Tuber melanosporum shapes *nir*S-type denitrifying and ammonia-oxidizing bacterial communities in *Carya illinoinensis* ectomycorrhizosphere soils

Zongjing Kang [1, 2] , **Jie Zou** [1, 2] , **Yue Huang** [1, 2] , **Xiaoping Zhang** [1, 2] , **Lei Ye** [1] , **Bo Zhang** [1] , **Xiaoping Zhang** [2] , **Xiaolin Li**
Corresp. [1]

[1] Soil and Fertilizer Institute, Sichuan Academy of Agricultural Sciences, Chengdu, China

[2] Department of Microbiology, College of Resources, Sichuan Agricultural University, Chengdu, China

Corresponding Author: Xiaolin Li
Email address: kerrylee_tw@sina.com

Background. *NirS*-type denitrifying bacteria and ammonia-oxidizing bacteria (AOB) play a key role in the soil nitrogen cycle, which may affect the growth and development of underground truffles. We aimed to investigate *nirS*-type denitrifying bacterial and AOB community structures in the rhizosphere soils of *Carya illinoinensis* seedlings inoculated with the black truffle (*Tuber melanosporum*) during the early symbiotic stage.

Methods. The *C. illinoinensis* seedlings inoculated with or without *T. melanosporum* were cultivated in a greenhouse for six months. Next-generation sequencing (NGS) technology was used to analyze *nirS*-type denitrifying bacterial and AOB community structures in the rhizosphere soils of these seedlings. Additionally, the soil properties were determined.

Results. The results indicated that the abundance and diversity of AOB were significantly reduced due to the inoculation of *T. melanosporum*, while these of *nirS*-type denitrifying bacteria increased significantly. Proteobacteria were the dominant bacterial groups, and *Rhodanobacter*, *Pseudomonas*, *Nitrosospira* and *Nitrosomonas* were the dominant classified bacterial genera in all the soil samples . *Pseudomonas* was the most abundant classified *nirS*-type denitrifying bacterial genus in ectomycorrhizosphere soils whose relative abundance could significantly increase after *T. melanosporum* inoculation. A large number of unclassified *nirS*-type denitrifying bacteria and AOB were observed. Moreover, *T. melanosporum* inoculation had little effect on the pH, total nitrogen (TN), nitrate-nitrogen (NO_3^--N) and ammonium-nitrogen (NH_4^+-N) contents in ectomycorrhizosphere soils. Overall, our results showed that *nirS*-type denitrifying bacterial and AOB communities in *C. illinoinensis* rhizosphere soils were significantly affected by *T. melanosporum* on the initial stage of ectomycorrhizal symbiosis, without obvious variation of soil N contents.

1 **_Tuber melanosporum_ shapes _nirS_-type denitrifying and**

2 **ammonia-oxidizing bacterial communities in _Carya_**

3 **_illinoinensis_ ectomycorrhizosphere soils**

6 Zongjing Kang [1,2#], Jie Zou [1,2#], Yue Huang[1,2], Xiaoping Zhang[1,2], Lei Ye [1], Bo Zhang[1], Xiaoping

7 Zhang[2], Xiaolin Li[1*]

8 [1] Soil and Fertilizer Institute, Sichuan Academy of Agriculture Sciences, Chengdu, China

9 [2] Department of Microbiology, College of Resources, Sichuan Agricultural University, Chengdu,

10 China

11 [#] Zongjing Kang and Jie Zou contributed equally to the work.

13 *Corresponding Author: Xiaolin Li

14 Email address: kerrylee_tw@sina.com

Abstract

Background. *NirS*-type denitrifying bacteria and ammonia-oxidizing bacteria (AOB) play a key role in the soil nitrogen cycle, which may affect the growth and development of underground truffles. We aimed to investigate *nirS*-type denitrifying bacterial and AOB community structures in the rhizosphere soils of *Carya illinoinensis* seedlings inoculated with the black truffle (*Tuber melanosporum*) during the early symbiotic stage.

Methods. The *C. illinoinensis* seedlings inoculated with or without *T. melanosporum* were cultivated in a greenhouse for six months. Next-generation sequencing (NGS) technology was used to analyze *nirS*-type denitrifying bacterial and AOB community structures in the rhizosphere soils of these seedlings. Additionally, the soil properties were determined.

Results. The results indicated that the abundance and diversity of AOB were significantly reduced due to the inoculation of *T. melanosporum*, while these of *nirS*-type denitrifying bacteria increased significantly. Proteobacteria were the dominant bacterial groups, and *Rhodanobacter*, *Pseudomonas*, *Nitrosospira* and *Nitrosomonas* were the dominant classified bacterial genera in all the soil samples. *Pseudomonas* was the most abundant classified *nirS*-type denitrifying bacterial genus in ectomycorrhizosphere soils whose relative abundance could significantly increase after *T. melanosporum* inoculation. A large number of unclassified *nirS*-type denitrifying bacteria and AOB were observed. Moreover, *T. melanosporum* inoculation had little effect on the pH, total nitrogen (TN), nitrate-nitrogen (NO_3^--N) and ammonium-nitrogen (NH_4^+-N) contents in ectomycorrhizosphere soils. Overall, our results showed that *nirS*-type denitrifying bacterial and AOB communities in *C. illinoinensis* rhizosphere soils were significantly affected by *T. melanosporum* on the initial stage of ectomycorrhizal symbiosis, without obvious variation of soil N contents.

39 Introduction

40 *Tuber* (Ascomycota, Pezizales) commonly known as the truffle, is an edible fungus which can

41 form symbiotic relationships with plants. To date, more than 200 species of truffles have been

42 discovered worldwide (Bonito et al., 2011), particularly in Sweden, France, Italy, Hungary, Spain,

43 the United Kingdom, and Russia (Wedén et al., 2004). *Tuber melanosporum*, native in France,

44 Italy and Spain, is a valuable and rare species of *Tuber* in the market (Donnini et al., 2014). *T.*

45 *melanosporum* attracted people primarily by its unique aroma compounds. As ectomycorrhizal

46 fungi, truffles need to form symbiotic association with the host plant to complete their life cycle

47 (Kües et al., 2011). Recently, the cultivation of *T. melanosporum* (as well as other species

48 including *T. aestivum* Vittad., *T. borchii* Vittad., and *T. indicum*) has been conducted worldwide

49 by planting truffle-mycorrhized plant hosts in nurseries (Reyna et al., 2014), and yet studies on the

50 cultivation of this black truffle species in China are seldom reported.

51   Truffles can form symbiotic relationships with trees from several genera, including *Corylus*,

52 *Quercus*, *Abies Pinus*, *Populus* and *Salix* (Healy et al., 2016; Wan et al., 2016). The pecan tree,

53 *Carya illinoinensis* (Wangenh.) K. Koch, is an indigenous and economically important tree that

54 grows naturally in moist bottom-land habitats in the United States (Sparks, 1992). However though

55 endemic to the Mississippi basin, *C. illinoinensis* is now cultivated globally (Wakeling et al., 2001;

56 Ruan et al., 1992). *Tuber lyonii* Butters was the first truffle species described in symbiotic

57 association with the pecan tree (Trappa et al., 1996). Bonito et al. (2011) hypothesized that pecan

58 orchards could be managed to optimize both truffle and pecan production simultaneously. In

59 general, the utilization of *C. illinoinensis* as a host plant for *T. melanosporum* has good application

60 prospects and is worthy of study.

61   Soil microorganisms and physicochemical properties in the rhizosphere of host plant can change

62   and vary with the growth of truffles, from the occurrence of ectomycorrhizae to the formation of

63   their mature fruiting bodies (Ponce et al., 2014; Barbieri et al., 2007; Garcia-Barreda et al., 2017).

64   The microbial communities of ectomycorrhizosphere soils are verified to be different from that of

65   rhizosphere soils in both artificial conditions and the wild (Fu et al., 2016; Li et al., 2018). There

66   were numerous classified and unclassified microorganisms detected in the ectomycorrhizosphere

67   soils of *Tuber* (Garcia-Barreda et al., 2012), which made it difficult to analyze the taxonomy and

68   function of all the bacteria simultaneously. *Paracoccus*, *Pseudomonas*, *Rhizobium*, *Bacillus*, and

69   *Nitrosomonadales* were some possible denitrifying bacteria and ammonia-oxidizing bacteria

70   (AOB) (Yoshida et al., 2010; Purkhold et al., 2000) already detected in the ectomycorrhizae and

71   ectomycorrhizosphere soil of *Tuber*-host associations (Yang et al., 2019; Li et al., 2019; Mello et

72   al., 2013). Nevertheless, the distribution of denitrifying bacteria and the structure of AOB

73   communities in ectomycorrhizosphere soils stay unclear.

74   The nitrogen (N) cycle is one of the most important nutrient cycles in an ecosystem (Thamdrup,

75   2012), which is vital for host plants and *Tuber*. Both denitrification and ammonia oxidation are

76   important biological processes in the N cycle. Denitrification is the main pathway of N_2O release

77   in farmland ecosystems (Conrad, 1997), and an important process that reduces N pools in farmland

78   soils (Seitzinger et al., 2006). By denitrification, denitrifying microorganisms gradually reduce the

79   nitrates or nitrous acid in the environment to gaseous products (NO, N_2O, and N_2) under multi-

80   step enzymatic catalysis. Most soil denitrification is performed by denitrifying microbes, including

81   bacteria, fungi, and archaea (Shapleigh, 2006). Denitrification-related enzymes containing nitrate,

82   nitrite, NO, and N_2O reductase, can be expressed by certain denitrifying bacteria such as

83   *Pseudomonas denitrificans* and *Bacillus stearothermophilus* (Gregory et al., 2003). The nitrite

84 reductase functional gene, which control the catalyzing of the reduction of nitrite to nitric oxide,

85 is the main molecular marker in denitrifying bacteria research (Braker et al., 2000). Nitrite

86 reductase has two different structural forms, one of them contains copper (Cu-*nir*) and is encoded

87 by the *nirK* gene, while the other contains the heme CD1 (cd1-*nir*) is encoded by *nirS* gene

88 containing (Zumft, 1997). More *nirS* gene are detected than *nirK* gene in most soil samples, and

89 *nirS*-type denitrifying bacteria species in the environment are affected by factors such as water,

90 plants, etc (Azziz et al., 2017). As the main driver of the ammonium oxidation process, AOB has

91 long been a focal and functional focus in environmental microbial ecology (Kowalchuk et al.,

92 2001). The AOB in β-Proteobacteria and γ-Proteobacteria has been long considered to be the most

93 active in ammonium oxidation in soils. The oxidation of ammonia to nitrite is a rate-limiting step

94 of nitrification controlled by many AOB ammonia monooxygenase (*amoA*) genes. These genes

95 were widely involved in the study of the relative abundance of AOB in ecosystems (Purkhold et

96 al., 2000; Wang et al., 2011). The efficiency of nitrification and denitrification in soils were related

97 to the number of microbial functional genes. The community structure of ammonia-oxidizing

98 microorganisms, however could in return be affected by the concentration of ammonium nitrogen

99 and organic matter (Petersen et al., 2012). Structures of *nirS*-type denitrifying bacterial and AOB

100 community in *Tuber* ectomycorrhizosphere soils is unclear. Only few studies have focused on the

101 relationship between these bacteria and properties of the ectomycorrhizosphere soils.

