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

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Abstract

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

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

1. Introduction

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

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

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

2. Materials and methods

2.1 Scene setting

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

rainy seasons (April-May and September-November) and the mean annual temperature is 11.8°C. The long-term experiment is based on a randomised block design with three replicates cultivated with maize (*Zea mays* L.), resulting in a total of 38 combinations of fertilisation, crop residue and rotation management. For this study, we selected the following treatments (each treatment plot measuring 75 m^2):

(1) Maize for silage with 250 kg mineral N ha^{-1} (crop residue removal, CRR)

(2) Maize for grain with 250 kg mineral N ha^{-1} (crop residue incorporation, CRI)

(3) Maize for silage with 250 kg bovine slurry N ha^{-1} (SLU)

(4) Maize for silage with 250 kg farmyard manure N ha^{-1} (FYM)

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

2.2 Soil sampling

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

2.3 Soil chemical characteristics

Total soil organic C concentrations of the soil samples were analysed by dry combustion in a LECO RC-612 TruMac CN (LECO Corp., St. Joseph, MI, USA) at 650°C (ÖNORM L1080). $KMnO_4$ determination of labile carbon was analysed according to Tatzber et al. (2015). Total N was determined according to ÖNORM L1095 with elemental analysis using a CNS (carbon, nitrogen, sulfur) 2000 SGA-

410–06 at 1250°C. Potential nitrogen mineralization was measured by the anaerobic incubation method (Keeney, 1982), as modified according to Kandeler (1993). Soil pH was measured electrochemically (pH/mV Pocket Meter pH 340i, WTW, Weilheim, Germany) in 0.01 M $CaCl_2$ at a soil-to-solution ratio of 1:5 (ÖNORM L1083). Carbonate content was measured gas-volumetrically (CO_2 evolution; ÖNORM L1084). Plant available phosphorous (P) and potassium (K) were determined by calcium-acetate-lactate (CAL) extraction (ÖNORM L1087). Substrate induced respiration using glucose as a substrate was carried out using the MicroResp method according to Campbell et al. (2003) and the MicroResp manual.

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

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

2.5 Sequence data and statistical analyses

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

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

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

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

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

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

3. Results

3.1 Soil chemical characteristics

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

depth, CAL-extractable phosphorous being under the detection limit below 50 cm in all treatments except in FYM. The differences between individual treatments in CAL-extractable phosphorous and potassium were significant at all soil depths for potassium and at 0-25 cm and 25-50 cm for phosphorous. The highest concentrations were always observed in the FYM treatment. The carbon and nitrogen characteristics (SOC, labile C, N_t, C/N ratio, potential N mineralization, substrate induced respiration) as well as CAL-extractable nutrients all correlated significantly negatively with soil pH and $CaCO_3$ and significantly positively with one another in the whole data set (S1). Significant correlations decreased down the soil profiles, but even at 75-100 cm positive correlations between potential N mineralization, SOC, C/N ratio and substrate induced respiration were observed (S1).

3.2 Soil bacterial community size, diversity and structure

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

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

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

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

The effect of treatments on bacterial community composition was smaller than the effect of soil depth but still significant (generalised discriminant analysis for weighted Unifrac distances p=0.0001) (Figure 2B). Treatments with inorganic and organic N sources differed from each other at p=0.0001 and mis-classification error of only 4.2%. This effect of N source was observed, with discriminant analysis $p\leq0.05$ and mis-classification error $\leq20\%$, in all soil depths except for 25-50 cm. The three field blocks, in contrast, did not host distinct bacterial communities (discriminant analysis P=0.064, mis-classification error 45.8%).

3.3 Bacterial taxa discriminating between amendments

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

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

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

4. Discussion

4.1 Soil chemical characteristics

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

showed that organic amendments with or without additional mineral fertilisation across Europe significantly increased the SOC concentrations in the topsoils. The higher SOC contents in organic treatments in the deeper soil depths, observed in the current study, is potentially explained by increased fresh carbon input by preferential flow and/or increased root growth or root depositions (Chabbi et al., 2009). In contrast to other agricultural studies (Blair et al., 2006; Tatzber et al., 2015), we did not detect any significant differences in labile C in any of the studied soil depths, most likely due to large variation. Total N expectedly showed no differences between the treatments except for higher N at FYM at 0-25 cm depth, given that all treatments were fertilised with similar amounts of N, even though in different forms. Due to the increases in SOC contents, the C/N ratios also differed significantly between the organic and mineral treatments at all depths. Both soil pH and $CaCO_3$ increased down the profiles, as expected, due to geogenic conditions. Interestingly, differences in soil pH and $CaCO_3$ between the organic and mineral nitrogen treatments were significant at 50-75 cm depth, indicating differences in the leaching patterns in these treatments or higher $CaCO_3$ input by organic fertilisers. The FYM treatment yielded the in highest CAL-extractable P and K, and this was the only treatment in which CAL-extractable P was detected below 50 cm. This agrees well with Vanden Nest et al. (2016), who showed that P from farmyard manure was more available as well as more prone to leaching compared to compost. Those authors explained this by observed decreases in orthophosphate sorption in farmyard manure amended soils. This may also be connected to enhanced microbial activity in the FYM treatment (Chen et al., 2003). Nonetheless, differences in the CAL-extractable P concentration have also been linked to a different surplus between treatments (Borda et al., 2011).

