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Distribution patterns of soil microbial eukaryotes suggests widespread algivory by phagotrophic protists as an alternative pathway for nutrient cycling

Christophe V.W. Seppey [a, *], David Singer [a], Kenneth Dumack [b], Bertrand Fournier [c], Lassaâd Belbahri [a], Edward A.D. Mitchell [a, d], Enrique Lara [a]

[a] Laboratory of Soil Biodiversity, University of Neuchâtel, Rue Emile-Argand 11, Neuchâtel, Switzerland
[b] Department of Terrestrial Ecology, University of Cologne, Zülpicher Strasse 47b, Köln, Germany
[c] Department of Biology, Concordia University, 7141 Sherbrooke Street West, Montreal, Canada
[d] Jardin Botanique de Neuchâtel, Chemin du Perthuis-du-Sault 58, CH-2000 Neuchâtel, Switzerland

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ABSTRACT

High-throughput sequencing (HTS) of soil environmental DNA (eDNA) allows assessing the full diversity of soil micro-eukaryotes. The resulting operational taxonomic units (OTUs) can be assigned to potential taxonomic and functional identities using increasingly complete reference databases. HTS of soil eDNA is revealing a high diversity and abundance of potential eukaryovorous protists, thus challenging the paradigm of the predominantly bacterivorous function of soil phagotrophic protists (i.e. microbial loop).

Using Illumina sequencing of soil eDNA and targeting the V9 region of the SSU rRNA gene, we investigated the taxonomic and functional diversities, distribution and co-occurrence patterns of soil micro-eukaryotes in three land-use categories: forests, meadows and croplands located in Switzerland. Each OTU was assigned to a broad functional category (phototrophs, phagotrophs, osmotrophs, or parasites).

Total OTU richness was similar in the three land-use categories, but community composition differed significantly between forests and other land-uses. The proportion of fungal sequences (especially Basidiomycota) was highest, and phototroph (i.e. soil microalgae) sequences least abundant in forests. Seven OTUs representing phagotrophic protists, together accounting for >25% of all phagotroph sequences, were significantly correlated to the total number of phototroph sequences, thus suggesting algivory. At least three of these OTUs corresponded to known algal predators.

These results suggest that beyond plants, soil microalgae represent a functionally significant but rarely considered input of carbon in soils that should be taken into account when modelling soil nutrient cycling.

1. Introduction

Our perception of the diversity and functional roles of protists is rapidly changing due mainly to the application of high-throughput sequencing (HTS) of environmental DNA (eDNA). HTS has revealed the extent of the huge unknown protist diversity in the photic zone of the world's oceans and shown that a large fraction of this diversity corresponded to mutualistic and parasitic symbionts (de Vargas et al., 2015). Likewise, studies performed on terrestrial habitats are revealing similarly high diversity of protists with a dominance of saprotrophs and parasites (Dupont et al., 2016; Geisen et al., 2014, 2015a). These studies also revealed that many protists feed on eukaryotes, thus questioning the long-held view that soil phagotrophs fed mainly on bacteria (i.e. soil microbial loop) (Dumack et al., 2016a,b; Geisen et al., 2015b, 2016; Geisen, 2016).

Soil microbial eukaryotes, including protists and fungi, are involved in numerous biotic interactions and recognised as key actors of biogeochemical cycling (Verni and Gualtieri, 1997; van der Wal et al., 2013), and are thus considered a key element in soil fertility. However, the first (and still often the only) recognised

* Corresponding author.
E-mail address: christophe.seppey@unine.ch (C.V.W. Seppey).

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functional role of soil protists was grazers of bacteria leading to the "soil microbial loop" paradigm, according to which phagotrophic grazing on soil bacteria releases labile compounds such as ammonium that stimulate plant growth (Bonkowski and Clarholm, 2012; Clarholm, 1985). Although feeding on bacteria is unquestionably widespread in phagotrophic microbial eukaryotes, there is increasing evidence that eukaryovory (i.e. the act of feeding partially or exclusively on other eukaryotes) is also common (Dumack et al., 2016a,b; Geisen et al., 2016). This implies that soil nutrient cycles are likely more complex than generally assumed.

Recent studies focusing on soil invertebrates have also questioned the origin of the carbon source feeding the soil communities, suggesting that very few soil invertebrates depend on litter (Pollierer et al., 2009) and suggesting that soil algae represent a functionally relevant source of soil carbon (Schmidt et al., 2016). The latter experimental study showed that autotrophic microbes contributed up to 17% of the body carbon of collembolan and 3% of earthworms over one week. However it is yet unclear to what extent this input is direct or if algae are first ingested by microbial grazers such as soil phagotrophs.

Several soil protists are known to be highly specialized predators of eukaryotes. For example, grossglockneriid ciliates feed exclusively on fungi (Petz et al., 1985). Parasitoids are also frequent in soils, including the widespread but still poorly studied *Rozella* group (also known as "Rozellida"; Lara et al., 2010 or Cryptomycota Jones et al., 2011) which prey on chytrids, oomycetes and green algae and also include endo-nuclear parasites of Amoebozoa that ultimately cause cell death and lysis (Corsaro et al., 2014). In those cases, nutrient release by protists does not rely on bacterivory, implying pathways for nutrient cycling alternative to the microbial loop. It is unclear how quantitatively relevant this pathway is but one way to assess this is to study the diversity and abundance of taxa involved in these trophic relationships using the now available data from massive sequencing of soil environmental DNA.

The true diversity of soil protists has long been poorly known, mainly due to methodological limitations for their isolation, culture and subsequent identification (Ekelund and Ronn, 1994; Foissner, 1999). Metabarcoding (environmental DNA amplicon based identification) of high-throughput sequencing data is now the golden standard for environmental screening of microbial diversity (Pawlowski et al., 2016). HTS data may also inform on the functioning of ecosystems based on the genetic identification of the organisms and knowledge on their lifestyles (de Vargas et al., 2015; Lara et al., 2015; Massana et al., 2014). The next step is to infer the biotic relationships between these organisms, which can be hypothesized when OTUs co-occur systematically across many samples, as can now be assessed by HTS. In practice, the nature of these relationships (i.e. trophic, but also symbiosis, competition, etc.) is not known, and co-occurrence data can thus be difficult to interpret in biological terms. Examples of known relationships taken from the literature can however illustrate well-supported co-occurrence and clarify the true nature of these relationships between organisms. Examples are manifold: predation of ciliates on fungi (Petz et al., 1985), of cercozoa on chlorophytes (Dumack et al., 2016a; Hess et al., 2012; Hess and Melkonian, 2013) but also symbioses, like between trebouxiophytes and testate amoebae (Gomaa et al., 2013). Putative relationships inferred from metabarcoding studies can also be explored by conducting new observations and experiments.