102 Therefore in our study, we infected *C. illinoinensis* with the spores of *T. melanosporum* and

103 tracked the formation of mycorrhizae during the next six months of cultivation. We also did Next-

104 generation amplicon sequencing for *nirS* and *amoA* genes, to analyze the denitrification and

105 ammonia oxidation microbial community structures in rhizosphere soils. Furthermore, the

106 physicochemical soil properties in the rhizosphere of *C. illinoinensis* seedlings were determined.

107   The study aims to investigate *nirS*-type denitrifying bacterial and AOB community structures

108   involving in the ammonium oxidation and denitrification processes in the *T. melanosporum*

109   ectomycorrhizosphere soils.

Materials & Methods

Cultivation of sterile *C. illinoinensis* seedlings

112   Seeds of *C. illinoinensis* were soaked in fresh-water for about 20 h and sterilized with 0.3%

113   potassium permanganate for 30 min, then the seeds were rinsed with distilled water until the last

114   wash solution became colorless. In order to increase the germination rate, the seeds were stored in

115   the sand which had been sterilized for 90 min at 121˚C in an autoclave. One month later, the

116   germinated *C. illinoinensis* seeds were sown in a plastic container filled with the sterilized

117   substrate (vermiculite, perlite and water at a ratio of 1:1:1, v/v/v) (Fig. S1) (Li et al., 2017). The

118   plastic container with seeds was then placed in a natural light plastic greenhouse and watered with

119   sterile water, to maintain a soil moisture content of 25%. The temperature of the greenhouse was

120   23°C–25°C in daytime and 16°C–20°C at night. The well-grown *C. illinoinensis* seedlings with

121   similar plant height and stem thickness were selected for inoculation.

Truffle inoculation treatment

123   *T. melanosporum* ascocarps were purchased from French truffle producing areas and used as spore

124   inoculum. The ascocarps were washed with sterile water and their surface sterilized by burning

125   briefly with 75% alcohol. Then the ascocarps were soaked in sterile water to stimulate spores

126   release and germination (Semeniuk, 2008). Finally, the treated *T. melanosporum* ascocarps were

127   smashed and blended using food chopper to obtain their paste. Next, the substrates with peat,

128   vermiculite, perlite organic soil and water at a ratio of 1:1:1:0.9 (v/v/v/v) were prepared and

129   sterilized for 90 min at 121˚C in an autoclave. The sterilized substrate (total nitrogen 2.05 g/kg,

130   nitrate-nitrogen 37.50 mg/kg and ammonium-nitrogen 20.16 mg/kg) was filled into nursery pots

131   and 1.5 g of the paste inoculum was mixed into the substrate of each pot, then one sterile seedling

132   was transplanted into each nursery pot. Thirty seedlings inoculated with *T. melanosporum* were

133   named group Ⅰ. At the same time, an equivalent number of *C. illinoinensis* seedlings were

134   transplanted into nursery pots without *T. melanosporum* inoculum, named group Ⅱ. Each group

135   had thirty replicates to ensure the survival of enough seedlings after 6 months cultivation. All pots

136   were maintained in a greenhouse under the same conditions without fertilization, and they were

137   watered with tap water every three days at 6:00 pm for 6 month (Geng et al., 2009).

138   **Sampling strategy and soil analyses**

139   Eight pots of group Ⅰ and four pots of group Ⅱ were randomly selected, and roots of these

140   seedlings were observed under a microscope. Morphological and anatomical analysis revealed that

141   the four seedlings in group Ⅱ were not colonized by ectomycorrhizal fungi, and only 4 of the 8

142   seedlings in group Ⅰ inoculated with truffle spores, showed ectomycrrhizae formation (Fig. 1)

143   (Marozzi et al., 2017). The four pots of group Ⅰ colonized by *T. melanosporum* were used as

144   experimental materials, and the four pots of group Ⅱ were used as controls, thus, each experimental

145   treatment had four replicates. Ectomycorrhizosphere soils (rhizosphere soils of *C. illinoinensis*

146   seedlings inoculated with *T. melanosporum*) and rhizosphere soils (rhizosphere soils of *C.*

147   *illinoinensis* seedlings without inoculating) samples were sampled and placed into 2 mL EP tubes.

148   The samples were cooled rapidly using liquid nitrogen and stored at -80°C. The remaining soils

149   around the *C. illinoinensis* roots were air dried for soil property analysis, including pH, organic

150   matter (OM), available potassium (AK), total nitrogen (TN), available phosphorus (AP), nitrate-

151   nitrogen (NO_3^--N) and ammonium-nitrogen (NH_4^+-N), which were determined following the

152 methods described in previous studies (Lu, 1999; Li et al., 2017). Ectomycorrhizosphere soils of

153 *C. illinoinensis* colonized with *T. melanosporum* were labeled as "FH" from Chines name of the

154 *Tuber*, "Faguo Heibao", and the rhizosphere soils of the *C. illinoinensis* seedlings without *T.*

155 *melanosporum* inoculation were labeled as "CK" standing for Control Check.

156 **DNA extraction, PCR amplification, and MiSeq sequencing**

157 DNA from rhizosphere soils was extracted using a Soil DNA Kit (D5625-01, Omega Bio-tek Inc.,

158 Norcross, GA, USA) following the manufacturer's instructions. The DNA concentration and

159 purity were measured on 1% agarose gels, adjusted to 1 ng/μL using sterile water and stored at -

160 20°C.

161 The universal primers Cd3aF (5'-GTSAACGTSAAGGARACSGG-3') (Michotey et al., 2000)

162 and R3cd (5'-GASTTCGGRTGSGTCTTGA-3') (Throbäck et al., 2004) were used to amplify

163 *nirS*-type denitrifying bacteria in PCR. The reaction consisted of 10 ng DNA template, 4 μL

164 FastPfu buffer, 2 μL dNTPs (2.5 mmol·L^{-1}), 0.4 μL FastPfu polymerase, 0.8 μL forward primer (5

165 μmol·L^{-1}), and 0.8 μL reverse primer (5 μmol L^{-1}), diluted to 20 μL with ddH$_2$O. The reaction

166 program was set as follows: pre-denaturation at 95°C for 4 min, followed by 28 cycles of

167 denaturation at 95°C for 40 s, annealing at 56°C for 30 s, extension at 72°C for 40 s, and then final

168 extension at 72°C for 10 min. The universal primers b-amoA-1F (5'-

169 GGGGTTTCTACTGGTGGT-3') and b-amoA-2R (5'-CCCCTCKGSAAAGCCTTCTTC-3')

170 (Mao et al., 2011) were used to amplify the AOB in a 30 μL reaction system, which were consisted

171 of 15 μL 2× Ex Taq MasterMix, 1.2 μL forward and reverse primers (10 μmol/L), 3 μL DNA

172 template and 9.6 μL RNase-free water. Samples were amplified by subjecting them to the

173 following PCR program: 95°C for 3 min followed by 40 cycles at 95°C for 60s, 55°C for 45 s and

174 72°C extension for 60 s. The samples were then mixed and analyzed by 1.5% agarose gel

175   electrophoresis, after which the PCR product was recovered using a gel recovery kit (Axygen,

176   USA) and eluted with Tris-HCl and 1.5% agarose gel electrophoresis (Kim et al., 2011). According

177   to the preliminary quantitative electrophoresis results, PCR products were quantified using

178   QuantiFluor^{TM}-ST Blue Fluorescence Quantitation System (Promega, USA), after which the

179   proportions were mixed according to template concentration for each sample. Finally, these

180   samples stored at -80°C.

181   High throughput sequencing was conducted at Shanghai Majorbio Bio-pharm Technology Co.,

182   Ltd. and sequenced according to manufacturer's recommendations with the Illumina MiSeq

183   sequencing platform. Raw data were submitted to the Sequence Read Archive (SRA) database

184   with the accession number SRR10522644–SRR10522659.

185   **High-throughput data analysis and statistical analysis**

186   Paired-end reads were obtained, and paired-end reads that overlapped were merged using FLASH

187   (Version 1.2.11) (Tanja & Salzberg, 2011). The high-quality sequences with mismatch rate of

188   overlap sequence lower than 20% and with barcode and primer sequence were filtered by QIIME

189   (Version 1.8.0) and Trimmomatic software (Caporaso et al., 2010), then correct sequence

190   direction. Uparse (Version 7.0.1090) software was used to analyze the high-quality sequences, and

191   these sequences with ≥97% similarity were clustered to the same OTU (Operational Taxonomic

192   Units) (Edgar, 2013). A representative sequence for each OTU was screened for further annotation,

193   and the taxonomic analysis of OTUs was performed using the RDP Classifier (Version 2.11;

194   http://sourceforge.net/projects/rdp-classifier/) (Wang et al., 2007) based on the FunGene

195   (Release7.3; http://fungene.cme.msu.edu/) database. Mothur (Version 1.30.2) was used to divided

196   OTUs, and alpha diversity analysis of *nirS*-type denitrifying bacterial and AOB population

197   (Coverage, Chao1, ACE, Shannon and Simpson index) were performed based on these OTUs.

198   Source code for the OTUs data is available at: https://github.com/catherinekang/bacterial-

199   community.git. Moreover, a rarefaction curve was used to evaluate the representativeness of

200   sequencing amount for the diversity of the original *nirS*-type denitrifying bacteria or the AOB. In

201   addition, barplots based on the R software (Version 3.1.3) (Core et al., 2011) were utilized to

202   cluster and analyze the phylum and genus to share the taxonomic composition of the soil microbial

203   communities. Principle component analysis (PCA) and clustering analysis were used to reflect the

204   beta diversity.

205   Alpha diversity estimators, soil properties and relative abundance of the taxa of AOB and *nirS*-

206   type denitrifying bacteria between the two treatments were compared with independent *t-test*,

207   which was performed in SPSS v21.0 (IBM Inc., Armonk, NY, USA). All the data were presented

208   as the mean value ± standard deviation (SD) of the four biological replicates in each treatment

209   group. All the significant differences were assessed at *P < 0.05*.

210   **Results**

211   **Soil property analysis**

212   The results of soil properties showed that the nitrate-nitrogen and available potassium contents of

213   FH were slightly higher than those of CK, and there were no significant different in pH, organic

214   matter, available potassium, total nitrogen, available phosphorus, nitrate-nitrogen and ammonium-

215   nitrogen between CK and FH (Table. 1).

