Long-term effects of contrasting tillage systems on soil C and N pools and on main microbial groups differ in relation to crop sequence

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Abstract

Individuation of the best conservation agricultural practices, leading to an increase of soil organic carbon (C) and hence of soil quality, is of paramount importance in the semi-arid Mediterranean environment, where soils are experiencing a continuous decline of organic matter. Therefore, the aim of this long-term study was to assess the combined effects of tillage systems and crop sequences on soil organic C and soil biochemical properties generally used as soil quality indicators. To this end, after 23 years of continuous contrasting tillage systems (conventional tillage, CT, versus no tillage, NT) and crop sequences (wheat monoculture versus wheat/faba bean rotation), soil samples were collected from topsoils (0–15 cm) and subsoils (15–30 cm) at three different sampling times during a cropping year. Soil samples were analysed for total and labile

organic C pools, microbial biomass C (MBC) and nitrogen (MBN), basal respiration and abundance of the main microbial groups by phospholipid fatty acids. Long-term NT increased total organic C (TOC) at a yearly rate of 0.17 g kg^{-1}. Such an increase, in turn, stimulated the microbial biomass, particularly gram-negative bacteria, suggesting a higher soil quality under NT, as also confirmed by the increase in MBC/TOC and the decrease in stress indices. On the contrary, no differences were observed with regard to fungal biomass. These findings suggested the need to reconsider the role of specific bacterial groups in organic C accumulation in soils of semiarid environments. Interestingly, the effects due to long-term NT appeared to be widely diversified as the crop sequence varied, while in the CT system the changes in the biochemical characteristics and in the main microbial groups due to the crop sequence appeared modest. Thus the interaction among the various aspects of agronomic management modulated the effects of substrate quality on the chemical and biological soil properties.

Keywords: no tillage, conventional tillage, wheat monoculture, wheat-faba bean rotation, biochemical soil properties, substrate quality

1. Introduction

Long-term experiments are valuable sources of information for a better understanding of which agronomic practices are more useful to maintain or even enhance soil organic matter and hence soil quality. Despite the high economic and labour costs to maintain this type of experiments over time, the consequent knowledge is irreplaceable for evaluating the sustainability of agricultural practices. This is even more true with regard to conservation agriculture practices, the effects of which on both cropping system and soil quality generally require a number of years of continuous application to become apparent (Johnston and Poulton, 2018; Mbuthia et al., 2015). Soils of arid and semi-arid regions are experiencing a continuous decline of the organic matter towards levels no longer able to sustain crop productivity. While soil protection and sustainability have become a worldwide task in

order to ensure food security and contrast climate change, the study of the long-term effects of conservation management practices (e.g. reduced or no tillage instead of intensive tillage, crop rotations instead of monoculture cropping) on soil microorganisms and carbon (C) and nitrogen (N) dynamics has become essential for assessing the effectiveness of such practices in supplying agroecosystem services (Chabert and Sarthou, 2020).

Soil biochemical properties, such as microbial biomass and activity, have been extensively used as reliable and sensitive indicators of soil functioning and quality, being linked to the living part of the soil, thereby responding quickly to any management practices that alter its characteristics (Gonzáles-Chávez et al., 2010; Laudicina et al., 2012, 2014). With regard to the effects of the tillage system on soil microbial biomass, conflicting results are reported in the literature: many studies have shown higher microbial biomass in no-tilled compared to conventionally tilled soils (Badagliacca et al., 2018a, 2018b; Helgason et al., 2010a) whereas others have reported no substantial differences with different tillage systems (Acosta-Martínez et al., 2007; Mbuthia et al., 2015). Also, studies about the effects of the tillage system on the main microbial groups are often contradictory, with unclear trends or no differences across the experiments, especially in the relative abundance of each microbial group (Helgason et al., 2010a; Sun et al., 2016). Therefore, the response of soil microorganisms is likely site-specific, depending upon the context in which tillage systems are adopted (climate, soil type and fertility, other management practices, etc.).

In addition to the tillage system, crop rotation can also have marked effects on the main soil microbial groups (Bünemann et al., 2008). Crop rotation can change the soil habitat by affecting soil nutrient status, C input from roots (via root exudates, mucilage, etc.), the amount and quality of crop residues, and aggregation/microbial habitat; these alterations in turn can affect soil microbial activity and diversity (Balota et al., 2004; Venter et al., 2016). Indeed, the soil microbial community can respond differently to root exudates of the different crops grown in rotation. Crop rotation including leguminous crops has been demonstrated to increase the microbial biomass C/total organic C ratio (microbial quotient) more than monoculture systems, due to the input of greater

organic residue variety (Anderson and Domsch, 2010). However, conflicting results are reported in the literature concerning the effects of crop rotation in place of monoculture cropping on the size, diversity and structure of the soil microbial community. For example, Lupwayi et al. (1998) showed that microbial diversity was significantly higher in a wheat/pea rotation than in continuous wheat culture. Tiemann et al. (2015) found that rotational diversity increased microbial community diversity and the relative abundance of fungi vs bacteria. In contrast, Navarro-Noya et al. (2013) observed no effect of crop sequence (maize/wheat rotation versus continuous maize) on soil microbial diversity. Finally, it has also be highlighted that crop rotation can influence the soil microbial community not only via the direct effects exerted by each crop as a result of its physiology, but also indirectly through the differing management practices (plant density, fertilization, weed control, etc.) applied to each crop.

Despite several studies having investigated the effects of soil tillage systems or crop sequences on soil C pools and soil biochemical properties, most of them have been performed under temperate conditions (e.g. Mbuthia et al., 2015; Wulanningtyas et al., 2021; Zhang et al., 2014). Studies conducted under semi-arid Mediterranean conditions are available (e.g. Madejón et al., 2007; Melero et al., 2009), but few of them have investigated how such soil properties change in response to the combined effects of tillage system and crop sequence. This is even more true for long-term experiments performed in the semi-arid Mediterranean region. Therefore, within the framework of a long-term soil tillage and crop sequence experiment established 23 years ago in Sicily (Italy), we conducted an in-depth study to evaluate the effects of contrasting tillage systems (no tillage, NT; conventional tillage, CT) continuously applied within different crop sequences (continuous wheat and wheat–faba bean rotation) on a range of chemical, biochemical and microbiological soil properties, all able to reflect the soil organic C dynamics. Multivariate statistical analyses were performed to verify if the various cropping systems (each resulting from a specific tillage system × crop sequence combination) were separated with respect to the canonical components (CDA) or the

variables themselves (classification tree) and to identify which variables were most correlated with the canonical components.

2. Materials and methods

2.1. Experimental site

The experiment was conducted under rainfed conditions at Pietranera Farm, which is located about 30 km North of Agrigento, Sicily, Italy (37°30′ N, 13°31′ E; 178 m a.s.l.), on a deep, well-structured soil classified as a Chromic Haploxerert (Soil Survey Staff, 2010). Soil characteristics, determined at the beginning of the experiment and related to the 0–40 cm layer, were: 525 g kg^{-1} clay, 216 g kg^{-1} silt, 259 g kg^{-1} sand, pH 8.1 (1:2.5 H_2O, w/v), 14.0 g kg^{-1} organic C, 1.29 g kg^{-1} total N, 36 mg kg^{-1} available P (Olsen), 340 mg kg^{-1} K_2O (exchangeable K), 35 $cmol_+$ kg^{-1} cation exchange capacity, and 0.38 cm^3 cm^{-3} water content at field capacity (matric potential = −0.01MPa) and 0.16 cm^3 cm^{-3} at permanent wilting point (matric potential = −1.5 MPa). The climate of the experimental site is semi-arid Mediterranean, with a mean annual rainfall of 585 mm (period of observation from 1983 to 2013), mostly in the autumn–winter period (September to February; 73%) and spring (March to May; 23%). Mean air temperatures are 15.9 °C in autumn, 9.7 °C in winter and 16.5 °C in spring.

2.2. Experimental design and crop management

The long-term field experiment, which began in autumn of 1991, was set up as a strip-plot design with two replications. Three soil tillage systems (CT, reduced tillage and NT) acted as vertical treatments and three crop sequences (continuous wheat, wheat–faba bean and wheat–berseem clover) as horizontal treatments; each year, both rotations (i.e., wheat–faba bean and wheat–berseem clover) were duplicated in reverse order to obtain, each year, data for all crops.

The tillage systems tested in this study were CT and NT, while crop sequences were continuous wheat ($_W$W) and wheat–faba bean rotation; soil samples were taken both from the wheat crop after

130 faba bean ($_{F}$W) and from the faba bean crop after wheat ($_{W}$F). In the CT treatment, one mouldboard
131 ploughing, carried out to a depth of 30 cm in the summer, was followed by two shallow (0–15 cm)
132 harrowing operations before planting. In NT, sowing was done by direct drilling. Plots were sized
133 18.5 m × 20.0 m. Glyphosate at a dose of 1066 g a.e. ha^{-1} was used in NT plots for weed control
134 before planting. Wheat plots (i.e., $_{W}$W and $_{F}$W) were broadcast-fertilized with 69 kg ha^{-1} of P_2O_5
135 before planting. Nitrogen fertilizer was broadcast on the soil surface at a rate of 120 kg N ha^{-1} in
136 $_{W}$W plots and of 80 kg N ha^{-1} in $_{F}$W plots. The total amount of N fertilizer was split into two
137 applications: 50% immediately before planting (as diammonium phosphate and urea) and 50% at
138 mid-tillering (end of March, before the second soil sampling) as ammonium nitrate. Faba bean plots
139 were broadcast-fertilized only with 46 kg ha^{-1} P_2O_5 before planting. Crop planting was done in
140 December using a no-till seed drill with hoe openers under both CT and NT, making the appropriate
141 sowing depth adjustments to ensure a homogeneous planting depth (3–5 cm). Faba bean cv. Gemini
142 was sown at 40 viable seeds m^{-2} with an inter-row spacing of 75 cm. No rhizobial inocula were
143 applied before planting because the soil had a native rhizobial population. Durum wheat, cv. Anco
144 Marzio, was planted in rows spaced 16 cm apart at 350 viable seeds m^{-2}. In $_{W}$W and $_{F}$W plots,
145 weeds were controlled by applying herbicide in post-emergence at the early growth stage of the
146 crop. In $_{W}$F plots, weeds were controlled mechanically by shallow hoeing (with minimum soil
147 disturbance) when plants were at the third-leaf stage. Faba bean was harvested in late June, leaving
148 standing straw and uniformly spreading crop residues. Wheat was also harvested in late June and
149 stubble (about 20–25 cm from the soil surface) was left standing. Wheat straw was baled and
150 removed from the field. The soil surface covered by mulch in the NT treatments was always > 30%.
151
152 *2.3. Soil sampling and analysis*
153 During the cropping season 2013–2014, two soil samples (each composed of three subsamples) per
154 plot were collected separately from the 0–15 cm (topsoil) and 15–30 cm (subsoil) soil layers at three
155 distinct times: December 2013 (before sowing), April 2014 (wheat heading/faba bean full

flowering) and July 2014 (at harvest), for a total of 144 soil samples. Visible pieces of crop residue and roots were removed by hand from soil samples. Soil samples were air-dried, sieved at 2 mm and stored in plastic bags at room temperature. For each soil sample, an aliquot was stored at 4 °C for determination of biochemical properties. All analyses were done within 1 month from sampling. Total organic C (TOC) was determined according to the Walkley–Black method (Nelson and Sommers, 1996).

