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Exposure of soil collembolans to microplastics perturbs their gut microbiota and alters their isotopic composition

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ABSTRACT

Effects of microplastics on aquatic organisms have been widely studied in recent years but effects on soil biota, and especially on the gut microbiota of soil animals, remain poorly understood. An experiment was therefore conducted using the common soil collembolan *Folsomia candida* exposed to microplastics for 56 days to investigate the effects of plastics on gut microbiota, growth, reproduction and isotopic turnover of collembolans in the soil ecosystem. A diverse microbial community was observed in the collembolan gut, consisting of (at phylum level) Actinobacteria (~44%), Bacteroidetes (~30%), Proteobacteria (~12%) and Firmicutes (~11%). Distinctly different bacterial communities and lower microbial diversity were found in the collembolan gut compared with the surrounding soil. We also found that exposure to microplastics significantly enhanced bacterial diversity and altered the microbiota in the collembolan gut. Moreover, collembolan growth and reproduction were significantly inhibited (by 16.8 and 28.8%, respectively) and higher $\delta^{15}N$ and $\delta^{13}C$ values were observed in the tissues after exposure to microplastics. These results indicate that exposure to microplastics may impact non-target species via changes in their microbiota leading to alteration of isotopic and elemental incorporation, growth and reproduction. The collembolan gut microbial data acquired fill a gap in our knowledge of the ecotoxicity of microplastics.

1. Introduction

Polymers are widely used in our daily life (Thompson et al., 2009; Wright and Kelly, 2017) and > 280 million tonnes of plastics are consumed annually (Duis and Coors, 2016). Plastic wastes have become major urban wastes (Zhao et al., 2015) because their degradation is very slow (Al-Salem et al., 2009; Wang et al., 2015). Polymers enter the environment and can then disintegrate with concomitant formation of small plastic particles (Rillig, 2012; Huerta Lwanga et al., 2016b). When the particle size is < 5 mm the material is defined as microplastics (Wright et al., 2013). Microplastic particles are accumulating in the seas and on land due to their durability and this has become a global problem of growing concern (Wright and Kelly, 2017). The effects of microplastics on marine organisms have been intensively studied (Lenz et al., 2016). Numerous studies so far have demonstrated that microplastics can harm aquatic organisms physically and also increase the accumulation of chemical pollutants in the tissues of organisms and disturb their metabolism (Auta et al., 2017; Sussarellu et al., 2017). However, the consequences of microplastics for soil organisms remain largely unknown (Maaβ et al., 2017). So far, only a few studies have investigated the effects of microplastics in soils and terrestrial systems (Huerta Lwanga et al., 2016a,b; Rodriguez-Seijo et al., 2017; Stamiatiadis and Dindal, 1983).

Microplastics can enter the soil environment by a variety of ways including the application of sewage sludge or the residues of plastic mulching films and evidence for the accumulation of microplasticss in soils is increasing (Huerta Lwanga et al., 2016b; Karen and Anja, 2016; Mahon et al., 2017). For instance, approximately 700 plastic particles per kg soil were found in European agricultural land (Barnes et al., 2009; Briassoulis et al., 2010). In north China a large number of agricultural sites are covered with plastic film to retain soil moisture and most of this material is discarded in soils in an unregulated manner (Huerta Lwanga et al., 2016a). It has been reported that soils in many (sub)tropical countries contain large amounts of plastic waste (Huerta

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Lwanga et al., 2016b). There is therefore an urgent need to evaluate the environmental risk of microplastics in soil ecosystems for the rational management of plastic wastes. Huerta Lwanga et al. (2016a) studied the exposure of earthworms to litter spiked with microplastics and observed that the plastic particles significantly lowered their survival and growth rates. Associated histopathological damage and atrophy or detachment of the gut epithelium have been confirmed in earthworms (Rodriguez-Seijo et al., 2017). Several recent studies have also shown that earthworms and collembolans in soils can transport microplastics through their activities (Huerta Lwanga et al., 2016a; Maaβ et al., 2017; Rillig et al., 2017). However, current knowledge of the effects of microplastics on the soil fauna remains inadequate for future ecological risk assessments of microplastics in soils.

Soil collembolans, or springtails, are micro-arthropods comprising a key group of soil mesofauna (Zhu et al., 2016; D'Annibale et al., 2017). They are widespread globally and can occur in high abundance in surface soils (e.g. 1000-100, 000 individuals m^{-2}) (Potapov et al., 2016). They play crucial functions by feeding on soil detritus, litter and microbiota to promote organic decomposition and nutrient cycling in soil ecosystems (Potapov et al., 2016). Furthermore, they are direct ecological vectors for soil pollutants (Chelinho et al., 2017). *Folsomia candida* is a model collembolan that has been frequently used in soil eco-toxicological and genomic studies (Agamennone et al., 2015; Princz et al., 2017). Rillig (2012) has suggested that collembolans may ingest microplastics and one recent study indicates that collembolans can transport microplastics (Maaβ et al., 2017). However, the effects of microplastics on collembolans remain largely unknown and this greatly restricts our understanding of the ecotoxicity of microplastics in soils.

