

Park JS, Simpson AGB. 2011. Characterization of *Pharyngomonas kirbyi* (= 'Macropharyngomonas halophila' nomen nudum), a Very Deep-branching, Obligately Halophilic Heterolobosean Flagellate. Protist 162: 691–709.

Pedersen AL, Winding A, Altenburger A, Ekelund F. 2011. Protozoan growth rates on secondary-metabolite-producing *Pseudomonas* spp. correlate with high-level protozoan taxonomy. FEMS Microbiology Letters 316: 16–22.

Pehl MJ, Jamieson WD, Kong K, Forbester JL, Fredendall RJ, Gregory GA, McFarland JE, Healy JM, Orwin PM. 2012a. Genes That Influence Swarming Motility and Biofilm Formation in Variovorax paradoxus EPS (R Manganelli, Ed.). PLoS ONE 7: e31832.

Peters R. H. 1986. The ecological implications of body size. Cambridge University Press, Vol2.

Pfandl K, Posch T, Boenigk J. 2004. Unexpected Effects of Prey Dimensions and Morphologies on the Size Selective Feeding by Two Bacterivorous Flagellates (*Ochromonas* sp. and *Spumella* sp.). The Journal of Eukaryotic Microbiology 51: 626–633.

Pieterse CMJ, de Jonge R, Berendsen RL. 2016. The Soil-Borne Supremacy. Trends in Plant Science 21: 171–173.

Queck S-Y, Weitere M, Moreno AM, Rice SA, Kjelleberg S. 2006. The role of quorum sensing mediated developmental traits in the resistance of Serratia marcescens biofilms against protozoan grazing. Environmental microbiology 8: 1017–25.

Rahdar M, Tavalla M, Ebadi S, Salehi M. 2016. The identification of free-living amoebae in water and soil from in and around Ahvaz, Southwest Iran. Biochemical and Cellular Archives 16: 379–382.

Rankin DJ, Bargum K, Kokko H. 2007. The tragedy of the commons in evolutionary biology. Trends in Ecology & Evolution 22: 643–651.

Rasamiravaka T, Labtani Q, Duez P, El Jaziri M. 2015. The Formation of Biofilms by *Pseudomonas aeruginosa* : A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms. BioMed Research International 2015: 1–17.

Ravanbakhsh M, Sasidharan R, Voesenek LACJ, Kowalchuk GA, Jousset A. 2018. Microbial modulation of plant ethylene signaling: ecological and evolutionary consequences. Microbiome 6: 52.

Reeder WHH, Sanck J, Hirst M, Dawson SC, Wolfe G V. 2015. The Food Web of Boiling Springs Lake Appears Dominated by the Heterolobosean *Tetramitus thermacidophilus* Strain BSL. Journal of Eukaryotic Microbiology 62: 374–390.

Rickard AH, McBain AJ, Ledder RG, Handley PS, Gilbert P. 2003. Coaggregation between freshwater bacteria within biofilm and planktonic communities. FEMS Microbiology Letters 220: 133–140.

Ríos-Castillo AG, Thompson KD, Adams A, Marín de Mateo M, Rodríguez-Jerez JJ. 2018. Biofilm formation of *Flavobacterium psychrophilum* on various substrates. Aquaculture Research 49: 3830–3837.

Ritz K, Griffiths BS. 1987. Effects of carbon and nitrate additions to soil upon leaching of nitrate, microbial predators and nitrogen uptake by plants. Plant and Soil 102: 229–237.

Robinson BS, Bamforth SS, Dobson PJ. 2002. Density and Diversity of Protozoa in Some Arid Australian Soils. The Journal of Eukaryotic Microbiology 49: 449–453.

Rønn R, Mccaig AE, Griffiths BS, Prosser JI. 2002. Impact of Protozoan Grazing on Bacterial Community Structure in Soil Microcosms. Applied and Environmantal Microbiology 68: 6094–6105.

Rosenberg K, Bertaux J, Krome K, Hartmann A, Scheu S, Bonkowski M. 2009. Soil amoebae rapidly change bacterial community composition in the rhizosphere of Arabidopsis thaliana. The ISME journal 3: 675–684.

de Ruiter PC, Neutel AM, Moore JC. 1995. Energetics, patterns of interaction strengths, and stability in real ecosystems. Science (New York, N.Y.) 269: 1257–60.

Ryan MP, Pembroke JT. 2018. Brevundimonas spp: Emerging global opportunistic

pathogens. Virulence 9: 480–493.

