

1 Invasive Earthworms Alter Forest Soil Microbiomes and Nitrogen Cycling

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20 **Competing Interests**

21 The authors declare no competing interests.

## 22 **Abstract**

23 Northern hardwood forests in formerly glaciated areas had been free of  
24 earthworms until exotic European earthworms were introduced by human activities. The  
25 invasion of exotic earthworms is known to dramatically alter soil physical, geochemical,  
26 and biological properties, but its impacts on soil microbiomes are still unclear. Here we  
27 show that the invasive earthworms alter soil microbiomes and ecosystem functioning,  
28 especially for nitrogen cycling. We collected soil samples at different depths from three  
29 sites across an active earthworm invasion chronosequence in a hardwood forest in  
30 Minnesota, USA. We analyzed the structures and the functional potentials of the soil  
31 microbiomes by using amplicon sequencing, high-throughput nitrogen cycle gene  
32 quantification (NiCE chip), and shotgun metagenomics. Both the levels of earthworm  
33 invasion and soil depth influenced the microbiome structures. In the most recently and  
34 minimally invaded soils, *Nitrososphaera* and *Nitrospira* as well as the genes related to  
35 nitrification were more abundant than in the heavily invaded soils. By contrast, genes  
36 related to denitrification and nitrogen fixation were more abundant in the heavily invaded  
37 than the minimally invaded soils. Our results suggest that the N cycling in forest soils is  
38 mostly nitrification driven before earthworm invasion, whereas it becomes denitrification  
39 driven after earthworm invasion.

40

## 41 **Introduction**

42 Earthworms are well-known ecosystem engineers that shape soil structure and  
43 drive nutrient dynamics in soil ecosystem [1]. They feed on litter and soil, burrow  
44 horizontally and vertically through soils, and release fecal materials to mix nutrients in  
45 soils, altering soil porosity, bulk density, water infiltration, gas emission, nutrient  
46 mineralization, and plant productivity [2].

47 Although earthworms are widely considered ubiquitous across the forest,  
48 grassland, agricultural, and garden ecosystems in the world, their global distribution is  
49 only beginning to be synthesized [3]. Glaciers and peri-glacial environments cleared out  
50 native earthworm populations from large areas in the northern USA and Canada as well  
51 as other Arctic areas in Eurasia during the last Ice Age [4]. Since then, most of these  
52 areas had remained earthworm-free until European earthworm species were introduced  
53 by human activities [5].

54 The earthworm invasion is now widely regarded as a force that substantially  
55 alters physical, geochemical, and biological properties of soils in northern hardwood  
56 forests [6, 7], and its ecosystem effects are believed to harm plant diversity [8] and be  
57 increasingly detrimental with ongoing changes in land uses and climates [9]. Invasive  
58 earthworms are known to reduce the litter layer (O horizon) while mixing organic matter  
59 with underlying minerals to create A horizon [10]. Presumably coupled with the loss of O  
60 horizon, invasion of European earthworms results in increased leaching of nitrates in  
61 the formerly glaciated deciduous forests [11]. Denitrification enzyme activity was also  
62 higher in the forest soils with earthworms than in those without earthworms [12].

63           A limited number of studies suggest that the invasion of earthworms could alter  
64 soil microbial communities. For example, Dempsey *et al.* [13] observed changes in soil  
65 microbial community composition based on the phospholipid fatty acid (PLFA) in a  
66 northern hardwood forest in New York, USA. Although PLFA analysis provides  
67 quantitative information, it cannot provide community compositions at low taxonomic  
68 (e.g., genus) levels. Hoeffner *et al.* [14] and de Menezes *et al.* [15] used terminal  
69 restriction fragment length polymorphism (T-RFLP) and high-throughput 16S rRNA  
70 gene amplicon sequencing, respectively, to analyze the effects of invasive earthworms  
71 on soil bacterial communities. However, these studies analyzed short-term impacts (e.g.,  
72 10-20 days [14] and 17 weeks [15]) by using soil mesocosms. Field investigation is  
73 essential to analyze the longer-term impacts of earthworm invasion in natural soil  
74 environments.

75           Previously, we studied the impacts of earthworm invasion on the soil  
76 physicochemical properties in a northern hardwood forest in Minnesota, USA [10, 16,  
77 17], which built on decades-long research on ecological processes and effects of  
78 earthworm invasion [18-21]. At this site, a gradient of earthworm density was observed  
79 within a 190-m distance, which most likely reflects the history of earthworm invasion [17].  
80 The invasion of earthworms has drastically changed the cycling of carbon, nitrogen, and  
81 other nutrients in soils [16, 17, 20]; however, it is unclear how it influenced soil microbial  
82 communities. Since microbes play crucial roles in soil C and N cycling in forest  
83 ecosystems [22], we hypothesize that the abundances of microbes important for C and  
84 N cycling have changed by the invasion of earthworms.

85           Consequently, the objectives of this study were to (1) elucidate the impacts of  
86 earthworm invasion on soil bacterial, archeal, and fungal communities at field conditions,  
87 (2) clarify the relationships between the levels of earthworm invasion, microbial  
88 communities, and soil physicochemical properties, and (3) analyze how earthworm  
89 invasion influenced the abundance of microbes/genes important for C and N cycles. To  
90 meet these objectives, we collected soil samples at different depths from three sites  
91 across an active earthworm invasion chronosequence in a hardwood forest in  
92 Minnesota. Our analyses, based on the amplicon sequencing, high-throughput nitrogen  
93 cycle gene quantification, and shotgun metagenomics, suggest that the structures and  
94 the functional potentials of the soil microbiomes altered by the invasion of earthworms.  
95 Microbial N cycling was most notably influenced. Our results suggest that the N cycling  
96 in forest soils is mostly nitrification driven before earthworm invasion, whereas it  
97 becomes denitrification driven after earthworm invasion.

98

## 99 **MATERIALS AND METHODS**

### 100 Soil sample collection

101           Soil samples were collected from a formerly glaciated northern hardwood forest  
102 in Minnesota, USA (Fig. S1). Earthworm biomass and species composition vary along a  
103 transect of ~200 m, while other environmental variables including climate, vegetation,  
104 geology, and topography are consistent within the transect as described in the  
105 supplementary information. We selected three sites along the transect: heavily invaded  
106 site (H), minimally invaded site (M), and the intermediate site (I). The minimally invaded  
107 site had the smallest earthworm biomass, dominated by epigeic earthworms (Fig. S2),

108 which live and feed in a surface litter [23]. The heavily invaded site had the largest  
109 earthworm biomass, dominated by anecic and endogeic species. While endogeic  
110 earthworms burrow horizontally through soils and feed on decomposed matter and  
111 mineral soils, anecic earthworms live in deep vertical burrows and feed at a soil surface  
112 [23]. Although the total amount of earthworm biomass at the site I was similar to that at  
113 the site H, the population of anecic earthworms was small. Each ecological group (i.e.,  
114 epigeic, endogeic, and anecic earthworms) differently influences soil ecosystems based  
115 on their feeding and moving behaviors [24].