Gut microbiota make important contributions to host health, metabolism and immunity (Agamennone et al., 2015; Berg et al., 2016). For example, gut microbiota play a key role in the absorption of nutrients by many arthropods (Engel et al., 2012). In addition, pollutants may alter the composition of animal gut microbiota but the pattern of shift in gut microbiota is different in different species (Brule et al., 2015; Pass et al., 2015; Raymann et al., 2017). Thus, more studies on responses of gut microbiota to environmental pollutants are needed. Histopathological damage in the earthworm gut were observed in an exposure study of soils spiked with microplastics (Rodriguez-Seijo et al., 2017). Previous studies on aquatic organisms also reveal that microplastics may scratch gut tissues and be retained in the gut (Grigorakis et al., 2017; Vendel et al., 2017). However, the effects of microplastic exposure on the gut microbiota of collembolans have never been investigated. Moreover, information about the composition of collembolan gut microbiota is also lacking. Studying the gut microbial community of *F. candida* will contribute to the identification of the core microbial community in the gut of collembolans and an understanding of the effects of the microbiota on animal health.

The nitrogen and carbon isotope ($\delta^{15}N$ and $\delta^{13}C$) composition of animal tissues are useful indicators of the trophic positions and feeding habits of animals (Ek et al., 2016; Zhu et al., 2016). Recently there has been increasing evidence that environmental pollutants may alter stable isotope contents of animal tissues by interfering with growth rate and metabolic turnover (Ek et al., 2015, 2016; Zhu et al., 2016) but different pollutants have different effects in this regard. The $\delta^{15}N$ and $\delta^{13}C$ values of daphnids exposed to lindane were significantly elevated compared to controls (Ek et al., 2015). In line with this, using collembolans fed cadmium-contaminated yeast, Zhu et al. (2016) found significantly higher $\delta^{15}N$ values in collembolans fed Cd-contaminated yeast than in controls, suggesting a slower rate of nitrogen turnover under the influence of Cd. Against that, Banas et al. (2009) observed that exposure to DDT did not change the $\delta^{15}N$ value of fish, and the $\delta^{15}N$ value of exposed snails generally decreased (Ek et al., 2016). In addition, the characteristics of microplastics are markedly different from those of other chemical pollutants (Costa et al., 2016). This suggests that further studies are needed to explore the effects of microplastics on the stable isotope values of organisms.

The aims of the present study were to identify the core microbial community of the collembolan gut, to compare the microbial communities in soil and in the collembolan gut, to evaluate the effect of microplastic exposure on the microbial community of the collembolan gut using 16S RNA gene high-throughput sequencing, and to explore the changes in the stable isotope composition of exposed collembolan tissues, with concomitant growth inhibition and metabolic imbalance. These results will contribute to enhancing our understanding of the ecological risk from microplastics in soil ecosystems and the inherent relationships between soil and collembolan gut microbial communities.

2. Materials and methods

2.1. Exposure treatment

The parthenogenetic collembolan *Folsomia candida* used in the present study was originally sourced from Aarhus University in Denmark and has been cultured for more than six months in our laboratory. Referring to the standardized methods of the Organization for Economic Co-operation and Development (Zhu et al., 2016), we arranged a suitable breeding environment for *F. candida* in Petri dishes with a layer of moist plaster of Paris/activated charcoal mixture (ratio 8:1 w/w) and obtained age-synchronized *F. candida* individuals. Before microplastic exposure, 7-9-day-old collembolan juveniles were transferred into the test soil (clay loam, WHC 46.8%, pH($CaCl_2$) 4.76, CEC 13.86 cmol kg^{-1}, OM content 24.6 g kg^{-1}, total N content 3.8 g kg^{-1}) from Ningbo, east China, which was not contaminated with microplastics, and pre-incubated for one week at 20 ± 2 °C and 75% relative humidity (RH) with a 16:8 h dark/light photoperiod (800 lux) to acclimate the animals to the new cultivation environment. Throughout the pre-incubation process the mortality of the animals was < 1% and we therefore used these collembolans to start the microplastic exposure experiment. Field conditions were mimicked by withholding food during pre-incubation and exposure experiments. Soil moisture content was maintained by adding distilled water twice weekly.

Commercial polyvinyl chloride (PVC) particles (K-value 72-71) purchased from Aladdin Industrial Corporation (Shanghai, China) were selected as model microplastics to conduct exposure experiments because PVC is one of the commonest polymers in use worldwide. Most of the test PVC particles were between 80 and 250 μm in diameter (Fig. S1) and their viscosity number was 135-127 mL g^{-1}. Uncontaminated PVC particles were obtained by removing any surface absorbed solvent-soluble plastic monomers and other allogenic materials using octane and pentane. The cleaned PVC particles were dried at 50 °C and stored at 4 °C before use.

Two experiments were conducted to test the effect of MP exposure on (1) growth, reproduction and isotope composition and (2) the gut microbiota of the collembolans. In experiment 1, eight 14-16-day-old pre-incubated collembolans were transferred into glass cylinders (inner diameter 5.3 cm, 6.5 cm high) containing 30 g moist soil. The experimental treatments consisted of a control (0 g microplastics kg^{-1} dry soil) and microplastic exposure (1 g microplastics kg^{-1} dry soil). Each exposure treatment was separately repeated five times and the whole exposure test lasted for 56 days. On days 0, 28 and 56, collembolan samples were collected to determine body weight (to reflect body size), elemental composition and isotope values. Moreover, reproduction was counted after 28 days of exposure. In experiment 2, 60 acclimated 14-16-day-old collembolans were exposed to 0 g kg^{-1} (control) or 1 g microplastics kg^{-1} with three replicates. The exposure experiment was conducted in the same cylinders each containing 65 g moist soil. After 56 days of exposure all collembolans were collected for analysis of gut microbiota. The microplastic exposure concentration was chosen on the basis of concentrations found in contaminated soils and the results of studies on the effects of microplastics on earthworms (Huerta Lwanga et al., 2016b).