Saleem M, Fetzer I, Dormann CF, Harms H, Chatzinotas A. 2012. Predator richness increases the effect of prey diversity on prey yield. Nature Communications 3: 1305–1307.

Sandon H. 1927. The composition and distribution of the protozoan fauna of the soil Soil Science 23.5: 415.

Schisler DA, Slininger PJ, Behle RW, Jackson MA. 2004. Formulation of Bacillus spp. for Biological Control of Plant Diseases. Phytopathology 94: 1267–1271.

Schlimme W, Marchiani M, Hanselmann K, Jenni B. 1997. Gene transfer between bacteria within digestive vacuoles of protozoa. FEMS Microbiology Ecology 23: 239–247.

Schneider FD, Scheu S, Brose U. 2012. Body mass constraints on feeding rates determine the consequences of predator loss. Ecology Letters 15: 436–443.

Schnider-Keel U, Seematter A, Maurhofer M, Blumer C, Duffy B, Gigot-Bonnefoy C, Reimmann C, Notz R, Défago G, Haas D, et al. 2000. Autoinduction of 2,4-Diacetylphloroglucinol Biosynthesis in the Biocontrol Agent *Pseudomonas fluorescens* CHA0 and Repression by the Bacterial Metabolites Salicylate and Pyoluteorin. Journal of Bacteriology 182: 1215.

Schulz-Bohm K, Geisen S, Wubs ERJ, Song C, de Boer W, Garbeva P. 2017. The prey's scent – Volatile organic compound mediated interactions between soil bacteria and their protist predators. The ISME Journal 11: 817–820.

Seppey CVW, Singer D, Dumack K, Fournier B, Belbahri L, Mitchell EAD, Lara E. 2017. Distribution patterns of soil microbial eukaryotes suggests widespread algivory by phagotrophic protists as an alternative pathway for nutrient cycling. Soil Biology and Biochemistry 112: 68–76.

Sherr BF., Sherr EB. 1991. Proportional distribution of total numbers, biovolume, and bacterivory among size classes of 2-20 μm nonpigmented marine flagellates. Mar. microb. Food Webs 5: 227–237.

Sherr BF, Sherr EB, Berman T. 1983. Grazing, growth, and ammonium excretion rates of a heterotrophic microflagellate fed with four species of bacteria. Applied and Environmental Microbiology 45: 1196–1201.

Simms EL, Lee Taylor D. 2002. Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. Integrative and Comparative Biology 42: 369–380.

Singer D, Lara E, Steciow MM, Seppey CVW, Paredes N, Pillonel A, Oszako T, Belbahri L. 2016. High-throughput sequencing reveals diverse oomycete communities in oligotrophic peat bog micro-habitat. Fungal Ecology 23: 42–47.

Singh BK, Dawson LA, Macdonald CA, Buckland SM. 2009. Impact of biotic

and abiotic interaction on soil microbial communities and functions: A field study. Applied Soil Ecology 41: 239–248.

Smirnov AV, Brown S. 2004. Guide to the methods of study and identification of soil gymnamoebae. Protistology 3: 148–190.

Smirnov AV, Chao E, Nassonova ES, Cavalier-Smith T. 2011. A Revised Classification of Naked Lobose Amoebae (Amoebozoa: Lobosa). Protist 162: 545–570.

Sogin ML, Gunderson JH. 1987. Structural Diversity of Eukaryotic Small Subunit Ribosomal RNAs. Annals of the New York Academy of Sciences 503: 125–139.

Somasundaram S, Bonkowski M, Iijima M. 2008. Functional Role of Mucilage - Border Cells: A Complex Facilitating Protozoan Effects on Plant Growth. Plant Production Science 11: 344–351.

Song C, Mazzola M, Cheng X, Oetjen J, Alexandrov T, Dorrestein P, Watrous J, van der Voort M, Raaijmakers JM. 2015. Molecular and chemical dialogues in bacteria-protozoa interactions. Scientific Reports 5: 12837.

Tedersoo L, Bahram M, Põlme S, Kõljalg U, Yorou NS, Wijesundera R, Ruiz LV, Vasco-Palacios AM, Thu PQ, Suija A, et al. 2014. Global diversity and geography of soil fungi. Science 346.

Thakur MP, Geisen S. 2019. Trophic Regulations of the Soil Microbiome. Trends in Microbiology 27: 771–780.

Trap J, Bonkowski M, Plassard C, Villenave C, Blanchart E. 2015. Ecological importance of soil bacterivores for ecosystem functions. Plant and Soil 398.1-2: 1-24.