116 After removing large undegraded leaves from the surface, three replicate soil  
117 cores (0-20 cm) were taken at each site by using a surface-disinfected soil probe. The  
118 soil core samples were divided into six segments (0-2 cm, 2-4 cm, 4-6 cm, 6-8 cm, 8-10  
119 cm, and 10-20 cm by depth) and placed in Whirl-Pak bags. A total of 54 soil samples  
120 were collected (three sites  $\times$  three soil cores  $\times$  six segments). Samples were kept on ice  
121 immediately after collection, and frozen with dry ice within 2 h of collection. Soil  
122 physicochemical properties (Soil pH, bulk densities, and carbon, ammonium, nitrate,  
123 and nitrite contents) were measured in this study or obtained from previous literature as  
124 described in the supplementary information.

125

#### 126 DNA extraction, PCR, and amplicon sequencing

127 Total DNA was extracted from 0.25 g of each soil sample by using a DNeasy  
128 PowerSoil Kit (Qiagen) and QIAcube (Qiagen) according to the manufacturer's  
129 instructions. From these DNA samples, the V4 region of the 16S rRNA gene and the

130 fungal internal transcribes spacer 2 (ITS2) region between 5.8S and 23S rRNA gene  
131 were amplified and sequenced as described in the supplementary information.

132 The paired-end raw sequence reads were quality-filtered, trimmed, and  
133 assembled using NINJA-SHI7 [25]. The assembled sequences were clustered into  
134 operational taxonomic units (OTUs) at 97% sequence similarity by using NINJA-OPS  
135 [26]. Taxonomic assignments of the archaeal/bacterial and fungal OTUs were done  
136 using the Greengenes database version 97 [27] and UNITE [28] reference data sets,  
137 respectively. The resulting OTU tables with taxonomic information were used for  
138 statistical analyses (see below).

139 Based on the fungal OTU sequence data, fungal trophic modes and functional  
140 guilds were predicted by using the FUNGuild software [29]. Only results with the  
141 confidence scores of “Probable” and “Highly Probable” were used for statistical  
142 analyses.

143

#### 144 Nitrogen Cycle Evaluation (NiCE) chip

145 High-throughput microfluidic qPCR was used to quantify nitrogen cycle-  
146 associated genes (Nitrogen Cycle Evaluation [NiCE] chip) [30]. Several assays were  
147 newly added to the NiCE chip system to increase the target coverage. A total of 43  
148 qPCR assays were included, targeting the genes associated with nitrification,  
149 denitrification, dissimilatory nitrate reduction to ammonium (DNRA), anaerobic  
150 ammonium oxidation (anammox), and nitrogen fixation (Table S1). Quantification was  
151 done using the standard curve method [31] as described in more detail in the  
152 supplementary information.

153

#### 154 Shotgun metagenomic sequencing

155 DNA extracted from surface soils (0-2 cm depth) were also used for shotgun  
156 metagenomics as described in the supplementary information. To identify N cycle-  
157 related genes, we mapped the high-quality metagenomic sequence reads against the  
158 NCycDB, a comprehensive nitrogen cycle protein sequence database [32], by using  
159 DIAMOND [33] with a minimum sequence identity of 70%, a minimum query coverage  
160 of 75%, and an E-value of  $<10^{-5}$ . To identify fungal denitrification-associated genes, we  
161 used the reference sequences reported by Higgins et al. [34] for the read mapping.  
162 Description of the top-hit sequence (i.e., aligned with the lowest E-value) was extracted  
163 for each mapped read, grouped according to their potential functions, and used to  
164 create a read count table. The number of reads mapped to each of the sequence  
165 groups (i.e., gene functions) was normalized by the total number of high-quality  
166 sequence reads for each sample and used for statistical analyses.

167 High-quality metagenomic sequence reads were also mapped against the  
168 Greengenes and UNITE databases to identify bacterial/archaeal and fungal  
169 communities, respectively, by using bowtie2 which is implemented in the NINJA-OPS  
170 pipeline [26]. The resulting read count data were used for statistical analyses.

171

172

#### 173 Statistical analyses

174 Statistical significances in the quantitative data obtained in this study were tested  
175 with the Kruskal-Wallis rank-sum test by using R version 4.0.2 (<https://www.r->



176 [project.org/](#)). Microbial community structures were analyzed by using R with *vegan* [35],  
177 *phyloseq* [36], and *DESeq2* packages [37]. Principal coordinates analysis (PCoA) with  
178 Bray-Curtis distance matrices were used to visualize the dissimilarities in microbial  
179 communities among sites (i.e., the levels of earthworm invasion) and soil depth.  
180 Differences in microbial community structures were tested using permutational  
181 multivariate analysis of variance (PERMANOVA).

182 Canonical analysis of principal coordinates (CAP) was done using Bray-Curtis  
183 distance matrices to identify environmental variables associated with the patterns in  
184 microbial communities [38]. Environmental variables that were correlated with other  
185 variables at the Spearman's  $\rho$  values of  $>0.80$  were removed from the CAP analysis.  
186 Multicollinearity among the environmental variables was also identified by calculating  
187 variance inflation factors (VIF). Variables with a VIF of  $>10$  were removed from the  
188 model in the CAP analysis. Furthermore, variables that were not significant ( $p > 0.05$ ) by  
189 PERMANOVA were removed from the model.

190 Taxa or genes that increased or decreased their relative abundance after  
191 earthworm invasion were identified by Spearman's rank correlation analysis between  
192 gene abundance and earthworm biomass, which was the largest in the heavily invaded  
193 soil and the smallest in the minimally invaded soils (Fig. S2). In addition, differentially  
194 abundant taxa across samples were identified using *DESeq2* with  $\alpha = 0.01$ .

195

#### 196 Nucleotide sequence accession numbers

197 The 16S rRNA gene and fungal ITS2 amplicon sequences as well as the shotgun  
198 metagenomics sequence reads were deposited to the GenBank database under the

199 BioProject number PRJNA504043. The SRA accession numbers are available in Table  
200 S2 and S3.

201

## 202 **RESULTS**

### 203 Abundance of microbes in soils

204 The abundances of archaea/bacteria and fungi were estimated by qPCR  
205 targeting the 16S rRNA gene (Fig. 1A) and the ITS2 region (Fig. 1B), respectively.  
206 While abundances of archaea/bacteria and fungi were not significantly different by the  
207 levels of earthworm invasion (i.e., sites), they were significantly different by depth ( $p$   
208  $<0.01$  by Kruskal-Wallis test). Both archaea/bacteria and fungi were most abundant in  
209 near-surface soils. Soil depth was negatively correlated with the copy numbers of the  
210 16S rRNA gene (Spearman's  $\rho = -0.77$ ,  $p <0.01$ ) and the ITS2 region (Spearman's  $\rho =$   
211  $-0.60$ ,  $p <0.05$ ). Although differences in the abundance of archaea/bacteria between the  
212 surface soil (0-2 cm) and soils at 10-20 cm were similar by site, those between the  
213 surface soil and soils at 8-10 cm depth was the larger in the minimally invaded soil (site  
214 M) than heavily invaded soil (site H) ( $p <0.05$  by Kruskal-Wallis test) (Table S4). The  
215 same trend was also seen for the differences in the fungal abundances between the  
216 surface soil (0-2 cm) and the soils at 8-10 cm (Table S4).

