

# Soil protist communities in burrowing and casting hotspots of different earthworm species

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## ABSTRACT

Biotic interactions in the soil food web have been increasingly recognized as crucial for ecosystem functioning, however there are still knowledge gaps in terms of the associations between macrofauna and microorganisms, particularly in soil hotspot microsites. A manipulative study was performed to investigate the protist community in the drilosphere and casts of three earthworm species (epigeic *Amyntas cortices*, anecic *Amyntas hupeiensis* and endogeic *Drawida gisti*) and a control soil without earthworms. Results showed that earthworm presence significantly increased the relative abundance of parasitic protists ( $p < 0.05$ ). The diversity of protists within drilosphere and casts depended on earthworm species, with *D. gisti* and *A. hupeiensis* increasing diversity and *A. cortices* decreasing protist diversity ( $p < 0.05$ ). Structural equation modelling indicated that protist abundance and diversity in the drilosphere were regulated indirectly via neutralized soil pH and accelerated C and N mineralization, while in casts were affected directly through earthworm gut processing and indirectly through elevated water content. In conclusion, the marked effects on soil protist community mediated by earthworms varied from distinct earthworm species and microsites. The present study suggests the necessity to consider interactions between protist and earthworms in evaluating the effects of soil fauna on soil biota and the related ecosystem processes.

## 1. Introduction

The belowground ecosystem harbors greater diversity of organisms than its aboveground counterpart, making it an arena supporting more intensive biotic interactions (Torsvik et al., 2002; Wardle, 2002, 2006). Such biotic interactions in turn play key roles in maintaining the belowground biodiversity and determining a range of ecosystem processes (Bardgett and Wardle, 2010; Thakur and Geisen, 2019). As such, there has been a growing body of studies about biotic interactions in soil ecosystems, where the interactions between soil macrofauna and microorganisms have been increasingly studied (Chang et al., 2016a; Hoeffner et al., 2018; Luo and Gu, 2018). In addition to bacteria and fungi in soil, the interaction between protists and macrofauna also plays an important role in soil ecological functioning but remains less understood.

Earthworms are known as ecosystem engineers and keystone species in soil (Blondel and Aronson, 1995; Brussaard et al., 2007; Alaoui et al.,

2011), and are functionally important due to their capability in regulating nearly all soil biotic and abiotic properties (Edwards, 2004; Blouin et al., 2013; Bertrand et al., 2015; Liu et al., 2019). They interact with and exert profound effects on microbial communities via complex processes, either directly or indirectly (Gómez-Brandón et al., 2011; Pass et al., 2015; Gong et al., 2019). As unavoidable constituents of earthworm diets, microbial patches are digested selectively after gut processing (Curry and Schmidt, 2007; Jayasinghe and Parkinson, 2009; Zirbes et al., 2011). Earthworms could also impact microorganisms through modifying the physicochemical properties of their habitats (Blouin et al., 2013). However, most studies have focused on the effects of earthworms on the whole soil (Brown et al., 2004; Gong et al., 2018), few investigated their effects on microbes within micro-scale drilosphere and casts (Brown, 1995; Devliegher and Verstraete, 1997; Zirbes et al., 2012; Schlatter et al., 2019a), especially for soil protists (Bonkowski and Schaefer, 1997; Sampedro, 2007; Andriuzzi et al., 2016).

Drilosphere and casts, serving as proxies for improved organic matter

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transformation (Moorhead et al., 2012), are resource-rich and biological-active hotspots derived from burrowing, feeding and casting activities of earthworms (Brown et al., 2004; Kuzyakov and Blagodatskaya, 2015; Hill et al., 2018; Schlatter et al., 2019b). The drilosphere has been defined as the layer of 2 mm thick soil around burrow walls with enriched carbon (C) and nitrogen (N) contents and elevated rates of nutrient turnover (Brown et al., 2000; Schrader et al., 2007; Moorhead et al., 2012; Stromberger et al., 2012). Additionally, earthworms mix soil organic matter and inorganic materials via gut processing and excrete them as casts, a great contribution to biological aggregation (Lipiec et al., 2015b; Haydu-Houdeshell et al., 2018). Together, microsites (i.e. drilosphere and casts) provide suitable shelters and abundant bioavailable resources for soil microbial communities (Jegou et al., 2001; Lipiec et al., 2015a). Though the magnitudes and ways of earthworms influencing soils vary in different hotspots, whether there exists a discrepancy of soil community between drilosphere and casts remains unknown. Past decades have witnessed increasing studies of bacterial and fungal communities within earthworm-mediated soil hotspots (Montecchio et al., 2015; Aira et al., 2016; Hoeffner et al., 2018). However, soil protists, as critical components of soil microflora and food web with huge biomass and enormous diversity (Bouwman and Zwart, 1994; Geisen et al., 2018), are largely neglected in previous studies. Though protists are considered as the main microbial consumers and also a driver of carbon and nutrient cycling in soil, the structure of protist communities is still poorly understood (Geisen et al., 2017; Fiore-Donno et al., 2019). Recently, high-throughput sequencing which allows the detection of a broader range of soil protist diversity (Bates et al., 2013; Mahe et al., 2017), has contributed to our knowledge of protist community (Bachy et al., 2013). The soil protist community is subject to soil physiochemical conditions such as moisture, pH, soil pore size and nutrient content (Tsyganov et al., 2013; Zahn et al., 2016). These conditions are notably influenced by earthworms and especially in the drilosphere and casts. Biotic effects such as earthworm feeding could affect specific taxa of protists via gut processing. For example, it has been reported that several free-living protists failed to adapt to the intestinal environment and disappeared after the digestion process of earthworms (Brown et al., 2004), while some parasitic protists and protists in resistant (cyst) stages were able to survive through the gut process (Ibrahim et al., 2016). Given the unique roles of earthworms and protists, particularly their interactions in soil ecosystem functionality, investigating the micro-scale responses of soil protist in earthworm-mediated drilosphere and casts would contribute to interpreting the soil protist community structure and distribution in distinct microsites.

