Highlights

1. Long term no tillage increased total organic and microbial C
2. The stimulation effect of no tillage on C pools depended on the cropping systems
3. Total organic C accumulation in no tillage was associated to bacterial community
4. Wheat crop stimulate the bacterial community, specifically the gram negative

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

[1]Giuseppe Badagliacca, [2]Vito Armando Laudicina*, [2]Gaetano Amato, [2]Luigi Badalucco, [2]Alfonso Salvatore Frenda, [2]Dario Giambalvo, [2]Rosolino Ingraffia, [3]Antonella Plaia, [2]Paolo Ruisi

[1]Department of Agriculture, Mediterranean University of Reggio Calabria, Feo di Vito, 89124 Reggio Calabria, Italy

[2]Department of Agricultural, Food and Forest Sciences, University of Palermo, Viale delle Scienze, 90128 Palermo, Italy

[3]Department of Economics, Business and Statistics, University of Palermo, Viale delle Scienze, 90128 Palermo, Italy

*Corresponding author: Vito Armando Laudicina, Tel +3909123897074; Fax +39091484035

E-mail address: vitoarmando.laudicina@unipa.it

Abstract

Determining the best conservation agriculture practices for increasing soil organic carbon (C) and hence soil quality is of paramount importance in the semi-arid Mediterranean environment, where soils are experiencing a continuous decline in organic matter. Therefore, the aim of this long-term study was to assess the combined effects of tillage system and crop sequence on soil organic C and biochemical properties of soil generally used as indicators of soil quality. After 23 years of continuous application of contrasting tillage systems (conventional tillage [CT], vs. no tillage [NT]) and crop sequences (wheat monoculture vs. wheat-faba bean rotation), soil samples were collected from topsoil (0–15 cm) and subsoil (15–30 cm) at three different times during a cropping year. Soil samples were analyzed for total and labile organic C pools, microbial biomass C (MBC) and

microbial biomass N, basal respiration, and the abundance of 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}. This in turn stimulated microbial biomass, in particular Gram-negative bacteria. This suggests a higher soil quality in NT, as was confirmed by the increase in MBC/TOC and the decrease in stress indices. In contrast, no differences were observed with regard to fungal biomass. These findings suggest the need to reconsider the role of specific bacterial groups in organic C accumulation in soils of semiarid environments. It is interesting that the effects of long-term NT varied widely by crop sequence, whereas in CT changes in biochemical characteristics and in the main microbial groups due to crop sequence were modest. Thus, the interaction among various aspects of agronomic management modulates the effects of substrate quality on chemical and biological properties of soil.

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

Abbreviations

CT: Conventional tillage

NT: no tillage

$_{W}W$: continuous wheat

$_{F}W$: wheat after faba bean

$_{W}F$: faba bean after wheat

TOC: Total organic carbon

MBC: Microbial biomass carbon

MBN: Microbial biomass nitrogen

C_{extr}: Extractable carbon

N_{extr}: Extractable nitrogen

BR: Basal respiration

qCO_2: Metabolic quotient

MBC/TOC: Microbial quotient

PLFAs: Phospholipid fatty acids

FAME: Fatty acid methyl esters

FAs: Fatty acids

B: Bacteria

F: Fungi

BAC^+: Gram positive bacteria

BAC^-: Gram negative bacteria

CDA: Canonical discriminant analysis

1. Introduction

Long-term experiments are valuable means of better understanding the agronomic practices useful for maintaining or even enhancing soil organic matter and hence soil quality. Despite the cost of maintaining experiments over time in terms of money and labor, the consequent knowledge is invaluable for evaluating the sustainability of agricultural practices. This is even more true with regard to conservation agriculture, the effects of which on both cropping systems 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 in organic matter levels no longer able to sustain crop productivity. Soil protection and sustainability have become worldwide tasks to ensure food security and mitigate the effects of climate change by increasing soil sequestered carbon (C). Thus, , 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 C and nitrogen (N) dynamics has become essential for assessing the effectiveness of such practices for supplying agroecosystem services (Chabert and Sarthou, 2020).

Biochemical properties of soil, such as microbial biomass and activity, have been extensively used as reliable and sensitive indicators of soil functioning and quality, as they are linked to the living part of the soil and thus respond quickly to any management practices that alter its characteristics (Laudicina et al., 2012; Panettieri et al., 2020; Piazza et al., 2020). Conflicting results have been reported with regard to the effects of tillage system on soil microbial biomass. Many studies have shown a higher microbial biomass in untilled compared to conventionally tilled soils (Badagliacca et al., 2018a, 2018b; Zuber and Villamil, 2016) whereas others have reported no substantial differences with different tillage systems (Acosta-Martínez et al., 2007; Mbuthia et al., 2015). Also, studies of the effects of tillage system on main microbial groups are often contradictory, with unclear trends or no differences across experiments, especially in the relative abundance of each microbial group (Helgason et al., 2010a; Sun et al., 2016). Therefore, the response of soil

89 microorganisms is likely site specific, depending on the contexts in which tillage systems are
90 adopted (climate, soil type and fertility, other management practices, etc.).
91 In addition to tillage system, crop rotation can also have marked effects on main soil microbial
92 groups (Bünemann et al., 2008). Crop rotation can change the soil habitat by affecting the soil
93 nutrient status, C input from roots (via root exudates, mucilage, etc.), the amount and quality of
94 crop residues, and aggregation/microbial habitats; these alterations in turn can affect soil microbial
95 activity and diversity (Liang et al., 2017; Venter et al., 2016). Indeed, the soil microbial community
96 can respond differently to root exudates of different crops grown in rotation. Crop rotation that
97 includes leguminous crops increases the microbial biomass C (MBC)/total organic C (TOC) ratio
98 (the microbial quotient) more than monoculture systems because of the input of organic residues of
99 different quality (Anderson and Domsch, 2010). However, conflicting results are reported
100 concerning the effects of crop rotation in place of monoculture cropping on the size, diversity, and
101 structure of the soil microbial community. For example, Lupwayi et al. (1998) showed that
102 microbial diversity was significantly higher in a wheat-pea rotation than in a continuous wheat
103 culture. Tiemann et al. (2015) found that rotational diversity increased the diversity of the microbial
104 community and the relative abundance of fungi vs. bacteria. In contrast, Navarro-Noya et al. (2013)
105 observed no effect of crop sequence (maize-wheat rotation vs. continuous maize) on soil microbial
106 diversity. Finally, crop rotation can influence the soil microbial community not only via the direct
107 effects exerted by each crop as a result of its physiology but also indirectly through the differing
108 management practices (plant density, fertilization, weed control, etc.) applied to each crop.
109 Most studies investigating the effects of soil tillage systems or crop sequences on soil C pools and
110 biochemical properties of soil have been performed in temperate regions (e.g., Mbuthia et al., 2015;
111 Wulanningtyas et al., 2021; Zhang et al., 2014). Studies conducted under semi-arid Mediterranean
112 conditions exist (e.g., Madejón et al., 2007; Melero et al., 2009), but few have investigated how soil
113 properties change in response to the combined effects of tillage system and crop sequence. This is
114 even more true for long-term experiments performed in the semi-arid Mediterranean region.

Although tillage system is the main factor affecting the distribution of soil microbial communities, several studies have identified crop type and development as the main driver of microbial responses to land management (Lopes and Fernandes, 2020). Thus, although it is clear that both factors (tillage and crop type) can influence soil microbial communities and thus processes such as C mineralization and sequestration, a marked disparity in results related to the relative effects of tillage and crop type on these organisms persists (Helgason et al., 2009). Therefore, within the valuable 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] vs. conventional tillage [CT]) continuously applied within different crop sequences (continuous wheat [wW] and wheat–faba bean rotation) on a range of chemical and biochemical properties of soil as well as on main microbial groups in the soil, all able to reflect soil organic C dynamics under Mediterranean semiarid conditions. Multivariate statistical analyses were performed to determine wheter the various cropping systems (each resulting from a specific tillage system × crop sequence combination) varied with respect to canonical components (canonical discriminant analysis [CDA]) or 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). Characteristics of the 0-40 cm soil layer determined at the beginning of the experiment were as follows: 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 phosphorus (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 = −0.01

MPa) 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 (from 1983 to 2013), mostly the autumn/winter period (September-February; 73%) and spring (March-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 (wW, wheat–faba bean, and wheat–berseem clover) acted 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, and the crop sequences were $_{W}W$ and wheat–faba bean rotation; soil samples were taken both from the wheat crop after faba bean ($_{F}W$) and from the faba bean crop after wheat ($_{W}F$). In CT, one moldboard plowing, carried out to a depth of 30 cm in the summer, was followed by two shallow (0–15 cm) harrowing operations before planting. In NT, sowing was done by direct drilling. Plots were 18.5 × 20.0 m. Glyphosate at a dose of 1066 g a.e. ha^{-1} was used in NT plots for weed control before planting. Wheat plots (i.e., $_{W}W$ and $_{F}W$) were broadcast-fertilized with 69 kg ha^{-1} P_2O_5 before planting. N fertilizer was broadcast on the soil surface at a rate of 120 kg N ha^{-1} in $_{W}W$ plots and 80 kg N ha^{-1} in $_{F}W$ plots. The total amount of N fertilizer was split over two applications: 50% immediately before planting (as diammonium phosphate and urea) and 50% at mid-tillering (end of March, before the second soil sampling) as ammonium nitrate. Faba bean plots were broadcast-fertilized only with 46 kg ha^{-1} P_2O_5 before planting. Crops were planted in December using a no-till seed drill with hoe openers under both CT and NT; the appropriate adjustments were made to the sowing depth to ensure a homogeneous planting depth (3–5 cm). Faba bean cv. Gemini was sown at 40 viable seeds m^{-2} with an inter-row spacing of 75 cm. No rhizobial inocula were applied before planting because the soil

had a native rhizobial population. Durum wheat cv. Anco Marzio was planted in rows spaced 16 cm apart at 350 viable seeds m^{-2}. In $_{W}W$ and $_{F}W$ plots, weeds were controlled with the application of herbicide in post-emergence in the early growth stage of the crop. In $_{W}F$ plots, weeds were controlled mechanically by shallow hoeing (with minimum soil disturbance) when plants were at the third-leaf stage. Faba bean was harvested in late June leaving standing straw and uniformly spreading crop residues. Wheat was also harvested in late June and stubble (about 20–25 cm from the soil surface) was left standing. Wheat straw was baled and removed from the field. The soil surface covered by mulch in the NT treatments was always >30%.

