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Title: Out of sight : Profiling soil characteristics, nutrients and bacterial communities affected by organic amendments down to one meter in a long-term maize experiment

Year: 2019

Version: Accepted version (Final draft)

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Please cite the original version:

Sandéna, T., Zavattaro, L., Spiegel, H., Grignani, C., Sandén, H., Baumgarten, A., Tirola, M., & Mikkonen, A. (2019). Out of sight : Profiling soil characteristics, nutrients and bacterial communities affected by organic amendments down to one meter in a long-term maize experiment. *Applied Soil Ecology*, 134, 54-63. <https://doi.org/10.1016/j.apsoil.2018.10.017>

1 **Out of sight: Profiling soil characteristics, nutrients and bacterial communities affected by**
2 **organic amendments down to one meter in a long-term maize experiment**

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14 **Abstract**

15 Common soil characteristics, nutrients and microbial activity at deeper soil depths are topics seldom
16 covered in agricultural studies. Biogeochemical cycles in deep soils are not yet fully understood. This
17 study investigates the effect of different mineral and organic fertilisation on soil organic matter
18 dynamics, nutrients and bacterial community composition in the first meter of the soil profiles in the
19 long-term maize cropping system experiment Tetto Frati, near the Po River in northern Italy. The
20 following treatments have been applied since 1992: 1) crop residue removal (CRR), 2) crop residue
21 incorporation (CRI), 3) crop residue removal with bovine slurry fertilisation (SLU), 4) crop residue
22 removal with farmyard manure fertilisation (FYM). A total of 250 kg N ha⁻¹ were applied annually as
23 mineral fertiliser in the first two and as organic fertilizer in the latter two treatments. Soil organic carbon
24 (SOC) was significantly higher in the treatments with organic amendments (CRI, SLU and FYM)
25 compared to CRR in 0-25 cm (11.1, 11.6, 14.7 vs. 9.8 g kg⁻¹, respectively), but not in the deeper soil. At
26 75-100 cm soil depth, SLU and FYM had the highest potential N mineralisation. Bacterial diversity
27 decreased down the soil profile much less than microbial biomass. Incorporation of crop residues alone
28 showed no positive effects on either biomass or diversity, whereas fertilisation by FYM instead of
29 mineral fertilizer did. Bacterial community composition showed depth-related shifts: Proteobacteria and
30 Actinobacteria dominated the topsoil, whereas Chloroflexi, Nitrospira and Thermotogae were relatively
31 more abundant deeper in the soil profile. Although the main factor determining soil bacterial community
32 composition in the entire dataset was soil depth, both the size and diversity of bacterial community, as
33 well as several discriminating taxa, were affected by organic N fertilisation down to 1 m depth. This
34 calls for continued efforts to study the deeper soil depths in the numerous long-term field experiments,
35 where mostly topsoils are currently studied in detail.

36 **Keywords:** deep soil, soil microbiome, organic amendments, farmyard manure fertilisation, long-term
37 experiment, bovine slurry fertilisation

38 **1. Introduction**

39 Closing nutrient cycles in agriculture by utilizing alternative fertiliser sources including crop residues,
40 bovine slurry and farmyard manure is needed in order to sustain the functioning of agricultural soils, to
41 maintain long-term fertility and to become less dependent on external mineral fertilisers. The
42 management practices applied strongly affect a soil's ability to produce biomass and subsequently to
43 provide other soil functions including carbon sequestration and climate regulation, water purification
44 and regulation, nutrient cycling and provision of habitat for biodiversity (Schulte et al., 2014). The
45 microbial community and its diversity play a key role in soil functioning (Schulte et al., 2014). The
46 focus of agricultural soil studies mainly lies within the most active, tilled topsoils (Sandén et al., 2018).
47 Accordingly, the functioning of the deep soils that are less explored by crop roots is a topic not often
48 covered in agriculture. Such soils are also more time consuming to sample. Soils are one of the most
49 biologically diverse habitats on Earth (Bender et al., 2016), even though the diversity and ecological
50 function of deep soils remain largely unknown. A key question is how microbial communities down the
51 soil profile are affected by agricultural management. Nevertheless, deep soils do matter (Harper and
52 Tibbet, 2013), even if their function and dynamics are not yet fully understood (Rumpel and Kögel-
53 Knabner, 2011). SOM in deep soils originates mainly from dissolved organic carbon, root products, and
54 transported particulates from the topsoils (Rumpel and Kögel-Knabner, 2011). Subsoils can contribute
55 up to more than half of the total soil C stocks, as was recently confirmed in England and Wales (Gregory
56 et al., 2014). Thus, their role, e.g. in C sequestration, should not be underestimated (Rumpel and Kögel-
57 Knabner, 2011). Molecular structure alone does not determine the stability of SOM; rather, physical
58 connections and disconnection between microorganisms and SOM control how much is stabilized in the
59 soil (Schmidt et al., 2011). The role of microorganisms in SOM dynamics has been highlighted in recent
60 literature (Liang et al., 2017), showing that the total amount of carbon in soils that has cycled through
61 the living biomass is far greater than that currently in the living microbial biomass. This microbially
62 derived carbon may become very stable upon sorption to mineral surfaces, incorporation into organo-
63 mineral complexes, or when inaccessible to microorganisms due to physical barriers (Liang et al., 2017,
64 and references therein).