Previous studies demonstrated that different ecotypes of earthworms modify soil properties distinctly (Ferlian et al., 2018; Xiao et al., 2018). Earthworms from different ecotypes affect microbial community through niche partition and resource allocation (Sheehan et al., 2008; Gómez-Brandón et al., 2012). Such species-specific effects also occur in species within the same ecotype due to other factors like feeding preference and body size (Chang et al., 2016b; Hoeffner et al., 2018). Although earthworm species identity has attracted increasing attention, there is limited knowledge about the effects of earthworm species identity on soil protists.

We aimed to explore protist patterns at the earthworm-mediated microsites (i.e. drilosphere and casts) of three earthworm species classified as three ecotypes (i.e. epigeic, anecic and endogeic). However, due to the limitation of incubation time and microcosm volume, the ecotype-associated effect on soil properties was weakened in our experiment and the differences among treatments of earthworm species could be attributed to species-specific effect (Ernst et al., 2008; Chang et al., 2016b). It is hypothesized that (1) the soil protist community in the drilosphere was altered indirectly by soil physiochemical properties such as moisture content, pH and nutrient bioavailability (Andriuzzi et al., 2016) and soil protist community in casts was altered directly mainly through earthworm gut processing (Drake and Horn, 2007),

which leads to the discrepancy of soil protist community between drilosphere and cast; (2) earthworm species identity could result in disparate effects on soil protist community via niche partitioning and distinct physiology pattern (Newington et al., 2004; Xiao et al., 2018).

## 2. Materials and methods

### 2.1. Earthworms and soils

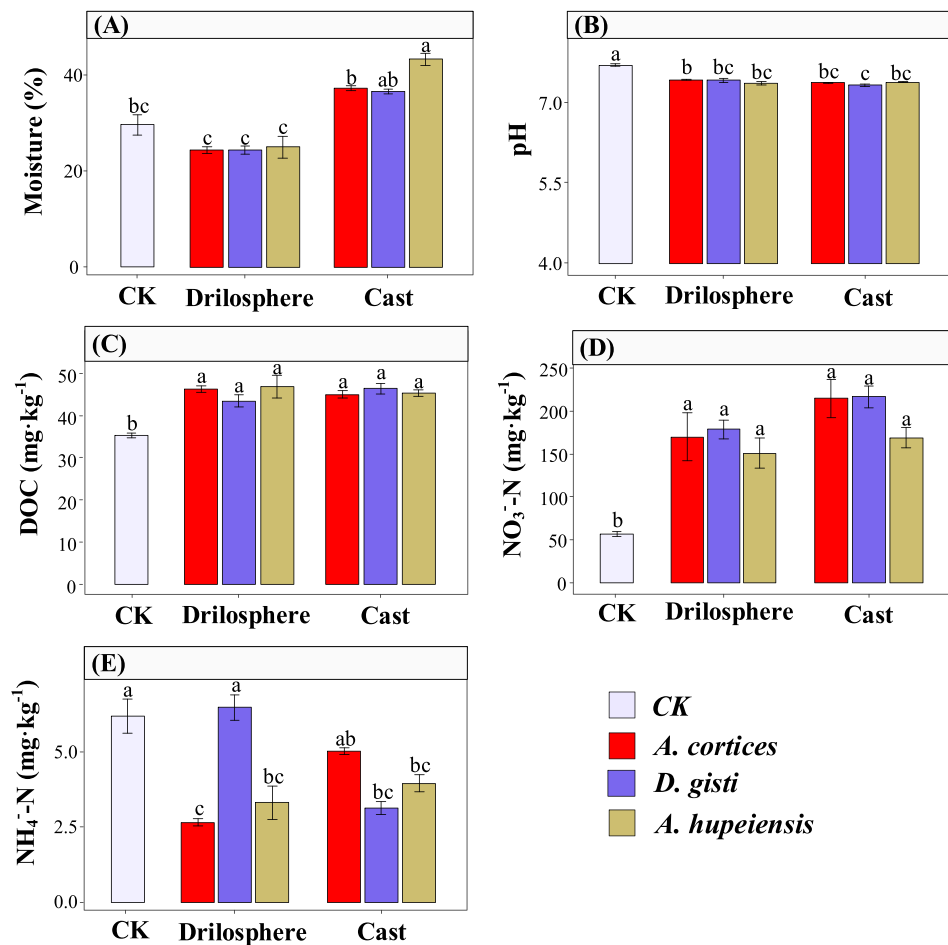
All earthworms were collected in June 2017 from Zijin Mountain (118°50'27" E, 32°30'52" N), Nanjing, China, a natural reserve grown with mixed forest vegetation composing of coniferous, evergreen and deciduous broad-leaved trees, located in a subtropical region. Local earthworm species including *Amyntas cortices*, *Amyntas hupeiensis* and *Drawida gisti* belonging to epigeic, anecic and endogeic ecotypes, respectively (Bouché, 1972), were collected. Individuals with the same body size for each species were selected (Appendix Table S1), transported immediately to the lab and kept in the respective experimental soils for preincubation and acclimation for one week in a climate chamber at 25 °C prior to the experiment. To eliminate the influence of earthworm activities, soil from the 5–20 cm layer and without obvious earthworm burrows and casts was collected. The sandy loam soil consisted of 30% sand (20–2000 µm), 34% silt (2–20 µm) and 36% clay (<2 µm). The soil was sieved through a 2 mm mesh after removing visible debris and fauna manually.