Phototrophic protists (i.e. eukaryotic algae) in soils include mostly exclusive free-living phototrophs (e.g. Bacillariophyta, Chrysophyceae, Xanthophyceae) and photosymbionts as in lichens (e.g. Trebouxiophyceae). Soil eukaryotic algae constitute an important part of the so-called cryptogamic crusts, which represent a significant carbon input in arid ecosystems (Elbert et al., 2012; Freeman et al., 2009; Frey et al., 2013). They are however also widespread in more humid soils but their functional role there is less well known and, consequently, has not been considered in the classical model of the soil microbial loop (Berard et al., 2005).

In order to assess the patterns of micro-eukaryotic taxonomic and functional diversities and address questions such as the possible role of soil algae as a carbon source it is useful to compare contrasted terrestrial ecosystems. Here we describe and compare the overall diversity and community structure of soil micro-eukaryotes in forest, meadow and cropland soils from 44 sites in Switzerland based on Illumina sequencing of the V9 region of SSU rRNA gene. Based on these data, we explored more specifically the abundance patterns of phototrophs and the co-occurrence patterns with their potential phagotroph predators. This trophic link was also explored by direct microscopic observations.

2. Material and method

2.1. Sampling

We collected 44 soil samples in permanent plots of the Swiss Biodiversity Monitoring program which aims to assess biodiversity all over Switzerland (BDM http://www.biodiversitymonitoring.ch/en/home.html). The sites included three land-uses which cover most of the Swiss territory (16 forests, 16 meadows and 12 croplands) (Fig. 1, Table S1) and spanned a diversity of soil types that could be arguably considered as representative of the entire country. Likewise, samples were collected in a range of altitudes covering most of the Swiss territory (excepted alpine sites). We expect therefore to cover a significant part of the microeukaryotic diversity present in Swiss soils. In this purpose, each sample was characterized using the typology of Swiss natural habitats (Delarze et al., 2015) (Table S1). Forests included both coniferous (e.g. *Picea abies*), or broadleaved trees (e.g. *Fagus sylvatica*). Most meadows were amended and used to produce fodder. Croplands were used for maize, cereals or tobacco cultivation. Meadows and croplands were designed as open habitats as much more light reach their soil surface than in forests. Sampling was performed over one month between September 27th, 2012 and October 31st, 2012. At each site, three topsoil cores (5 cm diameter x 5 cm depth) were taken along a circle of 1 m radius in the same land-use and pooled. Soil samples were kept cool (in an icebox) and DNA was extracted within 2–3 days.

2.2. DNA extraction, amplification and sequencing

DNA was extracted using the MoBio PowerSoil extraction kit (Carlsbad, CA, USA) according to the manufacturer instructions. The SSU rRNA V9 region was amplified using the broad spectrum

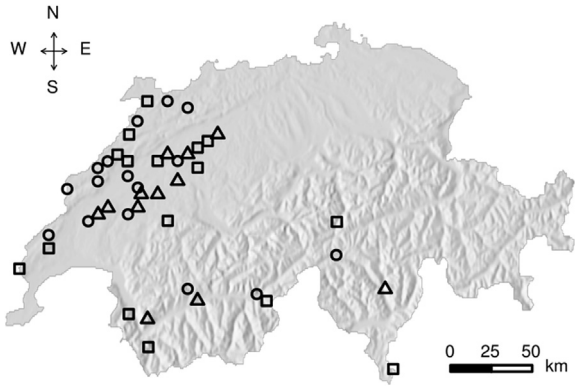

Fig. 1. Location of the 44 sampling sites in Switzerland. Squares, circles and triangles indicate forests, meadows, and croplands, respectively.

eukaryotic primers 1380F/1510R (CCCTGCCHTTTGTACACAC/CCTTCYGCAGGTTCACCTAC) (Amaral-Zettler et al., 2009). We used the smaller V9 region in this study instead of V4 because (1) we expected to have less taxonomical biases by using a fragment whose length is almost constant in all eukaryotes (as opposed to, for instance, the V4 region; de Vargas et al., 2015) and (2) because the fragment is shorter, the probability of generating artefactual diversity (i.e. chimeras) is lower (Valentini et al., 2009). PCR reactions were run in triplicates with a PTC-200 Peltier Thermo Cycler (BioConcept, Allswill, Switzerland) with 1 ng of environmental DNA, 6 μL of 10 x PCR buffer, 0.6 μL of each primer, 0.6 μL of each dNTP 400 μM (Promega, Dübendorf, Switzerland) and 0.2 μL of 0.05 U/μL GoTaq (Promega, Dübendorf, Switzerland). The volume was adjusted to 30 μL with ultra-pure water. Amplification was conducted with the following conditions: denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 57 °C for 60 s and 72 °C for 90 s and final extension at 72 °C for 10 min (Amaral-Zettler et al., 2009). PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and pooled together at the same concentration prior to sequencing. A DNA library was prepared using the New England Biolabs's kit NEBNext DNA Sample Prep Master Mix Set 1. Illumina HiSeq sequencing was done by Fasteris (Geneva, Switzerland) using an Illumina HiSeq 2000 technology to obtain paired-end reads (2 × 100bp).

2.3. Bioinformatic analyses

The PR^2 database (Guillou et al., 2013) was used as the reference database for a first taxonomic assignation of reads and OTUs; only sequences containing complete forward and reverse primers described above were retained. SSU sequences from bacteria and archaea were also added to the PR^2 database from the Silva database (Pruesse et al., 2007) in order to identify and remove eventual prokaryotic sequences from the analysis. Prokaryotic SSU sequences were truncated from the general primer 1389F (TTGTACACACCGCCC) (Amaral-Zettler et al., 2009) to the end of the SSU rRNA sequence and kept as ortholog of the eukaryotic V9 fragment. The truncated prokaryotic sequences were then de-replicated before being added to the PR^2 database.

Reads were merged using the program Flash (v. 1.2.9) (Magoc and Salzberg, 2011) and demultiplexed into samples using the program Sabre (https://github.com/najoshi/sabre). Only sequences containing complete forward and reverse primers described above were kept. Good quality sequences were selected according to the method used in de Vargas et al. (2015). Chimeric sequences were then discarded using the software Usearch (v. 7.0.1090) (Edgar et al., 2011) by comparing reads against the PR^2 (Guillou et al., 2013) and Silva (Pruesse et al., 2007) databases and against reads within the sample. In order to remove artefactual sequences, we kept only those that were found at least three times in two samples (de Vargas et al., 2015).

OTUs were clustered using the software Swarm (v. 1.2.5) (Mahé et al., 2014) with the default set-up. OTUs were then taxonomically assigned by aligning the dominant sequence of every OTU against the PR^2 database using Ggsearch (Fasta package v. 36.3.6 Pearson, 2000). The OTUs were considered as undetermined eukaryotes if their percentage identity with sequences of PR^2 was lower than 80% as in de Vargas et al. (2015). We also removed sequences belonging to prokaryote, Metazoa or Embryophyceae. In order to homogenize the number of reads present in all samples for further numerical analyses, we randomly selected 50′000 for each sample.

2.4. Assignation to functional groups and numerical analyses

We selected 41 taxa with well-characterized trophic function (i.e. 5 osmotrophs, 5 parasites, 6 phototrophs, 25 phagotrophs) in the list of divisions, classes and orders of the PR^2 assignation for the diversity analyses (Table S1).

