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Heavy metal contamination collapses trophic interactions in the soil microbial food web via bottom-up regulation

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ABSTRACT

The soil microbial food web plays a vital role in soil health, nutrient cycling and agricultural productivity. Notably, there is a distinct paucity of information regarding the effects of heavy metal contamination on trophic-level interactions within the microbial food web of agricultural soils, which experience appreciably metal contamination worldwide. Herein, we investigated trophic interactions among predators (protists), their preys (bacteria and fungi) and competitors (nematodes) under four metal contamination levels using high-throughput sequencing and a laboratory verification experiment. Metal contamination decreased growth of protist-preferential prey (e.g., small-sized and gram-negative bacteria), increased the growth of protist-nonpreferential prey (e.g., pathogenic fungi and *Actinobacteria*), and had a limited effect on the soil protist-competitor (i.e., nematodes). This resulted in a considerable decrease in the diversity and abundance of protistan consumers and a re-arrangement of their interactions with other organisms. From a systemic view, the direct link was weaker between heavy metal contamination and the protist community than the indirect linkage, i.e., metal-induced changes in the prey community. We further validated these results with laboratory incubation trials that documented growth inhibition of protist (*Colpoda*) and protist-preferential prey (*Spingomonas*) versus growth stimulation of protist-nonpreferential prey (*Arthrobacter*) under metal contamination. These findings indicate that metal contamination collapses trophic-level interactions within the soil microbial food web via bottom-up regulation, providing important implications for managing trophic interactions to maintain agricultural ecosystem services under the challenge of worldwide metal contamination.

1. Introduction

The soil microbial food web is the primary driver of biogeochemical cycles (e.g., carbon, nitrogen and phosphorus) and energy flows in terrestrial ecosystems (de Vries et al., 2013; Kardol et al., 2016). Organisms within soil microbial food webs are composed of billions of organisms per kilogram soil, including predators (e.g., protist and nematode) and their prey (e.g., bacteria and fungi) (Adl and Coleman, 2005; Paul, 2015). These organisms are regarded as mediators for a myriad of critical soil functions and as bioindicators for assessing soil health in response to environmental disturbances (Neher, 2001).

Many bacteria and fungi contribute to mineralization and fixation of nutrients and the development of plant roots (Finkel et al., 2020; Jiang et al., 2021), whereas some are notorious pathogens to plants (Carrión et al., 2019). Protists, as predators, are a central hub of the soil microbial food web feeding on bacteria and fungi, releasing nutrients assimilated

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by microbial biomass and suppressing plant pathogens, thereby promoting soil productivity and crop performance (Xiong et al., 2020). Additionally, protists compete for prey with other predators, such as nematodes, or cooperate with them for survival in extreme environments (Paul, 2015). There is a consensus that soil ecosystem functions (e.g., nutrient cycling, energy flows, etc.) are regulated by the integrated soil biota system rather than individual organism types (Fry et al., 2018). However, the responses of trophic interactions within the soil microbial food web to environmental perturbations remain a distinct knowledge gap (Wang et al., 2020), having several implications for regulation of trophic cascades among belowground organisms in their maintenance of ecosystem services.

The soil microbial food web generally displays resistance and resilience to environmental stressors, such as drought, nutrient deficiency and pollution by shifting resources acquisition pathways or by compensation of species with the same function (de Vries et al., 2012; Li et al., 2022; Qi et al., 2022). When environmental stress exceeds the tolerance range of a species and causes extinction of key species, resistance and resilience of the soil food web will be degraded or destroyed (Ives and Cardinale, 2004). As a result of trophic cascades, the effects of environmental stress will likely spread to other trophic levels (Barnes et al., 2017). In general, soil environmental perturbances trigger trophic cascades via two pathways: top-down or bottom-up (Thakur and Geisen, 2019). With top-down regulation, protists and nematodes structure bacterial and fungal communities via selective predation (Flues et al., 2017; Jiang et al., 2020). The dominance of top-down regulation is further modulated by the grazing intensity, such as predator populations and availability of prey (Lenoir et al., 2007). With bottom-up regulation, changes in bacterial and fungal communities induced by primary production, resource inputs or contaminants would be propagated to higher trophic levels (Kou et al., 2020; Nguyen et al., 2020). Changes in trophic cascades throughout the entire soil microbial food web by top-down or bottom-up regulation induced by environmental perturbance may further impact ecosystem functions, such as nutrient cycling and soil productivity (Kou et al., 2020; Thakur and Geisen, 2019). Parsing out the responsive keystones resulting in cascade effects among trophic interactions is critical for managing soil health and plant productivity in a world experiencing a multitude of human impacts to soil systems (Barnes et al., 2017).

Soil contamination resulting from anthropogenic activities, especially heavy metal contamination with high pervasion, persistence and toxicity, has been regarded as a global concern for human health and ecosystem services (Amundson et al., 2015; Hou et al., 2020; Sun et al., 2006). Herein, heavy metals are recognized as a universal environmental stress that filters soil biota and triggers cascade effects in belowground food webs (Huang et al., 2021). Recent studies are primarily restricted to the ecological risks of heavy metal contamination (e.g., diversity change, alteration of community composition and specific taxa growth inhibition). Notably, most studies only focused on one trophic level (e.g., bacterial or protist community) rather than the entire soil microbial food web containing both predators and prey (Wang et al., 2020). Hence, there is considerable uncertainty concerning how soil heavy metal contamination selectively alters multiple trophic levels via trophic cascades and which regulation pathway (i.e., top-down versus bottom-up) is dominant in affecting trophic interactions.

To address this knowledge gap, we identified the most responsive trophic levels (bacterial, fungal, protist and nematode communities) and investigated trophic interactions throughout the soil microbial food web, along a gradient of combined heavy metal contamination (background to high contamination levels). By using both sequencing and confirmatory laboratory trials, we addressed two key questions: (1) Are the responses of soil prey (bacteria and fungi) to combined metal contamination different from those of predators (protists and nematodes)? (2) How do trophic interactions among the four organism types respond to increasing metal contamination levels? Our overarching hypotheses were: (1) Combined metal contamination exerts a greater and more direct influence on prey communities than predator communities because species at lower trophic levels tend to be fast-growing *r*-strategists and can respond quickly to environmental disturbances (Jansson and Hofmockel., 2020; Wang et al., 2019); and (2) Heavy metal stress induces a bottom-up rather than top-down regulation on the trophic interactions by altering the more susceptible prey communities and attenuating the predation of predators, thereby leading to a trophic cascade throughout the entire soil microbial food web. This study provides important insights into the mechanisms that by which trophic relationships within the soil microbial food web respond to environmental stress factors (heavy metal contamination), and possible avenues for regulating trophic interactions to promote soil health and productivity.