Microbial biomass C (MBC) and N (MBN) were determined by the fumigation-extraction method (Brookes et al., 1985; Vance et al., 1987). Soil aliquots, remoistened at 50% of water holding capacity (WHC) and equivalent to 25 g oven-dry soil, were fumigated with alcohol-free chloroform in vacuum desiccators for 24 h in the dark. After removing the chloroform by repeated evacuations, the soil samples were extracted with 100 mL of 0.5 M K_2SO_4 for 45 min on a horizontal shaker (70 rpm). Non-fumigated soil samples were similarly extracted and used as controls. All soil extracts were filtered through Whatman 42 paper and then analysed for organic C by the acid-dichromate oxidation method and for total N by the Kjeldahl method. Organic C and total N held in non-fumigated soil extracts (C_{extr} and N_{extr}, respectively) were used as proxies for available C and N (Laudicina et al., 2013). MBC and MBN were estimated as the differences between the total organic C and total N extracted from fumigated and non-fumigated samples, respectively, multiplied by a conversion factor (k_{EC}) of 2.64 for MBC and of 2.22 (k_{EN}) for MBN. Basal respiration (BR) was determined by incubating 10 g of soil moistened at 50% of WHC for 24 h in 125 cm^3 air-tight glass bottles at 20 °C. The CO_2 evolved after 24 h of incubation was assessed by injecting a 1 mL aliquot of gas from the headspace of the bottles into a gas chromatograph (TRACE GC-Thermo Scientific, Milano, Italy) equipped with a thermal conductivity detector. The metabolic quotient (qCO_2) was calculated and expressed as mg CO_2-C g^{-1} MBC h^{-1}, whereas the microbial quotient (expressed as a percentage) was the MBC/TOC ratio (Anderson and Domsch, 2010).

Phospholipid fatty acids (PLFAs) were extracted from soils and analysed according to the modified Bligh and Dyer method (White et al., 1979). Lipids were extracted from 5 g of soil with a single-

phase mixture of chloroform–methanol–citrate buffer (1:2:0.8, v/v/v) as described by Wu et al. (2009). The resulting extract was fractionated into neutral lipids, glycolipids and polar lipids with 10 mL chloroform, 20 mL acetone and 10 mL methanol through a silicic acid column, respectively. The polar lipids were trans-esterified to the fatty acid methyl esters (FAMEs) by mild alkaline methanolysis (Guckert et al., 1985). The FAMEs were recovered with an n-hexane:chloroform mixture (4:1, v/v), reduced to dryness by rotavapor and re-dissolved in 200 mL of n-hexane. The FAMEs were detected by a gas chromatograph (FOCUS GC-Thermo Scientific, Milano, Italy) equipped with a flame ionization detector and a Mega-10 fused-silica capillary column (50 m long, 0.32 mm I.D., 0.25 μm film thickness). The GC temperature progression was as follows: initial isotherm at 115 °C for 5 min, increase at a rate of 1.5 °C per minute from 115 to 230 °C, and final isotherm at 230 °C for 2 min. Both the injection port and detector were set up at 250 °C, and helium at 1 mL min^{-1} in a constant flow mode was used as carrier. The injected volume was 1 mL in splitless mode. Nonadecanoic acid methyl ester (19:0; cat no. N-5377, Sigma-Aldrich Co.) was used as an internal standard for quantification of FAMEs. Identification of the peaks was based on comparison of retention times to known standards (Supelco Bacterial Acid Methyl Esters and Supelco 37 component Fatty Acid Methyl Esters). The abundance of each FAME was expressed as nanomoles per gram of dry soil and as mole percent (mol %) of total fatty acids. Fatty acids with fewer than 14 C-atoms or more than 20 C-atoms were excluded as considered to originate from non-microbial sources. The FAs i15:0, a15:0, 15:0, i16:0, i17:0, 17:0, cy17:0, 18:1ω7 and cy19:0 were used to represent bacterial biomass while the FA 18:2ω6,9 was used for fungal biomass (Frostegård and Bååth, 1996). The FAs i15:0, a15:0, i16:0 and i17:0 were chosen to represent Gram-positive bacteria, the FAs 18:1ω7, cy17:0 and cy19:0 for Gram-negative bacteria (Zelles, 1997). The cyclopropyl/precursor stress indices (cy17:0/16:1ω7 and cy19:0/18:1ω7) were calculated (Pettersson and Bååth, 2003).

207 *2.4. Statistical analysis*

208 Reported data are the arithmetic means of four samples (2 samples per plot × 2 replications) per
209 treatment and per three sampling times (n = 12) and are expressed on an oven-dry basis (105 °C) of
210 soil. Data were analysed considering a linear mixed model for a strip-plot design repeated in time
211 (Schabenberger and Pierce, 2002) and computed separately for the two layers.
212 Canonical discriminant analysis (CDA; Friendly and Fox, 2020) was carried out to separate
213 treatments and to identify the major sources of difference between groups. CDA effectively projects
214 the data into the space of linear combinations of the variables that account for the greatest
215 proportion of between-group variance relative to within-group variance. Also, another method of
216 nonparametric statistical analysis, i.e. a recursive partition technique that allows acquisition of a so-
217 called 'Classification tree' (Breiman et al., 1984), was performed. This approach has the advantage
218 that data transformation is unnecessary, normality conditions or covariance homogeneity are not
219 necessary, and variable selection is intrinsic to the methodology, i.e. the procedure identifies the
220 most important (discriminant) variables. The R package "ggplot2" (Wickham et al., 2016) was used
221 to prepare the figures. Statistical analyses were performed using R software (R Core Team, 2020).
222

223 **3. Results**

224 *3.1. Weather conditions*

225 Total rainfall in the 2013–2014 growing season was 603 mm, which was higher than the long-term
226 average for the area (Figure 1). Rainfall was well distributed over the growing season; about 25% of
227 the total rainfall occurred in the period from September to November (i.e., before crop sowing),
228 whereas the period from December to April was the wettest of the growing season (66% of the total
229 rainfall with 33 rainy days). Finally, the period from May to July was quite dry (8% of the total
230 rainfall with only 6 rainy days). The mean air temperature in the year during which this study was
231 carried out was 15.2 °C, which was lower than the long-term mean air temperature (15.9 °C; Figure
232 1).

233

234 *3.2. Soil chemical and biochemical properties*

235 Both tillage system and crop sequence significantly influenced most of the topsoil properties,
236 whereas few were affected in the subsoil (Table 1). In the topsoil, TOC was on average 32% higher
237 in NT than in CT (Figure 2). TOC was also affected by crops, with higher values in $_{F}$W than $_{W}$W
238 and $_{W}$F. In NT, TOC decreased with depth, whereas in CT it remained almost unchanged. The
239 effect of tillage system on TOC, however, varied with the crop sequence, being higher in NT than in
240 CT in $_{W}$F (+0.8 g C kg^{-1}), whereas no significant differences were observed between the two tillage
241 systems in both $_{F}$W and $_{W}$W. Still, in the subsoil of CT, TOC was 8% higher in $_{F}$W than $_{W}$F (Figure
242 2). Similarly, when the comparison was between the two wheat crops, in CT, TOC was significantly
243 higher in $_{F}$W than in $_{W}$W (+3%).

244 Also, C_{extr}, in the topsoil, was on average 26% higher in NT than in CT but the effect of tillage
245 system changed with crop sequence (Table 1). Indeed, C_{extr} was higher in NT than in CT in both
246 wheat crops but no significant differences between the two tillage systems were found in $_{W}$F (Figure
247 2). Significant differences for soil C_{extr} between $_{W}$F and $_{F}$W, within both the CT and NT systems,
248 were observed (with $_{W}$F > $_{F}$W under CT; and $_{F}$W > $_{W}$F under NT); on the other hand, no significant
249 differences were observed for this variable between the two wheat crops, under either CT or NT. In
250 the subsoil, C_{extr} was affected only by tillage system, being on average 32% higher in CT than in
251 NT (Figure 2). In the topsoil, N_{extr} was more affected by tillage system ($P = 0.021$) than crop
252 sequence ($P = 0.057$, Table 1). In NT, N_{extr} was on average 20% higher than in CT, while in $_{W}$W
253 and $_{F}$W it was on average 21% greater than in $_{W}$F (Figure 2). In the subsoil, N_{extr} values were higher
254 ($P = 0.099$) in $_{W}$W and $_{F}$W than $_{W}$F, regardless of the tillage system (Figure 2).

255 MBC, in the topsoil, was affected by both tillage system and crop sequence; it was 62% higher in
256 NT than in CT (Figure 3), with the wheat plots ($_{W}$W and $_{F}$W) showing higher values than the faba
257 bean plots ($_{W}$F) in both tillage systems (on average +39% and +30% under CT and NT,
258 respectively). In the subsoil of NT soils, MBC was lower than in the upper soil layer (Figure 3),

whereas no differences were evident between the topsoil and subsoil of conventionally tilled soils. The effect of crop interacted with tillage system (Table 1) but, on average, MBC was higher in wheat plots than in faba bean plots, regardless of the tillage system. MBN was affected only by the tillage system in the upper soil layer, showing on average values 46% higher in NT than in CT (Figure 3). BR was affected by both experimental factors only in the topsoil. Indeed, NT significantly increased BR compared to CT whereas, among crops, $_{W}$W showed higher BR values than the other two crops (Figure 4).

In the topsoil, the microbial quotient was significantly affected by both experimental factors, and also by their interaction, being higher in NT compared to CT in both $_{W}$W and $_{W}$F (Figure 4). Moreover, significant differences were found for the microbial quotient between the two crops grown in rotation, both within CT and NT systems (with $_{F}$W > $_{W}$F under both tillage systems). Also, when the comparison was made between the two wheat crops, microbial quotient in NT was significantly higher in $_{W}$W than $_{F}$W (+15%), whereas no differences were observed between the two wheat crops in CT. In the subsoil, crop sequence, both alone and interacting with tillage system, affected microbial quotient. The most significant differences occurred in continuous wheat, with NT showing higher microbial quotient values than CT, and for the rotation in CT, with $_{F}$W having higher values than $_{W}$F. The qCO_2 did not show a univocal pattern among treatments, with the only exception for $_{F}$W, in both soil layers and regardless of tillage system, that showed the lowest values (Figure 4).