Travisano M, Velicer GJ. 2004. Strategies of microbial cheater control. Trends in Microbiology 12: 72–78.

Turner TR, Ramakrishnan K, Walshaw J, Heavens D, Alston M, Swarbreck D, Osbourn A, Grant A, Poole PS. 2013. Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. The ISME journal 7.

Tyml T, Skulinová K, Kavan J, Ditrich O, Kostka M, Dyková I. 2016. Heterolobosean amoebae from Arctic and Antarctic extremes: 18 novel strains of *Allovahlkampfia, Vahlkampfia* and *Naegleria*. European Journal of Protistology 56: 119–133.

de Vargas C, Audic S, Henry N, Decelle J, Mahé F, Logares R, Lara E, Berney C, Le Bescot N, Probert I, et al. 2015. Ocean plankton. Eukaryotic plankton diversity in the sunlit ocean. Science (New York, N.Y.) 348: 1261605.

Venables WN, Ripley BD. 2013. Modern applied statistics with S-PLUS. Springer Science & Business Media.

Vestergård M, Bjørnlund L, Henry F, Rønn R. 2007. Decreasing prevalence of rhizosphere IAA producing and seedling root growth promoting bacteria with barley development irrespective of protozoan grazing regime. Plant and Soil 295: 115–125.

Visvesvara GS, Moura H, Schuster FL. 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris* , *Naegleria fowleri* , and *Sappinia diploidea*. FEMS Immunology & Medical Microbiology 50: 1–26.

Voisard C, Bull CT, Keel C, Laville J, Maurhofer M, Schnider U, Dfago G, Haas D. 1994. Biocontrol of Root Diseases by *Pseudomonas fluorescens* CHA0: Current Concepts and Experimental Approaches. In: Molecular Ecology of Rhizosphere Microorganisms. Weinheim, Germany: Wiley-VCH Verlag GmbH, 67–89.

Wagner MR, Lundberg DS, del Rio TG, Tringe SG, Dangl JL, Mitchell-Olds T. 2016. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nature Communications 7: 12151.

Walochnik J, Mulec J. 2009. Free-living amoebae in carbonate precipitating microhabitats of karst caves and a new vahlkampfiid amoeba, *Allovahlkampfia spelaea* gen. nov., sp. nov. Acta Protozoologica 48: 25–33.

Wang S, Brose U. 2018. Biodiversity and ecosystem functioning in food webs: the vertical diversity hypothesis (D Mouillot, Ed.). Ecology Letters 21: 9–20.

Wei Z, Yang T, Friman V-P, Xu Y, Shen Q, Jousset A. 2015. Trophic network architecture of root-associated bacterial communities determines pathogen invasion and plant health. Nature Communications 6: 8413.

Weidner S, Latz E, Agaras B, Valverde C, Jousset A. 2017. Protozoa stimulate the plant beneficial activity of rhizospheric pseudomonads. Plant and Soil 410: 509–515.

Weitere M, Bergfeld T, Rice SA, Matz C, Kjelleberg S. 2005. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environmental Microbiology 7: 1593–1601.

West SA, Griffin AS, Gardner A. 2007. Evolutionary Explanations for Cooperation. Current Biology 17: R661–R672.

West SA, Griffin AS, Gardner A, Diggle SP. 2006. Social evolution theory for microorganisms. Nature Reviews Microbiology 4: 597–607.

Wildschutte H, Wolfe DM, Tamewitz A, Lawrence JG. 2004. Protozoan predation, diversifying selection, and the evolution of antigenic diversity in Salmonella. Proceedings of the National Academy of Sciences of the United States of America 101: 10644–9.

Wilkinson DM, Mitchell EAD. 2010. Testate Amoebae and Nutrient Cycling with Particular Reference to Soils. Geomicrobiology Journal 27: 520–533.

Willems A, Busse ' J, Goor M, Pot ' B, Falsen E, Jantzen E, Hoste B, Gillis ' M, Kersters ' K, Auling G, et al. 1989. Hydrogenophaga, a New Genus of Hydrogen-Oxidizing Bacteria. Microbiology 39.3:319-333

Wootton EC, Zubkov M V., Jones DH, Jones RH, Martel CM, Thornton CA, Roberts EC. 2007. Biochemical prey recognition by planktonic protozoa. Environmental Microbiology 9: 216–222.

Xiong W, Jousset A, Guo S, Karlsson I, Zhao Q, Wu H, Kowalchuk GA, Shen Q, Li R, Geisen S. 2018. Soil protist communities form a dynamic hub in the soil microbiome. The ISME Journal 12: 634–638.