65 The long-term effects of crop residue incorporation, bovine slurry, and farmyard manure (FYM) have
66 been comprehensively studied (e.g. Lehtinen et al., 2014; Poeplau et al. 2015, 2017; Zavattaro et al.,
67 2017 and references therein). These management practices have been shown to increase the topsoil
68 organic carbon (SOC) contents, whereby the responses range from a 7% increase with incorporation of
69 crop residues (Lehtinen et al., 2014) to more than a 30% increase with FYM amendments (Zavattaro et
70 al., 2017). For total nitrogen the response has ranged between 2% for crop residue incorporation (Sandén
71 et al., 2018) and circa 20% for FYM amendments (Zavattaro et al., 2017). How these management
72 practices affect other soil quality attributes and soil microbiology, especially at deeper soil depths,
73 remains to be answered.

74 This study was designed to investigate the effects of different mineral and organic fertilisation on soil
75 organic matter dynamics, nutrients as well as the bacterial community composition down to 1 m depth
76 in a maize cropping system. Specifically, our objective was to disentangle how crop residue removal
77 (CRR) vs. crop residue incorporation (CRI), or application of bovine slurry (SLU) or farmyard manure
78 (FYM) affect the above-mentioned properties. Our specific research questions were: (i) To which depth
79 do organic amendments influence soil chemical characteristics and the soil bacterial community? and
80 ii) Which bacterial groups are affected by organic amendments in different soil depths? To answer these
81 questions we utilized the long-term experiment Tetto Frati, near the Po River in northern Italy. This site
82 has a known management history since 1992 and features management practices representing the local
83 agricultural situation.

84 **2. Materials and methods**

85 **2.1 Scene setting**

86 The long-term platform Tetto Frati at the Experimental Center of the University of Turin in NW Italy
87 (44°53'N 07°41'E) started in 1992. The site, soil and treatments have previously been described by
88 Grignani et al. (2007), Bertora et al. (2009a, 2009b) and Zavattaro et al. (2012, 2016). In brief, the site
89 is located in the western area of the River Po plain at 229 m.a.s.l. It is characterized by a deep, loamy,
90 calcareous and scarcely weathered alluvial soil. The mean annual precipitation is 792 mm with two main

91 rainy seasons (April-May and September-November) and the mean annual temperature is 11.8°C. The
92 long-term experiment is based on a randomised block design with three replicates cultivated with maize
93 (*Zea mays* L.), resulting in a total of 38 combinations of fertilisation, crop residue and rotation
94 management. For this study, we selected the following treatments (each treatment plot measuring 75
95 m²):

- 96 (1) Maize for silage with 250 kg mineral N ha⁻¹ (crop residue removal, CRR)
- 97 (2) Maize for grain with 250 kg mineral N ha⁻¹ (crop residue incorporation, CRI)
- 98 (3) Maize for silage with 250 kg bovine slurry N ha⁻¹ (SLU)
- 99 (4) Maize for silage with 250 kg farmyard manure N ha⁻¹ (FYM)

100 Plots also received mineral phosphorous (P) and potassium (K) fertilisation according to national
101 fertilisation practice. All fertilisers and manures were distributed in spring before tillage activities. For
102 more details of the treatments, see Table 1.

103 **2.2 Soil sampling**

104 Composite soil samples of 10-15 individual soil cores were collected in March 2015 from three field
105 replicates of each investigated treatment, from four depths in the first meter (0-25 cm corresponding to
106 the tillage depth, 25-50 cm, 50-75 cm, 75-100 cm) of the soil profiles. Soils were sieved through a 2 mm
107 stainless sieve in Italy and aliquots shipped to Austria for biochemical characterization and to Finland
108 for DNA-based analyses. The latter were kept at -20°C until extraction, whereas the former were air-
109 dried prior to further analyses, with the exception of soil samples for substrate induced respiration, which
110 were kept at 4°C.

