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Eukaryotes in soil aggregates across conservation managements: Major roles of protists, fungi and taxa linkages in soil structuring and C stock

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1   **Eukaryotes in soil aggregates across conservation managements: major roles of protists, fungi**

2   **and taxa linkages in soil structuring and C stock**

3

4   **Running title: eukaryotic diversity and roles in soil aggregates**

5

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12

13   **Abstract**

14   The stabilization of soil organic carbon (SOC) promoted by conservation agriculture (CA) depends

15   on soil aggregation. Aggregation protects SOC and creates heterogeneous microhabitats hosting

16   diverse soil biota which in turn promote aggregation. A long-term experiment, studying the

17   interaction of tillage with nitrogen (N) fertilization on a soybean-wheat rotation, was used to

18   investigate eukaryotic community diversity, composition, and structure within small

19   macroaggregates (sM) and occluded microaggregates (mM). Using high-throughput Illumina

20   sequencing, we found (i) a different eukaryote diversity response to management intensification

21   across soil aggregates and soil depths; (ii) a conserved core community composition of eukaryotes

22   across CA treatments and aggregates at surface and subsurface layers; (iii) a different effect of

23   tillage on eukaryotic community structure in sM and mM along the soil profile according to N

24   availability; (iv) a positive association of protists, and fungi with the amount of sM and mM, and

25   their SOC content; (v) a stronger complexity of within- and cross-domain networks (eukaryotes and

26   eukaryotes-prokaryotes) in mM than in sM at surface layer. Overall, our findings demonstrated for

27   the first time that protists together with fungi play major roles in soil structuring and C cycling, and

28   that Cercozoa represent hubs in soil biota aggregate networks.

29

30   **Keywords**

31   soil aggregates; soil eukaryotes; protists; Illumina sequencing; 18S rRNA gene amplicon; soil biota

32   linkages.

1. Introduction

Soil organic carbon (SOC) stability mainly depends on physical protection (Six et al., 2000; Six and Paustian, 2014), whereas molecular structure of plant residues and root exudates play a secondary role in SOC persistence (Schmidt et al., 2011; Lal et al., 2015). Organic carbon (C) is protected in soil aggregates by physically limiting the access of decomposers and enzymes and the diffusion of O_2.

According to the model of Tisdall and Oades (1982), primary particles (clay and silt particles, $\emptyset$ <53 µm) are bound together by persistent bacterial, fungal, and plant debris into free microaggregates ($\emptyset$ 53-250 µm). Free microaggregates are bound into macroaggregates ($\emptyset$ > 250 µm) by transient agents (i.e., microbial and plant polysaccharides) that are rapidly decomposed by microorganisms, and by temporary agents (i.e., roots, fungal hyphae and glomalin) that persist in the medium term. Labile SOC is mainly located in macroaggregates, while free microaggregates contain a more recalcitrant SOC pool (Elliott, 1986; Jastrow and Miller, 1998).

Intensive agricultural practices, such as tillage and fertilization, shorten the life cycle of macroaggregates and diminish the formation rate of new microaggregates, worsening soil structure (Six et al., 2000). In no-tillage systems (NT), the slower turn-over of macroaggregates resulted in more sequestration of crop-derived C in microaggregates formed within macroaggregates (occluded microaggregates, mM; $\emptyset$ 53-250 µm), and thus the amount of mM is crucial for the long-term C-sequestration in soils (Six et al., 2000; Denef et al., 2007; Sheehy et al., 2015). In this context, the application of conservation agriculture (CA) practices (i.e., minimum tillage/NT, crop rotation and mulching) may allow the establishment of microhabitats with variable nutrient availabilities for a diverse soil biota, acting as efficient binding agent (Kong et al., 2011; Gupta and Germida, 2015; Totsche et al., 2018; Piazza et al., 2019). Moreover, CA practices may also produce yields equivalent to or even greater than conventional systems (Rusinamhodzi et al., 2011; Aune, 2012; Pittelkow et al., 2015; Himmelstein et al., 2016).

58   In boreal climates, long-term NT and minimum tillage (MT) have been shown to increase the

59   amount of macroaggregates and mM as well as their SOC content in the shallow layer (surface soil

60   within horizon A) in comparison to conventional tillage (CT) (Franzluebbers and Arshad, 1997;

61   Sheehy et al., 2015). This was demonstrated under various soil textures and was more evident in

62   clay, clay-loam and silt-loam soils. Similarly, in humid tropical climates and sandy loam soils,

63   long-term application of MT significantly increased SOC content in large soil aggregates, whereas

64   the reverse was reported under CT (Onweremadu et al., 2007). Accordingly, Denef et al. (2007)

65   highlighted a promotion of mM fraction and mM-associated C stocks in NT and MT compared with

66   CT under similar climate. Moreover, nitrogen (N) fertilization was reported to increase SOC in

67   macroaggregates and free microaggregates by decreasing the activity of cellulolytic fungi and

68   bacteria (Ghosh et al., 2019; Duan et al., 2021). Recently, in a cold and humid Mediterranean area

69   and in a silt-loam soil, high N fertilization rates in combination with MT not only increased mM,

70   but also promoted a shift to low level, but more efficient C-cycling microbial enzyme activities,

71   which were correlated to a greater accumulation of SOC (Piazza et al., 2020). Overall, in four

72   regions across Europe the intensification of agriculture was reported to consistently reduce soil

73   biota diversity in bulk soil, making soil food webs less diverse and composed of smaller bodied

74   organisms (Tsiafouli et al., 2015).

75   Although the role of bacterial and fungal communities (including arbuscular mycorrhizal fungi,

76   AMF) in soil aggregation and SOC stabilization is widely recognized to be fundamental (Six et al.,

77   2004; Lehmann et al., 2017; Bach et al., 2018), the diversity and potential role of other soil biota

78   have received less attention. Soil biota diversity has indeed proved to be the major driver of C

79   sequestration and nutrient cycling in bulk soil (De Vries et al., 2013; Wagg et al., 2019; Delgado-

80   Baquerizo et al., 2020). Many studies demonstrated that earthworms and bacterivore nematodes are

81   directly involved in the formation of macroaggregates by incorporating fresh organic matter inside

82   mM and thus promoting SOC accumulation (Six et al., 2004; Pulleman et al., 2005; Bossuyt et al.,

83   2006; Fonte et al., 2007; Zhang et al., 2013; Delgado-Baquerizo et al., 2020). Moreover, an indirect

4

84 effect on SOC accumulation by earthworms and bactrivorous nematodes was also reported and

85 explained by the shift of soil microbial diversity through taxa regulating nutrient flow (Delgado-

86 Baquerizo et al., 2020). Thus, in this study, we investigate the diversity and related roles of the

87 eukaryotic component of soil biota within small macroaggregates (sM) and mM across CA

88 managements. Moreover, since a more connected soil biota network takes up more C (Morriën et

89 al., 2017), the study was extended to elucidate how eukaryotes are connected among each other, and

90 to the prokaryotic community. In this context, long-term CA field experiments in the Mediterranean

91 area, such as the one used in this study, provide a great opportunity for improving the understanding

92 of soil eukaryotic diversity and functionality in soil aggregates and C stocks.

93   The following hypotheses were tested: (1) conservation tillage and N fertilization shift soil

94 eukaryote community diversity, composition and structure, in soil aggregates along the soil profile;

95 (2) soil aggregates differentially shape the diversity, composition and structure of soil eukaryotes;

96 (3) some eukaryotic taxa are predictors for soil structuring and C stocks; (4) eukaryotes form

97 structured assemblages and distinctive networks in soil aggregates (within-domain networks); (5)

98 the traits of the eukaryotes-prokaryotes networks vary across aggregates (cross-domain networks),

99 and some network traits can predict soil structuring and C stocks.

100

101 **2. Materials and Methods**

102 *2.1. Field experiment*

103   A long-term CA field experiment on a bread wheat (*Triticum aestivum* L.) - soybean (*Glycine*

104 *max* L. Merr.) rotation was set up in 1993 at the Centro Interdipartimentale di Ricerche Agro-

105 Ambientali Enrico Avanzi (Pisa, Italy; 43°40' latitude N; 10° 19' longitude E; 1 m above sea level)

106 in an alluvial silt loam soil (131, 613 and 256 g kg^{-1} of sand, silt and clay, respectively). The

107 experiment was conducted comparing two tillage intensities and two N fertilization levels. The

108 tillage intensities were: conservation tillage (minimum tillage, MT: disk harrowing at 15-cm depth)

109 and conventional tillage (CT: mouldboard ploughing at 25-cm depth, disking and harrowing at 15-

110 cm depth). The N fertilization levels applied only to bread wheat were: 0 and 200 kg N ha^{-1} (N0 and

111 N200, respectively). The soil is classified as Typic Xerofluvent by USDA system (Soil Survey

112 Staff, 1975) and as Fluvisol by FAO (IUSS, 2006). Climate of the site is cold, humid Mediterranean

113 (Csa), according to the Köppen-Geiger climate classification (Kottek et al., 2006). The experiment

114 was arranged following a split-plot design, with tillage as main-plot factor and N fertilization as

115 subplot factor and three replicate plots (dimension: 11.5 x 14.5 m). The N fertilizer treatment was

116 applied as urea and the rate was split into three applications, before sowing (60 kg N ha^{-1}), at the

117 first detectable node (70 kg N ha^{-1}), and 15 days after this stage (70 kg N ha^{-1}). Under CT, almost

118 100% of the residues were incorporated in the 0-25 cm soil layer, whereas under MT approximately

119 50% of the crop residues were incorporated at 0-15 cm depth. Crops were managed applying pre-

120 emergence herbicide for weed control and no disease or insect treatments (Piazza et al., 2020).

121   .

