

# Implications of size-dependent predation of protists and nematodes on the composition and functionality of the prokaryotic soil microbiome

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## ABSTRACT

Protists and nematodes are dominant predators of prokaryotic communities in soil. Their strong difference in size suggests different feeding strategies and preferences. Here we analyzed the relevance of size-dependent predation by distinguishing in microcosm experiments the contribution of small-sized and large-sized protists and nematodes, respectively. Sterilized soils were inoculated with soil suspensions filtrated across 250 µm, 100 µm, 60 µm, 20 µm and 5 µm pore sized membranes and a non-filtrated control. After 60 days of incubation, only the soils inoculated with the 5 µm-filtrate showed lower abundances of bacteria, archaea and fungi, and a differently composed protistan community. The nematode communities were always dominated by the small-sized bacterivore genus *Acrobeloides*. Correlation analyses indicated that small-sized protists were associated with a wider range of prokaryotic taxa than larger sized protists. In contrast to small-sized nematodes, large-sized showed no correlation with specific prokaryotic taxa. The comparison of effects on prokaryotes at DNA and RNA levels suggested a preference of the soil predators for actively growing rather than resting cells. Small-sized protists (only Cercozoa) and nematodes had a more pronounced influence on the gene abundances of microbial groups and N cycling genetic potentials, i.e., bacterial *amoA*, *nirS*, and *nosZII* genes. Therefore, we conclude that the common ecological size-dependent predation theory also applies to trophic interactions of protists and nematodes with soil prokaryotes. The distinct feeding preferences can alter the prevalence of different N-functional genes, which could thus potentially modify the dynamics of the N cycling in soil.

## 1. Introduction

Soil biodiversity plays a vital role in regulating key ecosystem processes such as nutrient cycling (Wall et al., 2010), carbon storage (Lal, 2004), and plant growth (Saleem et al., 2019), all of which are essential for ecosystem health and sustainability (Bardgett and Van Der Putten, 2014). Different groups of organisms coexist with each other in soil ecosystems, including bacteria, archaea, fungi, protists and nematodes. These organisms interact with each other in many ways, forming intricate relationships that shape the emerging functions (Coleman, 2008). One of the most important interactions takes place in trophic chains, where energy and nutrients flow between organisms (Wardle et al., 2010). Soil bacteria and archaea, both prokaryotes, carry out many key steps in the biogeochemical cycling including nitrogen (Nelson et al., 2016). Nitrogen represents a major growth limiting factor in agricultural cropping systems. Beyond this functional role, nitrogen also serves as a major food source in soil trophic chains (Koller et al., 2013).

Protists and nematodes are major consumers of prokaryotes in soil (Bonkowski and Clarholm, 2012; Geisen et al., 2016; Neher, 2010). Predatory protists can, in fact, make up 75 % of the total protistan soil community (Oliverio et al., 2020), and bacterivores are the dominant feeding types of nematodes at the global scale (Van Den Hoogen et al., 2019). When feeding on bacteria, both protists and nematodes may exhibit some preferences for specific species or taxa (Geisen et al., 2023; Wood, 1973). Consequently, by preferring one taxon over another, they can potentially influence the soil microbial community composition. The protist *Cercomonas*, for example, was found to preferentially graze on Proteobacteria, i.e., one of the most dominant bacterial phyla in soils. Thus, grazing can reduce bacteria of this phylum, but it may also stimulate their regrowth (Amacker et al., 2022). Generally, predatory protists can discern among various prey organisms by assessing traits such as cell surface properties or responding to volatile organic compounds (Jousset, 2012; Schulz-Bohm et al., 2017). Furthermore, some bacteria, like *Pseudomonas fluorescens*, have evolved defenses against predators by

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producing phenolic compounds that are toxic to protists (Jousset et al., 2006). While there is ample evidence for complex interactions between predators and their bacterial prey, it is yet difficult to identify common rules that would allow to predict the impact of such interactions on the diversity, resilience and functionality of the soil microbiome.

Size-dependent predation is a well-established theory for above-ground animals. It states that the size of the predators has implications for the size of their prey (Cohen et al., 1993). Most of the smaller predators prefer smaller prey, because of their mouth opening size (Hambright, 1991). Larger predators are often unlikely to target overly small prey due to their limited nutritional value (Brose, 2010), capture efficiency (Brose et al., 2008), or search time (Portalier et al., 2019). Because of their smaller gut size, the digestion efficiency of small-sized predators is generally lower, and thus they require higher-quality food to meet their energy demands. This concept is known in general ecology as the Jarman–Bell principle (Garnick et al., 2018). While the size-dependent predation theory has been investigated for complex trophic chains with a focus on soil animals (Digel et al., 2014; Hohberg and Traunspurger, 2005), their relevance for controlling the diversity of the prokaryotic microbiome is not yet well established. Soil provides an intricate three-dimensional labyrinth that impedes the movement of predators towards their food resources (Erktan et al., 2020). Nematodes navigate in water-filled soil pores with a neck diameter of 30 to 90  $\mu\text{m}$ , while phagotrophic protists can move through smaller pores down to a diameter of ca. 5  $\mu\text{m}$  (Rønn et al., 2001; Feng and Balkcom, 2017). Thus, soil bacteria inhabiting micropores with a lower diameter can escape predation by larger protists and nematodes (Wright et al., 1995).

Feeding preferences of predators may not only be affected by the prokaryotic taxon, but also whether the prey is actively growing, resting or dead. In fact, it was estimated that the proportion of dead cells in the prokaryotic microbiome can be up to 40 % in temperate soil (Carini et al., 2016). Copiotrophic soil bacteria, i.e., Bacilli (Firmicutes) or Bacteroidota, are characterized by fast growth when an appropriate food source is available, but also by long periods of resting without food (Stone et al., 2023). The implications of these prokaryotic cellular conditions on predator preferences are not well understood. One of the reasons is that most studies on the composition of the soil prokaryotic community are based sequencing 16S rRNA gene PCR amplicons from soil DNA (Delgado-Baquerizo et al., 2018; Smets et al., 2016). However, this DNA includes both genetic information of active, inactive and even dead cells. Thus, it cannot give a good estimate about the composition of the actively metabolizing part of the microbiome (Carini et al., 2016; Lennon and Jones, 2011). To emphasize the proportion of active microbial cells, 16S rRNA has been used instead as a PCR target, because the number of ribosomes is usually higher in actively, protein-synthesizing growing cells as compared to resting or dead cells. Integrating RNA analysis into studies on trophic interactions could therefore help to understand whether certain predators preferentially feed on active, resting, or damaged bacterial cells (Ingham et al., 1985; Jiang et al., 2017). Only a few studies have yet examined such trophic interactions at the RNA level for agricultural soils (Fujino et al., 2023; Rürger et al., 2023).

Trophic interactions including protists and nematodes could play a fundamental role in regulating the abundance and diversity of the prokaryotes, providing key steps in the soil nitrogen cycle (Shaw et al., 2019; Zhao et al., 2019). Each of the different metabolic steps in the nitrogen cycle, i.e., nitrification, denitrification or nitrogen ( $\text{N}_2$ ) fixation can be provided by different bacteria or archaea. For example, the first step in nitrification is the oxidation of ammonia by either bacteria or archaea (Prosser and Nicol, 2012). Denitrification can be performed by many different bacterial and archaeal taxa (Krichels et al., 2025). The diversity of taxa carrying out these functions in soils depends on variables, i.e., the soil type, management or environmental conditions. The role of predation as another variable in this process is not clear. Due to the similar C:N ratios between predators and bacterial prey and their relatively low assimilation efficiency, protists utilize 10 to 40 % and

nematodes utilize 50 to 70 % of prey carbon for biomass production (Ferris et al., 1997; Griffiths, 1994), excreting the excess nitrogen as ammonia. This ammonia is readily available for plant roots, thereby promoting plant growth (Bonkowski, 2004).