2. Materials and methods

2.1. Study area and sample collection

We collected surface soil samples along a gradient of combined heavy metal contamination (Cd, Zn, Cu and Ni) created by an adjacent E-waste recycling facility. The agricultural field (c. 2000 × 2000 m) located in Taizhou (121°35′ E, 28°47’ N), Zhejiang Province has a typical subtropical marine monsoon climate with a mean annual temperature of 17.3 °C and precipitation of 1693 mm. Soils are classified as Ferrosols, equivalent to Oxisols/Ultisols in USDA Soil Taxonomy. We collected soil samples in one agricultural area to minimize heterogeneity in soil properties, climate and agricultural management, which would introduce several potential co-variates into the interpretation of soil biota response to metal contamination. A total of 37 soil plots (10 × 10 m) were sampled from a location nearest to the E-waste recycling facility to the furthest edge of the area. Distance between the sampling sites and latitude/longitude coordinates are shown in Fig. S1. At each plot, five soil cores from the 0–20 cm depth were uniformly mixed as a composite sample and transported to the laboratory on ice. A subsample of soil for DNA extraction was maintained at −80 °C prior to processing.

2.2. Physicochemical property measurements

Soils were air dried and passed through a 2-mm sieve for physicochemical analyses and a 0.15-mm sieve for heavy metal quantification. Soil pH was determined in a soil:water ratio of 1:2.5 by glass electrode-pH meter (Mettler, S220-Bio-CN, Germany). Total carbon (TC) and nitrogen (TN) were measured using an elemental analyzer (Elementar Vario EL Cube, Germany). Dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) were extracted with deionized water (1:10 soil:water) and analyzed with a TOC/TN_b (total bound nitrogen) analyzer (Analytic Jena multi N/C 3100, Germany). Total heavy metal concentrations were determined following digestion with a mixture of perchloric, nitric, hydrochloric and hydrofluoric acids (Yang et al., 2018). Available heavy metal concentrations were assessed as operationally-defined by extraction with 0.01 M calcium chloride (1:10 soil:water) (Song et al., 2018). Concentrations of cadmium (Cd), copper (Cu), nickel (Ni) and zinc (Zn) were quantified by ICP-MS (PerkinElmer Nexlon300X, USA).

A pollution index (PI) for individual heavy metals was calculated according to Nemerow (1974):

$$PI = \frac{C_i}{S_i} \tag{1}$$

where C_i is the concentration of the heavy metals in the test soil and S_i is the reference concentration of the corresponding heavy metals, and “i” is an individual heavy metal. The reference concentrations for each heavy metal are shown in Table S1.

A combined contamination risk from the four heavy metals was appraised using the integrated Nemerow index (PI_N) (Liu et al., 2021; Qi et al., 2022) as calculated by:

$$PI_N = \sqrt{\frac{MaxPI_i^2 + AvePI_i^2}{2}} \tag{2}$$

where $MaxPI_i$ and $AvePI_i$ are the maximum and average values from the individual pollution index (PI), respectively.

According to PI_N values, the soil samples were divided into four contamination levels: background ($0<PI_N\leq1$; n = 7), low ($1<PI_N\leq2$; n = 6), moderate ($2<PI_N\leq3$; n = 8) and high ($PI_N>3$; n = 16) (Chen et al., 2015). The soil properties of the four contamination levels are shown in Table S2.

2.3. DNA extraction, sequencing and qPCR

Soil nematodes were isolated from 80 g of field-moist soil in triplicate using a two-step process: (1) extraction from fresh soil using the modified Baermann method followed by (2) extraction with sugar-centrifugation floatation (Liu et al., 2008). Total nematode abundances were enumerated using an Olympus CKX53 inverse light microscope (40 × magnification) and expressed as individuals 100 g^{-1} dry soil. The nematode DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Germany). Genomic DNA from other organisms (bacteria, fungi and protists) was extracted using the MioBio PowerSoil DNA Isolation Kit (USA). Primer sets for the four organisms were supplied as indicated in Table S3. The 250 bp paired-end sequencing was conducted on an Illumina HiSeq 2500 platform (Illumina, USA).

To estimate absolute abundances of bacteria, fungi and protist, qPCR assays were conducted in triplicate using the fluorescent dye SYBR-Green approach on a LightCycler 480II (Roche, Germany), containing 8 μl sterile water, 10 μl SYBR MasterMix, 0.5 μl of each primer and 1 μl genomic DNA. PCR protocols were conducted as previously described (Zhao et al., 2019).

2.4. Metal-exposure incubation experiments

Metal-exposure trials were utilized to validate the potential mechanisms revealed by sequencing of field samples. From the 22 species isolated from the field soils (Table S4), we selected a typical bacterivorous protist strain (*Colpoda*), a protist-preferential prey (a small-sized, gram-negative bacterium, *Sphingomonas*) and a protist-nonpreferential prey (a bacterium in *Arthrobacter* from *Actinobacteria*) for the validation trials. We incubated the two bacteria and protist in a sterile transparent soil extract to simulate field-soil environmental conditions. To simplify the combined heavy metals effect, we selected Cd, which was identified as one of the most toxic heavy metals in previous toxicological tests (Gallego et al., 2007), as a single metal contamination stressor. The Cd was added in accordance with the available Cd concentrations of the field soils forming four contamination levels (0, 0.5, 0.7 and 1.11 mg kg^{-1} Cd for background, low, moderate and high contamination, respectively). Overall, six groups with four contamination levels and four replicates were applied: 1) S, *Sphingomonas*; 2) A, *Arthrobacter*; 3) C, *Colpoda*; 4) S + C, *Sphingomonas* + *Colpoda*; and 5) A + C, *Arthrobacter* + *Colpoda*; 6) A + S + C, *Arthrobacter* + *Sphingomonas* + *Colpoda*. The bacterial CFUs were counted on LB agar plates. Both the abundances for trophozoites and cysts of *Colpoda* were enumerated using an inverse light microscope at 400 × magnification after incubation for 48 h. Trophozoites and cysts of *Colpoda* were differentiated by morphology as shown in Fig. S2; the trophozoites were kidney shaped (c. 20 μm), whereas the cysts were spherical (c. 6 μm). Additional details concerning the validation experiments are provided in Supporting Information: Methods S1.

2.5. Bioinformatics analysis

The raw sequencing data were analyzed as follows. Sequences were clustered into OTUs using the UPARSE algorithm and a ≥97% similarity criterion after quality screening (Edgar, 2013); chimeras were discarded with UCHIME. Total OTU counts for each sample were rarefied to the level representing the lowest number of sequences per library. Bacteria and fungi OTUs were assigned against RDP (Cole et al., 2013) and UNITE databases (Nilsson et al., 2019), respectively. Taxonomic assignments for nematode and protist OTUs were determined by SINTAX classifier (Edgar, 2016) against the PR2 database (version 4.12). We calculated the alpha diversity index using the QIIME pipeline (alpha_diversity.py).

Propagule size and gram strain of bacteria were defined at the phylum level according to previous reports (Table S5) (Luan et al., 2020; Zinger et al., 2018). Trophic functional modes of fungi were classified by FUNGuild and the taxa with confidence rankings of "highly probable" and "probable" were retained (Nguyen et al., 2016). Functional guilds for nematodes and protists were identified at genus level based on summaries by Yeates et al. (1993) and Adl et al. (2019), respectively.