2.3. Soil sampling and analysis

During 2013–2014 cropping season, two soil samples per plot (each composed of three subsamples) were collected separately from the 0–15 cm (topsoil) and 15–30 cm (subsoil) soil layers at three 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 the soil samples. Soil samples were air-dried, sieved at 2 mm, and stored in plastic bags at room temperature. An aliquot of each soil sample was stored at 4°C for the determination of biochemical properties. All analyses were performed within 1 month of sampling. TOC was determined according to the Walkley–Black method (Nelson and Sommers, 1996).

MBC and microbial biomass 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 the chloroform was removed by repeated evacuation, the soil samples were extracted with 100 mL 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 analyzed for organic C by the acid-

dichromate oxidation method and for total N by the Kjeldahl method. Organic C and total N in the 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 TOC and total N extracted from fumigated and non-fumigated samples, respectively, multiplied by a conversion factor of 2.64 (k_{EC}) for MBC and 2.22 (k_{EN}) for MBN. We determined basal respiration (BR) 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. we assessed the CO_2 evolved after 24 h of incubation by injecting a 1 mL aliquot of gas from the headspace of the bottles into a gas chromatograph (TRACE GC; Thermo Scientific, Milan, 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 analyzed according to the modified Bligh and Dyer method (White et al., 1979). Lipids were extracted from 5 g 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, respectively, through a silicic acid column. The polar lipids were trans-esterified into 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 n-hexane. The FAMEs were detected by a gas chromatograph (FOCUS GC; Thermo Scientific) equipped with a flame ionization detector and a Mega-10 fused-silica capillary column (50 m long, 0.32 mm ID, 0.25 μm film thickness). The GC temperature progression was as follows: initial isotherm at 115°C for 5 min, increased of 1.5°C per minute from 115°C 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 a carrier. A total of 1 mL was injected in splitless mode. Nonadecanoic acid methyl ester (19:0; cat no. N-5377; Sigma-Aldrich, Milan, Italy) was used as an

internal standard for the quantification of FAMEs. Identification of the peaks was based on a 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 in nanomoles per gram of dry soil and as a mole percent (mol %) of the total fatty acids. Fatty acids with fewer than 14 C-atoms or more than 20 C-atoms were excluded, as they were considered to have originated 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 whereas the FA 18:2ω6,9 was used to represent 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 were chosen to represent 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).

2.4. Statistical analysis

Reported data are the arithmetic means of four samples (2 samples per plot × 2 replications) per treatment and per three sampling times (n = 12) and are expressed on an oven-dry basis (105°C) soil. Data were analyzed with a linear mixed model for a strip-plot design repeated in time (Schabenberger and Pierce, 2002) and computed separately for the two layers.

CDA (Friendly and Fox, 2020) was performed to differentiate treatments and to identify the major sources of difference between groups. CDA effectively projects data into the space of linear combinations of the variables that account for the greatest proportion of between-groups variance relative to within-groups variance. Also, another method of nonparametric statistical analysis, that is, recursive partitioning, which generates a classification tree (Breiman et al., 1984), was performed. The advantage of this approach include the fact that data transformation is unnecessary, normality conditions or homogeneity of covariance are not required, and variable selection is intrinsic to the methodology i.e. the procedure identifies the most important discriminant variables.

The R package "ggplot2" (Wickham et al., 2016) was used to prepare the figures. Statistical analyses were performed using R (R Core Team, 2020).

3. Results

3.1. Weather conditions

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

3.2. Soil chemical and biochemical properties

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

Also in the topsoil, C_{extr} was on average 26% higher in NT than in CT, but the effect of tillage system varied by crop sequence (Table 1). Indeed, C_{extr} was higher in NT than in CT in both wheat crops but no significant differences between the two tillage systems were found in $_{W}$F (Figure 2).

Significant differences in soil C_{extr} were observed between $_{W}F$ and $_{F}W$ in both the CT and NT systems ($_{W}F > _{F}W$ in CT, and $_{F}W > _{W}F$ in NT); however, no significant differences in this variable were observed between the two wheat crops, in either CT or NT. In the subsoil, C_{extr} was affected only by tillage system, being on average 32% higher in CT than in NT (Figure 2). In the topsoil, N_{extr} was affected more by tillage system ($P = 0.021$) than crop sequence ($P = 0.057$, Table 1). N_{extr} was on average 20% higher in NT than in CT, whereas it was on average 21% greater in $_{W}W$ and $_{F}W$ than in $_{W}F$ (Figure 2). In the subsoil, N_{extr} values were higher ($P = 0.099$) in $_{W}W$ and $_{F}W$ than $_{W}F$, regardless of tillage system (Figure 2).

In the topsoil, MBC was affected by both tillage system and crop sequence; it was 62% higher in NT than in CT (Figure 3), with the wheat plots ($_{W}W$ and $_{F}W$) showing higher values than the faba bean plots ($_{W}F$) for both tillage systems (on average +39% and +30% in CT and NT, respectively). In NT, MBC was lower in the subsoil than in the top soil (Figure 3), although no differences were evident between the topsoil and subsoil in CT. Crop interacted with tillage system (Table 1), but on average MBC was higher in wheat plots than in faba bean plots regardless of tillage system. MBN was affected only by tillage system in the upper soil layer, showing values on average 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 the 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 in both the CT and NT systems ($_{F}W > _{W}F$ in both tillage systems). Also, when the two wheat crops were compared, the microbial quotient in NT was significantly higher in $_{W}W$ than $_{F}W$ (+15%), although no differences were observed between the two wheat crops in CT. In the subsoil, crop sequence, both alone and through its interaction with tillage system, affected the microbial quotient. The most significant differences occurred in $_{W}W$, with NT showing higher

296 microbial quotients than CT, and for the rotation in CT, with $_{F}$W having higher values than $_{W}$F.
297 qCO_2 did not show a univocal pattern among treatments, with the sole exception that for $_{F}$W, in
298 both soil layers and regardless of tillage system, it showed the lowest values (Figure 4).
299
300 *3.3. Main soil microbial groups*
301 In the topsoil, regardless of crop sequence, total PLFAs were on average 54% higher in NT
302 compared to CT, but the difference between the two tillage systems interacted with crop sequence
303 to decrease PLFAs in the order $_{W}$W > $_{F}$W > $_{W}$F (Figure 5). A significant difference in total PLFAs
304 was observed between the two wheat crops CT ($_{F}$W > $_{W}$W) and similarly between the two crops
305 grown in rotation NT ($_{F}$W > $_{W}$F). In the subsoil, total PLFAs were significantly higher in NT than
306 in CT (Figure 5). A significant difference in total PLFAs was observed between the two wheat
307 crops in NT ($_{W}$W > $_{F}$W); moreover, higher PLFA values were found under both tillage systems in
308 $_{F}$W than in $_{W}$F.
309 In the subsoil, the abundance of the main microbial groups was affected by the interaction of the
310 tested factors (Table 1). Total and Gram-negative bacteria were on average higher in NT than in CT
311 (Figures 5 and 6). In CT, a significant difference in total and Gram-negative bacteria was observed
312 between the two wheat crops ($_{F}$W > $_{W}$W), whereas in NT a significant difference was found
313 between the two crops grown in rotation ($_{F}$W > $_{W}$F). Also, the BAC^{+}/BAC^{-} and cy19:0/cis18:1ω7
314 ratios were higher in CT than in NT in the wheat plots. The cy17:0/cis16:1ω7 ratio was affected
315 only by tillage system and showed higher values in CT than in NT (Figure 7).
316
317 *3.4. CDA and classification tree analysis*
318 Two CDAs were performed separately for the topsoil and the subsoil. The relationship of the
319 variables to the canonical dimensions are shown in Figure 8 by vectors. Each vector is defined by
320 the correlations it has with the canonical dimensions. In the topsoil (Figure 8A), both canonical
321 dimensions were significant according to a likelihood ratio stepdown test. Nearly 71% of between-

group mean differences were accounted for by the first canonical dimension (CAN1), which was positively influenced by all soil traits included in the analysis, but especially by TOC, MBC, and CO_2; moreover, CAN1 clearly distinguished CT from NT systems. The second canonical dimension (CAN2), which explained 16.8% of the variance, was positively influenced by TOC, BAC^+, and fungi and negatively influenced mainly by CO_2; CAN2 clearly differentiated the crop sequence treatments NT but not in CT.

In the subsoil (Figure 8B), both canonical dimensions were significant, according to a likelihood ratio stepdown test. Nearly 48% of between-groups mean differences were accounted for by CAN1, which was highly related to BAC^-, bacteria, and PLFAs. CAN2, which accounted for 27.3% of the variance, was highly related to MBC, MBN, and N_{extr}. A less clear distinction between the two tillage systems was obtained; however, CAN1 still discriminated NT wheat plots (both $_{W}$W and $_{F}$W) from all other plots.

