Deterministic processes dominate soil microbial community assembly in subalpine coniferous forests on the Loess Plateau

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Microbial community assembly is influenced by a continuum (actually the trade-off) between deterministic and stochastic processes. An understanding of this ecological continuum is of great significance for drawing inferences about the effects of community assembly processes on microbial community structure and function. Here, we investigated the driving forces of soil microbial community assembly in three different environmental contexts located on subalpine coniferous forests of the Loess Plateau in Shanxi, China. The variation in null deviations and phylogenetic analysis showed that a continuum existed between deterministic and stochastic processes in shaping the microbial community structure, but deterministic processes prevailed. By integrating the results of redundancy analysis (RDA), multiple regression tree (MRT) analysis and correlation analysis, we found that soil organic carbon (SOC) was the main driver of the community structure and diversity patterns. In addition, we also found that SOC had a great influence on the community assembly processes. In conclusion, our results show that deterministic processes always dominated assembly processes in shaping bacterial community structure along the three habitat contexts.

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2 **forests on the Loess Plateau**

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22   **Abstract** Microbial community assembly is influenced by a continuum (actually the trade-off) between

23   deterministic and stochastic processes. An understanding of this ecological continuum is of great significance

24   for drawing inferences about the effects of community assembly processes on microbial community structure

25   and function. Here, we investigated the driving forces of soil microbial community assembly in three different

26   environmental contexts located on subalpine coniferous forests of the Loess Plateau in Shanxi, China. The

27   variation in null deviations and phylogenetic analysis showed that a continuum existed between deterministic

28   and stochastic processes in shaping the microbial community structure, but deterministic processes prevailed.

29   By integrating the results of redundancy analysis (RDA), multiple regression tree (MRT) analysis and correlation

30   analysis, we found that soil organic carbon (SOC) was the main driver of the community structure and diversity

31   patterns. In addition, we also found that SOC had a great influence on the community assembly processes. In

32   conclusion, our results show that deterministic processes always dominated assembly processes in shaping

33   bacterial community structure in the three habitat contexts.

34   **Introduction**

35   Understanding the fundamental ecological mechanisms that drive the assembly processes of microbial

36   communities is a major challenge in community ecology (Shen et al. 2013), particularly microbial ecology.

37   The assembly processes of the microbial community in a local community are generally influenced by two types

38   of ecological processes, including deterministic and stochastic processes. First, deterministic factors, such as

39   organism traits, interspecies relationships (e.g., competition, predation, mutualisms, and trade-offs), and

40   environmental factors (e.g., pH, temperature, salt, and moisture) govern the community structure (Chase &

41   Myers 2011; Dumbrell et al. 2010; Ofiţeru et al. 2010). Ecologists have traditionally appreciated that the

42   environmental context determines the assembly processes of microbial communities: "Everything is everywhere,

43   but the environment selects" (Baas-Becking 1934). For example, environmental factors such as pH (Tripathi et

44   al. 2018), temperature (Anderson & Laurel 2013), or nitrogen levels (Xiong et al. 2016) may be major

45   determinants of microbial community structure.

46   For the other type of community assembly processes (i.e., stochastic processes), it is assumed that

47   community structures are independent of organism traits and are governed by birth, death, colonization,

48   extinction, drift, and speciation (Hubbell & BordadeAgua 2004). And it is hypothesized that species are all

49   ecologically equivalent (Woodcock et al. 2007). Previous studies have confirmed that both deterministic and

50   stochastic processes act concurrently to regulate the assembly of ecological communities (Diniandreote et al.

51   2016; Diniandreote et al. 2015; Zhou & Ning 2017), but the relative importance may vary in different

52   environmental contexts (Tian et al. 2017). This may be because the variation in ecological selection strength

53   and the rates of dispersal on different habitat contexts can influence the relative importance of deterministic and

54   stochastic processes across temporal and spatial scales, in addition to within entire ecosystems (Chisholm &

55   Pacala 2011, Jurburg et al. 2017). Therefore, investigation into community driving forces in different habitats

56   can enrich the understanding on the community assembly process.

57   In this study, soil was sampled from 23 soil plots in subalpine coniferous forests located on the Loess

58   Plateau in Shanxi province, China. The 16S ribosomal RNA genes of bacteria were analyzed using high-

59   throughput sequencing. To investigate the driving forces of soil microbial community assembly, we sampled

60   three sites having different environmental characteristics. Sampling was performed along three different

61   altitudinal gradients. This study can largely enrich the understanding on microbiology of subalpine mountains.

62   Our aims were as follows: (i) to quantify the relative roles of deterministic and stochastic processes in bacterial

63   community dynamics for three different habitat contexts; and more precisely (ii) to evaluate the effects of

64   environmental factors on microbial community assembly.

65   **Materials and methods**

66   **2.1 Site and sampling**

67   A total of 23 soil plots were sampled (Table S1, Figure S1) in August 2016 and August 2017. The sites

68   were selected because their vegetation was subalpine mountain coniferous forests and they were located between

69   1900 m and 3055 m above mean sea level (amsl). The study area has a warm temperate continental monsoon

70   climate, and mostly cinnamon soil.

71   This study focused on response patterns along environmental gradients rather than exploring differences

72   among treatment groups. Thus, we sampled along three altitudinal gradients without replicates. Previous studies

73   have shown that for continuous environmental drivers, gradient designs further allow for better extrapolation,

74   characterization of (nonlinear) response functions, and, consequently, quantitative outputs better suited for

75   ecological models than replicated designs (Cottingham et al. 2005).

76   To avoid the interference of vegetation factors, we sampled plots in the single vegetation type (i.e., *Larix*

77   *principis-rupprechtii* forests). These sites located on subalpine ecological environments possess pronounced

78   climatic gradients and climosequences within short distances, with a high level of environmental heterogeneity

79   (Siles & Margesin 2017). Therefore, the sites with different altitudinal gradients corresponded to different

80   environmental contexts and different environment characteristics.

81   Eight plots were sampled from the Wutai Mountain site (WT), which ranges between 1,900 m and 3,055 m

82   amsl. Ten plots were sampled from the Pangquangou Natural Reserve site (PQG), ranging from 1,950 m and

83   2,650 m amsl. The last, five plots were sampled from the Luya Mountain site (LY), which ranges between 2,000

84   m and 2,400 m amsl. The details of each sample plots were added in the supplemental files (Table S1). At each

85 sampling site, a 1 m × 1 m sampling plot was established *in situ* along the elevation gradient. Five soil cores at

86 a depth of 15 cm were taken at each sampling plot, and then combined to form a single independent soil sample.

87 Then, the soil samples were sealed in plastic bags and refrigerated, immediately transported to the laboratory

88 and sieved using a 2 mm mesh. The soil samples were then stored at -80 °C until further analysis.

89 The soil samples were subsampled for molecular analysis and the DNA from of 1 g of soil was extracted

90 using an E.Z.N.A.@ Soil DNA Kit (OMEGA, USA). The quality and quantity of the DNA extracts were

91 measured using an Infinite 200 PRO plate reader (TECAN, Switzerland). The DNA purity was assessed based

92 on the A260/A280 absorbance ratios, and only DNA extracts with absorbance ratios of 1.8~2.0 were used for

93 further analyses. Three DNA samples were extracted, from each soil sample, which were then combined and

94 sequenced at Shanghai Personal Biotechnology Co., Ltd. on an Illumina MiSeq sequencing platform based on

95 the bacterial v3–v4 hypervariable region using bacterial 16S universal primers (341F 5′-

96 ACTCCTACGAGGAGCA-3′ and 805R 5′-TTACCGCGGCTGCTGGCAC -3′) (Tripathi et al. 2018).

97 **2.2 Bioinformatics analysis**

98 The sequencing data were analyzed using QIIME pipeline (v1.8.0, http://qiime.org/) (Caporaso JG 2010).

99 The filtered sequence alignments were denoised by DeNoiser (Reeder et al. 2010) and then screened for

100 chimeras using UCHIME (Edgar et al. 2011). The Archaea and unknown sequences were removed. The

101 sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity level using the average

102 neighbor method and taxonomy was blast to SILVA database by k-mer searching using MOTHUR (Pruesse et

103 al. 2007). The OTU table was rarefied to 4020 sequences per sample. Ten independent maximum-likelihood

104 phylogenetic trees based on Jukes–Cantor distance were then constructed using FastTree2 (Price et al. 2009)

105 after the removal of gaps and hypervariable regions using a Lane mask supplied by QIIME to support

106 phylogenetic diversity calculations.