3.3. Main soil microbial groups

In the topsoil, regardless of the crop sequence, total PLFAs were, on average, 54% higher in NT compared to CT, but with the difference between the two tillage systems interacting with crop sequence and decreasing according to the order $_{W}$W > $_{F}$W > $_{W}$F (Figure 5). A significant difference in total PLFAs was observed between the two wheat crops within the CT system (with $_{F}$W > $_{W}$W) and, similarly, between the two crops grown in rotation within the NT system (with $_{F}$W > $_{W}$F). In

285 the subsoil, total PLFAs were significantly higher in NT than in CT (Figure 5). A significant
286 difference in total PLFAs was observed between the two wheat crops in NT (with $_{W}W > {}_{F}W$);
287 moreover, higher PLFA values were found under both tillage systems in $_{F}W$ than in $_{W}F$.
288 In the subsoil, the abundance of the main microbial groups was affected by the interaction of the
289 tested factors (Table 1). Total and Gram-negative bacteria were, on average, higher in NT than in
290 CT (Figures 5 and 6). Within the CT system, a significant difference in total and Gram-negative
291 bacteria was observed between the two wheat crops (with $_{F}W > {}_{W}W$), whereas in NT a significant
292 difference was found between the two crops grown in rotation (with $_{F}W > {}_{W}F$). Also, the
293 BAC^{+}/BAC^{-} and cy19:0/cis18:1ω7 ratios were higher in CT than in NT in wheat plots. The
294 cy17:0/cis16:1ω7 ratio was affected only by the tillage system and showed higher values in CT than
295 in NT (Figure 7).
296
297 *3.4. Canonical discriminant analysis (CDA) and classification tree analysis*
298 Two CDAs were performed separately for the topsoil and the subsoil. The relationship of the
299 variables to the canonical dimensions is shown in Figure 8 by vectors. Each vector is defined by the
300 correlations it has with the canonical dimensions. In the topsoil (Figure 8A), both canonical
301 dimensions were significant, according to a likelihood ratio stepdown test. Nearly 71% of between-
302 group mean differences were accounted for by the first canonical dimension (CAN1) that was
303 positively influenced by all the soil traits included in the analysis, but especially by TOC, MBC and
304 CO_2; moreover, CAN1 clearly distinguished CT from NT systems. The second canonical dimension
305 (CAN2), which explained 16.8% of the variance, was positively influenced by TOC, BAC^{+} and
306 fungi, and negatively mainly by CO_2; CAN2 clearly separated the crop sequence treatments inside
307 NT, but not in CT.
308 In the subsoil (Figure 8B), both canonical dimensions were significant, according to a likelihood
309 ratio stepdown test. Nearly 48% of between-group mean differences were accounted for by the first
310 canonical dimension CAN1, which was highly related to BAC^{-}, bacteria and PLFAs, whereas

311 CAN2, that accounted for 27.3% of the variance, was highly related to MBC, MBN and N_{extr}. A less
312 clear distinction between the two tillage systems was obtained; however, CAN1 still discriminated
313 NT wheat plots (both $_{W}$W and $_{F}$W) from all other plots.
314 The classification trees fitted to the two layers are shown in Figure 9. Each tree consists of a series
315 of splitting rules, starting at the top of the tree (root of the tree, containing all the units), each based
316 on a single variable. Each tree is characterized by some splits that produce branches and internal
317 nodes, while, at the bottom, terminal nodes or leaves can be found. Each split guarantees that the
318 partition of the units in the two child nodes is characterized by the maximum obtainable
319 homogeneity of its unit (with respect to the response variable, crop × tillage in this study). The
320 variables associated with each split are the most discriminant variables. Referring to the topsoil
321 (Figure 9A), coherent with the CDA results, the most discriminant variables were TOC, MBC and
322 CO_2, i.e. three classical soil variables linked exclusively to the C cycle. In each leaf (bottom of
323 tree), the distribution of the units is reported: for example, in the bottom leaf (Node 4) more than
324 80% of units are 'CT-$_{W}$F', and were characterized by 'TOC < 13.885 and MBC < 307.125'.
325 Moreover, it can be observed that 'TOC < 15.02' corresponds to CT, while 'TOC ≥ 15.02'
326 corresponds to NT. The results for the subsoil (Figure 9B), as expected, were not quite as good.
327 More than three variables were necessary to partition the units in leaves as homogeneously as
328 possible, and this result was coherent with the results shown in the CDA plot, where overlapping of
329 the ellipses for each group was evident. Here, however, it was noteworthy that the first discriminant
330 was BAC^{-}, i.e. a well-defined microbial group.

331

332 **4. Discussion**

333 The CDAs performed using both measured and derived chemical and biochemical data clearly
334 separated the NT systems from the CT ones, exhibiting also more marked differences among the
335 topsoils than the subsoils. Interestingly, the CDAs also highlighted that the NT systems were more

scattered than the CT systems over the diagrams, thus suggesting greater variability for the investigated soil parameters due to crop sequence.

In the topsoil, a considerable increase of TOC in NT compared to CT was observed; the differences were so marked that TOC was the parameter with the highest discriminative power, as highlighted by the classification tree analysis. Since the input of crop residues did not differ between the two tillage systems (see Badagliacca et al., 2018a), differences in TOC can be ascribed to the effects of the tillage system on the fate of crop residues. In tilled soils, crop residues are incorporated into and mixed with soil; this increases their accessibility to soil microorganisms (Laudicina et al., 2016), thus speeding up their mineralization. In NT soils, the maintenance of crop residues on the soil surface made them less accessible to soil microorganisms, thus slowing down the decomposition process and leading progressively to the accumulation of organic C in the first centimetres of the topsoil, as argued by Álvaro-Fuentes et al. (2008). Moreover, as suggested by Six et al. (2000), conservative tillage practices, by preserving the soil structure, contribute to the formation of C-enriched micro-aggregates within the macro-aggregates that can physically protect soil organic matter from mineralization. On the contrary, intensive tillage practices, by disrupting soil aggregates and increasing soil aeration, favour the oxidation processes of the previously physically protected soil organic matter through microbial attack (Laudicina et al., 2016). Furthermore, in NT topsoil, a greater content of both C_{extr} and N_{extr} was observed compared to CT. These two latter parameters, as argued by Tivet et al. (2013), may positively influence the formation of soil aggregates, thus protecting soil organic matter and establishing a virtuous circle which supports soil C sequestration. Such greater substrate availability in NT, according to Sun et al. (2016), increases MBC and the MBC/TOC ratio, as a consequence of higher C availability for microorganisms (Anderson and Domsch, 2010; Badalucco et al., 2010). In conjunction with the greater substrate availability, synergistically, crop residues accumulated on NT topsoil can reduce fluctuations of soil temperature and moisture, so making the topsoil more favourable for soil microorganisms (Turmel et al., 2015). Noteworthy, in NT, compared to CT, a consistent increase in the microbial biomass,

362 mainly ascribable to the increase of bacteria instead of fungi, was observed. The absence of higher
363 amounts of fungi in NT was an unexpected result since many authors have found more fungi in NT
364 treatments (Laudicina et al., 2016; Sharma-Poudyal et al., 2017; Sun et al., 2016). However, the
365 effect of tillage on the fungal community is controversial as it is related to the context in which the
366 experiments are carried out (Helgason et al., 2010b; Shi et al., 2012; Zhang et al., 2015). In the
367 context of this field experiment, regardless of the tillage system, bacteria dominated the microbial
368 community and this may be ascribed to many factors including the moderately alkaline soil reaction
369 and the low soil moisture for most of the year (Helgason et al., 2010a), as well as to the higher
370 substrate C and N availability compared to CT (Grayston et al., 2001). With regard to the latter
371 factor, bacteria and fungi have a different stoichiometry, with the former having a C to N ratio of
372 about 5 and the latter of about 10, on average (Moore et al., 2000). Therefore, bacteria and fungi are
373 expected to have, respectively, higher and lower nitrogen requirements. Consequently, if the access
374 to C is equivalent but N is limiting then a shift towards fungal dominance is expected, but if N is
375 not limited bacterial dominance is expected (Carney et al., 2007). Actually, in this study, it is
376 reasonable to assume that N was not a limiting factor since the element was applied yearly with
377 adequate inorganic fertilization or by the legume crop (faba bean); this consideration is confirmed
378 by a TOC/total N ratio below 10 in all of the treatments (see Badagliacca et al., 2018a), a C_{extr}/N_{extr}
379 ratio below 6 (which is similar to that of bacteria) and an MBC/MBN ratio below 7.7. Therefore, an
380 increase in TOC in NT over the long period was associated with an increase of the bacterial
381 community clearly higher than that of the fungal one. In this regard, the role of fungi in the C cycle
382 could be more related to absorption of the metabolites released by bacteria rather than to their direct
383 decomposition of soil organic matter (Zhang et al., 2013). This finding is noteworthy and should
384 promote a focus on the role of bacteria in C sequestration in the semi-arid Mediterranean
385 environment. Furthermore, it suggests that the role of F/B ratio as an indicator of C sequestration in
386 soils of the semi-arid Mediterranean environment should be reconsidered (Fanin et al., 2019).

387 The greater amount of total bacteria in NT than in CT was mainly attributable to BAC^{-}; this is in
388 contrast with the findings of some authors (e.g. Zhang et al., 2014) but agrees with those of other
389 studies carried out in warm and dry environments (Ali et al., 2018; Ma et al., 2014). The higher C
390 availability in NT soils may be the cause of the predominance of BAC^{-} (78% higher compared to
391 CT, on average) which grow more quickly and have greater ability than BAC^{+} to proliferate as soon
392 as nutrient availability increases (Feng and Simpson, 2009). Furthermore, Kramer and Gleixner
393 (2008) and Fanin et al. (2019) suggested that BAC^{-} prefer plant-derived C substrate rather than soil-
394 derived C. This hypothesis is coherent with what Laudicina et al. (2014) found in the same study
395 area, i.e. higher amounts of easily decomposable substrate in NT compared to CT. However, long-
396 term NT also promoted, albeit to a lesser extent than BAC^{-}, BAC^{+} (+58% in NT than CT, on
397 average), which are able to utilize older organic C substrates due to their capability to utilize
398 recalcitrant organic C (Fanin et al., 2019; Kramer and Gleixner, 2008). The stress indicators
399 calculated as the ratio of cyclopropane to monoenoic precursor fatty acids agreed with what is
400 stated above. Indeed, the higher cy17:0/cis16:1ω7 ratio in CT suggests stress conditions for soil
401 microorganisms, likely as a consequence of soil microbial adaptive mechanisms (Gil et al., 2011;
402 Stromberger et al., 2007) to C limitation which could have limited bacterial growth (Liu et al.,
403 2015). Still regarding topsoil, few variations of the chemical and biochemical parameters and main
404 microbial groups were observed due to the effect of crop sequences applied under the CT regime.
405 On the contrary, large differences among the main microbial groups were observed in the NT
406 regime, linked more to the effect of the crop than to the cumulative effect of the long period. The
407 higher values for total PLFAs and bacteria, particularly BAC^{-}, observed in the wheat plots than in
408 the $_{W}$F plots can be ascribed to the different plant density and morpho-physiological root traits
409 between species (higher root density and root exudate deposition in wheat than in faba bean;
410 Acosta-Martínez et al., 2007; Rich and Watt, 2013) and to the different N fertilization (Liu et al.,
411 2010), with $_{W}$W and $_{F}$W receiving, respectively, 120 and 80 kg N ha^{-1} and faba bean receiving no
412 mineral N through fertilization. This evidence agrees with other studies (Bünemann et al., 2008;

González-Chávez et al., 2010), confirming the ability of wheat to increase concentrations of the fatty acids 18:1ω7, cy17:0 and cy19:0, i.e. the bioindicators of BAC^{-}, in its rhizosphere, especially when it is grown in monoculture or in very narrow crop rotations. With regard to N fertilization, a similar stimulation effect on BAC^{-} was observed by Kirchmann et al. (2013) and Zhang et al. (2019), which may have been due to the combined effect of N availability for bacterial growth and of root exudation by the plants (Palazzolo et al., 2019; Wardle, 2002). Moreover, as argued by Steward et al. (2018), both the aforementioned factors can interact, since N fertilization can support an increase of wheat root exudation represented by sugars, organic acids and other ready-available C forms for microbes, including BAC^{-} such as ammonia-oxidizing and denitrifying bacteria, in accordance with the results obtained by Zhu et al. (2016) and Badagliacca et al. (2018a) in a previous in-depth study performed within the same long-term experiment. Therefore, it appears that NT favours BAC^{-}, especially when associated with the cultivation of wheat.