216   **AOB diversity analysis**

217   A total of 123,091 reads were obtained from the 8 samples after quality control procedures, most

218   of which were 401-500 bp, and there were 11,990-19,959 reads in each sample (Fig. 2a). All the

219   sequences were clustered into 251 OTUs, and the two treatments share 47 OTUs. The number of

220   unique OTUs which can only be detected in FH (77) was smaller than the unique OTUs which can

221   only be detected in CK (127) (Fig. 3a).

222   As shown in Table 2, the coverage in the two treatments were 99%, indicating that we sequenced

223   ammonia monooxygenase genes at the proper depth and the data were reliable. The richness

224   indices such as ACE (*P = 0.049*) and Chao1 (*P = 0.043*) estimators in CK was significantly greater

225   than these in FH. Shannon diversity in the two treatments ranged from 1.32 to 1.62, and was

226   significantly greater in CK treatment (*P = 0.047*), however, there was no significant difference

227   between FH and CK in the Simpson index and the observed species (OTU). For the observed

228   species, Shannon, Chao1, and ACE of FH were lower than those of CK, which showed that the

229   richness and diversity of AOB in FH was significantly lower than that in CK (*P < 0.05*) (Table.

230   2).

231   **Taxonomic analyses of AOB**

232   As shown in Figure 4a, the observed species mainly belonged to two phyla, and Proteobacteria

233   was the most abundant in each treatment. The average relative abundance of Proteobacteria in CK

234   (98.59%) was significantly greater than that in FH (84.93%) (*P = 0.049*) (Table. 3).

235   The results of classification showed that 251 OTUs were separated into more than six genera

236   (Fig. 5a). The primary three genera in FH treatment were *Nitrosospira* (57.90%), *Nitrosomonas*

237   (25.12%) and unclassified bacteria (15.07%); while those in CK were *Nitrosospira* (55.38%),

238   *Nitrosomonas* (39.99%) and an unclassified genus belong to Betaproteobacteria (1.93%) (Table.

239   4). Moreover, the relative abundance of *Nitrosospira* in both treatments was approximately the

240   same, while the relative abundance of *Nitrosomonas* in CK was slightly greater than that in FH.

241   *Nitrosococcus* was detected in CK, with significantly higher relative abundance than that in FH (*P*

242 $= 0.046$). Nevertheless, the most abundant unclassified AOB genus in FH was significantly more

243 abundant than that in CK (1.41%) ($P = 0.005$).

244 **NirS-type denitrifying bacterial diversity analysis**

245 A total of 139,493 reads were obtained from the 8 samples after quality control procedures, most

246 of which the sequencing length was 301-400 bp, and there were 10,424-19,961 reads in each

247 sample (Fig. 2b). All the sequences from the two treatments were clustered into 750 OTUs, which

248 were utilized to form a histogram of genera and phyla. Moreover, there were a total of 193 OTUs

249 shared by two treatments and the number of unique OTUs detected in FH (311) was more than the

250 unique OTUs detected in CK (246) (Fig. 3b).

251 The coverage of the denitrifying bacterial communities in the two treatments was 99% (Table.

252 2). The ACE ($P = 0.042$) and Chao1 ($P = 0.048$) estimators revealed that the richness of *nirS*-type

253 denitrifying bacteria in FH was significantly greater than that in CK. The Simpson index of two

254 treatments was similar, and the number of observed species of FH was slightly higher than that of

255 CK without significant difference. And the Shannon index of FH was significantly higher than that

256 of CK ($P = 0.037$) (Table. 2). The results demonstrated that the richness and diversity of *nirS*-type

257 denitrifying bacteria were significantly increased with *T. melanosporum* inoculation. Moreover,

258 the observed species of *nirS*-type denitrifying bacteria were far more than AOB in both treatments

259 (Fig S2), the Shannon, Chao1 and ACE index of *nirS*-type denitrifying bacteria were much higher

260 than AOB.

261 **Taxonomic analyses of *nirS*-type denitrifying bacterial communities**

262 As shown in Figure 4b, at the phylum level, the most abundant were unclassified bacteria and no

263 rank bacteria, followed by Proteobacteria. The average proportions of Proteobacteria in the two

264 treatments were 9.49% (CK) and 8.69% (FH), with Proteobacteria and an unclassified bacteria

265   phylum being slightly more abundant in CK than in FH (Table. 3). Nevertheless, there was no

266   significant difference in the relative abundance of the dominant phyla between FH and CK.

267   The results showed that these 750 OTUs belonged to more than nine genera, and the most

268   abundant classified genera were *Pseudomonas*, *Rhodanobacter*, *Magnetospirillum* and *Rubrivivax*

269   (Fig 5b). The main classified genera in FH were *Pseudomonas* (0.46%), while the main classified

270   genus in CK was *Rhodanobacter* (0.33%), and the relative abundance of *Pseudomonas* of FH was

271   significantly higher than that of CK (*P = 0.017*) (Table. 4). The relative abundance of the no rank

272   bacterial genus was similar in FH and CK, without significant difference. However, the relative

273   abundance of two unclassified genera observed in FH, belonging to Gammaproteobacteria (*P =*

274   *0.027*) and Betaproteobacteria (*P = 0.015*), were significantly lower than those observed in CK.

275   **Principle component analysis (PCA)**

276   The Unifrac-PCA analysis was used to visualize the similarities and differences of the bacterial

277   component in different soil samples. The shorter distance between two samples means more

278   similar *nirS*-type denitrifying bacterial and AOB communities in the different samples, and the

279   different shaded circles represented confidence ellipses. The results showed that both AOB and

280   *nirS*-type denitrifying bacteria in the FH were not similar to CK. In Figure 6, the distance between

281   FH and CK in AOB was greater than the distance between them in *nirS*-type denitrifying bacteria,

282   which could indicate that the difference of AOB communities between FH and CK was greater

283   than that of *nirS*-type denitrifying bacterial communities between them.

284   **Discussion**

285   This experiment amplified *nirS* and *amoA* genes partner to detected the denitrifying bacterial and

286   ammonia-oxidizing bacterial (AOB) communities from the rhizosphere soil of *C. illinoinensis* with

287   or without *T. melanosporum* inoculation. The study also explored the impacts of *T. melanosporum*

288   inoculation on the nitrogen cycling bacteria in microecology of *C. illinoinensis* rhizosphere within

289   6 months after inoculation.

290   Results of PCA analysis showed a significant difference of *nirS*-type denitrifying bacterial and

291   AOB communities between ectomycorrhizosphere soil and rhizosphere soil. This was in

292   accordance with the previous finding that the composition and function of bacterial communities

293   in ectomycorrhizosphere soil could be altered by the formation of ectomycorrhizae (Jung et al.,

294   2012). In our study, the total nitrogen, nitrate-nitrogen and ammonium-nitrogen contents of

295   ectomycorrhizosphere soil were not significantly different from rhizosphere soil similarly to the

296   contents of other determined properties. Previous studies reported that the ectomycorrhizal fungi

297   could increase available nitrogen in forest soil by producing enzymes or organic acids,

298   accelerating plant litter decomposition, and soil mineral dissolution (Sterkenburg et al., 2018;

299   Lindahl et al., 2006). The contrasting results from our study, however, suggested that the

300   ectomycorrhizae of *T. melanosporum* had little impact on the soil properties in the host plant

301   rhizosphere which did not contain plant litter at the initial stage of inoculation.

302   Moreover, the richness and diversity of AOB in the ectomycorrhizosphere soil were

303   significantly lower than those in rhizosphere soil (*P < 0.05*). The Proteobacteria was the most

304   abundant AOB in both ectomycorrhizosphere soil (84.93%) and rhizosphere soil (98.59%), which

305   was consistent with the in forest soils (Rosch et al., 2002). The most abundant genera of AOB

306   were *Nitrosospira* and *Nitrosomonas*. Li et al. (2018) reported that the *Nitrosospira* and

307   *Nitrosomonas* were the most abundant AOB in ectomycorrhizosphere soil of *Pinus massoniana*

308   Lamb colonized by *Pisolithus tinctorius* under field condition, which is basically the same with

309   the findings of our study. In addition, the relative abundance of *Nitrosococcus* and an unclassified

310   AOB genus of Betaproteobacteria in ectomycorrhizosphere soils was significantly greater than

311   that in rhizosphere soils while another unclassified genus was significantly lower than that in

312   rhizosphere soils ($P < 0.05$). The soil moisture might have an indirect effect on AOB communities

313   by changing the availability and mobility of nitrogen in the substrate (Gleeson et al., 2010; Hu et

314   al., 2015). However, *C. illinoinensis* seedlings in two treatments were watered equally in this work.

315   Previous studies have illustrated that the diversity of bacterial communities reduced in the brûlés

316   (an area devoid of herbaceous cover) and ectomycorrhizosphere soil (Mello et al., 2013; Zhang et

317   al., 2019). Nevertheless, some bacterial genera in ectomycorrhizosphere soils were significantly

318   more abundant than those in rhizosphere soils, such as *Mesorhizobium, Reyranena,*

319   *Rhizomicrobium* and *Nordella* (Li et al., 2017). These findings may imply that the *T.*

320   *melanosporum* ectomycorrhizae have different effect on different AOB genera (Doornbos et al.,

321   2012), and the richness and diversity of the AOB in rhizosphere soils were reduced by *T.*

322   *melanosporum* inoculation in general, though the ectomycorrhizae of *T. melanosporum* indeed

323   affect the relative abundance of *Nitrosospira* and *Nitrosomonas* little during early symbiotic stage.

324   In terms of the structural analysis *nirS*-type denitrifying bacterial community, Proteobacteria

325   was the dominant group both in rhizosphere and ectomycorrhizosphere soils. Previous studies

326   showed that most of the denitrifying bacteria which were detected in crop rhizosphere soils and

327   arable land soils belong to Proteobacteria (Chang et al., 2012; Hui et al., 2010; Hou et al., 2018),

328   which corroborates our results. Our work also showed that the *Rhodanobacter*, *Pseudomonas*,

329   *Magnetospirillum* and *Rubrivivax* were the dominant classified bacterial genera in all the soil

330   samples, which were detected in plant rhizosphere soils and arable land soils frequently (Cho et

331   al., 2017; Yu et al., 2018; Wen et al., 2016). The richness and diversity of *nirS*-type denitrifying

332   bacterial communities in ectomycorrhizosphere soil were significantly greater than those in

333   rhizosphere soil ($P < 0.05$). The land use and fertilizer regimes affected the biological activity of

334   denitrifying bacteria (Hui et al., 2010), and the abundance of *Rhodanobacter* was sensitive to pH

335   (Green et al., 2010). However, the differences in soil properties between two treatments were not

336   significant in the present study, which may illustrate that the abundance of some *nirS*-type

337   denitrifying bacterial communities in rhizosphere soils was increased due to *T. melanosporum*

338   inoculation. The previous study showed that the mycorrhiza and rhizobia assist plants with the

339   uptake of phosphorus and nitrogen, respectively (Heijden et al., 2008),which may suggest that the

340   *T. melanosporum* ectomycorrhizae accelerate the weathering of minerals and the degradation of

341   recalcitrant organic matter by enhancing the colonization of *nirS*-type denitrifying bacteria in

342   ectomycorrhizosphere soils (Berendsen et al., 2012; Deveau et al., 2016). Meanwhile, the relative

343   abundance of unclassified bacterial genera belonging to Gammaproteobacteria and

344   Betaproteobacteria were significantly lower in ectomycorrhizosphere soils ($P < 0.05$), which

345   demonstrated that the colonization of *T. melanosporum* decreased the relative abundance of some

346   *nirS*-type denitrifying bacterial genera in ectomycorrhizosphere soils.