The objective of this study was to investigate the relevance of size-dependent predation of protists and nematodes on the structural and functional composition of the soil prokaryotic microbiome. We conducted a soil microcosm experiment with a series of filtration treatments to assess the impacts of potential top-down regulation by predators according to their body size. The abundance of microbial phyla was characterized by quantification of bacterial, archaeal 16S rRNA partial genes from soil DNA using quantitative PCR (qPCR) and of fungi by targeting their genomic ITS-1 region. The compositions of the prokaryotic communities as well as protists and nematodes were characterized by PCR amplicon sequencing from total soil DNA extracted from the microcosms, based on the relative abundance of their respective amplicon sequence variants (ASV). Implications of the predator-prey interactions were also studied by analyzing total soil RNA of the microcosms, to highlight metabolically active prokaryotes (Blazewicz et al., 2013; Cazzaniga et al., 2023). To address implications of the predator-prey relationships on the functionality of the microbiome, we quantified seven genes contributing to three different steps in the biogeochemical cycling of nitrogen (N-functional genes), i.e., ammonium oxidation,  $\text{N}_2$ -fixation, and denitrification, respectively.

We hypothesized that the concept of size-dependent predation would also be applicable to multitrophic interactions in soil. More specifically, we expected (1) that filtration across smaller pores would reduce the diversity of predator communities more strongly than across larger pores. We hypothesized (2) that smaller sized protists and nematodes would have a broader feeding range as they could move more easily into soil micro-pores to access food. Furthermore, we hypothesized (3) that actively growing prokaryotic taxa would be a preferred prey. Finally, we expected (4) that the preying activity of smaller sized protists and nematodes would more strongly interact with the abundance of different N-functional genes than their larger sized counterparts because of their broader feeding range.

## 2. Materials and methods

### 2.1. Experimental design

The soil of this study was a Luvisol which originated from a cropland field site under conventional soil tillage management, located near Hildesheim in Lower Saxony, Northern Germany (52° 00' 29.2" N 9° 56' 22.6" E). The soil samples were collected in September 2022. Its primary particle composition was 17.1 % clay, 80.9 % silt and 2.0 % sand. Soil pH ( $\text{CaCl}_2$ ) was 6.8, total soil organic carbon (C) 1.6 % (wt/wt), and total N 1.1 %. The soil samples were collected from the upper 10 cm of the horizon (plough layer) and a composite soil sample was created by combining four spatial cores within a one-square-meter area in the center of the field. Upon arrival at the laboratory, the soil samples were air-dried and adjusted to 60 % saturation of the total water holding capacity (WHC). This soil was then used after sterilization to set up the microcosms.

### 2.2. Soil sterilization and inoculum preparation

A filtration method was utilized to manipulate the interaction rates between soil prokaryotes and their potential predators including protists and nematodes. Initially, the collected soil was sieved through a stainless steel 2-mm mesh (Retsch, Haan, Germany) to remove plant roots, debris and stones. For sterilization, soil samples and mesocosm components were autoclaved at 121 °C for 90 min as described elsewhere (Yang et al., 2022). A parent inoculum suspension was prepared by mixing 100 g of the same fresh soil with 200 mL of sterilized demineralized water. Following vortexing for 5 min (Laboshake; Gerhardt

GmbH, Königswinter, Germany) at 180 rpm at 23 °C and settling for 1 min, the entire upper soil suspension was designated as the parent inoculum. This soil suspension was then sequentially and manually filtered at room temperature through a series of decreasing mesh sieves with pore size diameters of 250 µm, 100 µm, 60 µm, 20 µm, and 5 µm, using polyester sieves (Merck, Germany). Presterilized soils were inoculated with different filtrated soil suspensions, including fractions < 5 µm, < 20 µm, < 60 µm, < 100 µm and < 250 µm, as well as a non-filtrated soil suspension. We also included a control with untreated soil. The absence of viable microorganisms in sterilized soil was confirmed by diluting the sample 10<sup>6</sup>-fold and plating onto R2A agar plates with no colonies detected after 7 days of incubation at 25 °C. Both filtered and unfiltered soil microbial suspensions were thoroughly mixed with the sterilized soil. Thus, a total of seven treatments was generated from each sample. Each treatment was replicated eight times. Microcosm incubations were started by adding 50 mL of different size filtrated parent inoculum suspensions to 500 g of sterilized soil in a 1-L screw-cap bottle within a clean bench. All microcosms were sealed with sterile cotton plugs to avoid the contamination and then incubated at 20 °C for 60 days, before the experiment was terminated and DNA and RNA were extracted from the samples to analyze the composition of the soil microbiome (see below). During the microcosm incubations, sterile distilled water was added to each screw-cap bottle twice a week to maintain a 60 % saturation of the total WHC. The microcosms were placed in a glasshouse chamber with natural light supplemented by 400 W high-pressure sodium lamps, maintaining a 16-h day and 8-h night at 20 °C (Wagg et al., 2021).

### 2.3. PCR amplification and Illumina sequencing

The centrifugal flotation method was employed to isolate the motile and immotile nematodes from the soil (Caviness and Jensen, 1955). Briefly, 250 g of soil was thoroughly mixed with 1000 mL sterilized distilled water, and a density of a 1.6 M aqueous MgSO<sub>4</sub> solution (higher density compared to nematodes) was added. The mixture was then centrifuged in four aliquots, each in a 1 L polypropylene container for 4 min at 1800 x g for 5 min, allowing nematodes to float to the top while heavier soil particles settled at the bottom. The upper layer, containing the nematodes, was carefully collected using a 20 µm sieve (stainless steel; Retsch) at 23 °C and thoroughly washed using sterile water. The presence of nematodes was examined on a counting slide at 25 and 40 × magnification using a dissecting microscope (Zeiss, Jena, Germany) and subsequently frozen for DNA extraction.

The soil and nematodes samples were initially stored at -80 °C until further processing. A total of 0.5 g of soil samples were collected to extract DNA from protists and prokaryotes. Nematode samples were collected for DNA extraction, each in securely sealed, DNA/DNase/RNase/PCR inhibitor-free tubes (Sarstedt, Nümbrecht, Germany) after confirming of their presence under a microscope. The FastDNA®SPIN Kit (MP Biomedicals, Eschwege, Germany) was utilized for both extracting DNA from protists and prokaryotes using soil samples, and for extracting DNA from nematodes using sterilized demineralized water samples containing nematodes. For DNA extraction from each soil and nematode pellet, the FastPrep FP120 bead beating system (MP Biomedicals) was utilized at a high-speed setting of 6.0 m s<sup>-1</sup> for 40 s to ensure efficient cell lysis of soil microbes and nematodes. DNA concentrations were quantified using the Quant-iT™ PicoGreen dsDNA assay kit from Thermo Fisher Scientific (Roskilde, Denmark) on a Cary Eclipse Fluorescence Spectrophotometer. Total soil RNA was extracted using the FastRNA™ Pro Soil-Indirect Kit (MP Biomedicals) following the manufacturer's protocol. The quality and concentration of RNA was analyzed with the NanoDrop System (Thermo Fisher Scientific). Reverse transcription of RNA into single-strand cDNA was conducted using the Maxima H Minus First Strand cDNA Kit (Thermo Fisher Scientific) with random hexamer primers. Synthesized single-strand cDNA and DNA were stored at -80 °C.