2.2. Sample collection, detection of microplastics and DNA isolation

After exposure the soil from each Experiment 2 treatment was mixed well to obtain representative sub-samples for DNA extraction. Living collembolan samples were collected from the remaining soils by a water flotation method to extract gut microbial DNA. In brief, each soil was transferred into a 250-mL glass beaker containing 100 mL deionized water. After gentle agitation with a glass rod the floating collembolans were immediately collected and killed with chloroform to retain their ingestion and excretion products. Collembolans were immersed in 2% sodium hypochlorite (NaOCl) for 10 s with gentle shaking to remove microbiota from the body surface and then washed five times with sterilized water. Finally, the animals were dissected under sterile conditions using sterile forceps to obtain the gut. Thirty guts were pooled to give a composite sample and placed into a 2-mL Eppendorf tube with 0.96 mL sterile phosphate buffer solution. Samples of soil and collembolan gut were preserved at −80 °C for subsequent DNA extraction. At the same time, one hundred guts were digested with sodium hydroxide and nitric acid, and microplastics in the collembolan gut were identified using Fourier transform infrared spectroscopy (Roch and Brinker, 2017).

Fresh soil (0.5 g) and thirty collembolan gut samples were used to isolate DNA using a FastDNA Spin Kit for soil (MP Biomedicals, Illkirch, France) following the manufacturer's instructions. The extraction of collembolan gut DNA was slightly different from that of soil. Prior to the bead-beating step, 20 μL proteinase-K (Thermo Fisher Scientific, Waltham, MA) was added to the spin column and incubated at 55 °C for 3 h. The 100 and 50 μL provided DES solutions were used to elute the isolated soil and collembolan gut DNA, respectively. After DNA extraction, 1.0% agarose gel electrophoresis and spectrophotometric analysis (Nanodrop ND-1000, Thermo Fisher) were used to check quality and concentration of the isolated DNA which was then preserved at -20 °C for further study.

2.3. 16S rRNA gene amplication, high-throughput sequencing, and bioinformatic analysis

The hypervariable V4-V5 region of the bacterial 16S rRNA gene was chosen as the target to amplify 16S rRNA gene fragments using the forward primer 515F: GTGCCAGCMGCCGCGG and reverse primer 907R labelled with unique barcodes: CCGTCAATCMTTTRAGTTT (initially 95 °C for 5 min to activate enzyme activity, and 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, 35 cycles) (Turner et al., 1999). PCR reactions and gel purification were conducted as described previously (Caporaso et al., 2010; Chen et al., 2016). The concentration of PCR purified product was determined using the NanoDrop spectrophotometer. Twelve barcoded samples of equal quality were pooled as a sequencing library and high-throughput sequencing of the library was conducted on the Illumina Hiseq2500 platform (Novogene, Tianjin, China) (Chen et al., 2016).

Quantitative Insights Into Microbial Ecology (QIIME, version 1.8.0) was used for the high-throughput sequencing data using online instructions (Caporaso et al., 2010). The raw paired-end reads were merged after the adaptor, ambiguous nucleotides, low-quality reads and labelled barcodes being filtered to obtain clean combined reads targeting the complete V4−V5 region of the 16S rRNA gene. In QIIME the operational taxonomic units (OTUs) were picked using 97% sequence similarity as the criterion to identify the OTUs by cluster analysis (Edgar, 2010). Before downstream analysis, OTUs with only one sequence (singletons) were removed. The RDP Classifier 2.2 was used to assign the taxonomy of each OTU according to reference sequences in the Greengenes 13.8 16S rRNA gene database (McDonald et al., 2012). The alignment of sequences was conducted via a PyNAST aligner (Caporaso et al., 2010). The metrics observed species (OTU) was used to estimate bacterial alpha diversity, and the PD Whole tree, Chao1 and rarefaction curves were calculated as indicators of bacterial alpha diversity by comparing the level of bacterial OTU diversity. Principal coordinate analysis (PCoA) based on the weighted Unifrac metric and Adonis test was used to determine differences among samples of different microbial communities.

2.4. Carbon and nitrogen stable isotope analysis

The collembolans collected from Experiment 1 were transferred into a new Petri dish covered with moist filter paper and incubated for 24 h to remove collembolan gut contents. The collembolans were then washed three times with deionized water and dried at 50 °C for 24 h. The dried collembolan samples were stored at 4 °C until further analysis. They were weighed and analyzed for ^{13}C and ^{15}N isotope signatures using a Flash EA 2000 Series Elemental Analyzer connected via a Conflo IV to a DeltaV Advantage isotope ratio mass spectrometer (FLASH-EA-DELTA-V, Thermo Finnigan, Waltham, MA). Quality control consisted of determining an internal reference (fish muscle tissue) every 10 samples. Overall precision of the ^{13}C and ^{15}N measurement was < 0.10‰. ^{13}C and ^{15}N isotope natural abundances are expressed by δX notation as described previously (Zhu et al., 2016).

2.5. Statistical analysis

Mean values, standard deviations, and standard errors of all data were determined using Microsoft Excel. The abundance of bacterial species and the C:N ratio of collembolan tissues are presented as mean ± standard error (SE), and weight, reproduction, and elemental and isotopic composition of collembolans are shown as mean ± standard deviation (SD). Effects of treatment and growth on the elemental composition and isotopic signatures and effects of treatment and %N on growth of collembolans were tested using the generalized linear model (GLM). PCoA based on the weighted Unifrac metric was used to determine the difference between different samples of microbial communities using R version 3.4.1. The Adonis test and diversity index (Shannon, Chao1 and PD Whole tree) were calculated in R version 3.4.1 with vegan 2.4–3 (Simpson et al., 2016). Single factor analysis of variance, least significant difference and t-tests were used to compare samples using the IBM SPSS version 22 statistical software package. Network analysis of collembolan samples was conducted using the Gephi software version 0.9.1 with the Force Atlas algorithm. Differences were considered to be significant in all statistical tests at the 0.05 level.