347   Our work revealed that the *Pseudomonas* was the most abundant classified *nirS*-type

348   denitrifying bacteria in ectomycorrhizosphere soils, which are widely distributed in plant

349   rhizosphere soils and truffle orchard soils (Prieme et al., 2002; Sharma et al., 2005). Furthermore,

350   *Pseudomonas* were also detected in fruiting bodies of *Tuber indicum*, *Tuber pseudoexcavatum*,

351   *Tuber sinoaestivum*, *Tuber huidongense* and *T. melanosporum* (Ye et al., 2018; Roux et al., 2016).

352   Though the ascocarp of *T. melanosporum* itself may not be rich in *Pseudomonas*, it can somehow

353   acquire *Pseudomonas* and massive other nitrogen cycle-related bacteria (Antony-Babu et al.,

354   2013). And though these nitrogen cycle-related bacteria were carried in the ascocarp inoculum and

355   brought into the substrate, we still found some *nirS*-type denitrifying bacteria such as Unclassified

356   Gammaproteobacteria and Unclassified Betaproteobacteria significantly less abundant in

357   ectomycorrhizosphere soils than in rhizosphere soils. These may imply that, if without the

358   bringing-in of these bacteria from the ascocarp to the soils, their relative abundance might be

359   lower. This further revealed that the presence of truffle mycelia inhibited the colonization of AOB

360   and some *nirS*-type denitrifying bacteria. The *Pseudomonas* such as *Pseudomonas fluorescens*

361   could enhance both the mycorrhization of *T. melanosporum* and the nutrient uptake of the host

362   plant (Dominguez et al., 2012; Citterio et al., 2001; Mamoun et al., 1992). The relative abundance

363   of *Pseudomonas* detected in ectomycorrhizosphere soils were significantly greater than that in

364   rhizosphere soils (*P < 0.05*), and the previous study also indicated *Pseudomonas* had greater

365   representation inside the brûlés compared with the outside (Mello et al., 2013). Therefore, we

366   hypothesized that *T. melanosporum* may improve the growth of the host plants by enhancing the

367   colonization of *Pseudomonas* in rhizosphere soils of the host plants in the initial symbiotic stage.

368   The ectomycorrhizosphere soils of the host plant contained a large number of *Pseudomonas*

369   species whose culture condition was unclear and needs further study. We also found that the

370   abundance of *nirS*-type denitrifying bacteria was much greater than that of AOB in the rhizosphere

371   soils of *C. illinoinensis*, and there were a large number of unclassified nitrogen cycling bacteria in

372   ectomycorrhizosphere soils. The dynamic evolution of other nitrogen cycling microorganism

373   including fungal and archaea communities over the process of ectomycorrhizal formation require

374   further exploration.

375   On account of sterilized seeds and substrates used in our study, bacteria in tap water and air

376   could then randomly get into the soil, and those who get early entrance may be more competitive

377   and dominant due to their earlier starts and more rapid reproduction. This may cause the levels of

378   specific AOB and *nirS*-type denitrifying bacteria vary greatly in different samples of the same

379   treatment. In fact, we did find that the number of certain OTUs differed greatly in 4 different

380   samples in the same treatment. Therefore, the results obtained when the sample size was small (n

381   = 4) may not comprehensively reflect the true situation of all plants under the same culture

382   conditions. It is difficult to guarantee that the significant differences in bacterial communities

383   between two treatments were not accidental. We will set a larger number of samples in a treatment,

384   while by measuring the rate of nitrification and denitrification, the mycorrhizal degree of *T.*

385   *melanosporum* mycorrhizal synthesis, and the abundance of the dominant AOB and *nirS*-type

386   denitrifying bacteria (especially *Nitrosococcus* and *Pseudomonas*, whose abundances differed

387   greatly in ectomycorrhizosphere and rhizosphere), we could conduct some in-depth researches.

388   The subsequent work could also focus on analyzing the correlation of the above three, and explore

389   whether the dominant bacteria related to the nitrogen cycle at the initial stage of inoculation could

390   affect the mycorrhizal synthesis by changing the content of available nitrogen in the soil, in

391   addition, isolating and culturing these unclassified bacteria and learning their biological

392   characteristics.

393   **Conclusions**

394   In the current study, we found *nirS*-type denitrifying bacterial and AOB communities in *C.*

395   *illinoinensis* rhizosphere soils were significantly affected by *T. melanosporum* inoculation at the

396   early symbiotic stage, though the total nitrogen, nitrate-nitrogen and ammonium-nitrogen contents

397   of ectomycorrhizosphere soils were not different from those of rhizosphere soils. There was a

398   lower-abundance of AOB and greater-abundance of *nirS*-type denitrifying bacterial communities

399   in *C. illinoinensis*-*T. melanosporum* ectomycorrhizosphere soils compared with control treatment.

400   Proteobacteria was the dominant bacterial phylum, and the abundance of *Nitrosomonas* (AOB)

401   was significantly reduced because of *T. melanosporum* inoculation, while the abundance of

402   *Pseudomonas* (*nirS*-type denitrifying bacteria) increased. This work furthers our understanding of

403   rhizosphere microecology and the cultivation of *T. melanosporum-C. illinoinensis*.

References

405   Antony-Babu S, Deveau A, Van-Nostrand JD, Zhou J, Le-Tacon F, Robin C, Frey-Klett P, Uroz

406   S. 2014. Black truffle associated bacterial communities during the development and maturation

407   of *Tuber melanosporum* ascocarps and putative functional roles. *Environmental Microbiology*,

408   16(9):2831-2847. DOI: 10.1111/1462-2920.12294

409   Azziz G, Monza J, Etchebehere C, and Irisarri P. 2017. *nirS*- and *nirK*-type denitrifier communities

410   are differentially affected by soil type, rice cultivar and water management. *European Journal*

411   *of Soil Biology* 78:20-28. DOI: 10.1016/j.ejsobi.2016.11.003

412   Barbieri E, Guidi C, Bertaux J, Freyklett P, Garbaye J, Ceccaroli P, Saltarelli R, Zambonelli A,

413   Stocchi V. 2007. Occurrence and diversity of bacterial communities in *Tuber magnatum* during

414   truffle   maturation.   *Environmental   Microbiology*   9(9):2234-2246   DOI: 10.1111/j.1462-

415   2920.2007.01338.x

416   Berendsen RL, Pieterse CMJ, Bakker PAHM. 2012. The rhizosphere microbiome and plant health.

417   *Trends in Plant Science* 17(8) DOI: 10.1016/j.tplants.2012.04.001

418   Bonito G, Brenneman T, Vilgalys R. 2011. Ectomycorrhizal fungal diversity in orchards of

419   cultivated   pecan   (*Carya   illinoinensis*;   Juglandaceae).   *Mycorrhiza*   21:601-612   DOI:

420   10.1007/s00572-011-0368-0

421   Bonito G, Trappe JM, Donovan S, Vilgalys R. 2011. The Asian black truffle *Tuber indicum* can

422   form ectomycorrhizas with North American host plants and complete its life cycle in non-native

423   soils. *Fungal Ecology* 4(1):83-93 DOI: 10.1016/j.funeco.2010.08.003

424   Braker G, Zhou J, Wu L, Devol AH, Tiedje JM. 2000. Nitrite reductase genes (*nirK* and *nirS*) as

425   functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine

426   sediment communities. *Applied and Environmental Microbiology*, 66(5):2096-2104 DOI:

427   10.1128/AEM.66.5.2096-2104.2000

428   Bremer C, Braker G, Matthies D, Reuter A, Engels C, Conrad R. 2007. Impact of plant functional

429   group, plant species, and sampling time on the composition of *nirK*-type denitrifier communities

430   in soil. *Applied and Environmental Microbiology* 73(21):6876-6884 DOI:

431   10.1128/AEM.01536-07

432   Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña

433   AG, Goodrich JK, Gordon JI. 2010. QIIME allows analysis of high-through put community

434   sequencing data. *Nature* 7(5): 335-336 DOI: 10.1038/nmeth.f.303

435   Citterio B, Malatesta M, Battistelli S, Marcheggiani F, Gazzanelli G. 2001. Possible involvement

436   of *Pseudomonas fluorescens* and *Bacillaceae* in structural modification of *Tuber borchii* fruit

437   bodies. *Canadian Journal of Microbiology* 47:264-268. 10.1139/w01-005

438   Conrad R. 1997. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4,

439   OCS, N_2O, and NO). *Microbiological reviews* 60(4):609-640 DOI: 10.1006/mpat.1996.0079

440   Core R, Null RDCT, Team R, Team TSUD, Coreteam R. 2011. A Language and Environment for

441   Statistical Computating. Available at https://www.R-project.org 14:12-21

442   Deveau A, Antony-Babu S, Le Tacon F, Robin C, Frey-Klett P, Uroz S. 2016. Temporal changes

443   of bacterial communities in the *Tuber melanosporum* ectomycorrhizosphere during ascocarp

444   development. *Mycorrhiza* 26, 389-399 DOI: 10.1007/s00572-015-0679-7

445   Dominguez JA, Martin A, Anriquez A, Albanesi A. 2012. The combined effects of *Pseudomonas*

446   fluorescens and *Tuber melanosporum* on the quality of *Pinus halepensis* seedlings. *Mycorrhiza*

447   22:429-436 DOI: 10.1007/s00572-011-0420-0

448   Donnini D, Benucci GMN, Bencivenga M, Falini LB. 2014. *Quality assessment* of truffle-

449   inoculated seedlings in Italy: Proposing revised parameters for certification. *Forest Systems*

450   23(2):385-393 DOI: 10.5424/fs/2014232-05029

451   Doornbos RF, van Loon LC, Bakker PAHM. 2012. Impact of root exudates and plant defense

452   signaling on bacterial communities in the rhizosphere. A review. *Agronomy for Sustainable*

