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Long-term conservation tillage and nitrogen fertilization effects on soil aggregate distribution, nutrient stocks and enzymatic activities in bulk soil and occluded microaggregates

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ABSTRACT

Conservation agriculture is globally recommended for increasing soil C and reducing greenhouse gases emissions by modifying soil physical, chemical and biological properties and processes. We assessed the impact of long-term conservation tillage and N fertilization in wheat-soybean rotation on soil aggregate distribution, nutrients stocks and functions of soil microorganisms related to C, N, P and S cycles at different aggregation scales in the Mediterranean area. A long-term field experiment was set up in a silt loam soil comparing two tillage intensities to bread wheat (*Triticum aestivum* L.) - soybean (*Glycine max* L. Merr.) rotation: conventional tillage (CT) and minimum tillage (MT). Two N fertilization levels were applied on bread wheat: 0 and 200 kg N ha^{-1} (N0 and N200, respectively). Under CT, almost 100% of the crop residues were incorporated in the 0–25 cm soil layer, whereas under MT approximately 50% were incorporated at 0–15-cm depth. Tillage was the most discriminant factor explaining 72% and 60% of total variance of soil parameters at 0–15 cm and 15–30 cm soil depth, respectively, whereas N fertilization explained 22% and 29% of total variance, respectively. All enzyme activities were higher under MT, whereas the majority of soil chemical parameters were higher under N200. Under MT a higher proportion of free microaggregates (+51%) was recorded compared to CT suggesting that in a silt loam soil MT had a greater potential to form macroaggregates. The proportion of small macroaggregates was not changed by tillage, but when this fraction was fractionated a higher proportion of occluded microaggregates was found under MT at 15–30 cm (+21%). In the occluded microaggregates, interaction of tillage x N fertilization explained 52% of total variance of soil parameters at 0–15 cm. The most discriminant parameters were the biochemical ones and SOC. All those parameters were higher in MT-N200. Specifically, SOC in occluded microaggregates was increased by N fertilization under MT (+16% at 0–15 cm; +84% at 15–30 cm), whereas it was decreased under CT (-46% at 0–15 cm; -15% at 15–30 cm). In addition, the synthetic indexes for enzymatic activities and for those involved in C-cycle were reduced by N fertilization under MT up to 37% and increased up to 87% under CT. In the Mediterranean area, maintaining or even increasing SOC conservation may require both reduced tillage systems and N fertilization, shifting microbial community towards toward taxa more effective in contrasting soil degradation.

1. Introduction

In conservation agriculture, minimal mechanical disturbance of soil, surface cover and crop rotation are the three key management strategies (Hobbs et al., 2008) that, together with nitrogen (N) fertilization, affect crop productivity, amount of carbon (C) returning to soil from crop residues, and rates of soil organic matter (SOM) mineralization (Cambardella and Elliott, 1993; Lal, 2004a). These management practices are globally recommended for increasing C stock in soil and reducing greenhouse gases emissions (Alvarez, 2005; Palm et al., 2014) by modifying soil physical, chemical and biological properties and processes (Halvorson et al., 1999; Marinari et al., 2000; Mbuthia et al.,

Abbreviations: MT, minimum tillage; CT, conventional tillage; N0, 0 kg N ha^{-1}; N200, 200 kg N ha^{-1}; SOC, soil organic carbon; Cell, β-cellobiohydrolase (cellulase); NAG, N-acetyl-β-glucosaminidase (chitinase); β-gluc, β-glucosidase; α-gluc, α-glucosidase; Xylos, β-xylosidase (xylosidase); Phosph, acid phosphaphase (phosphatase); Aryls, arylsulphatase (sulphatase); L.AP, leucine-aminopeptidase (leucine); SEI, Synthetic Enzyme Index; SEIc, Synthetic Enzyme Index involved in C-cycle

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2015; Ercoli et al., 2007, 2017a).

The loss of SOM is recognized as one of the major soil threats by the European Community (European Communities, 2012). Maps drawn by the Joint Research Centre indicated top layers having 1–2% soil organic C (SOC) and it is steadily declining in cultivated soils across Europe (Jones et al., 2005; Smith et al., 2005; Bindi and Olesen, 2011). In this context, no-tillage (NT) and minimum tillage (MT) are conservative practices largely recommended for their potential to increase SOC sequestration (Lal, 2004b, 2008; Corsi et al., 2012; Somasundaram et al., 2017), but published data on the magnitude of SOC build-up under NT and MT varied widely (Merante et al., 2017). No-tillage was estimated to increase soil C storage by 0.04-0.19 Mg C ha^{-1} $year^{-1}$ over four global climatic regions in comparison to conventional tillage (CT) (Smith, 2008). In detail, soil C sequestration rate increases by 0.1–1.0 Mg C ha^{-1} $year^{-1}$ in humid and cool climates and by 0.3-0.4 Mg C ha^{-1} $year^{-1}$ in humid and warm temperate climates (Lal, 2004b; Franzluebbers, 2005; Aguilera et al., 2013). Moreover, MT was estimated to increase soil C sequestration rate by 0.12-0.20 Mg C ha^{-1} $year^{-1}$ in semi-arid continental and tropical climates (López-Fando and Pardo, 2009; Dalal et al., 2011; Prasad et al., 2016). However, other studies on NT and reduced tillage did not record any effect in dry temperate and tropical climates (Halvorson et al., 2002; Ogle et al., 2012; Follett et al., 2013; Stockmann et al., 2013). Under NT a differential depth distribution of SOC was also highlighted compared to conventional tillage, with significant accumulation at the surface layer (< 10 cm), no difference at 10–20 cm and a significant decrease at 20–40 cm soil depth (Luo et al., 2010; Powlson et al., 2014).

Increasing N fertilization may change SOC accumulation, depending on the net effect between the increase of soil C inputs by crop production, residues and roots and the stimulation of SOC decomposition (Dalal et al., 2011; Geisseler and Scow, 2014; Stewart et al., 2017). Several studies have shown that incorporating crop residues together with an adequate rate of N fertilizer consistently increase SOC accumulation (Moran et al., 2005; De Sanctis et al., 2012; Aguilera et al., 2013). In contrast, in some studies high N availability in soil following N fertilization was associated with a decline of SOC accumulation due to SOC decomposition higher than residue deposition (Russell et al., 2009; Brown et al., 2014).

The molecular structure of organic matter has long been thought to determine SOC stabilization as persistence was explained mainly by chemical mechanisms (Sollins et al., 1996). However, it was recently demonstrated that the molecular structure of plant residues and the root exudates has a secondary role in determining SOC stability and persistence largely depends on physical protection (Six et al., 2000a; Schmidt et al., 2011; Six and Paustian, 2014). Thus, organic C is protected in soil aggregates by physical and biological mechanisms limiting the accessibility of decomposers and enzymes to the organic substrates and the diffusion of O_2 (Lehmann and Kleber, 2015). Following the conceptual hierarchical model of Tisdall and Oades (1982), mineral particles are bound together by bacterial, fungal, and plant debris into microaggregates, which in turn are bound into macroaggregates by transient agents (i.e., microbial and plant polysaccharides) that are rapidly decomposed by microorganisms, and by temporary agents (i.e., roots, fungal hyphae and glomalin) persisting for months to years. Carbon concentration increases with the increase of the size class of aggregates and younger and more labile organic matter is contained in macroaggregates than in microaggregates (Elliott, 1986; Jastrow, 1996). Thereafter, SOM turnover in agroecosystems and the rapid mineralization of labile SOC, after aggregates disruption in CT, were explained by the hierarchical model modified and implemented by Six et al. (2000a) (e.g., Six et al., 2004; Lagomarsino et al., 2009). According to this model, SOC is protected in the long-term into occluded microaggregates (microaggregates within macroaggregates) rather than into macroaggregates, and SOC stabilization mainly depends on macroaggregate turnover. Therefore, the occluded microaggregates fraction was proposed as an indicator of SOC storage capacity of best management practices, since 49–112% of the difference in total SOC between NT and CT occurred into this fraction (Six and Paustian, 2014). However, in the Mediterranean areas, where agricultural soils are characterized by low SOC levels (1–2%; Rial et al., 2017) and are highly vulnerable to extreme rainfalls and temperatures (Vallebona et al., 2015), the effects of tillage and N fertilization on SOC storage in the occluded microaggregates was scarcely investigated (Denef et al., 2007; Gentile et al., 2013). Few studies were performed on the effect of N fertilization on SOC storage and N-cycling microbial communities in the occluded microaggregates (Kong et al., 2005, 2010), and no studies investigated the response to tillage intensity in term of changes of SOC storage and soil enzymes in the occluded microaggregates. However, the effect of tillage intensities and N fertilization was investigated at micro-scale level on soil enzyme activities by Lagomarsino et al. (2009) in the Mediterranean area, and by Wang et al. (2018) in a subtropical humid area.