As a first comparison of community composition, we calculated the Shannon index and performed a non-metric multidimensional scaling (NMDS) analysis on OTU abundances. We assessed the difference in diversity among land-use types with a non-parametric multiple comparisons Nemenyi test (Hollander and Wolfe, 1999) (posthoc.kruskal.nemenyi.test function, package PMCMR v. 4.1 Pohlert, 2014), and also calculated NMDSs for each pairs of land-use and tested the community difference by a permutation test (envfit function vegan package v. 2.0–10, Oksanen et al., 2013). P-values were multiplied by three to take into account multiple tests adjustment (Holm, 1979).

We then assessed in which environment sequences belonging to phototroph organisms were most abundant using a Nemenyi test. To retrieve putative algae consumers, we measured the correlation between each of the 100 most dominant phagotroph OTUs and the total abundance of phototrophs, taking also into account land-use as second environmental variable in linear models (LMs). To normalize the distribution of both phagotroph OTUs and total phototroph abundance we log transformed their sequences abundances (decostand function, vegan package v. 2.0–10, Oksanen et al., 2013). The two environmental variables (i.e. total phototroph abundance, land-use) were tested independently in the LMs as none of the model tested showed significant interaction. We finally adjusted the p-values of the two environmental variables for the 100 models according to Holm (1979). We also verified if each of the LM respected conditions of residuals normality and homoscedasticity by performing a Shapiro test on model residuals and non-constant variance test (function shapiro.test and ncvTest, packages car v. 2.0–20; Fox and Weisberg, 2011, and stats v. 3.1–0; R Core Team, 2014 respectively). OTUs respecting the LM conditions and showing a significant correlation with phototroph abundance, were selected as putative alga consumers and their taxonomy was verified on GenBank by using Blast with the default parameters.

2.5. Isolation of protists and microscopic observation on algivorous behaviour

To illustrate the trophic interactions among selected protists and test if identified co-occurrences indeed could be interpreted in terms of trophic relationships, we documented by microscopical observations organisms from the same genus/species as the OTUs whose abundances were positively and significantly correlated with those of algae. *Rhogostoma* sp. was isolated from leaf surfaces (Cologne, Germany), *Leptophrys vorax* was isolated from a freshwater puddle (Cologne, Germany), and *Trinema* sp. appeared as a contamination in such protist cultures. All protists were morphologically determined. The illustrated organisms were identified morphologically based on unmistakable criteria, which were corroborated by taxonomic literature (Howe et al., 2011; Hess et al., 2012; Lara et al., 2007) but were not sequenced in the frame of this study.

The pictures of *Leptophrys vorax* were obtained from an individual directly taken from a natural sample. Other organisms were cultured in Waris-H medium (McFadden and Melkonian, 1986) at room temperature on a window bench and enriched with *Characium* sp. and an undetermined coccoid green alga. The cultures were checked for potential algal ingestion after three days of incubation, using an inverted microscope (Nikon Eclipse TS-100, Japan) at 100x and 400× magnification. Pictures were taken with a Nikon digital sight DS-U2 camera (program: NIS-Elements v 4.13.04) and a Nikon Eclipse 90i (DIC, up to 600× magnification).

3. Results

3.1. Data quality and overall diversity

The full dataset contained 15′365′116 raw reads, of which 93.9% passed the quality check, 87.5% were found at least three times in two samples, 87.4% were not considered as chimeras, and 77.4% were not considered as Metazoa, Embryophyceae or prokaryotes. Therefore, a total of 11′893′592 reads were left for further analyses. In the dataset adjusted to 50′000 sequences by sample, we retrieved a total of 18′586 OTUs, of which 87% could be taxonomically assigned unambiguously according to the assignation threshold; altogether, representing 97% of the reads and 75% of the OTUs (Fig. S1, Fig. S2). The most abundant supergroup of eukaryotes in all samples were Opisthokonta (Fungi), followed by Rhizaria (Cercozoa) and Stramenopiles.

The most noticeable difference in relative abundance of taxa could be observed between open and forest habitat, and was mostly due to a divergence in the abundance of Basidiomycota (Fig. S1). In contrast, richness did not differ deeply between land-use types, and varied between 2371 and 3516 OTUs. Richness was dominated by both Fungi and Rhizaria, more or less in equal proportions, followed by Stramenopiles (Fig. S2).

Shannon diversity and micro-eukaryotic community composition differed significantly between forest and open habitats (meadows and croplands) (Nemenyi test, and permutation test on NMDS after correction, $P < 0.001$) while diversity and communities did not differ significantly between meadows and croplands ($P > 0.05$; Fig. 2 and Fig. S3).

3.2. Diversity and abundance patterns

Fungi (the large majority of the osmotrophic taxa) represent more than 50% of the overall abundance and 43%, 51% and 67% of all reads in croplands, meadows and forests respectively (Fig. S1). The dominance of Fungi in forest samples was mostly due to the presence of a single Basidiomycota OTU (X3), which accounted for 38% of the totality of all reads in forests. Although taxonomic resolution of the SSU rRNA is too low to differentiate between fungal species, the OTU X3 could be assigned (100% match) to a wide array of Agaricomycotina, (e.g. *Leucopaxillus*, *Ampulloclitocybe*). In addition to Basidiomycota, the two next dominant groups of fungi were the Ascomycota and the Mucoromycota.

Potential parasites reached 3.9% of the overall abundance representing 2.7%, 4.4% and 4.8% of the reads of croplands, meadows and forests respectively. Among these numbers, Oomycota represented the large majority of parasites abundance regardless of the land-use (75%, 61%, 57% in croplands, meadows and forests respectively). The five most abundant Oomycota OTUs were assigned with confidence (≥94% of identity) to genera *Aphanomyces* (X99), *Pythium* (X53, X8), *Pythiopsis* (X31) and *Saprolegnia* (X30). Oomycota were followed in abundance by Mesomycetozoa, Gregarinasina and Phytomyxea, whose relative abundance varied depending on the land-use (8%–21%, 4%–17% and 5%–11% respectively).

Sequences belonging to OTUs assigned to phototrophic organisms accounted for 1.9% (42′475 sequences) of all sequences. This proportion was highest in open habitats, representing 3.5%, 1.8% and 0.9% of the sequences found in croplands, meadows and forests, respectively (Fig. 3). As for overall community patterns, this difference was statistically significant between forests and the other two land-uses (Nemenyi test after correction, $P < 0.01$; Fig. 3). The diversity of phototrophic micro-eukaryotes was largely dominated by Chlorophyceae, followed by diatoms (=Bacillariophyta), and Trebouxiophyceae or Xanthophyceae; the rest being shared by other typical subaerial algae like Ulvophyceae and other Archaeplastida (Fig. 3).