Soil DNA and cDNA concentrations were adjusted to 10 ng µL<sup>-1</sup> before PCR amplification. The 16S rRNA gene was amplified using the primer pairs 515F and 806R (Parada et al., 2016); the 18S rRNA V4 hypervariable region was amplified with the forward primer TAR-euk454FWD1 and a reverse primer TAREukREV3 (Stoeck et al., 2010). The primers NF-1 and 18Sr2b (Porazinska et al., 2009) targeting the 18S small subunit (SSU) ribosomal gene were used for amplifying the nematode community. A two-step PCR process was employed to reduce initial PCR product concentration and prevent non-specific amplification. In the first step, duplicate PCRs using a 25-µL reaction were performed with specific components, including 5 µL of 5 × Q5 reaction buffer, 0.5 µL of 0.2 mM of dNTPs, 1.25 µL of 0.5 µM of each primer, 0.25 µL of Q5 polymerase (0.02 U µL<sup>-1</sup>) and 1 µL DNA template. Details of primers used and PCR reaction conditions can be found in the Supplementary Table S1. Subsequently, the amplicons from the first step were 10-fold diluted and used as a template for a second PCR, which incorporated barcoded primers. Agarose gel electrophoresis (1 % agarose) was employed to evaluate PCR amplicon quality. The agarose (Roth, Karlsruhe, Germany) was dissolved in 1 × TAE buffer (Roth). The correct amplification size was verified by comparison to DNA size markers (Sigma-Aldrich, Merck Life Science, Taufkirchen, Germany) (Green and Sambrook, 2019). Purification and normalization of the PCR amplicons were carried out using the SequalPrep Normalization Plate Kit (Invitrogen, Karlsruhe, Germany) to achieve a concentration of 1–2 ng µL<sup>-1</sup> for each sample. The samples collected from bacterial, protistan, and nematode communities were individually combined, and libraries were generated using the Ovation Rapid DR Multiplex System 1–96 by NuGEN Technologies (Redwood City, CA, USA). Ligation of standard Illumina adapters and sequencing primers was performed using MyTaq (Bioline, Luckenwalde, Germany). Paired-end sequencing (2 × 300 bp) was conducted on the Illumina MiSeq platform by LGC Genomics (Berlin, Germany).

### 2.4. Quantification of gene abundances in soil

Bacterial and archaeal abundance in soil was estimated from soil extracted DNA (for more details see section below) by quantitative real-time PCR (qPCR) targeting the V4 region of their 16S rRNA genes (Table S2). For determining fungal abundance, primers were specific for a fragment of the chromosomal ITS-1 region. Furthermore, the following functional prokaryotic genes, all connected to nitrogen transformation potentials, were quantified: archaeal *amoA*, bacterial *amoA*, both encoding for ammonium monooxygenases, *nifH* encoding for a protein contributing to the nitrogenase complex for N<sub>2</sub> fixation, *nirS* and *nirK*, both encoding for distinct nitrite reductases, and *nosZI* and *nosZII*, both encoding for nitrous oxide reductases, respectively. qPCR was executed using the CFX96 Touch™ real time PCR detection system (Bio-Rad Laboratories), following a method described for 16S rRNA genes and ITS-1 elsewhere (Hemkemeyer et al., 2015). Details on primers and conditions can be found in the Supplementary Table S2. More specifically, the qPCR reaction mixture, with a volume of 10 µL, consisted of 5 µL SYBR Green Master Mix (Thermo Scientific), 0.5 µM each of forward and reverse primer (0.5 µL), 1 µL DNA template (normalized to contain 10 ng DNA), and 3 µL nuclease-free water (New England Biolabs, Frankfurt am Main). Standard curves for qPCR were generated genomic DNA extracted from pure cultures of *Bacillus subtilis*, *Methanobacterium oryzae*, *Fusarium culmorum* with plasmid DNA containing fragments of the respective N-functional genes (Liu et al., 2024).

### 2.5. Bioinformatic analysis

The standard operating procedure of the QIIME2 platform was employed to process the raw DNA sequences. Reorientation of forward and reverse reads was accomplished using an in-house Python script available at [github.com/DamienFinn/MiSeq\\_read\\_reorientation](https://github.com/DamienFinn/MiSeq_read_reorientation) (January 2025). The reoriented reads were then demultiplexed using the

cutadapt paired-end process (Martin, 2011). Subsequently, the vsearch join-pairs function was applied to merge the forward and reverse reads (Rognes et al., 2016). Quality filtration, dereplication, and denoising of the read data were performed using DADA2 (Callahan et al., 2016). Bacterial amplicon sequence variants (ASV) were assigned using Silva 138 (Quast et al., 2012; Yilmaz et al., 2014), and both the protistan and nematode ASV were assigned based on the Protist Ribosomal Reference (PR2) database (Guillou et al., 2013). It should be noted that this database also contains metazoan sequences. Other eukaryote-associated ASV, i.e., Rhodophyta, Streptophyta, Fungi, Opisthokonta\_X, other Metazoa, and ambiguous taxa, were excluded from the ASV table.

Notably, for prokaryotes a total of 7517 ASV were detected with DNA, and 5020 ASV with cDNA amplified from rRNA. For more detailed information on the numbers of samples analyzed, sequence reads, and rarefied sequences per sample, see Supplementary Table S3. Raw DNA sequences are deposited at the European Nucleotide Archive database (Project Accession number PRJEB70423).

## 2.6. Classification of microbial body sizes

Due to the inherent challenge in precisely assessing the body sizes of the various soil microbial groups at a fine scale, we categorized the body sizes of protists, and nematodes based on their taxonomic affiliations at the phylum or subphylum level. To estimate body sizes, we referred to literature-based propagule size data and incorporated recent research findings on the average microbial body sizes from 576 dominant genera (Li et al., 2022; Luan et al., 2020). In total, 19 groups were analyzed, comprising 6 protistan and 13 nematode taxa (Table S4). Our emphasis was primarily on the most abundant phyla or subphyla, defined by a relative abundance above 1 % or very close to it. This categorization approach relies on the assumption that body size and trophic status are generally conserved within microbial taxa at phylum level (Martiny et al., 2015). We then categorized the four phagotrophic protistan phyla into two groups: large-sized including Ciliophora and Conosa, and small-sized including Lobosa and Cercozoa. For nematodes, we adopted a functional group-based assignment since the body size of nematodes tends to remain consistent within each functional trait (Van Den Hoogen et al., 2019; Zhao et al., 2015). We incorporated fungivore, predator, and omnivore nematodes, recognizing that they commonly feed on organisms beyond their designated feeding group (Potapov et al., 2022). The three herbivorous groups were not considered, and the remaining ten predatory genera were also categorized into two body size groups, i.e. the large size class composed of *Anatonchus*, *Aporcelaimellus*, *Alaimus*, *Thonus*, and *Plectus*; the small sized encompassing *Ditylenchus*, *Filenchus*, *Eucephalobus*, *Aphelenchoides*, and *Acroboloides*.

## 2.7. Correlation-based co-occurrence networks between different trophic levels

Separate networks encompassing prokaryotes both at DNA and RNA levels, protists and nematodes rarefied ASV tables were constructed for untreated and sterilized soils inoculated with 250  $\mu\text{m}$ -, and the 5  $\mu\text{m}$ -filtrates. A total of eight samples for each treatment was used to construct each network. Furthermore, two networks, one with all DNA- (56 samples in total) and another with all RNA-based (56 samples in total) ASV prokaryotes, and all qualified protistan, and nematode ASV irrespective of the microcosms from which they were obtained, were constructed to enhance the robustness of the results. Spearman correlation was selected to assess the pairwise associations among the ASV. Initial thresholds were set to retrieve 1000 positive and 1000 negative edges for each measure, with 1000 permutations and 1000 bootstrap scores applied to mitigate compositionality bias. Method-specific  $P$  values were computed and subsequently merged using Brown's method (Newman, 2006). Edges with merged  $P$  values below 0.01 were retained after multiple testing via the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). An edge indicates a statistically significant

correlation ( $p < 0.01$ ) between ASV, with the correlation threshold set at  $p > 0.8$ . Final networks were visualized with CoNet 3.6.1 (Shannon et al., 2003).