The classification trees fitted to the two layers are shown in Figure 9. Each tree consists of a series of splitting rules, starting at the top of the tree (the root of the tree, containing all the units), each based on a single variable. Each tree is characterized by some splits that produce branches and internal nodes, whereas at the bottom terminal nodes or leaves can be found. Each split guarantees that the partitioning of the units into the two child nodes is characterized by the maximum obtainable homogeneity of the unit (with respect to the response variable, crop × tillage in this study). The variables associated with each split are the most discriminant variables. With regard to to the topsoil (Figure 9A), consistent with the CDA results, the most discriminant variables were TOC, MBC, and CO_2 (i.e., three classic soil variables linked exclusively to the C cycle). In each leaf (bottom of the tree), the distribution of units is reported: for example, in the bottom leaf (Node 4) more than 80% of units are CT-$_{W}$F, and are characterized by TOC < 13.885 and MBC < 307.125. Moreover, TOC < 15.02 corresponds to CT, whereas TOC ≥ 15.02 corresponds to NT. The results for the subsoil (Figure 9B), as expected, were not quite as good. More than three variables were necessary to partition the units into leaves as homogeneously as possible. This result was consistent

with the results shown in the CDA plot, where the overlapping of the ellipses for each group was evident. Here, however, it is noteworthy that the first discriminant was BAC^{-} (i.e., a well-defined microbial group).

4. Discussion

The CDAs performed with both measured and derived chemical and biochemical data clearly differentiated the NT systems from the CT systems and also showed more marked differences among the topsoils than the subsoils. It is interesting that the CDAs also highlighted the fact that the NT systems were more scattered over the diagrams than the CT systems, which suggests greater variability in the investigated soil parameters due to crop sequence.

In the topsoil, a considerable increase in TOC was observed in NT compared to CT. Such difference was so marked that TOC was the parameter with the highest discriminative power, as highlighted by the classification tree analysis. Because the input of crop residues did not differ between the two tillage systems (see Badagliacca et al., 2018a), the differences in TOC can be ascribed to the effects of tillage system on the fate of crop residues. Indeed, tillage can increase soil aeration and the rate of oxygen diffusion (Khan, 1996), which in turn can increase the degradation of organic matter and lower C sequestration. Moreover, by incorporating and mixing crop residues with soil, tillage increases their accessibility to soil microorganisms (Laudicina et al., 2016), thus speeding up their mineralization. The maintenance of crop residues on the surface of NT soil 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 centimeters of the topsoil (Álvaro-Fuentes et al., 2008). Moreover, as suggested by Six et al. (2000), by preserving the soil structure, conservative tillage practices contribute to the formation of C-enriched micro-aggregates in macro-aggregates, which can physically protect soil organic matter from mineralization. In contrast, by disrupting soil aggregates and increasing soil aeration, intensive tillage practices favor the oxidation

of previously physically protected soil organic matter through microbial attack (Laudicina et al., 2016). Furthermore, both C_{extr} and N_{extr} were higher in topsoil in NT compared to CT. These two 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 that supports soil C sequestration. Such greater availability of substrates in NT, according to Sun et al. (2016), increases MBC and the MBC/TOC ratio as a consequence of the greater availability of C for microorganisms (Anderson and Domsch, 2010; Badalucco et al., 2010). In conjunction with the greater availability of substrates, synergistically speaking, crop residues that accumulated on NT topsoil can reduce fluctuations in soil temperature and moisture, making the topsoil more favorable for soil microorganisms (Turmel et al., 2015). The lower MBC values in CT may also be due to enhanced exposure to the drying of microflora caused by the disruption of aggregates, as discussed before. It is remarkable that a consistent increase in microbial biomass, mainly ascribable to the increase in bacteria instead of fungi, was observed in NT compared to CT. The absence of greater amounts of fungi in NT was an unexpected result, as numerous studies have found more fungi in NT treatments (Laudicina et al., 2016; Sun et al., 2016; Wang et al., 2021). Also, in a global meta-analysis Chen et al. (2020) recently demonstrated that soil fungi and bacteria both respond positively to conservation tillage and are significantly associated with increases soil C content. However, the effect of tillage on the fungal community is controversial, as it is related to the context in which experiments are performed (Helgason et al., 2010b; Shi et al., 2012; Zhang et al., 2015). In this field experiment, regardless of tillage system, bacteria dominated the microbial community. This may be ascribed to many factors, including the moderately alkaline soil reaction and the low soil moisture for most of the year, as well as the higher availability of C and N substrates compared to CT. With regard to the latter factor, bacteria and fungi have different stoichiometry, with the former having a C/N ratio of about 5 and the latter a ratio of about 10 on average (Moore et al., 2000). Therefore, bacteria and fungi are expected to have, respectively, higher and lower N requirements. Consequently, if access to C is equivalent but N is limiting then a shift towards fungal dominance is expected, but if N is

399 not limited then bacterial dominance is expected (Carney et al., 2007). Actually, in this study, it is
400 reasonable to assume that N was not a limiting factor for bacteria because it was supplied yearly by
401 inorganic fertilization or by the legume crop (faba bean); this is confirmed by a TOC/total N ratio
402 below 10 in all treatments (see Badagliacca et al., 2018a), a C_{extr}/N_{extr} ratio below 6 (which is
403 similar to that of bacteria), and an MBC/MBN ratio below 7.7. Therefore, the increase in TOC in
404 NT over the long period was associated with the increase in the bacterial community, which was
405 greater than the increase in the fungal community. In this regard, the role of fungi in the C cycle
406 could be related more to the absorption of metabolites released by bacteria than to direct
407 decomposition of soil organic matter (Zhang et al., 2013). This finding is noteworthy and should
408 promote a focus on the role of bacteria in C sequestration in the semi-arid Mediterranean
409 environment. Furthermore, it suggests that the role of the F/B ratio as an indicator of C
410 sequestration in soils of the semi-arid Mediterranean environment should be reconsidered (Fanin et
411 al., 2019).
412 The greater amount of total bacteria in NT than in CT was mainly attributable to BAC^-. This
413 finding is in contrast with some studies (e.g. Zhang et al., 2014) but agrees with others performed in
414 warm and dry environments (Ali et al., 2018; Ma et al., 2014). One explanation for this result may
415 be that the higher C availability in NT soils promotes BAC^- (78% higher compared to CT, on
416 average) over BAC^+, as the former grow more quickly and are better able to proliferate as soon as
417 the availability of nutrients increases (Feng and Simpson, 2009). This hypothesis is compatible with
418 what Laudicina et al. (2014) found in the same study area (i.e., higher amounts of easily
419 decomposable substrate in NT compared to CT). Furthermore, Fanin et al. (2019) suggested that
420 BAC^- prefer plant-derived C substrate rather than soil-derived C. However, long-term NT also
421 promoted, albeit to a lesser extent than BAC^-, BAC^+ (+58% in NT than CT, on average), which are
422 able to use older organic C substrates due to their capability to utilize recalcitrant organic C (Fanin
423 et al., 2019). The stress indicators calculated as the ratio of cyclopropane to monoenoic precursor
424 fatty acids agreed with this. Indeed, the higher cy17:0/cis16:1ω7 ratio in CT suggests stress

425 conditions for soil microorganisms, likely as a consequence of soil microbial adaptation (Gil et al.,
426 2011) to limited C, which could have limited bacterial growth (Liu et al., 2015). Still regarding the
427 topsoil, little variations in chemical and biochemical parameters and main microbial groups were
428 observed due to the effects of the crop sequences applied in CT. In contrast, large differences
429 among the main microbial groups were observed in NT, which were linked more to the effect of
430 crop than to the cumulative effect of time. The higher values for total PLFAs and bacteria, in
431 particular BAC^{-}, observed in the wheat plots than in the $_{W}F$ plots can be ascribed to the different
432 plant densities and morpho-physiological root traits of the species (higher root density and root
433 exudate deposition in wheat than in faba bean; Acosta-Martínez et al., 2007; Rich and Watt, 2013)
434 and to the different N fertilization (Liu et al., 2010), with $_{W}W$ and $_{F}W$ receiving, respectively, 120
435 and 80 kg N ha^{-1} and faba bean receiving no mineral N through fertilization. This agrees with other
436 studies (Bünemann et al., 2008; González-Chávez et al., 2010), confirming the ability of wheat to
437 increase concentrations of the fatty acids 18:1ω7, cy17:0, and cy19:0 (i.e. the bioindicators of
438 BAC^{-}), in its rhizosphere, especially when it is grown in monoculture or in very short crop
439 rotations. With regard to N fertilization, a similar stimulation effect on BAC^{-} was observed by
440 Kirchmann et al. (2013) and Zhang et al. (2019), which may have been due to the combined effect
441 of N availability for bacterial growth and of root exudation by the plants (Palazzolo et al., 2019;
442 Wardle, 2002). Moreover, as argued by Steward et al. (2018), both factors can interact, as N
443 fertilization can support an increase in wheat root exudation represented by sugars, organic acids,
444 and other readily-available C forms for microbes, including BAC^{-} such as ammonia-oxidizing and
445 denitrifying bacteria, in accordance with the results obtained by Zhu et al. (2016) and Badagliacca
446 et al. (2018a) in a previous in-depth study performed as part of the same long-term experiment.
447 Therefore, it appears that NT favors BAC^{-}, especially when associated with the cultivation of
448 wheat.
449 The reason why the different crop sequences had pronounced effects on the main microbial groups
450 in NT but not in CT remains to be properly elucidated. A number of factors could have played a

role in this, 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 due to the varying soil tillage regimes (Panettieri et al., 2020), and differences in weed flora between CT and NT by crop sequence (as previously observed by Ruisi et al., 2015, in this same long-term experiment), which may have shaped the soil microbial community by releasing different root exudates and depositing residues of different quality. All these factors can also interact with one another and produce cumulative effects over time. Overall, this study suggests that the variations in TOC, C_{extr} and N_{extr} and other physical characteristics (bulk density and porosity; see Badagliacca et al., 2018a) induced by NT made this system much more responsive to stimuli deriving from changes in other management factors (i.e., crop sequence), with effects visible up to the main microbial groups. This is very interesting and certainly deserves further investigation, as it could suggest a greater resilience of the soil-plant system in NT as opposed to CT. It is interesting that, crop data obtained from the same long-term experiment showed that NT positively influenced the yield of crops and their efficiency of resource-use only in systems in which a proper crop sequence (i.e., cereal-legume rotation instead of continuous cereal cropping) was adopted and, in addition, when other crop management practices (weed control, N fertilization, etc.) were appropriately modulated (Amato et al., 2013; Ruisi et al., 2016). The results of this research provide a useful key for interpreting these effects.