4.2 Soil bacterial community size, diversity and structure

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

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

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

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

irrigated arid zone farmland, also reported the phylum Actinobacteria to decrease in relative abundance with depth (0-3 m), independent of fertilisation treatment. Curiously, in arid zone farmland, Alphaproteobacteria seemed to be relatively more abundant at depth than in topsoils (Li et al., 2014), whereas forest soils showed the same Alphaproteobacterial depth trend as the Tetto Frati agricultural plots (Eilers et al., 2012). Less well-known are the groups more abundant deeper in soil. This is because soil microbial research has focused on the topsoils accessible to perennial crop roots – and easier to sample. Among these groups, members of the diverse phylum Chloroflexi are often abundant in subsoil environments. Subsoil Chloroflexi have generally been regarded as heterotrophic anaerobic bacteria with an organohalide-respiring or fermentative energy metabolism. Cultivation-independent genomic analysis, however, has revealed their metabolic potential to span from the aerobic respiration of sugars and autotrophic CO_2 fixation (Hug et al., 2013). The other phylum especially abundant in the Tetto Frati deepest soil depth, Nitrospirae, has only one established family, Nitrospiraceae. It contains the predominant known nitrite oxidizers in the environment (chemolithoautotrophic *Nitrospira*), but also aerobic and anaerobic genera with the potential for autotrophic or heterotrophic growth on Fe and S transformations (Daims, 2014). In each soil depth except for 25-50 cm, bacterial communities were statistically significantly impacted by the treatments. Both the size and diversity of the bacterial community were positively affected by organic N fertilisation down to 1 m depth.

4.3 Bacterial taxa discriminating between amendments

Unexpectedly, changes in the relative abundance of specific bacterial taxa due to organic amendments were even more clearly detectable at depth than in the topsoil. The same finding was reported by Li et al. (2014) and may be related to typically greater heterogeneity of topsoils (Eilers et al., 2012) – even though unconstrained ordination of our samples in Figure 2 suggests differently. Most of the OTUs highlighted by differential abundance analysis were representatives of poorly known groups with no cultivated representatives and even spurious taxonomy. Out of the two groups more abundant under organic N application, FTL22 was classified as Methylomirabiliaceae (NC10 clade A), a candidate family known for its potential to anaerobically oxidise methane by reducing nitrite to N_2, growing autotrophically with CO_2 (Ettwig et al., 2010). Methylomirabiliaceae have recently been detected in various subsoil environments, including an agricultural field, with higher relative abundance deeper

down the soil profile (Shen et al., 2016). The curious positive effect of organic fertilisation on the relative abundance of autotrophic Methylomirabiliaceae (as well as on absolute abundance because organic N application also increased total biomass) are potentially explainable by more favourable N forms or available methane (produced by methanogenic archaea), or other chemical or biotic effects not measured in the current study (no difference in applied or measured total N between the two fertilisation types). In agreement with this interpretation, Bertora et al. (2009a) measured field greenhouse gas (GHG) emissions in the same Tetto Frati experiment and reported that manured soils oxidised CH_4 and acted as a sink rather than a source of methane. The environmental relevance of group FTL22 remains to be investigated; if our finding is replicated and if Methylomirabiliaceae prove to be more active under organic N fertilisation, then they could help improve denitrification in the subsoil. This would decrease nitrite leaching into the groundwater and possibly reduce CH_4 emissions. Interestingly, this potential to anaerobically oxidise methane by reducing nitrite to N_2 does not seem to be shared by all members of the NC10 candidate phylum. Hug et al. (2016) recently sequenced the first representative of wb-A12 (NC10 clade D) from deep river sediments. Based on their analysis, this other group (more abundant in Tetto Frati subsoils under organic than mineral N application) is potentially comprised of chemolitoautotrophs deriving energy from sulphur metabolism (sulfite oxidised to sulfate, and thiosulfate to H_2S to org. S), not from N transformations. This seems plausible given that organic fertilisation also increases the sulphur input into the soils.

Sequences belonging to group GAL15 were more abundant at depth in mineral compared to organic N application. The used Silva v.119 and v.123 reference databases classified GAL15 as Thermotogaceae, but according to Hug et al. (2016) this group belongs to the phylum Armatimonadetes. Hug et al. (2016) sequenced the first genome of this group, according to which GAL15, as in the case of Methylomirabiliaceae, are also autotrophs that reduce nitrite. However, only *nirK* was detected, meaning that the end product may be NO rather than N_2. Those authors also found a near-complete assimilatory sulphate reduction pathway, so GAL15 could derive energy by either N or S reduction in Tetto Frati soils as well. Chloroflexi P2-11E (subdivision 10) was the second group more abundant at depth under mineral versus organic N fertilisation. Very little is known about this group, which has been characterised solely based on 16S rDNA sequences. The nearest database matches of the P2-11E OTU

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

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

5. Conclusions

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

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

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