## 2.8. Statistical analyses

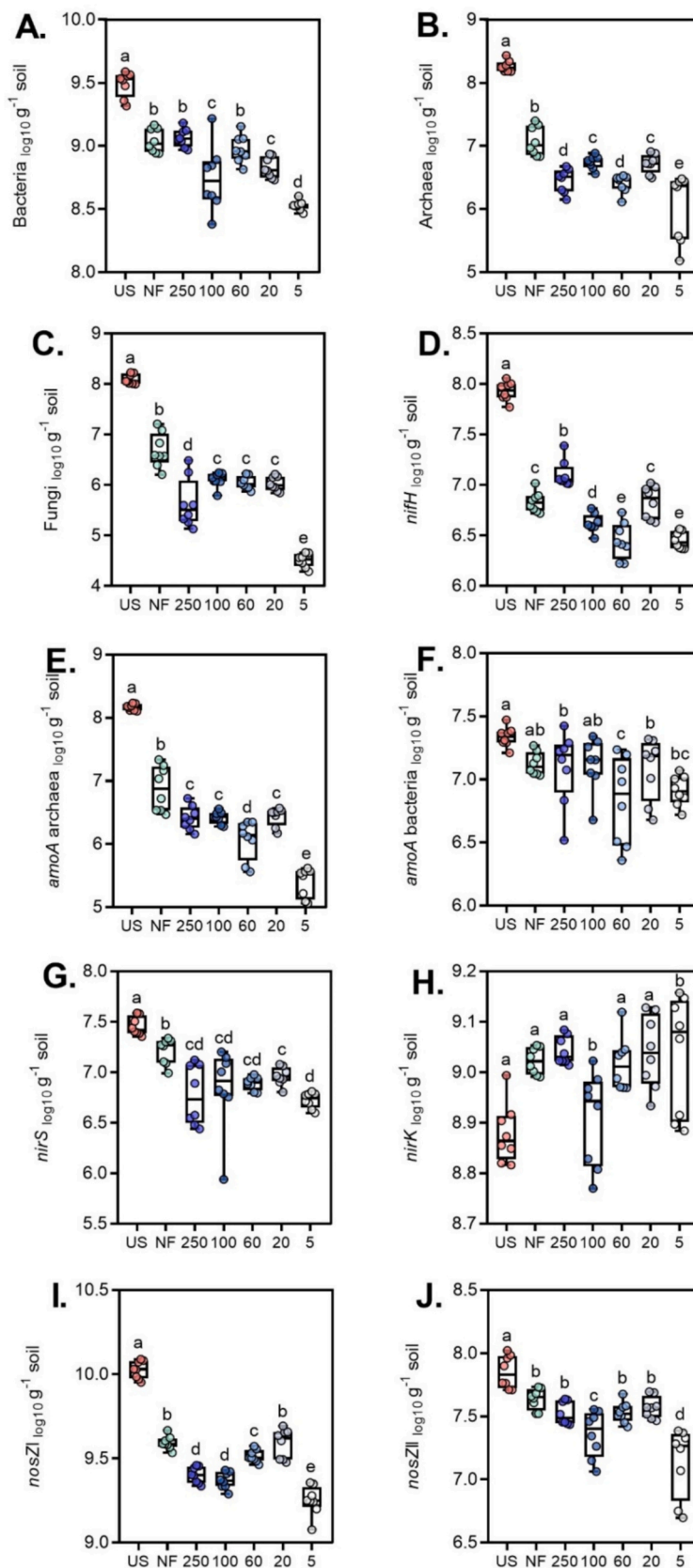
To test the significant differences of the relative abundances of prokaryotic phyla and families, protistan phyla and nematode genera, ANOVA tests were conducted using SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk (Shapiro and Wilk, 1965) and Levene's tests (Levene, 1960) were used to confirm normality and homogeneity of variances. If these assumptions were met, Tukey's multiple comparison test was applied to assess the statistical significance. Alpha-diversity (Shannon index, Chao 1 richness and Pielou's evenness) of soil prokaryotic (at both DNA and rRNA level), protistan and nematode communities were evaluated in R (version 4.1.2) using the Vegan package (Oksanen et al., 2020). A two-way ANOVA was performed, followed by Duncan's multiple comparison test, to assess the statistical significance (at a significance level of  $p < 0.05$ ) for diversity indices and gene copy numbers across various treatments. Pearson's correlation among gene abundance in bacteria, archaea, fungi, and N cycling, concerning different size categories of soil protists and nematodes, was conducted using *ggcor* package (Huang et al., 2020). Principal component analysis (PCA) based on the centre-log ratio-transformed relative abundance of soil prokaryotic (total and active), protistan and nematode communities was carried out using the "provenance" package in R (Gloor et al., 2016). Permutational multivariate analysis of variance (PERMANOVA) was conducted using the *adonis* function in the Vegan package to assess Bray-Curtis based differences in community structures for soil prokaryotes, protists, and nematodes. The absolute abundances of soil prokaryotic phyla and families were calculated by multiplying their relative abundances with the corresponding prokaryotic gene copy numbers (Tkacz et al., 2018). The functional traits of protistan ASV were assessed according to previous studies (Dumack et al., 2020; Xiong et al., 2021). The Nematode Indicator Joint Analysis (MINJA, available at <https://shiny.wur.nl/ninja/>) (Sieriebriennikov et al., 2014) was utilized to categorize feeding types (herbivores, fungivores, bacterivores, omnivores, and predators) at genus level.

## 3. Results

### 3.1. Impact of size-filtration on the abundance of microbial groups and N-functional genes in the soil microcosms

After 60 days of incubation, the bacterial abundance in the microcosms with sterilized soil amended with soil suspensions was significantly lower than in the untreated soils (Two-way ANOVA followed by Duncan's multiple comparison test,  $P < 0.05$ , Fig. 1A). The size filtration reduced the bacterial abundance in soils inoculated with 100  $\mu\text{m}$  ( $P < 0.05$ ), 20  $\mu\text{m}$  ( $P < 0.001$ ), and most pronounced, with 5  $\mu\text{m}$  ( $P < 0.001$ ) filtrates. In accordance, there was a gradual decline of archaea (Fig. 1 B) and fungi (Fig. 1 C) with significantly lower values in soils receiving the 5  $\mu\text{m}$  filtrates ( $P < 0.001$ ). For the comparison of soil inoculated with non-filtered and size-filtered fractions of soil suspensions, we found that gene abundances in the filtrates were significantly lower as compared to non-filtered soil suspensions ( $P < 0.05$ ), except for bacterial gene copies in the microcosms inoculated with the 250  $\mu\text{m}$  filtrate ( $P = 0.44$ ). This demonstrated that the filtration approach generated soil microbiomes of reduced microbial abundances as compared to the untreated soil and soil inoculated with non-filtered soil suspensions.