### 2.2. Incubation experiment setup

The experimental design was set up with two microsite levels including drilosphere (the layer of 2 mm thick soil around burrow walls) and casts across three earthworm species levels (*A. cortices*, *A. hupeiensis* and *D. gisti*) and a control without any earthworms, which summed to 7 treatments. Each treatment had 4 replicates leading to 28 experimental units in total. Earthworms were placed on filter paper soaked with sterile water for 48 h to evacuate gut contents and then washed by sterile water prior to inoculation into corresponding microcosms (Dalby et al., 1996). Earthworms were added to each microcosm with the same fresh weight of 8.0 g, which was considered to be suitable for earthworm growth and activities (Lowe and Butt, 2005). The microcosms were composed of 1 L white polyethylene containers (12 cm × 15 cm, height × diameter) filled with 600 g dry weight of soil adjusted to 60% water-holding capacity, and added 10 ml sterile water every two days to keep soil moist. All containers were sealed with nylon mesh preventing earthworms from escape and incubated in a climate chamber at 25 °C and 12/12 h light/dark periods.

### 2.3. Microsite soil sampling and analysis

After 5 days of incubation, sampling was included as following: the cast excreted by earthworms; drilosphere, the layer of 2 mm thick soil around burrow walls; soils from the control without earthworms. Soils from control were collected using a sterile shovel after the whole soil column being broken down and 50.0 g from each plot were fully mixed as one composite sample. The casts and drilosphere soil were sampled by the following method: Firstly, casts on the surface of the soil column in the container were carefully collected with tweezers; then, the whole container together with the soil column was turned upside down on the table and then the container was detached from the soil column and removed vertically exposing casts and drilosphere in the flank and bottom of the soil column. Sub-surface casts were carefully collected with tweezers followed by drilosphere soil which was gently scraped away using a sterile scalpel; finally, the soil column was broken up and the inner casts and drilosphere soil were collected using the same method. Soil for edaphic analysis were stored at 4 °C and analyzed within a week after sampling and soils for DNA extraction were archived at −70 °C.



**Fig. 1.** The effect of three earthworms on (A) soil moisture, (B) pH, (C) DOC, (D)  $\text{NO}_3^-$ -N and (E)  $\text{NH}_4^+$ -N within drilosphere and cast. CK represents control without earthworms. Bars represent mean values and error bars show standard errors (n = 4). Different lowercase letters represent significant difference among drilosphere, cast and control soil without earthworms (Tukey's HSD test;  $p < 0.05$ ).

Soil protists including ciliates, flagellates and amoebae were enumerated by most probable number method (Darbyshire et al., 1974) with following adaptations: Briefly, 3 g of fresh weight soil was suspended in 30 ml sterile Neff's Modified Amoeba Saline (NMAS) (Page, 1976) and vigorously shaken on a vertical shaker (HYL-C2, China) at 180 rpm for 30 min to detach protists from soil particles. Three-fold dilution series with tryptic soy broth (0.1 g/L) and NMAS at 1:9 v/v were added into 96-well microtiter plates. Plates were sealed with parafilm and incubated at 15 °C in darkness. Each well was inspected three times after 7, 14 and 21 days for presence of protists with an inverted microscope at 100 × to 400 × magnification.

Soil moisture content was determined gravimetrically on a dry weight basis after drying at 105 °C for 24 h (Gardner, 1983). Soil pH was measured by combining soils and boiled distilled water with a ratio of 1:2.5. Dissolved organic carbon was extracted by mixing soil with 50 ml ultrapure water and centrifuging (8000 rpm, 10 min). The obtained supernatant was then filtered through a 0.45 μm polyethersulfone membrane filters and analyzed by a total C analyzer (Elementar, Germany) and a continuous flow analyzer (Skalar, Holland). Ammonia and nitrate were extracted by 2 M KCl (1:5 ratio of soil to KCl) filtered through 0.45 μm paper filters and analyzed by a continuous flow analyzer (Skalar, Holland).