The reason why the adoption of different crop sequences had pronounced effects on the main microbial groups in NT but not in CT remains to be properly elucidated. A number of factors could have played a role in this finding, including different amounts of root exudates released by the same crop under different edaphic conditions (as argued by Ohwaki and Hirata, 1992), distinct fates of the different crop residues at varying the soil tillage management (Marschner et al., 2003), and differences in the weed flora between CT and NT systems at varying the crop sequence (as previously observed by Ruisi et al. 2015 in this same long-term experiment), which may have contributed to shape the soil microbial community by releasing different root exudates and leaving residues of different quality. All these factors can also interact with each other and produce cumulative effects over time. In any case, from this study it would appear that the variations induced by NT in TOC, C_{extr} and N_{extr} and some physical characteristics (bulk density and porosity; see Badagliacca et al., 2018a) made this system much more responsive to the stimuli deriving from changes in other management factors (i.e. the crop sequence), with effects visible up to the main microbial groups. This is a very interesting aspect that certainly deserves further investigation as it

could suggest a greater resilience of the NT system compared to the CT one. Interestingly, crop data obtained from the same long-term experiment had shown that the application of NT could positively influence the yield of crops and their resource-use efficiency only when it is applied in systems where a proper crop sequence (i.e. cereal/legume rotation instead of continuous cereal cropping) is adopted, and, in addition, when other crop management practices (weed control, N fertilization, etc.) are virtuously modulated (Amato et al., 2013; Ruisi et al., 2016). The results of this research provide a useful key to interpret these effects.

The joined analysis of data recorded in the subsoil made it possible to differentiate the conventional system from the NT one, although differences appeared less marked than those found in the topsoil. In particular, no difference was observed for TOC between CT and NT. However, overall, considering only the ploughed soil layer (0–30 cm depth), long-term NT allowed the COP21 target in the Mediterranean semi-arid environment to be reached (Arrouays and Horn, 2019; Minasny et al., 2017). Many authors have reported that the lower TOC in the topsoil of CT compared to NT is generally counterbalanced by a higher TOC in the subsoil (where crop residues are incorporated by tillage; Jantalia et al., 2007; Thomas et al., 2007). However, the present study does not confirm this finding; in fact, the C stock in the entire soil layer considered (0–30 cm) has increased progressively over the period of the experiment (23 years) in NT compared to CT (see Badagliacca et al., 2018a, 2018b). Moreover, a positive effect of CT over NT was observed in the subsoil with regard to soil C_{extr}; this should be ascribed mainly to the different stratification of the crop residues induced by the two tillage systems rather than to differences in C transfer between upper and deeper soil layers. With regard to the abundance of the main microbial groups, in the subsoil, the systems under study (tillage and crop sequence) showed effects similar to those observed in the topsoil, but to a lesser extent. Similar to what was observed in the topsoil, the different crop sequences resulted in effects with a similar trend on the microbial population in NT, but not in CT.

5. Conclusions

Overall, the results suggest that in the semi-arid Mediterranean environment, long-term NT, compared to CT, improves soil quality by increasing the soil organic C, microbial biomass and microbial quotient, thus enhancing the agroecosystem's contribution to mitigation and adaptation to climate change. Long-term NT increased soil organic C in the topsoil by 3.9 g kg^{-1} (on average among the crop sequences) corresponding to 0.17 g kg^{-1} per year. On average, considering the 0-30 cm soil layer, TOC increased by 0.08 g kg^{-1} per year, thus allowing the achievement of the COP21 target in the Mediterranean semi-arid environment.

The greater availability of organic substrates due to NT application, in turn, stimulated soil microbial biomass and in particular the bacterial community, mainly BAC^{-}, instead of the fungal one. This result is noteworthy and should promote a focus on the role of bacteria in C sequestration in cropped soils of the semi-arid Mediterranean environment. Furthermore, it suggests that the role of fungi to bacteria ratio as an indicator of C sequestration should be reconsidered at least for the semiarid Mediterranean environment.

The effects of NT were widely diversified as the crop sequences varied, while with CT the differences due to the different crop sequences were modest and not always appreciable. This underlines the importance of the interaction between the various aspects of agronomic management (tillage, crop sequence, fertilization etc.) in modulating the effects of substrate quality on the chemical and biological properties soil. The information obtained from this study may contribute to a more successful application of conservation agriculture practices in Mediterranean semiarid regions, in order to maintain or even enhance soil quality.

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727 **Figure captions**

728

729 **Figure 1.** Rainfall events (blue columns) and daily mean air temperature (red line) at the
730 experimental site during the 2013–2014 growing season (from September 2013 to July 2014).

731

732 **Figure 2.** Total organic carbon (TOC), extractable organic C (C_{extr}) and extractable organic N
733 (N_{extr}) as affected by tillage system (CT, conventional tillage: grey plots; NT, no tillage: coloured
734 plots) and crop ($_{W}$W, continuous wheat; $_{F}$W, wheat grown after faba bean; $_{W}$F, faba bean grown
735 after wheat) determined on soil samples collected from the 0–15 cm (topsoil) and 15–30 cm
736 (subsoil) soil layers. Circles inside plots represent means, with whiskers representing ± SE (n = 12).
737 The width of the plot shows the density distribution of values.

738

739 **Figure 3.** Soil microbial biomass C (MBC) and microbial biomass N (MBN) as affected by tillage
740 system (CT, conventional tillage: grey plots; NT, no tillage: coloured plots) and crop ($_{W}$W,
741 continuous wheat; $_{F}$W, wheat grown after faba bean; $_{W}$F, faba bean grown after wheat) determined
742 on soil samples collected from the 0–15 cm (topsoil) and 15–30 cm (subsoil) soil layers. Circles
743 inside plots represent means, with whiskers representing ± SE (n = 12). The width of the plot shows
744 the density distribution of values.

745

746 **Figure 4.** Soil basal respiration (BR), microbial quotient (MBC/TOC) and metabolic quotient
747 (qCO_2) as affected by tillage system (CT, conventional tillage: grey plots; NT, no tillage: coloured
748 plots) and crop ($_{W}$W, continuous wheat; $_{F}$W, wheat grown after faba bean; $_{W}$F, faba bean grown
749 after wheat) determined on soil samples collected from the 0–15 cm (topsoil) and 15–30 cm
750 (subsoil) soil layers. Circles inside plots represent means, with whiskers representing ± SE (n = 12).
751 The width of the plot shows the density distribution of values.

752

753 **Figure 5.** Total PLFAs, bacteria and fungi as affected by tillage system (CT, conventional tillage:
754 grey plots; NT, no tillage: coloured plots) and crop ($_{W}$W, continuous wheat; $_{F}$W, wheat grown after
755 faba bean; $_{W}$F, faba bean grown after wheat) determined on soil samples collected from the 0–15
756 cm (topsoil) and 15–30 cm (subsoil) soil layers. Circles inside plots represent means, with whiskers
757 representing ± SE (n = 12). The width of the plot shows the density distribution of values.
758
759 **Figure 6.** Gram-positive (BAC^{+}) and Gram-negative (BAC^{-}) bacteria and fungi to bacteria ratio
760 (F/B) as affected by tillage system (CT, conventional tillage: grey plots; NT, no tillage: coloured
761 plots) and crop ($_{W}$W, continuous wheat; $_{F}$W, wheat grown after faba bean; $_{W}$F, faba bean grown
762 after wheat) determined on soil samples collected from the 0–15 cm (topsoil) and 15–30 cm
763 (subsoil) soil layers. Circles inside plots represent means, with whiskers representing ± SE (n = 12).
764 The width of the plot shows the density distribution of values.
765
766 **Figure 7.** Gram-positive to Gram-negative bacteria ratio (BAC^{+}/BAC^{-}), and cy17:0/cis16:1ω7 and
767 cy19:0/cis18:1ω7 ratios as affected by tillage system (CT, conventional tillage: grey plots; NT, no
768 tillage: coloured plots) and crop ($_{W}$W, continuous wheat; $_{F}$W, wheat grown after faba bean; $_{W}$F, faba
769 bean grown after wheat) determined on soil samples collected from the 0–15 cm (topsoil) and 15–
770 30 cm (subsoil) soil layers. Circles inside plots represent means, with whiskers representing ± SE
771 (n = 12). The width of the plot shows the density distribution of values.
772
773 **Figure 8.** Canonical discriminant analysis (CDA) ordination biplots of the six cropping system
774 centroids separately for the 0–15 cm (topsoil, A) and 15–30 cm (subsoil, B) soil layers. CT,
775 conventional tillage; NT, no tillage; $_{W}$W, continuous wheat; $_{F}$W, wheat grown after faba bean; $_{W}$F,
776 faba bean grown after wheat. Within each biplot, the direction and length of each line (vectors)
777 indicate the canonical loadings of the determined soil properties on the first two canonical variables.

778 The plot shows the scores on the canonical dimensions and overlays 60% data ellipses for each
779 group.
780
781 **Figure 9.** Classification tree separately for the 0–15 cm (topsoil, A) and 15–30 cm (subsoil, B) soil
782 layers obtained by a recursive partition technique of statistical analysis carried out on the chemical
783 and biochemical soil properties. The most important (discriminant) soil properties are shown.
784 Threshold values discriminating the plots are reported. CT, conventional tillage; NT, no tillage;
785 $_{W}W$, continuous wheat; $_{F}W$, wheat grown after faba bean; $_{W}F$, faba bean grown after wheat.