All N-functional genes targeted in this study were detected at quantifiable levels in the untreated and presterilized soils soil suspension inoculated microcosms (Fig. 1 D. - J.). Their abundance showed a similar pattern as detected for bacteria and archaea, with a significantly reduced abundance in soils which had received the 5  $\mu\text{m}$  filtrate as compared to all other treatments ( $P < 0.05$ ). While the abundance of



**Fig. 1.** Quantification of structural and functional genes by qPCR of the soil microbiome as detected in the soil microcosms amplified from total DNA after 60 days of incubation. **A.** Bacteria, **B.** Archaea, **C.** Fungi, **D.** *nifH*, **E.** *amoA* archaea, **F.** *amoA* bacteria, **G.** *nirS*, **H.** *nirK*, **I.** *nosZI*, **J.** *nosZII*. US, Untreated soil (from which soil suspensions were obtained); soil microcosms inoculated with the non-filtrated soil suspension (NF), with a filtrate passing through 250  $\mu\text{m}$  membrane (250), a 100  $\mu\text{m}$  (100), a 60  $\mu\text{m}$  (60), a 20  $\mu\text{m}$  (20), and a 5  $\mu\text{m}$  (5) membrane, respectively. Each treatment is represented by eight replicates.

*nirS* in the microcosm soils correlated with the size-filtrates they had been inoculated with, its functional alternative gene *nirK* showed an opposite pattern with highest numbers detected in microcosms inoculated with the 5  $\mu\text{m}$  filtrate (Fig. 1 G. and H.). The abundance of the archaeal *amoA* gene was much lower in the 5  $\mu\text{m}$ -filtrate inoculated soils compared to the larger-sized filtrates ( $P < 0.001$ ), while for the bacterial *amoA*, there were no pronounced differences between the different size filtrates ( $P > 0.05$ ).

### 3.2. Impact of size filtration on the community composition of prokaryotes in the soil microcosms

For prokaryotes, soil suspensions as compared to untreated soil were always reduced in Shannon diversity, Chao 1 richness and Pielou evenness, at both DNA and RNA level, respectively (Supplementary Fig. S1 A. – F.). Soils inoculated with the  $<5$  and  $<20$   $\mu\text{m}$  size filtrates exhibited significantly lower diversity, richness and evenness ( $P < 0.05$ ) but this effect was only detectable at the DNA level. PCA (Supplementary Fig. S2 A. and B.) and PERMANOVA (Supplementary Table S5) showed clear differences between communities from untreated soil and the pre-sterilized soils inoculated with the size-filtered fractions and non-filtered soil suspensions at both DNA and RNA level ( $P < 0.05$ ). More pronounced differences were detected between the soils inoculated with the different size filtrates at the DNA level compared to RNA level.

As compared to untreated soils, the prokaryotic community of soils inoculated with the different size filtrates always showed at the DNA level a lower relative abundance of Actinobacteriota and Crenarchaeota, and a higher proportion of Proteobacteria, Bacteroidota and Firmicutes (Fig. 2 A.). The relative abundance of Bacteroidota was significantly

higher in the microcosm soils inoculated with the 5  $\mu\text{m}$  filtrate compared to the other size-filtrate treatments ( $P < 0.05$ , Supplementary Table S6).

In accordance with the DNA results, the RNA-based analyses showed a lower relative abundance of Actinobacteriota, Crenarchaeota and a higher relative abundance of Proteobacteria, as compared to untreated soil (Fig. 2 B.). With RNA, the proportion of Bacteroidota and Firmicutes was higher in the soils inoculated with the different size-filtrates. The overall effect of the different filtration steps, however, did not reveal contrasting prokaryotic community compositions, apart from Actinobacteriota which were relatively less abundant in soils inoculated with the 20  $\mu\text{m}$  and 5  $\mu\text{m}$  filtrates (as compared to US and NF,  $P < 0.05$ , Table S5).

The archaeal *Nitrososphaeraceae* was the most abundant prokaryotic family at the DNA level. In contrast, soils inoculated with the different size filtrates showed *Oxalobacteraceae*, *Hymenobacteraceae*, and *Micrococcaceae* as being the most abundant families (Fig. S3 A.). No significant differences between soils inoculated with different size filtrates were observed ( $P > 0.05$ , Table S7). At RNA level, the dominant families in soils inoculated with the size-filtrates were *Sphingobacteriaceae*, *Nitrososphaeraceae*, and *Bacillaceae* (Supplementary Fig. S3 B.) Overall, there were no tangible differences in community compositions at the family level in response to inoculations with the different size-filtrates ( $P > 0.05$ , Supplementary Table S7).

### 3.3. Impact of size filtration on the community composition of protists in the soil microcosms

Compared to the untreated soil, the soils inoculated with unfiltered and size-filtered fractions showed a lower protist diversity, richness and evenness, being most pronounced with the 20  $\mu\text{m}$  and 5  $\mu\text{m}$  filtrate

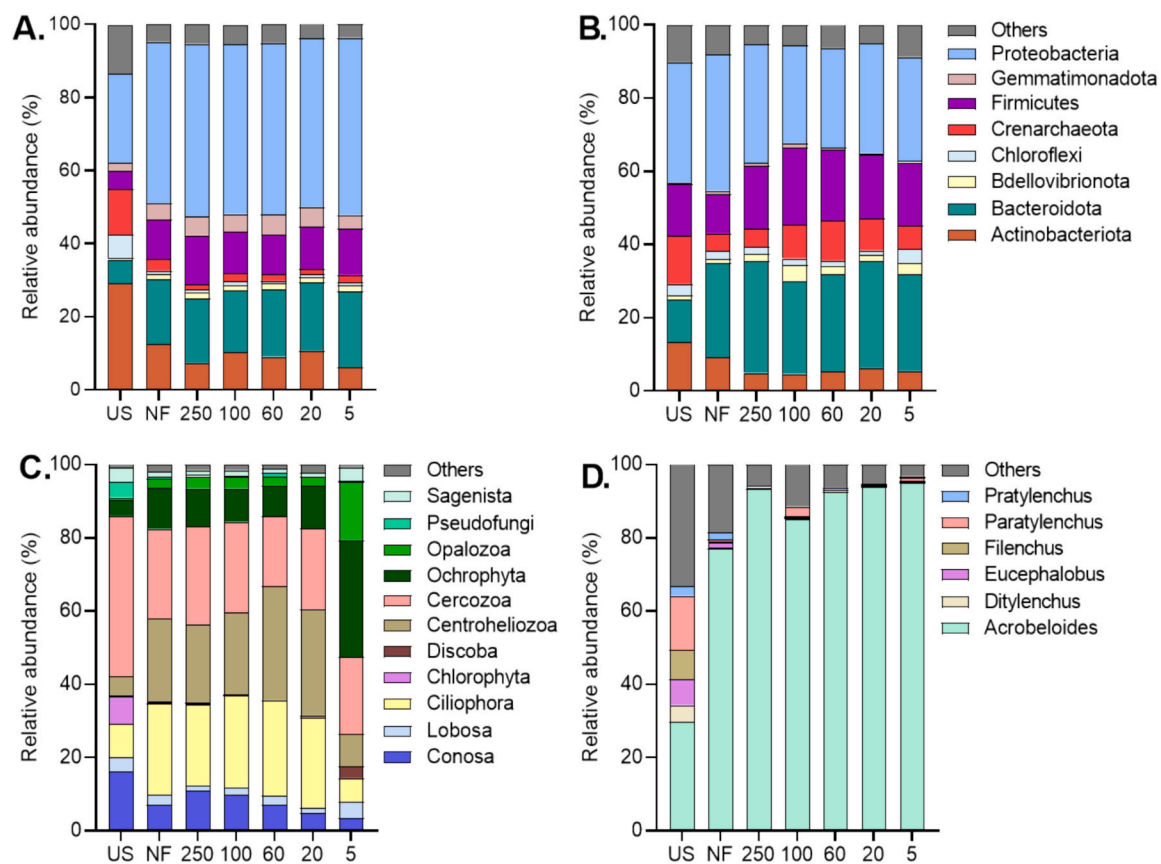


Fig. 2. Compositional analyses (relative abundances) of the microbial communities in the soil microcosms. The prokaryotic microbiome as characterized by their 16S rRNA genes (A.) and their 16S rRNA (B.); (C.) protists, and (D.) nematodes. A. – C. compositions are shown at phylum level, D. compositions are shown at genus level.