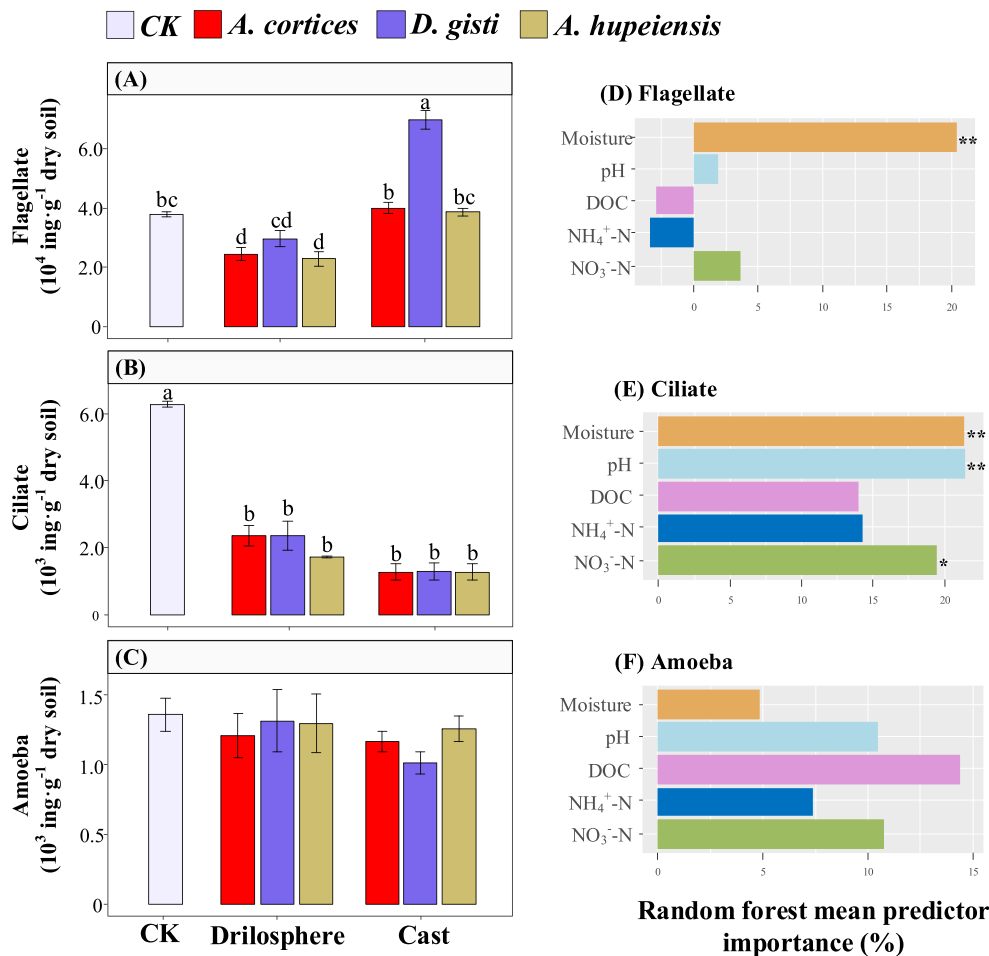
#### 2.4. Extraction of soil DNA and Illumina HiSeq sequencing

Total DNA from each soil sample was extracted from 0.25 g fresh weight soil using the DNeasy® PowerLyzer® PowerSoil® Kit according

to the manufacturer's instructions and checked for concentration by using UV/VIS spectrometer (NANODROP 2000; America). The obtained genomic DNA was used for Illumina HiSeq2000 sequencing on a HiSeq platform. Polymerase chain reaction (PCR) was conducted using primers 3NDf (5'-GGCAAGTCTGGTGCCAG-3') (Cavalier-Smith et al., 2009) and V4\_euk\_R2 (5'-ACGGTATCTRATCRTCCTCG-3') (Brate et al., 2010) attached with barcode flanking the eukaryotic V9 hypervariable regions of 18S rRNA gene. PCR amplification was performed in 25 μL volume with 30 ng of template DNA, 1 μL of each primer (10 μM), 3 μL of BSA (2 ng/μL), 12.5 μL of 2 × Taq PCR MasterMix and 7.5 μL of ddH<sub>2</sub>O. The PCR amplification program consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 45 s at 95 °C, 50 s at 55 °C, 45 s at 72 °C and ending with a final extension of 10 min at 72 °C. All amplified DNA samples were pooled and checked for successful amplification by running 3 μL of PCR products in a 2% agarose gel and then purified with a PCR purification kit. Paired-end amplicons were performed on the Illumina HiSeq platform of personal biotechnology company Allwegene Tech (Beijing, China). All the raw sequences were deposited in NCBI with accession number SRR10836915- SRR10836970.

#### 2.5. Data analysis

Raw sequences were assembled by unique barcode using QIIME version 1.9.1 (Caporaso et al., 2010). The split sequences were merged using FLASH (Magoc and Salzberg, 2011), after the low-quality sequences were discarded using QIIME. The retained sequences with a length lower than 100 bp or with a quality score lower than 0.1 were



**Fig. 2.** The left figures show the effect of three earthworms on total number of (A) flagellates, (B) ciliates and (C) amoebae within drilosphere and cast. CK represents control without earthworms. Bars represent mean values and error bars show standard errors ( $n = 4$ ). Different lowercase letters represent significant difference among drilosphere, cast and control soil without earthworms (Tukey's HSD test;  $p < 0.05$ ). The right figures show the random forest mean predictor importance (% increase MSE) of the edaphic physicochemical properties with respect to abundance of (D) flagellates, (E) ciliates and (F) amoebae. Significance levels of each predictor are represented by \* $p < 0.05$  or \*\* $p < 0.01$ .

removed. After discarding singleton reads, clustering of OTUs at 97% similarity was performed. Then we discarded chimerae by using UCHIME method (Edgar et al., 2011). Finally, the PR2 database (Guillou et al., 2013) was used as the reference for taxonomy assignments. The diversity of each sample was determined using Shannon index and the number of observed OTUs. Non-metric multidimensional scaling was conducted to visualize the protist community differences across different microsites of earthworm species. We assigned the top 100 OTUs accounting for 68% of the total sequences into function groups to predict potential protist functions according to a previous review of eukaryotic classification (Adl et al., 2012).