OTU assigned to phagotrophic organisms accounted for 32% (704′308 sequences) of all sequences. Seven of the 100 most dominant phagotroph OTUs showed a positive correlation to total phototroph sequence abundance, and respected the conditions of residuals normality and homoscedasticity (Fig. 4 Fig. S4 and Table S4). These OTUs belong to Cercozoa (X2, X117, X64, X54), Ciliophora (X321) and Stramenopiles (X12, X343) and together account for 27% of the phagotroph sequence abundance and 8% of the total abundance of all sequences of the dataset (Table S3). Apart from one OTU assigned to Labyrinthulea (X343), all other OTU sequences obtained a good match (≥97%) with sequences from the GenBank database (Table S3). In addition to these seven OTUs we observed that X34 (Glissomonadida, group of *Viridiraptor*) was correlated to the abundance of eukaryotic algae and among the ten most abundant phagotrophic OTUs despite the fact that the linear model did not respect the conditions of homoscedasticity (Fig. 4, Tables S3 and S4, and Fig. S4). In addition to these eight OTUs, seven other OTUs were also correlated to total phototroph sequence abundance but were rare and without a homoscedastic distribution (Table S4).

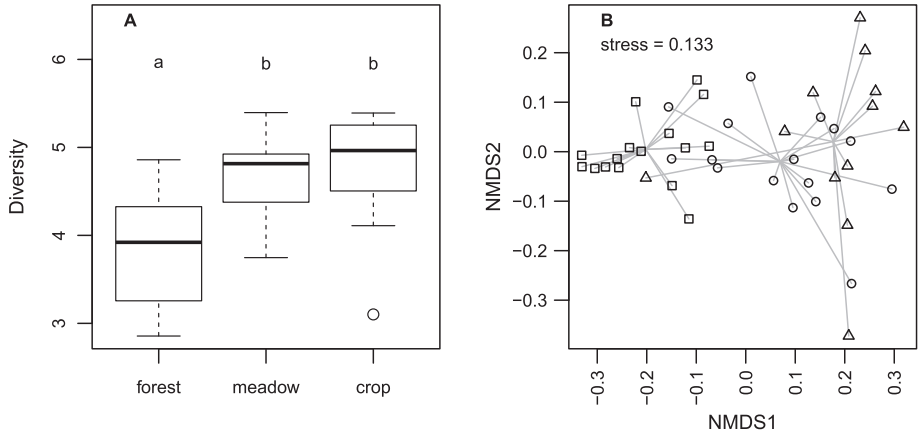

Fig. 2. (A) Shannon diversity of micro-eukaryotic OTUs in Swiss forests, meadows, and croplands - letters above the boxplots represent groups of environments expressing significant different diversity distribution according to a Nemenyi test ($P < 0.05$); (B) non-metric multidimentional scaling (NMDS) based on Bray-Curtis dissimilarities of 44 soil samples from Switzerland. The three land-uses are denoted by squares (forests), circles (meadows) and triangles (croplands).

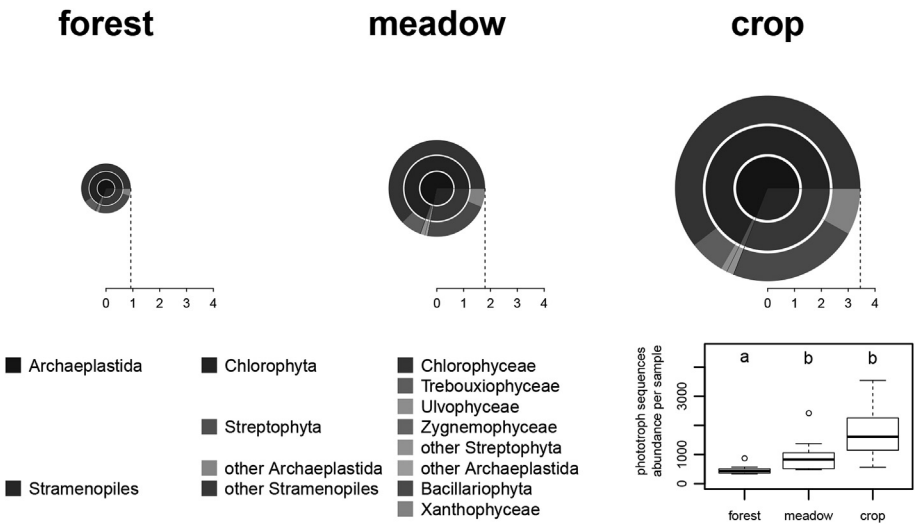

Fig. 3. Top: Relative abundance of phagotroph taxon sequences in Swiss forests, meadows, and croplands. The radius of the pie-chart represents the percentage of phototroph sequences in each land-use type. Taxa representing less than 1% of the land-use are represented in the "other Streptophyta" or "other Archaeplastida" sections. Bottom right: Abundance of phototroph sequences according to the land-use. Letters on the top part of the boxplots represent the groups of land-use formed according to the Nemenyi test ($P < 0.05$) on the phototroph sequences abundances.

3.3. Microscopic observations

We screened environmental samples to find organisms corresponding to the taxonomic assignation of OTUs significantly correlated to the sequence abundance of phototrophic protist taxa and found three (i.e. X2, X64, and X117) out of the seven identified by our analyses. All three organisms were observed with most likely ingested algal material, either in the natural samples (*Leptophrys vorax*, potentially linked with X64), or when incubated with algal cells (*Rhogostoma* sp., *Trinema* sp., potentially linked with X2 and X117) (Fig. 5). *Rhogostoma* spp. are characterized by the presence of a hyaline theca with a cleft-like apertural opening (not shown) and filopodia. Similar to *Rhogostoma* spp., *Trinema* spp. exhibit filopodia, but in contrast bear large circular scales and a subterminal, ovoid or round aperture. The filose genus *Leptophrys* vorax is characterized by being naked, sometimes with slightly orange cytoplasm, the ingestion of diverse groups of algae and the transformation between the isodiametric and expanded morphotype. Since all these features were observed in our isolates, we determined them as such.

4. Discussion

Metabarcoding studies are revealing not only a huge unknown diversity but also unsuspected trophic interactions in every studied environment (de Vargas et al., 2015). The predation of phototrophs by heterotrophic protists suggested by our data implies a carbon input to the soil ecosystem that was not taken into account by the traditional microbial loop model and is in line with a recent study focusing on soil invertebrates (Schmidt et al., 2016).

4.1. Overall diversity and community patterns

The diversity patterns of individual micro-eukaryotic groups across the three land-use types is coherent with the contrast among these habitats. Fungi dominate micro-eukaryotic communities in forest soils (Behnke et al., 2011; Geisen et al., 2015c; Glaser et al., 2015; Lesaulnier et al., 2008). In our data, this dominance was explained by the presence of the OTU X3, which is assigned to Fungi that build ectomycorrhiza (e.g. *Leucopaxillus*, *Ampulloclitocybe*) (Cairney and Chambers, 1999) and thus most likely establishes symbiotic relationships with trees. Alternatively, some Fungi represented also by OTU X3 (e.g. Auriculariaceae, Panaeolus) (Boddy et al., 2007) are known as wood decomposers, which would explain their high abundances in forests.