The comprehensive analysis of data for the subsoil made it possible to differentiate the conventional system from that of NT, 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, if we consider only the plowed 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 (0–30 cm) 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 could be due to the different stratification of crop residues induced by the two tillage systems rather than to differences in C transfer between upper and lower soil layers.

With regard to the abundance of the main microbial groups, the systems under study (tillage and crop sequence) showed effects in the subsoil 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 on the microbial population with a similar trend in NT but not in CT. Finally, further research is needed to elucidate, by molecular analysis, the changes induced by tillage systems and crop sequences on soil bacterial and fungal community structures after long-term continuous application.

5. Conclusions

Overall, these results suggest that in the semi-arid Mediterranean environment, long-term NT, compared to CT, improves soil quality by increasing soil organic C, microbial biomass, and the microbial quotient, thus potentially enhancing the contribution of the agroecosystem to mitigating and adaptating to climate change. Long-term NT increased soil organic C, thus allowing achievement of the COP21 target in this Mediterranean semi-arid environment.

The greater availability of organic substrates due to the application of NT in turn stimulated soil microbial biomass and in particular the bacterial community, mainly BAC^{-}, instead of the fungal community. 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 the fungi-to-bacteria ratio as an indicator of C sequestration should be reconsidered, at least for this environment.

The effects of NT varied widely by crop sequences, whereas those of CT were modest and not always appreciable. This underlines the importance of the interaction between various aspects of

agronomic management (tillage, crop sequence, fertilization, etc.) in modulating the effects of substrate quality on the chemical and biological properties soil. Moreover, the variations in total and labile soil C and N pools and in physical characteristics induced by NT allows NT systems to be much more responsive to stimuli deriving from changes in crop sequence, with effects visible up to the main microbial groups. This is very interesting and certainly deserves further investigation, as it could suggest a greater resilience of the soil-plant system in NT as opposed to CT. The information obtained from this study may contribute to a more successful application of conservation agriculture practices in Mediterranean semiarid regions, to maintain or even enhance soil quality.

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758

759 **Figure captions**

760

761 **Figure 1.** Rainfall events (blue bars) and daily mean air temperature (red line) at the experimental
762 site during the 2013–2014 growing season (September 2013-July 2014).

763

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

770

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

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

Figure 5. Total PLFAs, bacteria and fungi as affected by tillage system (CT, conventional tillage: grey plots; NT, no tillage: coloured plots) and crop ($_W$W, continuous wheat; $_F$W, wheat grown after faba bean; $_W$F, faba bean grown after wheat) determined on soil samples collected from the 0–15 cm (topsoil) and 15–30 cm (subsoil) soil layers. Circles inside plots represent means, with whiskers representing ± SE (n = 12). The width of the plot shows the density distribution of values.

Figure 6. Gram-positive (BAC^+) and Gram-negative (BAC^-) bacteria and fungi-to-bacteria ratio (F/B) as affected by tillage system (CT, conventional tillage: gray plots; NT, no tillage: colored plots) and crop ($_W$W, continuous wheat; $_F$W, wheat grown after faba bean; $_W$F, faba bean grown after wheat) in soil samples collected from the 0–15 cm (topsoil) and 15–30 cm (subsoil) soil layers. Circles inside plots represent means, with whiskers representing ± SE (n = 12). The width of the plot shows the density distribution of values.

Figure 7. Gram-positive to Gram-negative bacteria ratio (BAC^+/BAC^-), and cy17:0/cis16:1ω7 and cy19:0/cis18:1ω7 ratios as affected by tillage system (CT, conventional tillage: grey plots; NT, no tillage: coloured plots) and crop ($_W$W, continuous wheat; $_F$W, wheat grown after faba bean; $_W$F, faba bean grown after wheat) in soil samples collected from the 0–15 cm (topsoil) and 15–30 cm

(subsoil) soil layers. Circles inside plots represent means, with whiskers representing ± SE (n = 12). The width of the plot shows the density distribution of values.

Figure 8. Canonical discriminant analysis (CDA) ordination biplots of the six cropping system centroids for the 0–15 cm (topsoil; A) and 15–30 cm (subsoil; B) soil layers. In each biplot, the direction and length of the lines (vectors) indicate the canonical loadings of the soil properties on the first two canonical variables. The plot shows scores for the canonical dimensions and overlays 60% data ellipses for each group. CT, conventional tillage; NT, no tillage; $_{W}$W, continuous wheat; $_{F}$W, wheat grown after faba bean; $_{W}$F, faba bean grown after wheat.

Figure 9. Classification trees for the 0–15 cm (topsoil; A) and 15–30 cm (subsoil; B) soil layers obtained by recursive partitioning performed on chemical and biochemical properties of the soil. The most important (discriminant) soil properties are shown. Threshold values discriminating the plots are reported. CT, conventional tillage; NT, no tillage; $_{W}$W, continuous wheat; $_{F}$W, wheat grown after faba bean; $_{W}$F, faba bean grown after wheat.

Figure1

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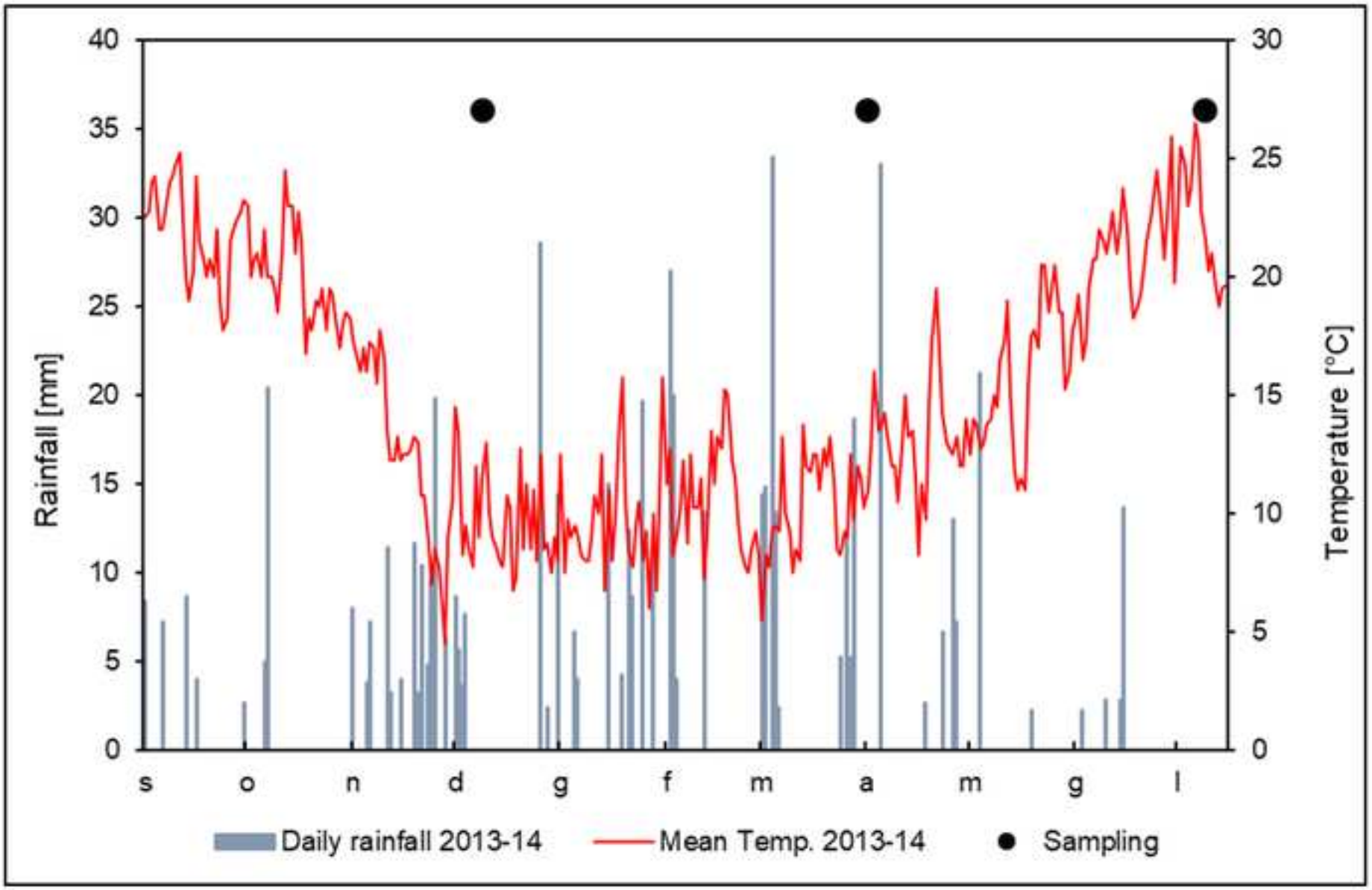