All statistical analysis was conducted using the R Version 3.4.4 (Team, 2013) and results were presented as mean values attached with standard errors. A two-way ANOVA was performed to test for the main and interactive effects of earthworms and microsites on soil physicochemical properties and protist abundance and diversity, followed by Turkey's HSD test. A classification random forest (RF) analysis (Breiman et al., 2001), conducted by R package "randomForest" (Liaw and Wiener, 2002), was performed to evaluate the most important and credible soil properties predicting the abundance of soil protists. The significance of RF model and the cross validated  $R^2$  was assessed with 5000 permutations using R package "A3" (Fortmann-Roe, 2013). The significance of each determined soil property was assessed using R package "rfPermute" (Archer, 2013). Structure equation modelling (SEM) was performed using SPSS and AMOS Version 24 (IBM Corporation, NY, USA) to evaluate the pathways how earthworms (presence or absence) affect soil physicochemical properties (pH, moisture, DOC,  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$ ) and soil protist abundance (the number of flagellates, ciliates and amoeba) and diversity within drilosphere and casts.

Three metrics including Chi-squared ( $\chi^2$ ) test, the comparative fit index (CFI) and the standardized root mean square residual (SRMR) was applied to calculate the adequacy of models (Schermelleh-Engel et al., 2003).

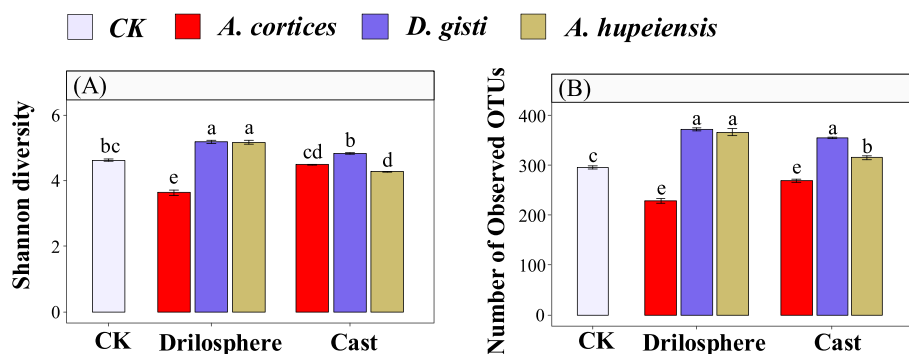
### 3. Results

#### 3.1. Soil physicochemical properties

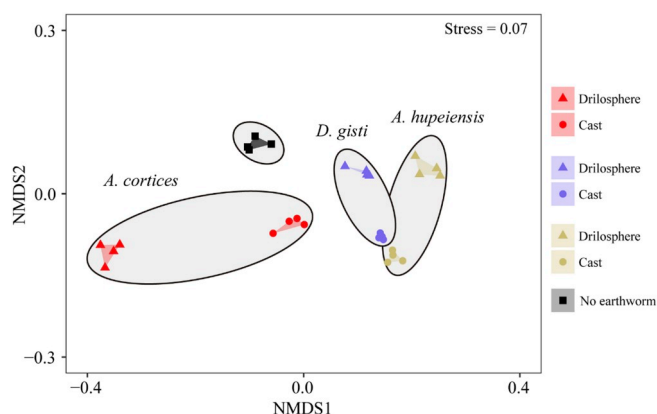
Soil moisture in the drilosphere was significantly lower than that in casts ( $p < 0.05$ , Fig. 1A). Regardless of microsites, earthworms decreased soil pH and increased the content of dissolved organic carbon and nitrate ( $p < 0.05$ , Fig. 1B, C and D). With *A. cortices*, ammonia content in the drilosphere was significantly lower than that in cast, whereas the reverse was true for *D. gisti* ( $p < 0.05$ , Fig. 1E).

#### 3.2. Soil protist community abundance and structure

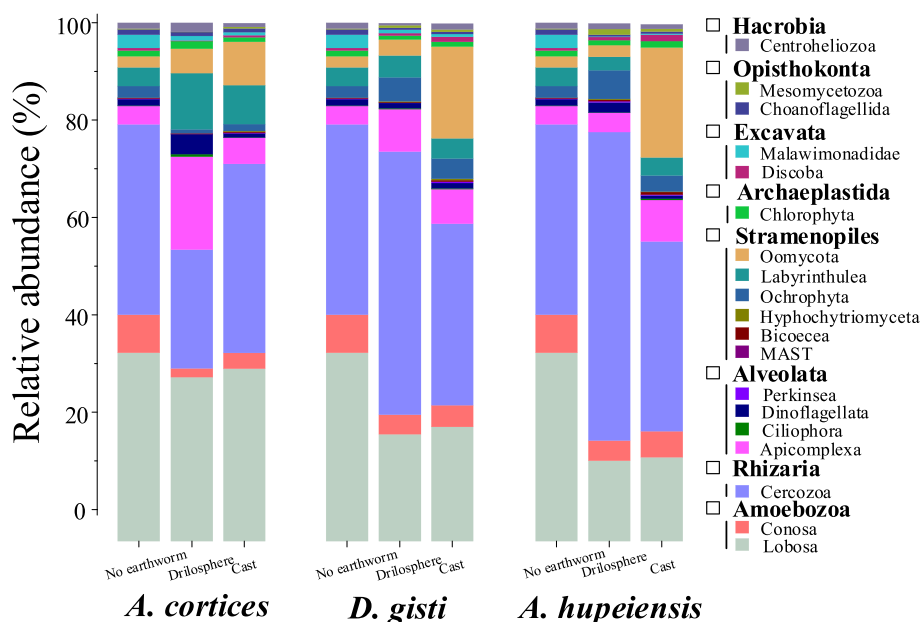
The abundance of flagellates in casts was higher than that in drilosphere for each earthworm species and the casts of *D. gisti* had the most flagellates among all the earthworm species ( $p < 0.05$ , Fig. 2A), showing a significant reliance of flagellate abundance on earthworm species (Table S2). Earthworms markedly decreased ciliates in both drilosphere and casts ( $p < 0.05$ , Fig. 2B). The abundance of amoeba showed no significant differences between drilosphere and cast (Fig. 2C). The result of Random Forest models demonstrated that the abundance of flagellates and ciliates were significantly influenced by soil moisture (Fig. 2D and E), while the soil properties in present study showed no significant influence on the abundance of amoeba (Fig. 2F).