Figure1

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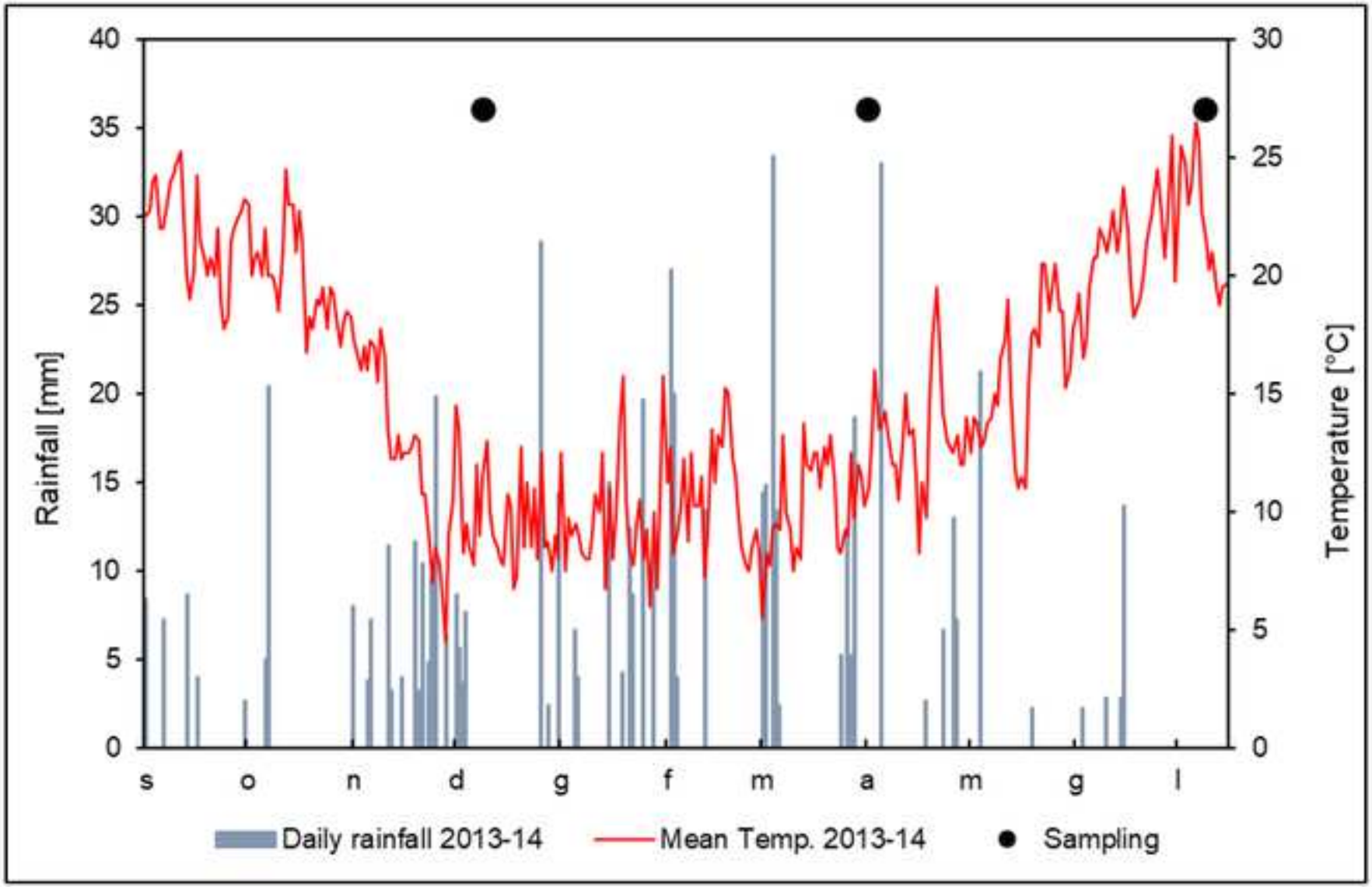

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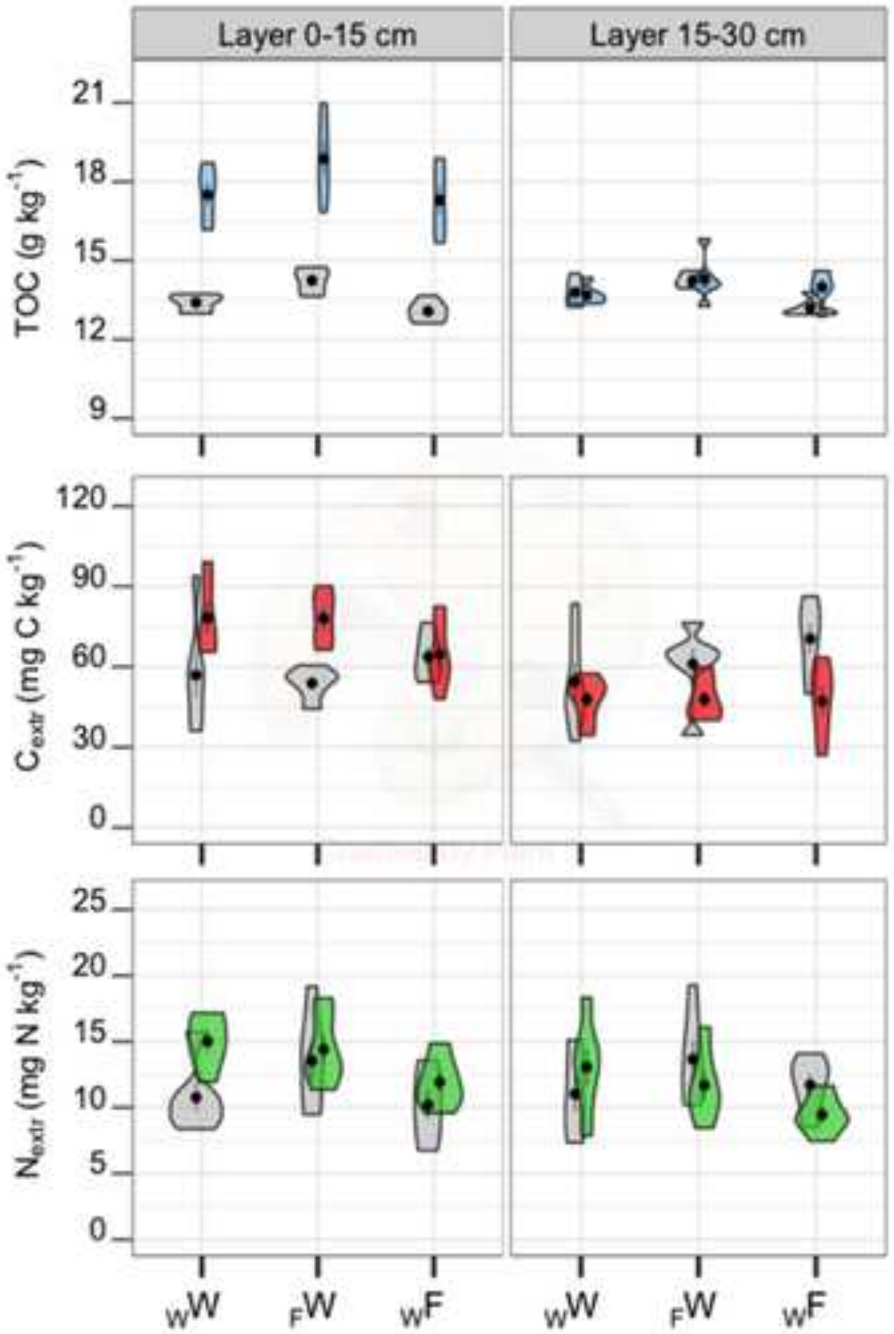

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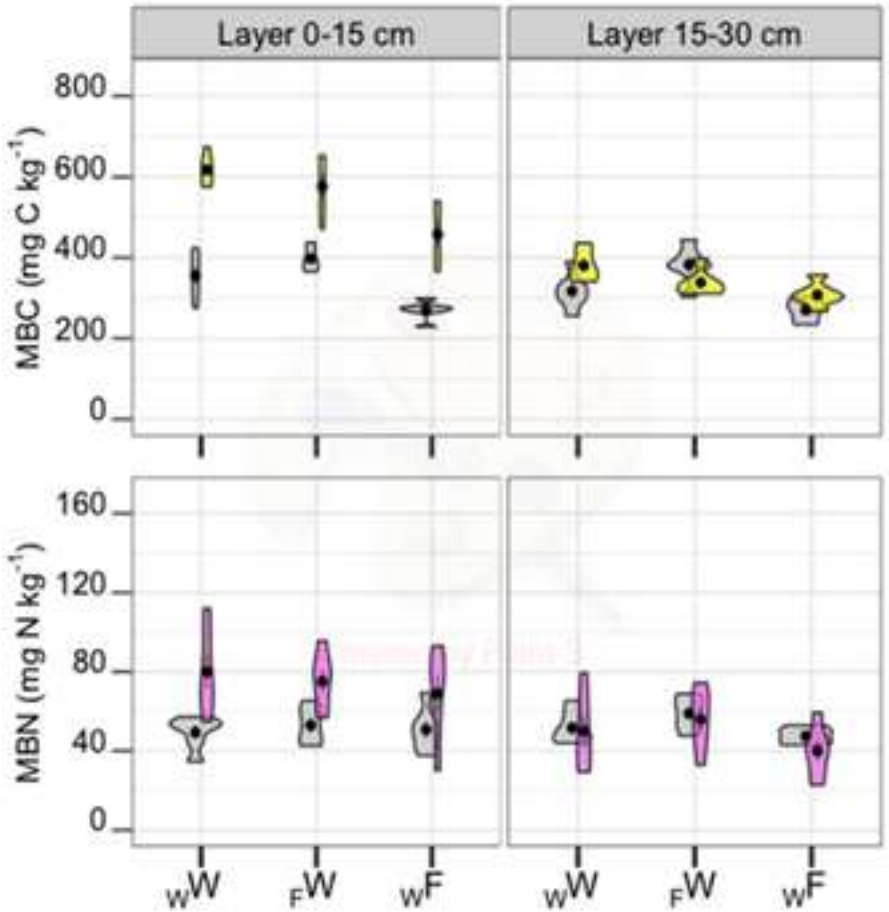