inoculated soils (Fig. S1 G.- I.). The community compositions of protists were generally significantly different between treatments, as indicated by PCA (Fig. S2C.) and PERMANOVA (Table S5). The pre-sterilized soils inoculated with the unfiltered and size-filtered fractions displayed a lower relative abundance of Cercozoa ( $P < 0.05$ ) and Chlorophyta ( $P < 0.05$ ) as compared to untreated soil (Supplementary Table S8). Irrespective of the size fractions, the protistan community exhibited dominance by Cercozoa, Centroheliozoa, Ciliophora, and Ochrophyta (Fig. 2 C.). The relative abundance of Conosa declined from 250  $\mu\text{m}$  to 5  $\mu\text{m}$  filtrate inoculated soils. Soils inoculated with the 5  $\mu\text{m}$  filtrate exhibited a higher relative abundance of Opalozoa ( $P < 0.05$ ), Ochrophyta ( $P < 0.05$ ), and Discoba ( $P < 0.05$ ) and a reduced relative abundance of Ciliophora ( $P < 0.05$ ) as compared to other filtrates. These contrasting compositions were confirmed at the family level with a strong increase of *Chrysophyceae* clade C (Ochrophyta) and a decrease of *Oxytrichidae* (Ciliophora) (Supplementary Fig. S3 C.). More specifically, soil DNA of the inoculated treatments showed a lower relative abundance of suspected phototrophs, plant pathogens, and saprotrophs (Supplementary Fig. S4 A.).

### 3.4. Impact of the size-filtration on the community composition of nematodes in the soil microcosms

Soils inoculated with the non-filtered and size-filtered soil suspensions showed lower diversity, richness and evenness of nematodes as compared to untreated soils (Fig. 2 D.; Fig. S1 J.-L.). Compositional differences between the untreated soils and soils inoculated with size-filtered soil suspensions were tangible by PCA and PERMANOVA ( $P < 0.01$ , Supplementary Fig. S2 D. and Table S5). Only soils inoculated with the 60 ( $P < 0.01$ ) and 250  $\mu\text{m}$  ( $P < 0.01$ ) filtrates exhibited significant difference as compared to soil inoculated with non-filtered soil suspensions. There was no evident separation of communities in soil inoculated with the different the size-filtrates ( $P > 0.05$ ). The relative abundance of *Acrobeloides* was 2- to 3-fold higher in the pre-sterilized soils inoculated with soil suspensions, than in the untreated soil ( $P < 0.001$ , Table S8). In contrast, *Ditylenchus* ( $P < 0.05$ ), *Eucephalobus* ( $P < 0.05$ ), *Filenchus* ( $P < 0.001$ ), and *Paratylenchus* ( $P < 0.05$ ), were found significantly lower relative abundance in the pre-sterilized soils inoculated with soil suspensions as compared to untreated soil. In regard to nematode functional traits, the pre-sterilized soils inoculated with the suspension or their filtrates showed a lower relative abundance of herbivores and fungivores, and a higher relative abundance of bacterivores (Supplementary Fig. S4 B.).

### 3.5. Body size distribution of soil protists and nematodes

For protists, Cercozoa showed the smallest body size and, with approx. five-fold difference, Ciliophora the largest (Supplementary Table S4A). The large sized Ciliophora displayed a lower and the small sized Lobosa a higher relative abundance in soils inoculated with the 5  $\mu\text{m}$  filtrate (Supplementary Fig. S5 A.). In addition, the relative abundance of the large-sized Conosa increased in soils inoculated with the larger sized filtrates. For nematodes, the smallest nematodes were represented by the herbivore *Paratylenchus* and the bacterivore *Acrobeloides*, while the largest by the predator *Anatonchus* (Supplementary Table S4 B.). The relative abundance of both the small-sized and the large-sized genera were not strongly affected by soils receiving the different filtrates (Supplementary Fig. S5 B.).

### 3.6. Co-occurrence network analyses of prokaryotes, protists and nematodes

To search for potential changes of associations between predators and prokaryotes, separate correlation networks were constructed (i) for the untreated soils, (ii) for pre-sterilized soils after inoculation with the unfiltered soil suspension, as well as (iii) for the 250  $\mu\text{m}$  and (iv) the 5

$\mu\text{m}$  filtrated suspensions. To consider differences between total and active prokaryotic taxa, separate networks based on prokaryotic ASV as detected at DNA and at level RNA were constructed (Supplementary Fig. S6).

Protist and nematode networks showed less complex networks than those from untreated soil. This was indicated by the lower number of nodes (Table S9 A.). While the number of prokaryotic nodes was not different between the size-filtrate treatments, nodes contributed by protists and especially nematodes were strikingly lower in soils which had received the 5  $\mu\text{m}$  filtrate. Here, the number of nodes from small-sized protists to networks was particularly high. Strikingly, only the small-sized but not the large-sized nematodes occurred as nodes in any of the networks, suggesting no taxon-specific link between them and their prey. As indicated by network edges, prokaryotes with most links to their potential predators were, irrespective of the DNA or RNA level, Proteobacteria, Bacteroidota and Firmicutes (Fig. S7 A.).