111 **2.3 Soil chemical characteristics**

112 Total soil organic C concentrations of the soil samples were analysed by dry combustion in a LECO
113 RC-612 TruMac CN (LECO Corp., St. Joseph, MI, USA) at 650°C (ÖNORM L1080). KMnO₄
114 determination of labile carbon was analysed according to Tatzber et al. (2015). Total N was determined
115 according to ÖNORM L1095 with elemental analysis using a CNS (carbon, nitrogen, sulfur) 2000 SGA-

116 410–06 at 1250°C. Potential nitrogen mineralization was measured by the anaerobic incubation method
117 (Keeney, 1982), as modified according to Kandeler (1993). Soil pH was measured electrochemically
118 (pH/mV Pocket Meter pH 340i, WTW, Weilheim, Germany) in 0.01 M CaCl₂ at a soil-to-solution ratio
119 of 1:5 (ÖNORM L1083). Carbonate content was measured gas-volumetrically (CO₂ evolution; ÖNORM
120 L1084). Plant available phosphorous (P) and potassium (K) were determined by calcium-acetate-lactate
121 (CAL) extraction (ÖNORM L1087). Substrate induced respiration using glucose as a substrate was
122 carried out using the MicroResp method according to Campbell et al. (2003) and the MicroResp manual.

123 **2.4 DNA extraction and high-throughput amplicon sequencing of bacterial 16S rRNA gene**

124 DNA was extracted with the MoBio Powerlyzer PowerSoil DNA Isolation Kit (MoBio Laboratories,
125 Carlsbad, CA, USA) according to kit instructions from 0.25 g (\pm 0.01 g) soil. Beat beating was done with
126 FastPrep FP120 (MP Biomedical) at 4 ms⁻¹ for 45 s. Two to four ng of soil DNA, quantified with Quant-
127 IT PicoGreen® dsDNA Assay Kit (Invitrogen), was used as a template in the amplification of the V1-
128 V2 fragment of the 16S rRNA gene with universal bacterial primers 27r
129 (AGAGTTTGATCMTGGCTCAG) and 338r (TGCTGCCTCCCGTAGGAGT). The PCR reaction of
130 25 µl consisted of Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific), 0.4 µM of
131 each primer (Sigma Aldrich), and 0.02% Bovine Serum Albumin (Thermo Scientific). Thermal cycling
132 consisted of 10 min initial denaturation at 95 °C, followed by 30 cycles of 95 °C for 30 s, 52 °C for 30
133 s and 72 °C for 60 s, and was conducted on the Bio-Rad CFX96 Real-Time System (Bio-Rad
134 Laboratories). Two µl of the product were used as a template in a second PCR, where Ion Torrent PGM
135 sequencing adapters and barcodes (IonA_IonXpressBarcode_27f and P1_338r) were added to the ends
136 in five additional cycles with conditions otherwise identical to the first amplification. Products were
137 purified with Agencourt AMPure XP (Beckman Coulter Life Sciences, Indianapolis, IN, USA),
138 quantified with Quant-IT PicoGreen dsDNA Assay Kit (Invitrogen), and pooled in equimolar quantities
139 for 400 bp library sequencing on an Ion Torrent PGM. The sequencing template was prepared with the
140 Ion PGM Hi-Q OT2 Kit and sequenced with the Ion PGM Hi-Q Sequencing Kit on Ion 316 Chip v2 (all
141 Life Sciences, Thermo Fisher Scientific).

142 **2.5 Sequence data and statistical analyses**

143 Sequence data was analysed with mothur v.1.36 (Schloss et al., 2009) following roughly the Standard
144 Operating Procedure for 454 amplicon data (Schloss et al., 2011). Sequences were trimmed allowing a
145 maximum 1 nucleotide (nt) difference with primer and barcode, maximum homopolymer length of 8 nt,
146 no ambiguous nt, minimum average quality of 20 in a rolling window of 10 nt, and minimum remaining
147 length of 200 nt. Sequences were aligned using the Silva v. 119 database, and chimeras were searched
148 and removed with the default commands (de novo chimera search with chimera.uchime). Remaining
149 good-quality sequences were classified against the Silva v. 119 database (Quast et al., 2013) using the
150 mothur implementation of the "Bayesian" classifier with 1000 iterations, and "contaminating" sequences
151 were removed (chloroplasts, mitochondria, Archaea, Eukaryota, and sequences not classified on the
152 Kingdom level). The number of remaining bacterial sequences, on which the taxonomic proportions are
153 based, varied from 8530 to 17119 sequences per sample.

154 Unique sequences (148 063) were clustered into operational taxonomic units (OTUs) at the 97%
155 similarity level, OTUs with only a single sequence in the entire dataset were discarded, and the
156 remaining OTU table was rarefied to an equal number of observations per sample (8093) before
157 calculation of OTU-based alpha diversity estimates. OTUs were classified and their representative (most
158 abundant) sequence was identified.

159 A phylogenetic tree was constructed from the unique sequences (148063) using the mothur
160 implementation of Clearcut (Evans et al., 2006), based on which phylogenetic diversity and weighted
161 UniFrac dissimilarity matrix (Lozupone et al., 2007) were calculated at a uniform sampling depth of
162 8530 sequences. Based on the weighted UniFrac dissimilarity matrix, multivariate unconstrained
163 ordination and generalised discriminant analysis (Anderson and Robinson, 2003) were done with
164 CAP12 (Anderson, 2004). Primer 6.1.12 (PRIMER-E Ltd) was used to calculate Spearman Mantel
165 correlations of distance matrices to connect univariates (Euclidian distances of square-root transformed
166 variables) to multivariate community data (weighted UniFrac dissimilarities).