122 *2.2. Soil sampling and analysis of soil physical and chemical parameters*

123   Soil sampling was carried out in Spring 2016 before soybean sowing. In each replicate plot, a

124 homogenized sample was obtained by mixing four soil cores collected at two soil depths (surface

125 layer: 0-15 cm; sub-surface layer: 15-30 cm). A total of twenty-four soil samples were collected (12

126 at the surface layer; 12 at the subsurface layer). Once in the laboratory, each sample was air-dried,

127 gently broken apart and then passed through an 8-mm sieve. The isolation of small macroaggregates

128 (sM; 250-2000 µm) was done from 80 g of the sieved soil samples by the wet sieving method (Six

129 et al., 1999). Occluded microaggregates (mM; 53-250 µm) were isolated from an additional

130 isolation of sM (i.e., starting from 80 g of the sieved soil samples) and utilising a device designed

131 and built by Piazza et al. (2020). Once collected, the fractions were freeze-dried (FreeZone 2.5

132 Labconco, Kansas City, MO, USA) for 48-72 h for dry weight determination and chemical and

133 molecular analyses. Both aggregate fractions of all samples were then analysed for SOC by CHN

134   combustion method (LECO, Italy) and SOC content was calculated and expressed in Mg ha^{-1}

135   (Bremner and Mulvaney, 1982; Piazza et al., 2020).

136

137   *2.3. Molecular analyses*

138   DNA was extracted from 0.25 g of sM (n=24) and mM samples (n=24) using the DNeasy

139   PowerSoil Kit (QIAGEN, Venlo, Netherlands). The DNA extracts were then quantified by a

140   spectrophotometer (NanoDrop Technology, Wilmington, DE) and stored at -20 °C. PCRs were

141   generated from 10 ng μL^{-1} genomic DNA in volumes of 25 μL with 0.125 U μL^{-1} of GoTaq® Hot

142   Start Polymerase (Promega Corporation, WA, USA), 0.5 μM of each primer, 0.2 mM of each

143   dNTP, 1 mM of MgCl$_2$ and 1x reaction buffer, using the PTC-200 96-well Peltier Thermal Cycler

144   (MJ Research, MA, USA). The primers were TAReuk454FWD1-ill (5'-

145   TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGANNNHNNNWNNNHCCAGCASCYGC

146   GGTAATTCC-3') and TAReukREV3-ill (5'-

147   GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTACTTTCGTTCTTGATYRA-3')

148   (modified from Stoeck et al., 2010). The primer pair has attached Illumina sequencing tags, and for

149   the forward primer a 13 bp random sequence was included in order to improve cluster definition on

150   the MiSeq slide. Primers target the hypervariable region V4 of the small subunit ribosomal RNA

151   (SSU rRNA 18S) gene fragment. The thermal cycler was programmed as follows: 95 °C for 2 min,

152   35 cycles at 94 °C for 30 s, 50 °C for 45 sec, 72 °C for 1 min and 30 s and a final extension step at

153   72 °C for 10 min. PCR products were examined by electrophoresis through a 1% agarose gel in 0.5

154   × TBE buffer, then purified with magnetic beads (Agencourt® AMPure® XP, Beckman Coulter,

155   USA) and freshly prepared 80% ethanol, and quantified by fluorimetry with the use of Quant-iT™

156   dsDNA HS (High-Sensitivity) Assay Kit (Invitrogen by Thermo Fisher Scientific, CA, USA),

157   following the instructions of the manufacturer. Cleaned and quantified amplicons of each library

158   were adjusted in an equimolar ratio (10 ng/μL) for the required Illumina P5 and P7 sequences

159   addition along with index sequences in a new PCR step. Indexing was performed using primers

160   from the Nextera® Index kit (sets A and D; Illumina Inc., CA, USA) and the resulting

161   metabarcoding libraries were sequenced on an Illumina MiSeq sequencer (2 * 300 bp paired-end

162   reads) at the Genomics and Bioinformatics Laboratory (Technology Facility, Department of

163   Biology, University of York, UK). Details are given in the Supplementary Methods 1.

164

165   *2.4. Bioinformatic analyses*

166   Raw data generated from the Illumina MiSeq sequencing run were processed and analyzed

167   following the pipelines of QIIME 2 (2018.4) and USEARCH (v10.0.240) (Edgar, 2010; Caporaso et

168   al., 2012). Forward and reverse paired-end sequences were assembled independently for each

169   sample using -fastq_mergepairs USEARCH command. Primer sequences were then trimmed off by

170   employing cutadapt plugin (2018.4) with default settings. To avoid potential errors in sequencing

171   data, quality of sequence reads was checked by -fastq_eestats2 USEARCH command, using the

172   expected number of errors in a read as a measure of quality for filtering (Edgar and Flyvbjerg,

173   2015). Reads were then trimmed at the length where the "drop-off" point for the maximum

174   expected error value occurred (250 bp). Quality filtered reads were de-replicated by -fastx_uniques

175   USEARCH command, then Operational Taxonomic Units (OTUs) were generated using

176   USEARCH by clustering sequence reads at the 97% similarity threshold. During the process,

177   chimeric sequences and singletons were removed from the dataset. For the curation, the sequences

178   were aligned using ClustalW and then Neighbor Joining (NJ) phylogenetic tree was built in

179   MEGA7 (Kumar et al., 2016) (https://www.megasoftware.net). The most abundant sequence of the

180   eukaryotic OTU in each cluster was selected, and used as representative sequence for that OTU

181   after branch collapsing. For the curation, the sequences were aligned using ClustalW and then

182   Neighbor Joining (NJ) phylogenetic tree was built in MEGA7 (Kumar et al., 2016)

183   (https://www.megasoftware.net). The most abundant sequence of the eukaryotic OTU in each

184   cluster was selected, and used as representative sequence for that OTU after branch collapsing. The

185   OTUs were phylogenetically assigned using the 18S SSU SILVA database (version 132, release

186   date 13.12.2017) (Quast et al., 2012; Yilmaz et al., 2013) by clustering sequence reads at the 97%

187   similarity threshold. After curation, the representative sequences were re-aligned using ClustalW

188   and the phylogenetic tree was built in MEGA7 using the Neighbor Joining (NJ) analysis with 1 000

189   boostrap replicates and the Kimura 2-parameter model (uniform rates).

190   The suitability of the eukaryotic community sampling was verified by rarefaction curves

191   plotting the number of eukaryotic classes/phyla *versus* the number of sequence reads, while

192   accumulation curves were calculated plotting the number of classes/phyla *versus* the number of soil

193   samples using the package Vegan in R (Oksanen et al., 2013). Since there was a high variability in

194   the number of reads per sample, sequencing depth per sample was standardized to the median

195   number of reads across the samples in each data matrix using the same package in R (standardized

196   datasets). All representative sequences were deposited in the NCBI GenBank database

197   (SUB5948379 submission: MN178662-MN178794 accession numbers).

198   Prokaryotic data were obtained from the same soil matrices and depths (Piazza, 2019) and were

199   based on the V4 region of the 16S rRNA gene sequenced using a MiSeq Illumina approach

200   (SUB5941754 submission: MN171543-MN172157 accession numbers).

201

202   *2.5. Statistical analyses*

203   To test hypothesis 1 - Conservation tillage and N fertilization shift soil eukaryote community

204   diversity, composition and structure in soil aggregates along soil profile - analysis of variance

205   (ANOVA), Venn diagrams and permutational analysis of variance (PERMANOVA) were applied.

206   Concerning diversity, richness, Shannon index (*H'*) and Simpson index ($\lambda = 1- \lambda'$) were calculated

207   at class level and analysed by two-way ANOVA, according to the experimental design. These

208   analyses were done in Vegan package in R and plotted by ggplot2 (Wickham and Chang, 2008).

9

209 Data were ln--transformed when needed to fulfil the assumptions of ANOVA. Post-hoc Tukey-B

210 significant difference test was used for comparison among treatments. Concerning composition,

211 Venn diagrams were drawn to visualize the OTUs unique to the treatments as well as the shared

212 ones. The standardized datasets were used to generate the Venn diagrams by the online tool

213 InteractiVenn (http://www.interactivenn.net; Heberle et al., 2015). Concerning structure, the

214 relative abundances of eukaryotes were calculated at class level and the permutational analysis of

215 variance (PERMANOVA) (Anderson and Braak, 2003) and the analysis of homogeneity of

216 multivariate dispersion (PERMDISP) were used to test the effect of treatments (Togerson, 1958;

217 Clarke and Gorley, 2006). Response data were square-root transformed to down-weight the

218 dominant taxa and the Bray-Curtis index of dissimilarity was used to measure ecological distance.

219 When PERMANOVA indicated a significant effect, the principal coordinate analysis (PCO) was

220 carried out (Anderson et al., 2008) to visualize the most relevant patterns in the response data. In

221 each PCO biplot, only the taxa with a strong correlation (r = 0.50-0.80) with the ordination scores

222 on each PCO axis were displayed. *P*-values were calculated using the Monte-Carlo test and

223 residuals were permuted according to the experimental model (Oksanen et al., 2013). Multivariate

224 analyses were performed using PRIMER 6 and PERMANOVA+ software (Clarke and Gorley,

225 2006; Anderson et al., 2008).

226 To test hypothesis 2 - Soil aggregates differentially shape the diversity, composition and

227 structure of soil eukaryotes - eukaryotic richness, *H'* and λ at phylum level and at both soil depths

228 were analysed by one-way ANOVA, using soil matrix (sM *vs* mM) as fixed factor, and tillage and

229 N fertilization as covariates. Analyses were performed in Vegan package in R and data were plotted

230 by ggplot2. Data were ln-transformed when needed to fulfil the assumptions of ANOVA, and the

231 post-hoc Tukey-B significant difference test was used for comparison among treatments. Moreover,

232 the effect of matrix on composition and structure were analysed at phylum level using the Venn

233 diagrams and PERMANOVAs, as described above.