453   *Development* 32(1):227-243 DOI: 10.1007/s13593-011-0028-y

454   Edgar RC. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads.

455   *Nature Methods* DOI: 10:184. 10.1038/NMETH.2604

456   Fu Y, Li X, Li Q, Wu H, Xiong C, Geng Q, Sun H, Sun Q. 2016. Soil microbial communities of

457   three major Chinese truffles in Southwest China. *Canadian Journal of Microbiology*:2016-2139

458   DOI: 10.1139/cjm-2016-0139

459   Garcia-Barreda S, Molina-Grau S, Forcadell R, Sánchez S, Reyna S. 2017. Long-term soil

460   alteration in historical charcoal hearths affects *Tuber melanosporum* mycorrhizal development

461   and environmental conditions for fruiting. *Mycorrhiza* DOI: 10.1007/s00572-017-0773-0

462   Garcia-Barreda S, Reyna S. 2012. Below-ground ectomycorrhizal community in natural *Tuber*

463   *melanosporum* truffle grounds and dynamics after canopy opening. *Mycorrhiza* 22:361-369

464   DOI: 10.1007/s00572-011-0410-2

465   Geng LY, Wang XH, Yu FQ, Deng XJ, Tian XF, Shi XF, Xie XD, Liu PG, Shen YY. 2009.

466   Mycorrhizal synthesis of *Tuber indicum* with two indigenous hosts, *Castanea mollissima* and

467   *Pinus armandii*. *Mycorrhiza* 19(7):461-467 DOI: 10.1007/s00572-009-0247-0

468   Gleeson DB, Müller C, Banerjee S, Ma W, Siciliano SD, Murphy DV. 2010. Response of ammonia

469   oxidizing archaea and bacteria to changing water filled pore space. *Soil Biology & Biochemistry*

470   42:1888-1891 DOI: 10.1016/j.soilbio.2010.06.020

471 Green SJ, Prakash O, Gihring TM, Akob DM, Jasrotia P, Jardine PM, Watson DB, Brown SD,

472   Palumbo AV, Kostka JE. 2010. Denitrifying bacteria isolated from terrestrial subsurface

473   sediments exposed to mixed-waste contamination. *Applied & Environmental Microbiology*

474   76:3244-3254 DOI: 10.1128/AEM.03069-09

475 Gregory LG, Bond PL, Richardson DJ, Spiro S. 2003. Characterization of a nitrate-respiring

476   bacterial community using the nitrate reductase gene (*narG*) as a functional marker.

477   *Microbiology* 149:229-237 DOI: 10.1099/mic.0.25849-0

478 Healy RA, Zurier H, Bonito G, Smith ME, Pfister DH. 2016. Mycorrhizal detection of native and

479   non-native truffles in a historic arboretum and the discovery of a new North American species,

480   *Tuber arnoldianum* sp. nov. *Mycorrhiza* 26:781 DOI: 10.1007/s00572-016-0713-4

481 Hu HW, Macdonald CA, Trivedi P, Holmes B, Bodrossy L, He JZ, Singh BK. 2015. Water

482   addition regulates the metabolic activity of ammonia oxidizers responding to environmental

483   perturbations in dry subhumid ecosystems. *Environmental Microbiology* DOI: 17.

484   10.1111/1462-2920.12481

485 Hui T, Yan K, Zhang L, Chi F, Li Q, Lian S, and Wei D. 2010. Diversity analysis of nitrite

486   reductase genes (*nirS*) in black soil under different long-term fertilization conditions. *Annals of*

487   *Microbiology* 60:97-104. 10.1007/s13213-009-0009-4

488 Jung SC, Martinez-Medina A, Lopez-Raez JA, Pozo MJ. 2012. Mycorrhiza-induced resistance and

489   priming of plant defenses. *Journal of Chemical Ecology* DOI: 38:651-664. 10.1007/s10886-

490   012-0134-6

491 Kim OS, Imhoff JF, Witzel KP, Junier P. 2011. Distribution of denitrifying bacterial communities

492   in the stratified water column and sediment–water interface in two freshwater lakes and the

493   Baltic Sea. *Aquatic Ecology* 45:99-112 DOI: 10.1007/s10452-010-9335-7

494   Kowalchuk GA, Stephen JR. 2001. Ammonia-oxidizing bacteria: a model for molecular microbial

495   ecology. *Annual Review of Microbiology* DOI: 55:485. 10.1146/annurev.micro.55.1.485

496   Kües U, Martin F. 2011. On the road to understanding truffles in the underground. 48(6):560 DOI:

497   10.1016/j.fgb.2011.02.002

498   Li Q, Zhao J, Xiong C, Li X, Chen Z, Li P, Huang W. 2017. *Tuber indicum* shapes the microbial

499   communities of ectomycorhizosphere soil and ectomycorrhizae of an indigenous tree (*Pinus*

500   *armandii*). *Plos One* 12(4):e175720 DOI: 10.1371/journal.pone.0175720

501   Li Q, Yan LJ, Ye L, Zhou J, Zhang B, Peng WH, Zhang XP. 2018. Chinese black truffle (*Tuber*

502   *indicum*) alters the ectomycorrhizosphere and endoectomycosphere microbiome and metabolic

503   profiles of the host tree *Quercus aliena*. *Front. Microbiol.* 9:2202 DOI:

504   10.3389/fmicb.2018.02202

505   Li XL, Zhang XP, Yang M, Yan LJ, Liu CY. 2019. *Tuber borchii* sapes the etomycorrhizosphere

506   mcrobial cmmunities of *Corylus avellana*. *Mycobiology* 47(2):1-11 DOI:

507   10.1080/12298093.2019.1615297

508   Li Y, Chen Z, He J, Wang Q, Shen C, Ge Y. 2018. Ectomycorrhizal fungi inoculation alleviates

509   simulated acid rain effects on soil ammonia oxidizers and denitrifiers in Masson pine forest.

510   *Environmental Microbiology* DOI: 10.1111/1462-2920.14457

511   Lindahl B, Ihrmark K, Boberg J, Trumbore S, Hogberg P, Stenlid J, Finlay R. 2006. Spatial

512   separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. *New*

513   *Phytologist* 173:611-620 DOI: 10.1111/j.1469-8137.2006.01936.x

514   Lu RK. 1999. Soil and agro-chemical analytical methods. China Agricultural Science and

515   Technology Press: Beijing, China.

516   Mamoun M, Olivier JM. 1992. Effect of soil *Pseudomonads* on colonization of hazel roots by the

517   ecto-mycorrhizal species *Tuber melanosporum* and its competitors. *Plant & Soil* 139:265-273

518   DOI: 10.1007/bf00009318

519   Mao Y, Yannarell AC, Mackie RI. 2011. Changes in N-transforming archaea and bacteria in soil

520   during the establishment of bioenergy crops. *Plos One* 6 DOI: 10.1371/journal.pone.0024750

521   Marozzi G, Sánchez S, Benucci G M N, Bonito G, Falini L B, Albertini E, and Donnini D. 2017.

522   Mycorrhization of pecan (*Carya illinoinensis*) with black truffles: *Tuber melanosporum* and

523   *Tuber brumale*. *Mycorrhiza* 27:303-309 DOI: 10.1007/s00572-016-0743-y

524   Mello A, Ding GC, Piceno YM, Napoli C, Tom LM, DeSantis TZ, Andersen GL, Smalla K, Paola

525   B. 2013. Truffle brûlés have an impact on the diversity of soil bacterial communities. *Plos One*

526   8(4):e61945 DOI: 10.1371/journal.pone.0061945

527   Michotey V, Méjean V, Bonin P. 2000. Comparison of methods for quantification of cytochrome

528   cd1-denitrifying bacteria in environmental marine samples. *Applied & Environmental*

529   *Microbiology* 66:1564-1571 DOI: 10.1128/AEM.66.4.1564-1571.2000

530   Petersen DG, Blazewicz SJ, Firestone M, Herman DJ, Turetsky M, Waldrop M. 2012. Abundance

531   of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates

532   across a vegetation gradient in Alaska. *Environmental Microbiology* 14(4):993-1008 DOI:

533   10.1111/j.1462-2920.2011.02679.x

534   Ponce RA, Agreda T, Agueda B, Aldea J, Modrego MP. 2014. Soil physical properties influence

535   "black truffle" fructification in plantations. *Mycorrhiza* DOI: 24. 10.1007/s00572-014-0558-7

536   Purkhold U, Pommerening-Röser A, Juretschko S. 2000. Phylogeny of all recognized species of

537   ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications

538   for molecular diversity surveys. Applied and Environmental Microbiology 12:5368-5382 DOI:

539   10.1128/AEM.66.12.5368-5382.2000

540   Prieme A, Braker G, Tiedje JM. 2002. Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments

541   in forested upland and wetland soils. *Applied & Environmental Microbiology* 68:1893-1900

542   DOI: 10.1128/AEM.68.4.1893-1900.2002

543   Reyna S, Garcia-Barreda S. 2014. Black truffle cultivation: A global reality. *Forest Systems*

544   23:317-328 DOI: 10.5424/fs/2014232-04771

545   Roux C, Tournier E, Lies A, Sanguin H, Chevalier G, Duponnois R. 2016. Bacteria of the genus

546   *Rhodopseudomonas* (Bradyrhizobiaceae): obligate symbionts in mycelial cultures of the black

547   truffles *Tuber melanosporum* and *Tuber brumale*. *Springerplus*, 5(1):1085 DOI:

548   10.1186/s40064-016-2756-6

549   Ruan YL, Wood BW, Payne JA. 1992. Chinese hickory: an emerging nut-tree crop. *Fruit Varieties*

550   *Journal*.

551   Seitzinger S, Harrison JA, Bã Hlke JK, Bouwman AF, Lowrance R, Peterson B, Tobias C, Van

552   Drecht G. 2006. Denitrification across landscapes and waterscapes: a synthesis. *Ecological*

553   *Applications* 16:2064-2090 DOI: 10.1890/1051-0761(2006)016[2064:dalawa]2.0.co;2

554   Semeniuk G. 2008. Common leafspot of alfalfa: ascospore germination and disease development

555   in relation to moisture and temperature. *Journal of Phytopathology* DOI: 110:281-289.

556   10.1111/j.1439-0434.1984.tb00068.x

557   Shapleigh JP. 2006. The dnitrifying pokaryotes. *Prokaryotes* 2006:769-792 DOI: 10.1007/0-387-

558   30742-7_23

559   Sharma S, Aneja MK, Mayer J, Munch JC, Schloter M. 2005. Diversity of transcripts of nitrite

560   reductase genes (*nirK* and *nirS*) in rhizospheres of Grain Legumes. *Appl Environ Microbiol*

561   71:2001-2007 DOI: 10.1128/AEM.71.4.2001-2007.2005

562   Sparks D. 1992. Pecan cultivars: the orchard's foundation.

563   Sterkenburg E, Clemmensen KE, Ekblad A, Finlay RD. 2018. Contrasting effects of

564   ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. DOI:

565   10.1038/s41396-018-0181-2.