Yera H, Zamfir O, Bourcier T, Viscogliosi E, Noël C, Dupouy-Camet J, Chaumeil C. 2008. The genotypic characterisation of Acanthamoeba isolates from human ocular samples. British Journal of Ophthalmology 92: 1139–1141.

Yubuki N, Leander BS. 2008. Ultrastructure and molecular phylogeny of Stephanopogon minuta: An enigmatic microeukaryote from marine interstitial environments. European Journal of Protistology 44: 241–253.

Zahn G, Wagai R, Yonemura S. 2016. The effects of amoebal bacterivory on carbon and nitrogen dynamics depend on temperature and soil structure interactions. Soil Biology and Biochemistry 94: 133-137.

Zancan S, Trevisan R, Paoletti MG. 2006. Soil algae composition under different agro-ecosystems in North-Eastern Italy. Agriculture, Ecosystems and Environment 112: 1–12.

Zhang S, Li Q, Lü Y, Sun X, Jia S, Zhang X, Liang W. 2015. Conservation tillage positively influences the microflora and microfauna in the black soil of Northeast China. Soil and Tillage Research 149: 46–52.

Zhang L, Lueders T. 2017. Micropredator niche differentiation between bulk soil and rhizosphere of an agricultural soil depends on bacterial prey. FEMS Microbiology Ecology 93: fix 103.

Zuber S, Carruthers F, Keel C, Mattart A, Blumer C, Pessi G, Gigot-Bonnefoy C, Schnider-Keel U, Heeb S, Reimmann C, et al. 2003. GacS Sensor Domains Pertinent to the Regulation of Exoproduct Formation and to the Biocontrol Potential of Pseudomonas fluorescens CHA0. Molecular plant-microbe interactions 16.7: 634-644.

Layman summary

Soil is a living natural resource and essential in the provisioning of food, clothing and building materials. Many soil properties are the product of soil-dwelling organisms, encompassing not only visible animals but also enormous diversity of soil microorganisms. Among soil microorganisms, bacteria and fungi have traditionally been the most described groups, however, this is only a part of the story going on in the soil, with the help of studies in soil food web, we are aware that soil bacteria/ fungi are under massive predation pressure by their predators – soil protists.

The best-known function of soil protists is as bacteria feeders, protists consume bacteria prey and further release the nutrients which were locked in bacteria biomass, those nutrients benefit the remaining bacteria and plant root. Moreover, protists do not feed on all prey bacteria equally, this selective feeding by protists can lead to shifting prey bacterial community structure, which may be linked with changes in soil community functioning. Given the lack of a generalized pattern of protists predation, the central question of this thesis is whether the protist predation follow certain patterns. I proposed to use protist traits as a means to address this question. Due to the focus of previous work on only one model protists and some closely related protists, I then collected and isolated 20 protists species with diverse morphotypes to investigate the central question.

I started by clarifying the taxonomic affiliations of the protists isolates used in this thesis, with a special focus on Heterolobosea amoeba, which are widespread and diverse in soil. Given the lack of morphologically useful characteristics in Heterolobosea amoeba, I further combined morphological characterization and molecular tools to identify these species and proposed a new species *Vahlkampfia soli*. This first soil *Vahlkampfia* species highlights the need for additional efforts in the future to cultivate previously unknown soil protists.

I continued to investigate the relationship between protist traits and their effects on prey bacterial community structure. I measured a range of protists traits, including growth rate, length, width, morphology and volume across my target 20 protist species covering the main phylogenetic lineages found in soil. I further used m experiments to assess the effect of each species on the structure of a semi-natural soil bacterial community. This work revealed that protists traits, especially cell volume, could be linked to their predation effect on the bacterial community. It was critical that cell volume calculations were not only based upon cell size but also considered geometric morphotype. Future studies seeking to detangle predation and

prey interactions as related to protist cell volume should, therefore, consider both cell size and morphotype.

To understand the functionality of protists, I then examined how protist predation and competition with other soil bacteria communities affect the stability of bacterial cooperative interactions. I utilized two types of bacterial strains, one is a cooperator bacterial strain that produces the public goods, the other one is a defector (cheater) bacterial strain that takes advantage of public goods without contributing to their production. I combined these two types of bacteria in the presence of competing soil bacterial communities and protists. This work revealed that multitrophic interactions strongly impacted microbial cooperation, in particular, competition increased the net benefit of cooperation at low and intermediate resource availabilities, however, predation followed the opposite pattern. Moreover, multitrophic interactions constrained the defector invasion and promote cooperation, but this effect strongly depended on resource availability.