217

### 218 Alpha diversity measures

219 Soil archaeal/bacterial and fungal communities were analyzed by sequencing the  
220 16S rRNA gene and ITS2 region, respectively. The number of sequences per sample  
221 ranged from 28 079 to 284 434 and from 967 to 53 610 for the 16S rRNA gene and

222 ITS2 region, respectively (Table S2). Numbers of sequences were normalized at the  
223 smallest number of sequences by random subsampling for the diversity analyses. The  
224 subsampled sequences provided sufficient resolution of the microbial communities, as  
225 indicated by Good's coverage ranging from 0.952 to 0.992.

226 Species richness was inferred by the numbers of observed OTUs for  
227 archaea/bacteria (Fig. 1C) and fungi (Fig. 1D). The numbers of archaeal/bacterial and  
228 fungal OTUs decreased by depth ( $p < 0.01$  and  $< 0.05$ , respectively, by Kruskal-Wallis  
229 test), but not by the levels of earthworm invasion. Soil samples collected at 10-20 cm  
230 had the smallest number of OTUs for both archaea/bacteria and fungi, and the values  
231 were similar across sites. Differences in the numbers of observed OTUs for  
232 archaea/bacteria between the surface soil (0-2 cm) and the soils at 8-10 cm depth was  
233 larger in the site M than the sites H and I (Table S5).

234 Shannon diversity index values calculated based on the archaeal/bacterial 16S  
235 rRNA gene sequences were also significantly different by soil depth ( $p < 0.01$  by  
236 Kruskal-Wallis test) (Fig. 1E), whereas those calculated based on the fungal ITS2  
237 region were not (Fig. 1F). Shannon index values for archaea/bacteria were the smallest  
238 in the soil samples collected at the 10-20 cm samples, and these values were similar  
239 across sites. Shannon diversity index values were not significantly different by the levels  
240 of earthworm invasion for both archaea/bacteria and fungi, although differences in the  
241 Shannon index values for archaea/bacteria between the surface soil (0-2 cm) and soils  
242 at 8-10 cm depth was greater in the site M than the sites H and I (Table S5).

243

244 Patterns in soil microbial communities

245 Principal coordinates analysis (PCoA) showed the grouping of soil  
246 archaeal/bacterial communities by the levels of earthworm invasions (site M vs. site I  
247 and H) and soil depth (Fig. 2A). Community dissimilarities among different levels of  
248 earthworm invasions and soil depths were supported by PERMANOVA ( $p < 0.01$ ). The  
249 archaeal/bacterial communities in the near-surface soil (0-2 cm) and those in the  
250 deepest soil (10-20 cm) were most distantly plotted to each other. Distances between  
251 the archaeal/bacterial communities in the soils with minimum earthworm invasion (site  
252 M) and those in the soils with intermediate and heavy invasions (site I and H,  
253 respectively) were larger in the shallow soils (0-10 cm depths) than the deep soils (10-  
254 20 cm). Archaeal/bacterial communities in the deep soils overlapped each other,  
255 indicating that their community structures were similar to each other.

256 Fungal communities in the soils with different levels of earthworm invasion or  
257 different soil depths overlapped each other on the PCoA plot (Fig. 2B). However, fungal  
258 communities in the soils with minimum earthworm invasion were more closely related to  
259 each other than those in the soils with intermediate and heavy invasions, resulting in  
260 significant community dissimilarities by the levels of earthworm invasion ( $p < 0.01$  by  
261 PERMANOVA). In contrast, fungal communities were not different by soil depth ( $p = 0.38$   
262 by PERMANOVA).

263

#### 264 Taxonomic composition

265 Major archaeal and bacterial phyla identified in this study include *Acidobacteria*,  
266 *Actinobacteria*, *Bacteroidetes*, *Crenarchaeota*, *Nitrospirae*, *Planctomycetes*,  
267 *Proteobacteria*, and *Verrucomicrobia* (Fig. 3A). Relative abundances of these phyla

268 were similar among the soils with different levels of earthworm invasions, except for  
269 *Crenarchaeota* and *Nitrospirae*. Relative abundances of *Crenarchaeota* and *Nitrospirae*  
270 were the largest in the soil with minimal invasion of earthworms (site M) ( $p < 0.01$  by  
271 Kruskal-Wallis test). Similar results were obtained by the 16S rRNA gene amplicon  
272 sequencing and the shotgun metagenomics (Fig. S3).

273 *Ascomycota*, *Basidiomycota*, and *Zygomycota* were the major fungal phyla  
274 identified in this study (Fig. 3B). Relative abundances of *Basidiomycota* were larger in  
275 the soils with more earthworm invasions (site I and H) than those with minimal invasions  
276 earthworm invasions ( $p < 0.01$  by Kruskal-Wallis test).

277

#### 278 Microbial taxa responsive to earthworm invasion

279 To further identify the taxa that increased or decreased their relative abundance  
280 after the invasion of earthworms, Spearman's rank correlation analysis was used (Table  
281 S6). Taxa that showed positive correlations with the levels of earthworm invasion  
282 included the genus *Mycobacterium* ( $\rho = 0.56$ ,  $p < 0.01$ ) in the phylum *Actinobacteria*.  
283 Taxa that showed negative correlations with the levels of earthworm invasion included  
284 the genus *Nitrososphaera* ( $\rho = -0.60$ ,  $p < 0.01$ ) in the phylum *Crenarchaeota*, the genus  
285 *Nitrospira* ( $\rho = -0.50$ ,  $p < 0.01$ ) in the phylum *Nitrospirae*, and the fungal order *Helotiales*  
286 ( $\rho = -0.57$ ,  $p < 0.01$ ) in the phylum *Ascomycota*.

287

#### 288 Quantities of the N cycle-associated genes

289 Some members of the genera *Nitrososphaera* and *Nitrospirae* play important  
290 roles in the N cycle, namely ammonia oxidation and nitrite oxidation, respectively. The

291 decrease in their relative abundances in the earthworm-invaded soils could influence  
292 the overall N cycling in the soils. To clarify this, we used a high-throughput N-cycle gene  
293 quantification tool called the NiCE chip. With this tool, we could quantify almost all  
294 genes associated with the N cycle, including archaeal *amoA* and *nxB* of *Nitrospira*. Of  
295 the 43 assays included in the NiCE chip, 18 assays showed quantitative results in >60%  
296 of the samples. These 18 assays targeted genes for nitrification (*amoA* and *nxB*),  
297 denitrification (*napA*, *nirK*, *nirS*, *norB*, *nosZ*), and nitrogen fixation (*nifH*). In general,  
298 denitrification-related genes were more abundant than nitrification-related genes (Fig.  
299 4A). Many of the denitrification- and nitrogen fixation-related genes were also more  
300 abundant in the soils with heavy earthworm invasions than the soils with minimal  
301 invasions ( $p < 0.05$  by Kruskal-Wallis test).

302 By contrast, the quantities of *nxB* of *Nitrospira* (measured by the *nxB169f*  
303 assay) were significantly greater in the soils with minimal invasion of earthworms than  
304 those heavily invaded by earthworms ( $p < 0.01$  by Kruskal-Wallis test) (Fig. 4B), in  
305 agreement with the changes in the relative abundance of *Nitrospira* measured by 16S  
306 amplicon sequencing. Interestingly, while quantities of *Nitrospira nxB* decreased by  
307 depth in the samples with minimal invasion of earthworms ( $p < 0.01$  by Kruskal-Wallis  
308 test), those in the samples with heavy/intermediate invasions increased by depth ( $p$   
309  $< 0.01$  by Kruskal-Wallis test).