234   To test hypothesis 3 - Some eukaryotic taxa are potential predictors for soil structuring and C

235   stocks - multiple regression analysis was applied using as independent variables the standardized

236   relative abundances (calculated as described above) of eukaryotic taxa at class level. To account for

237   the compositional nature of the data, an additive log-ratio transformation was applied (Gloor et al.,

238   2017). The dependent variables were sM and mM weights, and SOC content per unit of surface in

239   sM and mM. The assumptions of the linear regression model were verified (Supplementary Method

240   2) and the multiple linear regression analysis was applied using a stepwise method with the

241   following probability criteria: $P < 0.05$ to accept and of $P > 0.05$ to remove a phylum or a

242   within/cross-domain network traits. Multiple regressions were performed using the SPSS software

243   package version 25.0 (SPSS Inc., Chicago, IL, United States). Details about regression analysis are

244   reported in Supplementary Methods 2.

245   To test hypothesis 4 - Eukaryotes form structured assemblages and distinctive networks in soil

246   aggregates - we built networks using the SParse InversE Covariance estimation for Ecological

247   ASsociation   Inference   (SPIEC-EASI)   package   version   0.1   in   R

248   (https://github.com/zdk123/SpiecEasi/). SPIEC-EASI is a pipeline for inferring sparse inverse

249   covariance matrix within and between multiple compositional datasets, under joint sparsity penalty

250   (Kurtz et al., 2015). The within-domain analyses were performed on the standardized eukaryotic

251   dataset at class level for each soil matrix and depth. The neighborhood selection (MB method) was

252   applied as graphical inference model (Meinshausen and Bühlmann, 2010), since it has been shown

253   to better perform than other available methods (e.g., CCREPE, SPARCC, SPIEC-EASI glasso)

254   (Kurtz et al., 2015). The Stability Approach to Regularization Selection (StARS) was applied to

255   select the optimal sparsity parameter (Liu et al., 2010), and the StARS variability threshold was set

256   to 0.05 and nλ to 100 for all networks. We evaluated the weights of the edges in the networks using

257   SPIEC-EASI (frequency *versus* edge weights = modularity), and we plotted the degree distributions

258   of frequencies of the edges using adj2igraph (Kurtz et al., 2015). In the networks a node represents

259   a connected taxon, an edge the connection between taxa, a singleton an unconnected taxon and a

11

dyad two connected taxa. For the eukaryotic networks (within-domain network) we calculated the following parameters: number of nodes excluding singletons, number of edges, number of singletons and dyads, number of subnetworks (a subnetwork is a network composed by at least three nodes), mean nodes per subnetwork, linkage density (complexity: the average number of edges per node), percentage of positive interactions and modularity. Moreover, in each network, we calculated the frequency of the phyla within the subnetworks, the mean of edges and nodes, and the percentage of positive edges for each phylum. Details about trait calculations are given in Supplementary Methods 3.

To test hypothesis 5 - The traits of the eukaryotes-prokaryotes networks vary across aggregates we inferred the associations between eukaryotes and prokaryotes domains by the cross-domain extension of SPIEC-EASI (Kurtz et al. 2015; Tripton et al., 2018). The same traits calculated for within-domain networks, except for the positive edges, were assessed (Supplementary Methods 3). In addition, the percentage of eukaryotes/prokaryotes per subnetwork was calculated and the number of subnetworks with only eukaryotes, only prokaryotes and with both domains were counted. The significance of the cross-domain relationships was tested by the Mantel test (Mantel and Valand, 1970) on the standardized read data that were centered and normalized and using the function Jaccard in PC-ORD 5 to build the resemblance matrix (Grandin, 2006) and also by the co-Correspondence Analysis (CoCA) (ter Braak and Schaffers, 2004) in CANOCO 5 (ter Braak and Smilauer, 2012). To test if some network traits can predict soil structuring and C stocks - a multiple linear regression was performed after verification of the assumptions (Supplementary Methods 3). The analysis was performed using as independent variables the log(1+x)-transformed and normalized within- and cross-domain network traits in sM and mM (i.e., traits of eukaryotic networks and of eukaryotic-prokaryotic networks), and using as dependent variables sM and mM weights, and SOC content per unit of surface in sM and mM. Scripts for within and cross-domain network construction and analysis are available in Supplementary Methods 4.

286

287 **3. Results and discussion**

288 *3.1. Illumina sequencing information*

289   MiSeq sequencing yielded a total number of 2 940 322 reads from the 48 soil samples, and

290 following quality-filtering a total of 2 884 052 sequence reads having a length of 392 bp were

291 obtained. After BLAST against the 18S SSU SILVA database (Quast et al., 2012; Yilmaz et al.,

292 2013), we found 2 036 277 reads, ranging from 3 to 106 540 reads per sample that were assigned to

293 a total of 4 211 OTUs. After sequence curation and the removal of Plantae sequences, 863 809

294 reads, ranging from 6 421 and 46 429 reads per sample, were retrieved and assigned to 133 OTUs,

295 56 classes and 27 phyla (Fig. S1, Fig. S2). The rarefaction and accumulation curves demonstrated

296 that sampling effort was sufficient as the curves reached the asymptote (Fig. S3, Fig. S4).

297

298 *3.2. Effect of conservation management on eukaryotic diversity in soil aggregates*

299   To test if conservation tillage and N fertilization shift the diversity of soil eukaryotes in soil

300 aggregates (hypothesis 1), richness and diversity indices were determined along the soil profile in

301 small macroaggregates (sM) and occluded microaggregates (mM). A greater eukaryotic diversity in

302 sM was consistently found at both soil layers under CT compared to MT, as shown by the

303 significant increase of richness (+26%) and *H'* and λ (+9%) (Fig. S5). This higher eukaryotic

304 diversity might be due to larger root development and higher availability of root exudates, organic

305 matter (e.g., nutrients, organic acids), water and oxygen, reported under deep ploughing systems,

306 and which have been shown to promote microbial growth and soil biota diversity/functionality

307 (Guan et al., 2014; Edwards et al., 2015; Ercoli et al., 2017; Piazza et al., 2020), according to the

308 response of individual taxonomic units to habitat and trophic conditions (van Capelle et al., 2012).

309 At surface layer, N fertilization significantly increased λ under MT, suggesting an increase in

310 number of relative abundances of taxa regulated by N availability. Conversely, under CT, λ was

13

311 high and not modified by N fertilization, suggesting non-limiting N availability due to improved

312 plant growth and higher mineralization rate of residues. A low eukaryotic diversity was previously

313 found in bulk soil under N fertilization (Lentendu et al., 2014), and within soil aggregates a higher

314 microbial diversity was found at low nutrient availability and NT compared with high nutrient

315 availability and ploughing (Lagomarsino et al., 2012; Zhang et al., 2013; Bach et al., 2018).

316 In mM an opposite pattern was found at surface layer, and no effect of N fertilization alone or

317 in interaction with tillage was observed at both soil depths (Fig. S5). At surface layer, the

318 eukaryotic diversity indices increased by 6% under MT compared to CT. Under tillage

319 intensification, macroaggregates are indeed disrupted and occluded microaggregates became free in

320 the soil (Six et al., 2000), potentially reducing diversity and C sequestration. This is supported by

321 the highest SOC accumulation observed in mM under MT (Piazza et al., 2020), and by the high soil

322 biota diversity found in the present study within mM.

323 To test if soil aggregates differentially shape the diversity of soil eukaryotes (hypothesis 2),

324 richness, H' and λ were determined in sM and mM. Overall eukaryotic diversity was significantly

325 higher in mM than in sM (Fig. S5) (i.e., at surface layer: richness +16%, H' +6%, and λ +2%; at

326 subsurface layer: richness +20 and H' +5%). These results are in accordance with the higher

327 richness and H' of bacteria and fungi found in free microaggregates compared to large

328 macroaggregates (Bach et al., 2018). Our findings support the fact that soil aggregates are distinct

329 habitat spaces with eukaryotes adapted to SOM resources, pore-space network, and water and

330 oxygen availability characteristic of sM and mM.

331

332 *3.3. Effect of conservation management on eukaryotic composition in soil aggregates*

333 To test if conservation tillage and N fertilization shift the composition of soil eukaryotes in soil

334 aggregates (hypothesis 1) and how soil aggregates shape their composition (hypothesis 2), this

335 parameter was evaluated along the soil profile in sM and mM. Across management practices and

336 soil depths, the eukaryotic phyla Cercozoa (21%), Ciliophora (13%), Chlorophyta (11%), Nematoda

14

337   (11%) and Glomeromycota (9%) were predominant in sM, whereas in mM the predominant phyla

338   were Ciliophora (19%), Cercozoa (18%) Chlorophyta (15%), and Ascomycota (11%) (Fig. 1a). The

339   other phyla showed an abundance $\leq$ 8%. Sun et al. (2021) found that protists were the most

340   dominant eukaryote (33.9% of the total eukaryotic sequences) in bulk soil. By contrast, Treonis et

341   al. (2018) analysing the whole eukaryotic structure in bulk soil found a high abundance of fungi,

342   Arthropoda, Nematoda and Anellida (40, 20, 20 and 11%, respectively), and a low abundance of

343   protists (0.63%). Among protists, Rhizaria was the group with the highest relative abundance in

344   arable soil, comprising as dominant taxa Cercozoa and Amoebozoa (Bates et al., 2013; Degrune et

345   al., 2019a; Santos et al., 2020). Similarly, in another study, fungi were reported to be the most

346   abundant (i.e., Ascomycota, Basidiomycota, fungi Incertae sedis and Glomeromycota), followed by

347   Alveolata, Metazoa, Rhizaria, Stramenopiles, and Viridiplantae (Chen et al., 2012). Therefore, we

348   can assume that the differences between bulk soil and soil aggregates depend on the variability of

349   pH, moisture and organic nutrient availability that shift soil biota at multiple trophic levels.