Finally, I synthesized existing knowledge on soil protists and demonstrate their importance as regulators of the rhizosphere microbiome. I summarized the different reported interactions between protists predators and prey in the rhizosphere. I then addressed the known and hypothesized consequences of protists on microbiome functionality and plant performance. I presented a framework to guide efforts to harness protists as a microbiome enhancer in sustainable agriculture.

Overall, this thesis started with investigating protists traits, showing that protist predation was related to protists traits, namely, protist cell volume was informative concerning protist impacts on bacterial prey communities. Furthermore, this thesis has shown that predation by protists can reinforce cooperation in soil, and such cooperation may be linked to positive effects on ecological functions including plant growth and plant disease suppression.

中文简要

土壤是有生命力的自然资源，对人类的衣食住行至关重要。许多土壤特性归功于土壤中的生物，不仅包括可见的大型土壤动物还包括种类极其丰富的土壤微生物。目前，研究最多的土壤微生物是细菌和真菌，但是由于技术或培养方法的限制，有一部分微生物并未受到足够重视。前人通过对土壤中食物网的研究发现，土壤细菌和真菌都面临着来自土壤原生生物的捕食压力。

土壤原生生物最广为人知的功能是细菌的捕食者。原生生物捕食细菌并进一步释放细菌体内被固化的养分，这些释放的养分有利于土壤内其他细菌和植物根系的生长。但是原生生物不能机会均等地摄食所有细菌，它们对细菌的捕食是具有选择性的，这一选择性摄食会导致细菌群落结构的变化，从而影响土壤群落功能。但是鉴于对原生动物捕食模式的认知较少，本论文的中心问题是原生动物捕食是否遵循某种可预测的模式。由于前人的研究只集中在单一原生生物和一些亲缘关系近的原生生物上，本论文利用原生生物的生物性状作为选择依据，收集并分离了20种具有不同形态型的原生生物以研究本论文提出的问题。

首先，本论文阐明了目标原生生物分离株的分类学隶属关系，着重研究在土壤中广泛分布的异叶足纲（Heterolobosea）变形虫。鉴于异叶足纲变形虫缺乏可通过肉眼鉴别的形态学特征，本论文结合了形态学表征和分子特性来鉴定这些物种，提出了简便虫属（*Vahlkampfia*）中一个新种并命名为 *Vahlkampfia soli*。这个新物种是土壤分离出的第一个简便虫属物种，这个新物种强调了后续工作对分离土壤内未知新物种的重要性。

接着，本论文继续探究原生生物性状对其猎物细菌群落结构的影响。为此，本论文检测了一系列原生生物性状，其中包括原生生物的生长速率、细胞长度、细胞宽度、形态和体积。本论文中利用的20株原生生物均在土壤中广泛分布。论文进一步通过“微宇宙”系统来评估每个原生生物物种对半天然土壤中细菌群落结构的影响。结果表明，原生生物的性状尤其是细胞体积，与其与细菌群落的捕食作用有关。至关重要的是，本论文中细胞体积的计算不仅基于细胞的大小，还考虑了原生生物的几何形态。该结果表明，在后续对原生生物与细菌的互作的研究中应同时考

虑原生生物的细胞大小和形态。

为了了解原生生物的生态系统功能，本论文研究了原生生物的捕食作用及其与土壤细菌群落的竞争作用是如何影响细菌合作的稳定性。论文中使用了两种类型的细菌菌株：一种是产生公共物品的合作细菌，另一种是利用公共物品而不付出贡献的作弊（欺诈者）细菌，将两种细菌混合在一起并置于与其它土壤细菌群落的竞争和原生生物捕食状态下。研究表明，在中和低资源可利用性条件下，多营养级互作影响了微生物的合作关系，特别是与土壤内其他细菌的竞争增加了合作的净收益。但是，原生生物捕食遵循的是相反的模式。此外，多营养级互作限制了作弊细菌的入侵进一步促进了微生物之间的合作，但是这种效果在很大程度上取决于资源的可用性。

最后，本论文综合了有关土壤原生生物的现有研究，阐明了土壤原生生物调控根际微生物群落的重要性。总结了根际中原生生物捕食者与细菌之间相互作用，并且讨论了原生生物对微生物组功能和植物性能的已知或可能影响。最后提出了以利用原生生物作为可持续农业中的微生物群落增强剂一个工作框架。