310 Quantities of archaeal *amoA* were not significantly different among the samples  
311 with different levels of earthworm invasion (Fig. 4C). In minimally invaded soils,  
312 archaeal *amoA* levels were significantly greater in the surface 0-2 cm soils than the soils  
313 collected at 10-20 cm depth ( $p < 0.05$  by Kruskal-Wallis and Mann's *post hoc* test).

314

315 Environmental variables associated with the patterns in microbial communities

316 Canonical analysis of principal coordinates (CAP) was used to clarify the  
317 relationship between environmental variables and the patterns in microbial communities.  
318 To select environmental variables for the CAP analysis, we first did a correlation  
319 analysis (Fig. S4). Most denitrification genes correlated with each other at Spearman's  $\rho$   
320  $>0.8$ . Assay nirK\_FlaCu was selected as the representative assay for denitrification  
321 because this assay produced quantitative values for all samples. Based on the  
322 correlation analysis, 13 variables were selected and used for the CAP analysis. After  
323 the VIF analysis and PERMANOVA, seven and five variables survived in the final CAP  
324 models for archaeal/bacterial and fungal communities, respectively (Fig. 5). Earthworm  
325 biomass, ammonium concentration, soil bulk density, and the quantities of denitrification  
326 and nitrogen fixation genes (nirK\_FlaCu and nifHF assays, respectively) were  
327 commonly identified as the variables significantly associated with the patterns in both  
328 archaeal/bacterial and fungal communities ( $p < 0.05$  by PERMANOVA). For  
329 archaeal/bacterial communities, additional two variables, quantities of nitrification genes  
330 (Arch\_amoA\_for and nxrB169f) were also identified.

331

332 Functional potentials of the soil microbial communities

333 To further assess the functional potential of the soil microbial communities, we  
334 used the shotgun metagenomics approach. Surface soils (0-2 cm) were used for the  
335 metagenomic analysis because microbiomes in these soils were most significantly  
336 different between sites H, I, and M. Similar to the NiCE chip results, the relative

337 abundances of the genes responsible for denitrification (*nar*, *nap*, *nor*, and *nos*) and  
338 nitrogen fixation (*nif*) were positively correlated with the levels of earthworm invasion  
339 (Spearman's  $\rho > 0.75$ ,  $p < 0.05$ ) (Fig. 6). The relative abundances of bacterial/archaeal  
340 nitrite reductase genes (*nirK* and *nirS*) and fungal nitrite reductase gene (fungal *nirK*)  
341 were also positively correlated with the levels of earthworm invasion (Spearman's  $\rho =$   
342 0.57 and 0.69, respectively), although they were not statistically significant ( $p = 0.143$   
343 and 0.057, respectively) (Table S7).

344 By contrast, the abundance of bacterial and archaeal ammonia monooxygenase  
345 genes for nitrification was negatively associated with the earthworm invasion levels  
346 (Spearman's  $\rho < -0.80$ ,  $p < 0.05$ ). The relative abundance of nitrite oxidoreductase gene  
347 (*nxr*) was also negatively yet insignificantly correlated with the levels of earthworm  
348 invasion (Spearman's  $\rho = -0.57$ ,  $p = 0.143$ ). The relative abundance of glutamate  
349 dehydrogenase was positively correlated with the levels of earthworm invasion  
350 (Spearman's  $\rho = 0.76$ ,  $p < 0.05$ ), although other genes for nitrogen assimilation, such as  
351 glutamine synthetase or asparagine synthase were not (Table S7).

352 To assess the potential functions of the soil fungal communities, we used the  
353 FUNGuild approach. The relative abundance of symbiotrophs (e.g., ectomycorrhizae)  
354 was positively correlated with the levels of earthworm invasion (Spearman's  $\rho > 0.46$ ,  $p$   
355  $< 0.01$ ); whereas those of saprotrophs and pathotrophs were negatively correlated with  
356 the levels of earthworm invasion (Spearman's  $\rho < -0.41$ ,  $p < 0.01$ ) (Table S8).

357

## 358 **Discussion**



359           While the invasion of earthworms itself did not influence the abundances of  
360 archaea/bacteria and fungi as well as the  $\alpha$  diversity measures, soil depths had  
361 significant impacts on the quantities and  $\alpha$  diversities of soil microbiota. Effects of soil  
362 depths on microbial communities have been well documented in temperate forest soils  
363 [39, 40]. Impacts of soil depth were greater in the minimally invaded soil (site M) than  
364 more earthworm-invaded soils (sites H and I), as we found larger differences in the  
365 quantities and diversities of soil archaeal/bacterial populations between the surface (0-2  
366 cm) and deep (8-10 cm) soils in minimally invaded soil than in heavily invaded soils.  
367 This is likely due to the mixing effects by soil-dwelling earthworms such as endogeic  
368 and anecic earthworms that were abundantly present in sites H and I. These  
369 earthworms vertically mix organic materials with minerals to form A horizon [16, 17].  
370 Interestingly, differences in the quantities and  $\alpha$  diversities of soil microbiota between  
371 the surface (0-2 cm) and the deepest (10-20 cm) soils were not significantly different by  
372 the level of earthworm invasion, suggesting that the impacts of earthworm invasion on  
373 soil microbial communities were minimal in the soils at 10-20 cm depth. This agrees  
374 with our field observations that A horizons rarely exceed 10 cm depth [17].

375           Both soil depth and the level of earthworm invasion had significant impacts on  $\beta$   
376 diversity. The impact of earthworm invasion on  $\beta$  diversity of soil archaeal/bacterial  
377 communities was greater in shallow soils (0-10 cm) than in deep soils (10-20 cm).  
378 Indeed, archaeal/bacterial communities in the deep soils were similar to each other  
379 regardless of the levels of earthworm invasion. Although some anecic earthworms are  
380 known to dig deep burrows of up to 2 m [7], our results suggest that the invasion of  
381 earthworms had limited influence on the archaeal/bacterial community structures in the

382 soils at 10-20 cm. This is also supported by the minimal presence of earthworm burrows  
383 below 10 cm as seen in the previous soil excavations to the depth of 1.5 m along the  
384 study transect including the site heavily infested with anecic *L. terrestris* [17]. The lack of  
385 deep earthworm activities may be due to the dry sandy loess layer and the dense clay-  
386 rich B horizon that underly the newly formed A horizon.

387 *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, *Proteobacteria*,  
388 and *Verrucomicrobia* occupied relatively large proportions of the archaeal and bacterial  
389 populations. Similar to this study, these phyla have been commonly detected in soils of  
390 temperate deciduous forests [41, 42]. Relatively large proportions of *Crenarchaeota* and  
391 *Nitrospirae* were also detected in this study, especially in the minimally invaded soils.  
392 These phyla are not frequently detected in other forest soils (e.g., [41, 42]), most of  
393 which presumably contained earthworms. *Nitrososphaera* spp. in the phylum  
394 *Crenarchaeota* and *Nitrospira* spp. in the phylum *Nitrospirae* were more abundant in the  
395 minimally invaded soils than the other soils. These microbes may play important roles in  
396 nitrification [43-46]. Interestingly, the vertical distribution of *Nitrospira* was different  
397 between the minimally invaded soils and more earthworm-invaded soils. *Nitrospira* spp.  
398 were more abundant near the surface of minimally invaded soils, probably because  
399 substrate for nitrification (ammonium and nitrite) were more available near the surface  
400 (O horizon). By contrast, *Nitrospira* spp. were more abundant in deeper soils of  
401 earthworm-invaded sites, probably because of the mixing soil microbes by earthworms  
402 and/or the leaching of nitrite downward.