167 For Sunburst taxonomic visualizations and identification of discriminating OTUs, sequence data were
168 imported to CLC Genomics Workbench v. 9.5.1 (Qiagen). Primer, quality and length-based (>200bp)
169 trimming were done with the default options. Sequences automatically trimmed to the same length (272
170 bp) were clustered into OTUs at the 97% similarity level based on Silva v.123 (99%) and Greengenes

171 v. 13.5 (99%) reference taxonomies, using default chimera removal and allowing formation of new
172 OTUs. “Contaminating” sequences were filtered out as in mothur, resulting in taxonomic classifications
173 based on 6325-11238 sequences per sample. Differential Abundance Analysis for OTUs discriminating
174 the different treatments was done with the Silva-based OTU table with the CLC defaults settings, with
175 correction for soil depth and multiple comparisons (Bonferroni correction). Classification of the
176 discriminating candidate taxa with spurious higher-level classification was checked with the SINA
177 Online Aligner (Pruesse et al., 2012) based on Silva v.128 and Greengenes v. 13.5 reference taxonomies
178 with relaxed Search and classify criteria (minimum identity 90, 5 neighbors). Bacterial 16S rRNA gene
179 sequences with MIMARKS details can be found in NCBI Sequence Read Archive under BioProject
180 NNNN (pending). No modelling of results was done in this study.

181 Univariate statistical analyses were performed using the IBM SPSS Statistics 20 software package. The
182 normality of data was checked with the Shapiro-Wilk’s test. The effects of the different treatments were
183 investigated with analysis of variance with Tukey’s significance test ($p < 0.05$) as a post hoc test and
184 the difference between inorganic and organic N fertilisation with the t-test. Correlations between
185 variables were tested with Pearson correlation.

186 **3. Results**

187 **3.1 Soil chemical characteristics**

188 SOC concentration and labile C decreased down the soil profile (Table 2). However, only for SOC were
189 significant differences recorded between the mineral N and organic N application treatments (0-25 cm,
190 50-75 cm and 75-100 cm soil depth). In 0-25 cm soil depth the SOC concentrations were significantly
191 higher in SLU and FYM treatments compared to crop residue removal. The total N concentration in 0-
192 25 cm soil depth was significantly higher in FYM compared to CRR and SLU (on average 1.70 g kg^{-1} ,
193 1.43 g kg^{-1} and 1.40 g kg^{-1} , respectively). The C/N ratio also decreased down the soil profile, being
194 significantly higher in the organic N application treatments than in the mineral N application rates at
195 each soil depth. Soil pH, as well as CaCO_3 , increased with depth. The differences between the mineral
196 N and organic N application treatments were significant in 50-75 cm soil depth for both characteristics,
197 and in 0-25 cm depth only for CaCO_3 . CAL-extractable phosphorous and potassium decreased with

198 depth, CAL-extractable phosphorous being under the detection limit below 50 cm in all treatments
199 except in FYM. The differences between individual treatments in CAL-extractable phosphorous and
200 potassium were significant at all soil depths for potassium and at 0-25 cm and 25-50 cm for phosphorous.
201 The highest concentrations were always observed in the FYM treatment. The carbon and nitrogen
202 characteristics (SOC, labile C, N, C/N ratio, potential N mineralization, substrate induced respiration)
203 as well as CAL-extractable nutrients all correlated significantly negatively with soil pH and CaCO₃ and
204 significantly positively with one another in the whole data set (S1). Significant correlations decreased
205 down the soil profiles, but even at 75-100 cm positive correlations between potential N mineralization,
206 SOC, C/N ratio and substrate induced respiration were observed (S1).

207 **3.2 Soil bacterial community size, diversity and structure**

208 The effect of soil depth on microbial biomass and genetic diversity (Figure 1) as well as community
209 composition (Figure 2) was notable. Microbial biomass – substrate-induced respiration (SIR) and DNA
210 yield – decreased logarithmically down the soil profile. In contrast, the diversity of the bacterial
211 communities was surprisingly high also at deeper depths: operational taxonomic unit (OTU) richness
212 decreased in the profiles on average by 24% (range 13-34%) and phylogenetic diversity (PD) only by
213 19% (range 10-31%), whereas both biomass estimators decreased on average by 88% one meter down
214 the soil profiles (DNA yield by 61-97%, SIR by 81-95%). In topsoils, both organic N application
215 treatments increased SIR compared with CRR (Tukey's post-hoc test $p=0.01$ for both). Crop residue
216 incorporation alone showed no positive effect on bacterial abundance or diversity (Tukey's post-hoc test
217 $p>0.05$ for CRR and CRI for all biomass and diversity measures), whereas the treatments with organic
218 N application did (Figure 1). The highest values were typically recorded in FYM, which significantly
219 increased DNA yield at 50-75 cm and SIR at 75-100 cm compared to both mineral N application
220 treatments (Tukey's post-hoc test $P<0.05$).

221 The genetic diversity of the bacterial community correlated positively with biomass (SIR or DNA yield
222 vs. Richness or PD: $r>0.64$, $p<0.001$ for all). Higher biomass thus did not reduce diversity by favouring
223 just a few positively selected taxa. This finding did not reflect analytical bias (less diversity in less
224 genetic material) because a similar amount of template (in ng DNA) was used in all PCR

225 amplifications. The correlations were also positive in the deepest soil depth alone (SIR vs. PD, DNA vs.
226 PD and DNA vs. Richness: $r > 0.60$, $p < 0.05$ for all).

227 Soil depth was also the dominating discriminating factor in sequence-based unconstrained ordination of
228 the samples (principle coordinate analysis for weighted Unifrac distances) (Figure 2A). The average
229 weighted Unifrac distance from 0-25 cm to 75-100 cm bacterial community in one profile was 35 (range
230 29-38), whereas the average distance between any two topsoil samples was 18 (range 13-27).
231 Differences in the bacterial community structure also correlated with the measured depth-dependent
232 univariates, including soil pH, TOC, labile C, and DNA yield (Mantel correlation Spearman $\rho = 0.83$,
233 0.83, 0.74 and 0.84, respectively, at $p = 0.0001$). Bacterial community composition at each soil depth,
234 averaged from the four treatments, is shown in Figure 3. The soil depths were notably similar on higher
235 taxonomic levels as well, except for the classes Alphaproteobacteria and Actinobacteria being
236 systematically relatively more abundant in topsoil than at the deepest depth (ranges 18-23% vs. 7-11%,
237 and 6-16% vs. 2-6%, respectively), and the phyla Chloroflexi, Nitrospirae and Thermotogae being
238 systematically relatively less abundant in topsoil than at 75-100 cm (ranges 5-9% vs. 12-17%, 2-5% vs.
239 8-15%, and 0% vs. 2-6%, respectively).

240 The effect of treatments on bacterial community composition was smaller than the effect of soil depth
241 but still significant (generalised discriminant analysis for weighted Unifrac distances $p = 0.0001$) (Figure
242 2B). Treatments with inorganic and organic N sources differed from each other at $p = 0.0001$ and mis-
243 classification error of only 4.2%. This effect of N source was observed, with discriminant analysis
244 $p \leq 0.05$ and mis-classification error $\leq 20\%$, in all soil depths except for 25-50 cm. The three field blocks,
245 in contrast, did not host distinct bacterial communities (discriminant analysis $P = 0.064$, mis-
246 classification error 45.8%).

247 **3.3 Bacterial taxa discriminating between amendments**

248 Differential abundance analysis for treatments (with correction for soil depth) was done to identify
249 OTUs that discriminate the organic amendments. After Bonferroni correction, no OTUs were detected
250 that differed between the two treatments with inorganic N fertilisation (CRR and CRI). Interestingly,
251 the OTUs that discriminated inorganic N application treatments (CRR and CRI) from organic N
252 application treatments (SLU and FYM) were typically the same for all four treatment pairs. Based on

253 these discriminating OTU classifications, taxa with differential abundance were retrieved from full
254 taxonomy tables. Curiously, most of the differences between treatments were detected in the two deeper
255 soil depths, not in topsoil (Figure 4).

256 Both candidate taxa that showed higher relative abundance in treatments with organic N fertilizer, wb1-
257 A12 and FTL22, were classified as members of Nitrospirae by the Silva v. 119 reference database.
258 However, when checking the classification of the OTU representative sequences of all the non-singleton
259 OTUs, each of them was, based on the more updated nomenclature of the Greengenes v. 13.5 taxonomy,
260 classified as a member of the NC10 candidate phylum. The relative abundance of candidate phylum
261 NC10 increased down the four depth ranges, from an average 0% to 1% to 2% to 4%, but the increase
262 was attributed mostly to treatments with organic N fertiliser (Figure 4C,D). OTUs relatively more
263 abundant in treatments with inorganic N fertiliser included two groups with no cultivated
264 representatives, GAL15 and P2-11E. The relative abundance of these groups also increased down the
265 soil profile, but much more in SLU and FYM than in CRR and CRI (Figure 4A,B). The two taxa that
266 discriminated SLU from all other treatments showed different depth profiles (Figure 4E,F). *Bacillaceae*
267 were notably more abundant in soil fertilised with bovine slurry than any other treatment, but only in
268 topsoil. Actinobacterial *Gaiellales*, in contrast, increased down the profile in other treatments but were
269 less abundant at 75-100 cm in SLU.

270 **4. Discussion**

271 **4.1 Soil chemical characteristics**

272 Fertilisation with mineral fertilisers is widely practiced, even though organic fertilisers are also valuable
273 sources of nutrients (Zavattaro et al., 2017). Intensive agriculture with regular tillage, removal of crop
274 residues and lack of organic amendments can led to SOM depletion (Franko and Spiegel, 2016; Spiegel
275 et al., 2018), degraded soil structure (compaction, poorer infiltration and aeration) and poorer nutrient
276 cycling potential of agricultural soils. The effect of organic versus mineral nitrogen input in the current
277 study was detected for several soil biochemical characteristics. SOC concentrations were higher under
278 organic nitrogen input, except at 25-50 cm depth. This is in line with Zavattaro et al. (2017), who
279 compared organic amendments with mineral inputs in a review of 80 long-term experiments. They

280 showed that organic amendments with or without additional mineral fertilisation across Europe
281 significantly increased the SOC concentrations in the topsoils. The higher SOC contents in organic
282 treatments in the deeper soil depths, observed in the current study, is potentially explained by increased
283 fresh carbon input by preferential flow and/or increased root growth or root depositions (Chabbi et al.,
284 2009). In contrast to other agricultural studies (Blair et al., 2006; Tatzber et al., 2015), we did not detect
285 any significant differences in labile C in any of the studied soil depths, most likely due to large variation.
286 Total N expectedly showed no differences between the treatments except for higher N at FYM at 0-25
287 cm depth, given that all treatments were fertilised with similar amounts of N, even though in different
288 forms. Due to the increases in SOC contents, the C/N ratios also differed significantly between the
289 organic and mineral treatments at all depths. Both soil pH and CaCO₃ increased down the profiles, as
290 expected, due to geogenic conditions. Interestingly, differences in soil pH and CaCO₃ between the
291 organic and mineral nitrogen treatments were significant at 50-75 cm depth, indicating differences in
292 the leaching patterns in these treatments or higher CaCO₃ input by organic fertilisers. The FYM
293 treatment yielded the in highest CAL-extractable P and K, and this was the only treatment in which
294 CAL-extractable P was detected below 50 cm. This agrees well with Vanden Nest et al. (2016), who
295 showed that P from farmyard manure was more available as well as more prone to leaching compared
296 to compost. Those authors explained this by observed decreases in orthophosphate sorption in farmyard
297 manure amended soils. This may also be connected to enhanced microbial activity in the FYM treatment
298 (Chen et al., 2003). Nonetheless, differences in the CAL-extractable P concentration have also been
299 linked to a different surplus between treatments (Borda et al., 2011).

300 **4.2 Soil bacterial community size, diversity and structure**

301 Little is still known about the factors shaping depth gradients in microbial community structure (Eilers
302 et al., 2012). Even if the main root biomass of most crops does not penetrate below the ploughed and
303 typically sampled top 30 cm of soil, microbes below this depth, impacted by agricultural practices, still
304 play a role in controlling nutrient leaching to aquifers and in the production of greenhouse gasses (e.g.
305 Butterbach-Bahl et al., 2013). In the current study, we focused sequencing efforts on the bacterial part
306 of the microbial community, as bacteria are the most abundant and metabolically active microbial
307 kingdom in tilled agricultural soils. Bacteria are also the kingdom of choice to track vertical

308 compositional changes in soil microbial community; fungi may be abundant in surface soils but not at
309 anaerobic depths, and especially methanogenic archaea can be relatively active in deeper depths but
310 typically form minority of prokaryotic community in surface soils.

311 We observed the effect of sampling depth to surpass the effect of organic amendments in terms of
312 bacterial community size and diversity. Biomass declined logarithmically in the 1-meter depth profile,
313 decreasing even more (on average by 90%) than SOC (on average by 70%) or labile C (on average by
314 80%). As the C/N ratio decreased down the profile, indicating relatively more available N resources, the
315 buildup of biomass deeper in the soil was constrained, probably due either to a lack of electron acceptors
316 (such as oxygen, nitrate and sulfate, not measured in the current work) or of phosphorus, which was
317 under the detection limit below 50 cm depth in all other treatments except FYM. Importantly, microbes
318 deeper in the soil were alive and potentially active: the depth decline curves were relatively similar for
319 SIR and DNA, and the potential N mineralization per unit of microbial biomass (DNA yield) actually
320 increased with depth (from an average 6 to 16 mg N/mg DNA 7 d⁻¹). Interestingly, OTU richness and
321 phylogenetic diversity decreased down the soil profile much less than biomass, on average only 20%.
322 These results – an exponential decrease in biomass but minor decrease in diversity in agricultural soil
323 profiles – agree with Eilers et al. (2012), who studied Colorado upper montane forest soils. Higher
324 diversity per unit biomass (or lower dominance) in deeper depths than in topsoils may be a common
325 characteristic of soils. Dispersal, or growth rates and diversification, are unlikely to increase down the
326 profile and explain the higher diversity/biomass ratio, but the phenomenon may be related to more
327 pronounced spatial isolation (due to lack of abiotic or biotic mixing) and less competition, which reduce
328 effective selection deeper down.

329 Our finding that soil depth surpassed the effect of treatment is not unique; Eilers et al. (2012) reported
330 the depth-related microbial community differences in one soil profile to equal or exceed differences
331 between topsoils from very different biomes. In Tetto Frati topsoil, Actinobacteria and
332 Alphaproteobacteria were the two most abundant bacterial classes, and systematically more abundant in
333 topsoil than at deeper depths. They are highly versatile heterotrophs with the potential to utilize also
334 recalcitrant organic substrates and, together with Acidobacteria (which showed no depth-dependent
335 trend), are considered as signature bacterial taxa in soils (Janssen, 2006). Li et al. (2014), studying

336 irrigated arid zone farmland, also reported the phylum Actinobacteria to decrease in relative abundance
337 with depth (0-3 m), independent of fertilisation treatment. Curiously, in arid zone farmland,
338 Alphaproteobacteria seemed to be relatively more abundant at depth than in topsoils (Li et al., 2014),
339 whereas forest soils showed the same Alphaproteobacterial depth trend as the Tetto Frati agricultural
340 plots (Eilers et al., 2012). Less well-known are the groups more abundant deeper in soil. This is because
341 soil microbial research has focused on the topsoils accessible to perennial crop roots – and easier to
342 sample. Among these groups, members of the diverse phylum Chloroflexi are often abundant in subsoil
343 environments. Subsoil Chloroflexi have generally been regarded as heterotrophic anaerobic bacteria
344 with an organohalide-respiring or fermentative energy metabolism. Cultivation-independent genomic
345 analysis, however, has revealed their metabolic potential to span from the aerobic respiration of sugars
346 and autotrophic CO₂ fixation (Hug et al., 2013). The other phylum especially abundant in the Tetto Frati
347 deepest soil depth, Nitrospirae, has only one established family, Nitrospiraceae. It contains the
348 predominant known nitrite oxidizers in the environment (chemolithoautotrophic *Nitrospira*), but also
349 aerobic and anaerobic genera with the potential for autotrophic or heterotrophic growth on Fe and S
350 transformations (Daims, 2014). In each soil depth except for 25-50 cm, bacterial communities were
351 statistically significantly impacted by the treatments. Both the size and diversity of the bacterial
352 community were positively affected by organic N fertilisation down to 1 m depth.

353 **4.3 Bacterial taxa discriminating between amendments**

354 Unexpectedly, changes in the relative abundance of specific bacterial taxa due to organic amendments
355 were even more clearly detectable at depth than in the topsoil. The same finding was reported by Li et
356 al. (2014) and may be related to typically greater heterogeneity of topsoils (Eilers et al., 2012) – even
357 though unconstrained ordination of our samples in Figure 2 suggests differently. Most of the OTUs
358 highlighted by differential abundance analysis were representatives of poorly known groups with no
359 cultivated representatives and even spurious taxonomy. Out of the two groups more abundant under
360 organic N application, FTL22 was classified as Methyloirabaliaceae (NC10 clade A), a candidate
361 family known for its potential to anaerobically oxidise methane by reducing nitrite to N₂, growing
362 autotrophically with CO₂ (Ettwig et al., 2010). Methyloirabaliaceae have recently been detected in
363 various subsoil environments, including an agricultural field, with higher relative abundance deeper

364 down the soil profile (Shen et al., 2016). The curious positive effect of organic fertilisation on the relative
365 abundance of autotrophic Methyloirabillaceae (as well as on absolute abundance because organic N
366 application also increased total biomass) are potentially explainable by more favourable N forms or
367 available methane (produced by methanogenic archaea), or other chemical or biotic effects not measured
368 in the current study (no difference in applied or measured total N between the two fertilisation types).
369 In agreement with this interpretation, Bertora et al. (2009a) measured field greenhouse gas (GHG)
370 emissions in the same Tetto Frati experiment and reported that manured soils oxidised CH₄ and acted as
371 a sink rather than a source of methane. The environmental relevance of group FTL22 remains to be
372 investigated; if our finding is replicated and if Methyloirabillaceae prove to be more active under
373 organic N fertilisation, then they could help improve denitrification in the subsoil. This would decrease
374 nitrite leaching into the groundwater and possibly reduce CH₄ emissions. Interestingly, this potential to
375 anaerobically oxidise methane by reducing nitrite to N₂ does not seem to be shared by all members of
376 the NC10 candidate phylum. Hug et al. (2016) recently sequenced the first representative of wb-A12
377 (NC10 clade D) from deep river sediments. Based on their analysis, this other group (more abundant in
378 Tetto Frati subsoils under organic than mineral N application) is potentially comprised of
379 chemolithoautotrophs deriving energy from sulphur metabolism (sulfite oxidised to sulfate, and
380 thiosulfate to H₂S to org. S), not from N transformations. This seems plausible given that organic
381 fertilisation also increases the sulphur input into the soils.