**Fig. 3.** The effect of three earthworms on (A) Shannon diversity and (B) the number of observed OTUs within drilosphere and cast. CK represents control without earthworms. Bars represent mean values and error bars show standard errors ( $n = 4$ ). Different lowercase letters represent significant difference among drilosphere, cast and control soil without earthworms (Tukey's HSD test;  $p < 0.05$ ).

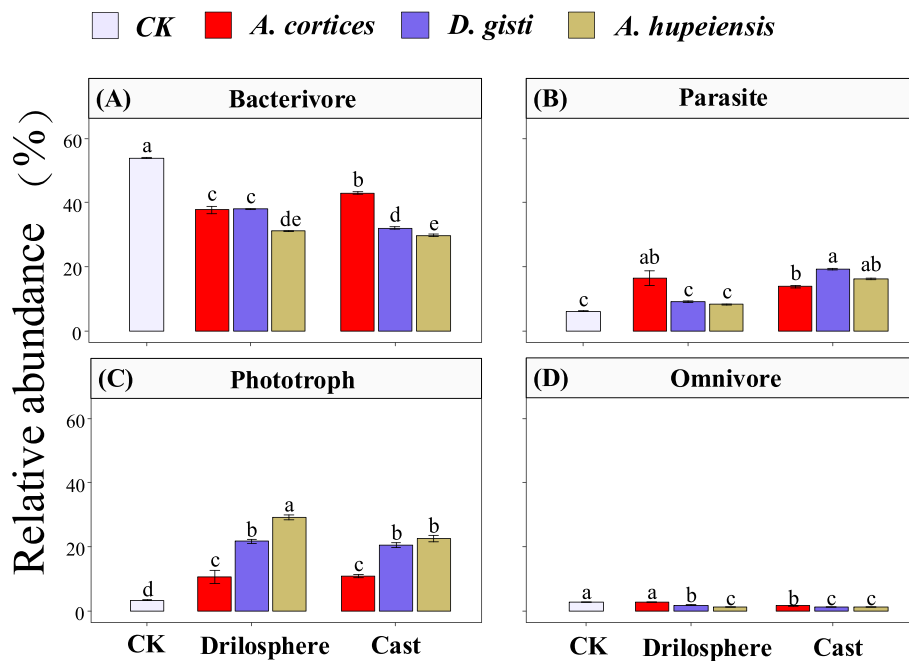


**Fig. 4.** Non-metric multidimensional scaling analysis based on protist OTU composition of phylum level in earthworm drilosphere, cast and soil without earthworms. The symbols filled with red, blue and yellow color represent *A. cortices*, *D. gisti* and *A. hupeiensis*, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 5.** Taxa plots summarizing the relative abundances of taxon-assigned OTUs identified for the earthworm drilosphere, cast and soil without earthworm. Taxon appears at phylum level of the PR2 database and represents  $>0.1\%$  of the total data. Both graph and legend share the same order, sequential from bottom to top.





**Fig. 6.** The effect of three earthworms on relative abundance of protist functional group (A, Bacterivore; B, Parasite; C, Phototroph; D, Omnivore) within drilosphere and cast. CK represents control without earthworms. Bars represent mean values and error bars show standard errors (n = 4). Different lowercase letters represent significant difference among drilosphere, cast and control soil without earthworms (Tukey's HSD test;  $p < 0.05$ ).

### 3.3. Mechanism of protist community response to earthworms in drilosphere and casts

The pathway of soil protist abundance and diversity responding to earthworms in the drilosphere was different from that in casts (Fig. 7A and B). In the drilosphere, earthworms indirectly affected protist abundance and diversity by lowering soil pH and ammonia content as well as increasing dissolved organic carbon and nitrate content. In casts, earthworms directly and negatively correlated with protist diversity and indirectly affected protist abundance by increasing soil moisture.

## 4. Discussion

Hotspots such as drilosphere and casts, which were created by earthworms in a short time (Stromberger et al., 2012; Hill et al., 2018; Schlatter et al., 2019b), showed distinct soil properties and this influence would diffuse to the surrounding bulk soil over time (Mudrák and Frouz, 2017). In our study, the experiment lasted only a few days which enabled us to compare the effects of earthworms and casts with very little effect on the remaining soil. However, the earthworm-induced effect on soil hotspots in such controlled laboratory system was probably larger than that under the field condition.