2.6. Accession numbers

All sequencing data are available in the National Center for Biotechnology Information Sequence Read Archive under the accession number SRP114845.

3. Results

3.1. Characterization of the F. candida gut microbiota

The structure and composition of the bacterial community of all collembolan gut microbiota (six samples) were analyzed by high-throughput sequencing of the bacterial 16S rRNA gene. A total of 310,792 high quality sequences were obtained through assembling and quality filtering, and sequencing number per sample ranged from 32557 to 74215. The 3% dissimilarity level was used to cluster OTUs, and 1242 and 1828 OTUs were identified in the control and microplastic-treated collembolan gut samples, respectively.

The most abundant category in the collembolan gut microbiota at the phylum level was Actinobacteria (44.2% of the total on average) and other abundant phyla were Bacteroidetes (29.7%), Proteobacteria (12.3%) and Firmicutes (11.2%). These four phyla comprised 97.4% of collembolan gut bacterial OTUs. Actinobacteria (44.1%) and

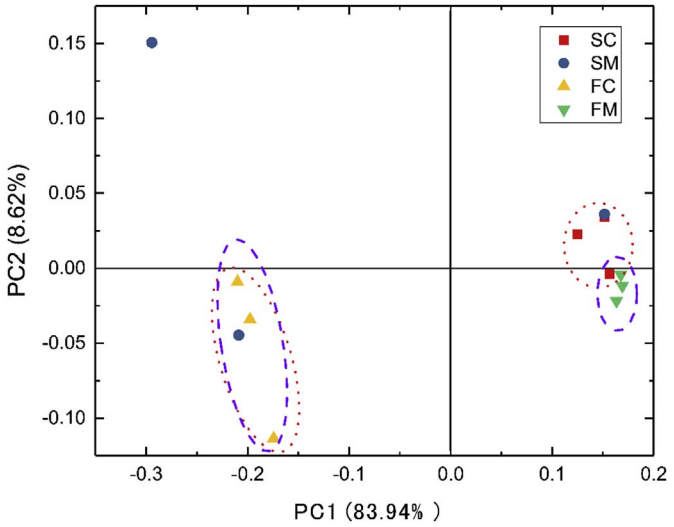

Fig. 1. Weighted UniFrac principal coordinate analysis (PCoA) plots describing the differences between samples. Contrasting the *Folsomia candida* gut (FC) and soil (SC) microbial community and the difference in *F. candida* gut microbial community subjected to microplastics exposure. SC, SM, FC, and FM indicate soil microbiota of the control, the microbiota of soil with microplastics, collembolan gut microbiota of the control, and collembolan gut microbiota after microplastic exposure, respectively. The microbiota of each sample is represented as a point.

Sphingobacteria (29.4%) were the two predominant classes in the collembolan gut microbiota, and the two dominant classes of Firmicutes were Clostridia (8.3%) and Bacilli (2.9%). In addition, Proteobacteria consisted mainly of Alphaproteobacteria (62.6%) and Gammaproteobacteria (29.3%), and 81.8% of Proteobacteria were Rhizobiales. The most abundant families were Sphingobacteriaceae (29.4 ± 1.8%), Streptomycetaceae (25.3 ± 6.7%) and Thermomonosporaceae (12.7 ± 5.7%). There were 11 genera with relative abundancs > 1%.

3.2. Comparative analysis of collembolan gut and soil microbiota

Principal coordinate analysis (PCoA) based on the weighted Unifrac metric reveals a significant separation between collembolan gut and soil microbiota (Adonis test, $P < 0.01$) along with the primary principal coordinate (explaining 83.94% of the total variance) (Fig. 1) in the control. At a sequencing depth of 32557 we summarize the alpha-diversity of the bacterial community in Fig. S2. The phylogenetic diversity metrics (PD whole tree) analysis shows a pronounced reduction in bacterial diversity in the collembolan gut compared with the surrounding soil (LSD test, $P < 0.05$) which was demonstrated by Chao1 richness and rarefaction curves of OTUs. The relative abundance of the four predominant phyla (Actinobacteria, Bacteroidetes, Proteobacteria and Firmicutes) all generated significant shifts from soil to collembolan gut (*t*-test, $P < 0.05$). Differences in bacterial composition at family level between soil and collembolan gut are described in Fig. 2. The relative abundances of Streptomycetaceae, Sphingobacteriaceae and Thermomonosporaceae in collembolan guts were significantly higher than those in surrounding soils (*t*-test, $P < 0.05$). A large number of families were shared between soil and collembolan gut (Fig. 2).

3.3. Effect of microplastic exposure on the gut microbial community of F. candida

High throughput sequencing of the bacterial 16S rRNA gene reveals that microplastic exposure significantly altered the microbial community of the collembolan gut (Adonis test, $P < 0.01$). Differences in bacterial communities between control and microplastic-exposed collembolan guts were observed using the PCoA of weighted Unifrac metrics analysis (Fig. 1). Moreover, alpha-diversity increased significantly in microplastic-exposed collembolan guts compared with the control (Fig. S2, LSD-test, $P < 0.01$) and this was confirmed by the PD whole tree, Chao1 richness and rarefaction curves of OTUs. The relative abundance of Bacteroidetes declined from 29.7 to 2.0% in collembolan guts due to microplastic exposure but the relative abundance of Firmicutes was significantly elevated from 11.2 to 34.0% (*t*-test, $P < 0.01$). Within the Firmicutes the family Bacillaceae increased dramatically after microplastic exposure from 1.5 to 20.6% (Fig. 3). The Venn diagrams show that 350 OTUs were shared in all three control samples, 716 OTUs in all microplastic exposure samples, and 302 OTUs were found in all collembolan samples (Fig. 4). The number of unshared OTUs in exposed collembolan gut (414) was much greater than in the control (48). The 17 most abundant collembolan-associated OTUs (> 1% abundance) were used to construct the network (Fig. 5). Seven of the 17 abundant OTUs were shared with all collembolan guts, and seven of the 17 abundant OTUs were associated with microplastic exposure, but only three were associated with the control. Network analysis of collembolan gut microbiota further demonstrates that more unique bacteria were identified in collembolan guts exposed to microplastics.