综上，本论文从研究原生生物的生物性状开始，表明原生生物的捕食与其性状有关，即原生生物的细胞体积可解释原生生物对细菌群落的影响。此外，本论文还表明原生生物的捕食可以加强土壤中细菌的合作，而这种合作可能对生态功能起积极的影响，包括促进植物生长和抑制植物病害。

Nederlandse samenvatting

De bodem is een levende natuurlijke hulpbron en essentieel bij de voorziening van voedsel, kleding en bouwmaterialen. Veel bodemeigenschappen zijn het product van bodem organismen, die niet alleen zichtbare dieren omvatten, maar ook een enorme diversiteit aan bodem micro-organismen. Bacteriën en schimmels zijn van oudsher de meest beschreven groepen van bodem micro-organismen, maar deze zijn slechts een deel van het verhaal in de bodem. Met behulp van studies over het bodem voedselweb zijn we ons ervan bewust dat bodembacteriën en - schimmels onder enorme predatiedruk staan door hun predatoren (natuurlijke vijanden), de bodemprotisten.

De bekendste functie van bodemprotisten is als predator van bacteriën. Protisten consumeren bacteriën en ontsluiten daarna de voedingsstoffen die waren opgesloten in de biomassa van bacteriën, waarna die voedingsstoffen de resterende bacteriën en planten ten goede komen. Bovendien consumeren protisten niet alle prooibacteriën in gelijke mate. Doordat protisten selectief consumeren kan de structuur van deze bacteriële gemeenschap verschuiven, waardoor er mogelijk een verband is met veranderingen in het functioneren van de bodemgemeenschap. Gezien het ontbreken van een algemeen patroon van predatie, is de centrale vraag in dit proefschrift of de protistenpredatie bepaalde patronen volgt. Mijn voorstel was om de eigenschappen van protisten te gebruiken als een middel om deze vraag te beantwoorden. Omdat de focus van eerder onderzoek voornamelijk op één model protist en enkele nauw verwante soorten lag, heb ik 20 soorten protisten met verschillende morphotypes verzameld en geïsoleerd om te gebruiken als een middel om deze vraag te beantwoorden.

Ik begon met het verduidelijken van de taxonomische connecties van de protist isolaten die in dit proefschrift worden gebruikt, met een speciale focus op Heterolobosea amoeba, die wijdverspreid en divers in de bodem zijn. Gezien het ontbreken van morfologisch bruikbare kenmerken in Heterolobosea amoeba, heb ik verder morfologische karakterisering en moleculaire hulpmiddelen gecombineerd om deze soorten te identificeren en een nieuwe soort *Vahlkampfia soli* voorgesteld. Deze eerste bodem *Vahlkampfia* soort benadrukt de behoefte aan extra inspanningen in de toekomst om voorheen onbekende bodemprotisten te cultiveren.

Ik ging door met het onderzoeken van de relatie tussen protist eigenschappen en hun effecten op de structuur van de prooi bacterie gemeenschap. Ik heb een reeks protist eigenschappen gemeten, waaronder groeisnelheid, lengte, breedte,

morfologie en volume van mijn 20 target protist soorten die de belangrijkste fylogenetische lijnen in de bodem omvatten. Ik gebruikte verder microcosm experimenten om het effect van elke soort op de structuur van een semi-natuurlijke bodembacteriegemeenschap te beoordelen. Uit dit werk bleek dat eigenschappen van protisten, met name het cel volume, kunnen worden gekoppeld aan hun predatie-effect op de bacteriegemeenschap. Het was van cruciaal belang dat berekeningen van het cel volume niet alleen gebaseerd waren op de celgrootte, maar dat ook het geometrische morfotype in beschouwing werd genomen. Toekomstige studies die proberen predatie en prooi-interacties te ontwarren met relatie tot het cel volume van protisten, moeten daarom zowel de celgrootte als het morfotype overwegen.

Om de functionaliteit van protisten te begrijpen, onderzocht ik vervolgens hoe protist predatie en concurrentie met andere bodembacteriëngemeenschappen de stabiliteit van bacteriële coöperatieve interacties beïnvloedden. Ik gebruikte twee soorten bacteriestammen; één is een coöperatieve bacteriestam die de publieke goederen produceert, de andere is een defector (valsspeler) bacteriestam die voordeel haalt uit de publieke goederen zonder bij te dragen aan hun productie. Ik combineerde deze twee soorten bacteriën in aanwezigheid van concurrerende bodembacteriegemeenschappen en protisten. Uit dit onderzoek bleek dat multitrofe interacties een sterke invloed hadden op microbiële samenwerking, met name concurrentie verhoogde het netto voordeel van samenwerking bij lage en gemiddelde beschikbaarheid van middelen. Predatie echter volgde het tegenovergestelde patroon. Bovendien beperkten multitrofe interacties de invasie van de defector en bevorderden de samenwerking , maar dit effect hing sterk af van de beschikbaarheid van middelen.