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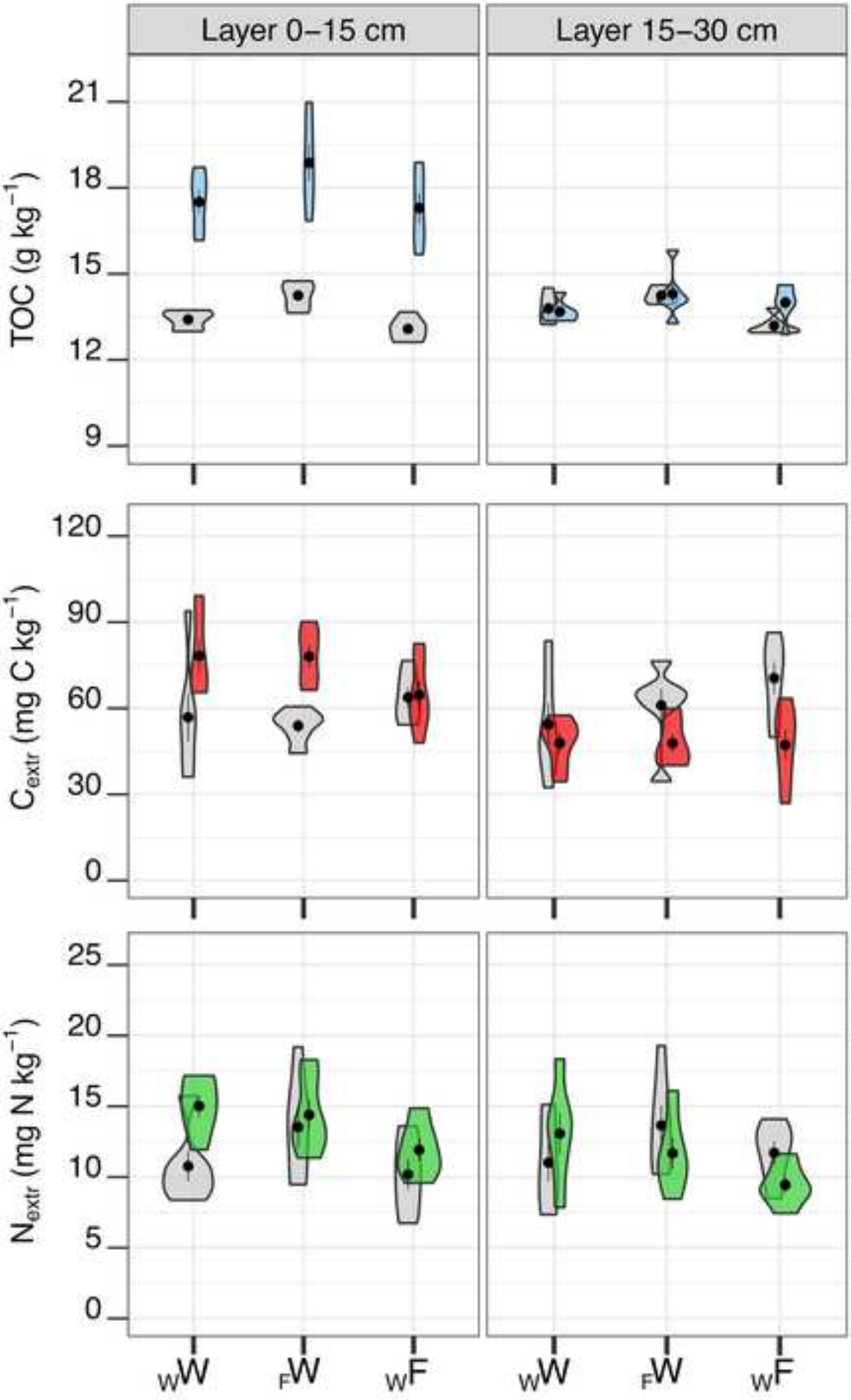

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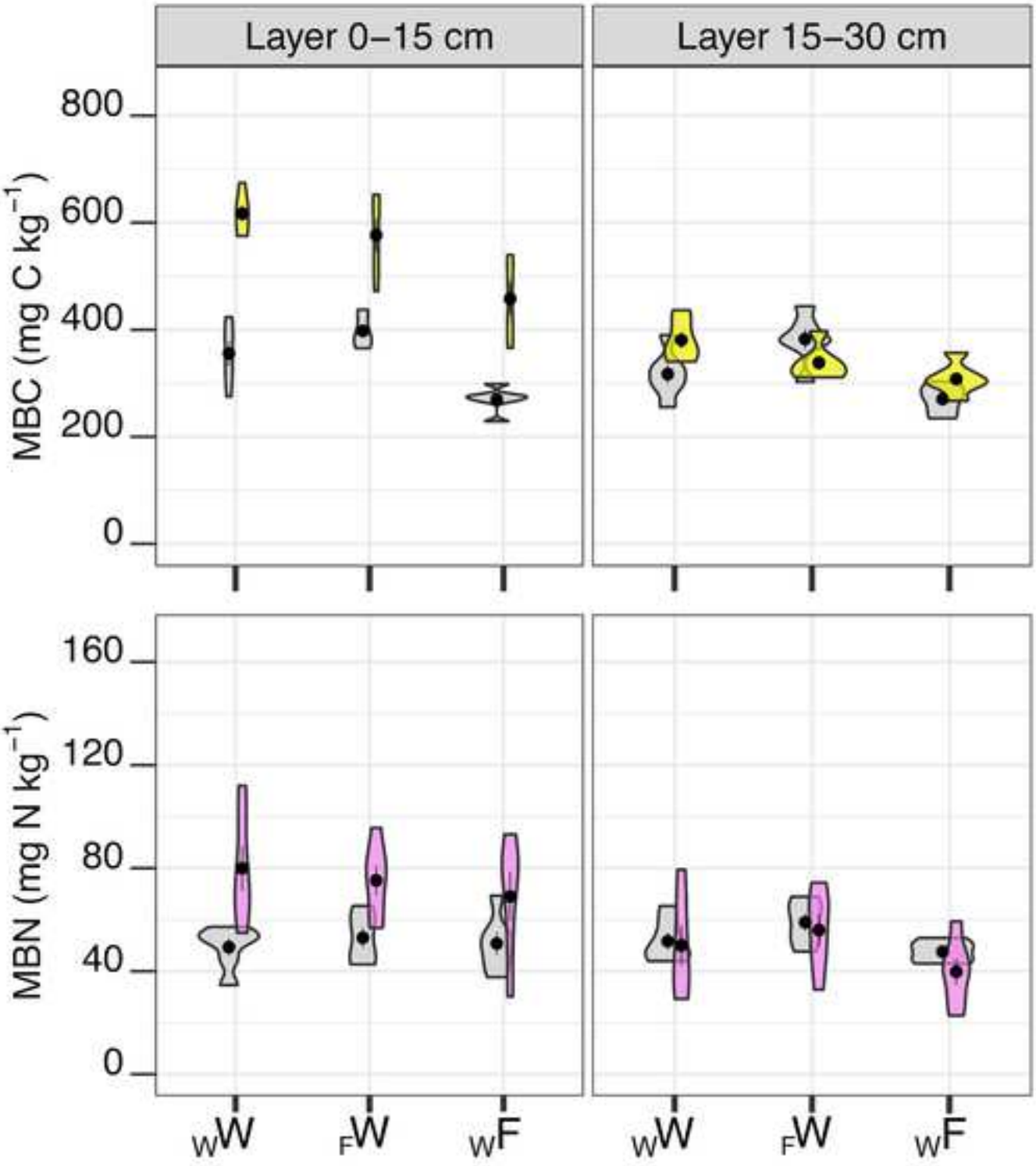

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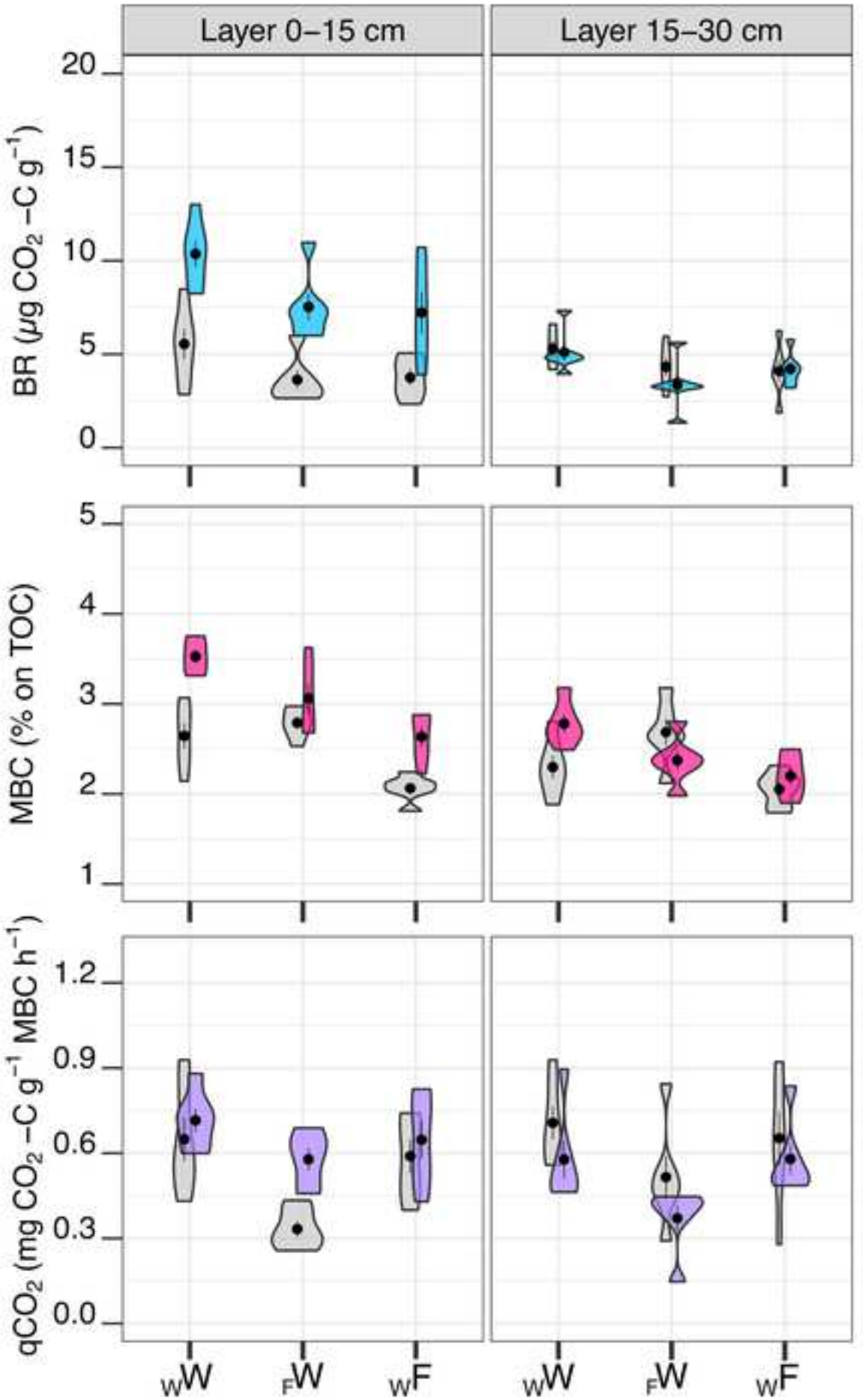