3.4. Growth, reproduction, and elemental and isotopic composition of F. candida

Low collembolan mortality was observed in all tests (< 8%) but a significant decrease in reproduction (by 28.8%) was found in the microplastic exposure treatment compared with the control (*t*-test, $P < 0.05$). The body weights of the collembolans which were introduced at the start of the experiment increased significantly with time (LSD test, $P < 0.05$) in all treatments. On day 56, microplastic exposure significantly decreased the body weight of introduced collembolans at the start of the experiment compared to the control (Table 1 and Fig. 6A). However, there was no evidence of ingestion of microplastics by the animals by observing the gut contents. Significant effects of microplastics on the carbon and nitrogen contents of collembolans were found in all treatments (Table 1 and Fig. 6B). Microplastic exposure did not have a statistically significant effect on the C:N ratio of collembolan tissues ($P > 0.05$, Fig. S3). Moreover, microplastic exposure significantly enriched $\delta^{15}N$ (by 18.9% on day 28 and 17.1% on day 56) and $\delta^{13}C$ (by 2.6% on day 28 and 8.6% on day 56) of collembolan tissues, and these values were related to the body weight of introduced collembolans at the start of the experiment (Table 1, Fig. 6C and D).

4. Discussion

It is generally recognized that soil animal gut microbiota are subject to host selection and edaphic effects (Pass et al., 2015; Berg et al., 2016) and soil animal guts consist of transient soil and inherently host-related microbiota. Therefore, compared to extraction methods for other soil animal (such as earthworm (Pass et al., 2015) and nematode (Berg et al., 2016)) microbiomes, we collected collembolan gut samples containing intact gut contents (ingested allogenic material) in order to reflect the real-time microbial community of the collembolan gut at the time point of sampling in the present study.

4.1. The collembolan gut microbiota

The predominant microbiota of the collembolan gut was first identified in the soil environment in this study, revealing a diverse bacterial community. Actinobacteria and Proteobacteria were observed in the core microbiota of earthworms (Pass et al., 2015) and Bacteroidetes, Proteobacteria and Firmicutes were also found in the core microbial community of termites (Otani et al., 2014). More OTUs were found in our samples from the soil environment compared with the

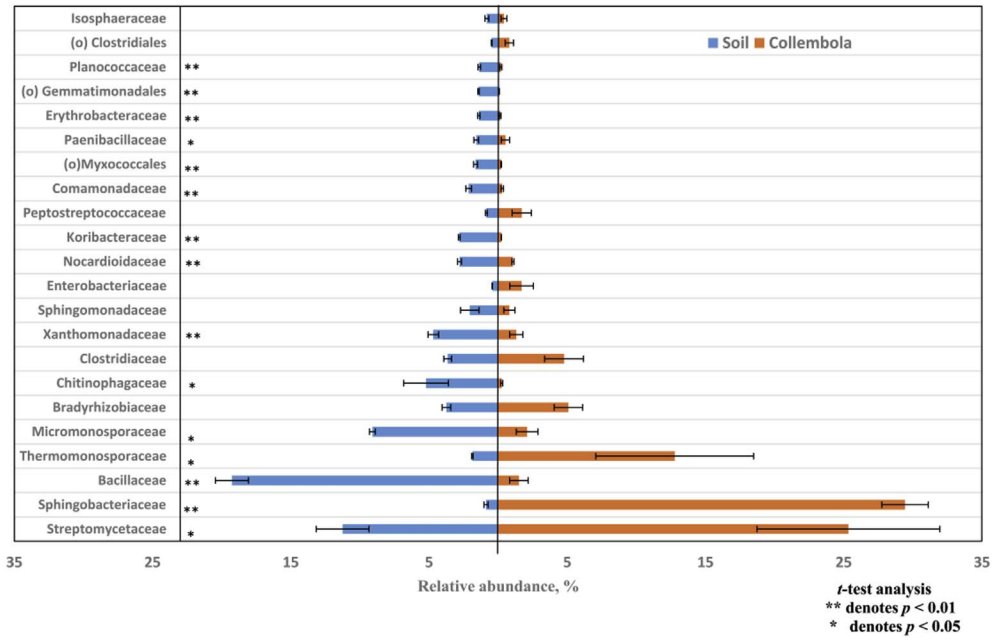

Fig. 2. A significant difference in bacterial families was observed between the collembolan gut and the soil. When family level annotation of microbiota was not possible the order was selected denoted by (o). Families with > 1% relative abundance in the collembolan gut and the soil are presented.