Tenslotte heb ik bestaande kennis over bodemprotisten gesynthetiseerd en hun belang als regulatoren van het rhizosfeer microbioom aangetoond. Ik heb de verschillende gerapporteerde interacties tussen predator en prooi in de rhizosfeer samengevat. Vervolgens heb ik de bekende en veronderstelde gevolgen van protisten op de microbioom functionaliteit en op prestatie van planten besproken. Ik presenteerde ook een kader om de inspanningen te sturen om protisten als microbioom verbeteraar te benutten in duurzame landbouw.

Samengevat begon dit proefschrift met het onderzoeken van eigenschappen van protisten, waaruit bleek dat predatie door protisten gerelateerd was aan eigenschappen van protisten. Met name het cel volume van de protisten was informatief wat betreft de impact van protisten op de bacteriële prooi gemeenschap. Verder heeft dit proefschrift aangetoond dat predatie door protisten de samenwerking in de

bodem kan versterken, en dergelijke samenwerking kan worden gekoppeld aan positieve effecten op ecologische functies, waaronder plantengroei en plantenziekten bestrijding.

Doing PhD is a challenging adventure, but I am still glad to get on this journey four years ago, most importantly, along this journey I am so lucky to be supported, inspired and loved by you all.

George, I am grateful to have your supervision in the past four years, the way how you think will forever influence me in science and life. Thank you for always supporting my ideas and guiding me how to tell my scientific story clearly. I will never forget your saying of 'science is art', encouraging myself to treat my work as a piece of art.

Alex, thank you for introducing me to this cute protist world, since then they are a part of my life, I not only see them in the lab but also in my dream. Thank you for instructing me to think from *impossible* to *I'm possible*, as well as caring for me in my daily life. You are a terrific second 'Dad' to me.

Stefan, I am so grateful that you agreed to be a part of the supervision team three years ago, thank you for sharing me so much protist knowledge and experience that I can not learn from books. I would not be confident to say I am a protistologist without your input and support.

I would like to thank all current and former members of Ecology and Biodiversity group, the moments together with all of you were my treasure in the Netherlands. Special thanks to Peter, thanks for all your help and support for everything along the way, I can not imagine our lab without you; Betty, thanks for your Dutch inputs, I can pronounce Rabarber better now; Gerrit, thank you for the lekker appelolaakoekmix, I still owe you one homemade appeltaart; Bedankt Bertus en Boudewijn voor het aanmoedigen van mij om Nederlands te spreken; Merel, thanks for those interesting hallway conversations about China; Thanks Mariet for sharing so much soil knowledge with me; Thanks Marijke for nice discussion during coffee break; Thanks Yann for always being helpful with statistics. Ida, I was so lucky to work with you at very beginning of my PhD, thank you for being my mentor in science, which made my start much easier; Tianjie, we already had such a special connection at the first time we met at E&B corridor, thank you for being my life mentor, showing me how to survive in the Netherlands; Erqin, you are smart, thank you for guiding me through many casual or scientific talks, talking with you is joyful and open-minded, you are already a wise Prof. Li to me; Thanks Hujie for helping me to get through those tough moments, especially seeing me cry for R, life is getting better and better now; Thanks Nathalie, for your positive energy, cheering me up always, also thank you for still being friends with me after crazy 504 hours in Brazil; Xiong, thanks for all your kind help along the way, and of course your spicy food! Limei, Enjoy our

house in Klaverstraat; Floriana, I enjoyed those moments that we used exaggerated body language for talking and thank you for making me laugh; Thanks Prof. Li for being friends with us, see you in Nanjing! Thanks Pengfei for your fitness tips and good luck with your career; Thanks Mohammad, for your always relaxed attitude; Amber, thanks for your nice advice on how to overcome difficulties during PhD; Beste Anouk, Bedankt voor de Chinese-Nederlandse uitwisseling, vooral hoe te vermijden om Nederlands 'rrrrrr….' te spreken; I also would like to thank Eirini, Edwin, Pilar, Carmen, Ana, Rens, Joeri, Monique, Joost, Susanna, Heinjo for nice interactions inside or outside group.