Therefore, we hypothesized that under Mediterranean climatic conditions in a silt loam soil MT at low N availability would increase the proportion of occluded microaggregates and the physical protection of SOC from microbial decomposition. To verify this hypothesis, we measured in a long-term field experiment soil aggregate fractions, SOC, N stocks and enzymatic activities in bulk soil and occluded microaggregates along the soil profile. In addition, the production of wheat and soybean was assessed to evaluate the 23-year (1993–2016) average effect of conservation tillage and N fertilization.

2. Materials and methods

2.1. Experimental site and treatments

The long-term field experiment was set up in 1993 comparing two tillage intensities to a bread wheat (*Triticum aestivum* L.) and soybean (*Glycine max* L. Merr.) cropping system: conventional tillage (CT), mouldboard ploughing at 25-cm depth, disking and harrowing at 15-cm depth; minimum tillage (MT), disk harrowing at 15-cm depth. Two N fertilization levels were also applied on bread wheat: 0 and 200 kg N ha^{-1} (N0 and N200, respectively). These treatments were applied arranged following a split-plot design with tillage as main-plot factor and N fertilization as subplot factor, with three replicate plots (dimension: 11.5 x 14.5 m). The experiment was conducted at the Centro Interdipartimentale di Ricerche Agro-Ambientali Enrico Avanzi (San Piero a Grado, Pisa, Italy; 43°40′ lat. N; 10°19′ long. E; 1 m above sea level) of the University of Pisa on an alluvial silt loam soil (13.1, 61.3 and 25.6% of sand, silt and clay, respectively in the 0–30 cm soil layer). The soil is classified as Typic Xerofluvent by USDA system (Soil Survey Staff, 1975) and as Fluvisol by FAO (IUSS working group WRB, 2006). Climate of the site is cold, humid Mediterranean (Csa), according to the Köppen-Geiger climate classification (Kottek et al., 2006). Nitrogen fertilizer treatment was applied to wheat as urea and the rate was split into three applications before seeding (60 kg N ha^{-1}), at first node detectable (70 kg N ha^{-1}), and 15 days after this stage (70 kg N ha^{-1}). Under CT, almost 100% of the residues were incorporated in the 0–25 cm soil layer, whereas under MT approximately 50% of the crop residues were incorporated at 0–15 cm depth. Crops were managed following the common agronomical technique applied in the area, comprising pre-emergence herbicide application for weed control, whereas no disease or insect treatment was applied. Details about treatments and management practices are given in Table S1.

2.2. Crop sampling and determinations

In the period 1993–2016, at physiological maturity, plants of bread wheat and soybean were manually cut at ground level from 1-m^2 area. For bread wheat, plants were partitioned into culms, leaves, chaff and grain and for soybean into leaves, stems, pods and pod components (shell and seeds). For dry weight determination samples of all plant

parts were oven dried at 65 °C until constant weight.

2.3. Soil sampling

For the evaluation of soil quality in stable systems following long-term changes in management practices, it is important to avoid sampling close to treatments (Picci and Nannipieri, 2002; Pellegrino et al., 2011). Therefore, soil sampling was carried out in spring 2016 (May) before soybean sowing in order to avoid to sample close to the main tillage and fertilization performed in late August 2015 and early March 2016, respectively. Moreover, spring is also considered the best time to assess microbiological parameters in the study area (i.e., soil enzyme activities) since the land is dry enough to access and soil temperature is optimal for microbial growth.

In each replicate plot, a homogenized sample was obtained by mixing the content of four soil cores collected at depths of 0–15 cm and 15–30 cm. In addition, undisturbed soil samples were taken in order to determine bulk density at both soil layers. When in the laboratory, 12 g of field-moist soil samples were immediately frozen at − 20 °C, whereas the remaining part of each sample was air-dried, gently broken apart and then divided in two subsamples: one subsample was sieved at 8 mm, and the second one was sieved at 2 mm.

2.4. Soil physical fractioning

For the physical fractionation of soil samples, the wet-sieving method was performed as described by Six et al. (1999). Briefly, 80 g of dried and 8-mm sieved soil was sprinkled for 5 min in a 2-mm sieve inside a basin filled up with distilled water above 1 cm the sieve mesh (slaking process). Then, the soil was sieved for 2 min and the content of the sieve (large macroaggregates; > 2000 μm) was collected. Particles < 2 mm and water were poured onto a 250-μm sieve inside a new basin and the sieving procedure was repeated, collecting the content of the sieve (small macroaggregates; 250–2000 μm). The remaining part of the sample was poured in a 53-μm sieve inside another basin and thereafter, both the top particles (free microaggregates; 53–250 μm) and what went through the mesh (s + c = silt and clay; < 53 μm) were separately collected. Each fraction obtained was oven-dried at 60 °C to constant weight for later quantification.

Small macroaggregates were further separated according to the microaggregate isolation method of Six et al. (2000a). To isolate occluded microaggregates (microaggregates within macroaggregates; 53–250 μm), a device was drawn, built and utilized (Fig. S1). Briefly, the small macroaggregates fraction, previously isolated from 80 g soil sample, was dispersed in 50 ml of distilled water for 20 min. After mounting the device on a shaker, small macroaggregates and water mixture were poured on top of a 250-μm mesh screen put at the base of the device and gently shaken (150 rpm) with 50 metal beads (Ø 4 mm) for 5 min. Continuous and steady water flow through the device ensured that microaggregates were immediately flushed onto a 53-mm sieve and were not exposed to any further disruption by the beads. After all small macroaggregates were broken up, the material on the 53-μm sieve was sieved for 2 min to ensure that the isolated microaggregates were water stable, then collected and oven dried at 60 °C until constant weight for later weighting and quantification. The isolation procedure of occluded microaggregates was repeated using as last step freeze-drying method (FreeZone 2.5 Labconco, Kansas City, MO, USA) instead of the oven-drying step, in order to obtain the samples for biochemical analyses (Stemmer et al., 1998). The materials that remained on the top of the 250-μm mesh (cPOM = coarse particulate organic matter and sand) and went through the 53-μm sieve (s + c M = occluded silt and clay) were also oven dried until constant weight.

2.5. Soil physical, chemical and biochemical analyses

Samples collected for soil bulk density were dried at 105 °C until constant weight and soil bulk density was then calculated by dividing the dry weight by the soil core volume (Blake and Hartge, 1986). Soil samples sieved at 2 mm (bulk soil) and the occluded microaggregates fraction were analysed for SOC, total N, and available P. All these analyses were carried out using three replicates. In addition, for SOC determination the analyses were carried out using three analytical replicates per sample. Soil OC was measured by CHN combustion method (LECO, Italy), while total N was evaluated by macro Kjeldahl digestion procedure (Bremner and Mulvaney, 1982). Available P was determined by colorimetry using a solution of sodium bicarbonate (Olsen et al., 1982). For the determination of the labile forms of N, the moist soil stored at − 20 °C was used for the extraction with 1 M KCl using a 1:10 soil:extractant ratio and 30-min shaking time. Ammonium (NH_4-N) concentration was then assessed photometrically using the Spectroquant® kit (Merck Millipore Corp., Billerica, MA, USA) and nitrate (NO_3-N) concentration was assessed using the ultraviolet spectrophotometric technique (Goldman and Jacobs, 1961). The content in one hectare of soil at 0–15 and 15–30 cm was calculated for each chemical parameter as follow: [soil chemical concentration x (soil bulk density x volume of soil)].