350   In both soil aggregates, the majority of taxa were common to all managements across soil

351   depths, whereas some were unique to certain managements, as shown by the Venn diagrams at class

352   resolution (Fig. 2b-c,e-f). This is also shown by the pie charts representing the proportion of the 56

353   classes retrieved in each management (Fig. 2a,d). Moreover, focusing on the shared taxa between

354   sM and mM, ca. 80% of eukaryotes were common to both soil aggregates, averaging soil depths

355   (Fig. 3a,b). Accordingly, a large conserved core community of soil prokaryotes and fungi was

356   found across managements at the same site in bulk soil (Piazza et al., 2019). This is also consistent

357   with the findings obtained in other studies in different soil types and managements in bulk soil as

358   well as in specific rhizocompartments (Lentendu et al., 2014; Edwards et al., 2015; Pershina et al.,

359   2018).

360   However, the exclusive presence of some eukaryotic taxa in the different systems and soil

361   aggregates (Fig. 2) suggests that long-term tillage and N fertilization may drive the development of

362   communities of specialized taxa putatively having specific functions (e.g., soil aggregate and/or

363   SOC accumulation and nutrient cycling). As example, at the surface layer, in sM the classes

364   *Nassophorea* and *Perkinsea* were exclusively found in MTN0, whereas *Pezizomycetes* and

365   *Gastrotricha* in CTN0 (Fig. 2a,b), while many taxa were exclusively found in mM, such as

366   Chlorophyta, *Eutardigrada*, *Eurotiomycetes*, *Nassophorea* and *Thecofilosea* in MTN0; Alveolata,

367   *Chilopoda* and *Rhabditophora* in MTN200 and *Dictyostelia* and *Pezizomycetes* in CTN200 (Fig.

368   2d,e). A more in-depth description of the eukaryotic composition and exclusiveness across

369   managements and aggregates is reported in the Supplementary Results and Discussion 1.

370

371   *3.4. Effect of conservation management on eukaryotic community structures in soil aggregates*

372   To test if conservation tillage and N fertilization shift the structure of soil eukaryotes in soil

373   aggregates (hypothesis 1), the relative abundance pattern of taxa was determined in soil aggregates.

374   Despite the high degree of similarity among treatments in term of community composition, we

375   highlighted a strong effect of the interaction between tillage and N fertilization on the eukaryotic

376   community structures in sM at both soil layers, and in mM only at the surface layer (Table 1, Fig.

377   1). Similarly, soil fungal community structure in bulk soil was strongly shaped by the interaction

378   between tillage and N fertilization at surface and subsurface layers (Piazza et al., 2019). However,

379   to our knowledge no studies have focused on the effect of the interaction of these practices on the

380   eukaryotic communities in soil aggregates, whereas a huge number of studies was performed to

381   assess the effect of tillage or N fertilization on the diversity/abundance and functionality of single

382   eukaryotic group in bulk soil (e.g., fungi: Jansa et al., 2003; Wang et al., 2019; Zhao et al., 2019;

383   micro-arthropods, nematodes and protozoa: Adl et al., 2006; Zhang et al., 2012; Briones and

384   Schmidt, 2017; Cai et al., 2020; protists: Zhao et al., 2019; Sun et al., 2021).

385   In the PCO plots, CTN0 and CTN200 showed similar community structures within sM at

386   surface layer (Fig. 1b) that were characterised by *Colpodea* and OTU1*Spirotrichea* (Ciliophora,

387   Alveolata) and *Conoidasida* (Apicomplexa, Alveolata). This supports that under ploughing N

388   availability is not limiting and the community structures are not affected by N fertilization. The

389   class *Colpodea* is a well-known dominant clade of mainly bacterivorous protists (Foissner, 1998)

390   and, according to our results, they were shown highly abundant in disturbed soils. By contrast,

391   *Spirotrichea* were found in more stable environments (Lüftenegger et al., 1985), not supporting the

392   large abundance found within sM under ploughed soils. This result not supported by literature may

393   suggest a high resistance of *Spirotrichea* to natural and anthropogenic stresses. Moreover,

394   Apicomplexa that are the third most abundant protistan group in soil, after Cercozoa and Ciliophora

395   (Fierer, 2017) are described as putative parasites of invertebrates (Del Campo et al., 2019). This is

396   in agreement with the lower occurrence of the invertebrates *Chromadorea* and *Enoplea* found in

397   our study under CT compared with MT. In MT, N fertilization determined a strong shift at the

398   surface layer (Fig. 1b), with community structure within sM under MTN200 characterized by a

399   large abundance of taxa belonging to Cercozoa, *Tubulinea* (Lobosa, Amoebozoa) and *Chromadorea*

400   (Nematoda) together with fungi, (i.e., OTU1Microbotryo: *Microbotryomicetes*, Ascomycota and

401   Mortierellomycota), and under MTN0 characterized by a large abundance of taxa belonging to

402   *Imbricatea* (Cercozoa), *Enoplea* (Nematoda) and OTU1*Nassophorea* (Ciliophora, Alveolata). The

403   dominance of Ascomycota in sM under MTN200 is in accordance with their higher abundance in

404   macroaggregates under mineral fertilization compared with no fertilization (Liao et al., 2018; Wang

405   et al., 2021). This confirms the importance of fungi as binding agents in soil aggregates (Six et al.,

406   2000). Cercozoa were reported to be affected by several abiotic factors, as soil moisture, clay

407   content and N availability (Lentendu et al., 2014; Fiore-Donno et al., 2019). However, although our

408   analyses did not allow discrimination of which cercozoan classes were favoured under MTN200

409   (Fig. 1b), the detection of *Imbricatea* within sM under MTN0 supports that N availability is a major

410   driver of cercozoan communities. This class had unexpectedly high abundance in sM under MTN0

411   (Fig. 1b), although it was shown to be highly favoured by organic fertilizers (Lentendu et al., 2014).

412   According to our results (Fig. 1b), the heterotroph lineage Tubulinea are dominant in highly N-

413   fertilizer soils (Sun et al., 2021), and the ciliate *Nassophorea*, characterizing the community

17

414 structures of sM under MTN0, supports their role in energy transfer between trophic levels under

415 low N availability (Gao et al., 2016). Finally, the dominance in sM under MT of *Enoplea* and

416 *Chromadorea* known to be plant and animal nematode parasites is consistent with their general

417 trend in soil aggregates (Jiang et al., 2017). Their abundance in N0 and N200, respectively, might

418 be explained by specific predator-prey interactions occurring within intra-aggregate pores at

419 differential N availabilities.

420   At the subsurface layer (Fig. 1c), N fertilization drove a stronger shift of the eukaryotic

421 community structure in sM under CT compared with MT that showed similar structures irrespective

422 to N fertilization, highlighting an opposite pattern as compared with the one observed at surface

423 layer (Fig. 1b). Under CTN0, sM was characterised by high abundance of *Chilopoda* (Arthropoda,

424 Animalia) and *Sarcomonadea* (Cercozoa, Rhizaria), and under CTN200 by high abundance of

425 *Colpodea* and *Conoidasida* (Alveolata) (Fig. 1c). Indeed, under ploughing, the subsurface layer is

426 less compacted than under MT and shows a lower bulk density, resulting in an increase of pore size

427 and aeration (Berisso et al., 2012). This may allow a larger root development under N fertilization

428 and a higher variation in soil moisture and temperature compared with no fertilization (Piazza et al.,

429 2020). Small macroaggregates are inaccessible to living centipedes (*Chilopoda*), thus their

430 abundance in this fraction under CTN0 can be only related to a role as binding agents or as dead

431 biomass consumed by decomposers. By contrast, *Sarcomonadea*, previously found in soil as the

432 dominant class within the phylum Cercozoa (Degrune et al., 2019b), are likely to play an active role

433 also in sM at low N availabilities. The abundance of *Colpodea* in CTN200 at the subsurface layer

434 (Fig. 1c) is consistent with their dominance at the surface layer (Fig. 1b) and can be explained by

435 bacterial pray changes following N application, while no information is available on *Conoidasida*

436 trophic functional role.

437   In MTN0 and MTN200 at subsurface layer sM were characterized by *Chromadorea*

438 (Nematoda), *Vampyrellidea* (Cercozoa), OTU1*Nassophorea* (Ciliophora, Alveolata) and many

439 fungi (e.g., *Glomeromycetes*, *Sordariomycetes*) (Fig. 1c). The abundance of *Choromadorea* and

18

440 *Nassophorea* is consistent with surface layer observations. Moreover, *Vampyrellidea,* observed for

441 the first time in sM, are fungivores that may control the parasitic rust fungus of wheat under MT

442 (Adl and Gupta, 2006). Finally, the abundance of *Glomeromycetes* and *Sordariomycetes* confirms

443 their crucial role in driving soil aggregates under undisturbed conditions (Rillig et al., 2015; Wang

444 et al., 2021).

445 In mM, a strong interaction effect between tillage and N fertilization was found on the

446 eukaryotic community structures only at surface layer, consisting in a strong shift of the structure

447 under CTN0 compared with the other treatments (Table 1, Fig. 1d). This effect is in line with the

448 aggregate distribution of mM found by Piazza et al. (2020). The shifts of aggregate distribution and

449 eukaryotic community structure toward more mM and distinct soil biota communities under CT at

450 low N availability can be related to a lower sequestration of C within mM and thus in differences of

451 the related functional soil biota. By contrast, the lack of effect at subsurface layer is unexpected

452 since the percentage of mM was significantly decreased by tillage intensification (CT < MT; - 21%)

453 (Piazza et al., 2020). However, this inconsistency could be due to the coverage of the V4 region

454 primer set, its taxonomic resolution or limitation in amplifying rare taxa or taxa with lower

455 proportions of template DNA in DNA extracts (Choi and Park, 2020). Moreover, considering

456 aggregate pore size and animal body size, the presence of traces of animal DNA (i.e., nematodes,

457 Arthropoda) within aggregates is likely not attributable to the occurrence of living animals, but to

458 the process of aggregate formation which utilises organic decaying material as binding agent.