403 While the relative abundance of *Nitrososphaera* spp. and *Nitrospira* spp.  
404 decreased by earthworm invasion, that of *Mycobacterium* (*Actinobacteria*) increased.

405 Specific bacteria can be enriched in the earthworm guts, in which C, N, and other  
406 nutrients are more abundant than in soils [47, 48]. Indeed, *Mycobacterium* was  
407 frequently isolated from the guts of anecic earthworms (*L. terrestris*) [49] and was also  
408 identified as one of the bacterial taxa residing in earthworm gut walls of endogeic  
409 earthworms (*Aporrectodea* spp.) [24]. In addition, *Mycobacterium* was present at a  
410 significantly larger proportion in the guts of epi-endogeic earthworms (*L. rubellus*) than  
411 in soils [50]. These results collectively suggest that earthworms likely enriched  
412 *Mycobacterium* in their guts and contributed to the increased abundance of  
413 *Mycobacterium* in earthworm-invaded soils.

414 Metagenomic analysis revealed that archaeal *amoA* was more abundant in the  
415 minimally invaded soils than in the other soils, further supporting that archaea including  
416 *Nitrososphaera* spp. play ammonia oxidation in the minimally invaded soils. The qPCR  
417 analysis (i.e., NiCE chip), however, did not show a significant difference in the archaeal  
418 *amoA* abundance among the soils. Similarly, a discrepancy was also observed for the  
419 abundance of *nxB* of *Nitrospira*: while NiCE chip results showed a significant difference  
420 in the abundance of *nxB* of *Nitrospira* among the soils, no difference was detected by  
421 metagenomics. The discrepant results obtained by the two approaches may be due, in  
422 part, to (1) the large variations seen in the qPCR results, which was likely caused by the  
423 relatively low abundance of archaeal *amoA* and *Nitrospira nxB* in the samples, (2)  
424 biases caused by the PCR primers (i.e., not all target genes are necessarily amplified  
425 by qPCR), or (3) the difference in the quantitative nature of the methods: while qPCR  
426 can provide absolute quantification (copies/g soil), metagenomics approach can provide  
427 only relative quantification (counts per million reads) in this study [51].

428 Both metagenomics and the NiCE chip analyses showed that many of the  
429 denitrification-related genes were more abundant in the earthworm-invaded soils than in  
430 the soils with minimal invasions. This may appear contradictory to the fact that the  
431 concentration of denitrification substrate (i.e., nitrate) was higher in the minimally  
432 invaded soil than the heavily invaded soils as seen in this study as well as Hale *et al.*  
433 [20]. However, it is probable that nitrate may have been consumed by denitrifiers in the  
434 earthworm-invaded soils, and therefore, became low compared to that in the minimally  
435 invaded soils. Similar to this study, greater denitrification activities were observed in  
436 earthworm-invaded forest soils than non-invaded soils [12, 52]. Higher denitrification  
437 activity was also reported in earthworm excreta (i.e., casts) than in the surrounding soils  
438 [53]. The larger abundance of denitrifying organisms in earthworm-invaded soils might  
439 be due, in part, to the enrichment of denitrifiers in earthworm intestines and/or the  
440 presence of more anoxic areas in soils. Earthworm intestines are known to promote  
441 denitrification, most likely due to the presence of anoxic area and readily assimilable  
442 carbon (i.e., mucus) [47, 48, 54, 55]. Earthworm-invaded soils could also have more  
443 anoxic micro-sites than the minimally invaded soils. Along our earthworm invasion  
444 chronosequence, both soil bulk density and the size and strength of soil aggregation are  
445 positively related with the level of earthworm invasion [16, 20], which is likely to offer  
446 more anoxic micro-sites that favor denitrification in the heavily invaded soils.

447 In addition to denitrification-related genes, the genes related to nitrogen fixation  
448 (*nif*) were also enriched in the earthworm-invaded soils in this study. The impact of  
449 earthworm invasion in nitrogen fixation is not well known; however, since total N content

450 can decrease by earthworm-associated activities [56], the soil environment may become  
451 N-limited, which provides a selective advantage for N-fixing microbes.

452       Regarding the soil fungal communities, the relative abundance of *Basidiomycota*  
453 increased by earthworm invasion. This might be related to the increased abundance of  
454 symbiotrophs identified by the FUNGuild analysis because some fungi in *Basidiomycota*  
455 are known as being ectomycorrhizae and symbiotically associate with trees [57].  
456 Ectomycorrhizae receive C mostly from their host plants instead of degrading complex  
457 organic matter in soils [57]. By contrast, the relative abundance of the order *Helotiales*  
458 (*Ascomycota*) decreased by the invasion of earthworms, which may be related to the  
459 decrease of the saprotrophs in the earthworm-rich soils. Most members of *Helotiales*  
460 live as soil saprophytes and degrade dead woods or other organic matter [58]. Similar to  
461 this study, Dempsey *et al.* [13] detected higher and lower levels of mycorrhizae and  
462 saprophytic fungi, respectively, in the earthworm-invaded soils than in the earthworm-  
463 free soils in a northern hardwood forest in New York, USA, by using the PLFA analysis.  
464 Collectively, these results suggest that soil fungal communities would shift from  
465 saprophytic to symbiotrophic communities by the invasion of earthworms, most likely  
466 due to the observed decrease in the organic C contents after earthworm invasions.

467       In conclusion, this study clearly shows that the invasion of earthworms alters soil  
468 microbial communities and ecosystem functioning. Earthworms mix soils, change soil  
469 physical structures, decrease the levels of C, N, and other nutrients in soils, secrete  
470 mucus, and enrich specific microbes in their guts, all of which can influence the  
471 microbial activities in soils. The most notable changes that earthworm invasion causes  
472 include the shift in the soil N cycling. Before the earthworm invasion, the N cycling in

473 forest soils is mostly nitrification driven, for which AOA and *Nitrospira* play key roles.

474 After earthworm invasion, the N cycling can become denitrification driven, which may

475 cause N-limited conditions and the increased importance of the N fixing populations.

476 The invasion of earthworms is ongoing. They are actively spreading in formerly

477 glaciated areas such as Alaska [59] and northern Scandinavia [60] in addition to

478 Minnesota. To better understand the impacts of “global worming” on the ecology of soil

479 systems, future research is necessary including the analysis of soil microbiomes at non-

480 invaded and recently invaded soils on a global scale.

481

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488

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- 657

658 **Figure Legends**

659 **Figure 1.** Abundance and diversities of microbes in the soil samples. Quantities of (A)  
660 16S rRNA gene and (B) fungal ITS2 region measured by qPCR. Numbers of  
661 observed OTUs for (C) archaea/bacteria and (D) fungi. Shannon diversity  
662 index values calculated based on (E) the archaeal/bacterial 16S rRNA gene  
663 and (F) fungal ITS2 region sequences. Legend: M, the site with minimal  
664 invasion of earthworms; I, the site with an intermediate invasion of earthworms;  
665 and H, the site with a heavy invasion of earthworms.