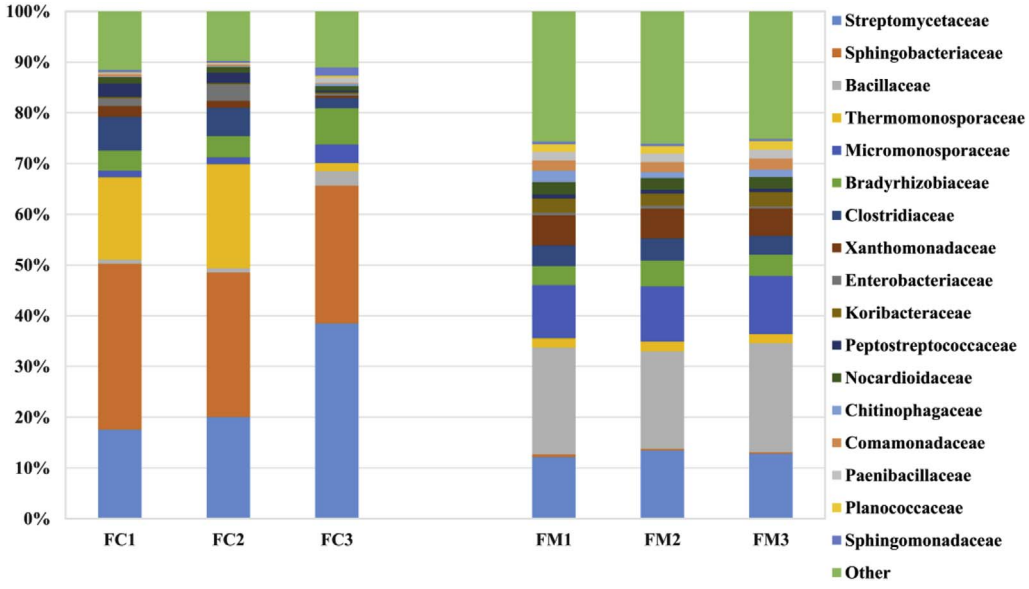

Fig. 3. Column chart presenting the bacterial OTUs counted in the different treatments of *F. candida*. Control (left: FC1, FC2 and FC3) and microplastic exposure (right: FM1, FM2 and FM3). The taxonomic composition of collembolan bacteria is displayed at the family level unless otherwise indicated. OTUs with < 1% of the total number of reads are categorized into 'Other'.

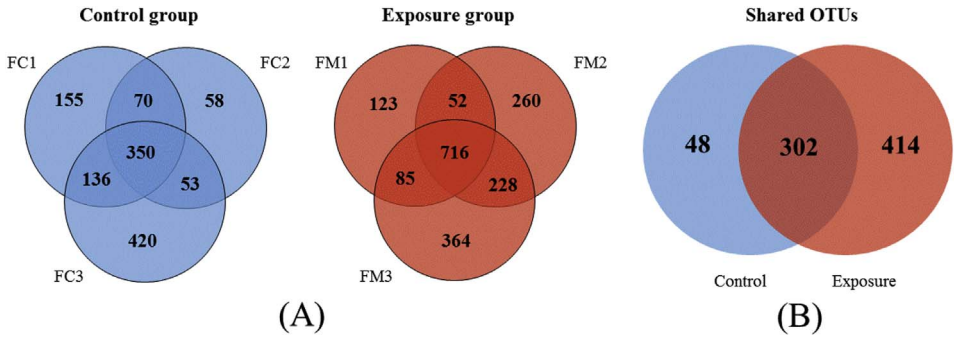

Fig. 4. Venn diagrams displaying the number of microbial OTUs shared within and between groups of samples. (A) The number of shared OTUs was counted between samples for each of the two groups, control (FC1, FC2 and FC3) and microplastic exposure (FM1, FM2 and FM3). (B) The number of shared OTUs was observed between the control and microplastic groups.

results of previous collembolan microbiomes using plate culture (Agamennone et al., 2015). This suggests that a complex external environment may contribute more microbiota to the host and possibly alter the microbial community of the host. Numerous previous studies indicate that host-associated microbiota are substantially affected by environmental factors (Staubach et al., 2013; Chassaing et al., 2015; Martinson et al., 2017) and that long-term laboratory rearing can alter the gut microbiota of the animals (Zouache et al., 2009; Agamennone et al., 2015).