2.6. Statistical analyses

Differences in soil properties, alpha diversity and abundance of soil organisms between the four metal contamination levels were assessed by one-way ANOVA and Tukey's HSD post-hoc test if the data met normality and homogeneity of variance requirements (Table S6 and Table S7). If conditions were not met, the differences were assessed by Kruskal-Wallis's test followed by Wilcoxon post-hoc test. Variations in communities based on Weighted UniFrac distance among different groups were evaluated with PERMANOVA using the *GUniFrac* package (Chen et al., 2012). Relationships between soil factors and the four organism communities were evaluated by the Mantel test and presented with *ggcor* package (Huang et al., 2020). Mantel tests were also used to analyze the pairwise relationships between bacteria, fungi, protist and nematode community composition using *vegan* package (Oksanen et al., 2022). A confirmatory factor analysis (CFA) model was constructed with *lavaan* package (Rosseel, 2012), wherein soil properties and biota communities were represented by the first axis of PCoA and all variables were standardized by Z transformation to improve normality (Zhao et al., 2019). We brought our potential hypotheses into the CFA model (step 1) and applied the "modificationindices" function in *lavaan* package (step 2) to represent the more important potential relationships for further model evaluation (Rosseel, 2012). Steps (1) and (2) were repeated until the model fitting indices were optimized. Bioindicators were selected by *Deseq2* at genus level (by comparison of background versus metal-polluted treatments) with $P < 0.05$ (FDR<5% under the Benjamini-Hochberg correction) and $|\log_2$ fold change$|>1$, which resulted in two catagories: sensitive and tolerant bioindicators (Love et al., 2014).

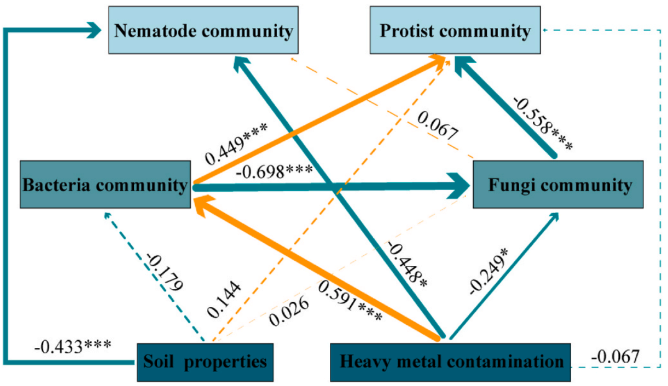
Bipartite networks were constructed under different contamination levels using Spearman correlation values to remove interactions between OTUs of the same species. To improve the robustness of network inferences, counts were transformed into relative abundances before network analysis (Faust, 2021). For consistent comparison across networks, cutoff values of $Padj<0.05$ and $\rho > 0.8$ were applied and the same number of OTUs (all 7940 OTUs) were chosen for all samples of each group to fully access relationships within the soil microbial food web. Networks were visualized in *Gephi* and the topological properties were calculated with *igraph* package (Csárdi and Nepusz, 2006). Heatmaps were applied to display the degree of nodes for taxa in the networks. All analyses were conducted in R (version 4.0.4).

3. Results

3.1. Diversity and community composition of soil biota

Metal contamination increased the diversity of bacteria and fungi, decreased the diversity of protist, and had comparably little effect on overall nematode diversity. Bacterial and fungal diversity in the metal-contaminated groups were significantly higher as compared to the background level (Fig. S3a). Conversely, protist diversity followed an opposite trend, showing a striking reduction for the three contamination groups versus background (Fig. S3a). There were no significant differences for nematode alpha diversity among the four contamination levels (Fig. S3a). Within the OTUs constituting bacterial diversity, a greater number of bacterial OTU types was observed in contaminated treatments (Fig. S4a). A total of 27.3% and 18.2% of fungal genera in the background group were saprotrophs and symbiotrophs (Fig. S4b), and 20% and 15% of the protist OTUs in the background group were bacterivores and omnivores, respectively (Fig. S4c). Absolute abundances of the four biota types did not vary significantly among the four metal contamination levels, except for the background vs. high contamination treatment for fungi abundance (Fig. S3b). The community composition of bacteria, fungi and protist in the background group was significantly different from that in the low, moderate and high contamination groups (PERMANOVA, $P < 0.01$), but showed no significant differences among the three contamination levels (Table S8 and Fig. S5, PERMANOVA, $P > 0.05$).

Bacterial, fungal and protist communities displayed significant correlations with the Nemerow index and heavy metal concentrations (Fig. 1). The links of bacterial and fungal communities experiencing metal contamination were stronger than for the protist community, as revealed by higher Mantel's r (Fig. 1). Conversely, the nematode community was more strongly correlated with other soil properties (e.g., pH, TC, TN) (Fig. 1). The bacterial ($r = 0.61$, $P < 0.001$) and fungal ($r = 0.50$, $P < 0.001$) community compositions had significant correlations with the protist community composition, but not with nematode community composition (Fig. S6), inferring a potential trophic cascade between the prey (both bacteria and fungi) and predator (only protist) communities under metal contamination. A confirmatory factor analysis (CFA) model showed that heavy metal contamination affected protist community composition mainly via changing its prey (bacterial and fungal) community composition (Fig. 2).



Chisq = 0.108; $P = 0.742$; GFI = 0.999; CFI = 1.000; AIC = 287.292; SRMR = 0.005; RMSEA = 0.000

Fig. 2. Direct and indirect effects of metal contamination on nematode, protist, bacterial and fungal community composition. The effects of heavy metal contamination were illustrated by a structural equation model (SEM). Continuous and dashed arrows indicate significant and non-significant relationships, respectively. The width of the arrow is proportional to the path coefficient (adjacent number). Biota community and soil properties are represented by PCoA1 (the first principal coordinate). Notations *, ** and *** indicate significant correlations at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

3.2. *Sensitive and tolerant taxa in soil microbial food web*

Metal contamination inhibited the growth of protist-preferential prey and favored the growth of protist-nonpreferential prey. Given the significant differences in protist, bacterial and fungal communities between background and metal-polluted groups, we identified the indicative genera by comparing the background with metal-polluted groups to determine which responsive genera accounted for the community alterations (Fig. S7). We further divided these indicators into different functional groups to gain insights into the general resource exploitation characteristics of the indicative genera (Figure S7 and Fig. 3). Bacterivorous and omnivorous genera identified as dominant sensitive protists