666 **Figure 2.** Principal coordinate analysis (PCoA) plots showing the Bray-Curtis  
667 dissimilarities among (A) archaeal/bacterial communities and (B) fungal  
668 communities. Each community is labeled with the site (M, the site with minimal  
669 invasion of earthworms; I, the site with an intermediate invasion of earthworms;  
670 and H, the site with a heavy invasion of earthworms) and soil depth (0-2, 2-4,  
671 4-6, 6-8, 8-10, and 10-20 cm from the surface).

672 **Figure 3.** Relative abundance of (A) archaeal/bacterial phyla and (B) fungal phyla in the  
673 soil samples as assessed by the 16S rRNA gene and ITS2 sequencing  
674 analyses. Legend: M, the site with minimal invasion of earthworms; I, the site  
675 with an intermediate invasion of earthworms; and H, the site with a heavy  
676 invasion of earthworms.

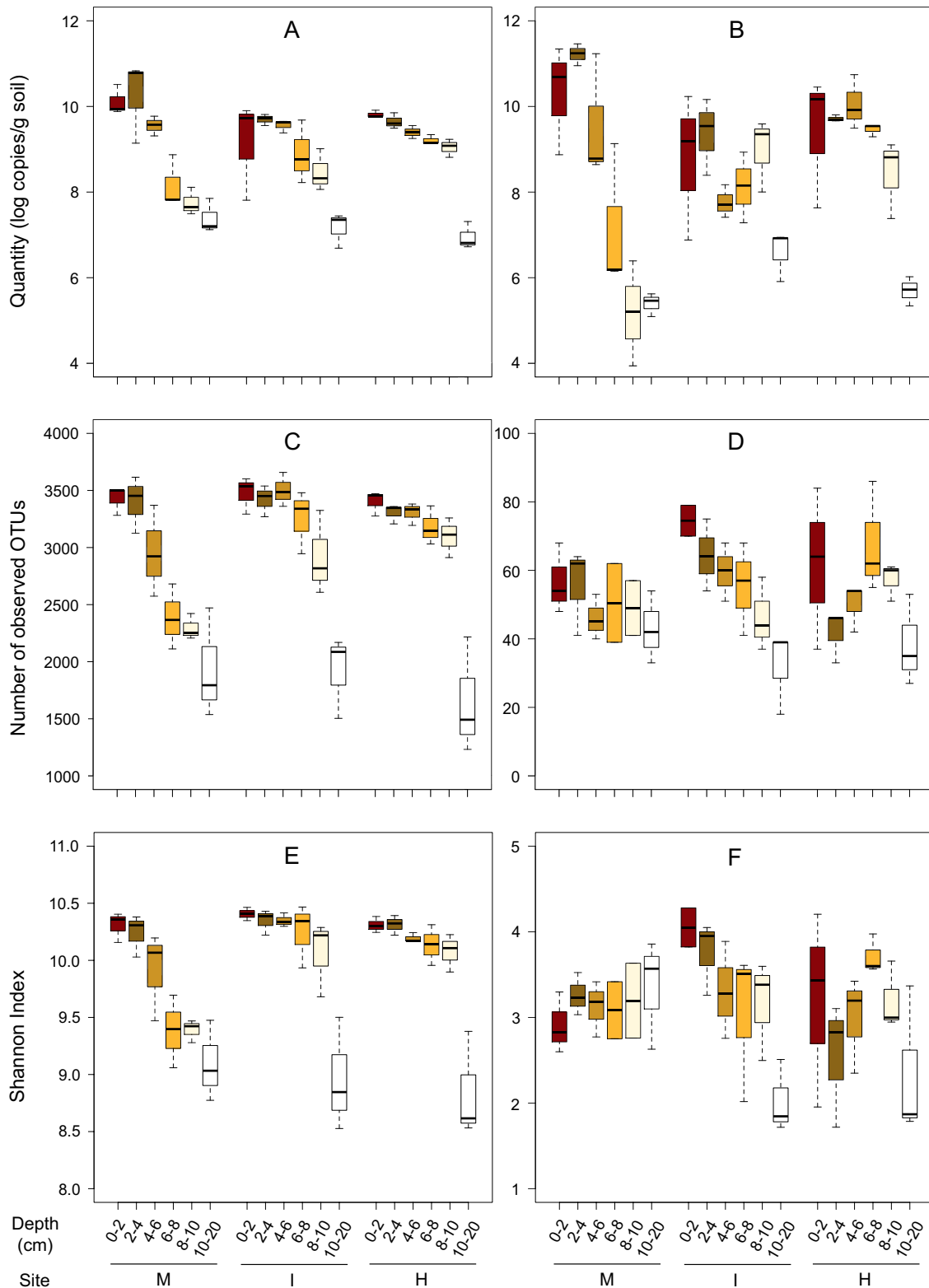
677 **Figure 4.** Quantities of N cycle-related genes in the soil samples. (A) Pseudo-heatmap  
678 showing the Nitrogen Cycle Evaluation (NiCE) chip results. Assays producing  
679 positives in >60% of samples are shown. (B) Quantities of nxrB of Nitrospira,  
680 based on the nxrB169f assay. (C) Quantities of archeal amoA based on the

681 Arch\_amoA\_for assay. Legend: M, the site with minimal invasion of  
682 earthworms; I, the site with an intermediate invasion of earthworms; and H, the  
683 site with a heavy invasion of earthworms.

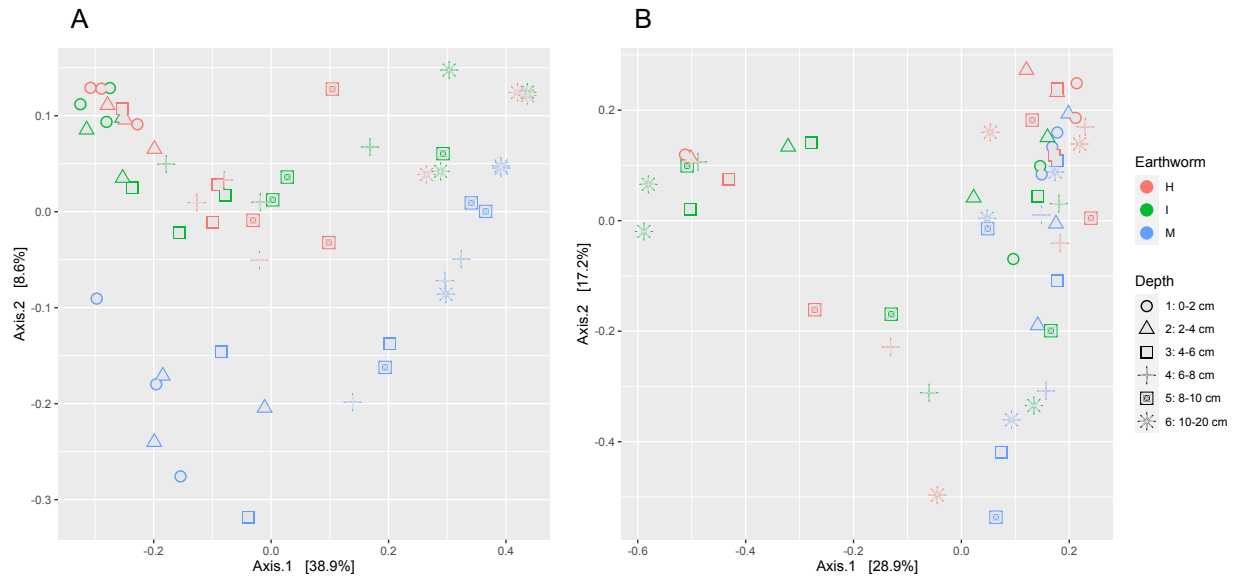
684 **Figure 5.** Canonical analysis of principal coordinates (CAP) plots showing the  
685 associations between environmental variables and the patterns in (A)  
686 archaeal/bacterial communities and (B) fungal communities. Microbial  
687 communities were analyzed using Bray-Curtis distance matrices. All  
688 environmental variables shown in the plots had significant effects ( $p < 0.05$ ) on  
689 the microbial community patterns based on PERMANOVA. Each community is  
690 labeled with the site (M, the site with minimal invasion of earthworms; I, the  
691 site with an intermediate invasion of earthworms; and H, the site with a heavy  
692 invasion of earthworms) and soil depth (0-2, 2-4, 4-6, 6-8, 8-10, and 10-20 cm  
693 from the surface).