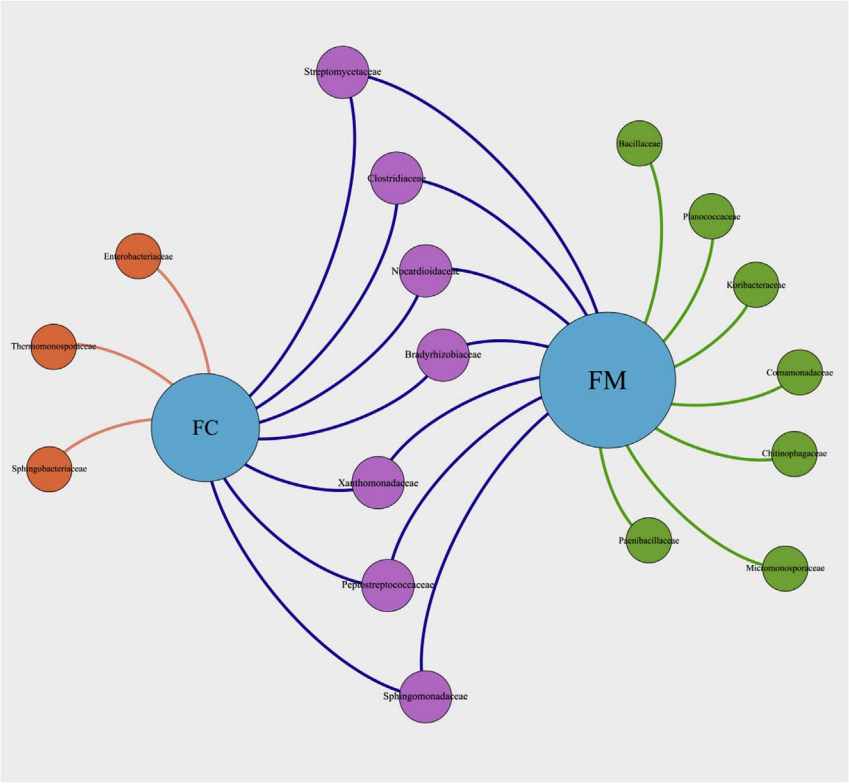

Fig. 5. Network analysis of *F. candida* samples with the abundance of associated OTUs at the family level. OTUs with > 1% abundance are presented in network association with collembolan guts (*F. candida*, blue circles). The FDR correction indicates Benjamini-Hochberg False Discovery Rate correction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Generalized linear models testing effects of treatment and DW on the elemental composition and isotopic signatures of *Folsomia candida* and effects of treatment and %N on DW [a].

Variable	Estimate	SE	*t*	*P* value	Partial η^2
(A) $\delta^{15}N$					
Treatment	1.977	0.451	4.388	0.001	0.616
DW	0.888	0.260	3.414	0.005	0.493
Treatment × DW	−0.594	0.165	−3.609	0.004	0.521
(B) $\delta^{13}C$					
Treatment	−5.921	0.389	−15.229	< 0.001	0.951
DW	−4.686	0.224	−20.877	< 0.001	0.973
Treatment × DW	2.889	0.142	20.347	< 0.001	0.972
(C) %N					
Treatment	10.640	1.029	10.337	< 0.001	0.899
DW	6.835	0.594	11.502	< 0.001	0.917
Treatment × DW	−4.337	0.376	−11.541	< 0.001	0.917
(D) %C					
Treatment	−31.830	3.634	−8.758	< 0.001	0.865
DW	−18.030	2.098	−8.593	< 0.001	0.860
Treatment × DW	12.385	1.327	9.332	< 0.001	0.879
(E) DW					
Treatment	−7.835	3.835	−2.043	0.044	0.258
%N	7.285	2.214	3.290	0.006	0.474
Treatment × %N	−1.735	1.400	−1.239	0.239	0.113

[a] The control was used as a reference. Treatment, microplastics exposure, 1 g kg^{-1}; DW, dry weight of collembolan; %N, nitrogen content; %C, carbon content. Identity function was selected for response variables (A) $\delta^{15}N$, (B) $\delta^{13}C$ and (D) %C, and for response variables (C) %N and (E) DW log link was conducted.

4.2. Differences between soil and gut microbiota

The microbial community of the collembolan gut was distinct from the surrounding soil microbiota. Specific conditions in the gut (e.g. anoxia, pH, and enrichment with nutrients) may exert a large effect on the gut microbiota resulting in selection for certain bacteria from the soil. Studies on nematodes have also shown significant differences between the gut microbiota and the soil (Berg et al., 2016). However, a large number of OTUs occurred in both the soil and the collembolan gut, indicating that soil bacteria may make an important contribution to the gut microbiota. Rhizobiales (~10%) likely from the soil microbiota (Depkatjakob et al., 2013) were found in the gut and this supports the explanation above. In addition, a notable reduction in microbial diversity was observed in the gut compared with the soil microbiota and similar results have been found for other soil fauna such as earthworms (Pass et al., 2015). This is likely to be because the specialized gut habitat has selected the microbiota from the soil. The abundances of the families Streptomycetaceae, Sphingobacteriaceae and Thermomonosporaceae were significantly enhanced by passing soil through collembolan gut due to stimulation of these bacteria by the gut habitat. Streptomycetaceae may contribute to increased nutrient utilization by collembolans because the addition of Streptomycetaceae enhances the degradation of cellulose in the earthworm gut (Pass et al., 2015).

4.3. Changes in growth, reproduction and elemental contents

Significant reductions in body weight and reproduction of the introduced collembolans at start of the test were observed as a result of exposure to microplastics and the carbon and nitrogen contents increased significantly in collembolan tissues (day 28) in our study. This suggests that exposure to microplasticss may disturb the metabolism and carbon and nitrogen elemental absorption of the collembolan. However, our previous studies and others suggest that microplastics are not ingested by the collembolan (Maaβ et al., 2017). Therefore, the effects of exposure to microplastics are likely due to habitat change and effects on nutrient absorption. We suspect that the presence of microplastics may alter feeding behavior and the ability to find high quality food as the plastics may change the soil structure (Rillig, 2012), thus influencing the activity of the collembolan. Moreover, damage to the animals (reduction in body weight and changes in elemental composition) is closely related to nutrient absorption. In addition, avoidance behavior in collembolans was observed in a microplastic exposure test (unpublished data) and this supports the above explanation. Similarly, when soil contains biochar, earthworms also show avoidance behavior