The next most represented groups (Stramenopiles, Rhizaria) comprise also organisms that can be encountered often in soils, such as Oomycota, Cercomonadida and Chrysophytes (Lesaulnier et al., 2008). Fungi, despite of being by far, the most abundant microbial eukaryotes in soils, had a richness which was comparable to Rhizaria (Fig. S2). This is most probably due to the highly ramified hyphae, and high biomass, in comparison to the mostly small and unicellular Rhizaria.

The distribution of parasites follows the different land-use characteristics. The deceasing abundance of Arthropod parasites (Mesomycetozoa and Gregarines) from forests to meadows to is in line with the corresponding decline in plant biomass, habitat complexity and diversity of ecological niches for their hosts. Additionally, pesticide use is highest in cropland and further reduces insect diversity and biomass (Lachat et al., 2011). Croplands are associated with an increase in Oomycota abundance, where OTUs assigned with acknowledged crop diseases (X99: 100% identity with *Aphanomyces euteiches*; X53: 100% identity with a crop pathogen group of *Pythium* sp.).

4.2. Phagotroph vs phototroph

The abundance of eight OTUs representing phagotrophs was strongly and significantly correlated to the total abundance of phototroph OTUs (Fig. 4). Six of these could be assigned with confidence to known genera, their sequences having over 97% identity with cultured organisms (Table S3). Amongst these taxa, four (*Rhogostoma, Platyreta*, *Trinema* and *Pseudochilodonopsis*) are relatively large sized protists (>20 μm) - and thus potential predators of micro-eukaryotes, including phototrophs. We illustrate three of these species in the act of predating algae (Fig. 5). *Rhogostoma* spp. (Rhogostomidae, Thecofilosea) (X2) are closely related to recently characterized eukaryvores that have been shown to avoid feeding on bacteria (Dumack et al., 2016a, b). Although some strains of *Rhogostoma* spp. can live exclusively on a

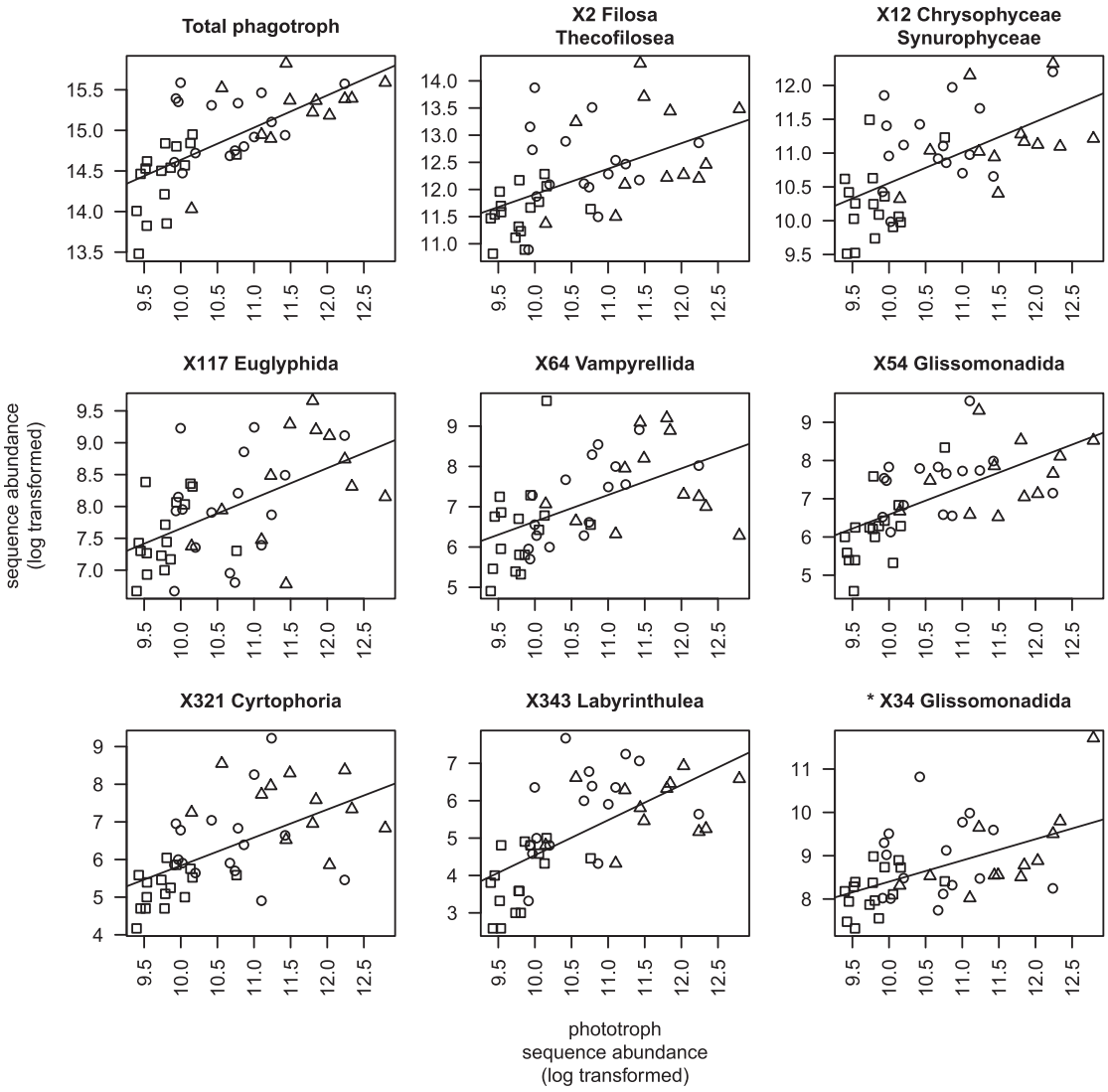

Fig. 4. Biplots showing the regression between the abundance of total phagotroph and eight OTUs and phototroph abundance. The code and taxonomic assignation of each OTU is shown on the top of each biplot. The asterisk indicates the OTU which belongs to the ten most dominant phagotroph OTUs and was significantly correlated to total phototroph abundance despite having an heteroscedastic distribution. The distribution of all other illustrated OTUs is homoscedastic.