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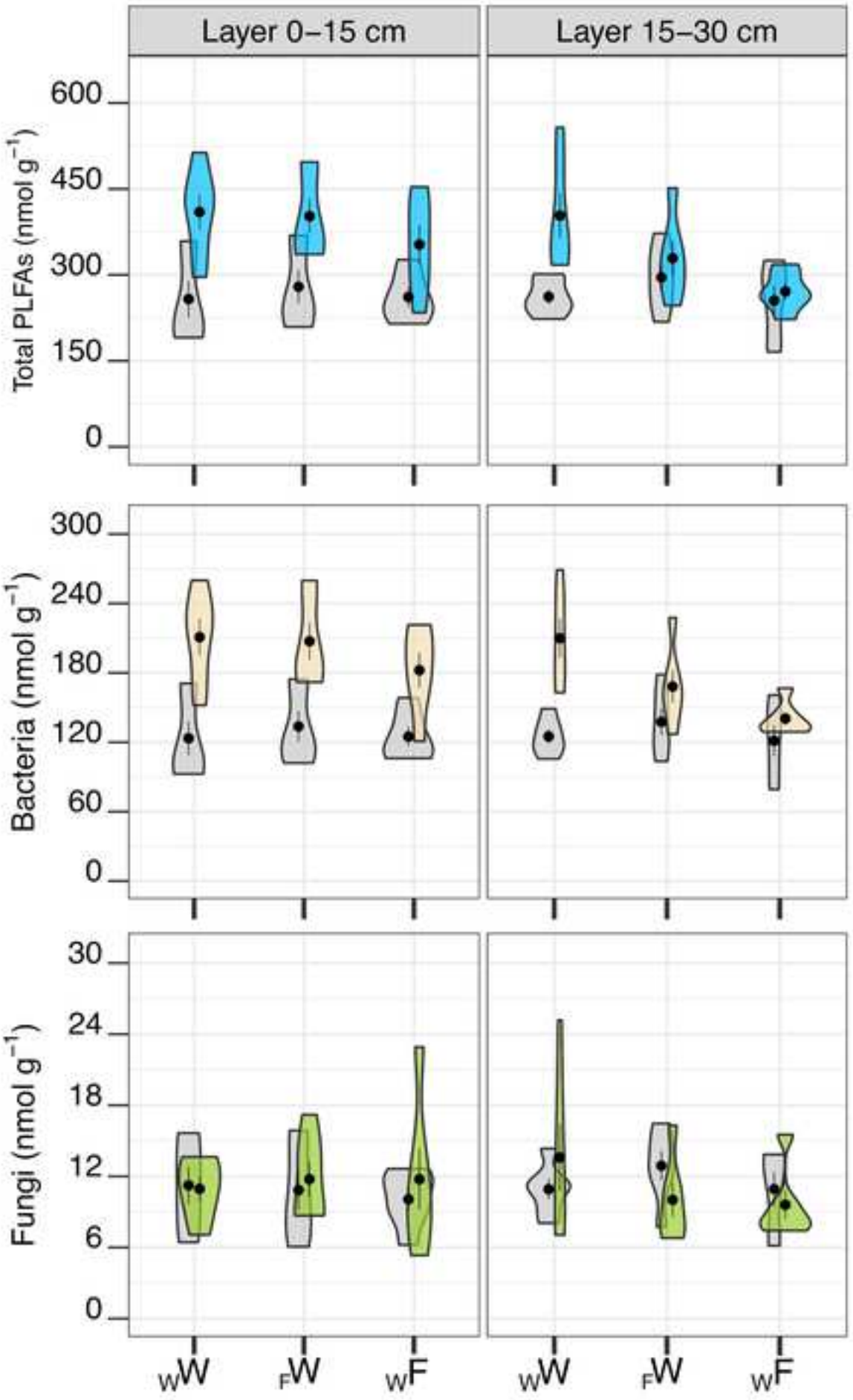

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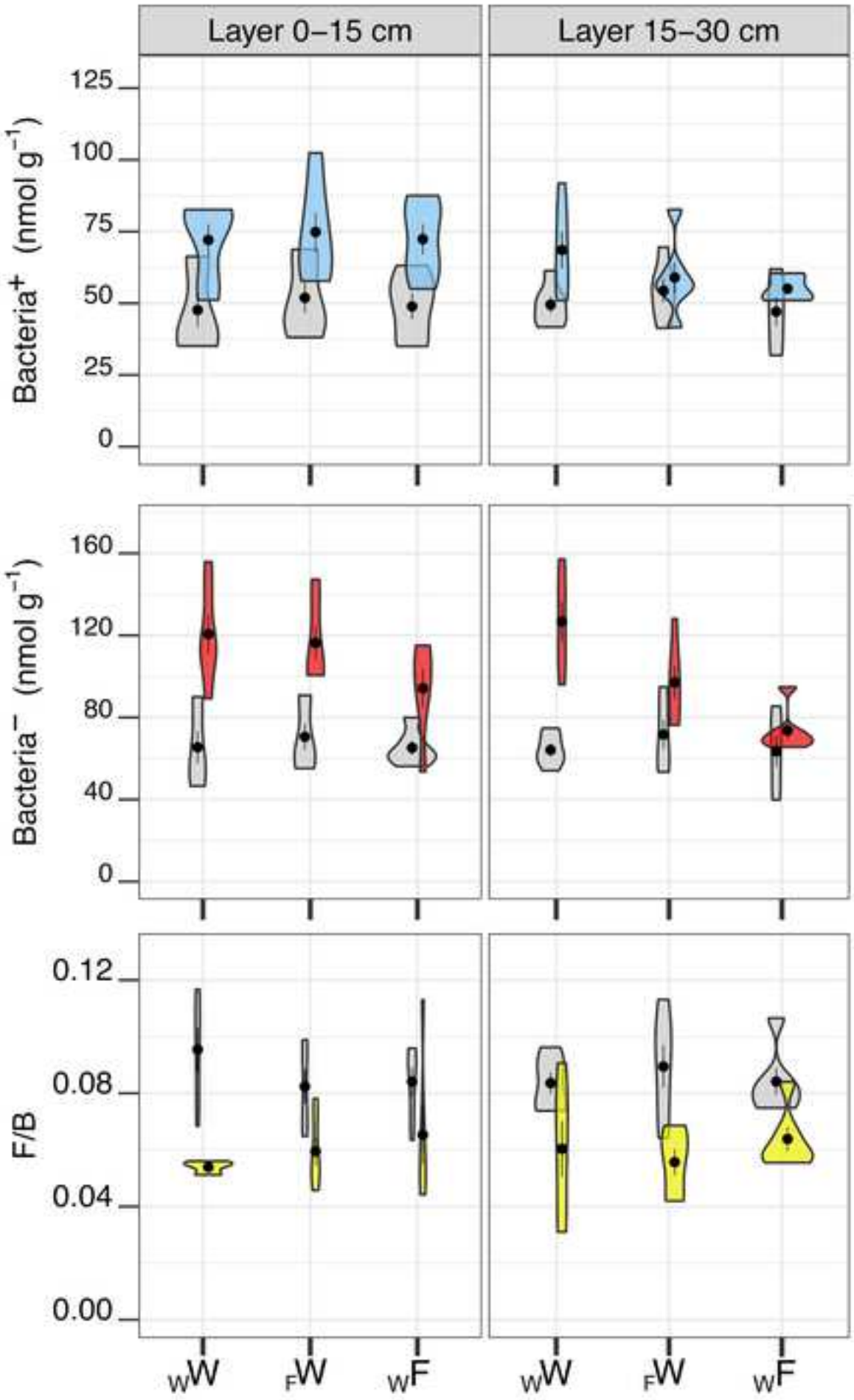