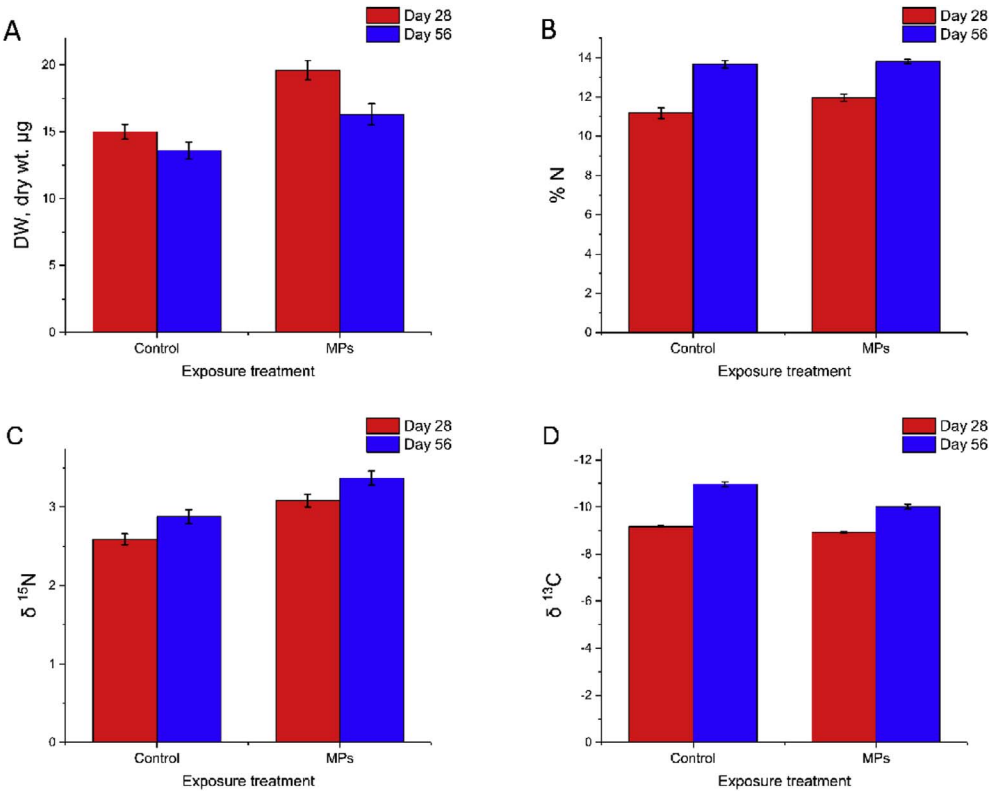

Fig. 6. (A) Accumulation of nitrogen (%N) and (B) biomass of the collembolan (dry weight, DW) and (C) incorporation of δ ^{15}N and (D) δ^{13}C along with microplastics (MPs) exposure. All data are presented as the mean ± standard deviation (n = 4).

(Li et al., 2011). The reduction in collembolan reproduction may be partly due to a shift in collembolan oviposition sites. Studies of aquatic organisms show that large numbers of animal eggs occur on the surfaces of microplastics, and we therefore assume that the presence of microplastics may affect the hatching of eggs (Goldstein et al., 2012; Majer et al., 2012; Ja and Costa, 2014), and this may partly explain the decrease in reproduction after exposure to microplastics.

4.4. Effects of microplastics on the collembolan gut microbiota

Using high throughput sequencing of the collembolan gut microbiota, our study demonstrates that microplastic treatment can alter the community composition and structure of the collembolan gut microbiota in the soil environment. The collembolan gut microbiota is initially derived from the external environment and is shaped by filtration and selection by the conditions in the gut habitat (Pass et al., 2015; Berg et al., 2016). Ingestion is the principal pathway for the recruitment of gut bacteria (Daly et al., 2010; Saraf et al., 2017). Since microplastics can affect collembolan activity, exposure to them may alter feeding behavior leading to changes in the gut microflora. In addition, exposure to microplastics affected collembolan growth and metabolism, perhaps also contributing to the changing gut microbiota. Unexpectedly, the collembolan gut microbial diversity increased significantly after exposure to microplastics compared with the control, likely due to ingestion of more bacterial taxa. This is supported by the large number of unique OTUs that were observed in the collembolan gut after exposure to microplastic particles.

4.5. Effects of microplastics on tissue isotopic composition

Far fewer studies have focused on the effects of pollutants than on the effects of food and temperature on isotopic turnover in the animal body, especially in the case of soil fauna (Ek et al., 2015; Zhu et al., 2016). We demonstrate that exposure to microplastics significantly increased δ^{15}N and δ^{13}C values of collembolan tissues. It is well known that both growth rate and metabolic turnover make an important contribution to isotopic turnover (Ek et al., 2016). Thus, the changes in δ^{15}N and δ^{13}C values in collembolan tissues are likely due to the response of growth rate and metabolic turnover due to microplastics. In addition, diet has a key influence on the δ^{15}N and δ^{13}C values of animals (Cherel et al., 2005; Ek et al., 2016). As described above, microplastics may alter collembolan feeding behavior and this will likely lead to alteration of δ^{15}N and δ^{13}C values in the tissues. The gut microbiota make an important contribution to the absorption of nutrients in invertebrates (Agamennone et al., 2015; Zheng et al., 2017), and the close correlation between the shift in gut microbiota and the isotopic turnover of the collembolan induced by microplastics may suggest gut bacteria-mediated effects of microplastics on the isotopic composition of the collembolan in the soil ecosystem. However, this requires further systematic investigation.

5. Conclusions

In general, a diverse microbial community is harbored in the collembolan gut, and the collembolan gut microbiota is significantly different from that of the surrounding soil due to filtering and shaping by the prevailing conditions in the collembolan gut. Microplastics can disturb the collembolan gut microbiota and enhance the diversity of gut bacteria, likely due to a shift in collembolan feeding behavior after exposure to microplastic particles. Moreover, exposure to microplastics can reduce collembolan growth and reproduction, but increase δ^{15}N and δ^{13}C values in the collembolan tissues. It is possible that microplastics can impact non-target species via changes in their microbiota leading to alteration of isotopic incorporation, growth and reproduction. These results contribute to our understanding of the effects of microplastics on the soil ecosystem and extend our knowledge regarding the composition of, and shifts in, the collembolan gut microbiota in natural and polluted soil ecosystems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.10.027.

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