566   Tanja M, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome

567   assemblies. *Bioinformatics*:21 DOI: 10.1093/bioinformatics/btr507

568   Thamdrup B. 2012. New pathways and processes in the global nitrogen cycle. *Annual Review of*

569   *Ecology Evolution and Systematics* 43:407-428 DOI: 10.1146/annurev-ecolsys-102710-145048

570   Throbäck IN, Enwall K, Jarvis Å, Hallin S. 2004. Reassessing PCR primers targeting, and genes

571   for community surveys of denitrifying bacteria with DGGE. *Fems Microbiology Ecology*

572   49:401-417 DOI: 10.1016/j.femsec.2004.04.011

573   Trappa JM, Jumpponen MA, Cázares E. 1996. NATS truffle and truffle-like fungi. Ⅱ. *Tuber lyonii*

574   (=*T. Texense*), with a key to the spiny-spored *Tuber* species groups. *Mycotaxon*. 60: 365-372

575   DOI: 10.1109/JSSC.2003.814437

576   Van der Heijden MGA, Bardgett RD, and Van Straalen NM. 2008. The unseen majority: soil

577   microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*

578   11:296-310 DOI: 10.1111/j.1461-0248.2007.01139.x

579   Wakeling LT, Mason RL, D'Arcy BR, Caffin NA. 2001. Composition of pecan cultivars Wichita

580   and Western Schley grown in Australia. *Journal of Agricultural and Food Chemistry* 49(3):

581   1277-1281 DOI: 10.1021/jf000797d

582   Wan SP, Yu FQ, Tang L, Wang R, Wang Y, Liu PG, Wang XH, Zheng Y. 2016. Ectomycorrhizae

583   of *Tuber huidongense* and *T. liyuanum* with *Castanea mollissima* and *Pinus armandii*.

584   *Mycorrhiza* 26:1-8.

585   Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive bayesian classifier for rapid assignment

586   of rRNA sequences into the new bacterial taxonomy. *Applied & Environmental Microbiology*

587   73:5261-5267 DOI: 10.1128/aem.00062-07

588   Wang S, Wang Y, Feng X, Zhai L, Zhu G. 2011. Quantitative analyses of ammonia-oxidizing

589   archaea and bacteria in the sediments of four nitrogen-rich wetlands in China. *Applied*

590   *Microbiology and Biotechnology* 90(2), 779-787 DOI: 10.1007/s00253-011-3090-0

591   Wedén C, Chevalier G, Danell E. 2004. *Tuber aestivum* (syn. *T. uncinatum)* biotopes and their

592   history on Gotland, Sweden. *Mycological Research* 108(3):304-310 DOI:

593   10.1017/s0953756204009256

594   Wen X, Meng W, Ti J, Yao W, Fu C. 2016. Bacterial community composition in the rhizosphere

595   of maize cultivars widely grown in different decades. *Biology & Fertility of Soils* 53:1-9 DOI:

596   10.1007/s00374-016-1169-6

597   Yang M, Zou J, Liu C, Xiao Y, Zhang X, Yan L, Ye L, Tang P, Li XL. 2019. Chinese white truffles

598   shape the ectomycorrhizal microbial communities of *Corylus avellana*. Annals of Microbiology

599   69:553-565 DOI: 10.1007/s13213-019-1445-4

600   Ye L, Li Q, Fu Y, Pierre S, Tan H, Zou J, Zhang B, Li XL. 2018. Host species effects on bacterial

601   communities associated with the fruiting bodies of *Tuber* species from the Sichuan Province in

602   Southwest China. Mycological Progress DOI: 10.1007/s11557-018-1397-2

603   Yoshida M, Ishii S, Otsuka S, Senoo K. 2010. *NirK*-harboring denitrifiers are more responsive to

604   denitrification-inducing conditions in rice paddy soil than *nirS*-harboring bacteria. *Microbes &*

605   *Environments* 25:45-48 DOI: 10.1264/jsme2.ME09160

606   Yu Z, Liu J, Li Y, Jin J, Liu X, Wang G. 2018. Impact of land use, fertilization and seasonal

607   variation on the abundance and diversity of *nirS*-type denitrifying bacterial communities in a

608   Mollisol in Northeast China. *European Journal of Soil Biology* DOI: 85:4-11.

609   10.1016/j.ejsobi.2017.12.001

610   Zhang X, Ye L, Kang Z, Zou J, Zhang X, Li X. 2019. Mycorrhization of *Quercus acutissima* with

611   Chinese black truffle significantly altered the host physiology and root-associated microbiomes.

612   *Peer J* 7:e6421 DOI: 10.7717/peerj.6421

613   Zumft WG. 1997. Cell biology and molecular basis of denitrification. *Microbiology and Molecular*

614   *Biology Reviews* 61(4):533-616 DOI: 10.1016/j.ccr.2004.08.030

615

Physical and chemical properties of soil samples.

OM, organic matter; TN, total nitrogen; NH_4^+-N, ammonium-nitrogen; NO_3^--N, nitrate-nitrogen; AK, available potassium; AP, available phosphorus. FH, ectomycorrhizosphere soil (the rhizosphere soil of *Carya illinoinensis* mycorrhized with *Tuber melanosporum*); CK, the rhizosphere soil of *Carya illinoinensis* without *Tuber melanosporum* partner. Values are mean ± standard deviation (*n=4*). There is no significant difference between two treatments (*P > 0.05*).

Sample	pH	OM (g/kg)	TN (g/kg)	NH_4^+-N (mg/kg)	NO_3^--N (mg/kg)	AK (mg/kg)	AP (mg/kg)
CK	9.48±0.06	37.23±1.63	1.08±0.03	10.80±0.45	11.36±0.12	199.52±31.15	18.20±0.51
FH	9.40±0.02	34.19±1.23	0.95±0.04	12.09±0.07	11.97±0.04	244.74±10.09	18.92±1.49

Community richness and diversity indices of ammonia-oxidizing bacteria and *nirS*-type denitrifying bacteria in rhizosphere soil with or without *Tuber melanosporum* partner.

FH, ectomycorrhizosphere soil (the rhizosphere soil of *Carya illinoinensis* mycorrhized with *Tuber melanosporum*). CK, the rhizosphere soil of *Carya illinoinensis* without *Tuber melanosporum* partner. Values are mean ± standard deviation (*n=4*). "*" indicates significant difference between samples (*P < 0.05*).

Samples		Observed species	Simpson	Shannon	ACE	Chao1	Coverage
AOB	CK	56.75±15.21	0.32±0.04	1.62±0.15	58.68±2.61	57.22±6.63	0.99
	FH	46.00±10.92	0.45±0.15	1.32±0.19*	48.13±8.17*	46.55±5.09*	0.99
	T-statistic	1.148	-1.722	2.482	2.460	2.551	0.069
	P-value	0.295	0.136	0.048	0.049	0.043	0.947
***nirS*-type denitrifying bacteria**	CK	175.00±14.38	0.16±0.05	2.55±0.16	188.43±10.19	179.61±14.37	0.99
	FH	193.50±40.51	0.13±0.03	2.80±0.08*	204.27±7.73*	196.39±40.51*	0.99
	T-statistic	-0.861	0.928	-2.675	-2.584	-2.475	-0.047
	P-value	0.422	0.389	0.037	0.042	0.048	0.964

The relative abundance of the most abundant ammonia-oxidizing and *nirS*-type denitrifying bacterial phyla in rhizosphere soil with or without *Tuber melanosporum* partner

FH, ectomycorrhizosphere soil (the rhizosphere soil of *Carya illinoinensis* mycorrhized with *Tuber melanosporum*). CK, the rhizosphere soil of *Carya illinoinensis* without *Tuber melanosporum* partner. Values are mean ± standard deviation ($n=4$). "*" indicates significant difference between samples ($P < 0.05$).

	Phyla	CK	FH	T-statistic	P-value
AOB	Proteobacteria	98.59%±0.01	84.93%±0.13*	2.467	0.049
	Unclassified Bacteria	1.41%±0.01	15.07%±0.13*	-2.594	0.041
***nirS*-type denitrifying bacteria**	Proteobacteria	9.49%±0.12	8.69%±0.02	0.135	0.897
	No rank Bacteria	33.25%±0.08	51.10%±0.12	-2.154	0.074
	Unclassified Bacteria miscellaneous	0.04%±0.00	0.03%±0.00	0.308	0.769
	Unclassified Bacteria	57.22%±0.14	40.18%±0.11	1.934	0.101

The relative abundance of the most abundant ammonia-oxidizing and *nirS*-type denitrifying bacterial genera in rhizosphere soil with or without *Tuber melanosporum* partner.

FH, ectomycorrhizosphere soil (the rhizosphere soil of *Carya illinoinensis* mycorrhized with *Tuber melanosporum*). CK, the rhizosphere soil of *Carya illinoinensis* without *Tuber melanosporum* partner. Values are mean ± standard deviation ($n=4$). "*" indicates significant difference between samples ($P < 0.05$).

	Genera	CK	FH	T-statistic	P-value
AOB	*Nitrosospira*	55.38%±0.42	57.90%±0.38	-0.089	0.932
	Nitrosomonas	39.99%±0.41	25.12%±0.28	0.600	0.573
	Nitrosococcus	0.12%±0.00	0.00%±0.00*	2.509	0.046
	Unclassified Betaproteobacteria	1.93%±0.02	0.85%±0.01*	2.533	0.044
	Unclassified Bacteria	1.41%±0.01	15.07%±0.13*	-4.409	0.005
	Unclassified Nitrosomonadales	0.98%±0.02	0.97%±0.01	0.011	0.991
nirS-type denitrifying bacteria	No rank Bacteria	33.25%±0.08	51.10%±0.12	-1.940	0.102
	Unclassified Bacteria	57.22%±0.14	40.18%±0.11	1.934	0.101
	Unclassified Proteobacteria	3.44%±0.02	7.16%±0.01	-2.268	0.095
	Unclassified Gammaproteobacteria	3.6%±0.07	0.00%±0.00*	2.910	0.027
	Unclassified Betaproteobacteria	1.98%±0.03	0.51%±0.00*	5.040	0.015
	Rhodanobacter	0.33%±0.01	0.35%±0.00	-0.061	0.953
	Pseudomonas	0.03%±0.00	0.46%±0.00*	-2.541	0.017
	Magnetospirillum	0.01%±0.00	0.01%±0.00	-0.049	0.963
	Rubrivivax	0.01%±0.00	0.04%±0.00	-0.981	0.396

Figure 1

Morphological features of *Carya illinoinensis* root in association with or without *Tuber melanosporum*.