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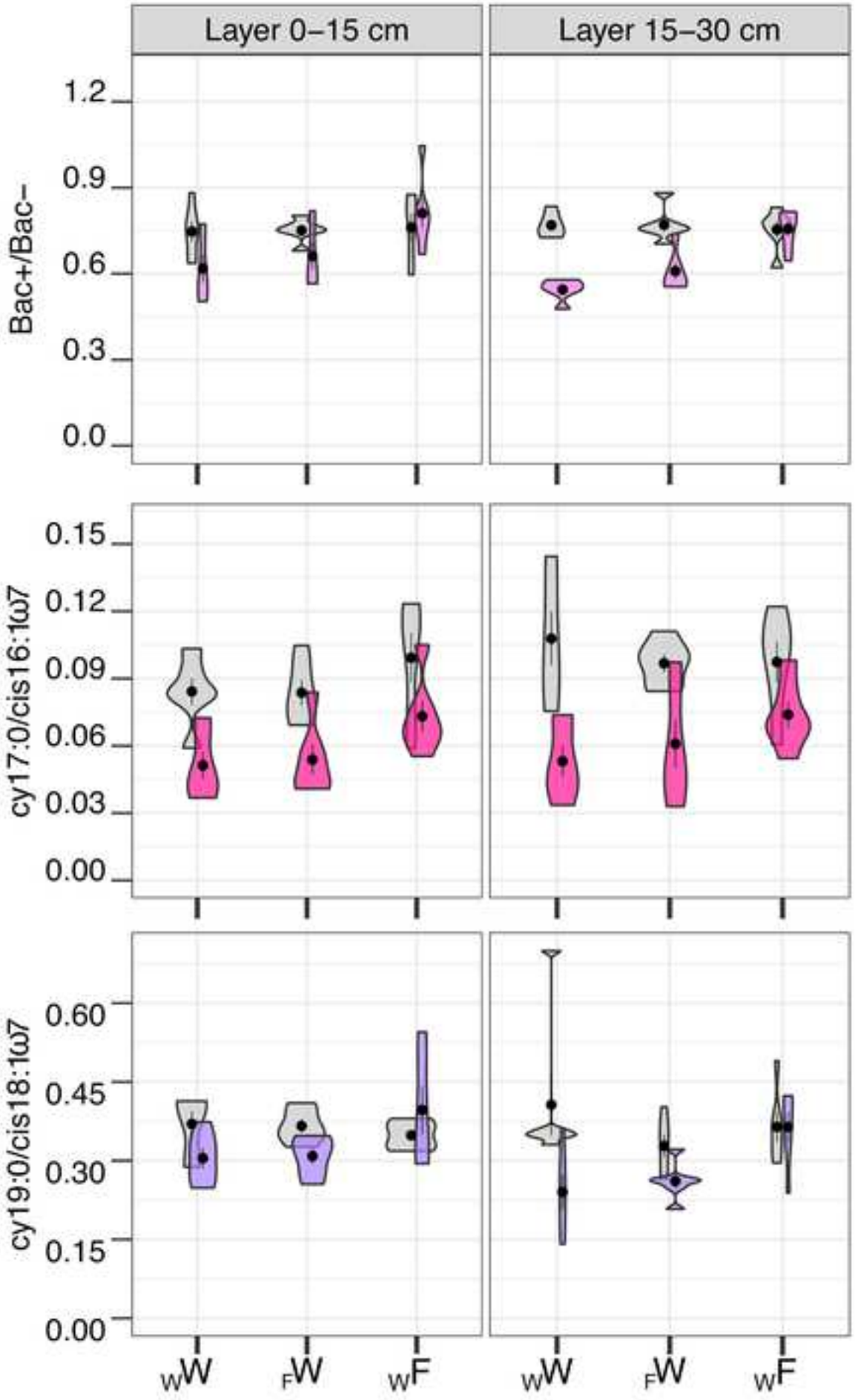

Figure8A

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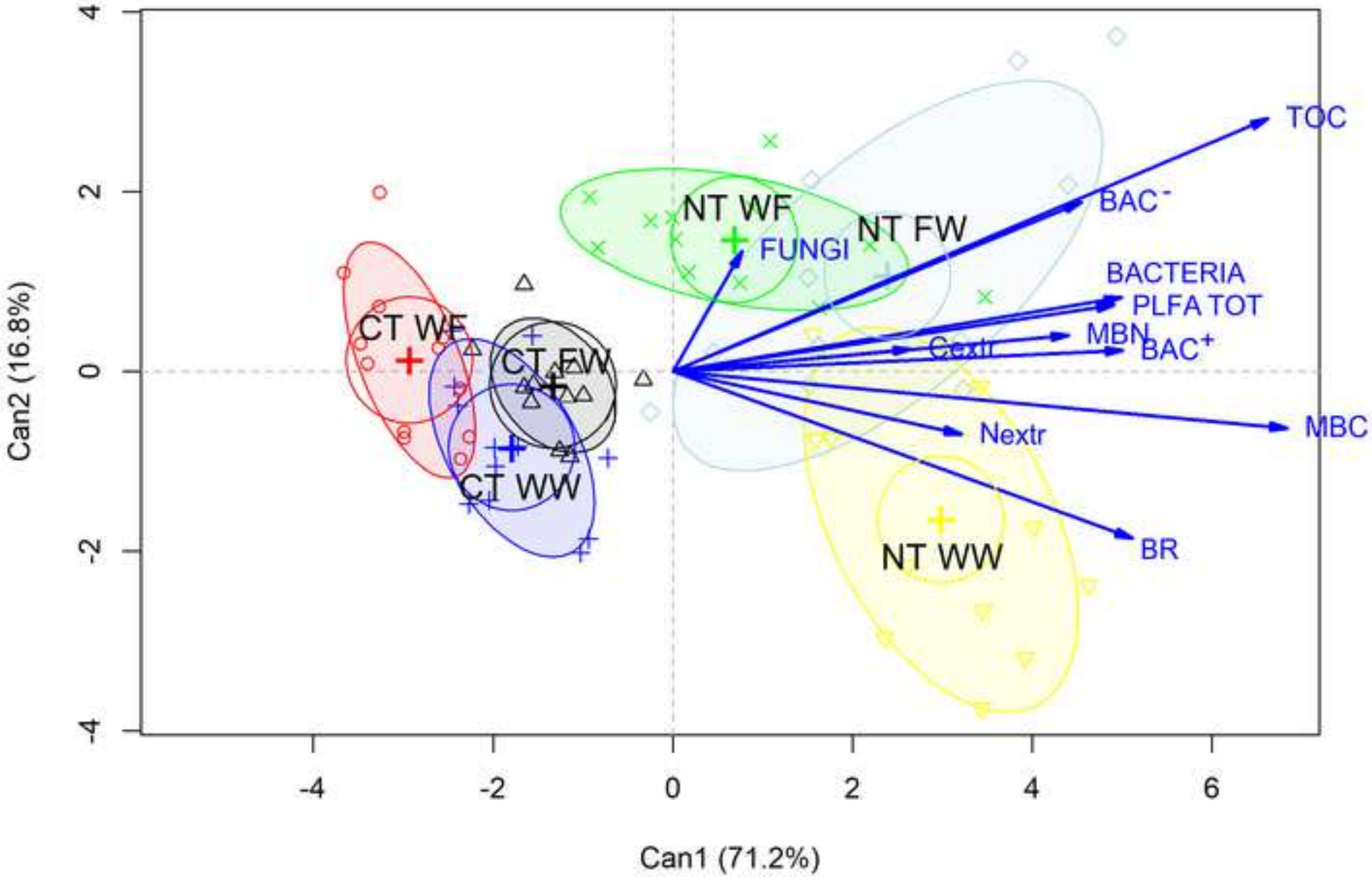

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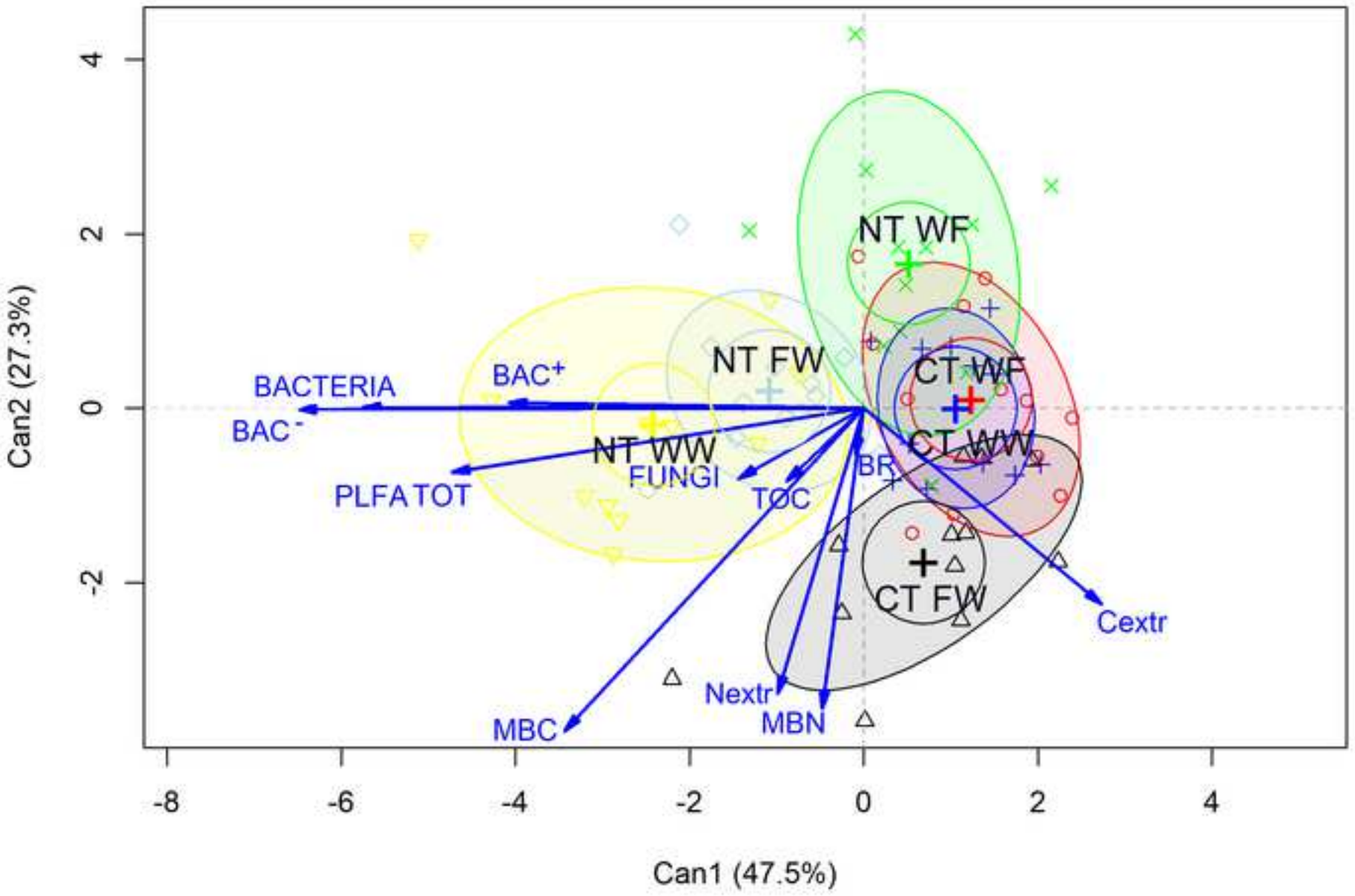