Soil enzyme potential activities were measured in samples of bulk soil and occluded microaggregates fraction using the fluorogenic methylumbelliferyl (MUF)-substrates method (Marx et al., 2001; Vepsäläinen et al., 2001). The following hydrolytic enzymes, known to be involved in C, N, P and S biogeochemical cycles (Nannipieri et al., 2002), were analysed for β-cellobiohydrolase = cellulase (Cell; EC 3.2.1.91), N-acetyl-β-glucosaminidase = chitinase (NAG; EC 3.2.1.30), β-glucosidase (β-gluc; EC 3.2.1.21), α-glucosidase (α-gluc; EC 3.2.1.20), β-xylosidase = xylosidase (Xylos; EC 3.2.2.27), acid phosphatase = phosphatase (Phosph; EC 3.1.3.2), arylsulphatase = sulphatase (Aryls; EC 3.1.6.1) and leucine-aminopeptidase = leucine (L.AP; EC 3.4.11.1). The respective substrates were 4-MUF β-D-cellobioside, 4-MUF-N-acetyl-β-glucosaminide, 4-MUF β-D-glucoside, 4-MUF α-D-glucoside, 4-MUF-7-β-D-xyloside, 4-MUF-phosphate, 4-MUF-sulphate and L-leucine-7-amino-4-methylcoumarin (AMC). Before starting the analysis, all soil samples were adjusted at 60% water holding capacity and kept at 25 °C for 3 days in the dark. Thus, a suspension of soil was obtained by homogenizing 2 g of each samples with 50 ml sterile water using an Ultra Turrax (IKA, Staufen, Germany) at 9600 rpm for 3 min. Aliquots of 50 μl were withdrawn and dispensed into 96-well black microplates (three analytical replicates per sample per substrate). Sodium acetate buffer 0.5 M pH 5.5 was used for Cell, NAG, β-gluc, α-gluc, Xylos, Phosph and Aryls, whereas TrisHCl buffer 0.05 M pH 7.8 was used for L.AP. One hundred μl of 1 mM substrate solution was added to all samples, obtaining a final substrate concentration of 500 μM. Plates were kept at 30 °C for 30 min and then fluorescence (excitation 360 nm; emission 450 nm) was measured with an automated fluorimetric plate-reader (Fluoroskan Ascent, Labsystem GmbH, Frankfurt, Germany) after 0, 30, 60, 120 and 180 min (Marinari et al., 2013).

The results were expressed as nmoles of product (MUF or AMC) of each enzymatic reaction released per g of soil per unit of time in relation to a standard curve performed with increasing MUF or AMC concentrations and incubated at the same experimental conditions. The SEI (Synthetic Enzymatic Index), expressed as sum of all enzymatic activities, has been calculated for all soils as a synthetic measure of microbial functional capacity. Synthetic enzyme index for the C-cycle (SEIc) was calculated by the sum of the enzymatic activity values of Cell, NAG, β-gluc, α-gluc and Xylos. Microbial functional diversity was assessed by calculating the Shannon diversity index (*H'*) defined as:

$$H' = -\Sigma\ p_i * \ln p_i$$

where p_i is the ratio of the activity of a particular enzyme to the sum of all enzymatic activities (Shannon and Weaver, 1949).

Since the eight enzymes tested here did show activity in all the analysed samples, then the diversity recorded reflects only the "evenness" or distribution of the enzyme activities (Bending et al., 2002).

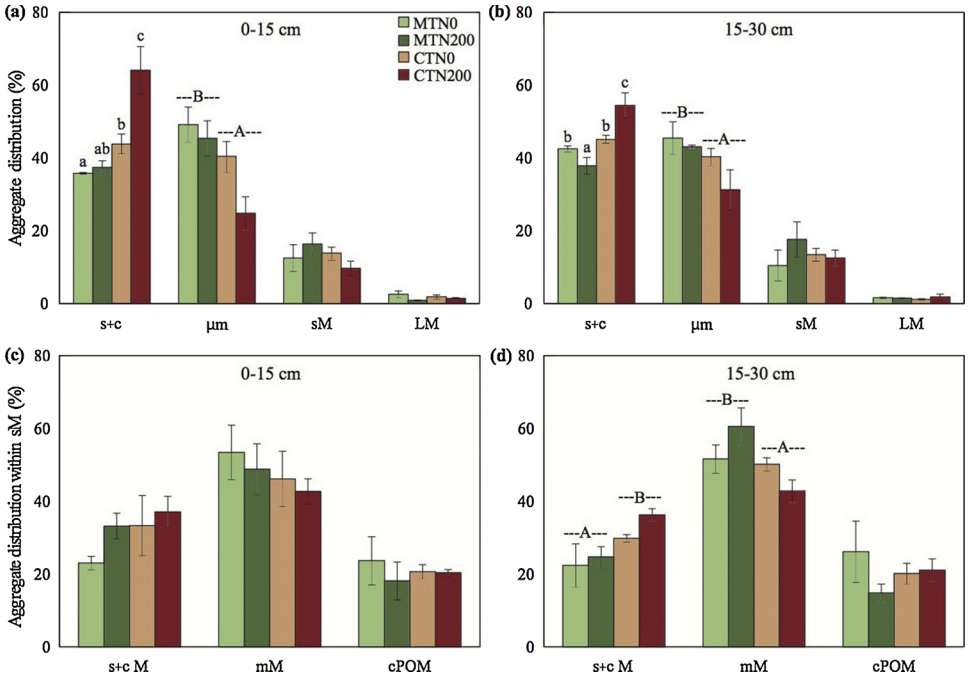

Fig. 1. Long-term effect of tillage and N fertilization on aggregate distribution in bulk soil (%) at 0–15 and 15–30 cm soil depths (a, b) (s + c, silt and clay; µm, free microaggregates; sM, small macroggregates; LM, large macroaggregates) and in the sM fraction at 0–15 and 15–30 cm depths (c, d) (s + c M, occluded silt and clay; mM, occluded microaggregates; cPOM, coarse particulate organic matter) in a wheat-soybean rotation. Values are means ± SE of three replicate plots per treatment. Within each fraction, different small letters represent significant differences among treatments (MTN0, minimum tillage and 0 kg N ha^{-1}; MTN200, minimum tillage and 200 kg N ha^{-1}; CTN0, conventional tillage and 0 kg N ha^{-1}; CTN200, conventional tillage and 200 kg N ha^{-1}), while capital letters represent significant differences between tillage treatments (MT, CT) (Tukey-B test, $P \leq 0.05$).

Finally, the ecoenzymatic C/N and N/P acquisition activities were measured by the ratios of β-glucosidase/(chitinase + leucine) [β-gluc/(NAG + L.AP)] and (chitinase + leucine)/phosphatase activities [(NAG + L.AP)/Phosph], respectively (Sinsabaugh et al., 2009).

Soil DNA extraction was performed on bulk soil and occluded microaggregates samples using the DNeasy PowerSoil Kit (QIAGEN, Venlo, Netherlands), following the instructions of the manufacturer. Then, the concentration of the extracted DNA for each sample was quantified by a spectrophotometer (NanoDrop Technology, Wilmington, DE, USA). Results were expressed on soil dry weight basis.

2.6. Statistical analysis

Data were analysed by a two-way ANOVA following the split-plot experimental design, using tillage as main-plot factor and N fertilization as subplot factor. Data were ln- and arcsine-transformed when needed to fulfil the assumptions of the ANOVA. Post-hoc Tukey-B significant difference test was used for comparisons among treatments. Means and standard errors given are for untransformed data. All the analyses were performed using the SPSS software package version 21.0 (SPSS Inc., Chicago, IL, USA).

The datasets of bio-/chemical parameters were square root transformed, standardized by variables and by the maximum, and Bray-Curtis' coefficients of similarity were calculated between samples in order to compute the Principal Coordinates Analysis (PCoA; Torgerson, 1958), allowing the observation of the most relevant patterns and structures among the factors. In the PCoA plots only the parameters having a Pearson correlation (r) higher o equal to 0.4 were shown. To test the simultaneous response of the bio-/chemical parameters against the two factors (tillage and N fertilization: fixed factors) the permutation analysis of variance (PERMANOVA) was utilised (Anderson, 2001). P-values were calculated using the Monte Carlo permutation test (999 permutations) together with the permutation of residuals under an unrestricted permutation model. Since PERMANOVA is sensitive to differences in multivariate location (among group variability) and dispersion (within group variability), the analysis of homogeneity of multivariate dispersion (PERMDISP; Anderson, 2006) was also performed (Anderson et al., 2006). When P-value is higher than 0.05, no difference occurs in the dispersion among groups and the results from PERMANOVA are reliable. All these analyses were performed using the software Primer 6 and PERMANOVA plus (Anderson et al., 2008).

3. Results

3.1. Crop production

Averaged over the period 1993–2016, grain yield of bread wheat was 3.8 Mg ha^{-1}, irrespective of tillage and N fertilization treatments. Grain yield of wheat was significantly affected by tillage ($P = 0.03$) and N fertilization ($P < 0.001$) (Table S2). Wheat yield increased from 3.4 Mg ha^{-1} in MT to 4.1 Mg ha^{-1} under CT (+21%) and from 2.6 Mg ha^{-1} under N0 to 5.0 Mg ha^{-1} under N200 (+92%). Conversely, soybean yield was not affected either by tillage or N fertilization that was applied to bread wheat and, averaged over treatments, was 2.6 Mg ha^{-1} (Table S2).

3.2. Aggregate distribution

Tillage intensity and N fertilization significantly affected aggregate distributions at both soil depths (Fig. 3a, b). Overall, silt and clay and free microaggregates were the predominant size classes in all treatments at both soil depths, accounting for more than 80% of the total soil dry mass. Small macroaggregates accounted for 10–18% and large macroaggregates for less than 3%.

At 0–15 cm soil depth, the proportion of the silt and clay fraction under MT was not influenced by N fertilization and it contributed on average 37% to the total soil dry mass, while under CT it increased in N200 (+46%) (Fig. 1a; Tables S3 and S4). At the same depth, the proportion of free microaggregates was greatly increased (+45%) by the reduction of tillage intensity, whereas the proportion of small and large macroaggregates was not affected either by tillage or N fertilization.

At 15–30 cm soil depth, the proportion of the silt and clay fraction did not change with tillage in N0 (on average 44% of the total soil dry mass), while in N200 it was higher under CT than MT (Fig. 1b; Tables S3 and S4). Moreover, under MT the proportion of silt and clay was higher at N0, while under CT was higher at N200. A greater proportion of free microaggregates was found under MT than CT (+24%) irrespective of N fertilization, while the proportion of small and large macroaggregates was not modified.

Following the separation of small macroaggregates into occluded silt and clay, microaggregates and coarse particulate organic matter and sand, it was shown that the pattern of distribution at 0–15 cm soil depth was not modified either by tillage or N fertilization (Fig. 1c; Tables S3 and S4). Overall, microaggregates were the predominant size class in all treatments, accounting for 48% and 51% of the small macroaggregate fraction at 0–15 cm and 15–30 cm soil depths, respectively. By contrast, at 15–30 cm soil depth, the proportion of silt and clay and microaggregates were significantly affected by tillage (Fig. 1d; Tables S3 and S4). Under MT the proportion of silt and clay decreased by 40% compared to CT, whereas the proportion of microaggregates increased by 21%.

3.3. Chemical parameters in bulk soil and occluded microaggregates

At 0–15 cm soil depth, tillage did not affect chemical parameters in bulk soil, whereas N fertilization only affected the labile forms of N (Tables S5 and S6). Indeed, NH_4-N and NO_3-N were 14% and 13% higher in N200 than N0, respectively. At 15–30 cm soil depth, tillage decreased only soil total N content from 4.15 ± 0.19 Mg ha^{-1} under CT to 3.71 ± 0.19 Mg ha^{-1} under MT. Moreover, at the same depth (15–30 cm), SOC and total N content significantly increased in N200 compared to N0 (+6% and +19%, respectively) (Tables 1, S5, S6). Available P content was not affected by N fertilization and was on average 69.4 and 63.3 Mg ha^{-1} at 0–15 cm and 15–30 cm soil depths, respectively (Tables S5 and S6).

At both soil depths, SOC in occluded microaggregates was increased by N fertilization under MT (+16% at 0–15 cm; +84% at 15–30 cm), whereas was decreased under CT (–46% at 0–15 cm; –15% at 15–30 cm) (Fig. 2a, b; Tables S7 and S8). Total N and Avail P in occluded microaggregates were not affected by tillage and N fertilization and were on average 0.29 Mg N ha^{-1} and 5.1 Mg P ha^{-1} at 0–15 cm depth, and 0.29 Mg N ha^{-1} and 5.9 Mg P ha^{-1} at 15–30 cm depth (Tables S7 and S8).

3.4. Biochemical parameters in bulk soil and occluded microaggregates

As regards bulk soil, tillage and N fertilization significantly affected most of the enzymes involved in C, N, P and S soil cycles at both soil depths (Table S9). In detail, tillage explained the highest percentage of total variance of all soil enzymes (Fig. S2a, b). By contrast, at 15–30 cm soil depth, β-gluc was strongly affected also by N fertilization that explained 44% of total variance (Fig. S1b).

At 0–15 cm soil depth, the reduction of tillage intensity from CT to MT significantly increased in bulk soil C-cycle enzyme activities, such as Cell (+81%), β-gluc (+92%), α-gluc (+54%), Xylos (+81%), as well as P- and S-cycle activities, such as Phosph (+57%) and Aryls (+88%) at 0–15 cm soil depth. Similarly, enzyme activities were increased at 15–30 cm soil depth, although the increases were lower (+49%, +36%, +46%, +58%, +45% and +38%, respectively) (Tables 2 and S10). Moreover, the reduction of tillage intensity increased NAG activity (+66%) only at 0–15 cm soil depth. Shannon diversity index (*H'*) and the C/N acquisition activity [β-gluc/(NAG + L.AP)] were increased by 6% and 60%, respectively, only at 0–15 cm soil depth. By contrast, the N/P acquisition activity [(NAG + L.AP)/Phosph] was significantly decreased (–18%) by tillage (Tables 2, S9, S10). SEI and SEIc were increased by 55% and 79%, respectively, at 0–15 cm soil depth and by 34% and 40%, respectively, at 15–30 cm soil depth.

At 0–15 cm soil depth, the increase of N fertilization from N0 to N200 increased in bulk soil Cell, β-gluc and NAG activity by 55%, 92% and 28%, respectively (Fig. 3a–c; Tables S9 and S10). At 15–30 cm soil depth, the increase of N fertilization increased both Cell and β-gluc activity by 34%. SEI was increased under N200 by 22% only at 0–15 cm soil depth and SEIc by 42% and 25% at both soil depths (Fig. 3d, e; Tables S9 and S10). Moreover, the C/N acquisition activity [β-gluc/(NAG + L.AP)] was significantly increased by 39% and 37% at 0–15 cm and 15–30 cm soil depth, respectively (Fig. 3f; Tables S9 and S10). Finally, neither tillage nor N fertilization modified L.AP at both soil depths (Tables S9 and S10).

PERMANOVA analyses showed that bio-/chemical parameters in bulk soil were significantly different between MT and CT and between N0 and N200 at both soil depths (Table S11). In the PCoA analyses, the first two principal coordinates explained 84% of the total variance at both soil depths (Fig. 4a, b). Tillage was the most discriminant factor since the samples clearly clustered in two groups along the first axis and explained 72% and 60% of total variance at 0–15 and 15–30 cm soil depth, respectively (Fig. S3). In addition, samples clustered according to N fertilization along the second axis, and the treatment explained 22% and 29% of total variance at 0–15 cm and 15–30 cm depth, respectively. All biochemical parameters, except the N/P acquisition activity [(NAG + L.AP)/Phosph], were higher under MT than CT at both soil depths, whereas chemical parameters were higher under N200 than N0 (Fig. 4a, b).

DNA concentration in bulk soil was significantly affected by tillage only at 0–15 cm depth and did not vary according to N fertilization at both soil layers. This parameter was 56% higher under MT compared to CT at 0–15 cm (40.6 ± 7.2 vs. 25.7 ± 2.6 ng g^{-1}; $P \leq 0.032$), whereas at 15–30 cm it was on average 34.1 ± 2.5 ng g^{-1}.

As regards occluded microaggregates, at 0–15 cm depth, tillage explained 75% of total variance for Phosph activity (Fig. S2 c), whereas the interaction of tillage and N fertilization explained the highest percentage of total variance for the majority of enzymatic activities (43%–81%). At 15–30 cm depth, N fertilization explained the highest percentages of total variance for Cell (46%), NAG (64%), β-gluc (44%), Xylos (81%), Aryls (83%) and L.AP (67%) activities (Fig. S2d). By contrast, tillage explained the highest percentages of total variance for α-gluc and Phosph activities (59% and 87%, respectively).

At 0–15 cm soil depth, N/P acquisition activity was significantly increased in occluded microaggregates from CT to MT by 100% (Fig. 5c, d), while C/N acquisition activity was increased due to N fertilization by 38% (Fig. 5f). The synthetic indices SEI and SEIc and all enzymes, except NAG, Xylos and Cell were similarly affected by the interaction of tillage and N fertilization (Tables S7 and S12). Specifically, N fertilization decreased the enzyme activities under MT, whereas it increased the activities under CT (Fig. 2c–j).

At 15–30 cm soil depth, N/P acquisition activity was significantly increased in occluded microaggregates from CT to MT by 80% (Fig. 5c, d), whereas α-gluc activity was decreased due to N fertilization by 29% (Fig. 5e). Moreover, the reduction of tillage intensity from CT to MT significantly decreased α-gluc (–38%) and Phosph activities (–70%) (Fig. 5a, b; Tables S10 and S12). The synthetic indices SEI and SEIc and all enzymes, except NAG, Xylos, Cell, β-gluc and L.AP (Tables S7 and S12), were decreased by N fertilization under MT and increased under CT.

Table 1
Long-term effect of N fertilization on soil organic carbon (SOC), total nitrogen (Tot N), ammonium (NH_4-N) and nitrate (NO_3-N) contents of bulk soil in a wheat-soybean rotation.

N fertilization[a]	SOC (Mg C ha^{-1})	Tot N (Mg N ha^{-1})	NH_4-N (kg N ha^{-1})	NO_3-N (kg N ha^{-1})
		0–15 cm depth		
N0	39.3 ± 1.2 a	3.8 ± 0.2 a	2.9 ± 0.0 a	15.5 ± 0.5 a
N200	41.8 ± 0.8 a	4.0 ± 0.1 a	3.3 ± 0.0 b	20.6 ± 0.5 b
		15–30 cm depth		
N0	39.3 ± 1.0 a	3.6 ± 0.1 a	3.2 ± 0.0 a	18.6 ± 0.4 a
N200	41.8 ± 0.6 b	4.3 ± 0.2 b	3.6 ± 0.0 b	22.2 ± 0.8 a

[a] N0: 0 kg N ha^{-1}; N200: 200 kg N ha^{-1}.

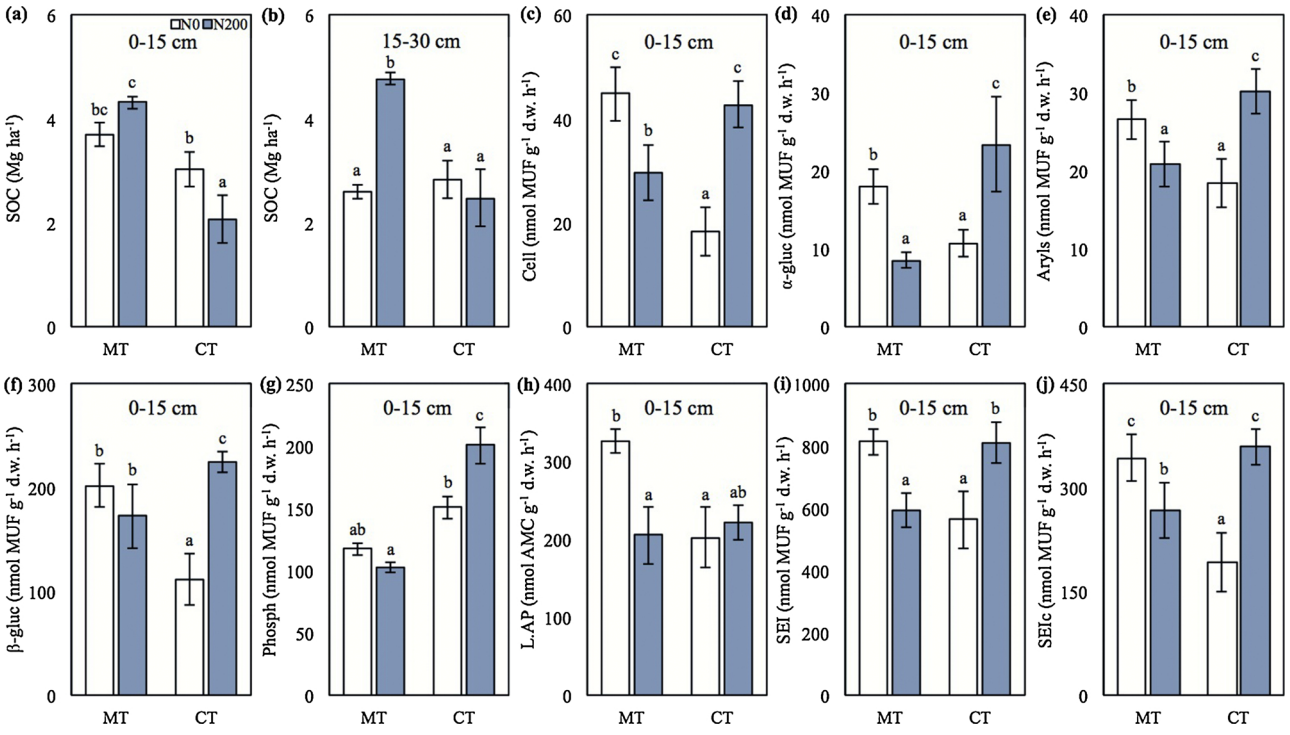

Fig. 2. Long-term effect of interaction between tillage and N fertilization on bio-/chemical parameters in occluded microaggregates. SOC (soil organic carbon content) at 0–15 and 15–30 cm depths (a, b); Cell (β-cellobiohydrolase, cellulase) (c); α-gluc (α-glucosidase) (d); Aryls (arylsulphatase) (e); β-gluc (β-glucosidase) (f); Phosph (acid phosphatase, phosphatase) (g); L.AP (leucine-aminopeptidase, leucine) (h); SEI (Synthetic Enzyme Index) (i); SEIc (Synthetic Enzyme Index involved in C-cycle) (j) at 0–15 cm depth. The parameters refer to soil in a wheat-soybean rotation. Values are means ± SE of three replicate plots per treatment. Within each parameter, different small letters represent significant differences among treatments (MT, minimum tillage; CT, conventional tillage; N0, 0 kg N ha^{-1}; N200, 200 kg N ha^{-1}) (Tukey-B test, $P \leq 0.05$).

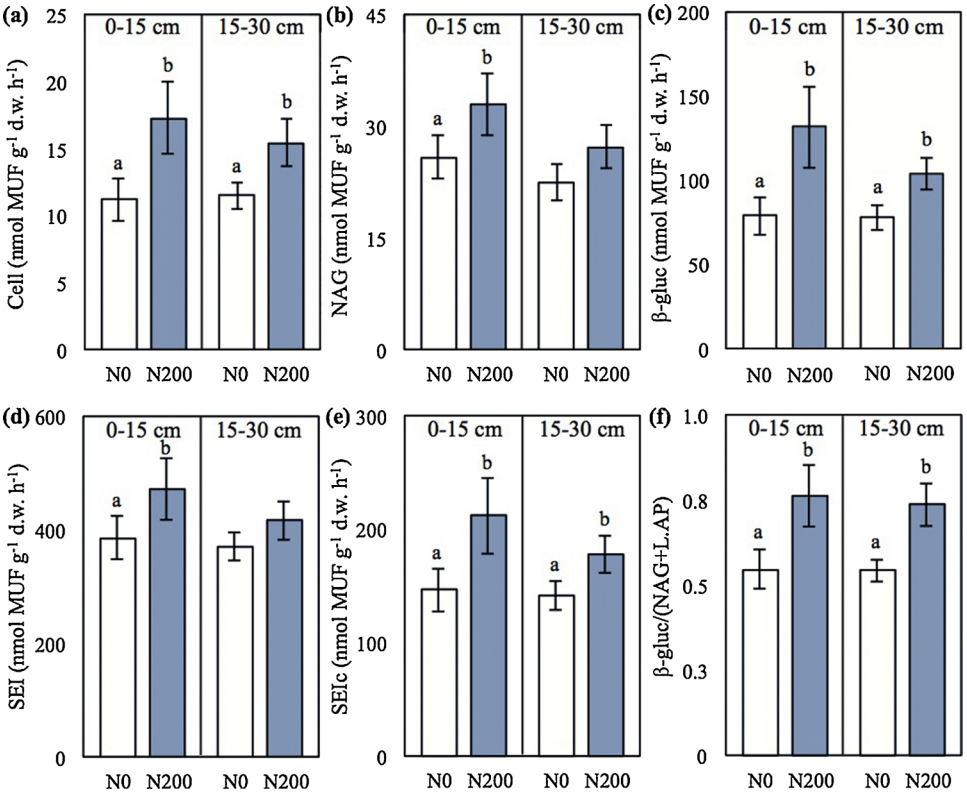

Fig. 3. Long-term effect of N fertilization on Cell (β-cellobiohydrolase, cellulase) (a), NAG (N-acetyl-β-glucosaminidase, chitinase) (b), β-gluc (β-glucosidase) (c), SEI (Synthetic Enzime Index) (d), SEIc (Synthetic Enzime Index involved in C-cycle) (e), β-gluc/(NAG + L.AP) (C/N acquisition activity) (f). The parameters refer to bulk soil at 0–15 and 15–30 cm depths in a wheat-soybean rotation (N0, 0 kg N ha^{-1}; N200, 200 kg N ha^{-1}). Values are means ± SE of three replicate plots per treatment. Within each depth, different letters represent significant differences between treatments (Tukey-B test, $P \leq 0.05$).

Table 2
Long-term effect of tillage on biochemical parameters in bulk soil in a wheat-soybean rotation.

Tillage[a]	Cell[b] (nmol MUF[c] g^{-1} $d.w.\,h^{-1}$)	NAG (nmol MUF g^{-1} $d.w.\,h^{-1}$)	β-gluc (nmol MUF g^{-1} $d.w.\,h^{-1}$)	α-gluc (nmol MUF g^{-1} $d.w.\,h^{-1}$)	Xylos (nmol MUF g^{-1} $d.w.\,h^{-1}$)	Phosph (nmol MUF g^{-1} $d.w.\,h^{-1}$)	Aryls (nmol MUF g^{-1} $d.w.\,h^{-1}$)	SEI (nmol MUF g^{-1} $d.w.\,h^{-1}$)	SEIc (nmol MUF g^{-1} $d.w.\,h^{-1}$)	Shannon diversity index (*H'*)	β-gluc/ (NAG + L.AP)	(NAG + L.AP)/ Phosph
						0–15 cm depth						
MT	18.3 ± 2.3 b	36.7 ± 2.6 b	138.3 ± 22.1 b	19.0 ± 1.6 b	18.8 ± 1.2 b	127.4 ± 6.1 b	26.7 ± 1.0 b	520.4 ± 37.5 b	231.0 ± 26.4 b	1.8 ± 0.0 b	0.8 ± 0.1 b	1.4 ± 0.1 a
CT	10.1 ± 1.1 a	22.1 ± 1.9 a	72.2 ± 8.5 a	12.3 ± 0.9 a	10.4 ± 0.5 a	80.9 ± 2.5 a	14.2 ± 0.7 a	336.5 ± 16.9 a	128.9 ± 10.8 a	1.7 ± 0.0 a	0.5 ± 0.0 a	1.7 ± 0.1 b
						15–30 cm depth						
MT	16.1 ± 1.6 b	29.0 ± 3.6 a	104.6 ± 5.8 b	19.6 ± 1.5 b	16.8 ± 1.4 b	118.1 ± 9.1 b	19.7 ± 1.3 b	450.3 ± 20.9 b	186.1 ± 11.7 b	1.7 ± 0.0 a	0.7 ± 0.0 a	1.4 ± 0.1 a
CT	10.8 ± 0.8 a	20.7 ± 1.9 a	77.0 ± 10.1 a	13.4 ± 0.7 a	10.6 ± 0.6 a	81.6 ± 3.8 a	14.3 ± 1.4 a	337.2 ± 16.7 a	132.5 ± 11.7 a	1.7 ± 0.0 a	0.6 ± 0.1 a	1.6 ± 0.1 a

[a] MT: minimum tillage; CT: conventional tillage.
[b] Cell: β-cellobiohydrolase (cellulase); NAG: N-acetyl-β-glucosaminidase (chitinase); β-gluc: β-glucosidase; α-gluc: α-glucosidase; Xylos: β-xylosidase (xylosidase); Phosp: acid phosphatase (phosphatase); Aryls: arylsulphatase; SEI: Synthetic Enzime Index; SEIc: Synthetic Enzyme Index involved in C-cycle; *H'*: Shannon diversity index; β-gluc/(NAG + L.AP): C/N acquisition activity; (NAG + L.AP)/Phosph: N/P acquisition activity.
[c] MUF: fluorogenic methylumbrelliferyl product.

Fig. 4. Principal Coordinates Analysis (PCoA) biplot on the long-term effect of tillage and N fertilization on soil bio-/chemical parameters in bulk soil at 0–15 (a) and 15–30 cm (b) depths, and in occluded microaggregates at 0–15 cm depth (c) in a wheat-soybean rotation. Treatments are: MTN0, minimum tillage and 0 kg N ha^{-1}; MTN200, minimum tillage and 200 kg N ha^{-1}; CTN0, conventional tillage and 0 kg N ha^{-1}; CTN200, conventional tillage and 200 kg N ha^{-1}. In the biplot only the parameters having a Pearson correlation > or equal to 0.4 were plotted. Data were square root transformed, standardized by variables and the maximum, and Bray-Curtis' coefficients of similarity were calculated between samples.

Finally, *H'* did not vary among treatments at both soil depths.

The results from PERMANOVA also supported by PERMDISP showed that bio-/chemical parameters in occluded microaggregates significantly differed accordingly to the interaction between tillage and N fertilization at 0–15 cm soil depth ($P = 0.017$), whereas at 15–30 cm depth no difference was observed (Table S11). In the PCoA biplot the first two principal coordinates explained 78% of total variance (Fig. 4c) and tillage x N fertilization 52% of total variance (Fig. S5). The most discriminant parameters were the biochemical ones and SOC, and all parameters were higher in MT-N200, except for N/P acquisition activity [(NAG + L.AP)/Phosph], which was higher in CT-N200.

Soil DNA concentration in occluded microaggregates was not

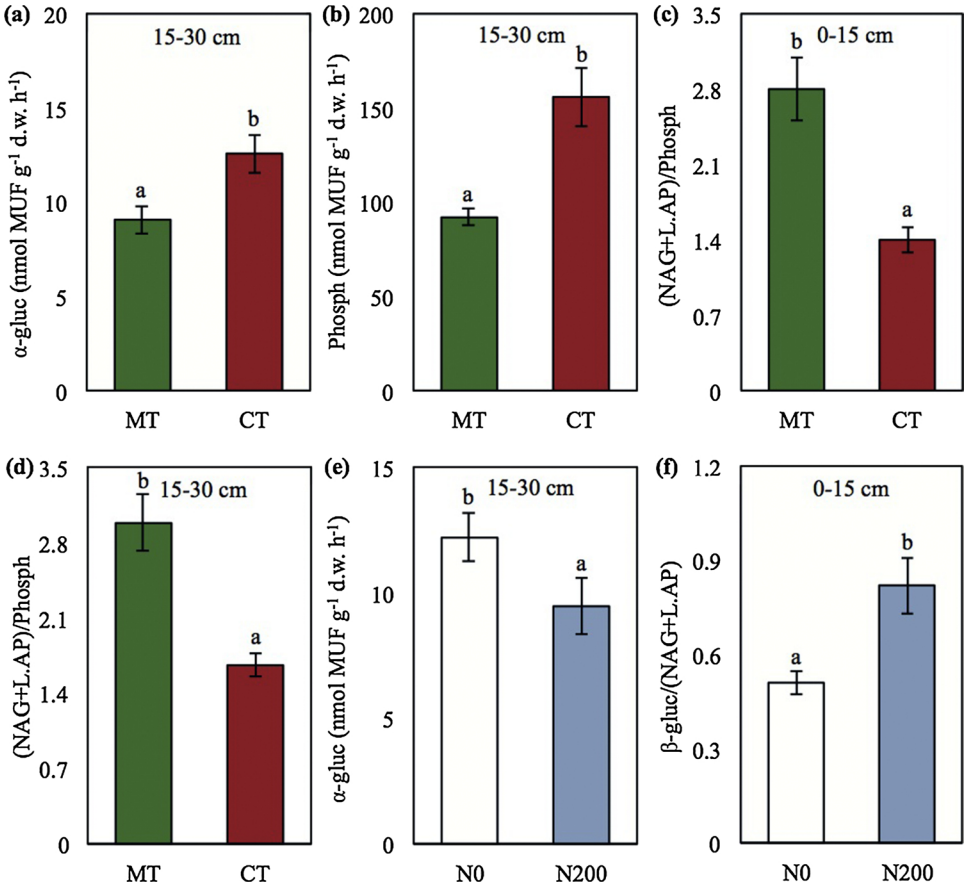

Fig. 5. Long-term effect of tillage and N fertilization on biochemical parameters in occluded microaggregates. Tillage: α-gluc (α-glucosidase) and Phosph (acid phosphatase, phosphatase) at 15–30 cm depth (a, b); (NAG + L.AP)/Phosph (N/P acquisition activity) at 0–15 and 15–30 cm depth (c, d). N fertilization: α-gluc at 15–30 cm depth (e); β-gluc/(NAG + L.AP) (C/N acquisition acitivity) at 0–15 cm depth (f). The parameters refer to soil in a wheat-soybean rotation. Values are means ± SE of three replicate plots per treatment. Within each parameter, different small letters represent significant differences between treatments (MT, minimum tillage; CT, conventional tillage; N0, 0 kg N ha^{-1}; N200, 200 kg N ha^{-1}) (Tukey-B test, $P \leq 0.05$).

affected either by tillage or fertilization at both soil layers, averaging 50.5 ± 10.1 ng g^{-1} at 0–15 cm depth and 33.1 ± 3.1 ng g^{-1} at 15–30 cm.

4. Discussion

4.1. Crop production

The results obtained in the soybean-wheat rotation experiment carried out in a Mediterranean climate revealed a yield disadvantage of bread wheat due to the reduction of tillage intensity and N fertilization (average of 23 years: –20% and –94%, respectively). Conversely, soybean yield did not respond either to tillage or to the residual effect of N fertilization to wheat. A meta-analysis carried out to synthetize the effects of the application of conservation tillage techniques in Europe under different soil conditions revealed that crop yield of annual field crops (potato, maize, winter and spring cereals and sugar beet) was decreased on average by 5% under reduced tillage (RT) compared to conventional tillage (CT) (van den Putte et al., 2010). In long-term experiments carried out in silty clay and clay soils located in typical Mediterranean conditions in Spain and South Italy, no substantial differences were recorded in wheat and faba bean yields under MT compared to CT, whereas, in line with our data, strong decreases in wheat yield were found in N0 respect to 180 kg N ha^{-1} (López-Bellido et al., 1996; Basso et al., 2010; Amato et al., 2013; Seddaiu et al., 2016). The variability of yield response to tillage and N fertilization in Southern Europe could be attributed to site-specific conditions, such as soil texture, pest and weed occurrence, and biological fertility of soil, whose relative importance varies according to climatic conditions (Pellegrino et al., 2011; Ruisi et al., 2014; Ercoli et al., 2017a). Reduced tillage performed even better than CT in dry climates or dry years and clay soils, and this is attributable to the presence of crop residues on soil surface enhancing water storage in soil (van den Putte et al., 2010; Amato et al., 2013).

4.2. Aggregate distribution

Despite the modest effects on crop yield and C storage in bulk soil, long-term conservation tillage and N fertilization strongly modified aggregate distribution: under MT a higher proportion of free microaggregates and a lower proportion of the silt and clay fraction were recorded, while the proportion of small macroaggregates was similar across the management treatments. Moreover, when small macroaggregates were further fractionated, a higher proportion of occluded microaggregates was detected under MT, and this effect was particularly evident in the undisturbed deeper soil layer. Thus, under MT it is evident a greater potential to form macroaggregates, as free microaggregates are the building blocks of aggregates, and consequently MT shows a higher potential to develop soil structure through the aggregate stabilization (Six et al., 2000a, 2004). Previous research comparing NT to conventional ploughing showed a great increase in microaggregates and a corresponding decrease in macroaggregates demonstrating the disrupting effect of tillage on soil structure (Six et al., 2000b; Mikha et al., 2013; Sheehy et al., 2015). Consistently, our data suggest that aggregate distribution shifted toward more microaggregates and fewer macroaggregates with decreasing tillage intensity and this effect was related to the greater soil C concentration in microaggregates.

4.3. Chemical parameters in bulk soil

Our data showed a slight SOC accumulation rate (0.16 Mg C ha^{-1} $year^{-1}$ at 0–30 cm) under MT, although the increase over CT was not significant. In line with our results, many studies on the effect of RT did not find significant SOC changes in comparison with CT up to 25 cm

soil depth in soils located in New Zealand, Canada (Québec and Ontario), USA (Oklahoma) and Japan (Horne et al., 1992; Angers et al., 1993; Lal et al., 1994; Yang and Kay, 2001; Koga and Tsuji, 2009). However, in maritime and continental Mediterranean areas, the response in SOC accumulation under MT over CT varied and ranged from –0.18 to 0.66 Mg ha^{-1} $year^{-1}$ (López-Fando and Pardo, 2011; González-Sánchez et al., 2012). Kong et al. (2005), across 10 Mediterranean cropping systems, observed a significant increase in SOC sequestration only when annual C input was 8.9 Mg C ha^{-1} or over. This can well explain the fact that in our experiment an effective SOC sequestration was not found under MT. Indeed, the amount of C returning to soil from aboveground residues and roots did not exceed 2.9 Mg C ha^{-1} y^{-1}. However, concerning total N, we found a differential pattern of response to tillage between surface and sub-surface layer. At 0–15 cm soil depth, soil total N was not affected by tillage and this could be determined by the incorporation in CT of a greater quantity of residues with a lower C:N ratio in comparison to MT (Sainju et al., 2011). In contrast, in the sub-surface layer, the significant increase of 0.4 Mg N ha^{-1} found under CT compared to MT could be determined by the incorporation of residues due to tillage.

Our results indicated that long-term N fertilization increased SOC content by 6% at 0–30 cm soil depth, and this corresponds to a SOC accumulation rate of 0.11 Mg C ha^{-1} $year^{-1}$. Similar accumulation patterns after N fertilization (+4–8%) were estimated in the long-term at global scale (Lu et al., 2011; Aguilera et al., 2013) due to the promotion of plant growth and the physical and chemical stabilization of SOC (Six et al., 2000a; Snyder et al., 2009).

Nitrogen fertilization not only affected SOC storage, but also increased the content of labile forms of N, and this could trigger a higher N leaching and a lower N use efficiency, factors affecting economic profitability and energy efficiency of crops, and also microbial soil abundance and structure (e.g., López-Bellido and López-Bellido, 2001; Koch et al., 2004; Morell et al., 2011; Ercoli et al., 2017b). Therefore, this effect must be considered especially in the Mediterranean area where rainfall are mainly concentrated in autumn, and N fertilization should be optimized through an appropriate combinations of N rates, time and number of applications (Ercoli et al., 2008, 2013).

4.4. Biochemical parameters in bulk soil

The reduction of tillage intensity induced a general positive effect on all enzymes in the bulk soil, increasing their activities at both soil depths. Accordingly, positive effects on soil enzymes due to reduced or no tillage have been widely reported (Lagomarsino et al., 2009; Nivelle et al., 2016; Zuber and Villamil, 2016; Chen et al., 2019). The nutrient acquisition activity in terms of C, P and S was more pronounced at the soil surface, where the organic substrates, composed by plant residues and SOC, are mainly accumulated (Stockmann et al., 2013). Although in this study no significant changes of SOC were observed owing to tillage, the general increase of enzyme activities recorded under MT may be ascribable either to i) higher presence of labile compounds through residue decomposition (Cotrufo et al., 2013; Nivelle et al., 2016) or to ii) reduced disturbance induced by MT on the microbial metabolic activities (van Capelle et al., 2012; Huang et al., 2013; Ciccolini et al., 2015). The positive effect of MT was particularly evident for SEIc, indicating a higher utilization of the C substrates. Similarly, Lagomarsino et al. (2011) reported increases in C cycling enzymes in no-tilled vineyard, hypothesizing a different chemical composition of organic substrates. It is worth to emphasize that enzymatic hydrolysis concerns the breakdown of complex organic polymers and does not necessarily lead to the complete mineralization of substrates, but can also lead to anabolic pathways for biosynthetic processes (i.e., humification and/or microbial immobilization) (Moscatelli et al., 2018). Accordingly, the significant increase of soil DNA found here under MT may confirm a microbial immobilization process.

No significant effects were evident on N cycle enzymes in response to tillage. However, in the surface layer, MT significantly increased the N/P acquisition activity and slightly decreased the C/N acquisition activity, which indicate microbial limitation for N with respect to P and C. Indeed, the N/P and C/N acquisition activity are consistent with the thresholds reported by Zeglin et al. (2013) (N/P > 0.44 and C/N < 1.41) for primary microbial N limitation. Moreover, following the fact that the availability of mineral N is known to prime microbial decomposition activity (priming effect), in particular triggering enzymes involved in C acquisition (Kuzyakov et al., 2000), N fertilization significantly increased NAG at 0–15 cm soil depth and Cell and β-gluc at both soil depths. Finally, N fertilization significantly increased the C/N acquisition activity at both depths. Indeed, the observed significant increase confirms less investment toward N acquisition relative to C. However, the products of enzymatic hydrolysis are likely to become the main precursors of stable SOC by promoting aggregation and the formation of strong chemical bonding to the mineral soil matrix (Cotrufo et al., 2013).

Microbial functional diversity increased significantly at 0–15 cm under MT. It is widely ascertained that conventional tillage heavily affects soil structure causing a strong disturbance to soil biota either as numbers of organisms or community structure (Wang et al., 2017; de Graaff et al., 2019). In particular, the complex network of fungal hyphae that enmesh microaggregates into larger aggregates (Tisdall and Oades, 1982; Bedini et al., 2009) is disrupted by ploughing and this negatively impact the fungal contribution to soil microbial biomass not only in terms of size but, mainly, in terms of specific metabolic processes (Drijber et al., 2000; Thiele-Bruhn et al., 2012). Although soil DNA concentration does not specifically inform on microbial composition, its significant increase in the surface layer combined to the enhancement of Aryls and NAG (indicators of fungal biomass) (Miller et al., 1998; Ercoli et al., 2012), may suggest that under MT the fungal component of soil biota is promoted. Minimum tillage could thus allow the establishment of suitable conditions for fungi, such as arbuscular mycorrhizal fungi (AMF), developing and promoting soil aggregation and structure on one side, and providing additional diverse biochemical functions on the other hand (Rillig, 2004; Six et al., 2006). The parallel increase of Phosph under MT may also point to the hypothesized positive effects on fungal biomass, specifically mycorrhizas (Elbon and Whalen, 2015). In contrast, N fertilization did not affect DNA concentration in soil, suggesting that microbial biomass is not modified, although an increase of microbial diversity was reported in several soil and climatic conditions (de Graaff et al., 2019).

4.5. Chemical and biochemical parameters in occluded microaggregates

In this study, SOC in occluded microaggregates was increased by N fertilization under MT and decreased under CT, suggesting that at high N availability SOC was immobilized under MT, whereas it was decomposed under CT. The differential effect of tillage according to N availability could be explained by the physico-chemical features in soil aggregates, which are extremely diverse according to tillage and consequently by the modification of microbial community composition and structure. In contrast to the deep knowledge on the role and impact of microbial activity on soil aggregation, the degree to which the microaggregate structure affects the microbial community is much less known (Six et al., 2004; Ciccolini et al., 2016). Since value of DNA concentration in soil can be considered a proxy to soil microbial biomass (Marstorp et al., 2000), the similarity in DNA concentration recorded in our research across tillage and fertilization indicates that management did not affect total microbial biomass. However, as the DNA concentration of microorganisms differs among microbial species, the management-induced responses in the compositions of microbial communities should be further elucidated in order to clarify the role of the microorganisms involved in residue degradation and SOC conservation (Piazza et al., 2019).

The enzymatic activities were differently affected in the occluded

microaggregates by the two treatments, confirming that the characteristics of occluded microaggregates deeply modify soil environment and thereby affects microbial activity (Jastrow and Miller, 1998). Along with this, it has been shown that the size, stability, and chemical properties of soil aggregates influence the spatial distribution, functioning and diversity of microbial populations at the microscale (Kong et al., 2011). The importance of microbial biomass for the formation of stable SOC has been recognized for over a decade and a growing body of evidence demonstrates that microbes are the largest contributor to stable SOC (e.g. Cotrufo et al., 2013) and that the quantity and strength of organo-mineral bonds are the major drivers of long-term SOM stabilization (Six et al., 2002; Six and Paustian, 2014). Indeed, under NT, the shift of the microbial community in soil toward fungal-dominated community is associated to an accumulation of SOC primarily due to the improvement of soil structural stability and the concurrent deposition of fungal-derived C in occluded microaggregates (Simpson et al., 2004; Kong et al., 2011).

The results obtained here confirm that occluded microaggregates, which significantly increased under MT and N200 as percentage of small macroaggregates, is an ecologically relevant micro-environment since it drives soil biological activity and SOC turnover (Six and Paustian, 2014). At the same soil layer, almost all enzymatic patterns showed opposite trends in response to N addition in MT (decrease) and CT (increase). In well-aerated and loosely structured soils (CT), the higher availability of organic matter made accessible by tillage practices, combined with N addition, triggers microbial activity enhancing decomposition processes (e.g., priming effect) and leading to SOC loss. On the other hand, MT likely reduced the availability of organic matter and diffusion of C substrates in the occluded microaggregates (Chen et al., 2015), decreasing enzymes activity thus providing evidence of SOC physical protection in the study (Schmidt et al., 2011). This effect, combined with the peculiar conditions characterizing these micro-environments, such as anaerobiosis, low water content, scarce substrate diffusion (Wang et al., 2018), may have prevented microorganisms from using the available N, switching their activity towards the immobilization processes that further lead to SOC increase found at both depths.

To explain the general decrease of enzymatic activities under N fertilization and MT, and the significant interaction between these two factors found only in the occluded microaggregates, several hypotheses may be drawn. Among these: N availability may differently affect the balance between fungal/bacterial ratio (Coleman, 2008) or active/dormant microorganisms according to the physical condition induced by tillage (Blagodatskaya and Kuzyakov, 2013). Furthermore, it should be emphasized that likely changes within the proportion of immobilized enzymes to the total enzyme activity may occur in occluded microaggregates, thus affecting their contribution to overall activity (Nannipieri et al., 2002).

5. Conclusions

In cold, humid Mediterranean conditions, long-term minimum tillage and N fertilization increased soil fertility and enzymes activities, but produced modest effects on grain yield of wheat and soybean. Under minimum tillage, the microbial biomass and the enzyme activity related to C, N and P cycles were largely increased, but no substantial changes in C accumulation were found in bulk soil. However, minimum tillage under high N fertilization promoted soil aggregation and SOC accumulation in occluded microaggregates together with a reduction of the activities of soil enzymes. These results suggest that microbial compounds produced during the degradation of labile plant products accumulate in soil through the efficient use by soil microbes and the stabilization by the soil matrix. Moreover, the differential response to N fertilization of conventional and minimum tillage may be attributable to the modification of microbial community composition. Therefore, in Mediterranean area, maintaining or even increasing SOC conservation may require both minimum tillage systems and adequate N fertilization rates promoting the shift of the soil microbial community structure toward taxa effective in contrasting soil degradation. In this context, molecular studies aiming to gain insight into microbial community structure are needed to identify the role of specific microbial taxa in C stabilization and mineralization.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.still.2019.104482.

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