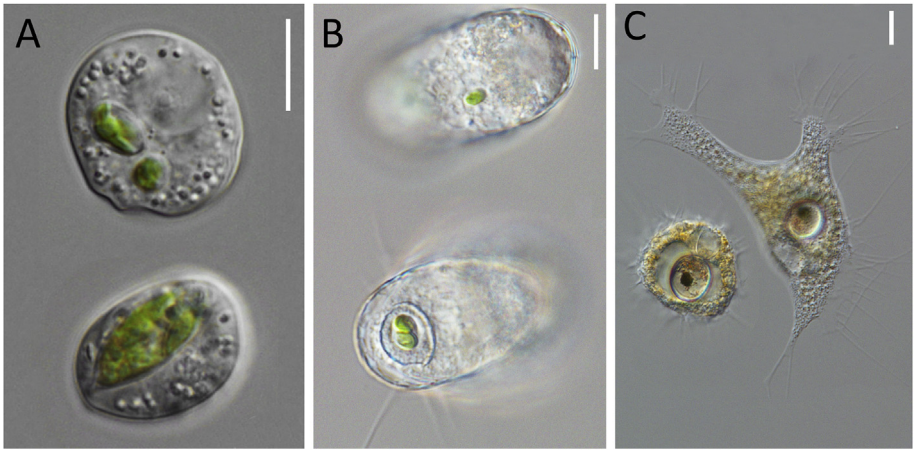

Fig. 5. Light microscopy (differential interference contrast – Nomarski) images of three selected organisms, closely related to the found OTUs correlating to the phototroph sequence abundances (*Rhogostoma* sp. (a), *Trinema* sp.(b), *Leptophrys vorax* (c)). The scale bar represent 10 μm.

bacterial diet (Howe et al., 2009), we could show that at least certain species of genus *Rhogostoma* do feed on algae. OTU X64 is a member of the exclusively eukaryvorous Leptophryidae (Vampyrellida). Members of this family have been reported to be algal predators to a large extent (see Fig. 5, represented by the closely related *Leptophrys vorax*) (Bass et al., 2009; Gong et al., 2015; Hess et al., 2012).

Co-occurrence patterns and observational data provide two lines of evidence that suggest that X64 actually feeds on algae. The same conclusions can be drawn for *Trinema* spp. (Euglyphida) (X117) where larger members of the genus feed to a large extent on micro-algae (Cyanobacteria and/or pigmented Eukaryotes) (Meisterfeld, 2000; Santibañez et al., 2011). Our observations confirmed the ingestion of algal material (Fig. 5). *Pseudochilodonopsis* (X321) are considered as exclusive algivores specialized on diatoms (Hamels et al., 2004). Labyrinthulomycetes branching within the Amphifilidae (X343) are a diverse group (Pan et al., 2016) including bacterivores such as *Sorodiplophrys stercoraria* and *Amphifila marina* (Anderson and Cavalier-Smith, 2012; Tice et al., 2016). The taxonomic as well as functional diversity of this group is however only marginally documented, and the existence of algivorous forms is thus possible.

The group of *Spumella*-like Chrysophyte (X12) is composed of small phagotrophic flagellates having lost their photosynthetic abilities secondarily. However, it has been shown that transitions between phagotrophic and phototrophic strategies occurred often in the evolutionary history of Chrysophytes. It is possible therefore that the *Spumella*-like Chrysophyte X12 is actually mixotrophic like many Chrysophyceae (Boenigk et al., 2005), and therefore shares higher light requirements with other phototrophs. Alternatively, it is possible that the *Spumella*-like Chrysophyte X12 feeds preferentially on bacteria that are associated to phototrophs and their exudates. Bacterial communities associated to algae are highly influenced by the host in aquatic systems (Sapp et al., 2007). A similar explanation could possibly be given for *Allapsa* (X54), a genus of small Cercozoan flagellates formerly collectively classified under the name "*Heteromita globosa*" (Howe et al., 2009).

To the contrary, OTU X34 is assigned to the Viridiraptoridae, a family of highly specialized Cercozoans feeding as yet known exclusively on phototrophic organisms (Hess and Melkonian, 2013). The linear model obtained for this OTU did not respect the conditions of homoscedasticity because of its high abundance in two samples. Such high sequence abundance may correspond to local blooms of these small flagellates, which are reported as frequent (Hess and Melkonian, 2013).

Altogether, phagotroph sequences belonging to an OTU co-occurring with phototrophs reached 26.9% of all phagotrophs (28.1% if X34 is considered). Thus, if only those organisms that we observed eating algae are actually playing that role, then 19.8% of all phagotrophs could actually feed (to various degrees) on phototrophs. Based on this, we estimate the total proportion of algal-feeders to account for between one fifth and one third of all phagotrophic sequences, an amount which is far from being negligible. It is noteworthy that, out of the 100 best represented phagotrophic OTUs, only seven were robustly correlated to phototroph abundance. This low number suggests that the correlations observed are probably highly specific, as demonstrated for X34 (Viridiraptoridae; Hess and Melkonian, 2013). Although it is known that organisms such as *Trinema* can feed opportunistically on various eukaryotes such as fungal conidia (Santibañez et al., 2011), the food regime of all members of this very diverse genus (Lara et al., 2016) has not been surveyed and it is still possible that some members are specialized in eating algae, at least to a certain extent. At this point, only experimental evidence can demonstrate if the selected OTUs represent organisms that are exclusive algal predators or not.

Whatever percentage of environmental sequences from phagotrophic organisms interacting with phototrophs is taken as a reference, the corresponding number of phototroph sequences is by far lower. As rRNA gene sequence numbers can be considered to providing reasonably accurate estimations of the relative biomass of the organisms in DNA environmental surveys (Giner et al., 2016), this suggests that the standing biomass of soil microalgae is lower than that of their predators. By analogy to aquatic ecosystems, this can be explained by the faster turnover of phototrophs. Indeed, most potential algal predators are large protists and can therefore be expected to have relatively longer generation times.

Trophic relationships inferred from correlative analysis of metabarcoding data need to be further explored, possibly with new statistical tools and datasets including other climatic zones and soil types. Nevertheless, we argue that what is now most needed is to characterise the many unknown OTUs, and conducting good observations and experimentation on these organisms to provide useful natural history background needed for sound interpretation of HTS data. As suggested by our study, we believe that future studies providing exact identities of the huge amount of unknown OTUs and revealing their life styles and ecology will provide sound interpretation of the ever-increasing massive sequencing data.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.05.002.

References

Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W., Huse, S.M., 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. PLoS One 4, e6372.

Anderson, O.R., Cavalier-Smith, T., 2012. Ultrastructure of diplophrys parva, a new small freshwater species, and a revised analysis of Labyrinthulea (Heterokonta). Acta Protozoologica 51, 291–304.

Bass, D., Chao, E.E.Y., Nikolaev, S., Yabuki, A., Ishida, K., Berney, C., Pakzad, U., Wylezich, C., Cavalier-Smith, T., 2009. Phylogeny of novel naked filose and reticulose cercozoa: granofilosea cl. n. and proteomyxidea revised. Protist 160, 75–109.

Behnke, A., Engel, M., Christen, R., Nebel, M., Klein, R.R., Stoeck, T., 2011. Depicting more accurate pictures of protistan community complexity using pyrosequencing of hypervariable SSU rRNA gene regions. Environmental Microbiology 13, 340–349.

Berard, A., Dorigo, U., Humbert, J.F., Martin-Laurent, F., 2005. Microalgae Community Structure Analysis Based on 18S RDNA Amplification from DNA Extracted Directly from Soil As a Potential Soil Bioindicator. Agronomy for Sustainable Development 25, 285–291.

Boddy, L., Frankland, J., van West, P., 2007. Ecology of Saprotrophic Basidiomycetes. British Mycological Society Symposia Series Elsevier Science.

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.