(a) Ectomycorrhizae of *Tuber melanosporum*, *Scale bar* =500 μm; b Root of *Carya illinoinensis* without *Tuber melanosporum*, *Scale bar* =700 μm; (c) Mantle with cystidia , *Scale bar* =65 μm; (d) Mantle cell of ectomycorrhizae, *Scale bar* =10 μm.

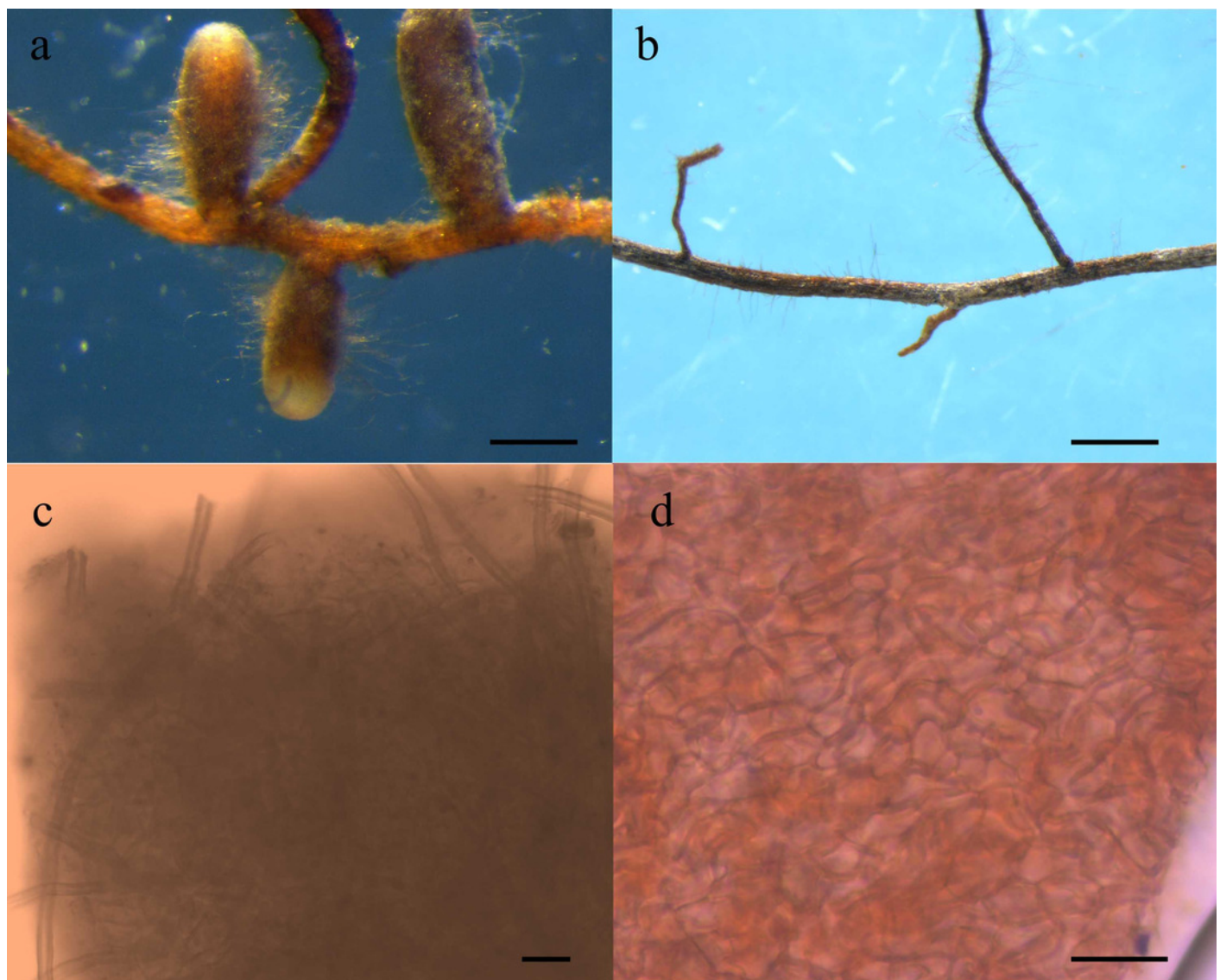



Figure 2

Rarefaction curves for ammonia-oxidizing bacterial (a) and *nirS*-type denitrifying bacterial (b) OTU diversity in different samples.

FH, ectomycorrhizosphere soil (the rhizosphere soil of *Carya illinoinensis* mycorrhized with *Tuber melanosporum*). CK, the rhizosphere soil of *Carya illinoinensis* without *Tuber melanosporum* partner.

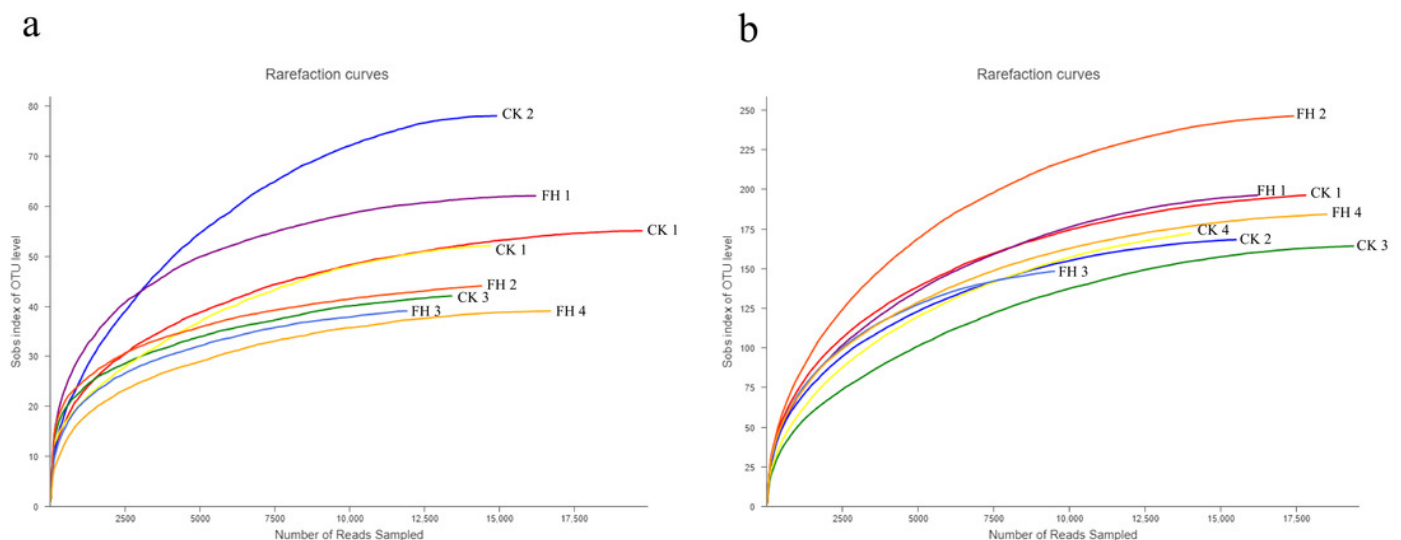



Figure 3

Numbers of shared and unique ammonia-oxidizing bacterial (a) and *nirS*-type denitrifying bacterial (b) Operational Taxonomic Units (OTUs).

FH, ectomycorrhizosphere soil (the rhizosphere soil of *Carya illinoinensis* mycorrhized with *Tuber melanosporum*). CK, the rhizosphere soil of *Carya illinoinensis* without *Tuber melanosporum* partner.

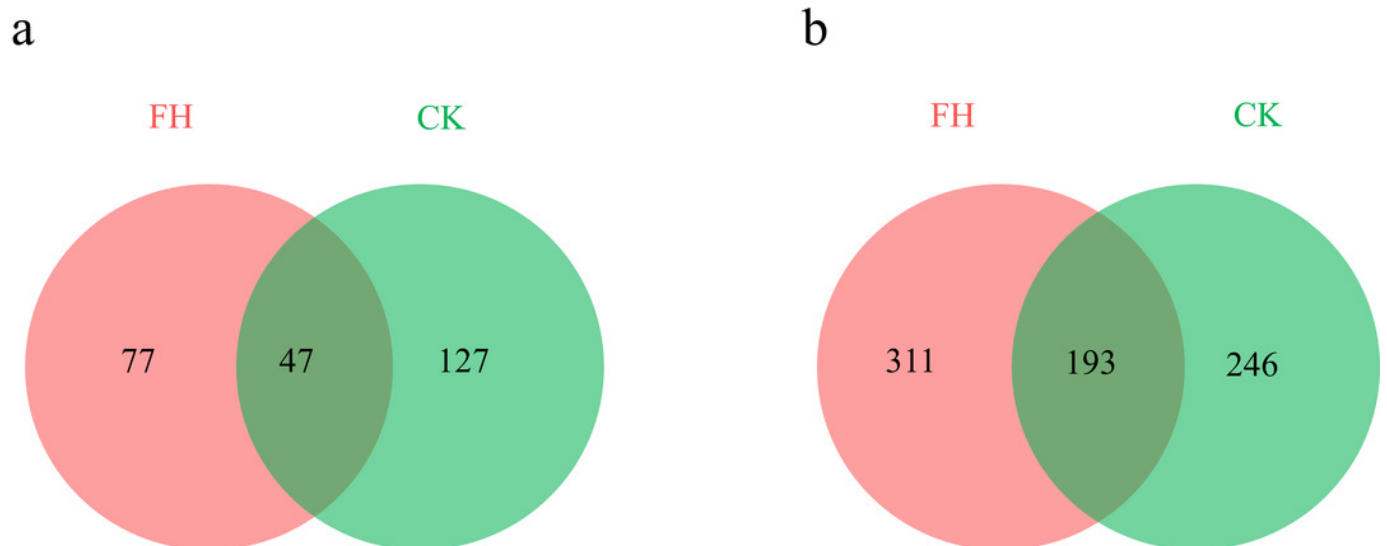



Figure 4

Taxonomic composition of ammonia-oxidizing bacterial (a) and *nirS*-type denitrifying bacterial (b) communities at the phylum levels.

FH, ectomycorrhizosphere soil (the rhizosphere soil of *Carya illinoinensis* mycorrhized with *Tuber melanosporum*). CK, the rhizosphere soil of *Carya illinoinensis* without *Tuber melanosporum* partner.