459 Nitrogen fertilization determined a strong shift at the surface layer in the eukaryotic community

460 structure of mM under CT (Fig. 1d) from *Glomeromycetes* (Glomeromycota), *Imbricatea*,

461 *Sarcomonadea* and *Vampyrellidea* (Cercozoa) in CTN0 to OTU1*Xantho* (Ochrophyta),

462 *Oligohymenophorea* (Ciliophora), Stramenopiles (Chromista) and the fungus *Tremellomycetes* in

463 CTN200. This is the first time that Glomeromycota have been detected within mM fraction.

464 Previously, using a cloning approach targeting the long-fragment SSU-ITS-LSU (Krüger et al.,

465 2009) we could not detect AMF within mM (data not shown), and this was also supported by

19

466 several works reporting their major roles only in macroaggregates (e.g., Miller and Jastrow, 2000,

467 Rillig et al., 2002). However, in this study, the observed large proportion of Glomeromycota (14%)

468 in mM under CTN0 supports the fact that tillage under unfertilized conditions may not negatively

469 affect the development of the extraradical mycelium, potentially improving the production of

470 glomalin and enhancing soil aggregate stability (Bedini et al., 2002). Similarly, the high abundance

471 of Cercozoa in mM under CTN0 suggests for the first time that this phylum plays a major role

472 within mM under ploughed and no fertilized conditions at surface layer. This is consistent with the

473 findings of Degrune et al. (2019a) that highlighted under ploughing and at topsoil distinct cercozoan

474 communities in microhabitats (i.e., drilosphere and rhizosphere) compared with bulk soil.

475 Moreover, the distinct eukaryotic community found at surface layer in mM under CTN200

476 additionally supports that, under ploughing, nutrient availabilities in microhabitats allow the

477 dominance of functional protists (Alveolata: *Oligohymenophorea*; Chromista: OTU1*Xantho* and

478 Stramenopiles), potentially contributing to OM decomposition and mineralization through several

479 functional groups. In addition, scarce information is available on the functional roles in agricultural

480 soils of *Tremellomycetes*, a heterogeneous group comprising saprotrophs, animal parasites, and

481 fungicolous species.

482 Similar community structures were observed at surface layer within mM in MTN0 and

483 MTN200 [e.g., *Sarcomonadea*1 (Cercozoa), and fungi such as *Dothideomycetes* (Ascomycota) and

484 OTU1Cystobasidio (Basidiomycota)] (Fig. 1d). These results support the hypothesis of a major role

485 played by Cercozoa together with distinct classes of fungi also within mM under MT. However, it

486 is well known that the 18S barcoding utilised in this work is less efficient compared with the ITS

487 for detecting many groups of fungi (Schoch et al., 2012).

488 To test if soil aggregates differentially shape the structure of soil eukaryotes (hypothesis 2), the

489 relative abundance pattern of taxa was determined in sM and mM. Significant differences among

490 matrices (sM *vs* mM) were found and supported by PERMANOVAs (Table 1). PCO biplots

491 showed that at both soil layers more phyla were linked to mM as compared with sM (Fig. 3c,d).

20

492 Recently, Liao et al. (2018) and Wang et al. (2021) used an Illumina sequencing approach for

493 studying at phylum and class level the bacterial and fungal community structures within soil

494 aggregates across different fertilization treatments. Although differences in community structure

495 were detected for both bacteria and fungi, fungal community in sM and free microaggregates

496 differed more than bacteria (Liao et al., 2018; Wang et al., 2021). Our findings support that some

497 unresolved taxa belonging to Glomeromycota and Ascomycota are positively associated with mM,

498 as previously reported in free microaggregates for unclassified Ascomycota (Wang et al., 2021) and

499 for a group of unclassified *Glomerales* (Lu et al., 2018). Similarly to the results of Jiang et al.

500 (2017), the total abundance of nematodes increased with increasing aggregate size. Finally, the

501 alveolate Apicomplexa, Ciliophora, and Dinoflagellata were preferentially found in mM, whereas

502 the amoebozoan Conosa and Lobosa in sM. This result additionally confirms the functional role

503 played by protists within microenvironments. Moreover, Mollusca in mM and Arthropoda and

504 Anellida in sM at both soil layers can be considered as preferential binding agents for aggregate

505 fractions.

506

507 *Eukaryotic taxa predictors for soil structuring and C stocks*

508 To test if some eukaryotic taxa are predictors for soil structuring and C stock (hypothesis 3) we

509 utilised a multiple regression analysis that allowed to identify the eukaryotic taxa that were good

510 predictors for the amount of sM and mM and their SOC content, irrespective of management and

511 soil depth (Table S1). Specifically, *Microbotryomycetes* and Alveolata were moderately strongly

512 related to the amount of sM, with *Microbotryomycetes* identified as best predictor. Similarly,

513 Cercozoa and *Chytridiomycetes* were related to the amount of mM, with Cercozoa playing the

514 major role. Moreover, *Microbotryomycetes*, Cercozoa and Alevolata were moderately related to

515 SOC in sM, with *Microbotryomycetes* consistently found to be the best predictor. Finally,

516 *Chytridiomycetes* and Cercozoa were moderately related to SOC in mM, with *Chytridiomycetes* the

517 best predictor. Previously, Bach et al. (2018) identified bacterial and fungal indicators in free and

518 large macroaggregates. However, it is the first time that *Microbotryomycetes* and *Chytridiomycetes*

519 have been shown to be correlated with the amount of sM and mM and their SOC content,

520 respectively. Both fungal classes correlated with the pattern of C-cycling enzymes and SOC content

521 in bulk soil (Piazza et al., 2019), and their abundance was high in sM and mM, respectively

522 (Degrune et al., 2019b). *Microbotryomycetes* were also recently identified as good predictors of

523 slow and passive SOC decomposition parameters (Hale et al., 2019). Our findings on the positive

524 association of protists, Alveolata and Cercozoa, with the amount of sM and mM and their SOC

525 content, support the multiple agroecological roles of protists found in bulk soil (Cavalier-Smith and

526 Chao, 2003; Delgado-Baquerizo et al., 2020). Moreover, our results confirm previous works

527 reporting that protists are shaped by pore size reduction and soil aeration (Berisso et al., 2012;

528 Degrune et al., 2019a), features related to soil aggregates. Thus, we can assume that Alveolata are

529 playing major role in soil, promoting sM formation and slowing down the decay of SOM within

530 sM, while Cercozoa are crucial microorganisms in mM taking part to long-term sequestration and

531 storing of SOC.

532

533 *3.6. How eukaryotes are interlinked among each other and to prokaryotes in soil aggregates, and*

534 *network traits predictor for soil structuring and C stocks*

535 To test if eukaryotes form structured assemblages and distinctive networks in soil aggregates

536 (hypothesis 4), and how eukaryotes are linked to prokaryotes (hypothesis 5), within- and cross-

537 domain networks were built for sM and mM. At the surface layer within- and cross-domain

538 networks were more complex in mM than in sM, whereas at the subsurface layer they did not vary

539 (Table 2, Fig. 4, Fig. S6). In the cross-domain networks, both sM and mM showed a general trend

540 toward a higher percentage of eukaryotes per subnetwork compared to prokaryotes at the surface

541 layer compared with the subsurface layer (Table 3, Fig. 4b,d). Moreover, at both soil depth, in the

542 sM cross-domain networks the majority of subnetworks were composed of both eukaryotes and

543 prokaryotes, whereas in the mM cross-domain networks the subnetworks were half composed of

544 eukaryotes and half of prokaryotes, and few subnetworks were mixed (Table 3). This is the first

545 study that demonstrated that eukaryotes, components of soil biota communities usually studied

546 separately, formed structured associations within each other and with prokaryotes in soil

547 aggregates. Moreover, mM consistently had tighter connectivity compared with sM in both within-

548 and cross-domain networks (Fig. 4, Fig. S6). This might be related to microhabitat conditions (i.e.,

549 wetter and more nutrient rich microhabitats) in mM that shift biotic interactions from facilitation to

550 competition, leading to higher correlations between eukaryotic taxa or eukaryotic and prokaryotic

551 taxa, as previously reported for fungi and bacteria in bulk soil and roots across land uses and

552 agricultural managements with a gradient of nutrient availabilities (e.g., SOC, P levels) and pH (de

553 Menezes et al., 2015; Banerjee et al., 2016, 2018; Wang et al., 2021). Other explanations could be a

554 higher proportion of viable cells and spores and a lower niche heterogeneity (i.e., nutrients) in mM

555 respect to sM, leading to tighter within- and cross-domain networks. Finally, a higher plant residue

556 diversity in mM could also explain the within- and cross-domain mM network traits, as previously

557 shown for plant community composition or host selectivity against microbial network complexity

558 (Xiong et al., 2021).

559   In the within-domain networks of sM and mM and at both soil layers, Cercozoa were highly

560 co-occurring in the subnetworks respect to the other eukaryotic phyla, as shown by the network

561 traits (Table 3, Supplementary Results and Discussions 2). It is noteworthy the high percentage of

562 taxa belonging to Cercozoa (21%) involved in the largest subnetworks, composed of 33, occurring

563 at surface layer in the within-domain mM networks. In addition, while fungi, mainly Ascomycota

564 and Basidiomycota, were highly co-occurring in the within-domain sM subnetworks at both soil

565 layer, protists, as Ochrophyta and Stramenopiles together with Chlorophyta, were co-occurring in

566 the within-domain mM subnetworks at surface and subsurface layer, respectively (Table 2).