Bonkowski, M., Clarholm, M., 2012. Stimulation of plant growth through

interactions of bacteria and Protozoa: testing the auxiliary microbial loop hypothesis. Acta Protozoologica 51, 237–247.
Cairney, J.W.G., Chambers, S.M., 1999. Ectomycorrhizal Fungi: Key Genera in Profile, first ed. Springer-Verlag, Berlin Heidelberg.
Clarholm, M., 1985. Interactions of bacteria, protozoa and plants leading to mineralization of soil-nitrogen. Soil Biology & Biochemistry 17, 181–187.
Corsaro, D., Walochnik, J., Venditti, D., Mueller, K.D., Hauroeder, B., Michel, R., 2014. Rediscovery of *Nucleophaga amoebae*, a novel member of the Rozellomycota. Parasitology Research 113, 4491–4498.
de Vargas, C., Audic, S., Henry, N., Decelle, J., Mahé, F., Logares, R., Lara, E., Berney, C., Le Bescot, N., Probert, I., Carmichael, M., Poulain, J., Romac, S., Colin, S., Aury, J.M., Bittner, L., Chaffron, S., Dunthorn, M., Engelen, S., Flegontova, O., Guidi, L., Horak, A., Jaillon, O., Lukes, J., Malviya, S., Morard, R., Mulot, M., Scalco, E., Siano, R., Vincent, F., Zingone, A., Dimier, C., Picheral, M., Searson, S., Kandels-Lewis, S., Acinas, S.G., Bork, P., Bowler, C., Gorsky, G., Grimsley, N., Hingamp, P., Iudicone, D., Not, F., Ogata, H., Pesant, S., Raes, J., Sieracki, M., Speich, S., Stemman, L., Sunagawa, S., Weissenbach, J., Wincker, P., Karsenti, E., 2015. Eukaryotic plankton diversity in the sunlit ocean. Science 348.
Delarze, R., Gonseth, Y., Eggenberg, S., Vust, M., 2015. Guide des milieux naturels de Suisse: Écologie, menaces, espèces caractéristiques. Bussigny, Switzerland.
Dumack, K., Baumann, C., Bonkowski, M., 2016a. A bowl with marbles: revision of the thecate amoeba genus lecythium (Chlamydophryidae, tectofilosida, cercozoa, Rhizaria) includinga description of four new species and an identification key. Protist 167, 440–459.
Dumack, K., Mueller, M.E.H., Bonkowski, M., 2016b. Description of lecythium terrestris sp nov (Chlamydophryidae, cercozoa), a soil dwelling protist feeding on fungi and algae. Protist 167, 93–105.
Dupont, A.O.C., Griffiths, R.I., 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.
Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.
Ekelund, L., Ronn, R., 1994. Notes on protozoa in agricultural soil with emphasis on heterotrophic flagellates and naked amoebae and their ecology. FEMS Microbiol Reviews 15, 321–353.
Elbert, W., Weber, B., Burrows, S., Steinkamp, J., Buedel, B., Andreae, M.O., Poeschl, U., 2012. Contribution of cryptogamic covers to the global cycles of carbon and nitrogen. Nature Geoscience 5, 459–462.
Foissner, W., 1999. Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples. Agriculture Ecosystems & Environment 74, 95–112.
Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression, second ed. Sage Thousand Oaks CA. http://socserv.socsci.mcmaster.ca/jfox/Books/Companion.
Freeman, K.R., Pescador, M.Y., Reed, S.C., Costello, E.K., Robeson, M.S., Schmidt, S.K., 2009. Soil CO_2 flux and photoautotrophic community composition in high-elevation, 'barren' soil. Environmental Microbiology 11, 674–686.
Frey, B., Buehler, L., Schmutz, S., Zumsteg, A., Furrer, G., 2013. Molecular characterization of phototrophic microorganisms in the forefield of a receding glacier in the Swiss Alps. Environmental Research Letters 8.
Geisen, S., Fiore-Donno, A.M., Walochnik, J., Bonkowski, M., 2014. Acanthamoeba everywhere: high diversity of Acanthamoeba in soils. Parasitology Research 113, 3151–3158.
Geisen, S., Laros, I., Vizcaino, A., Bonkowski, M., De Groot, G.A., 2015a. Not all are free-living: high-throughput DNA metabarcoding reveals a diverse community of protists parasitizing soil metazoa. Molecular Ecology 24, 4556–4569.
Geisen, S., Rosengarten, J., Koller, R., Mulder, C., Urich, T., Bonkowski, M., 2015b. Pack hunting by a common soil amoeba on nematodes. Environmental Microbiology 17, 4538–4546.
Geisen, S., Tveit, A.T., Clark, I.M., Richter, A., Svenning, M.M., Bonkowski, M., Urich, T., 2015c. Metatranscriptomic census of active protists in soils. ISME Journal 9, 2178–2190.
Geisen, S., 2016. The bacterial-fungal energy channel concept challenged by enormous functional versatility of soil protists. Soil Biology & Biochemistry 102, 22–25.
Geisen, S., Koller, R., Huenninghaus, M., Dumack, K., Urich, T., Bonkowski, M., 2016. The soil food web revisited: diverse and widespread mycophagous soil protists. Soil Biology & Biochemistry 94, 10–18.
Giner, C.R., Forn, I., Romac, S., Logares, R., de Vargas, C., Massana, R., 2016. Environmental sequencing provides reasonable estimates of the relative abundance of specific picoeukaryotes. Applied Environmental Microbiology 82, 4757–4766.
Glaser, K., Kuppardt, A., Boenigk, J., Harms, H., Fetzer, I., Chatzinotas, A., 2015. The influence of environmental factors on protistan microorganisms in grassland soils along a land-use gradient. Science of the Total Environment 537, 33–42.
Gomaa, F., Mitchell, E.A.D., Lara, E., 2013. Amphitremida (poche, 1913) is a new major, ubiquitous labyrinthulomycete clade. PLoS One 8.
Gong, Y., Patterson, D.J., Li, Y., Hu, Z., Sommerfeld, M., Chen, Y., Hu, Q., 2015. Vernalophrys algivore gen. Nov., sp nov (Rhizaria: cercozoa: Vampyrellida), a new algal predator isolated from outdoor mass culture of scenedesmus dimorphus. Applied Environmental Microbiology 81, 3900–3913.
Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., Boutte, C., Burgaud, G., de Vargas, C., Decelle, J., del Campo, J., Dolan, J.R., Dunthorn, M., Edvardsen, B., Holzmann, M., Kooistra, W.H.C.F., Lara, E., Le Bescot, N., Logares, R., Mahé, F., Massana, R., Montresor, M., Morard, R., Not, F., Pawlowski, J., Probert, I., Sauvadet, A.L., Siano, R., Stoeck, T., Vaulot, D., Zimmermann, D., Christen, R., 2013. The Protist Ribosomal Reference database (PR^2): a catalog of unicellular eukaryote Small Sub-Unit rRNA sequences with curated taxonomy. Nucleic Acids Research 41, D597–D604.
Hamels, I., Mussche, H., Sabbe, K., Muylaert, K., Vyverman, W., 2004. Evidence for constant and highly specific active food selection by benthic ciliates in mixed diatoms assemblages. Limnology and Oceanography 49, 58–68.