### 4.1. Pathways of earthworm effects on protist community within drilosphere and casts

Structural equation modeling analysis indicated different mechanisms driving the protist community structure in drilosphere and casts. These differential effects might result from the differences in ways and extent of earthworms interacting with soils through burrowing and cast production. In the drilosphere, earthworms affected soil protists indirectly via changing soil physicochemical properties. Our study also detected a changed soil pH in the drilosphere. A number of studies have emphasized the role of soil pH in determining microbial diversity at continental scales (Ferrenerg et al., 2013) as well as at micro-scale such as aggregatosphere and rhizosphere (Jiang et al., 2017; Fan et al., 2018; Gong et al., 2019). In this study, earthworms neutralized soil pH in the

drilosphere, probably due to their acidic epidermal mucus (Zhang et al., 2009). The neutral soil pH facilitated the formation of diverse soil microbial community such as bacteria (Tripathi et al., 2018), which could have an impact on soil protist diversity via trophic chain interactions (Rønn et al., 2002; Clarholm, 2005; Thakur and Geisen, 2019). In addition, the drilosphere contains abundant labile organic matter including epidermal mucus and is featured with high microbial enzyme activities, resulting in the accelerated C and N transformation (Tiunov and Scheu, 1999; Stromberger et al., 2012). Therefore, the elevated dissolved organic carbon and nitrate deposited in the drilosphere probably led to priming effects of native soil organic matter. The increased bioavailable resource is favorable to the microbial community including bacteria and fungi, which subsequently drives protist community via trophic interactions (Trap et al., 2016; Thakur and Geisen, 2019; Xiong et al., 2019). It should be noted that the increased content of dissolved organic carbon might be partly derived from some taxa of protists that failed to survive the drilosphere, leading to the negative relationship with protist abundance (Marschner and Kalbitz, 2003; Saleem et al., 2016). Moreover, the concentration of ammonia nitrogen was positively related to protist diversity and abundance. This phenomenon could be attributed to the diverse protist community with a higher C:N ratio than their bacterial prey, resulting in the release of extra nitrogen mainly in form of  $\text{NH}_3$  (Sherr et al., 1983; Coleman and Wall, 2015).

In casts, protist diversity was affected by earthworm gut-associated processes. The processes occurring in the intestinal environment strongly affects the microbial communities in the excreted casts (Domínguez, 2004). We observed a prominent decline of protist diversity in casts of *A. cortices* and relative abundance of bacterivores from functional group analysis as well as the number of ciliates from MPN analysis in casts of all earthworm species, indicating selective digestion of free-living protists in the gut (Pierce and Phillips, 1980; Cai et al., 2002). Recent studies highlighted ubiquitous host-associated protists lodging in the digestive track of earthworms (Wegener Parfrey, 2015; Nana et al., 2018; Borovičková et al., 2019), which was confirmed here by the high parasitic protist abundance in casts (Fig. 6B). In addition to direct effects, earthworms might affect protists indirectly via altering

(A)  $\chi^2 = 10.202$   $P = 0.116$   $df = 6$   $CFI = 0.97$   $RMSEA = 0.216$

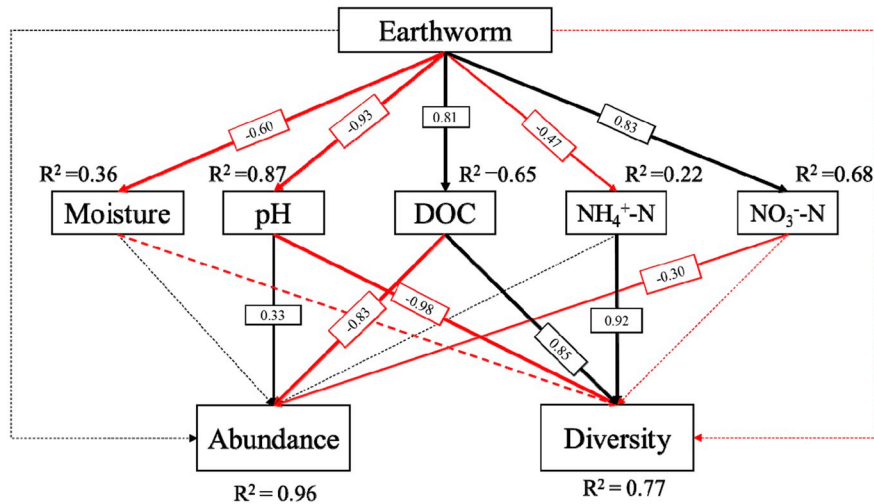
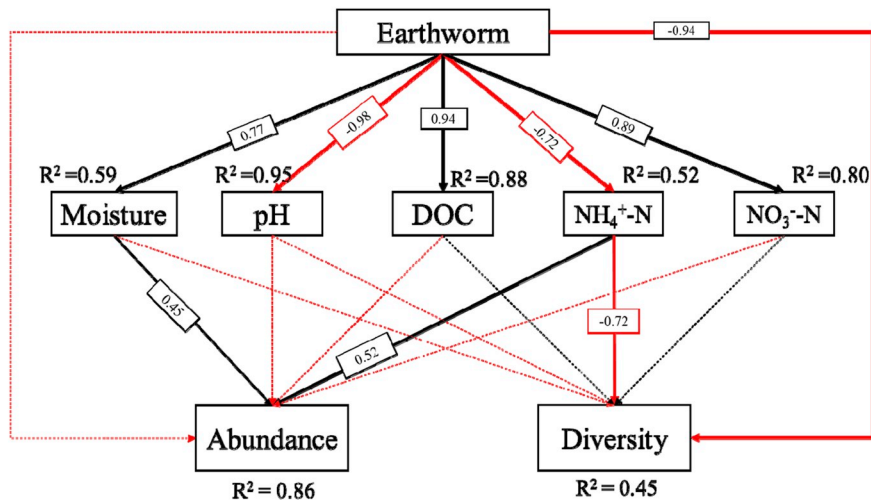