694 **Figure 6.** Nitrogen cycling in the forest soil influenced by earthworm invasion. Red and  
695 blue arrows indicate the genes that increased and decreased their relative  
696 abundances by earthworm invasion, respectively, based on Spearman's  
697 correlation analysis of the shotgun metagenomics reads ( $p < 0.05$ ). Black solid  
698 arrows indicate the genes that did not change their abundance by earthworm  
699 invasion. Black dashed arrows indicate the genes that were not detected.

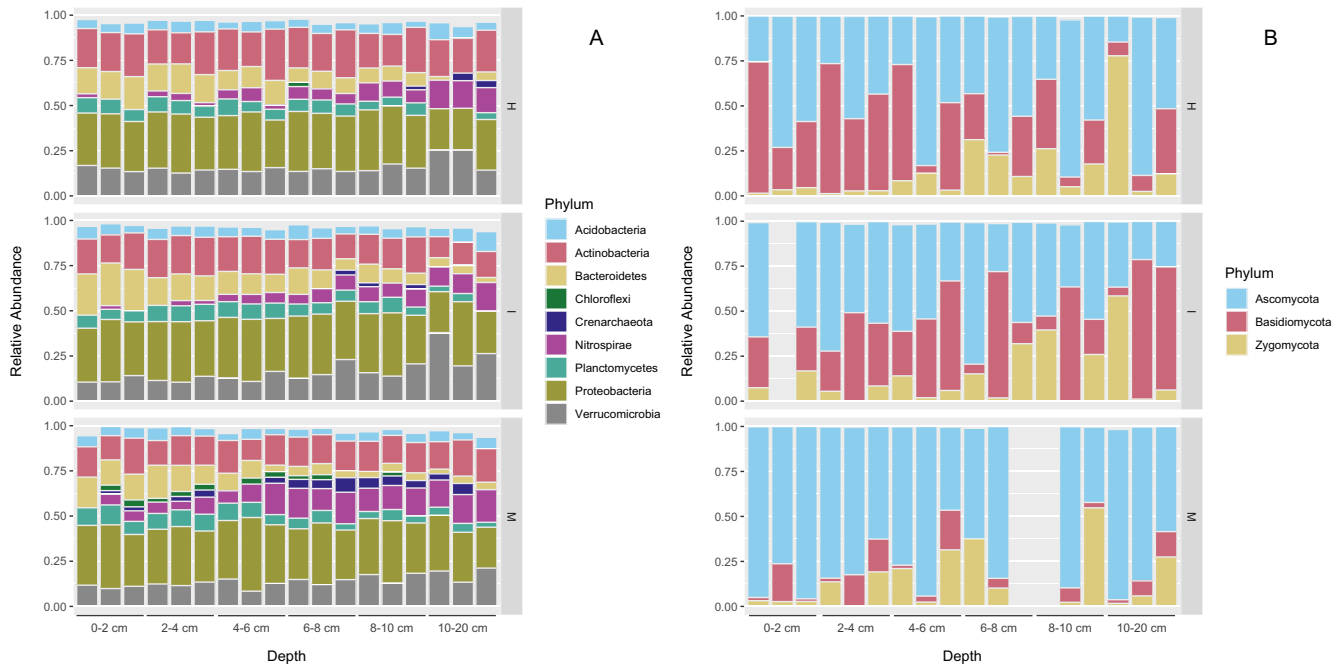




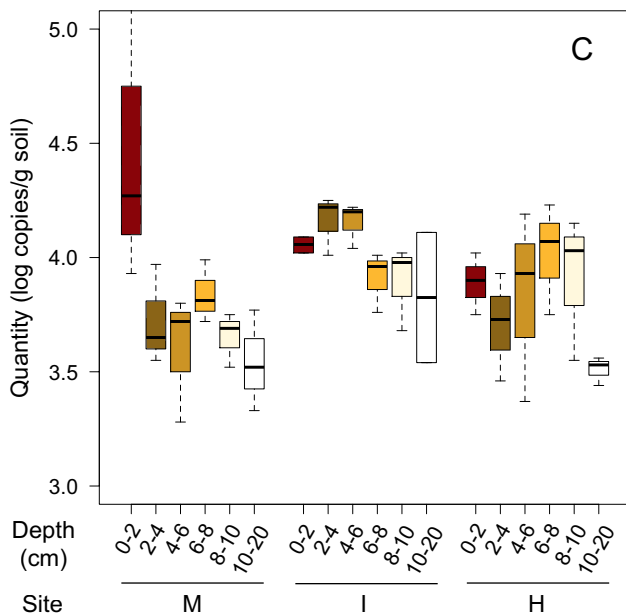
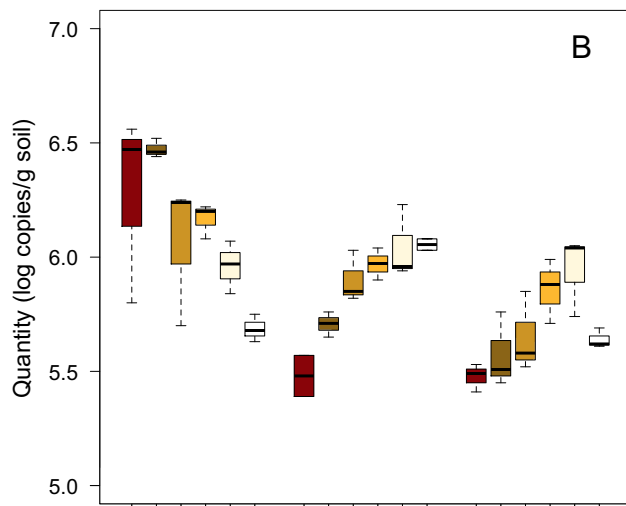
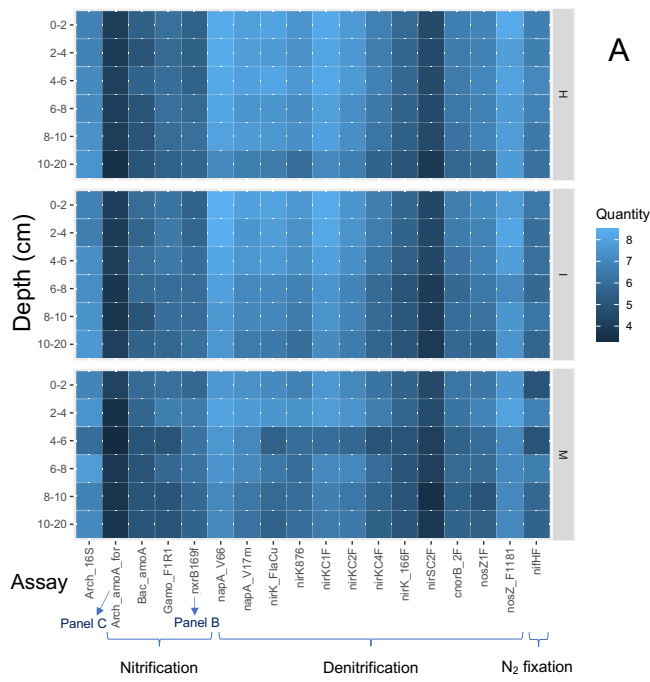
**Figure 1.** Abundance and diversities of microbes in the soil samples. Quantities of (A) 16S rRNA gene and (B) fungal ITS2 region measured by qPCR. Numbers of observed OTUs for (C) archaea/bacteria and (D) fungi. Shannon diversity index values calculated based on (E) the archaeal/bacterial 16S rRNA gene and (F) fungal ITS2 region sequences. Legend: M, the site with minimal invasion of earthworms; I, the site with an intermediate invasion of earthworms; and H, the site with a heavy invasion of earthworms.



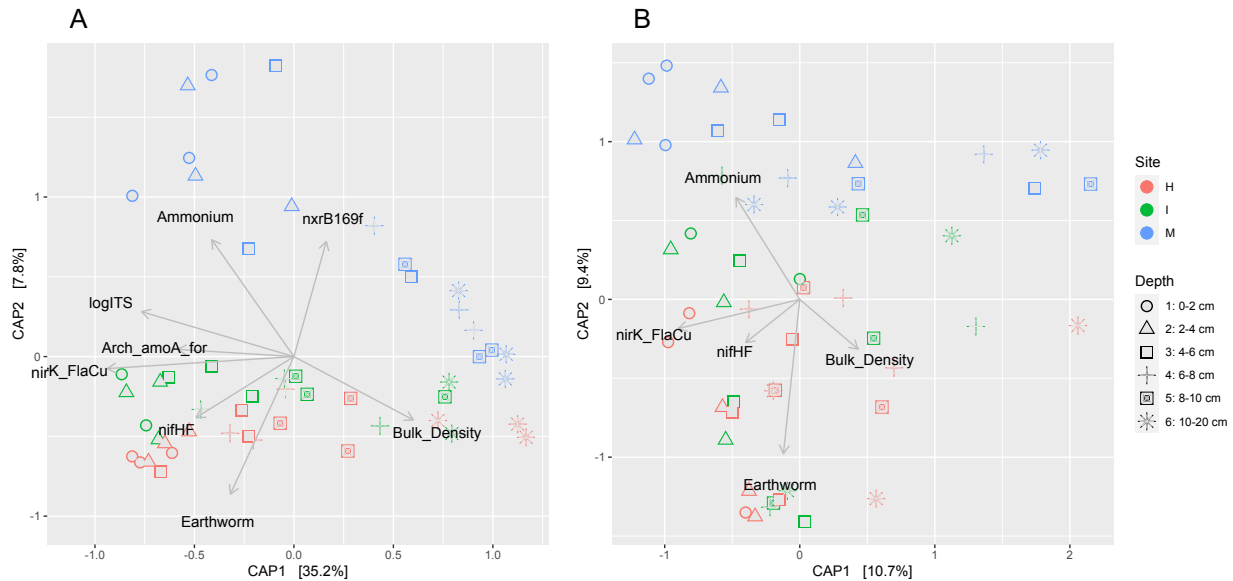
**Figure 2.** Principal coordinate analysis (PCoA) plots showing the Bray-Curtis dissimilarities among (A) archaeal/bacterial communities and (B) fungal communities. Each community is labeled with the site (M, the site with minimal invasion of earthworms; I, the site with an intermediate invasion of earthworms; and H, the site with a heavy invasion of earthworms) and soil depth (0-2, 2-4, 4-6, 6-8, 8-10, and 10-20 cm from the surface).



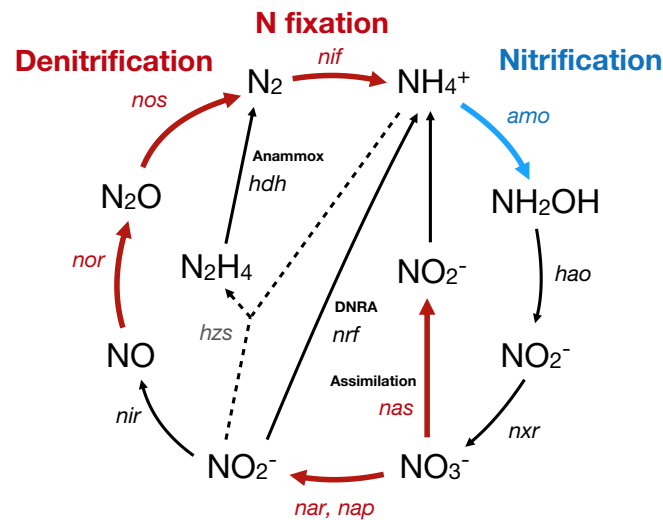
**Figure 3.** Relative abundance of (A) archaeal/bacterial phyla and (B) fungal phyla in the soil samples as assessed by the 16S rRNA gene and ITS2 sequencing analyses. Legend: M, the site with minimal invasion of earthworms; I, the site with an intermediate invasion of earthworms; and H, the site with a heavy invasion of earthworms.



**Figure 4.** Quantities of N cycle-related genes in the soil samples. (A) Pseudo-heatmap showing the Nitrogen Cycle Evaluation (NiCE) chip results. Assays producing positives in >60% of samples are shown. (B) Quantities of *nrxB* of *Nitrospira*, based on the *nrxB169f* assay. (C) Quantities of archeal *amoA* based on the *Arch\_amoA\_for* assay. Legend: M, the site with minimal invasion of earthworms; I, the site with an intermediate invasion of earthworms; and H, the site with a heavy invasion of earthworms.



**Figure 5.** Canonical analysis of principal coordinates (CAP) plots showing the associations between environmental variables and the patterns in (A) archaeal/bacterial communities and (B) fungal communities. Microbial communities were analyzed using Bray-Curtis distance matrices. All environmental variables shown in the plots had significant effects ( $p < 0.05$ ) on the microbial community patterns based on PERMANOVA. Each community is labeled with the site (M, the site with minimal invasion of earthworms; I, the site with an intermediate invasion of earthworms; and H, the site with a heavy invasion of earthworms) and soil depth (0-2, 2-4, 4-6, 6-8, 8-10, and 10-20 cm from the surface).



**Figure 6.** Nitrogen cycling in the forest soil influenced by earthworm invasion. Red and blue arrows indicate the genes that increased and decreased their relative abundances by earthworm invasion, respectively, based on Spearman's correlation analysis of the shotgun metagenomics reads ( $p < 0.05$ ). Black solid arrows indicate the genes that did not change their abundance by earthworm invasion. Black dashed arrows indicate the genes that were not detected.