Hess, S., Sausen, N., Melkonian, M., 2012. Shedding light on Vampires: the phylogeny of Vampyrellid amoebae revisited. PLoS One 7.
Hess, S., Melkonian, M., 2013. The mystery of clade X: *Orciraptor* gen. Nov and *Viridiraptor* gen. Nov are highly specialised, algivorous amoeboflagellates (glissomonadida, cercozoa). Protist 164, 706–747.
Hollander, M., Wolfe, D.A., 1999. Nonparametric Statistical Methods. Wiley.
Holm, S., 1979. A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6, 65–70.
Howe, A.T., Bass, D., Vickerman, K., Chao, E.E., Cavalier-Smith, T., 2009. Phylogeny, taxonomy, and astounding genetic diversity of glissomonadida ord. Nov., the dominant gliding zooflagellates in soil (Protozoa: cercozoa). Protist 160, 159–189.
Howe, A.T., Bass, D., Scoble, J.M., Lewis, R., Vickerman, K., Arndt, H., Cavalier-Smith, T., 2011. Novel cultured protists identify deep-branching environmental DNA Clades of cercozoa: new genera *Tremula, Micrometopion, Minimassisteria, Nudifila, Peregrinia*. Protist 162, 332–372.
Jones, M.D.M., Forn, I., Gadelha, C., Egan, M.J., Bass, D., Massana, R., Richards, T.A., 2011. Discovery of novel intermediate forms redefines the fungal tree of life. Nature 474, 200–U234.
Lachat, T., Pauli, D., Gonseth, Y., Klaus, G., Scheidegger, C., Vittoz, P., 2011. Evolution de la biodiversité en Suisse depuis 1900: Avons nous touché le fond? Haupt Verlag, Bern.
Lara, E., Heger, T.J., Mitchell, E.A.D., Meisterfeld, R., Ekelund, F., 2007. SSU rRNA reveals a sequential increase in shell complexity among the euglyphid testate amoebae (Rhizaria : Euglyphida). Protist 158, 229–237.
Lara, E., Moreira, D., Lopez-Garcia, P., 2010. The environmental clade LKM11 and Rozella form the deepest branching clade of fungi. Protist 161, 116–121.
Lara, E., Seppey, C.V.W., Gonzalez Garraza, G., Singer, D., Quiroga, M.V., Mataloni, G., 2015. Planktonic eukaryote molecular diversity: discrimination of minero-trophic and ombrotrophic peatland pools in Tierra del Fuego (Argentina). Journal of Plankton Research 37, 645–655.
Lara, E., Roussel-Delif, L., Fournier, B., Wilkinson, D.M., Mitchell, E.A.D., 2016. Soil microorganisms behave like macroscopic organisms: patterns in the global distribution of soil euglyphid testate amoebae. Journal of Biogeography 43, 520–532.
Lesaulnier, C., Papamichail, D., McCorkle, S., Ollivier, B., Skiena, S., Taghavi, S., Zak, D., van der Lelie, D., 2008. Elevated atmospheric CO_2 affects soil microbial diversity associated with trembling aspen. Environmental Microbiology 10, 926–941.
Magoc, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957–2963.
Mahé, F., Rognes, T., Quince, C., de Vargas, C., Dunthorn, M., 2014. Swarm: robust and fast clustering method for amplicon-based studies. Peerj. 2, e539.
Massana, R., del Campo, J., Sieracki, M.E., Audic, S., Logares, R., 2014. Exploring the uncultured microeukaryote majority in the oceans: reevaluation of ribogroups within stramenopiles. ISME Journal 8, 854–866.
McFadden, G.I., Melkonian, M., 1986. Use of hepes buffer for microalgal culture media and fixation for electron-microscopy. Phycologia 25, 551–557.
Meisterfeld, R., 2000. Order Arcellinida kent, 1880. In: Lee, J., Leedale, G., Bradbury, P. (Eds.), An Illustrated Guide to the Protozoa. Society of Protozoologists Lawrence, Kansas, pp. 827–859.
Oksanen, J., Blanchet, G.F., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Wagner, H., 2013. Vegan: Community Ecology Package. http://CRAN.R-project.org/package=vegan. R package version 2.0-10.
Pan, J., del Campo, J., Keeling, P.J., 2016. Reference Tree and Environmental Sequence Diversity of Labyrinthulomycetes. The Journal of Eukaryotic Microbiology 64, 88–96.
Pawlowski, J., Lejzerowicz, F., Apotheloz-Perret-Gentil, L., Visco, J., Esling, P., 2016. Protist metabarcoding and environmental biomonitoring: time for change. European Journal of Protistology 55, 12–25.
Petz, W., Foissner, W., Adam, H., 1985. Culture, food selection and growth-rate in the mycophagous ciliate *Grossglockneria acuta* Foissner, 1980-1st evidence of autochthonous soil ciliates. Soil Biology & Biochemistry 17, 871–875.
Pohlert, T., 2014. The Pairwise Multiple Comparison of Mean Ranks Package (PMCMR). http://CRAN.R-project.org/package=PMCMR (R package).
Pollierer, M.M., Langel, R., Scheu, S., Maraun, M., 2009. Compartmentalization of the soil animal food web as indicated by dual analysis of stable isotope ratios (N-15/N-14 and C-13/C-12). Soil Biology & Biochemistry 41, 1221–1226.
Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Gloeckner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35, 7188–7196.
R Core Team, 2014. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing Vienna, Austria. http://www.R-project.org/.
Santibañez, A., P.A, Kohshima, S., Scheihing, A., R.A, Silva, R., R, Jaramillo, M., J.I, Labarca, P., P.J, Casassa, R., G, 2011. First record of testate amoebae on glaciers and description of a new species puytoracia jenswendti nov sp (Rhizaria, Euglyphida). Acta Protozoologica 50, 1–14.
Sapp, M., Schwaderer, A.S., Wiltshire, K.H., Hoppe, H., Gerdts, G., Wichels, A., 2007.

Species-specific bacterial communities in the phycosphere of microalgae? Microbial Ecology 53, 683–699.
Schmidt, O., Dyckmans, J., Schrader, S., 2016. Photoautotrophic microorganisms as a carbon source for temperate soil invertebrates. Biology Letters 12.
Tice, A.K., Silberman, J.D., Walthall, A.C., Le, K.N., Spiegel, F.W., Brown, M.W., 2016. *Sorodiplophrys stercorea*: another novel lineage of sorocarpic multicellularity. The Journal of Eukaryotic Microbiology 63, 623–628.
Valentini, A., Pompanon, F., Taberlet, P., 2009. DNA barcoding for ecologists. Trends in Ecology & Evolution 24, 110–117.
van der Wal, A., Geydan, T.D., Kuyper, T.W., de Boer, W., 2013. A thready affair: linking fungal diversity and community dynamics to terrestrial decomposition processes. FEMS Microbiology Reviews 37, 477–494.
Verni, F., Gualtieri, P., 1997. Feeding behaviour in ciliated protists. Micron 28, 487–504.