I am glad to have awesome officemates in the past four years, Simone and Violeta, my dear sisters, thank you for those unforgettable moments we spent either inside or outside the office. Simone, you are my special and important witness for my changes during PhD, thank you for always being on my side and sharing your experience on how to say No, now I am quite addicted to it; Violeta, you are sweet and considerate, thank you for caring for me all the time! Good luck with finishing PhD without too much stress, you can do it! Robert, our office brother, thank you for always being nice and bearing us, I know three girls can create a lot of noise in the office; I also would like to thank my short-term office mate; Erik, Rob, Annemarie for the short but enjoyable office time together.

I would like to thank all my students; Xueyang, Giel, Fabian, Giovanna, Qiqi, you are great students, thank you for your great work!

Along this PhD jorney, I also received support and help from colleagues in our department, and I enjoyed time during IEB meetings and other activities, thank you all: Giannis, Gilles, Yang, Joyce, thanks again Hao for listening me complaining (PMI group), Sarah, Chrysoula and Tom (EvP), Freek and Peter Jan (Microbiology group).

Dan Dan, my crazy buddy, I always feel brave when doing stupid things together with you, thank you! Look forward to you going back to Europe; Sofija, you are sunshine, stay shining! Good luck with your PhD and look forward to seeing you again! Ruth, I am so glad to live with you for more than two years, thank you for being my family here and caring for me so much along the way; Thanks my JDC crew for dancing together for the show!

Voor mijn Nederlandse familie; bedankt dat jullie zoveel voor me hebt gezorgd, bedankt voor de gezellige tijd met wintersport, oliebollen, koffie en eten …. Jullie

hebben me hier erg thuis laten voelen. Natuurlijk zal ik nooit belangrijke Nederlandse woorden vergeten die jullie me hebben geleerd, opzouten heeft niets met zout te maken. My special and profoundest thanks to Julian, thank you for being there for me always! I count myself lucky to have you in my life, you always have ways to cheer me up with your unique mumbling talking, thank you for reminding me there is still a life besides PhD, and pushing me away from my PhD (in a good way), especially training me ice skating for Beijing 2022 Winter Olympic. I will try! Most importantly, thank you for loving me as I am, I love you!

感谢潘，龙哥， 邓博士，丁彧四年来的照顾，支持与鼓励；

感谢王依冬老乡四年多不间断给我带来家乡美味，以解我思乡之情；

感谢笑纯老弟在荷兰带我疯带我玩儿；

感谢段老师追随我来荷兰读博，在格村要坚强；

感谢我老铁霜霜线上相伴写论文，老铁等你的答辩好日子；

感谢三人游的大宝和昆哥，期待再次相聚；

感谢任荻秋女士一直以来的陪伴，愿你的美漂生活幸福安康；

最后感谢我的家人，谢谢你们支持我所有的选择，你们是我最暖的避风港；特别感谢我远在家乡的老父亲老母亲，感谢我彭姐的“严格管教”，感谢我高哥的“差不多就得了”，你们是我最坚强的后盾，比心！

最最后，致敬并致谢我已故的姥爷！

以上。

This thesis is printed CO_2 neural by planting two trees via Tree for All

About the Author

Zhilei Gao (高之蕾) was born on 22nd October 1990 in Beijing, China. In September 2009, she started her bachelor study at China Agricultural University, with a specialization in Horticulture. In September 2012, she joined the Master program of Agricultural Extension in China Agricultural University, this project closely collaborated with the Institute of Plant Protection in the Chinese Academy of Agricultural Sciences. During her Master internship, she isolated bacterial antagonists in soil against pathogen of crown gall disease in peachtree and further tested their efficiency in the field. However, several isolates worked well in the lab but poorly performed in the field. This made her realize the complex in soil and encourage her to learn further in ecology. In October 2015, she joined the Ecology and Biodiversity group at Utrecht University as a PhD candidate, with the supervision of Prof.dr.George Kowalchuk, Dr. Alexandre Jousset and Dr. Stefan Geisen (Wageningen University). She investigated the soil protists' traits and their effects on ecosystem functions. This thesis is the result of her PhD project. In October 2019, she started at Ecostyle as a scientific researcher.

Publication

Gao Z, Karlsson I, Geisen S, Kowalchuk G, Jousset A. 2019. Protists: Puppet Masters of the Rhizosphere Microbiome. Trends in Plant Science 24: 165–176.

Want to watch how protists move? Scan me!