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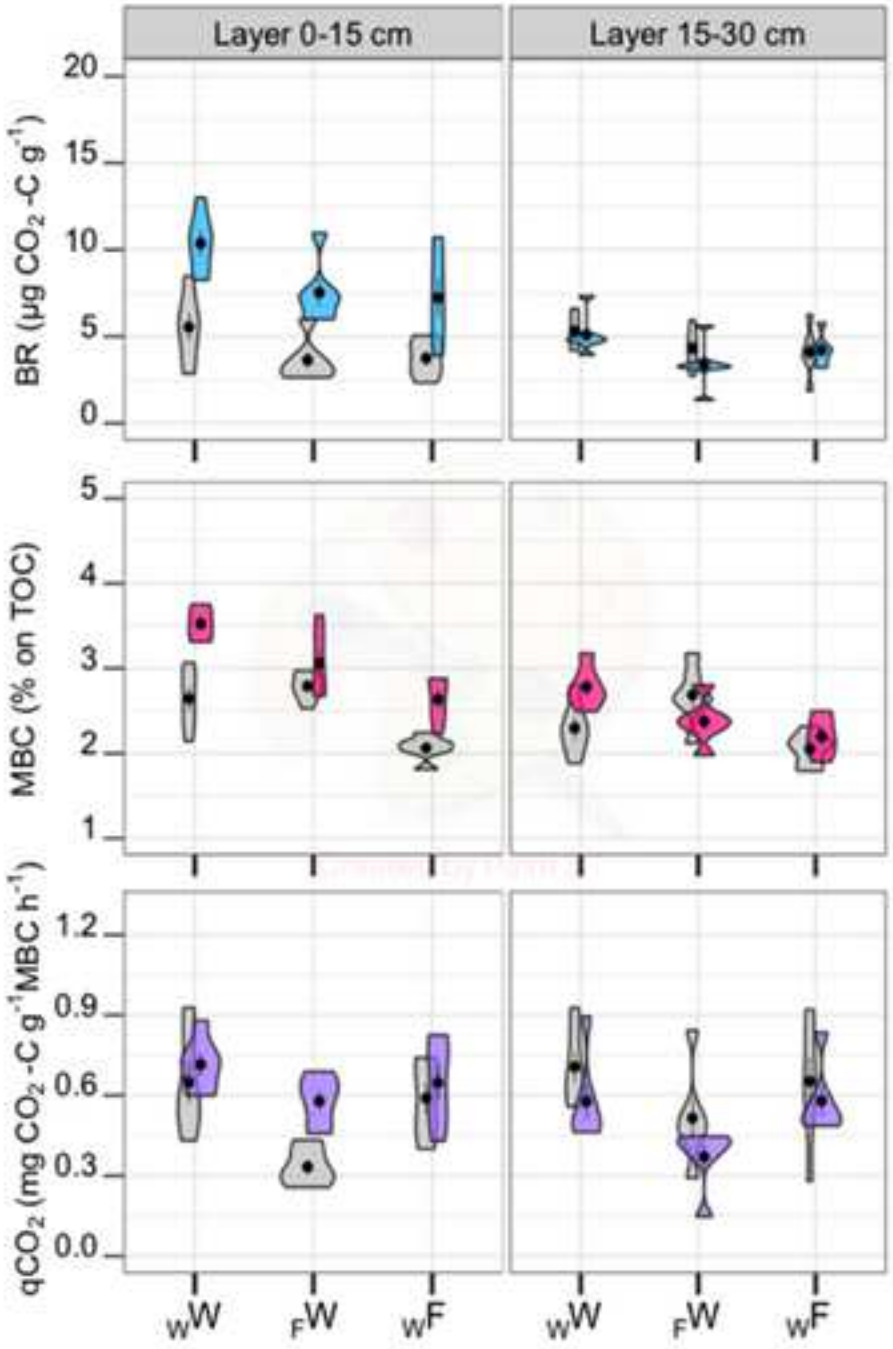

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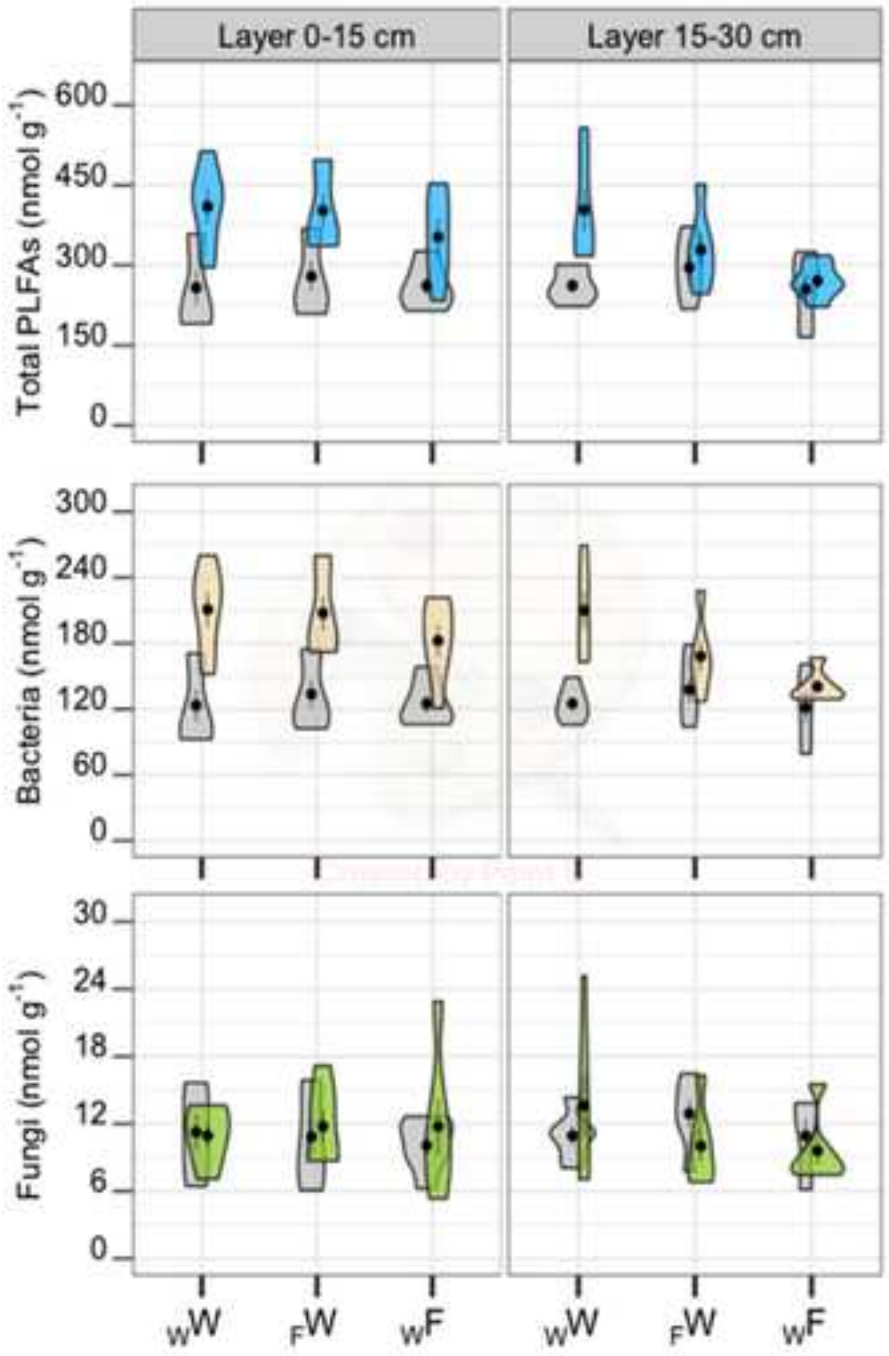

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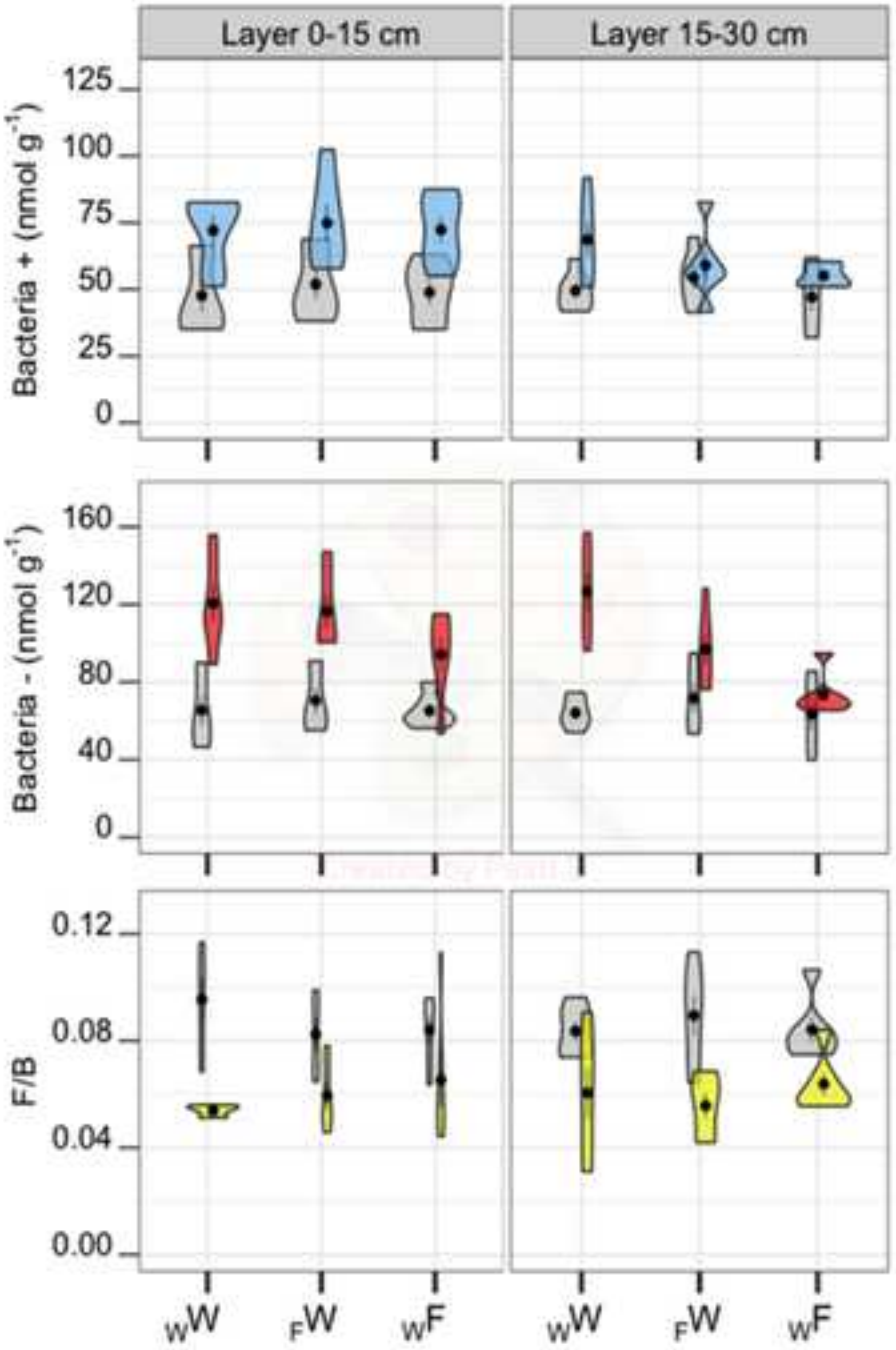