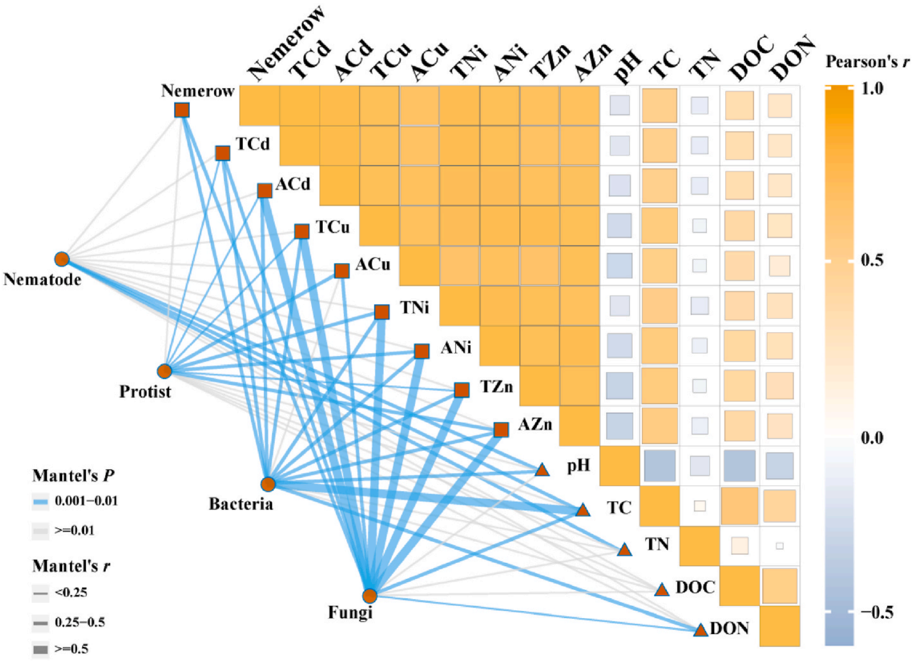


Fig. 1. Pairwise comparisons of soil chemical properties with a yellow-blue gradient denoting Pearson's correlation coefficient. Community composition was related to each soil property by Mantel tests. Width of edges represents the Mantel's r-value. Color of edges denotes Mantel's P. Abbreviations: TCd, Total Cd; ACd, Available Cd; TCu, Total Cu; ACu, Available Cu; TNi, Total Ni; ANi, Available Ni; TZn, Total Zn; and AZn, Available Zn.

to metal contamination contributed 66.4% of the sensitive protist abundance (Fig. 3a). *Sandonidae_X*, *Spongospora*, *Sandona*, *Thraustochytriaceae_X*, *Heterococcus*, *Limnofilidae_X*, *Mb5C-lineage_X* and *Echinamoeba* were shared and dominant sensitive genera. The relative abundances of these genera declined exponentially as the Nemerow index increased from 0 to 2, and then slightly declined as the Nemerow index further increased from 2 to 6 (Fig. S8).

As for sensitive bacteria genera, *Acidobacteria*, *Alphaproteobacteria* and *Deltaproteobacteria* contributed 79% of the sensitive bacteria abundance (Fig. 3b). The *Gp6*, *Geobacter*, *Gp2*, *Gp4*, *Sphingomonas*, *Nitrospira*, *Streptomyces* and *Latescibacteria_genera_incertae_sedis* were shared and dominant sensitive genera. The relative abundance of these genera declined exponentially as the Nemerow index increased from 0 to 2, and then remained constant with further increases in the Nemerow index (Fig. S9). As for tolerant bacteria genera, large-sized bacteria (*Actinobacteria* and *Betaproteobacteria*) contributed 33% of the tolerant bacteria abundance (Fig. 3b). The relative abundance of 8 tolerant genera (*Nocardioides*, *Arthrobacter*, *Intrasporangium*, *Jatrophihabitans*, *Angustibacter*, *Kutzneria*, *Nakamurella* and *Catenulispora*) from *Actinobacteria* in the background group was 2–12 times lower than those in the metal-polluted groups (Fig. S10).

The greatest differences between the proportions of sensitive and tolerant taxa were found for fungi. The proportion of tolerant fungi (12.96%) was 52% higher than that of sensitive fungi (8.51%) (Fig. 3c). Symbiotrophic fungi were only enriched in the background soil. In contrast, the proportion of obligate pathogenic fungi increased substantially in contaminated soils and took the place of symbiotrophic fungi as the second most dominant fungi in the tolerant fungi group (Fig. 3c). Moreover, pathotrophs contributed to 32.9% of the common tolerant fungi species number (Fig. S4b). The relative abundances of pathogenic fungi (e.g., *Cochliobolus, Edenia, Mycosphaerella* and *Setosphaeria*) in the metal-polluted groups were significantly higher than those in the background group (Fig. S11).

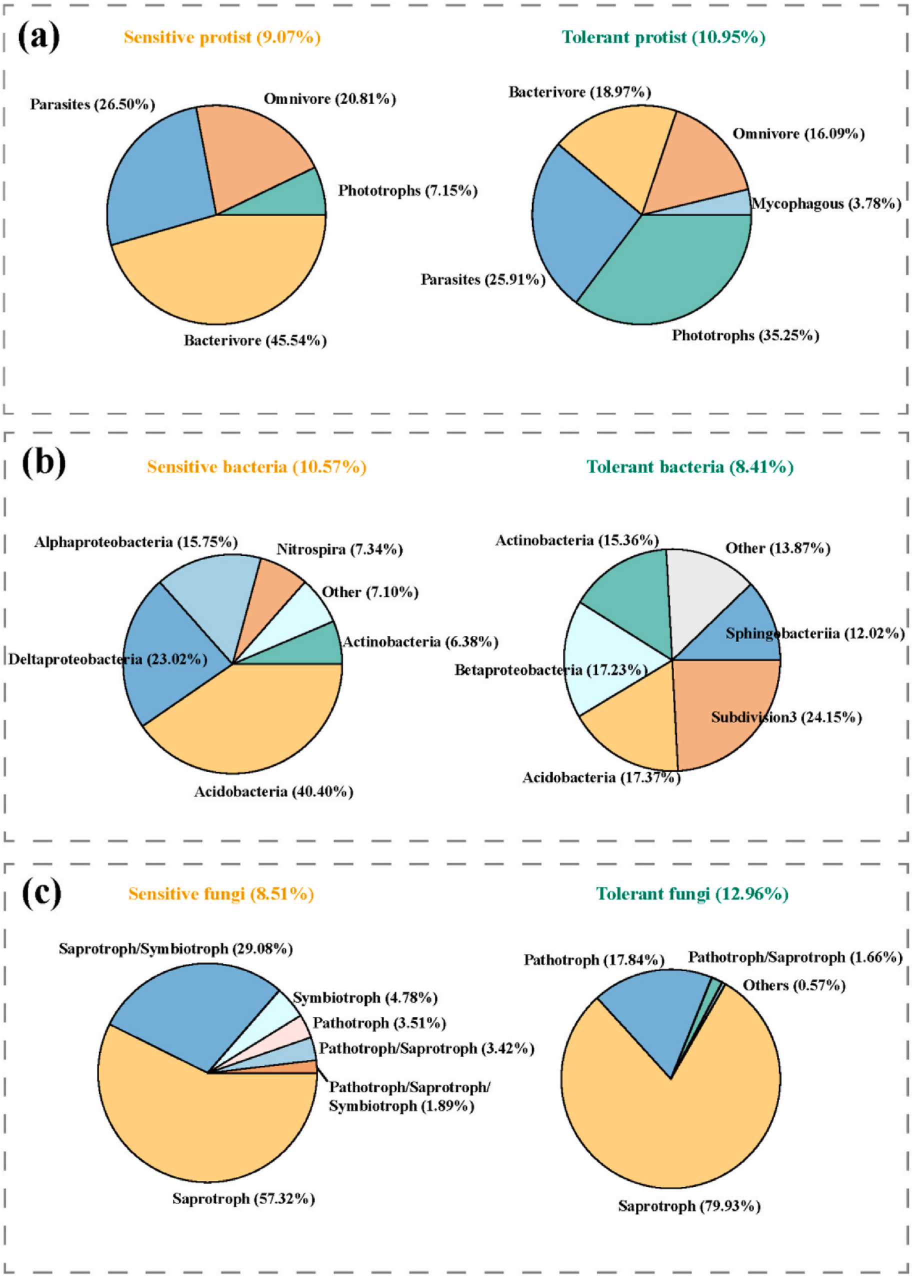


Fig. 3. Pie charts showing the proportions of sensitive (left) and tolerant (right) taxa at the trophic level for protist (a), phylum level for bacteria (b) and functional guild level for fungi (c). The responsive genera (sensitive genera and tolerant genera) were identified by *Deseq2* (by comparison of background versus metal-polluted treatments) with $P < 0.05$ (FDR<5% under the Benjamini-Hochberg correction) and $|\log_2$ fold change$|>1$ and further divided based on functional groups for protist and fungi. Pie charts do not contain the undefined genera because the unidentified genera occupied a large portion of the figure but provided limited information. The responsive nematode genera were not identified as there were minimal differences in nematode communities between background and metal contamination treatments. The proportions of sensitive and tolerant taxa were given at the top of each panel in yellow and green characters, respectively.

3.3. Potential multi-trophic interactions in soil microbial food web

Metal contamination decreased potential trophic-level interactions between predators and preys in the soil microbial food web as reflected by the decreasing number of nodes, edges and average degree. There was a trend for a progressive decrease in the number of nodes and edges with increasing metal contamination (Fig. 4 and Table S9). The node number for all four organisms decreased progressively with increasing metal contamination, wherein nematodes only contributed 2–3% of the total network nodes (Table S9). Average degrees in the network followed a power-law distribution ($P < 0.001$) and sharply declined with an increase in contamination level (Fig. 4 and Table S9). Significant differences were observed for the distribution patterns of degree, betweenness, closeness and transitivity among the four contamination levels (Table S10, $P < 0.001$) as determined by the Kolmogorov-Smirnov test, indicating a significant difference for whole network topological attributes among the four networks.

Metal contamination exerted effects on selected groups to influence the network interactions. The node degrees of both sensitive and tolerant genera decreased with increasing metal contamination (Fig. S12). In the background-level network, 41% of the degrees were observed in the sensitive bacterivorous protists and small-sized bacteria nodes (Fig. S12a). The connections between the sensitive species (e.g., bacterivorous protist) and other genera disappeared gradually along the metal contamination gradient (Fig. S12a). For instance, *Sandonidae_X* (a sensitive bacterivorous protist) had 126 links with other genera in the background network, but had no links with others in the high contamination network (Fig. S12b). Although the relative abundance of some species in *Actinobacteria* increased dramatically, such as *Arthrobacter*, *Intrasporangium*, *Micromonospora* and *Nocardioides*, their degrees did not increase with increasing metal contamination (Fig. S12b).

We defined taxa with the top 20° as key players in the networks and extracted out the sub-networks of three bacterivorous protists (*Sandonidae_X*, *Eocercomonas* and *Thraustochytriaceae_X*) with the most degrees to explore how the predator-prey relationships changed along the metal contamination gradient (Fig. S13). Potential predator-prey relationships became simpler when the contamination level increased from background to moderate, just as observed for the entire co-occurrence networks (Fig. S13). Three bacterivorous protists tended to form links with their preferential prey of small-sized bacteria, such as *Acidobacteria*

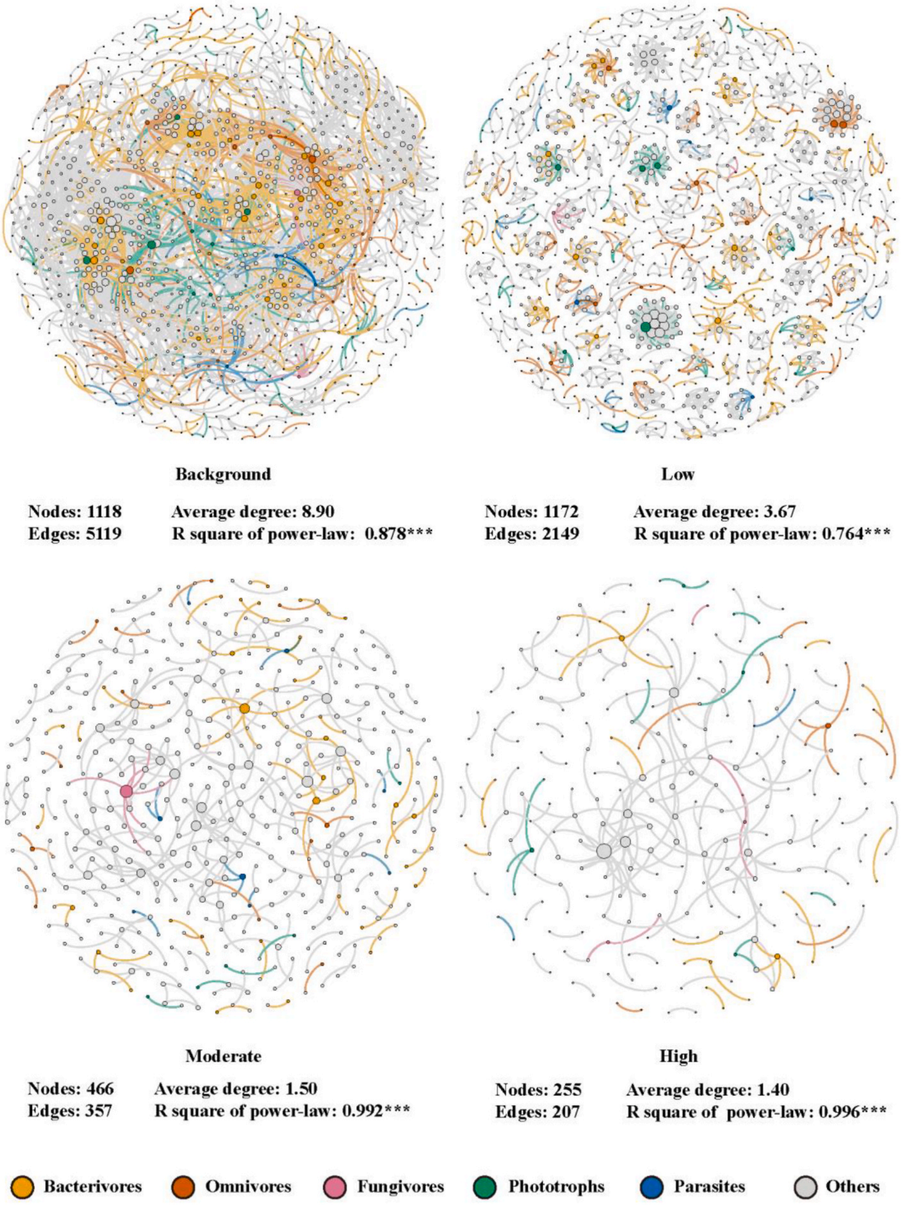


Fig. 4. Trophic interactions of bacteria, fungi, protists and nematodes decreased along metal contamination gradient. The node size is proportional to the node degrees and shows the connections of one taxon with other taxa. Node colors indicate the nodes belong to bacterivores (yellow nodes), omnivores (orange nodes), fungivores (pink nodes), phototrophs (green nodes), parasites (blue nodes) and other taxa (grey nodes). Edge colors represent the functional guilds using the same color scheme as for node colors.

subdivisions (*Gp*) and *Alphaproteobacteria,* with this tendency unchanged under metal pollution stress (Fig. S13).

3.4. Metal effects on trophic-level interactions in incubation experiments

Validation incubation trials revealed that metal contamination inhibited the growth of protist (*Colpoda*) via decreasing the abundance of protist-preferential prey (*Sphingomonas*) and stimulating the abundance of protist-nonpreferential prey (*Arthrobacter*) (i.e., bottom-up regulation). In the *Colpoda* (protist) monoculture system, the abundance of *Colpoda* trophozoites and its cysts did not decrease when metal contamination was introduced and there were no significant differences in *Colpoda* density among the three metal contamination levels (Fig. 5). The bacteria monoculture systems showed contrasting results with the abundance of *Sphingomonas* (protist-preferential prey) significantly decreasing with increasing contamination levels (Fig. 5). Moreover, the abundance of *Arthrobacter* (protist-nonpreferential prey) increased as the contamination increased from low to high metal levels (Fig. 5). In the *Colpoda* + *Sphingomonas* co-incubation system, abundance of *Sphingomonas* decreased significantly when the contamination level increased from background to higher metal contamination levels (Fig. 6a). Similarly, the abundance of *Colpoda* decreased up to 14-fold when the contamination level increased from background to higher metal contamination levels (Fig. 6a). In the *Colpoda* + *Arthrobacter* co-incubation system, the abundance of *Arthrobacter* significantly increased from low to high contamination levels (Fig. 6b). By contrast, the abundance of *Colpoda* trophozoites and its cysts declined progressively by up to 18 and 35 times, respectively (Fig. 6b). When *Colpoda* was co-incubated with *Arthrobacter* and *Sphingomonas*, the trend for *Colpoda* trophozoites showed a gradual decrease with increasing pollution levels, similar to that found for the *Arthrobacter* + *Colpoda* and *Sphingomonas* + *Colpoda* co-incubation systems (Fig. 6c). Accordant with *Colpoda* trophozoites, the abundance of *Sphingomonas* progressively declined with increasing pollution level (Fig. 6c). Conversely, the abundance of *Arthrobacter* gradually increased when the pollution level increased from low to high (Fig. 6c).

4. Discussion

4.1. Metal contamination alters predator-prey diversity and community composition

Metal stress has been shown to affect soil overall bacterial and fungal diversity (Yu et al., 2021). Our study further showed the specific taxa contributing to the increase in bacteria and fungi diversity under metal contamination stress. The protist-nonpreferential prey in *Actinobacteria* mainly contributed to the increase in bacteria diversity. Under metal contamination stress, some bacteria in *Actinobacteria* have the capacity to tolerate and absorb heavy metals (Alvarez et al., 2017), thereby allowing them to become enriched in metal-rich environments (Li et al., 2017). In contrast, small-sized bacteria and gram-negative bacteria (e.g., *Gp6*, *Geobacter* and *Sphingomonas*) were sensitive to metal contamination stress, resulting in their decrease in diversity and abundance under metal stress. These findings are consistent with previous studies that

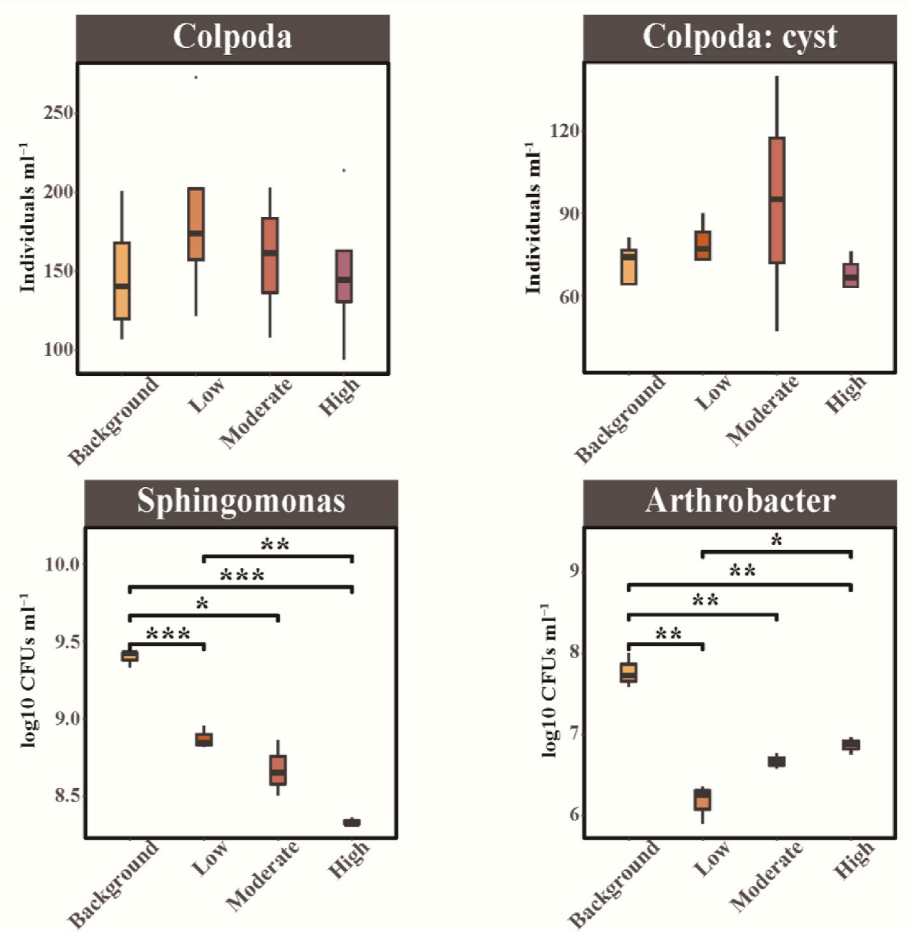


Fig. 5. Changes in the abundances of trophozoites and cysts of *Colpoda*, *Sphingomonas* and *Arthrobacter* across background, low, moderate and high metal contamination levels in the monoculture system. The density of bacteria is logarithmically normalized. Notations *, ** and *** indicate significant differences at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

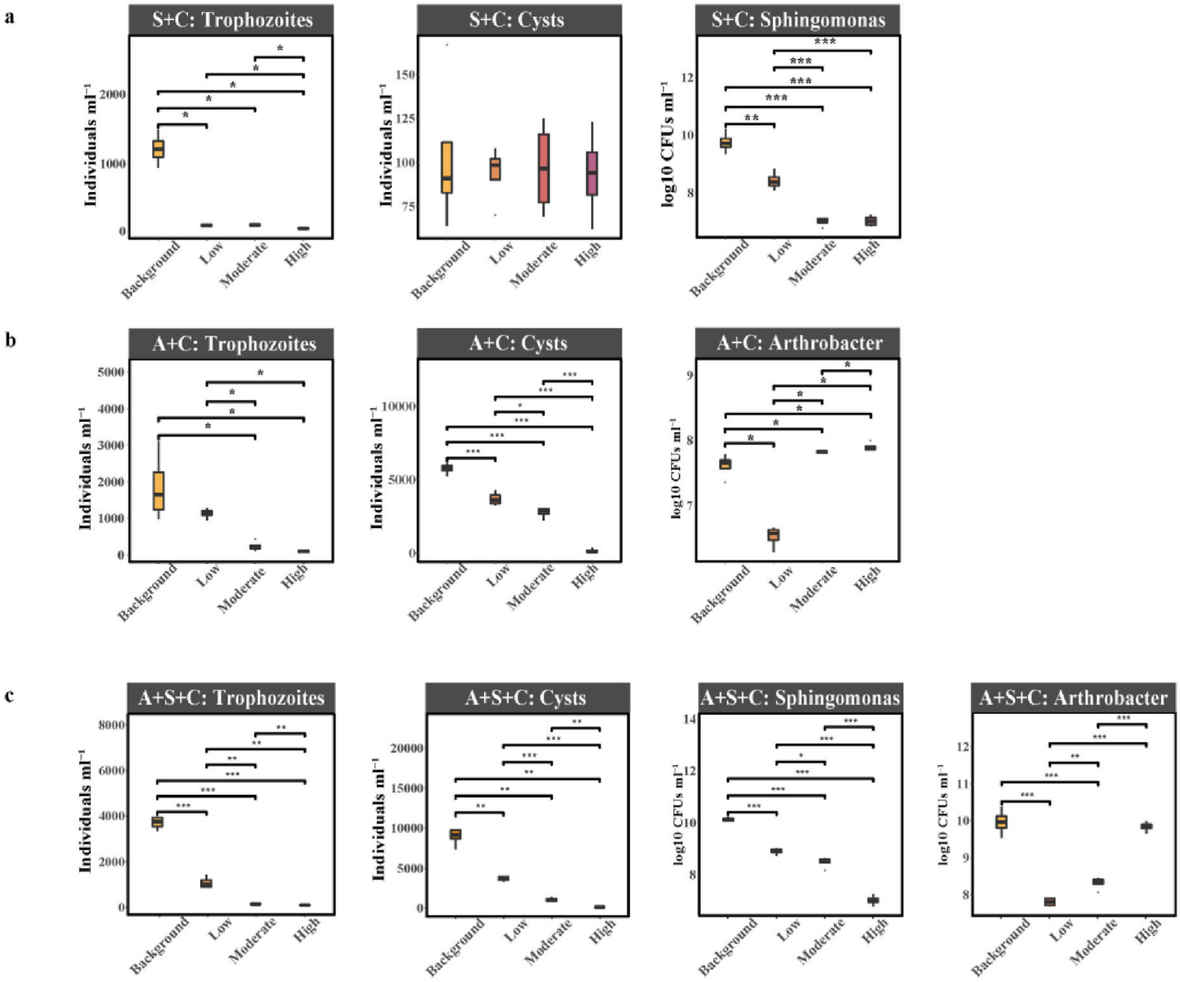


Fig. 6. Changes in the abundances of trophozoites and cysts of *Colpoda*, *Sphingomonas* and *Arthrobacter* across background, low, moderate and high metal contamination levels when co-incubated *Colpoda* with its preferential prey (*Sphingomonas*) or/and nonpreferential prey (*Arthrobacter*). The density of bacteria is logarithmically normalized. S + C, *Sphingomonas* + *Colpoda*; A + C, *Arthrobacter* + *Colpoda*; A + S + C, *Arthrobacter* + *Sphingomonas* + *Colpoda*. Notations *, ** and *** indicate significant differences at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

demonstrated *Acidobacteria* was the most sensitive biomarker in a metal-polluted soil (Wang et al., 2019), and *Geobacter* was predominant in unpolluted soil but disappeared in soils polluted with E-waste metals (Liu et al., 2015).

Fungi diversity increased due to an increase in both type and abundance of pathogenic fungi. For instance, the genera *Cochliobolus* and *Exserohilum*, identified as tolerant genera enriched in metal-contaminated soils (Fig. S11), comprise many destructive plant and human pathogens (Adler et al., 2006; Manamgoda et al., 2011). In contrast, arbuscular mycorrhizal fungi (*Claroideoglomus*, *Diversispora*, *Funneliformis*, *Glomus*, *Paraglomus* and *Rhizophagus*) that enhance plant growth and mitigate phytotoxicity (Li et al., 2006; Riaz et al., 2021) were depleted in the metal-polluted soils. The decrease in the diversity of beneficial taxa together with the increasing growth of potentially harmful taxa under metal contamination may alter the soil microbial food web highlighting an ecological risk to soil health and productivity.

The decrease in protist diversity under metal contamination stress was attributed to a decrease in both types and abundances of bacterivores and omnivores. These protistan consumers are more easily affected by environmental perturbations than other groups of protists, as their prey are more sensitive to environmental change (Nguyen et al., 2020; Zhao et al., 2020). In contrast, the nematode community was relatively less impacted by metal contamination than the protist community. We posit that nematodes are strongly affected by other soil properties (e.g., pH, TC, TN) as shown by the significant correlations between selected soil properties and the nematode community.

Our sampling strategy minimized heterogeneity in other environmental factors that have a strong influence on soil biota by focusing on a metal contamination gradient within an otherwise uniform agricultural site. The diversity of soil bacteria, fungi and protist changed markedly when the combined metal contamination level increased from background (0<Nemerow index≤1) to low contamination (1<Nemerow index≤2), but showed no significant changes at higher contamination levels (Fig. S3). Additionally, the relative abundance of sensitive protist and bacteria genera decreased exponentially when the Nemerow index increased from 0 to 2, with the negative response remaining constant at higher Nemerow index values (Figs. S8 and S9). Based on the responses of microbial diversity and abundance to combined heavy metal contamination, a "contamination warning range" was identified at the background−low contamination boundary corresponding to Nemerow index values of 0–2. Although the "warning range" may vary depending on the composition of heavy metals and exceedance multiples for a given heavy metal, the "warning range" provides a valuable reference as metal contaminated sites are typically mutually polluted by Cd, Zn, Cu and other heavy metals (Johansen et al., 2018; Zhang et al., 2021).

4.2. *Metal contamination collapses trophic-level interactions by bottom-up regulation*

Predators usually structure prey communities through top-down regulation under natural conditions (Flues et al., 2017; Jiang et al., 2020). In contrast, our results infer a bottom-up regulation in the microbial food web under heavy metal contamination stress, that is metal contamination affected the higher trophic level (predator community) via alteration of the lower trophic level (prey communities) (Fig. 2). This was supported by 1) the concurrent changes in the abundances of predators and prey; 2) the trophic interactions between protist and their prey revealed by network analysis; and 3) the confirmatory laboratory incubation trials. This finding provides novel insights regarding the role of environmental stress from heavy metal contamination on shaping trophic-level relationships through bottom-up regulation.