Fig. 7. Effects of earthworms (presence or absence) and soil physicochemical properties on protist abundance and diversity as indicated by structural equation modelling in (A) drilosphere and (B) cast. Significant effects ( $p < 0.05$ ) are plotted by solid lines, and non-significant effects are plotted by dotted lines. Black arrows indicate positive relationship and red arrows indicate negative relationship. Numbers at arrows are standardized path coefficients. The proportion of variation explained by the model ( $R^2$ ) are shown next to each endogenous variable. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(B)  $\chi^2 = 13.131$   $P = 0.216$   $df = 10$   $CFI = 0.98$   $RMSEA = 0.144$



moisture content of casts. Random forest analysis revealed that soil moisture had the highest mean predictor for determining the abundance of soil ciliates and flagellates (Fig. 2D and E), which is consistent with previous results that the abundance of protists decreased in soil with low moisture (Geisen et al., 2015a). A recent review demonstrated that soil water availability controls the development of phototrophs (Holzinger and Karsten, 2013), which agreed with their increased relative abundance along with increasing moisture in casts (Fig. 6C). A similar result was found for Oomycota and Apicomplexa (Fig. 5), which was supported by several studies revealing that some plant and animal parasites varied with soil water availability (Chadfield and Pautasso, 2012; Kolman et al., 2015; Cohen and Ben-Naim, 2016). Thus, the close relationship between soil water availability and soil parasitic protists inhabiting the earthworm gut may provide a broader insight into soil health and ecosystem function.

#### 4.2. Earthworm species-specific effect on soil protist community within soil hotspots

The results supported our second hypothesis that the microscale

effects of earthworms on protists are species-specific. Notably, the interaction of earthworm species and microsites significantly affected protist diversity, stressing the discrepancy of protist diversity in drilosphere and casts induced by earthworm species. Previous studies revealed that it is necessary to explicitly consider species identity when interpreting the mechanisms of how earthworms affect soil abiotic and biotic properties (Postma-Blaauw et al., 2006; Gómez-Brandón et al., 2012; Chang et al., 2016a, 2016b). The anecic *A. hupeiensis* and endogeic *D. gisti* was capable of redistributing organic materials through vertical mixing and excreting mucus of low molecular weight (Pan et al., 2010), strongly increasing resource availability and activating other microorganisms (Lavelle, 1994), which could subsequently lead to the increase of protist diversity. However, the situation was opposite in *A. cortices* with a decline of protist diversity observed, which may be due to its epidermal mucus proteins containing components of innate immunity and inhibiting the growth of some protists directly (Kauschke et al., 2007; Wang et al., 2011). Moreover, the abundance of flagellates was significantly dependent on earthworm species, confirming a strong species identity effect on protist abundance.

Recent studies revealed that soil hotspots such as rhizosphere or

detritosphere harbor a distinct protist assemblage compared with surrounding soil (Cleven, 2004; Kramer et al., 2016; Guo et al., 2018; Gao et al., 2019). The present study adds evidence that earthworm drilosphere and casts, serving as well-known biopore-induced hotspots (Kuzakov and Blagodatskaya, 2015; van Groenigen et al., 2018), could affect the protist assemblage. The NMDS analysis showed that the protist structure of *A. cortices* demonstrated a divergence compared with that of *D. gisti* and *A. hupeiensis*, also suggesting the central role of earthworm identity in shaping protist assemblage. The SAR group comprised of Stramenopiles, Alveolata and Rhizaria dominated the protist taxa across all treatments, which was in line with previous studies (Geisen et al., 2015b; Murase et al., 2015). The relative abundance of Apicomplexa increased in the drilosphere of all three earthworm species, indicating that such taxa of protists could infect animals (Rueckert et al., 2011). Moreover, the main subclass of the taxa in the current study was Coccidia, which was able to infect the intestinal tract of earthworms (Velavan et al., 2010; Owa et al., 2013). The introduction of earthworms also stimulated the taxon of Oomycota, which have been recognized as potential plant pathogens (Presti and Kahmann, 2017). Thus, earthworms could promote parasitic protists in the drilosphere and casts, facilitating the growth of animal parasitic protists as well as the translocation of some plant pathogens due to their movements within soil (Mahe et al., 2017).

## 5. Conclusion

The effects and mechanisms of earthworms on soil protists varied with earthworm species and their derived hotspots. Generally, earthworms reshaped the protist community by increasing the proportion of parasites within the drilosphere and in casts. Specifically, protist diversity increased after inoculation of *D. gisti* and *A. hupeiensis*, but decreased with *A. cortices*, highlighting that earthworm-mediated effects on protist diversity varied from different earthworm species. Protist abundance and diversity in the drilosphere were affected indirectly by earthworms neutralizing soil pH and increasing resource availability, while those in casts were regulated by direct gut processing and indirect elevated water availability. Together, the divergent protist community occurring in burrows and cast of distinct earthworm species paves the way towards extrapolation of protist community pattern from micro-scale to large-scale. These results help to unfold the protist community structure at microscale created by earthworms and understand the top-down effects of macro soil fauna impacting on micro biota.

## 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.

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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.2020.107774>.

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