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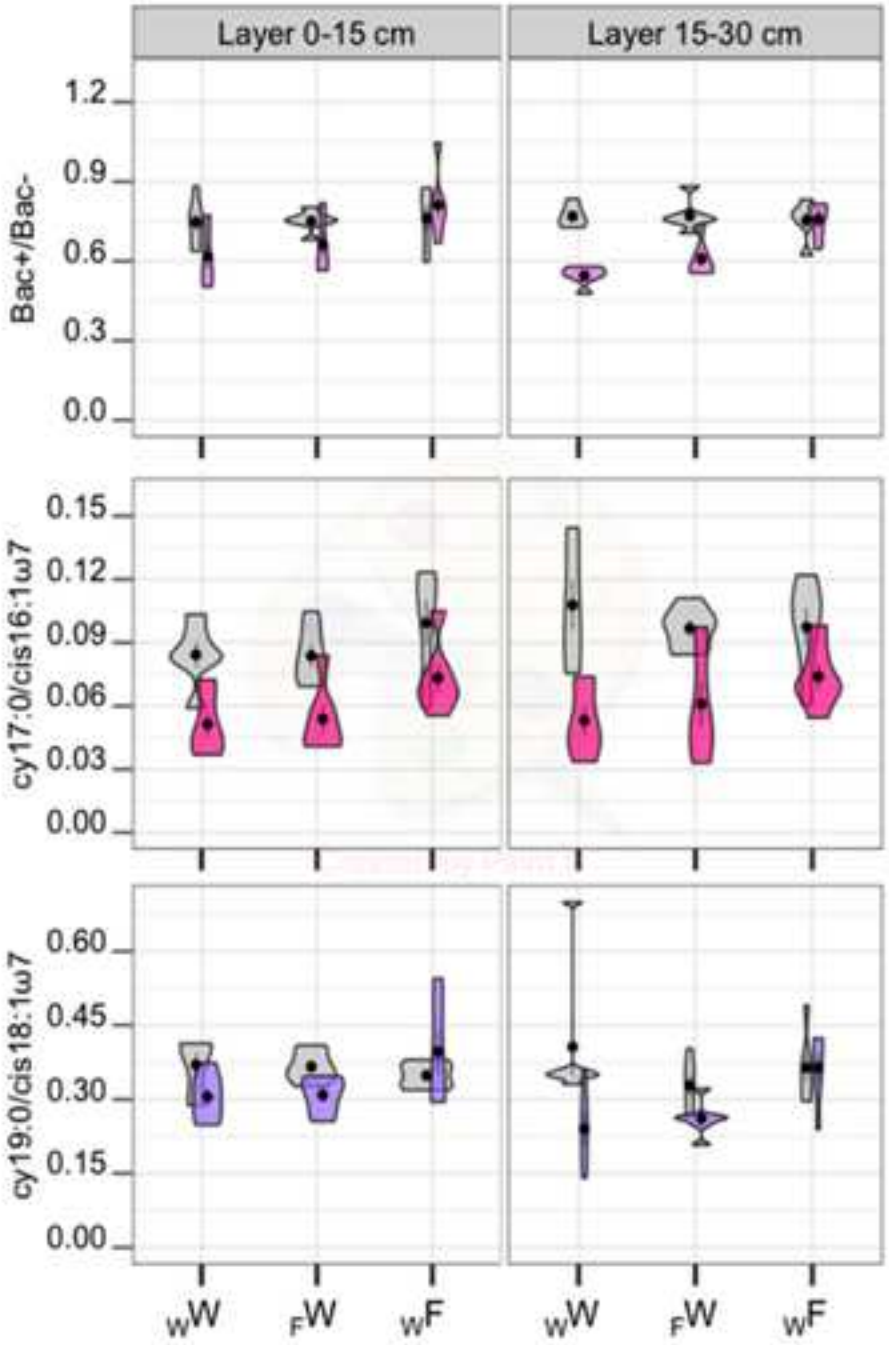

Figure8A

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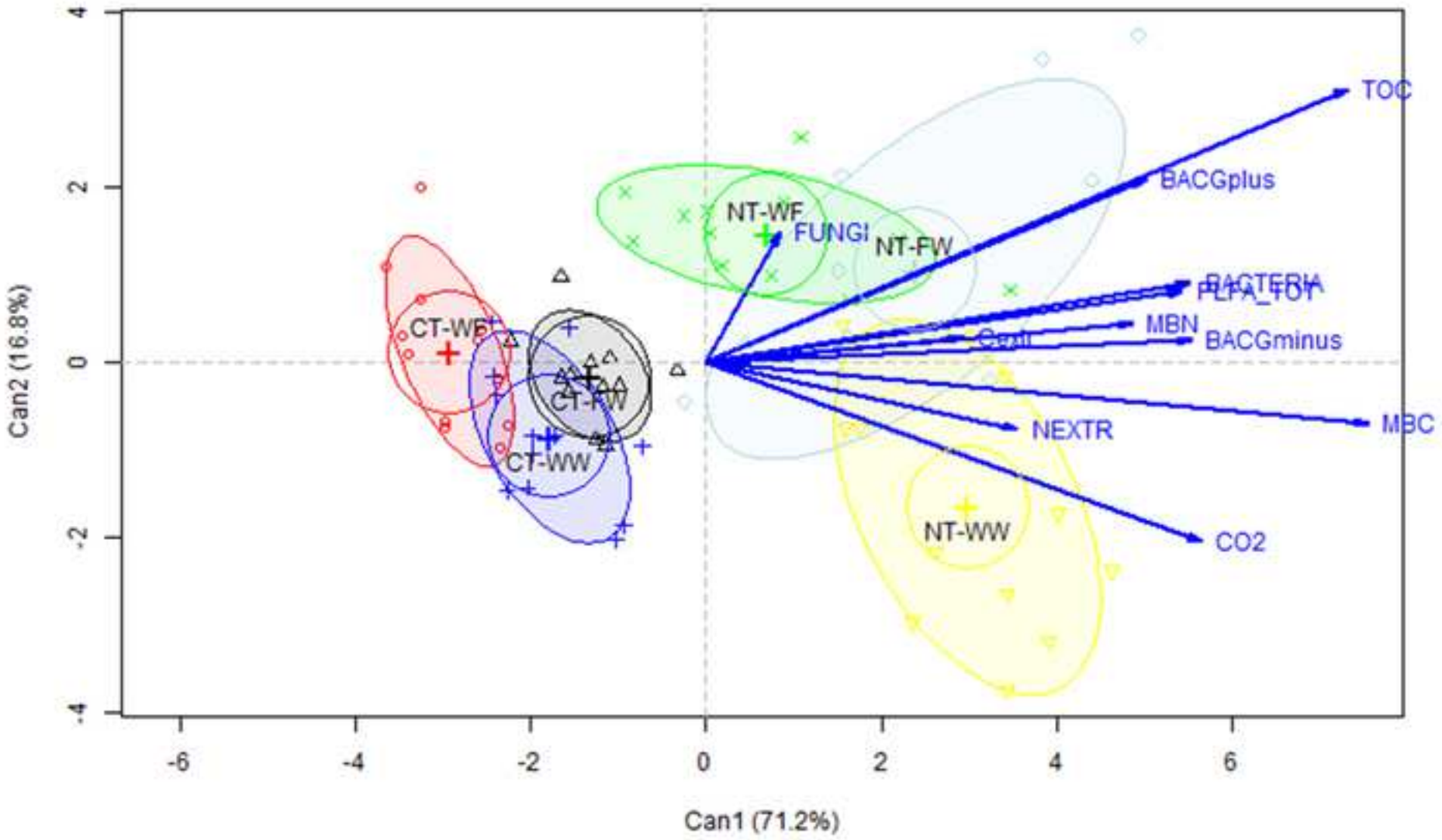

Figure8B

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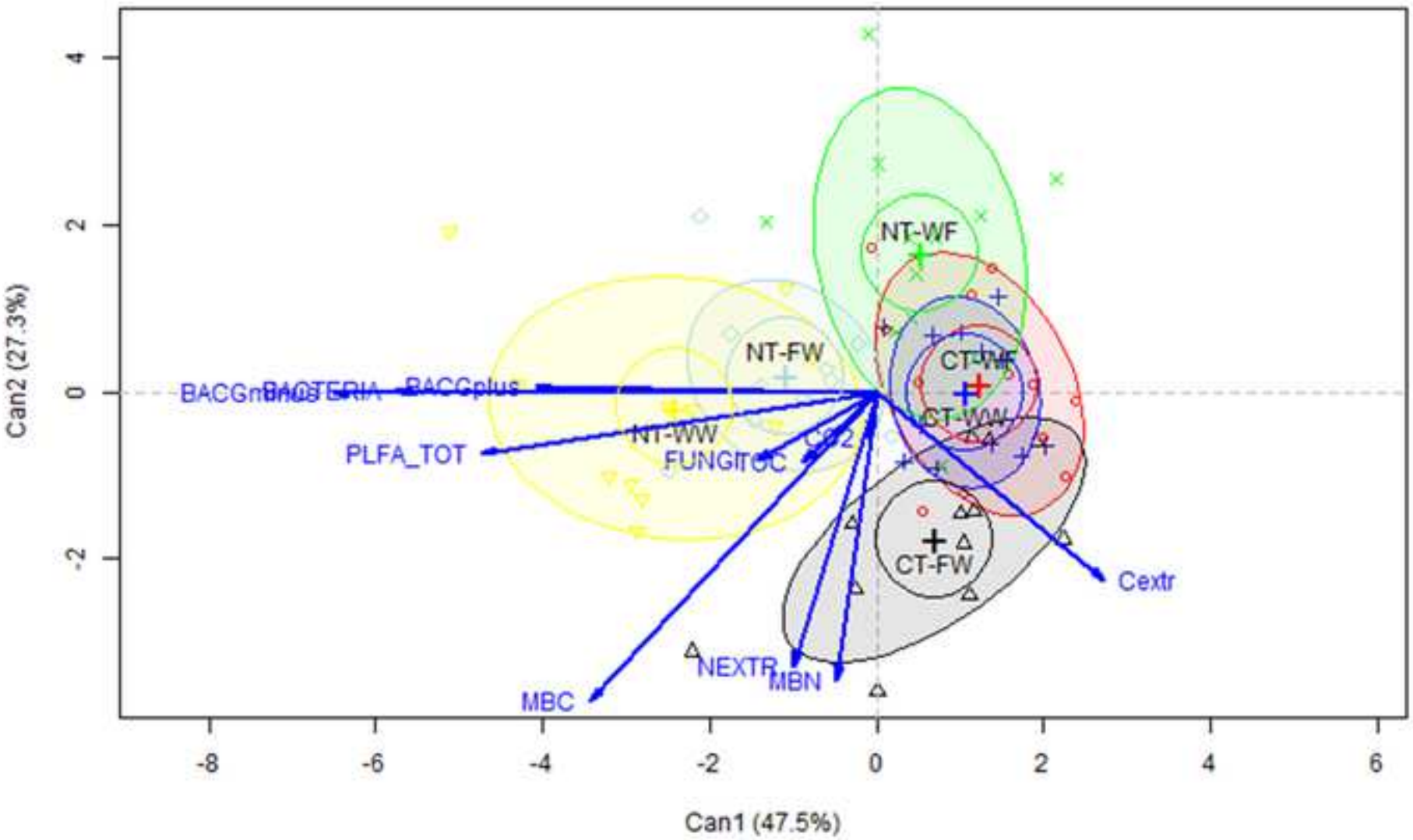