382 Sequences belonging to group GAL15 were more abundant at depth in mineral compared to organic N
383 application. The used Silva v.119 and v.123 reference databases classified GAL15 as Thermotogaceae,
384 but according to Hug et al. (2016) this group belongs to the phylum Armatimonadetes. Hug et al. (2016)
385 sequenced the first genome of this group, according to which GAL15, as in the case of
386 Methyloirabillaceae, are also autotrophs that reduce nitrite. However, only *nirK* was detected, meaning
387 that the end product may be NO rather than N₂. Those authors also found a near-complete assimilatory
388 sulphate reduction pathway, so GAL15 could derive energy by either N or S reduction in Tetto Frati
389 soils as well. Chloroflexi P2-11E (subdivision 10) was the second group more abundant at depth under
390 mineral versus organic N fertilisation. Very little is known about this group, which has been
391 characterised solely based on 16S rDNA sequences. The nearest database matches of the P2-11E OTU

392 representative sequences were from rhizosphere and subsoils around the world. Even though the
393 physiology and ecology of P2-11E remain enigmatic, findings similar to ours have been reported in
394 agricultural soils. Chávez-Romero et al. (2016) demonstrated that the relative abundance of P2-11E in
395 arable soil was affected by improved management practises, being lower in the fertilised versus
396 unfertilised soil (tillage-crop residue burned treatment). Zhang et al. (2013) reported the relative
397 abundance of P2-11E to correlate negatively with the manure ratio. Together, these results indicate that
398 P2-11E may be relatively more competitive under conditions of less C and N input.

399 Bovine slurry fertilisation was clearly separate from the other treatments in terms of Bacillaceae
400 abundance in the topsoil. This is likely an effect of direct inoculation rather than modification of the soil
401 physical-chemical environment by the amendment; *Bacillus* has been found to be abundant in fresh
402 livestock manure (McGarvey et al., 2004) and to increase in abundance during the initial thermophilic
403 phase of composting of feces (Maeda, 2010 and references therein). Interestingly, the difference in
404 Bacillaceae abundance did not extend to deeper soil depths. Even though organic amendments can travel
405 one meter down the soil profile – as evidenced by the difference in soil carbon and biomass between
406 mineral and organic N at 75-100 cm – bacteria apparently do not. The lower abundance of
407 Actinobacterial Gaiellales, at depth under bovine slurry application probably reflected changes in soil
408 physical-chemical conditions. Hermans et al. (2017) reported a strong negative correlation between the
409 soil C/N ratio and the relative abundance Gaiellales, comprised of strictly aerobic chemo-organotrophs.
410 Based on the C/N ratio, Gaiellales should have been equally rare under farmyard manure as under bovine
411 slurry, so this parameter does not explain our observation. The percent-range abundance of a supposedly
412 aerobic group half a meter below tillage depth is puzzling. This means that there is much more to learn
413 about the ecology of assumedly well-known bacterial taxa as well.

414 **5. Conclusions**

415 Our study at the Tetto Frati long-term maize cultivation experiment demonstrated the influence of
416 organic amendments on soil biochemical characteristics and the soil bacterial community down to one
417 meter depth. For example, the C/N ratio was significantly higher in the organic N application treatments
418 compared to the mineral N applications in each investigated soil depth, also reflecting the higher SOC

419 contents in organic treatments at depth. This is potentially explained by increased fresh carbon input
420 from the organic amendments by preferential flow and/or increased root growth or root depositions. In
421 topsoils, Actinobacteria and Alphaproteobacteria were the two most abundant bacterial classes, whereas
422 Acidobacteria showed no depth-dependent trend. In each of the investigated soil depths except for 25-
423 50 cm, we demonstrated statistically significant impacts of the treatments on bacterial communities.
424 Unexpectedly, however, changes in the relative abundance of specific bacterial taxa due to organic
425 amendments were even more clearly detectable in the deeper depths than in the topsoils. We still know
426 very little about even the main bacterial, let alone other microbial, groups residing and most likely
427 functioning below the plough depth in our agricultural soils - or how these are shaped by agronomic
428 practises. Further studies are needed to confirm or contest our findings at other long-term managed field
429 sites, and to expand to archaea and possibly fungi too. Altogether, the poorly known subsurface
430 microbial groups may contribute significantly to biogeochemical cycles of carbon and nutrients, but
431 have thus far been neglected in fertiliser budget calculations and in greenhouse gas considerations.

432 **Acknowledgements**

433 The research received funding for the soil sampling from the ExpeER (Experimentation in Ecosystem
434 Research) project, which received funding from the European Union's Seventh Programme for research,
435 technological development and demonstration under grant agreement No 262060, as well as funding
436 from the European Research Council (ERC) under the European Union's Seventh Framework
437 Programme (FP/2007-450 2013, grant agreement no. 615146) awarded to M.T.

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