The attenuation of protist predation in the networks was attributed to selective predation based on the prey characteristics. Predator feeding preference is related to prey traits (i.e., cell size, gram stain and chemical exudates), with small-sized prey preferred because they are easy to ingest (Liu et al., 2017). Additionally, consumers tend to prey on gram-negative bacteria because their cell membrane is much thinner than that of gram-positive bacteria (Mai-Prochnow et al., 2016), which renders gram-negative bacteria more easily digested by bacterivores (Petters et al., 2021). Conversely, some taxa may produce chemicals to inhibit the growth of predators. For example, *Actinobacteria* and pathogenic fungi, which were the main protist-nonpreferential prey and enriched in the metal polluted soils in our study, can secrete antibiotics and mycotoxin to kill predators (Richter et al., 2019; Thakur and Geisen, 2019; Winter et al., 2019; Yan et al., 2021). Consumers can also distinguish pathogenic taxa from non-harmful taxa by experience and avoid predating pathogenic species (Zhang et al., 2005). Thus, combined metal contamination exerted a bottom-up regulation on the protist community by diminishing palatable prey (e.g., small-sized and gram-negative bacteria) and enriching unsavory prey (e.g., *Actinobacteria* and pathogenic fungi). Notably, the protist non-preferential prey were enriched in the metal contaminated soils as a direct consequence of contamination and indirectly from competition release and predation. It has been reported that the protist non-preferential prey (mainly belong to *Actinobacteria* and pathogenic fungi) are tolerant species that can thrive under metal contamination (Akhtar and Shoaib, 2019; Alvarez et al., 2017; Li et al., 2017). In addition, the susceptible protist preferential prey (e.g., *Alphaproteobacteria* and *Deltaproteobacteria*) were inhibited by heavy metal contamination and consumed by protist (Czarny et al., 2020) (Fig. S13), which provids more opportunities for the protist non-preferential prey to thrive under metal contamination stress. Moreover, the depleted gram-negative bacteria were dominated by *Alphaproteobacteria* and *Deltaproteobacteria* (Fig. S4), which are copiotrophs (Fierer et al., 2007). The gram-positive bacteria enriched under metal contamination were mainly assigned to *Actinobacteria,* which are oligotrophs (Fierer et al., 2007). Copiotrophs and oligotrophs are equipped with different life strategies and tend to use different nutrients (Fierer et al., 2007). The higher edibility of gram-negative bacteria (mainly copiotrophs) in our research may further exert effects on soil nutrient dynamics.

Network analysis is a very useful tool to decipher microbial interactions and key players (Jordán et al., 2015). Average degree represents network complexity most intuitively (Wagg et al., 2019; Xiong et al., 2021). Network analysis showed a power-law distribution of node degrees, indicating that some nodes possessed much higher links than others (Banerjee et al., 2019). The disappearance of some key nodes could catastrophically crash the network into many disconnected sub-networks. Consistently, when heavy metal stress was introduced, key player nodes such as small-sized or gram-negative bacteria (*Acidobacteria* subdivisions, *Geobacter* and *Sphingomonas*) with the highest degrees were depleted, and consequently the node degree of key player protist consumers (*Sandonidae_X*, *Eocercomonas, Thraustochytriaceae_X* and *Sandona*) also decreased. Conversely, taxa such as large-sized bacteria (*Actinobacteria*) and pathogenic fungi were enriched in contaminated soils, but still had lower degrees because they are protist-nonpreferential prey and experienced few interactions with consumers in all of the networks. Taken together, the average degrees of the network decreased with increasing metal contamination levels and as a result, the whole network complexity decreased. A healthy biotic system contains complex interactions, providing a strong resistance to disturbance (Ives and Cardinale, 2004). Our study implies that under metal contamination stress, trophic interactions in the soil microbial food web were impaired, making the soil system less resilient and more susceptible to additional stressors.

We chose three representative taxa, one typical predator (*Colpoda*) and two distinct preys (*Sphingomonas* and *Arthrobacter*), to construct a simplified incubation system to validate the bottom-up regulation. *Colpoda* was the most prevalent protist identified in a global-scale survey (Oliverio et al., 2020) and has been widely used for toxicity bioassays and predation tests (Asiloglu et al., 2021; Darbyshire et al., 1994; Madoni, 2000). Similarly, *Sphingomonas* and *Arthrobacter* have been identified as sensitive and tolerant indicators for heavy metal contamination (Figs. S9 and S10) and typical protist-preferential and protist-nonpreferential prey, respectively. The validation test underpinned our inferences that heavy metal contamination inhibited the growth of protist by decreasing the abundance of protist-preferential prey and stimulating the abundance of protist-nonpreferential prey in co-incubation systems. When the predator-prey relationship was formed, the protists became dependent on the prey, and their abundances were 10 times higher than those in the monoculture system (Figs. 5 and 6). Due to the tight predator-prey relationship, growth of predators was highly associated with changes in the abundance of preferential and non-preferential prey caused by metal contamination, thereby confirming bottom-up regulation. By contrast, the growth of protist in a monoculture system was not inhibited by metal contamination. Validation research involving more taxa merits further exploration.

We demonstrated that protists were comparably more responsive to heavy metal contamination than nematodes based on co-occurrence networks. This was most likely attributed to their body size. Larger body size provides restricted access by nematodes (avg. body size ~1 mm) to their prey inhabiting microsites within the soil matrix as compared to the smaller protists (avg. body size ~20 μm) (Petters et al., 2021). Additionally, the abundance of nematodes in our study was much lower than found in other soil systems (van den Hoogen et al., 2019), which results in weaker predation by nematodes.

5. Conclusions

Our study provides novel insights into the role of anthropogenic perturbation (combined heavy metal contamination) on exerting a bottom-up regulation on trophic-level interactions within the soil microbial food web. We investigated the diversity, abundance and predation relationships of organisms within the soil microbial food web and identified a heavy metal “contamination warning range” corresponding to a Nemerow index range of 0–2 caused potential ecological risks to the soil ecosystems. Heavy metal contamination exerted a bottom-up control on the protist consumers by decreasing the abundance of protist-preferential prey and their interactions with other organisms, resulting in a dramatic reduction of interactions in soil microbial food web. The mechanism of bottom-up regulation was further verified by incubation experiments. The decreased trophic-level interactions in the microbial food web under metal contamination stress impose potentially far-reaching ecological risks on soil ecosystem services. Further investigations of bottom-up regulation by environmental stressors on soil functions carried out in the context of the entire microbial food web are strongly warranted to assess linkages to several ecological services, such as sustainable agriculture, biogeochemical cycling and climate regulation.

Author contributions

J.X. designed the study. X.W., Z.D., J.S., H.Y., and J.X. performed field sampling. X.W., H.Z., H.Y., and Y.Y. conducted the experiment. L. H., X.C., M.L., and X.K. provided experimental methods for soil fauna extraction and incubation. X.W., Z.D., J.L., and B.M. conducted the data analyses. X.W., Z.D., R.A.D. and J.X. wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2023.109058.

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