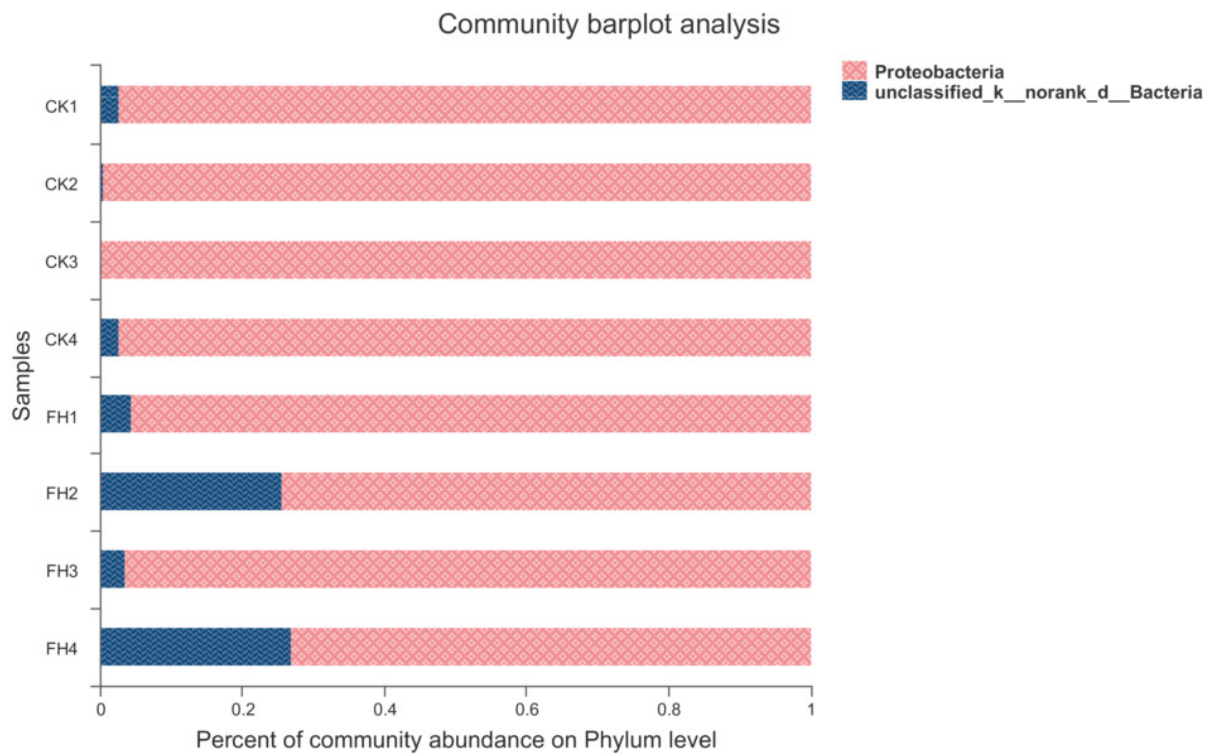
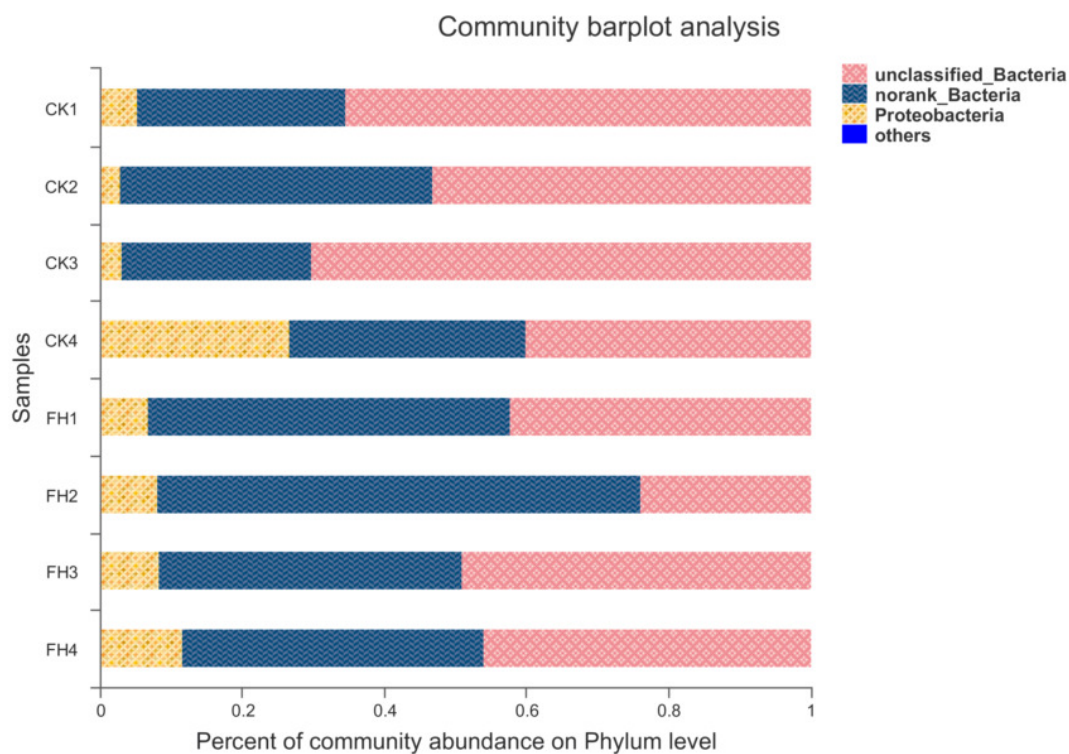
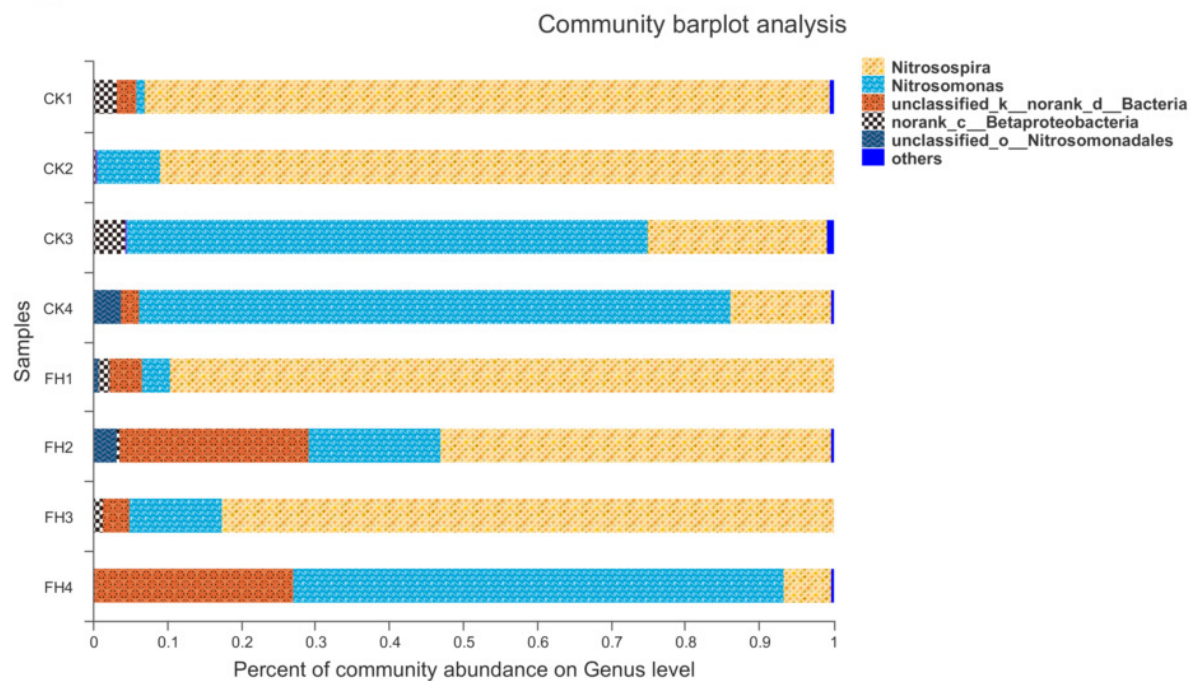



Figure 5

Taxonomic composition of ammonia-oxidizing bacterial (a) and *nirS*-type denitrifying bacterial (b) communities at the genus levels.

FH, ectomycorrhizosphere soil (the rhizosphere soil of *Carya illinoinensis* mycorrhized with *Tuber melanosporum*). CK, the rhizosphere soil of *Carya illinoinensis* without *Tuber melanosporum* partner.

a

b

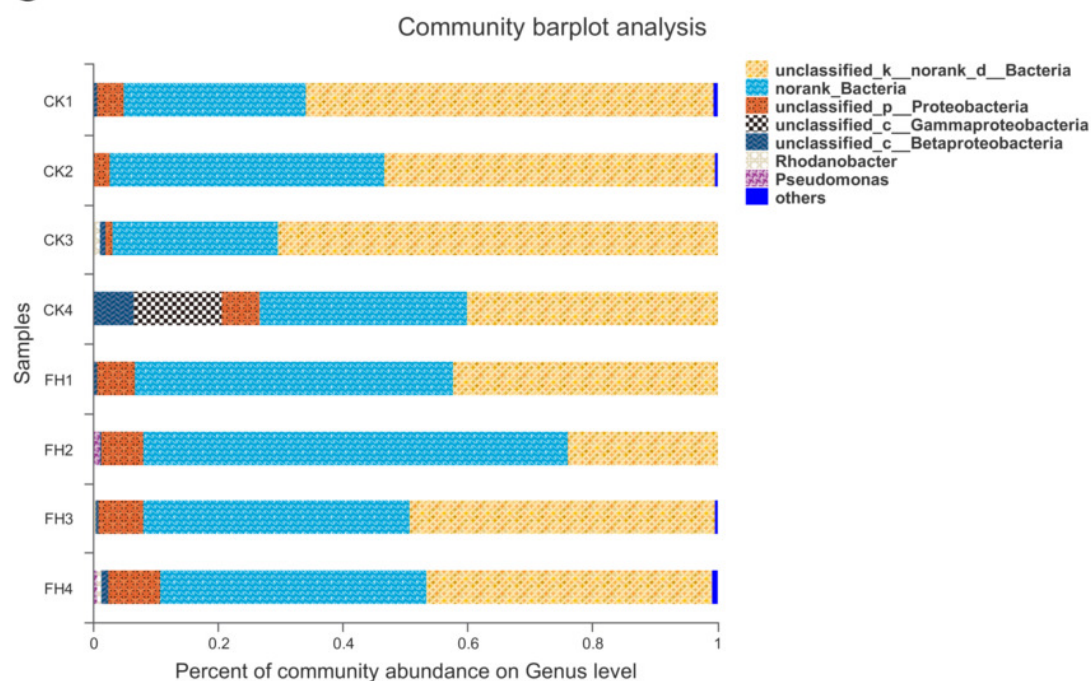



Figure 6

Principal component analysis (PCA) of ammonia-oxidizing bacterial (a) and *nirS*-type denitrifying bacterial (b) communities.

FH, ectomycorrhizosphere soil (the rhizosphere soil of *Carya illinoinensis* mycorrhized with *Tuber melanosporum*). CK, the rhizosphere soil of *Carya illinoinensis* without *Tuber melanosporum* partner.

a

b