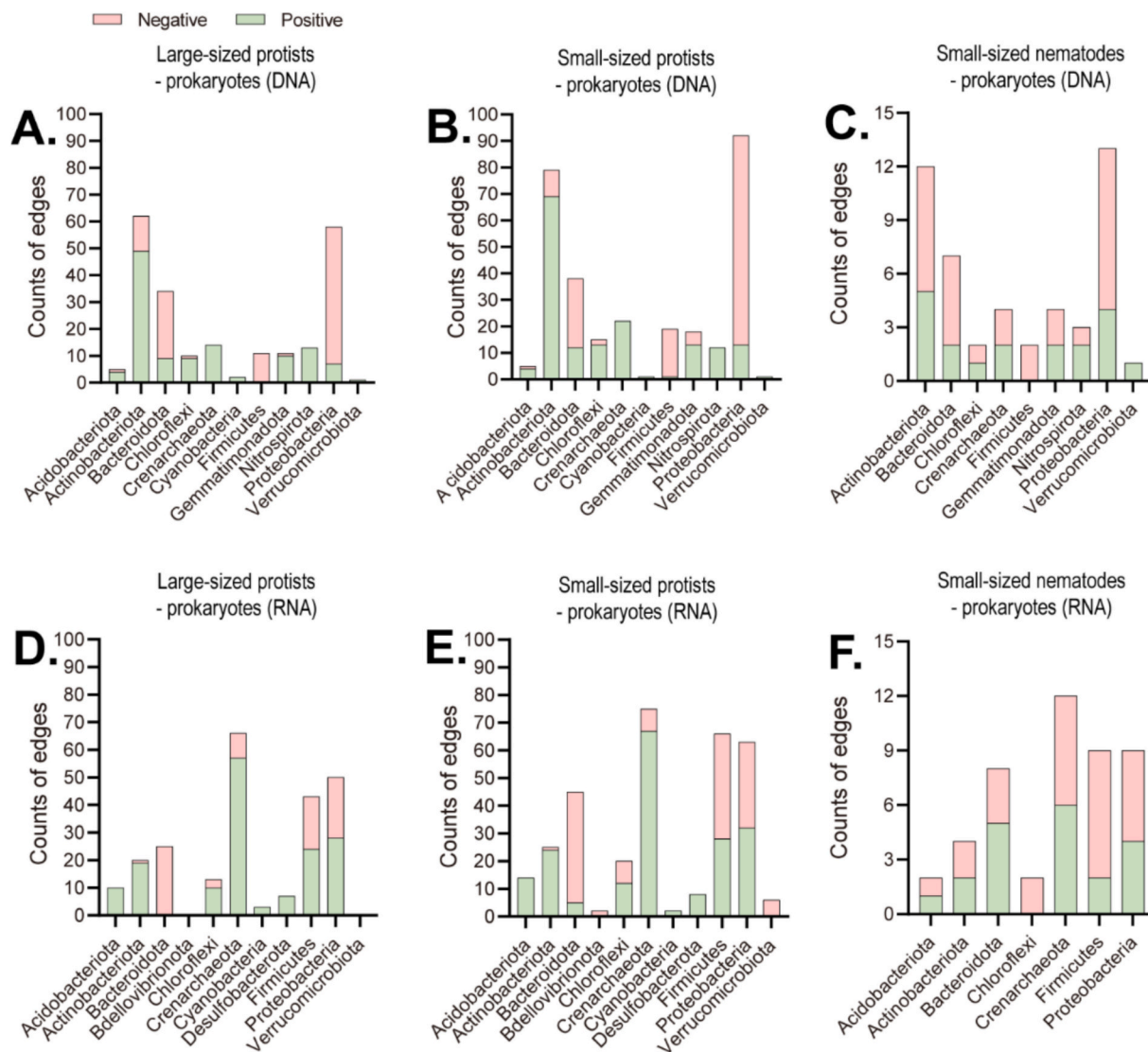
In order to increase the robustness of the networks, we combined all co-occurrence data of predators (protists and nematodes) with the prokaryotic ASV; one network included all DNA-derived ASV, and another included the RNA-derived (Fig. S8). Overall, both networks were similar in regard to the numbers of edges, nodes, as contributed by prokaryotes, protists, and nematodes (Table S9 B.). At the RNA level, irrespective of their body sizes, there were more links (network edges) between prokaryotes and protists as compared to DNA-based ASV (Fig. 3; Table S9 B.). In contrast, there were no pronounced differences in the number of links at DNA and RNA level for nematodes with prokaryotes. Irrespective of the DNA or RNA level, there was a smaller number of links between prokaryotes and large-sized protists as compared to the small-sized (Table S9 B.). For both small-sized and large-sized protists, as well as the small-sized nematodes, the highest number of edges with prokaryotic ASV at the DNA level were with members of the Actinobacteriota, Bacteroidota, and Proteobacteria, while at the RNA level these ASV were mainly from Crenarchaeota, Firmicutes and Proteobacteria (Fig. 3).

### 3.7. Implications of trophic interactions for the prokaryotic N-functional potentials

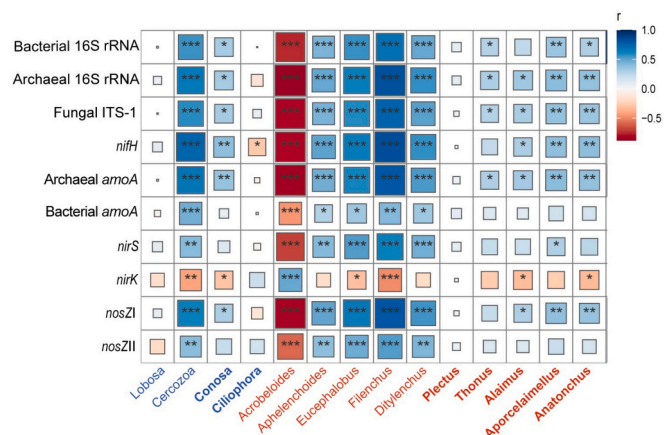
Potential associations among different size groups of protists and nematodes with the total abundance of bacteria, archaea, fungi, and of prokaryotic N-functional genes were investigated by Pearson's correlation analyses. Among the small-sized protists, Cercozoa showed significant correlations with all target genes ( $P < 0.01$ , Fig. 4), while Lobosa showed no correlations ( $P > 0.05$ , Fig. 4). Similarly, for large-sized protists, Conosa showed with the exception of the bacterial *amoA* ( $P > 0.05$ ), *nirS* ( $P > 0.05$ ) and *nosZII* ( $P > 0.05$ ), significant correlations, while the relative abundance of Ciliophora only correlated with one single gene (*nifH*,  $P < 0.05$ ). Small-sized nematodes showed across all genera strong correlations with all targeted genes (except for 2 of 50 pairwise analyses,  $P < 0.05$ ). Interestingly, *Acrobeloides* showed opposite positive and negative links as compared to the other four small-sized nematode genera ( $P < 0.001$ ). For the five large-sized nematodes, there were no correlations between the targeted genes and *Plectus* ( $P > 0.05$ ), but the other genera correlated with several target genes, even though these correlations were clearly less pronounced as compared to the small-sized genera ( $P < 0.05$ ). As compared to the other targeted genes, *nirK* exhibited a contrasting pattern in regard to positive and negative correlations.

## 4. Discussion

Microcosms were used in this study to evaluate the implications of size-dependent predation on the prokaryotic soil microbiome. As predators we selected protists and nematodes. Both encompass many taxa feeding on soil bacteria and archaea (Rønn et al., 2012). A size-filtration approach, intended to fractionate predators, was applied to inoculate



**Fig. 3.** Characterization of co-occurrence network properties, as obtained from combined networks of all treatments. The prokaryotic microbiomes were characterized as ASV of 16S rRNA genes (DNA) or ASV of 16S rRNA (RNA), as well as the relative abundance of ASV from protists and nematodes. The original networks are shown in the Supplementary Fig. S8, the size classification of protist and nematode taxa is indicated in the Supplementary Table S4.



**Fig. 4.** Co-occurrence heatmap showing correlations of structural and functional prokaryotic genes with phagotrophic protists (blue letters, bold for large-sized protists) and nematodes (red letters, the large-sized in bold). Stars indicate the significance of correlations.

previously sterilized soil with soil suspensions carrying microbial cells extracted with water from the same untreated soil. Not surprisingly, the composition of the prokaryotic community which evolved over 60 days of incubation was less diverse compared to untreated soil. A major difference was the lower abundance of Actinobacteriota, which was probably caused either by a less efficient extraction of bacteria from this phylum, due to a stronger soil particle attachment (Hemkemeyer et al., 2018) or by slower growth as compared to other phyla harboring more copiotrophic taxa, i.e., Proteobacteria and Bacteroidota (Fierer et al., 2007). Conditions for the latter two phyla were favorable because the autoclaving process most likely released soil bound nutrients supporting their growth. Despite these nutrients, the total populations sizes of bacteria, archaea, and also fungi in the inoculated soils never reached as high as they were in the untreated soils. The autoclaving process probably modified the organic compounds gluing soil aggregates, thereby reducing the number of micro-habitats available for microbial colonization (Berns et al., 2008).

The overall community compositions of the prokaryotic microbiomes detected in the microcosms were not strongly affected by the inoculated different size-filtrates. It always showed Proteobacteria, Bacteroidota and Firmicutes as their dominant phyla. Considering that

the size filtrates of 250  $\mu\text{m}$  down to 5  $\mu\text{m}$  fractionated the potential predators according to their body sizes, the lack of strong differences may have been either caused by the 60 days incubation period, or by a low predatory/prey ratio, or other unfavorable conditions for predators. The fact that the microcosms inoculated with the 5  $\mu\text{m}$ -filtrates had a lower prokaryotic cell abundance and, thus, a lower density of prey suggests that the incubation period were not sufficient to grow up to cell densities detected with the larger size-filtrates. This could explain why the protist communities from microcosms inoculated with the 5  $\mu\text{m}$ -filtrates showed a clearly different composition.