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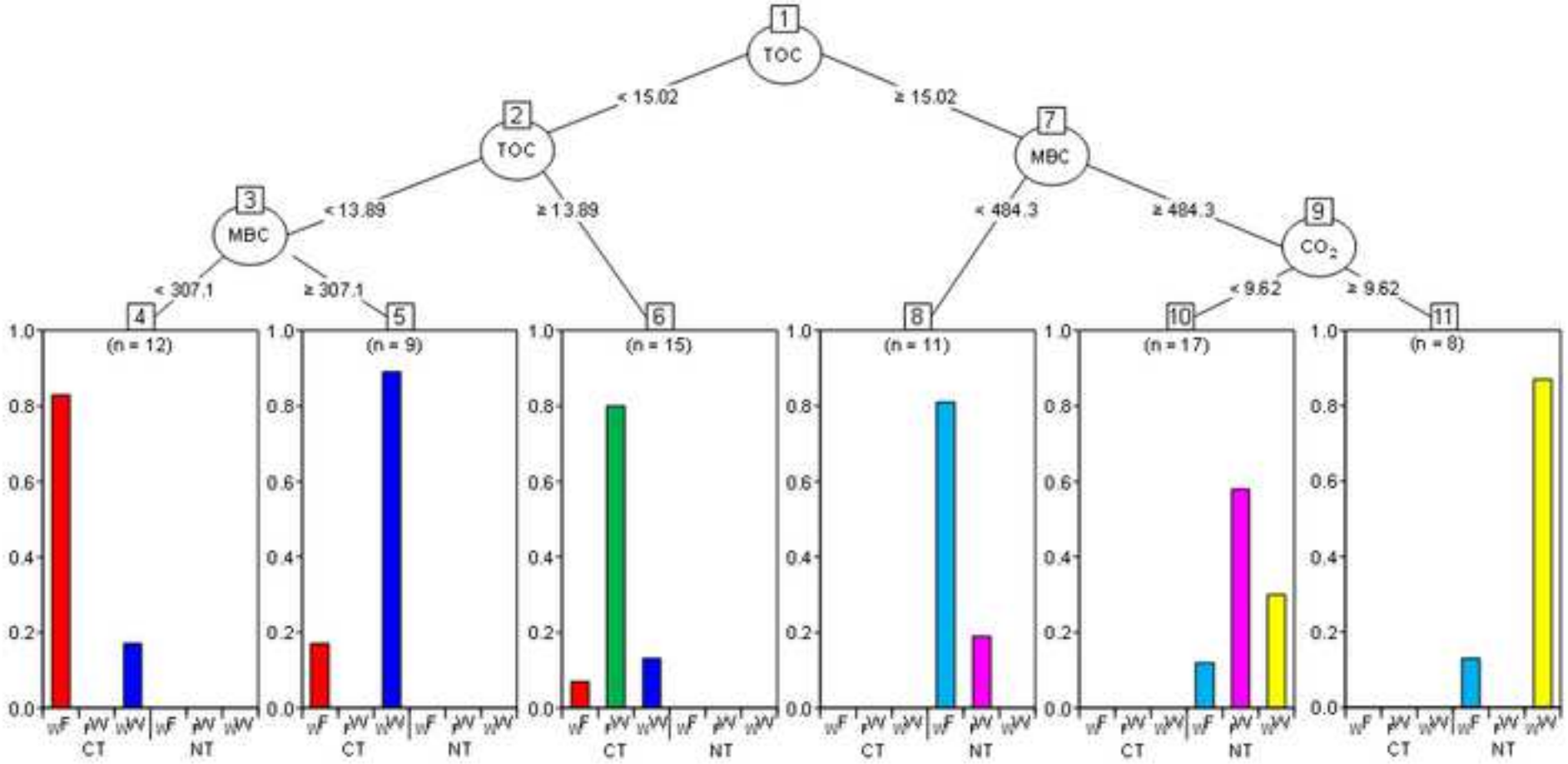

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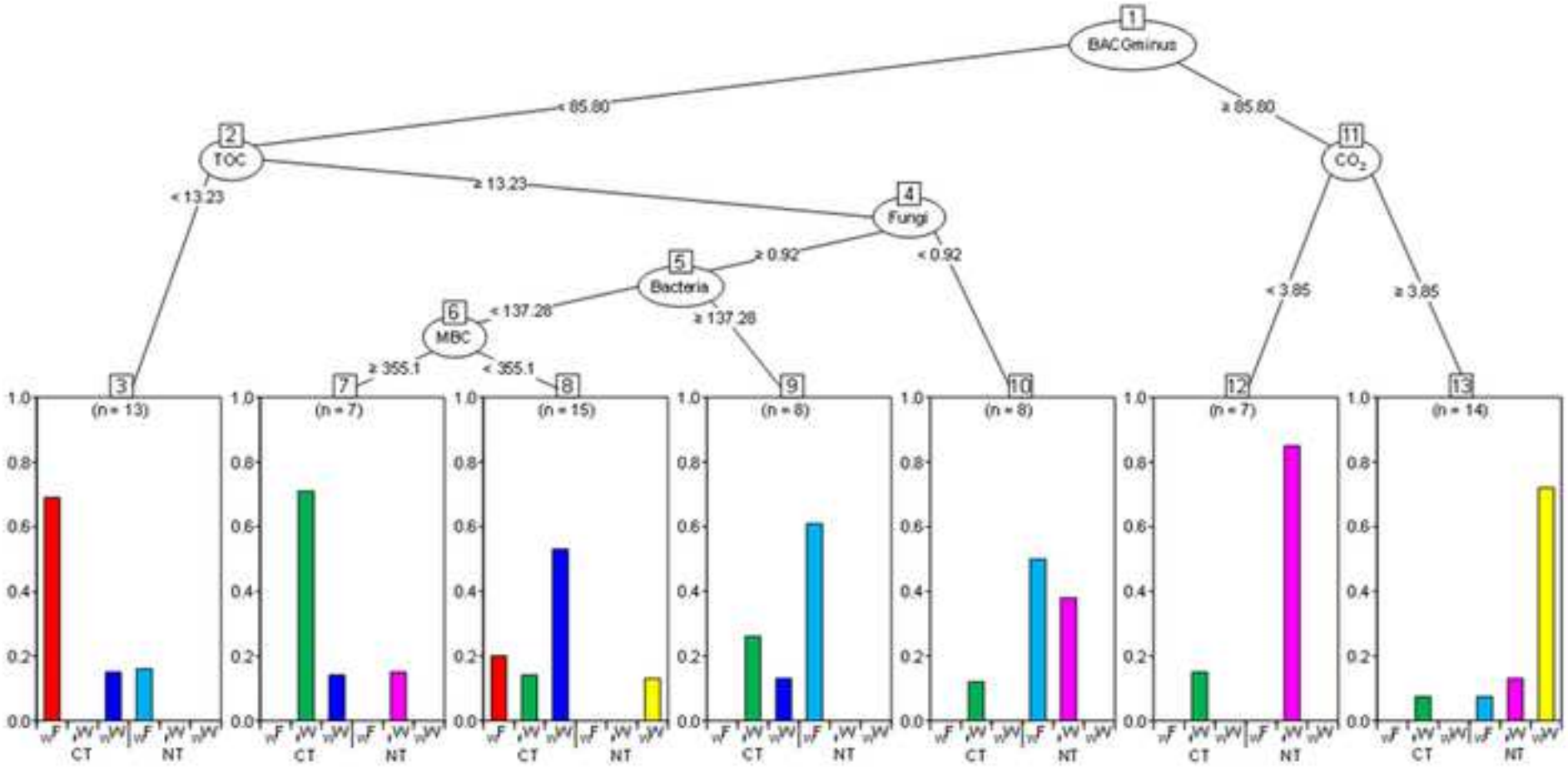

table

Table 1. Analysis of variance: P-values for the effects of the applied treatments (tillage system and crop sequence) on the chemical and biochemical properties of soil samples collected from the 0–15 cm (topsoil) and 15–30 cm (subsoil) soil layers. TOC, total organic C; C_{extr}, extractable organic C; N_{extr}, extractable organic N; MBC, microbial biomass C; MBN, microbial biomass N; BR, basal respiration; MBC/TOC, microbial quotient; qCO_2, metabolic quotient; total PLFAs; total bacteria; Gram-positive (BAC^+) and Gram-negative (BAC^-) bacteria, fungi, fungi to bacteria ratio (F/B), Gram-positive to Gram-negative bacteria ratio (BAC^+/BAC^-), and cy17:0/cis16:1ω7 and cy19:0/cis18:1ω7 ratios determined for soil samples collected from the 0–15 cm (topsoil) and 15–30 cm (subsoil) soil layers.

	0-15 cm soil layer			*15-30 cm soil layer*		
	Tillage System (TS)	Crop (C)	TS × C	Tillage System (TS)	Crop (C)	TS × C
df	1	2	2	1	2	2
TOC	≤ 0.001	0.009	0.708	0.204	0.034	0.048
C_{extr}	0.084	0.858	0.041	0.027	0.603	0.201
N_{extr}	0.021	0.057	0.248	0.538	0.099	0.122
MBC	≤ 0.001	≤ 0.001	0.098	0.209	≤ 0.001	0.004
MBN	0.001	0.83	0.476	0.202	0.104	0.727
BR	0.002	0.035	0.44	0.297	0.09	0.458
MBC/TOC	0.018	≤ 0.001	0.018	0.285	≤ 0.001	0.004
qCO_2	0.076	0.023	0.188	0.025	0.078	0.806
Total PLFAs	0.053	0.149	0.048	0.149	0.074	≤ 0.001
Bacteria	0.03	0.122	0.035	0.072	0.029	≤ 0.001
BAC+	0.062	0.345	0.856	0.175	0.161	0.016
BAC-	0.025	0.061	0.003	0.033	0.008	≤ 0.001
Fungi	0.525	0.688	0.192	0.685	0.557	0.022
F/B	0.138	0.203	0.32	0.063	0.787	0.963
BAC+/BAC-	0.453	0.089	≤ 0.001	≤ 0.001	0.013	≤ 0.001
cy17:0/cis16:1ω7	0.01	0.22	0.699	0.043	0.943	0.114
cy19:0/cis18:1ω7	0.671	0.246	≤ 0.001	0.177	0.07	0.047