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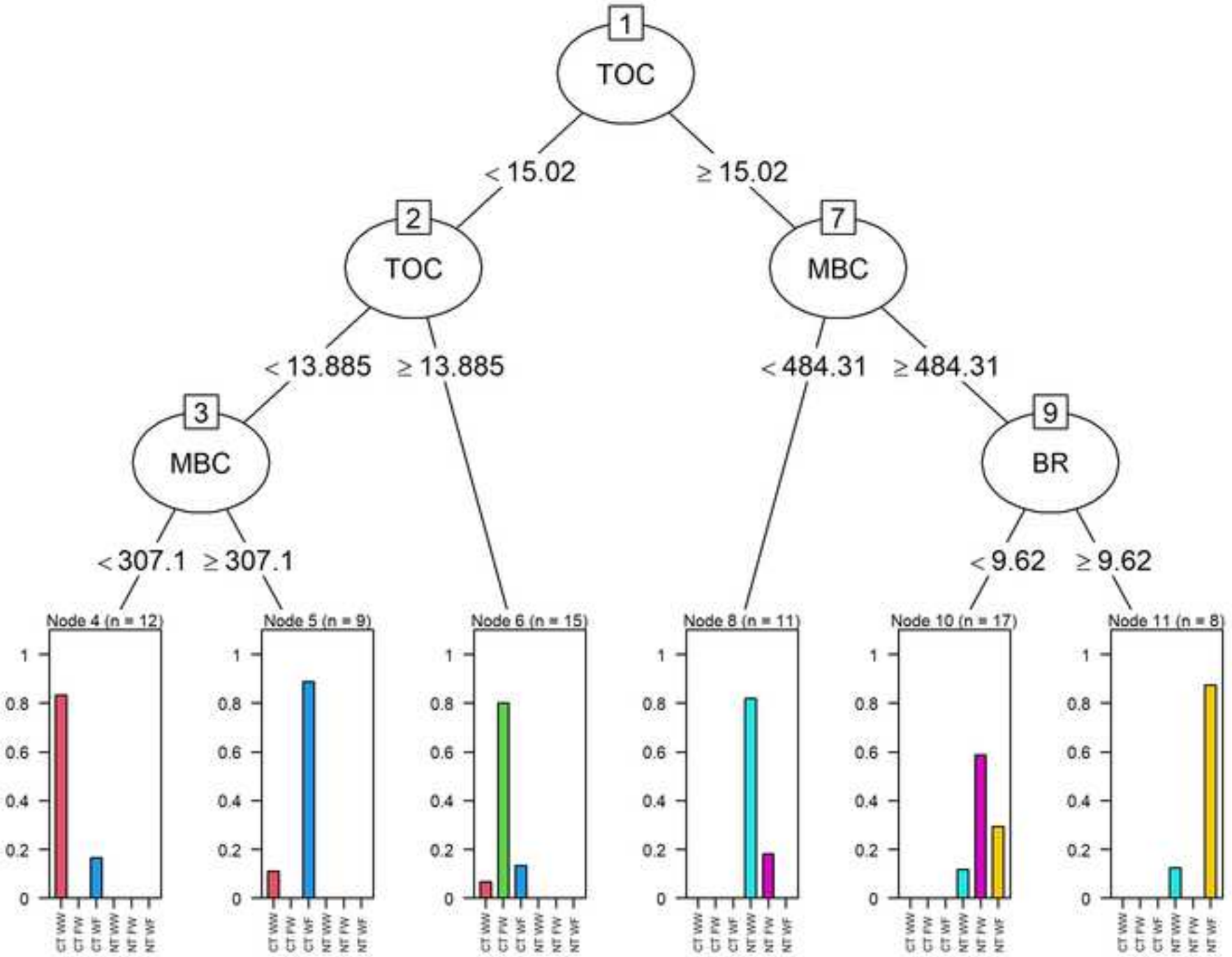

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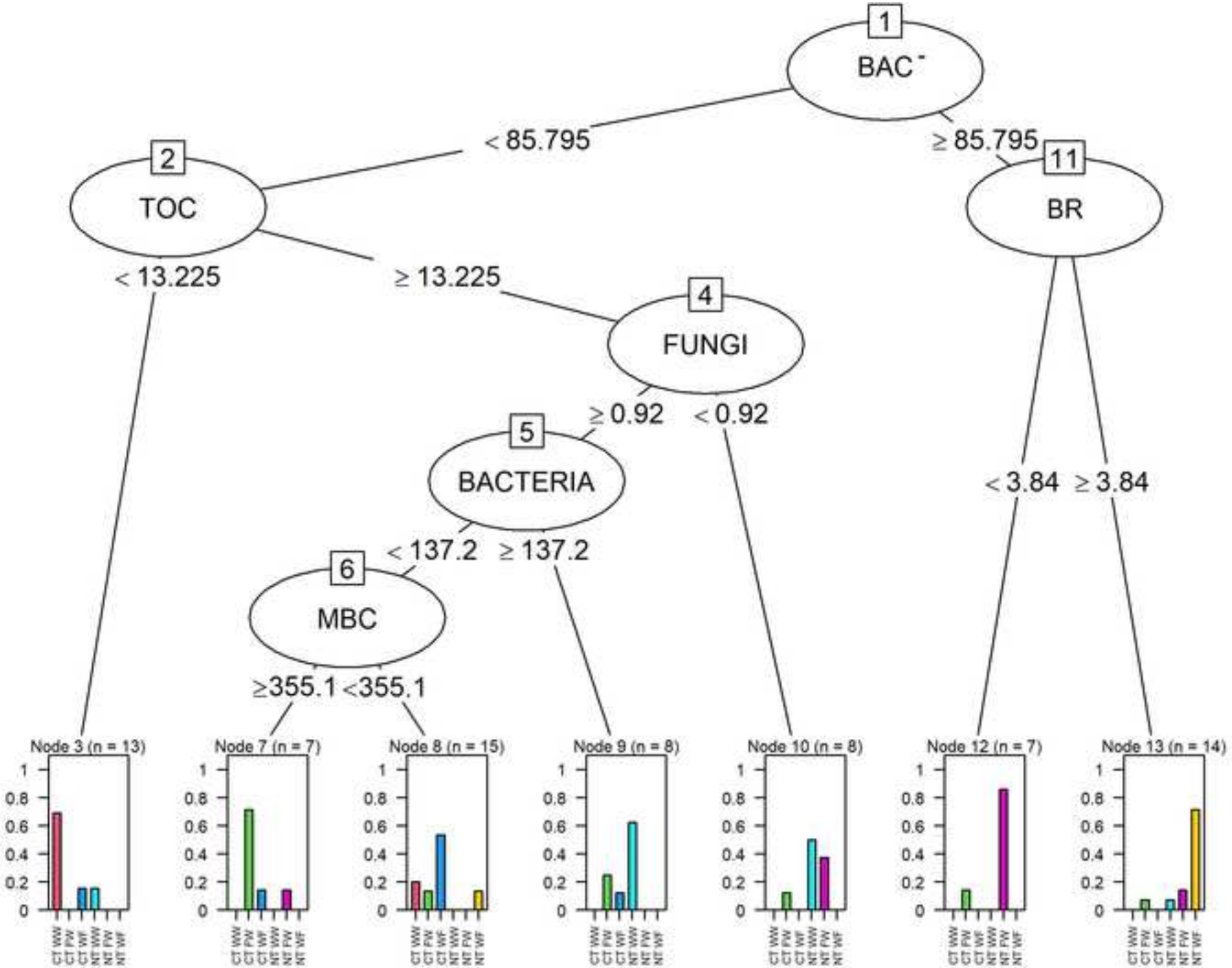

Table 1. Analysis of variance: P-values for the effects of the applied treatments (tillage system and crop sequence) on the chemical and biochemical properties of soil samples collected from the 0–15 cm (topsoil) and 15–30 cm (subsoil) soil layers. TOC, total organic C; C_{extr}, extractable organic 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