When testing hypothesis (1), filtration through smaller pores significantly reduced protistan community diversity compared to larger. The relative abundance of Opalozoa, Ochrophyta and small-sized Lobosa was higher in microcosms inoculated with the 5  $\mu\text{m}$ -filtrates, indicating that growth conditions for heterotrophic members of these groups were favorable (Chen et al., 2024; Lenzi et al., 2024; Smith, 1993). In contrast, larger sized protists, i.e., Centroheliozoa, and Ciliophora, showed a lower relative abundance. While the larger sized Conosa showed a gradual decline in microcosms inoculated with size filtrates from 250  $\mu\text{m}$  down to 5  $\mu\text{m}$ , this did not strongly impact on the prokaryotic community composition. Interestingly, the overall relative abundance of the typically soil dominant Cercozoa was not affected by filtration, but a higher taxonomic resolution indicated compositional differences within this phylum. This may have resulted from adaptations to different densities of prey.

Also, for nematodes, soils inoculated with the non-filtered and size-filtered soil suspensions showed lower diversity as compared to untreated soils. However, inconsistent with our hypothesis (1), their diversity was not reduced with filtration of decreasing pore size. Microcosms inoculated with soil suspensions showed a strong increase in the relative abundance of *Acrobeloides* and a decline of several other groups, e.g., *Paratylenchus*. This difference could be a result of different extraction efficiencies, damaging of cells during the extraction procedure, or less suitable conditions for growth in the inoculated microcosms, e.g., due to a lack of food sources (plant material in case of *Paratylenchus*). The strong dominance of small-sized bacterivore nematodes of the genus *Acrobeloides* was not different in soils inoculated with the different size filtrates. Other studies also detected this genus as a dominant representative of soil nematodes (Chen et al., 2020; Zhao et al., 2015). Apparently, even the filtration across membranes with 5  $\mu\text{m}$  pore diameter could not exclude this taxon. One reason could be that not adults but eggs were prevalent in the initial inoculant. The 60 days of incubation were probably sufficient to establish a dominant population with the nematode community. It should be noted that we used in this study DNA sequencing instead of morphology identification of the nematodes, despite known limitations like incomplete databases and primer bias (Ahmed et al., 2019). Sequencing allows to estimate the species diversity efficiently because it does not require knowledge on classical nematode taxonomy (Hayden et al., 2025). We acknowledge that the relative abundance of nematode genera in our study only reflects sequence read counts and not actual abundance.

While the different size-filtrates did not cause strong changes in the overall prokaryotic community compositions, they generated variation between predator and prey relationships which could further be exploited for generating two distinct combined networks of all treatments. The DNA level network, quantifying the abundance of genes, was suspected to give a good overall picture of the total diversity with the caveat that it may also detect some extracellular DNA released from dead microbial cells (Carini et al., 2016). Co-occurrence networks indicated with a higher number of nodes that the small-sized protists were more versatile in grazing on different prokaryotic taxa compared to the large sized. These results support hypothesis (2), indicating that smaller protists and nematodes had a broader feeding range than larger ones. Smaller protists can feed on a wider range of prokaryotic cells, including those which occur in small soil pores, which are not accessible for larger sized protists or nematodes (Adl and Gupta, 2006). Thus,

predation of small-sized and large-sized protists had different effects on the composition of the prokaryotic soil microbiome.

The total number of taxa (ASV) detected with the DNA level network was 50 % higher as compared to RNA, the number of nodes contributing to the networks was 7 % higher with RNA. This result supports hypothesis (3), suggesting that both protists and nematodes preferentially feed on actively growing prokaryotes. Actively growing bacterial cells will have a higher protein content than resting or senescent cells, and their cells walls will be thinner and less rigid (Navarro Llorens et al., 2010). Thus, they probably have higher nutritional value and are more palatable to both protists and nematodes. In addition, the increased metabolic activity and faster growth of active bacterial cells make them easier for protists and nematodes to catch (Amacker et al., 2022).

Correlation analyses also showed distinct patterns between the abundance of different predatory protists and nematodes with the microbial groups and the different prokaryotic functional genes. While small-sized nematodes showed highly pronounced correlations with almost all N-functional genes, these were less pronounced with the larger sized nematodes. This is consistent with a previous study showing that, although being from the same trophic group, the tested nematodes exhibited different foraging strategies and rates based on size, thereby ultimately affecting nitrogen cycling processes (Zhu et al., 2018). With the protists, Lobosa showed no significant correlations with any of the genes and Ciliophora only with *nifH*, correlations between most of the targeted genes with Cercozoa and Conosa were significant. Considering that size classification putting Lobosa and Cercozoa in the small-sized groups and Conosa and Ciliophora in the larger sized, this is an indication of size-dependent predation for nematodes but not for protists.

The alternative versions of the *nirK* and *nosZI*, i.e. *nirS* and *nosZII*, did not correlate equally with predators, suggesting that predation can result in compositional changes of prokaryotic N-functional genes. E.g., an increased relative abundance of *nirK* by grazing of *Acrobeloides* could reduce *nirS*, thus, shifting the contribution of the heme to copper-containing cytochrome nitrite reductases (Philippot et al., 2007; Ming et al., 2024). If abundance would simply be the explanation of the probability of serving as prey, the bacterial version of *amoA* should have been more strongly affected than the archaeal *amoA*. This however was not the case. In fact, Conosa and all small-sized nematodes (except for *Plectus*) showed a significant correlation with the archaeal but not with the bacterial *amoA* gene. Also, the *nifH* gene showed more pronounced correlations with protists and small sized nematodes than with the much more prevalent *nirK* gene. Therefore, these results partially support hypothesis (4), suggesting that smaller nematodes have a stronger impact on the abundance of different N-functional genes due to their broader feeding range.

In summary, the different size-filtrate treatments produced sufficient variation to generate a complex dataset between the abundance and diversity of microbial groups and prokaryotic functional genes with small-sized and large-sized protists and nematodes. Correlation-based analyses indicated that small-sized protists associated with a wider range of prokaryotic taxa than larger sized protists and small-sized nematodes. In contrast to these three groups, large-sized nematodes showed no feeding preferences for specific prokaryotic taxa. These results align with previous studies that protist populations thrive in coarse-textured soils due to higher predation rates on bacteria, while fine-textured soils with small pores (< 2–3  $\mu\text{m}$ ) provide a refuge for bacteria (Jones and Thomasson, 1976; Rutherford and Juma, 1992). Large-sized ciliate predation has been reported to be more pronounced in larger (6–30  $\mu\text{m}$ ) than in smaller pores (<6  $\mu\text{m}$ ) (Wright et al., 1995). The comparison of effects on prokaryotes at the DNA and RNA level suggested a preference of the soil predators to actively growing rather than resting cell stages, which can be explained by their suspected higher nutritional value. It should be noted, that our correlation based network analyses does not fully capture direct trophic interactions and no causal relationships (Geisen et al., 2023). Therefore, future research using pure culture incubation experiments should be helpful to validate

our findings on the specific predator-prey relationships as suggested by our networks.

In conclusion, our results indicate that the size-dependent predation theory also applies to potential interactions between protists and nematodes feeding on the prokaryotic soil microbiome. The soil pore structure will have a strongly modifying effect, because it limits predator-prey interactions. The distinct feeding preferences of protists and nematodes can alter the prevalence of different N-functional genes and, therefore, a better understanding of such predator-prey relationships should be considered for modelling the temporal and spatial dynamics of the prokaryotic nitrogen cycling in soil.

#### CRedit authorship contribution statement

**Haotian Wang:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stefan Geisen:** Writing – review & editing, Methodology, Conceptualization. **Christoph C. Tebbe:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no conflict of interest.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2025.106201>.

#### Data availability

Data will be made available on request.

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