567 Following the definition of keystone taxa by Banerjee et al. (2018), we can support the

568 agroecological theory that some Cercozoa, as *Sarcomondea* and *Vampyrillidea* (Fig. 4c), can be

23

569 suggested as "hubs" (keystone taxa) within mM. Consistently, in the sM and mM cross-domain

570 networks at the surface layer, Cercozoa can be considered as "hubs" as they are directly connected

571 to prokaryotes in the largest subnetworks (Fig. 4b,d). Moreover, protists were involved in the

572 largest subnetworks and showed variable direct connections (Fig. 4b,d, Fig. S6b,d). Indeed, the

573 linkages of protists, according to their feeding versatility (Geisen, 2016), varied from direct

574 connections to many prokaryotes, mainly belonging to Chloroflexi in the cross-domain sM

575 networks (Fig. 4b, Fig. S6b), to direct linkages to other eukaryotes (e.g., fungi) or other protists in

576 mM networks (Fig. 4d, Fig. S6d). So far, studies on the functional roles of soil biota in the

577 formation of soil aggregates have mainly focused on the role played by a single functional group,

578 e.g. AMF, earthworms, nematodes, termites and microarthropods (mites and collembolans) (e.g.,

579 Lee and Foster, 1991; Pulleman et al., 2005; Rillig and Mummey, 2006; Siddiky et al., 2012a,b;

580 Zhang et al., 2016). However, only recently, Cercozoa and other protists, as Lobosa and Ciliophora,

581 were shown to be positively related with ecosystem services, i.e. nutrient cycling and OM

582 decomposition (Delgado-Baquerizo et al., 2020). Details about traits and taxa co-occurrences in

583 within- and cross-domain networks and the significance of cross-domain relationships (Mantel test

584 and CoCA) are given in Supplementary Results and Discussion 2, Fig. S7 and Fig. S8.

585   To test if some network traits can predict soil structuring and C stocks, we utilized a multiple

586 regressions analysis that identified the number of edges and mean nodes per network as predictors

587 for the amount of sM and mM and their SOC content, irrespective of management and soil depth

588 (Table S2). Although network analysis is now largely applied to study soil biota co-occurrence and

589 plant-microbe associations across different treatments (e.g., de Menezes et al., 2015; Farrer et al.,

590 2019; Feng et al., 2019), little is known about soil biota networks within aggregates (Jiang et al.,

591 2015, 2017) and few studies have dissected the predictable power of the network traits (topological

592 properties) on ecosystem services. Jiang et al. (2015, 2017) indicated that aggregate fractions (large

593 and small macroaggregates, and free microaggregates) showed a strong effect on the association

594 networks of nematodes and bacteria and using the topological properties they could identify large

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595 macroaggregates network as organized soil food web, showing functional interrelationships

596 between bacterivorous nematodes and bacteria. In accordance with our results, the topological

597 properties of soil biota networks should be taken into consideration for dissecting soil structuring as

598 well as C cycling.

599

600 **4. Conclusions**

601   We have shown that soil aggregation is essential for a complete 'multifunctional' perspective

602 of soil biota. A full understanding of relationships between soil biota and soil functions requires

603 analyses emphasizing the feedbacks between soil structure and soil biota, rather than a

604 unidirectional approach simply addressing the roles of single key functional groups. Next

605 generation sequencing tools have been confirmed in this study to be crucial in the understanding of

606 eukaryotic structures and soil biota networks and have the potential to further reveal their

607 contributions to soil functions. Indeed, our findings demonstrate for the first time that protists

608 together with fungi play major roles in soil structuring and C cycling, and that Cercozoa represent

609 hubs in the soil biota aggregate networks. This supports the fact that their conservation is

610 fundamental to prevent soil degradation and to enhance SOC accumulation in agroecosystems.

611

612 **Declaration of competing interest**

613   The authors declare that they have no known competing financial interests or personal

614 relationships that could have appeared to influence the work reported in this paper.

615

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950

951   **Figure captions**

952

953   **Fig. 1.** Long-term effect of conservation management on community composition of eukaryotes in

954   two soil matrices: small macroaggregates (sM) and occluded microaggregates (mM). Managements

955   were: MTN0 (minimum tillage and 0 kg N ha^{-1}), MTN200 (MT and 200 kg N ha^{-1}), CTN0

956   (conventional tillage and 0 kg N ha^{-1}) and CTN200 (CT and 200 kg N ha^{-1}). Neighbor-joining (NJ)

957   tree of 56 eukaryotic taxon representative sequences (classes) found in (a) sM and (d) mM. NJ trees

958   are based on the sequences obtained from the amplification of the V4 region (18 SSU rRNA gene).

959   The eukaryotic taxa were assigned to Operational Taxonomic Unit (OTU) (at class phylogenetic

960   resolution) by BLAST against the 18S SSU SILVA database by clustering sequence reads at the

961   97% similarity threshold. For each OTU, the proportion of sequences retrieved in each management

962   (MTN0, light green; MTN200, dark green; CTN0, light red; CTN200, dark red) and soil depth (0–

963   15 cm: light grey; 15–30 cm: dark grey) are shown in the pie charts. Venn diagrams of eukaryotic

964   classes uniquely retrieved and shared across managements in sM at (b) 0-15 cm and (c) at 15-30

965   cm, and in mM at (e) 0-15 cm and (f) at 15-30 cm.

966

967   **Fig. 2.** Long-term effect of conservation management on relative abundances and community

968   structures of eukaryotes in two soil matrices: small macroaggregates (sM) and occluded

969   microaggregates (mM). Managements were: MTN0 (minimum tillage and 0 kg N ha^{-1}), MTN200

970   (MT and 200 kg N ha^{-1}), CTN0 (conventional tillage and 0 kg N ha^{-1}) and CTN200 (CT and 200 kg

971   N ha^{-1}). (a) Relative abundances of eukaryotes at phylum level across treatments, matrices and soil

972   depths. (b) Principal Coordinates Analysis (PCO) biplots on the interaction of tillage and N

973   fertilization on the eukaryotic community structure at class level in sM at 0-15 cm and (c) at 15-30

974   cm, and (d) in mM at 0-15 cm. The output of the PCO biplots is based on the significant effect of

975 treatments following the permutational analysis of variance (PERMANOVA). We displayed only

976 the taxa with a strong correlation (r = 0.50-0.70) with the ordination scores on each PCO axis.

977

978 **Fig. 3.** Venn diagrams of eukaryotic phyla uniquely retrieved and shared in sM and mM at 0-15 cm

979 (a) and at 15-30 cm (b). Principal Coordinates Analysis (PCO) biplots on effect of soil matrix

980 (small macroaggregates sM *vs* occluded microaggregates mM) on eukaryotic community structure

981 at phylum resolution at (a) 0-15 cm and at (b) 15-30 cm soil depth. The PCO biplots are based on

982 the significant effect of soil matrix according to the permutational analysis of variance

983 (PERMANOVA). In the biplots, only the taxa with a strong correlation (r = 0.50-0.70) with the

984 ordination scores on each PCO axis were displayed.

985

986 **Fig. 4.** Eukaryotic networks (within-domain networks) and eukaryotic-prokaryotic networks (cross-

987 domain Associations networks) (at class phylogenetic resolution) in small macroaggregates (sM)

988 (a,b) and occluded microaggregates (mM) (c,d) at surface layer (0-15 cm soil depth). Within-

989 domain networks were built using the SParse InversE Covariance estimation for Ecological

990 ASsociation Inference (SPIEC-EASI) package version 0.1 in R

991 (https://github.com/zdk123/SpiecEasi/), while cross-domain networks were built by the cross-

992 domain extension of SPIEC-EASI (Kurtz et al., 2015; Tipton et al., 2018). Details about network

993 construction are given in Material and Methods and Supplementary Methods 3, and R scripts are

994 provided in Supplementary Methods 4.

Table 1

Results of PERMANOVA and variation partitioning of the long-term effect of conservation management (tillage and N fertilization) within small macroaggregates (sM) and occluded microaggregates (mM) and of the effect of matrix (sM *vs* mM) on eukaryotic community structure at 0-15 and 15-30 cm soil depths.

	df	Pseudo-F	P (perm)	Variance explained	df	Pseudo-F	P (perm)	Variance explained
			0-15 cm				15-30 cm	
Eukaryotes at class level - sM								
TIL[†]	1	2.24	**0.025[‡]**	**11.96**	1	4.02	**0.003**	**22.69**
N fert	1	2.11	**0.017**	**10.76**	1	2.21	**0.012**	**9.05**
TIL x N fert	1	1.99	**0.024**	**19.13**	1	2.55	**0.019**	**23.20**
PERMDISP								
TIL			0.693				0.071	
N fert			0.078				0.359	
Eukaryotes at class level - mM								
TIL	1	4.60	**0.003**	**19.94**	1	1.05	0.411	-
N fert	1	3.90	**0.002**	**16.02**	1	1.64	0.127	-
TIL x N fert	1	3.79	**0.006**	**30.85**	1	0.90	0.569	-
PERMDISP								
TIL			0.237				-	
N fert			0.362				-	
Eukaryotes at phylum level - sM vs mM								
Matrix[§]	1	1.07	**0.001**	**38.31**	1	11.18	**0.001**	**42.51**
TIL x N fert	1	2.90	**0.004**	**3.74**	1	1.22	0.287	4.51
N fert	1	1.54	0.140	10.69	1	2.36	**0.014**	**2.84**
PERMDISP								
Matrix			**0.025**				0.069	

[†]PERMANOVA was performed following a split-plot design with tillage (TIL) as main-plot factor and N fertilization (N fert) as subplot factor and with three replicate plots per treatment: TIL (minimum tillage and conventional tillage) and N fert (0 kg N ha^{-1} and 200 kg N ha^{-1}).

[‡]In bold statistically significant values (P $\leq$ 0.05).

[§]PERMANOVA was performed using the matrix as fixed factor, TIL and N fert as covaribales and 12 replicate plots per matrix.