2.3 Environmental variables

108 In the laboratory, soil total carbon (TC), total nitrogen (TN), and total sulfur (TS) were measured using an

109 elemental analyzer (Vario EL/ MACRO cube, Elementar, Hanau, Germany); nitrate nitrogen ($NO_3^-\_N$),

110 ammonium nitrogen ($NH_4^+\_N$), and nitrite nitrogen ($NO_2^-\_N$) were measured by an Automated Discrete

111 Analysis Instrument (CleverChem 380, Germany). After shaking the soil: water suspension (1:2.5 mass/volume)

112 for 30 mins, the soil pH was measured using a pH meter (Hl 3221, Italy). The soil organic carbon in each soil

113 sample was measured using the potassium dichromate volumetric method (Nelson et al. 1982).

2.4 Null model analysis

115 A null model was constructed to account for changes in β-diversity while controlling for stochastic variation

116 and associated changes in α-diversity (i.e., local species richness; 999 iterations) (Chase et al. 2011). We

117 considered the null deviation as the relative difference between the observed *β*-diversity and the null-model *β*-

118 diversity (Tucker et al. 2016). As such, null deviation values may represent communities that are more similar

119 than expected by chance (a negative null deviation value), less similar than expected by chance (a positive

120 null deviation value), or close to the chance expectation (values near zero) (Tucker et al. 2016).

2.5 Phylogenetic analysis

122 Our study used phylogenetic turnover between communities to infer ecological processes (Stegen et

123 al. 2015). To quantify phylogenetic turnover between communities, we used the between community mean-

124 nearest-taxon-distance (βMNTD) metric. βMNTD was calculated using the R function 'comdistnt'

125 (abundance.weighted = TRUE; package "picante"). Then, we evaluated β-Nearest Taxon Index (βNTI),

126 which expresses the difference between observed βMNTD and the mean of the null distribution in units of

127   standard deviations (Stegen et al. 2013).

128   In addition, to distinguish more details in the assembly processes, we used the Raup–Crick metric

129   (Chase et al. 2011), extended to incorporate species' relative abundances; referred to as RC_{bray}. The R script

130   of RC_{bray} can be found at https://github.com/stegen/Stegen_etal_ISME_2013.

131   In a given community, we estimated the relative influence of variable selection or homogeneous selection

132   as the fraction of their comparisons with $\beta NTI > +2$ or $\beta NTI < -2$, respectively. We regard the fraction of the

133   between community comparisons with $|\beta NTI| < 2$ and $RC_{bray} > +0.95$ as dispersal limitation, while $|\beta NTI| < 2$

134   and $RC_{bray} < -0.95$ is considered homogenizing dispersal (Diniandreote et al. 2015; Stegen et al. 2013; Stegen et

135   al. 2015).

136   **2.6 Network analysis**

137   The co-occurrence network was constructed based on the Spearman correlation matrix offered in the

138   'psych' package in R. In this network, the nodes represent OTUs and the edges that connect these nodes

139   represent correlations between OTUs. Only those connections with correlation coefficients > 0.6 and $P <$

140   0.05 were used in the network. Thus, positive correlations indicate co-occurring OTUs based on

141   abundances, whereas negative correlations indicate that the OTUs are mutually exclusive (Barberán et al.

142   2012). P-values were false discovery rate (FDR) adjusted to control for the analysis (FDR < 0.05). The

143   network analysis was completed using the 'igraph' package in R.

144   **2.7 Statistical analysis**

145   All statistical analyses were performed in the R environment using the 'vegan', 'ggplot2', 'ggpubr', and

146   'corrplot' packages. A Venn diagram was used to visualize the shared OTUs among the sites. A correlation

147   matrix graph was used to demonstrate the correlation between soil physicochemical factors and was

148   constructed using the 'corrplot' packages in R. Multivariate regression tree analysis (MRT) was used to

149   explain the relationship between bacterial α-diversity estimates and environmental variables in a visualized

150   tree, and diversity indices were normalized to the same mean before performing MRT analysis (Ge et al.

151   2008). Based on the longest gradient lengths from the results of detrended correspondence analysis (DCA),

152   we selected redundancy analysis (RDA) to quantify the effects of environmental variables on microbial

153   community composition (Mo et al. 2018). Forward selection of PCNM variables based on permutation tests

154   was chosen to identify 2 of the 23 extracted PCNM variables that significantly ($P < 0.05$) explained the

155   spatial structure. The PCNM eigenfunctions, which represent the 'spectral decomposition of the spatial

156   relationship across sampling locations', can be considered as the spatial variables in the ordination-based

157   analysis. The contributions of environmental filtering and the space variable (PCNM) to the variation in

158   bacterial community composition were calculated by using variance partitioning analysis (VPA)

159   (CANOCO for Windows Version 5.0). The mantel test was performed in the R environment using the 'vegan'

160   packages.

161   **Results**

162   **Physicochemical properties of the soils from the different sites**

163   The soil physicochemical properties varied across the different sampling sites (Figure 1). Briefly, the

164   contents of ammonium nitrogen and nitrite nitrogen were the highest at LY sites (36.91 and 0.16 mg·kg-1,

165   respectively), and were lowest at WT sites (17.41 and 0.04 mg·kg^{-1}, respectively). The contents of nitrate

166   nitrogen (6.45 mg·kg^{-1}), SOC (70.29 mg·g^{-1}), TC (6.4%), and TN (0.51%) were the highest at WT sites, and

167   were the lowest at LY sites.

168   TN was significantly positively correlated with TC and SOC ($P < 0.05$) and significantly negatively

169   correlated with pH value ($P < 0.05$; Figure 2). TC and pH showed a significant negative correlation ($P < 0.05$).

170   SOC was significantly positively correlated with nitrate nitrogen ($P < 0.0.5$) and significantly negatively

171   correlated with nitrite nitrogen ($P < 0.05$). This indicated that the sites sampled had different environment

172   characteristics.

173   **Dynamics of bacterial community composition and diversity**

174   A total of 4258 OTUs were identified from 1,062,241 high-quality sequences recovered from 23 soil

175   samples. Good's coverage ranged from 95.19% to 99.75%, indicating that the identified sequences were

176   representative of most of the bacterial sequences in the collected soil samples. Rarefaction curve analyses,

177   which generally yielded asymptotic curves, indicated that the numbers of sampling plots were enough.

178   Detailed information of the sequencing results is provided in Table S2.

179   The soil microbial community composition varied across the different sampling sites (Figure 3). There were

180   15 bacterial phyla with relative abundances of more than 0.01% (Figure 3a). As shown in the Venn diagram,

181   869 bacterial shared OTUs were observed in all sampling sites. There were 46 bacterial phyla identified (Figure

182   3b). The abundance of *Proteobacteria* at all sites was the highest (mean relative abundance = 30.59%), and

183   followed by *Acidobacteria* (19.63%), *Actinobacteria* (16.51%) and *Chloroflexi* (13.22%). Briefly, the mean

184   relative abundance of *Proteobacteria* was the most at PQG (34.39%), and that of *Actinobacteria* was the highest

185   at LY (26.29%). The mean relative abundances of *Acidobacteria* (28.68%) *and Chloroflexi* (16.09%) were the

186   highest at WT. There were 31 bacterial families with relative abundances of more than 0.01% (Figure 3c). Based

187   on the clustering graph, the sampling plots of each of the sites roughly clustered together (Figure 3d). The

188   community α-diversity indices varied at the different sites (Figure 4). Briefly, the phylogenetic diversity (pd)

189   and the number of observed species (sobs) were the highest at WT sites ($P < 0.05$). There was no significant

190   difference in the ACE index, Chao index, Shannon index and Simpson index at the different sites (P > 0.05).

191   This indicated that the sites sampled had different soil microbial community structure.

192   **Effects of environmental factors on microbiome dynamics**

193   Based on the results of the DCA (axis length =1.02), we used RDA to identify the abiotic environmental

194   drivers that influenced bacterial community composition (Figure 5; permutation test, *P* < 0.01). The results

195   demonstrated that *Proteobacteria*, *Bacteroidetes,* and *Cyanobacteria* were mainly driven by pH, while SOC,

196   TC, and TN were the main abiotic drivers of *Parcubacteria* and *Planctomycetes*.

197   In the MRT analysis (Figure 6), we observed that the diversity indices (normalized) were mainly split by

198   SOC, explaining 36.75% in the first spilt. The correlation analysis showed similar results: SOC was significantly

199   correlated with bacterial communities at the phylum level (e.g., *Proteobacteria*, *Bacteroidetes*, and *Chloroflexi*).

200   Given its contribution to explaining community distribution patterns, SOC was further used as a descriptor for

201   the environmental gradients.

202   The variation partitioning analysis showed that environmental variables (20.3%) explained more variation

203   of microbial community structure than spatial variables (1.9%). This suggested that both deterministic and

204   stochastic processes were involved in the assembly of microbial communities, and that deterministic processes

205   were dominant. The unexplained variable was 78.6% (Figure 7).

206   **Nonrandom co-occurrence patterns of the microbial community**

207   Network analysis was applied to explore the interspecific relationship patterns in the microbial communities

208   (Figure 8). Compared with the LY- and WT- network, the PQG-network exhibited more edges (87), more

209   vertices (40), more modularity (0.691), higher average degree (4.35) and average clustering coefficients (0.858),

210   but less the numbers of modules (6) (Table S3). Strong positive correlations were observed at all sites, while

211   negative correlations were rare. The size of the nodes corresponds to betweenness centralization values.

212   **The bacterial community assembly processes**

213   According to the null model analysis, our results demonstrated that the null deviation values varied at

214   different sites (ranging from 0.29 to 0.57; Figure 9a). The bacterial communities at WT deviated significantly

215   from the null expected value (relative null deviation = 0.45) and were greater than that at LY site and PQG site

216   (relative null deviation = 0.32 and 0.34, respectively) ($P<0.05$).

217   Most importantly, we observed that the microbial community was more greatly shaped by variable

218   selection (βNTI > +2) (Figure 9b). From LY to WT, we observed a gradual increase in the relative role of

219   deterministic processes compared to stochastic processes (Figure 9c). Based on the regression analysis of the

220   environmental variables with assembly process parameters, we found that SOC had a great influence on

221   community assembly processes (Figure 9d). The mantel test between βNTI and SOC matrices indicated the

222   similar conclusion ($P < 0.05$, R = 0.509).

223   **Discussion**

224   Compared to LY, the microbial community at WT was more greatly driven by deterministic processes. The

225   driving effects of the deterministic processes gradually increased from LY to WT. Given this, we inferred that a

226   continuum existed between deterministic and stochastic processes in the assembly of microbial communities in

227   the study area. This is consistent with previous studies (Chase et al. 2011; Tucker et al. 2016; Jurburg et al. 2017;

228   Tian et al. 2017), which pointed out the relative importance of the two processes varied in the different

229   environmental contexts. For example, in terms of plants, aggregation in temperate forests reflect stronger

230   environmental correlations, suggesting a key role for species-sorting processes (deterministic processes) (Myers

231   et al. 2013). In terms of microorganisms, previous studies have noted that bacterial community assembly is

232   largely governed by stochastic processes in early successional soils, with the relative roles of deterministic

233   processes increasing progressively in later successional soils (Diniandreote et al. 2015; Ferrenberg et al. 2013;

234   Hanson et al. 2012).

235   Previous research has confirmed this continuum could be dependent on varying environmental

236   conditions and the characteristics of organisms (Zhou et al. 2013). Environmental factors, such as salinity

237   (Lozupone & Knight 2007), pH (Fierer & Jackson 2006; Griffiths et al. 2011), C/N ratio (Bates et al. 2011), soil

238   C (Drenovsky et al. 2004), nitrogen levels (Xiong et al. 2014), and the structure of the plant community

239   (Lundberg et al. 2012) may be major determinants of microbial community structure. Our results demonstrated

240   that pH, SOC, TC, and TN were the main abiotic drivers of microbial community compositions. More

241   importantly, based on the integrated results of the MRT analysis, RDA, and correlation analysis, we identified

242   SOC as a general descriptor that encompassed the environmental gradients by which the communities responded

243   to.

244   Our results demonstrated that SOC differed significantly at different sites, and was significantly correlated

245   with nitrate nitrogen, nitrite nitrogen and TN ($P < 0.05$). This indicated that SOC was closely related to soil

246   fertility and possessed the highest weighting. Litters from the trees will impact SOC, which in turn will impact

247   the community assembly structure, and this is perhaps the reason explaining why variable selection increases

248   from LY to WT sites. The relationships between SOC and bacterial community assembly have also been reported

249   across a broad range of microbial ecosystems (Bastida et al. 2013). Most importantly, we also observed that SOC

250   was closely associated with the community assembly process. Similar results reported that the relative roles of

251   stochastic and deterministic processes can vary with the successional age of soils and can primarily be attributed

252   to the covariance of soil pH with age (Tripathi et al. 2018). The unexplained variation in VPA (78.6%) could be

253   due to stochastic influences [e.g. drift or speciation (Caruso et al. 2011)], unmeasured soil physicochemical

254   properties [e.g. metal ion concentration (Gombeer et al. 2015)] or interactions between species [e.g. competition

255   (Caruso et al. 2011)]. In fact, in other studies of microbial communities using VPA, the unexplained portions

256   may also account for more than 50 % (Liao et al. 2016; Mo et al. 2018).

257   In deterministic processes, not only environmental filtering, but also interspecies interactions have a great

258   influence on community assembly. Ecologists recently accepted that competition and environmental

259   processes act simultaneously (Zhang et al. 2018). In the network analysis, the higher modularity indicates that

260   the network became denser, suggesting that the microbial communities are highly complex (Olesen et al. 2007).

261   Interestingly, the modularity was the highest at PQG (0.691). This may be related to the greater sampling scales

262   and elevation gradients, and thus greater environmental heterogeneity at PQG. The average path distance

263   represents the shortest path between two nodes (Wang et al. 2016), which demonstrated irregular variation at

264   WT (Zheng et al. 2017). Strong positive correlations were observed among sites, while negative correlations

265   were rare (Figure 8a–c). This implied that microbes might cooperate in order to adapt to similar niches. In the

266   network, positive links could be attributed to niche overlap and cross-feeding, while negative relationships could

267   be attributed to competition and amensalism (Faust & Raes 2012). From an ecological perspective, the

268   peripherals may represent specialists, whereas module hubs and connectors may be more generalists and network

269   hubs may be super-generalists (Figure 8d–f) (Deng et al. 2012). It is interesting to observe that the module hubs

270   and connectors differed at the different sites.

271   **Conclusion**

272   We quantified the importance of the deterministic and stochastic processes driving the bacterial

273   community assembly on different sites in subalpine coniferous forests, and showed that deterministic processes

274   prevailed. Moreover, SOC was closely related to microbial community structure and greatly influenced the

275   processes of community assembly.

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403

Bar plots indicating the soil physicochemical factors at different sites.

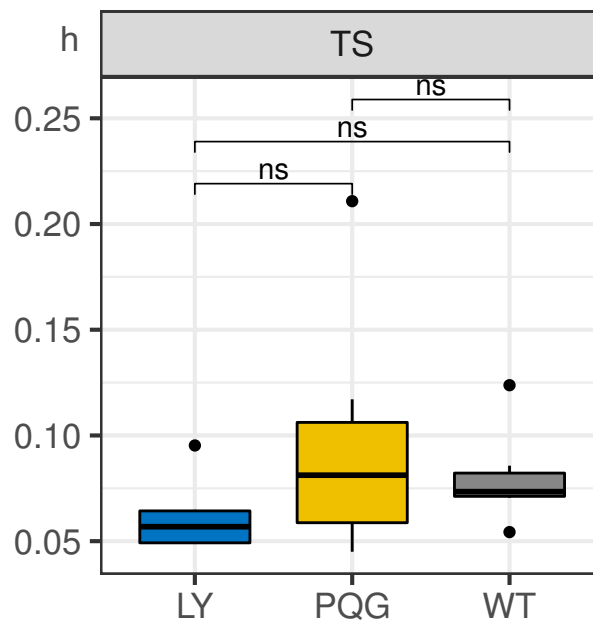
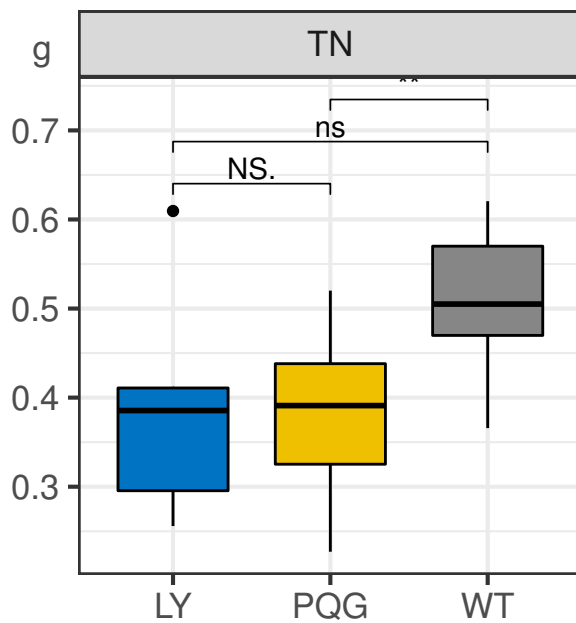
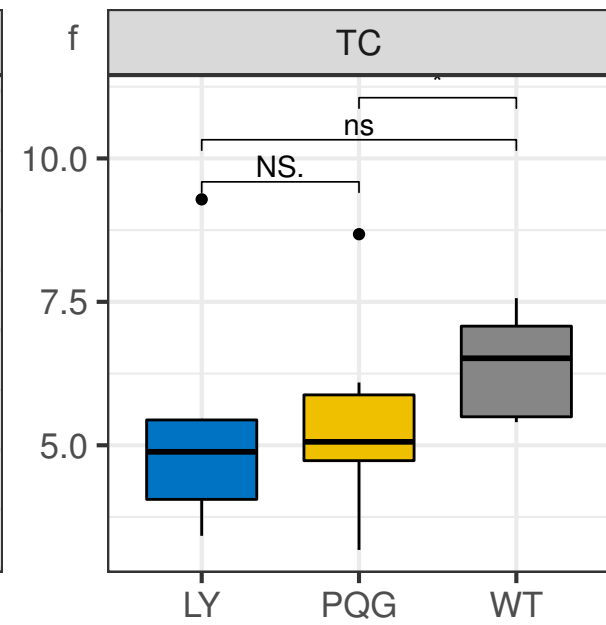
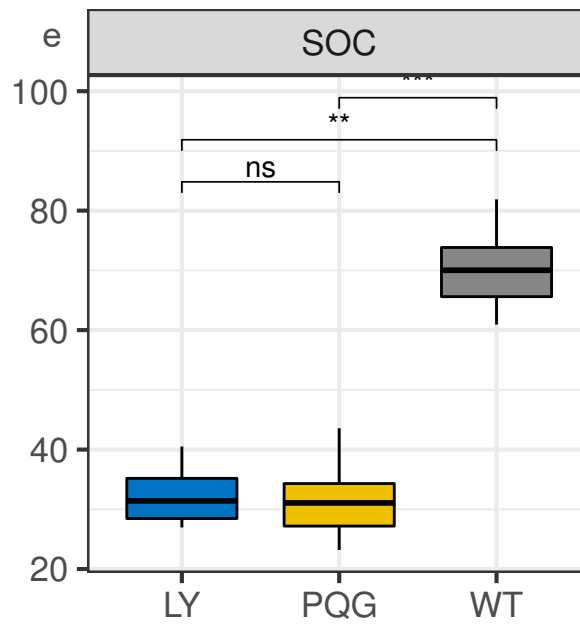
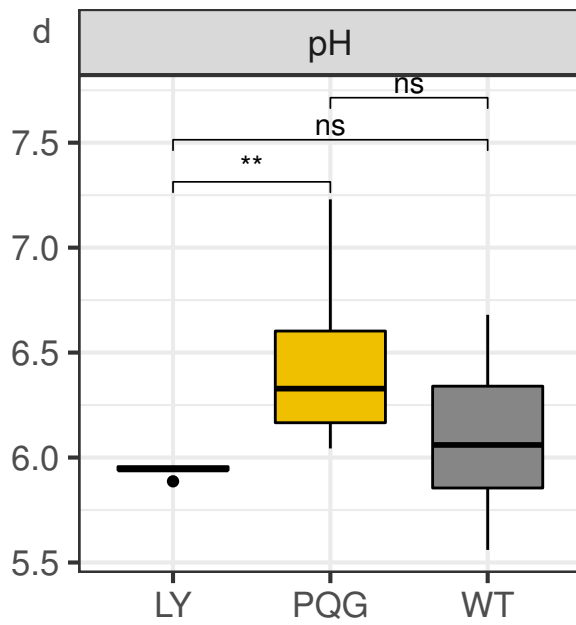
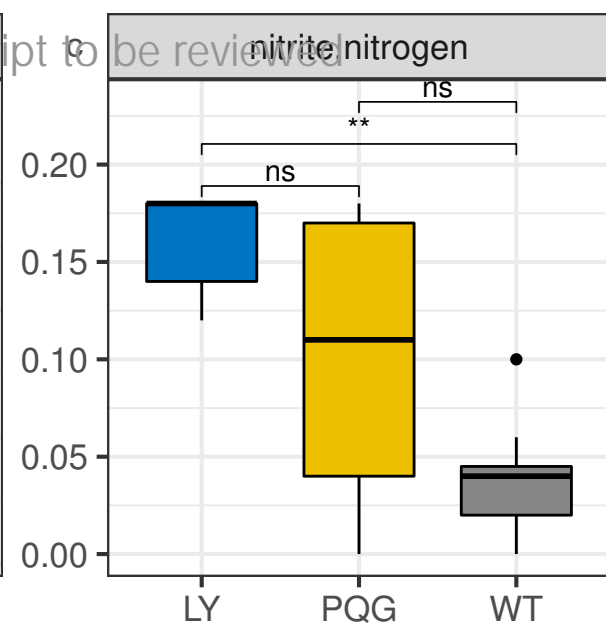
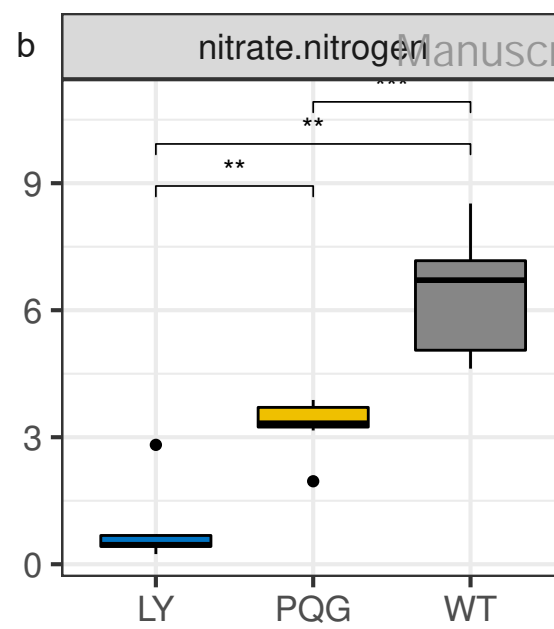
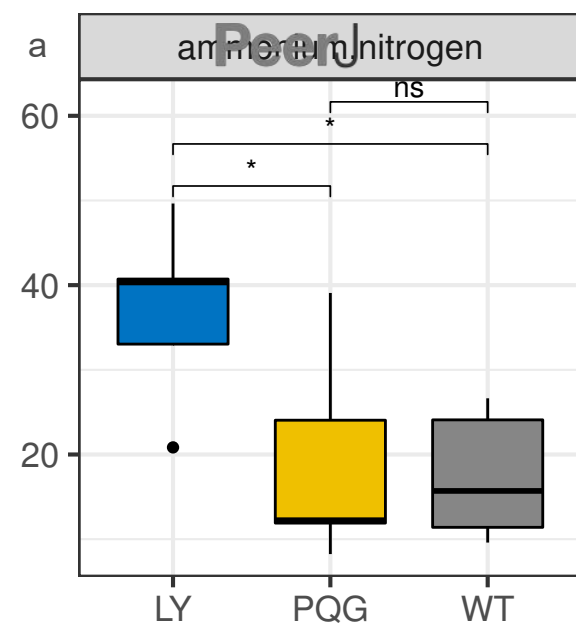



Figure 2

Correlation matrix graph indicating the correlation between soil physicochemical factors. Only the environmental factors with significantly difference represented in the figure.

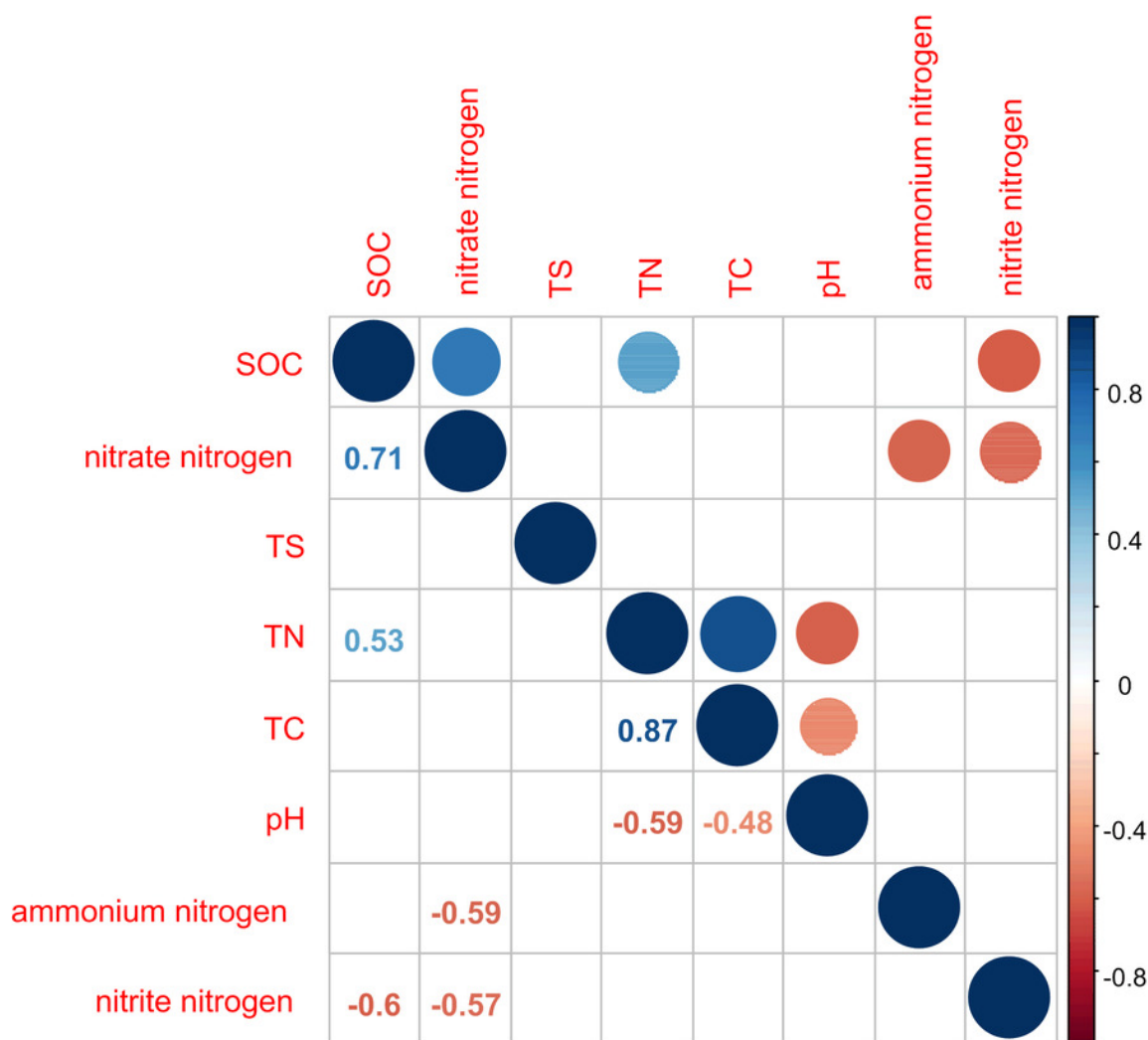
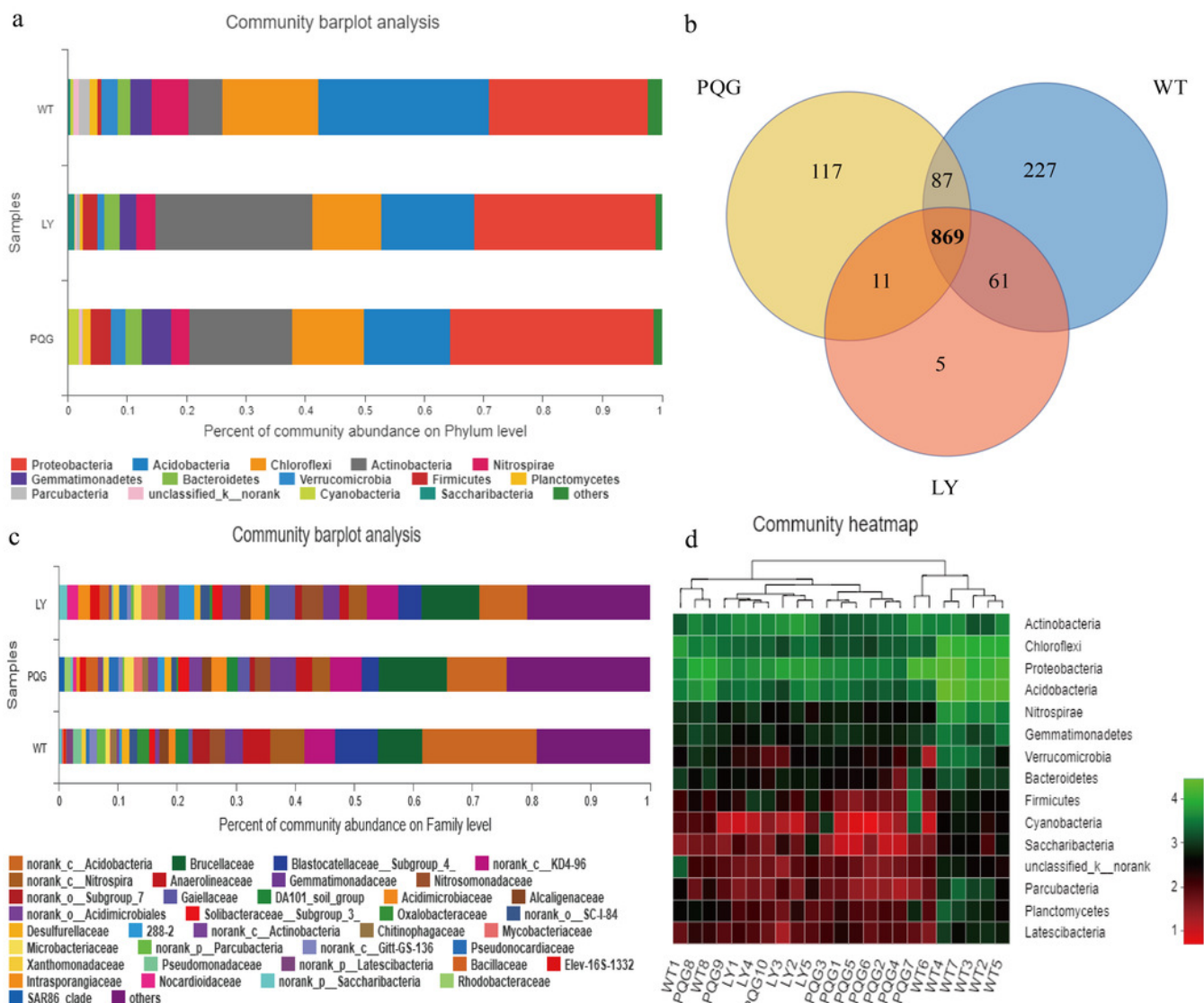
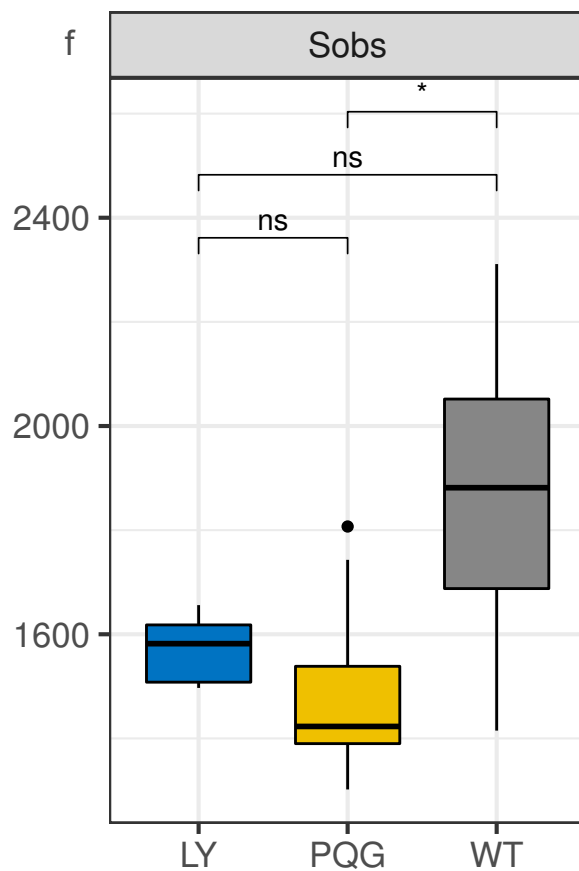
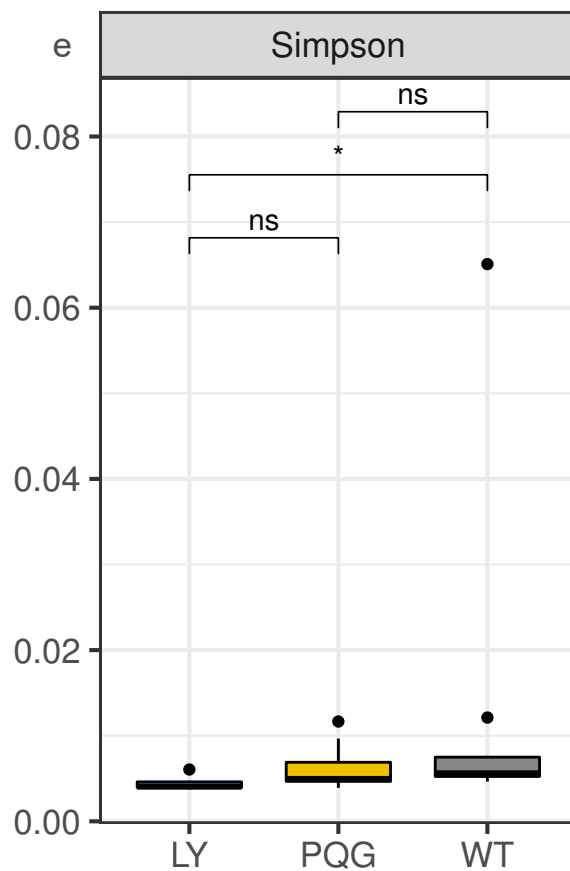
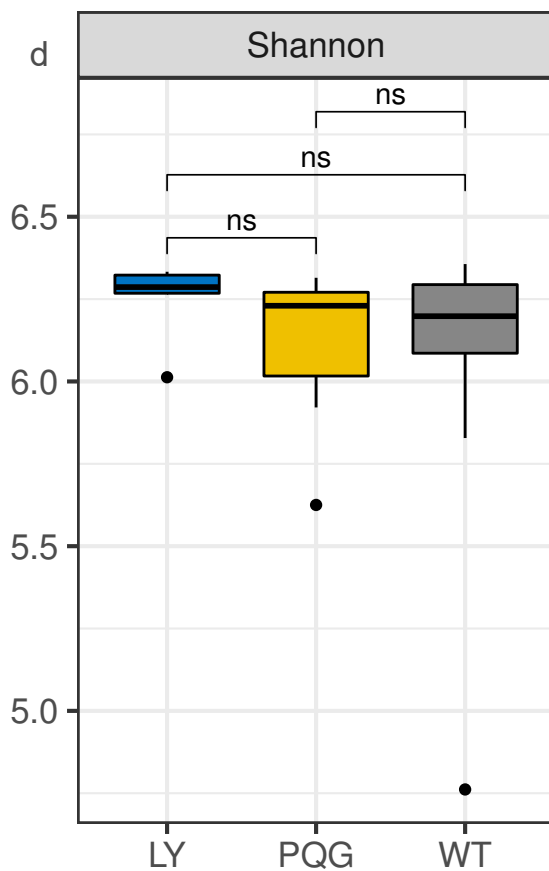
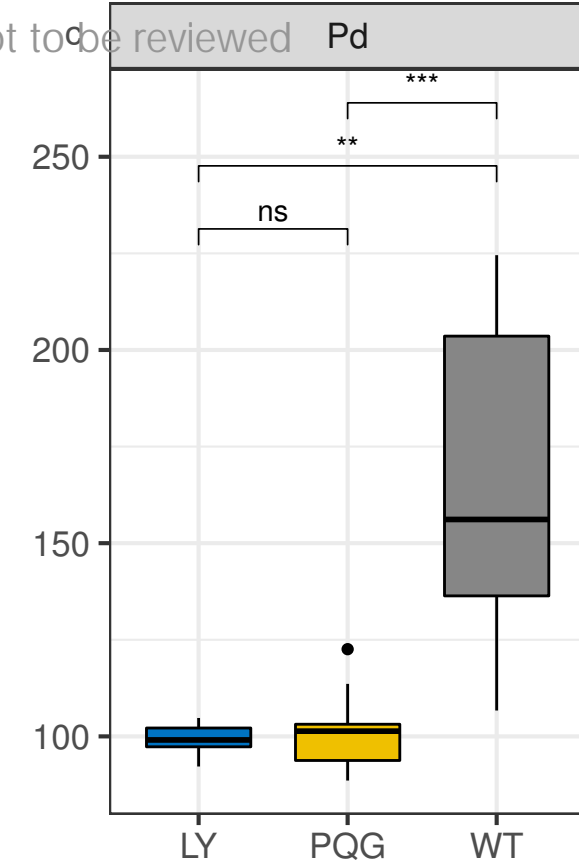
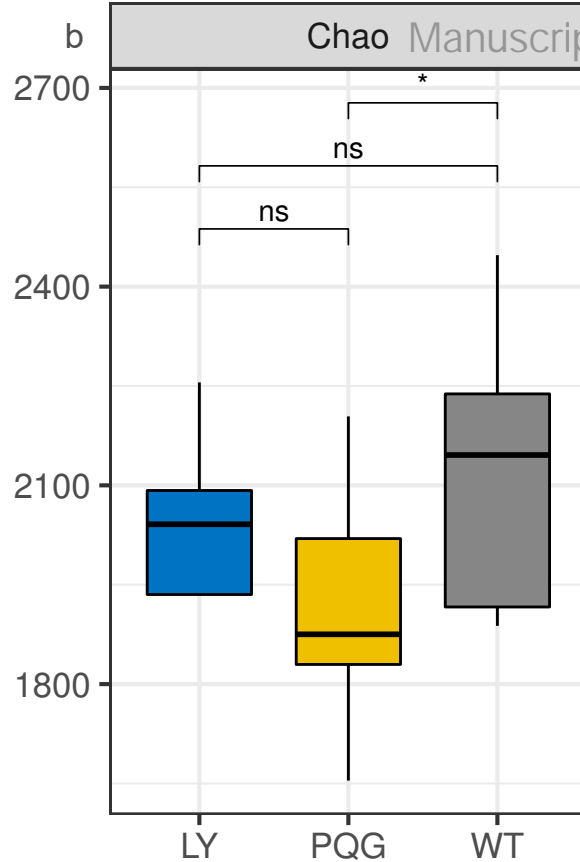
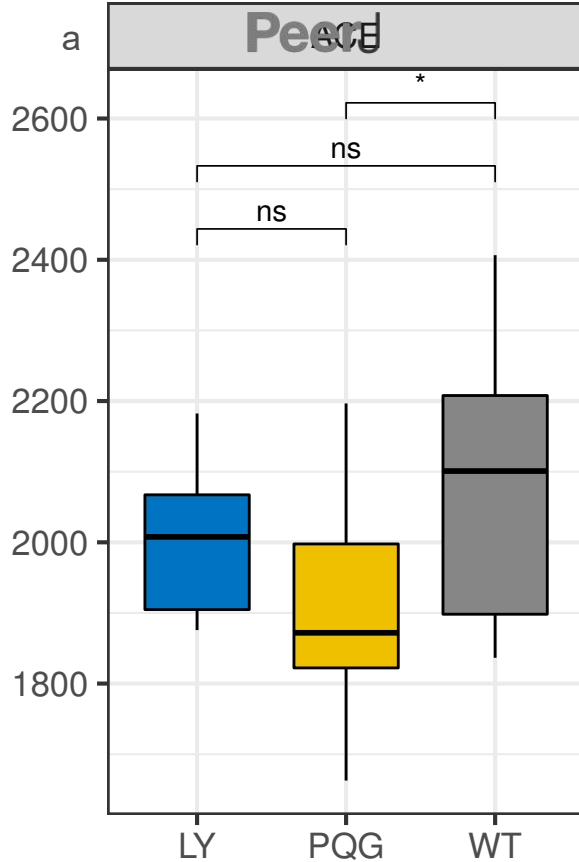



Figure 3

Microbial community composition and structure.

Relative abundance of the dominant bacterial phyla (a) and family levels (c) across the sites. Venn diagram (b) showing the shared OTUs in all plots. In the heat map (d), the horizontal coordinate represents the sample name, and the vertical coordinate represents the species name. A color gradient is used to represent the proportion of species. The value on each site represent average values of sampling plots.

Bacterial community diversity at the different sites.

RDA of the bacterial communities and the response of these communities to significant soil physicochemical properties.

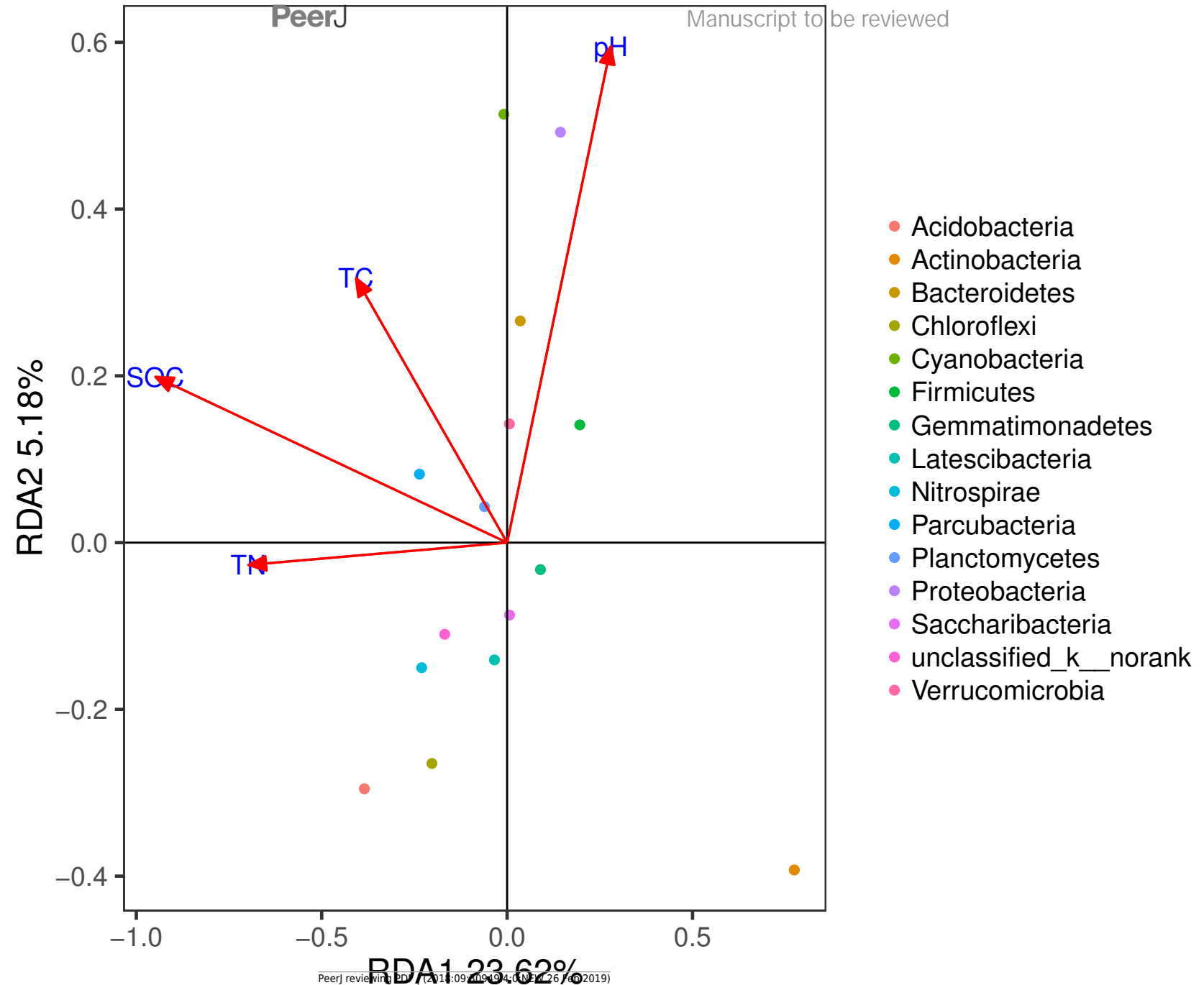
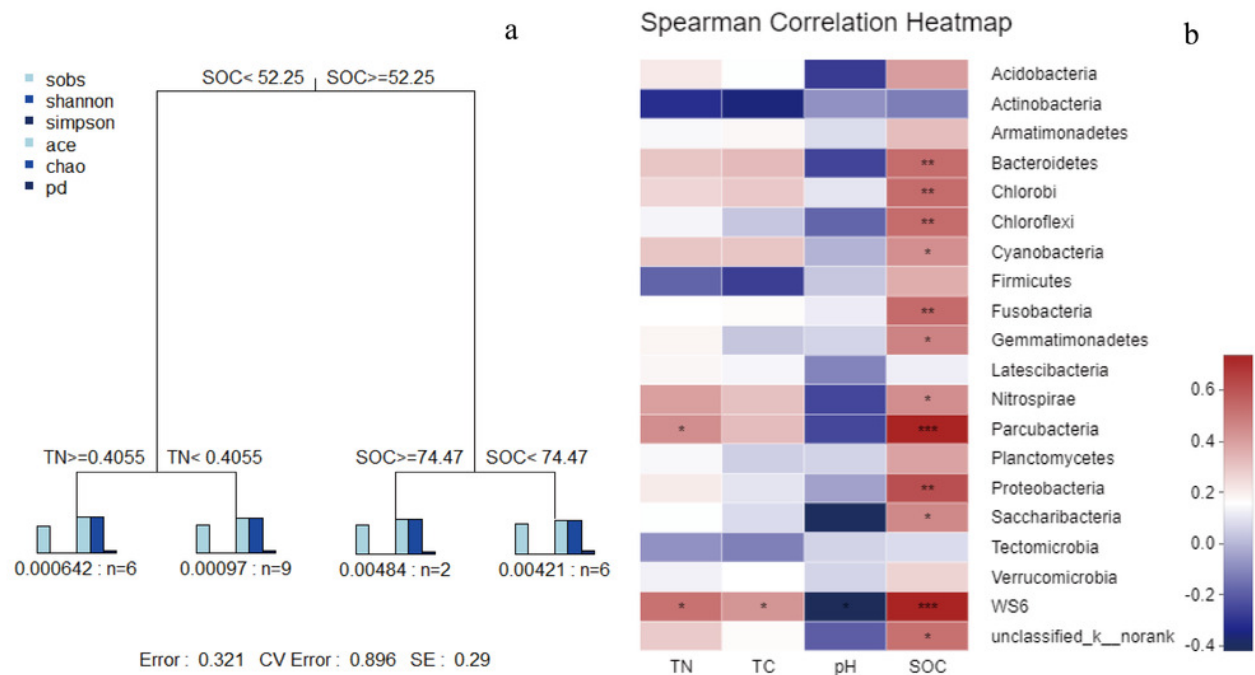



Figure 6

MRT of bacterial α-diversity data associated with key environmental factors (a). Correlation analysis (b) based on spearman correlation of microbial community composition and soil physicochemical factors.

Variation partitioning analysis showing the percentages of variance in bacterial communities explained by environment factors and spatial variable (PCNM).

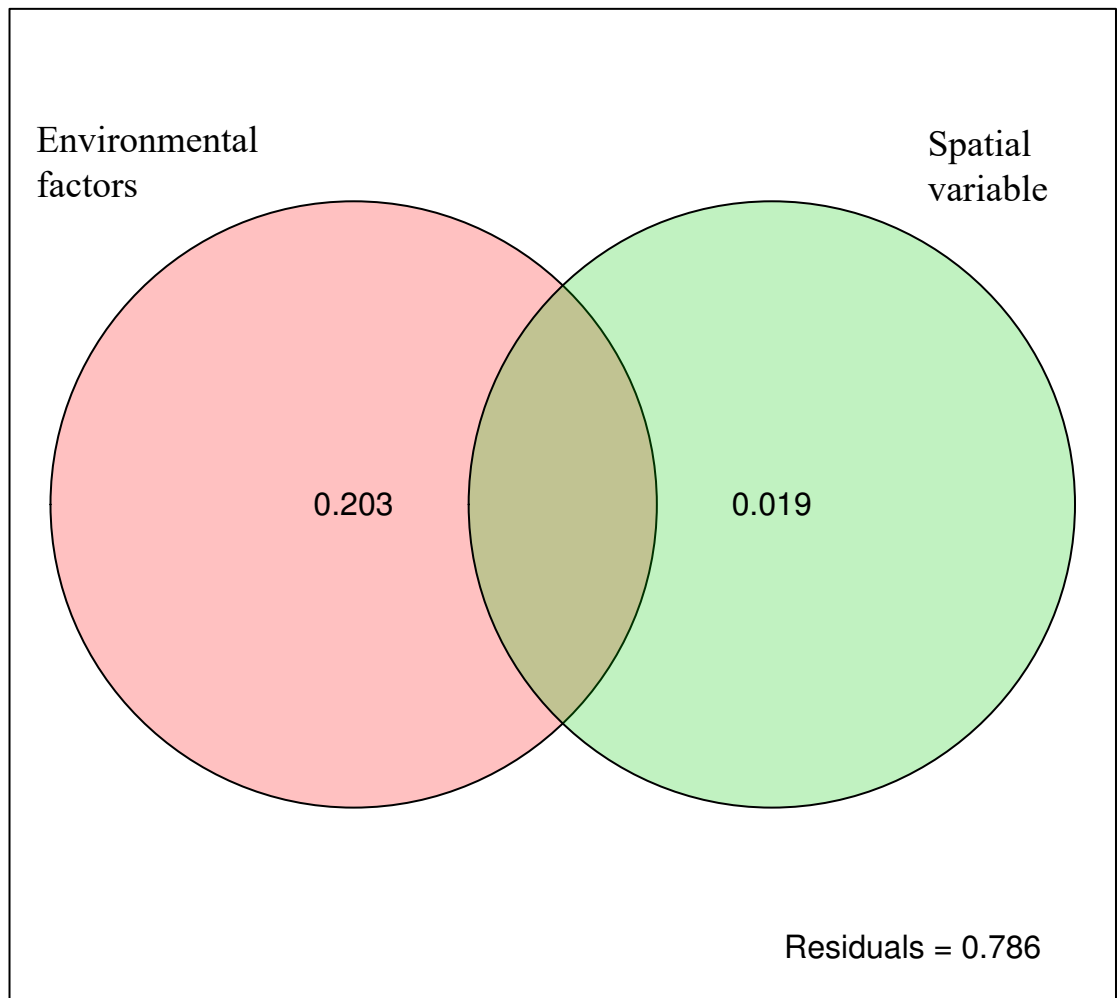



Figure 8

Network of co-occurring OTUs.

The letters a and d represent the network of the microbial community for LY; b and e for PQG; c and f for WT. Furthermore, a, b and c represent the network analysis colored by phylum, where d, e and f represent the network analysis colored by modular class. A red line indicates a positive interaction between two individual nodes, while a blue line indicates a negative interaction. The size of the nodes corresponds to betweenness centralization values.

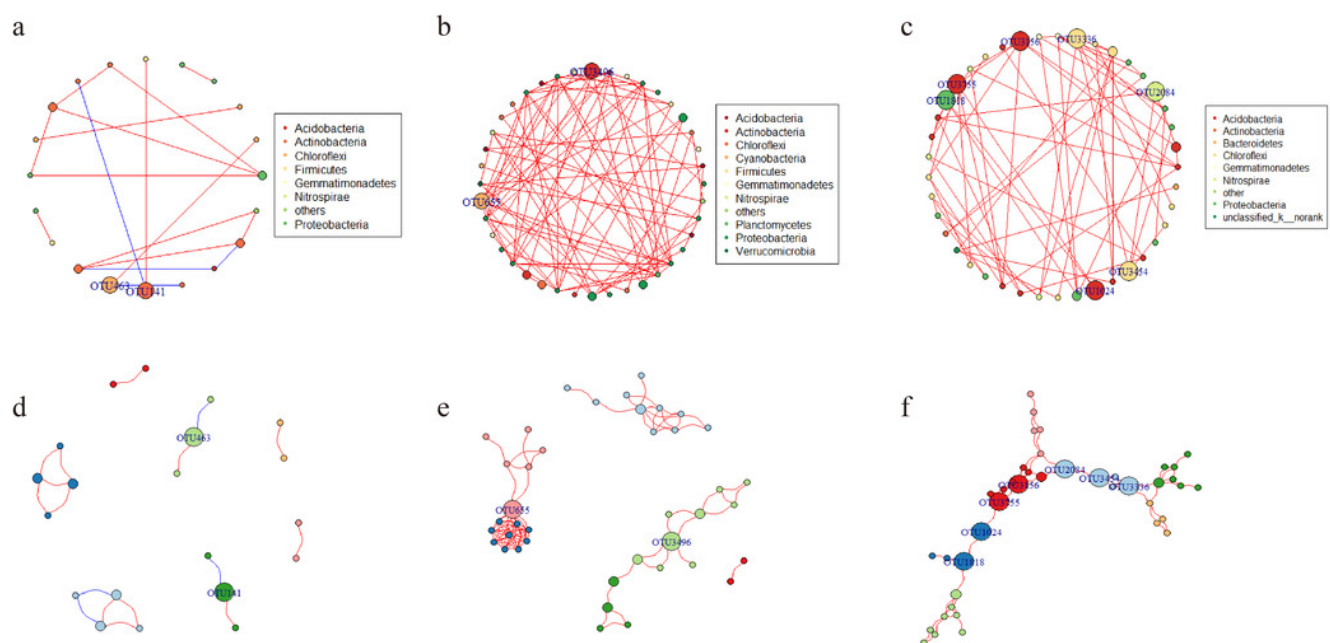



Figure 9.Microbial community assembly processes

The β-diversity null model analysis showing the null deviation of the bacterial communities at different sites (a). A null deviation close to zero suggests that stochastic processes are more important in structuring the community, whereas larger positive or negative null deviations suggest that deterministic processes play more important roles. Bar plot indicates that βNTI values varied among sites, but were all greater than +2 (b). Regression analysis of the environmental variables based on the results of the assembly processes parameters (c, d). We used the analysis of variance (ANOVA) to evaluate differences in the different indices. ns: not significantly; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.