1

Table 2
Traits of within- (eukaryotes) and cross-domain (eukaryotes - prokaryotes) network in macroaggregates (sM) and occluded microaggregates (mM) at 0-15 and 15-30 cm soil depth (for the network diagrams see Fig. 4 and S10).
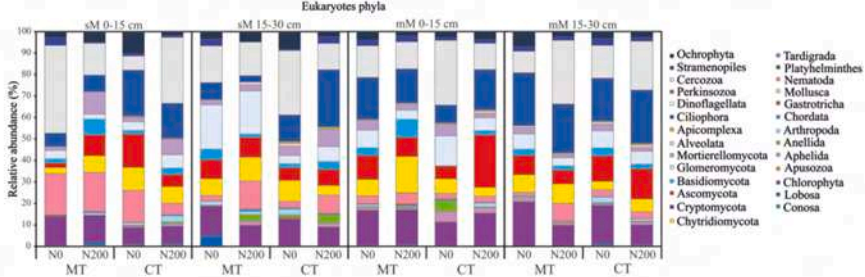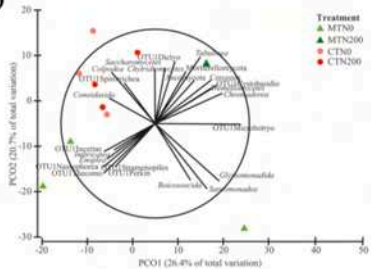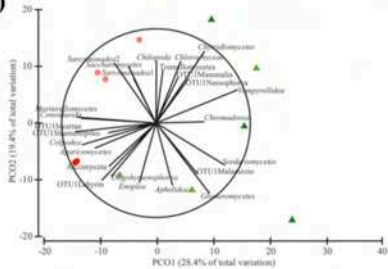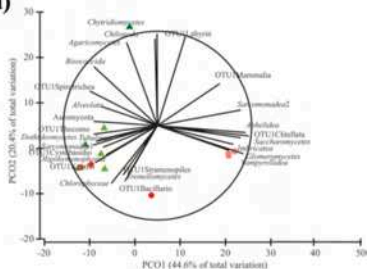
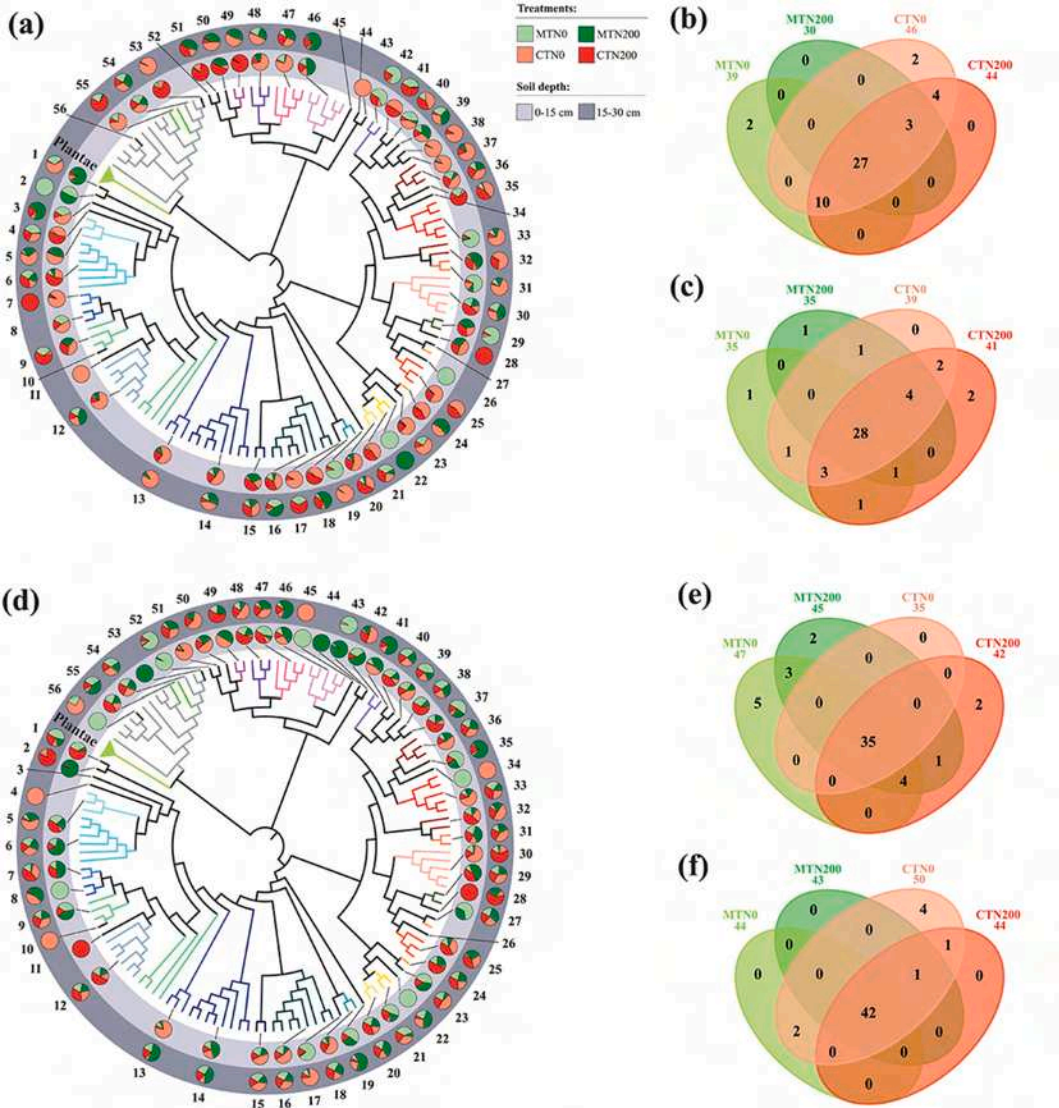| Traits	sM 0-15	sM 15-30	mM 0-15	mM 15-30
Eukaryotes				
Number of nodes excluding singletons	37	33	46	39
Number of edges	29	25	44	31
Number of singletons	12	16	8	15
Number of dyads	3	6	1	6
Number of subnetworks	6	5	4	5
Mean nodes per subnetwork	5.17 ± 0.60	4.20 ± 0.49	11.0 ± 7.34	5.40 ± 1.03
Linkage density (complexity)	1.57 ± 0.11 a	1.51 ± 0.13	1.89 ± 0.14 b	1.56 ± 0.13
% Positive interactions	78.4	75.8	95.7	79.5
Modularity	3	5	4	5
Identity of phyla with a frequency within the subnetworks ≥ 10%	Basidiomycota 15.9% Cercozoa 16.8%	Ascomycota 20% Basidiomycota 10% Cercozoa 11.7%	Cercozoa 11.6% Ochrophyta 16.7%	Chlorophyta 12% Cercozoa 21.7% Stramenopiles 16.7%
Eukaryotes - Prokaryotes				
Total number of nodes excluding singletons	79	83	96	67
Number of eukaryotic nodes excluding singletons	37	38	49	30
Number of prokaryotic nodes excluding singletons	42	45	47	37
Number of edges	74	72	109	54
Number of singletons	22	18	6	36
Number of dyads	3	8	2	5
Number of subnetworks	7	4	8	11
Mean nodes per subnetwork	10.43 ± 4.43	8.38 ± 2.74	23.50 ± 19.50	5.18 ± 0.70
Linkage density (complexity)	1.87 ± 0.11 a	1.76 ± 0.11	2.26 ± 0.12 b	1.61 ± 0.11
Modularity	9	9	11	9
Percentage of eukaryotes per subnetwork	68.39 ± 12.26	50.79 ± 12.03	74.70 ± 14.61	39.01 ± 14.69
Percentage of prokaryotes per subnetwork	31.61 ± 12.26	49.21 ± 12.03	25.30 ± 14.61	60.99 ± 14.69
Number of subnetworks with only eukaryotes	2	1	2	4
Number of subnetworks with only prokaryotes	0	1	0	5
Number of mixed subnetworks	5	6	2	2

2

Table 3

Traits of within- (eukaryotes) and cross-domain (eukaryotes - prokayotes) network in macroaggregates (sM) and occluded microaggregates (mM) at 0-15 and 15-30 cm soil depth (for the network diagrams see Fig. 4 and S10).

Traits	sM 0-15	sM 15-30	mM 0-15	mM 15-30
Eukaryotes				
Number of nodes excluding singletons	37	33	46	39
Number of edges	29	25	44	31
Number of singletons	12	16	8	15
Number of dyads	3	6	1	6
Number of subnetworks	6	5	4	5
Mean nodes per subnetwork	5.17 ± 0.60	4.20 ± 0.49	11.0 ± 7.34	5.40 ± 1.03
Linkage density (complexity)	1.57 ± 0.11 a	1.51 ± 0.13	1.89 ± 0.14 b	1.56 ± 0.13
% Positive interactions	78.4	75.8	95.7	79.5
Modularity	3	5	4	5
Identity of phyla with a frequency within the subnetworks ≥ 10% [†]	Basidiomycota 15.9% Cercozoa 16.8%	Ascomycota 20% Basidiomycota 10% Cercozoa 11.7%	Cercozoa 11.6% Ochrophyta 16.7%	Chlorophyta 12% Cercozoa 21.7% Stramenopiles 16.7%
Eukaryotes - Prokaryotes				
Total number of nodes excluding singletons	79	83	96	67
Number of eukaryotic nodes excluding singletons	37	38	49	30
Number of prokaryotic nodes excluding singletons	42	45	47	37
Number of edges	74	72	109	54
Number of singletons	22	18	6	36
Number of dyads	3	8	2	5
Number of subnetworks	7	4	8	11
Mean nodes per subnetwork	10.43 ± 4.43	8.38 ± 2.74	23.50 ± 19.50	5.18 ± 0.70
Linkage density (complexity)	1.87 ± 0.11 a	1.76 ± 0.11	2.26 ± 0.12 b	1.61 ± 0.11
Modularity	9	9	11	9
Percentage of eukaryotes per subnetwork	68.39 ± 12.26	50.79 ± 12.03	74.70 ± 14.61	39.01 ± 14.69
Percentage of prokaryotes per subnetwork	31.61 ± 12.26	49.21 ± 12.03	25.30 ± 14.61	60.99 ± 14.69
Number of subnetworks with only eukaryotes	2	1	2	4
Number of subnetworks with only prokaryotes	0	1	0	5
Number of mixed subnetworks	5	6	2	2

[†] For details about the traits of within-domain networks for each eukaryotic phylum occurring in sM and mM at both soil depths see Table S5.
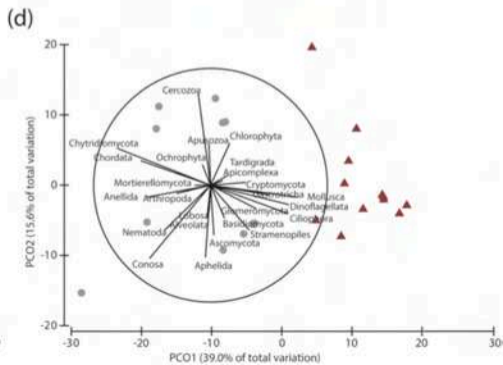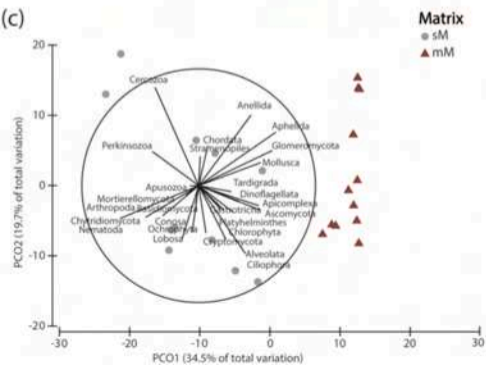
(a)

Eukaryotes phyla

sM 0-15 cm   sM 15-30 cm   mM 0-15 cm   mM 15-30 cm

Legend:
- Ochrophyta
- Stramenopiles
- Cercozoa
- Perkinsozoa
- Dinoflagellata
- Ciliophora
- Apicomplexa
- Alveolata
- Mortierellomycota
- Glomeromycota
- Basidiomycota
- Ascomycota
- Cryptomycota
- Chytridiomycota
- Tardigrada
- Platyhelminthes
- Nematoda
- Mollusca
- Gastrotricha
- Chordata
- Arthropoda
- Anellida
- Aphelida
- Apusozoa
- Chlorophyta
- Lobosa
- Conosa

(b) (c) (d)

Treatment: MTN0, MTN200, CTN0, CTN200

Eukaryotic taxa:

1. *Cystobasidiomycetes*
2. *Microbotryomycetes*
3. *Malasseziomycetes*
4. *Exobasidiomycetes*
5. *Tremellomycetes*
6. *Agaricomycetes*
7. *Dothideomycetes*
8. *Eurotiomycetes*
9. Ascomycota
10. *Lecanoromycetes*
11. *Pezizomycetes*
12. *Sordariomycetes*
13. *Saccharomycetes*
14. *Chytridiomycetes*
15. Mortierellomycota
16. *Glomeromycetes*
17. Cryptomycota
18. *Aphelidea*
19. *Thecomonadea*
20. Colpodea
21. *Oligohymenophorea*
22. *Nassophorea*
23. *Spirotrichea*
24. Alveolata
25. *Conoidasida*
26. *Perkinsea*
27. *Dinophyceae*
28. Dictyostelia
29. *Tubulinea*
30. *Sarcomonadea1*
31. *Sarcomonadea1*
32. Cercozoa
33. *Sarcomonadea2*
34. *Thecofilosea*
35. Incertae sedis
36. *Imbricatea*
37. Bacillariophyceae
38. *Xanthophyceae*
39. *Chrysophyceae*
40. Stramenopiles
41. *Labyrinthulomycetes*
42. *Boicosoecida*
43. *Gastrotricha*
44. *Rhabditophora*
45. *Eutardigrada*
46. *Chromadorea*
47. *Enoplea*
48. *Arachnida*
49. Chilopoda
50. *Mammalia*
51. *Clitellata*
52. *Gastropoda*
53. *Ulvophyceae*
54. *Chlorophyceae*
55. *Trebouxiophyceae*
56. Chlorophyta

Eukaryotic taxa:

1. *Dictyostelia* - Conosa
2. *Tubulinea* - Lobosa
3. Chlorophyta
4. *Chlorophyceae* - Chlorophyta
5. *Ulvophyceae* - Chlorophyta
6. *Trebouxiophyceae* - Chlorophyta
7. *Thecomonadea* - Apusozoa
8. *Aphelidea* - Aphelida
9. *Clitellata* - Annelida
10. *Arachnida* - Arthropoda
11. *Chilopoda* - Arthropoda
12. *Mammalia* - Chordata
13. *Enoplea* - Nematoda
14. *Gastrotricha*
15. *Chromadorea* - Nematoda
16. *Gastropoda* - Mollusca
17. *Rhabditophora* - Platyhelminthes
18. *Eutardigrada* - Tardigrada
19. *Chytridiomycetes* - Chytridiomycota

20. Cryptomycota
21. Ascomycota
22. *Dothideomycetes* - Ascomycota
23. *Eurotiomycetes* - Ascomycota
24. *Lecanoromycetes* - Ascomycota
25. *Pezizomycetes* - Ascomycota
26. *Sordariomycetes* - Ascomycota
27. *Saccharomycetes* - Ascomycota
28. *Agaricomycetes* - Basidiomycota
29. *Tremellomycetes* - Basidiomycota
30. *Cystobasidiomycetes* - Basidiomycota
31. *Microbotryomycetes* - Basidiomycota
32. *Exobasidiomycetes* - Basidiomycota
33. *Glomeromycetes* - Glomeromycota
34. *Mortierellomycetes* - Mortierellomycota
35. Alveolata
36. *Conoidasida* - Apicomplexa
37. *Colpodea* - Ciliophora
38. *Nassophorea* - Ciliophora

39. *Oligohymenophorea* - Ciliophora
40. *Spirotrichea* - Ciliophora
41. *Dinoflagellata*
42. *Sarcomonadea1* - Cercozoa
43. *Sarcomonadea2* - Cercozoa
44. *Imbricatea* - Cercozoa
45. *Incertae sedis* - Cercozoa
46. *Cercozoa* - Cercozoa
47. *Thecofilosea* - Cercozoa
48. *Vampyrellidea* - Cercozoa
49. Stramenopiles
50. *Bicosoecida* - Cercozoa
51. *Labyrinthulomycetes* - Stramenopiles
52. *Chrysophyceae* - Stramenopiles
53. *Bacillariophyceae* - Ochrophyta
54. *Xanthophyceae* - Ochrophyta
55. *Malasseziomycetes* - Basidiomycota
56. *Perkinsea* - Perkinsozoa

Prokaryotic taxa:

57. *Alphaproteobacteria* - Proteobacteria
58. *Deltaproteobacteria* - Proteobacteria
59. *Gammaproteobacteria* - Proteobacteria
60. *Betaproteobacteria* - Proteobacteria
61. *Blastocatellia* - Acidobacteria
62. Acidobacteria
63. *Holophagae* - Acidobacteria
64. Chlamydiae
65. *Clostridia* - Firmicutes
66. *Bacilli* - Firmicutes
67. Gemmatimonadetes
68. Cloacimonetes
69. *Spirochaetia* - Spirochaetes
70. *Deinococci* - Deinococcus Thermus
71. *Anaerolineae* - Chloroflexi
72. *Dehalococcoidia* - Chloroflexi
73. Chloroflexi
74. *Ardenticatena* - Chloroflexi

75. *Caldilineae* - Chloroflexi
76. *Ktedonobacteria* - Chloroflexi
77. *Chloroflexia* - Chloroflexi
78. *Thermomicrobia* - Chloroflexi
79. *Elusimicrobia* - Elusimicrobia
80. *Thermoleophilia* - Actinobacteria
81. Actinobacteria 1
82. *Rubrobacteria* - Actinobacteria
83. *Acidimicrobia* - Actinobacteria
84. *Nitriliruptoria* - Actinobacteria
85. Actinobacteria 2
86. *Verrucomicrobia* - Verrucomicrobia
87. Verrucomicrobia
88. *Spartobacteria* - Verrucomicrobia
89. *Phycisphaerae* - Planctomycetes
90. Planctomycetes
91. *Planctomycetacia* - Planctomycetes
92. Omnitrophica

93. Saccharibacteria
94. Parcubacteria
95. Peregrinibacteria
96. Cyanobacteria
97. *Chlorobia* - Chlorobi
98. *Ignavibacteria* - Chlorobi
99. *Cytophagia* - Bacteroidetes
100. Bacteroidetes
101. *Flavobacteria* - Bacteroidetes
102. *Sphingobacteria* - Bacteroidetes
103. Candidatus Berkelbacteria
104. Microgenomates
105. *Methariobacteria* - Euryarchaeota
106. *Methanomicrobia* - Euryarchaeota
107. *Thermoplasmata* - Euryarchaeota
108. Thaumarchaeota

Highlights

- Eukaryote diversity responded differently to managements across soil aggregates

- A core community of eukaryotes was found across managements, aggregates and depths

- Tillage shifted eukaryotic structure in sM and mM according to N availability

- Protists and fungi positively correlated with the amount of sM, mM and SOC content

- Within- and cross-domain networks of mM at 0-15 cm showed the highest complexity

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: