Fertilization promotes microbial growth and minimum tillage increases nutrient-acquiring enzyme activities in a semiarid agro-ecosystem

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ABSTRACT

Microorganisms respiratory and enzymatic activities provide sensitive indicators of changes in soil properties, such as those caused by interactive effects of tillage and fertilization regimes or other agricultural practices. However, the rapid, adaptive microbial growth, respiratory and enzymatic responses to changes in soil environments induced by specific agricultural practices are not well understood. Thus, to explore these adaptations we compared effects of contrasting environments on functional microbial traits (growth and enzyme kinetic parameters) in a Mediterranean agro-

ecosystem. These environments differed in long-term disturbance (no, minimum, or conventional tillage), nitrogen-richness (fertilization with 90 kg N ha^{-1} versus no fertilization), and resource scarcity (increasing with soil depth in 0-30, 30-60 and 60-90 cm layers). Reducing soil disturbance from conventional to minimum tillage promoted microbial growth through shorter T_{lag} and larger active biomass fraction and induced increases in N- and P-acquiring enzyme activities by increasing nutrients limitation. Fertilization stimulated increases in fast-growing microorganisms with low substrate-affinity enzyme systems, microbial biomass, enzymatic activities, and turnover rates of soil organics. In contrast, increasing scarcity of resources with soil depth strongly reduced microbial biomass and activity. A lack of correlation between soil and enzymatic stoichiometric ratios raises concern regarding the applicability of eco-enzymatic stoichiometric indexes in Mediterranean agro-ecosystems. We conclude that decomposition and turnover of organic substrates under contrasting agricultural practices are mediated by microbial communities with distinct functional traits (active fraction, growth parameters) and enzyme properties (V_{max}, K_m), which need to be considered in smart land use regimes.

Keywords: tillage, fertilization, soil depth, microbial growth parameters, enzyme activities, eco-stoichiometric approach

1. INTRODUCTION

The enzyme activity in soils is considered an early and sensitive indicator for assessing and comparing soil quality and degradation under different soil management (Cardelli et al., 2019; Kabiri et al., 2016), because soil enzymes quickly respond to environmental changes (Sherene, 2017). Soil enzymes are intimately involved in catalysing the basic processes of nitrogen, carbon and phosphorus transformation (Piotrowska-Długosz et al., 2021), as well as mediating the decomposition of organic matter (Nottingham et al., 2019). Production of these extracellular enzymes, which play key roles in microbial performance, is negatively related to soil nutrient availability and energy demand

(Sinsabaugh et al., 2009). Thus, their activities tend to increase with reductions in soils' nutrient contents (Bending et al., 2004; Paudel et al., 2011). Among the soil enzymes, glycosidases break down low molecular weight carbohydrates to produce glucose, which microorganisms use as an energy source (Zhang et al., 2015). In particular, β-glucosidase (BG) contributes to late steps of cellulose degradation by cleaving cellobiose and releasing simple sugars readily usable by microorganisms. N-acetyl-β-glucosaminidase (NAG) or chitinase is a key enzyme involved in the hydrolysis of chitin into amino sugars that represent the major source of mineralizable N in soils (Ekenler and Tabatabai, 2004). Acid phosphatase (AP) catalyses the hydrolysis of complex and unavailable forms of organic P into assimilable ones (Margalef et al., 2017). As working pH range for alkaline phosphatase is above 9, the AP is more widespread than alkaline phosphatase at soil pH values found in most natural soils (Margalef et al., 2017), and this justified our choice. Finally, leucine aminopeptidase (LAP) catalyses release of leucine and several other amino acids from polypeptides' N terminals (Wang et al., 2020) and it is involved in the microbial acquisition of N.

In addition to individual enzymatic activity, the set of functionally distinct enzymes was recently recommended for eco-enzymatic stoichiometric approach to identify nutrient limitation in soil, because BG, NAG and AP can be used as indicators of microbial resource allocation to the acquisition of C, N, and P from soil, respectively (Bai et al., 2021). Even if it is assumed that the stoichiometry of extracellular enzymes in soil provides indications of microbial nutrient acquisition rates and availability of limiting resources (Cui et al., 2019; Deng et al., 2019; Li et al., 2020; Zhou et al., 2020), direct links between experimental variables and environmental characteristics are not necessarily demonstrated (Prosser, 2020). In fact, evidence that highly diverse enzymes play similar roles in stepwise decomposition of organic substrates in soil is often ignored in enzymatic studies focusing on specific enzymes related to a single function or process. Limited numbers of enzymes have been analysed, with little consideration of the complex interactions between them in soil, and the applicability and sensitivity of the indexes is not always evident (Loeppmann et al., 2016; Mori, 2020). Moreover, roles of specific enzymes in C/nutrient cycling are often misinterpreted, so the

application of eco-enzymatic stoichiometry indexes to determine nutrient availability and carbon use efficiency has been intensely debated and criticised (Nannipieri et al., 2018).

As extracellular soil enzymes are mainly of microbial origin, enzymatic activity is closely linked to biomass and especially to the functional traits of active fraction of microbial community (Blagodatskaya et al., 2021). As the most labile components of soil organic matter (SOM) implied in the carbon cycle (Xu et al., 2020), microbial biomass, and dissolved organic carbon are sensitive to changes in soil management and agronomic practices (García-Orenes et al., 2013). For example, higher microbial biomass under no tillage treatment with respect to the conventional tillage was explained by the higher content of organic matter incorporated into the soil and the reduced disturbance in the former (Mbuthia et al., 2015). In contrast, reductions in microbial biomass have been reported after nitrogen fertilization in croplands, due to the increased microbial respiration during SOM mineralization and the resulting carbon loss (Alam et al., 2014; Jian et al., 2016). Thus, the microbial respiration during decomposition of organic compounds strongly influences the soil carbon cycle, because the carbon is lost as atmospheric CO_2 (Kleber et al., 2015). Generally, most ecologically relevant biogeochemical processes are mediated by physiologically active soil microorganisms, which comprise a small fraction of the total population (Blagodatskaya and Kuzyakov, 2013). In contrast, more than 80–90% of soil microorganisms are usually in a dormant or inactive state, in which they have minimal respiratory activity (Joergensen and Wichern, 2018). Microbial growth parameters such as specific growth rate, active fraction or growing microbial biomass (GMB) and lag period (T_{lag}) are used to identify functional traits and the successional changes in microbial communities (Blagodatskaya et al., 2007).

Furthermore, to identify the optimal strategy for sequestering carbon in soil and minimize the negative effects of crop production practices in agro-ecosystems, it is extremely important to understand not only the dynamics of total carbon reserves in soil under different soil tillage and fertilization regimes but also the associated changes in microbial functional traits, biomass and enzymatic activities. Therefore, the aim of our study was to compare microbial functional traits and

104 enzymes kinetic parameters in contrasting semiarid Mediterranean agro-ecosystems. These
105 environments differed in soil tillage (no, minimum, and conventional tillage), nitrogen-richness
106 (fertilization with 90 kg N ha^{-1} versus no fertilization), and resource scarcity (increasing with soil
107 depth in 0-30, 30-60 and 60-90 cm layers). We focused on the functional microbial traits developed
108 under these regimes since a previous study (De Mastro et al., 2020) demonstrated that cultivable
109 bacterial and fungal taxa are strongly affected by tillage and fertilization treatments. We hypothesized
110 greater microbial biomass under minimum versus conventional tillage, reduced enzymatic activity in
111 fertilized versus non-fertilized plots and decreasing fraction of active microorganisms with soil depth.
112 We also aimed to test the applicability of eco-enzymatic stoichiometric approach in Mediterranean
113 agro-ecosystems correlating the eco-enzymatic ratios to the corresponding soil nutrient ratios.
114
115 **2. MATERIALS AND METHODS**
116 *2.1. Field trials and soil analyses*
117 The experimental site is located at Policoro (Matera, Italy; 40°10′20″ N; 16°39′04″ E) and belongs
118 to the University of Bari. Policoro is characterized by a typical Mediterranean climate, with 16.8 °C
119 mean annual temperature and 539 mm average annual rainfall (Pantanelli experimental
120 meteorological station, Policoro). The experiment was carried out over one growing season (2015-
121 2016) in a field cultivated with wheat (*Triticum turgidum* L. var *durum*) that was sown in November
122 and harvested in June for grain. The crop was part of a 2-year rotation alternating with broad bean
123 (*Vicia faba* var. *equina* Pers.) cv Prothabat 69 in a split-block design with four replications, with
124 tillage as main factor and fertilization as sub-plot factor. Plots received either nitrogen fertilization as
125 100 kg ha^{-1} of a fertilizer with 18% N, 8.7% P, 2.9% Ca and 5.6% S before sowing, and 150 kg ha^{-1}
126 of urea at stem jointing, or no fertilization. At the end of the crop cycle, the wheat straw aerial residues
127 were removed from the experimental plots. Soil samples were taken at three depths (0-30, 30-60 and
128 60-90 cm) and stored in aerated polyethylene bags at 4 °C during transportation to the laboratory.
129 Before use, soils were homogenized and sieved through a 2 mm mesh. The treatments included three

levels of long-term disturbance (no tillage, minimum tillage, and conventional tillage: NT, MT and CT, respectively), two levels of nitrogen enrichment (90 kg N ha^{-1} versus no fertilization), and three levels of depth-related resource scarcity (0-30, 30-60 and 60-90 cm). The dose of 90 kg N ha^{-1} was chosen considering the results of a previous research (Ali et al., 2019), while the three sampling depths are based on the root system of the crops (Fan et al., 2016) and the depth of tillage adopted in the trial. Details about the tillage practices have been reported by De Mastro et al. (2019 a, b).

The main chemical soil properties were determined according to standard methods (Sparks et al., 1996). The soil pH was measured in 1 M KCl suspension at 1:2.5 soil to liquid ratio, while the electrical conductivity (EC) was measured in a filtrate from 1:2 soil to water ratio. The organic carbon (OC) content was measured by the Walkley-Black method, and the total nitrogen was determined by the Kjeldahl method. According to Olsen method, the available phosphorus (P_{ava}) was determined by ultraviolet and visible (UV–vis) spectrophotometry, while the exchangeable potassium (K_{exc}) was quantified using the inductively coupled plasma optical emission spectroscopy.

2.2. Enzyme Assays

Following Pritsch et al. (2004), enzyme activities were assayed using fluorogenically labelled substrates: 4-methylumbelliferone (MUF) for β-Glucosidase (BG), N-acetyl-β-Glucosaminidase (NAG) and acid phosphomonoesterase (AP), and 7-amino-4methylcoumarin (AMC) for leucine aminopeptidase (LAP) (Table 1).

All chemicals and substrates were purchased from Sigma (Germany). Enzyme activities were determined with a range of substrate concentrations (0, 5, 10, 20, 50, 100, 200 and 400 μmol g^{-1} soil of the 0-30 cm layer; 0, 2.5, 5, 10, 20, 50, 100, 200 μmol g^{-1} soil of the deeper layers).

Suspensions of 0.5 g soil (dry weight equivalent) with 50 mL deionized water were prepared separately for each of four incubated replicates using low-energy sonication (40 J s^{-1} output energy) for 2 min (Koch et al., 2007; Stemmer et al., 1998). Following sonication, 50 μL portions of soil

suspensions were added to 100 µL portions of aqueous solutions of each substrate at a range of concentrations and 50 µL of buffer (MES or TRIZMA, see Table 1) in a 96-well micro-plate (Puregrade, Germany). Fluorescence in the wells was induced and monitored for 2 h using a Victor3 1420-050 multi-label counter (Perkin Elmer, USA), with excitation and emission wavelengths of 355 and 460 nm, respectively, and 25 nm slit width.

Enzyme activities were expressed in terms of amounts of MUF or AMC released in nmol per g dry soil per hour (nmol g^{-1} dry $soil^{-1}$ h^{-1}). In addition, enzyme activities in each of every set of four field replicates were assayed at each substrate concentration in three analytical replicates (and thus in 12 micro-plate wells).

2.3. Enzyme kinetics

To quantify kinetic parameters of the selected enzymes we determined their Michaelis constant (K_m) and potential activity (V_{max}) under each treatment using the Michaelis-Menten equation:

$$v = V_{max} \times [S] / (K_m + [S])$$

where v is the reaction rate (as a function of enzyme substrate concentration), [S] is the substrate concentration, K_m is the substrate concentration at half-maximal rate, and V_{max} is the maximum reaction rate (Michaelis and Menten, 1913; Segel, 1975). The V_{max} of an enzyme indicates the splitting velocity or rate of dispersion of the enzyme-substrate complex into enzyme and reaction products, while K_m reflects the enzyme's affinity for the substrate (Zhang et al., 2009).

Three soil eco-enzyme ratios were calculated, according to Yang et al. (2020). These were the soil enzyme C:N, C:P and N:P ratios obtained by calculating Ln (BG)/Ln (LAP+NAG), Ln (BG)/Ln (AP) and Ln (LAP+NAG)/Ln (AP) activity ratios, respectively. Substrate turnover times (T_t) were calculated according to Panikov et al. (1992) with the following equation: T_t (hours) = $(K_m + S)/V_{max}$, where S is the substrate concentration. We calculated T_t values at substrate concentrations corresponding to both substrate deficits and excess substrate contents ($S = K_m/10$ and $S = 10*K_m$, respectively.

182

183 *2.4. Microbial biomass and kinetics of substrate-induced respiration*

184 Substrate-induced microbial growth rate (SIGR) kinetics were determined by monitoring rates of
185 CO_2 emission following application of growth substrates, glucose and nutrients, to estimate microbial
186 growth parameters according to the model proposed by Panikov and Sizova (1996). The model
187 simulates the transition of soil microorganisms from maintenance to an active state, including both
188 the lag and exponential growth phases. The SIGR approach enables estimation of the specific
189 microbial growth rate (μ) as well as the sustaining and growing fractions of microbial biomass
190 (Blagodatsky et al., 2000; Panikov, 1995). Briefly, 1 g samples (dry weight) of soil were placed in an
191 incubation vessel with 3 mL of 1 M NaOH in the bottom to trap the CO_2 and measure its production
192 rate. To each soil sample we then added 0.1 mL of a solution containing glucose (4 mg C g^{-1}) and
193 mineral salts: 1.9, 2.25 and 3.8 mg g^{-1} of $(NH_4)_2SO_4$, K_2HPO_4, and $MgSO_47H_2O$, respectively. The
194 amounts of mineral salts selected were based on the pH and buffer capacity of the soil, to ensure that
195 the pH changed less than 0.1 units during microbial growth. After the glucose addition, the vessels
196 were closed air-tight and electrical impedance was automatically recorded at 10-min intervals by a
197 RABIT respirometer system at 22 °C for 72 h.

198 The kinetics of microbial growth was estimated by fitting the parameters of Eq. 1 to the measured
199 CO_2 evolution rate (Blagodatsky et al., 2000; Panikov and Sizova, 1996):

200

201 $CO_2(t) = A + B*\exp(\mu * t)$ (1)

202 where A is the initial respiration rate uncoupled from ATP generation, B is the initial rate of the
203 growing fraction of total respiration coupled with ATP generation and cell growth, μ is the maximal
204 specific growth rate of soil microorganisms, and t is the time. The parameters of Eq. 1 were optimized
205 by minimizing the least-square sum using Model Maker-3 software (Cherwell Scientific Publishing
206 Ltd., Oxford, UK). Four replicate curves of respiration under each treatment were acquired. Fitting

was restricted to the initial phase of each curve, corresponding to unlimited exponential growth (Wutzler et al., 2012).

Other parameters of microbial growth kinetics were calculated from the optimized parameters of the fitted respiration curves (Eq. 1). In each case, T_{lag} was determined as the time between the moment of glucose addition and the moment when the increasing rate of growth-related respiration, B *exp(μ * t), reached the rate of respiration uncoupled from ATP generation (A). This was calculated using parameters of the approximated curve of the respiration rate of microorganisms (Eq. 2):

$$T_{lag} = \ln (A/B)/ \mu. \quad (2)$$

The maximal specific microbial growth rate (μ) derived from Eq. (1) was used as an intrinsic property of microorganisms to estimate relative contributions of fast-growing (r-strategists) and slow-growing (K-strategists) populations in the soil microbial community. According to Andrews and Harris (1986) and Pianka (1970), μ reflects the domination of r-strategists relative to K-strategists. This approach is based on widely accepted links between microbial community structure and substrate availability (Fierer et al., 2007; Panikov, 2010) and has been validated against other physiological parameters of total microbial community, such as enzymes' affinity to their substrates (K_m) and/or substrate use efficiency (Blagodatskaya et al., 2007, 2009). The total microbial biomass (TMB) and growing microbial biomass (GMB) before substrate addition were calculated using Eqs. 3 and 4, respectively.

$$TMB = B/r0Q \quad (3)$$

$$GMB = TMB \times r0 \quad (4).$$

The parameter r0, the physiological state index of microbial biomass (MB) at time zero (before substrate addition), was calculated from the ratio between parameters A and B, using Eq. 5 (Panikov and Sizova, 1996).

$$r0 = B (1-\lambda)/(A+B (1-\lambda)) \quad (5)$$

where λ is a basic stoichiometric constant, which has an accepted value of 0.9 (Panikov and Sizova, 1996).

2.5. Statistical analysis

To identify significant differences in microbial parameters and enzyme activities among treatments (tillage, fertilization, and depth), we applied three-way ANOVA using R (version 3.1.1) software at $\alpha < 0.05$. When significant differences were found, we conducted post hoc multiple comparisons using the Tukey HSD test. All presented results are means of four replicates ± standard error (SE).

3. RESULTS

3.1. Enzymatic activities as indicators of soil chemical properties

Responses of the measured soil chemical properties to the tillage, fertilization and resource deficiency treatments varied. The only measured soil property that was significantly influenced by tillage was exchangeable potassium content, which was 42.9% lower under CT than under the NT treatment (Table 2). Fertilized plots had about 40% lower exchangeable potassium contents, more than five times higher P_{ava} contents and slightly but significantly higher pH (0.1 units; Table 2) than unfertilized plots. In accordance with the anticipated increasing scarcity of resources, the organic carbon (OC), total nitrogen (TN) and available phosphorous (P_{ava}) contents decreased with depth from the 0-30 to the 60-90 cm layer by 60.9, 52.9 and 82.2%, respectively. The opposite trend was observed for the pH, electrical conductivity, and exchangeable potassium content. The interactions between soil fertilization and soil depth were significant for EC ($p=0.047$) and exchangeable K ($p=0.013$). Soil disturbance by conventional tillage reduced the activity of most tested enzymes (Fig. 1). This effect was strongest in unfertilized topsoil, where V_{max} values obtained for BG and NAG were almost two times higher under MT than under CT (Fig. 1). In addition, V_{max} and K_m values were significantly

higher under NT than under conventional tillage for the nutrient-acquiring enzymes, LAP and AP, respectively (Table 3, Fig. 1).

Despite increasing nutrient levels, fertilization did not generally affect enzyme activities significantly (Table 3). However, it had strong effects in topsoil under NT, raising V_{max} values of all tested enzymes 2- to 3.5-fold relative to those in unfertilized plots (Fig. 1). Thus, the tillage treatments affected enzymatic activities more strongly than the fertilization treatment.

Depletion of resources with soil depth was the factor that most strongly influenced activities of enzymes decomposing primary and secondary substrates (plant and microbial residues, respectively). V_{max} values of BG and NAG declined up to 5-fold from the topsoil to the deepest layer (Table 3). This effect was most pronounced under MT. The K_m values of BG and NAG were similar at each depth in unfertilized soils. However, the nutrient-acquiring enzymes LAP and AP had up to 3-fold lower and up to 2-fold higher K_m values in the lowest depth than in the other depths (Table 3, Fig. 2).

In summary, soil disturbance caused by tillage was the main factor reducing activities of the nutrient-acquiring enzymes LAP and AP (Table 3). In contrast, increases in resources deficiency with soil depth mainly affected kinetic parameters of enzymes contributing to decomposition of plant and microbial residues. Remarkably, fertilization was not among the main drivers of enzymatic activity. Thus, despite the enzyme-specific effects of agricultural practices, generally fertilization did not strongly affect enzymatic activities, apart from indirect effects through acceleration of microbial growth under NT.

3.2. Microbial biomass and kinetics of substrate-induced respiration

The application of glucose with nutrients to the soil induced an exponential increase in the CO_2 evolution rate within a few hours (Fig. 3). Microbial activity (as manifested by CO_2 release) clearly decreased with soil depth and was markedly lower in the deepest soil layer than in the upper layers. No significant tillage effect was detected, but soil fertilization stimulated microbial activity, CO_2 release, and hence respiration, in the top and deepest soil layers. The difference in rates of CO_2 fluxes

between the fertilized and unfertilized topsoil was highest under the NT treatment, so we show effects of fertilization under this type of tillage in Figure 3. The respiration rates 24 hours after glucose addition were 90.6% higher in samples from fertilized plots than in samples from unfertilized plots under the NT treatment. This effect gradually decreased with increasing tillage impact. Smaller differences between fertilized and unfertilized plots under MT and CT treatments were probably due to greater homogenization of the soil. Remarkably, the contrasting pattern of respiratory curves were not explained by microbial specific growth rates (μ) which did not differ significantly between treatments. In contrast, total and growing fraction of microbial biomass as well as lag time sensitively mirrored the differences in environmental conditions caused by fertilization, tillage, and soil depth (Fig. 4, Table 4).

Minimum tillage slightly increased, whereas the CT treatment decreased, total microbial biomass (TMB) (Fig. 4, Table 4). Consequently, TMB was about 59 and 19% higher in the unfertilized topsoil under the MT treatment than under the CT and NT treatments, respectively. Fertilization generally significantly decreased the TMB, which also decreased passing from the first layer to the underlying layers.

The growing microbial biomass (GMB) was much more sensitive to agricultural management than total biomass. Similarly to TMB, minimum tillage was the most favourable treatment for active microorganisms, as GMB was about 45.4 and 37.2% higher in the topsoil under MT than under the CT and NT treatments, respectively (Fig. 4). However, in contrast to its effects on TMB, fertilization resulted in twice as high GMB in the topsoil layer in the fertilized plots than in unfertilized plots (0.81 μg Cg^{-1} versus 0.43 μg Cg^{-1}). GMB was also 46.8% lower in the deepest soil layer than in the top layer.

The lag period (T_{lag}) was slightly longer under NT than under both tillage treatments (Table 4). It was also shorter in samples from fertilized plots than in corresponding samples from unfertilized plots (Table 4). Thus, fertilization reduced the time required for microorganisms to start growing. Finally,

308 T_{lag} was about 2 h longer in samples from the deepest layer than in samples from the topsoil layer,
309 and we detected a negative correlation between the active biomass and lag period (Fig. 4).
310 In summary, both TMB and GMB were 30-40% higher under MT than under CT and NT
311 treatments. Soil fertilization decreased TMB but increased GMB, suggesting that N was not the
312 limiting factor for active microbial biomass in these soils.
313 *3.3. Soil extracellular enzymes' stoichiometry*
314 The soil enzymatic C/N and C/P ratios were markedly lower under MT than under NT and CT
315 treatments (Table 5, Fig. 5). They were also significantly higher in the topsoil layer of fertilized CT
316 plots than in the same layer of unfertilized plots (Fig. 5). No fertilization effect on enzymatic ratios
317 was detected under the MT and NT treatments. No significant between-layer differences were
318 detected in any of the examined soil enzyme ratios, with the exception of C/P and C/N enzymes ratios,
319 which were lower in the first soil layer of unfertilized CT plots than in the other soil layers. Moreover,
320 the C/N, C/P and N/P stoichiometric ratios determined in soil were not consistent with those
321 determined for the soil enzymes (r=0.3095, r=-0.0612, r=0.2781 for C/N, C/P and N/P, respectively).
322 In contrast to enzymatic ratios, the soil C/N ratio was strongly influenced by depth, and the N/P and
323 C/P ratios were significantly affected by fertilization. In addition, the tillage treatments had no
324 significant effects on the nutrient ratios, although tillage was the factor that most strongly affected
325 enzymatic ratios.
326 Generally, the BG, NAG and AP substrate turnover times increased with soil depth, but the LAP
327 substrate turnover time decreased with depth (Fig. 6). No significant differences in turnover times
328 associated with differences in tillage were detected, but substrate turnover of the plant- and microbial-
329 residue decomposing enzymes (BG and NAG) was slower in the deepest layers of fertilized plots
330 than in their top layers under NT and CT treatments (Fig. 6).
331 **4. DISCUSSION**
332 Among the different treatments, the MT was the most favourable for the microbial communities,
333 resulting in the highest total biomass and abundance of active microorganisms. Minimum tillage also

resulted in the highest activities of C- and N-acquiring enzymes and selected distinct acid phosphatase enzyme systems, with differing K_m values from those under the CT and NT treatments. The higher V_{max} values of BG and AP under NT than under CT and, to a lesser degree MT, were presumably due to lower soil disturbance (Mendes et al., 2003; Pandey et al., 2014; Roldán et al., 2005), which mitigates runoff of residual soil nutrients (Sinsabaugh et al., 2008).

Increases in nutrient availability through fertilization generally promoted microbial growth and activities, production of enzyme systems with low substrate affinities, high microbial biomass, and fast turnover of soil organics. A remarkably strong effect of fertilization on V_{max} of all examined enzymes was detected in the topsoil in the absence of tillage (Table 3, Fig. 1), which could be related to the mineral form of fertilizer used. Organic, but not mineral, fertilization can reportedly accelerate activities of hydrolytic enzymes, e.g., glucosidases (Saha et al., 2008; Tiwari et al., 2019) and acid phosphatases (Nannipieri et al., 2011; Piotrowska-Dlugosz and Wilczewski, 2014). This implies that energy inputs, such as those associated with labile organic compounds, are essential for decomposition processes in soil (De Mastro et al., 2019b). De Mastro et al. (2020) found that fertilization increased fungal population by 40% and we found in this study that it increased amounts of P_{ava} 5.4-fold (Table 2) relative to the unfertilized plots. Therefore, stimulation of fungal activity may explain the strong observed increases in catalytic efficiency of all enzymes in the upper soil of fertilized plots under the NT treatment. No such between-layer effects of fertilization under the MT and CT treatments were detected, probably because the greater tillage-mediated homogenization and higher aeration of soil limited hindrance of microbial activity in the deeper layers. High activities of enzymes (especially BG and NAG) in the upper soil layer can be ascribed to the higher contents of OC and nutrients (TN and P_{ava}) and the intensive decomposition of soil organic matter (Eivazi and Tabatabai, 1990). This is because the availability of nutrients influences soil microbial communities' diversity, enzyme production, and hence V_{max} values (Allison and Martiny, 2008; Kujur et al., 2012; Nemergut et al., 2008; Stone et al., 2011).

359 Reponses of the microbial community to depletion of resources with soil depth included reductions
360 in total and growing biomass, activities (V_{max}) of plant- and microbial-residue decomposing enzymes
361 as well as increases in activities and changes in substrate-affinities (K_m values) of nutrient-acquisition
362 enzyme systems. This is consistent with expectations, as enzymatic activities are generally reduced
363 in deeper soil layers by the higher bulk density, lower oxygen availability (Davidson et al., 2012;
364 Kleber, 2010; Schnecker et al., 2015), and lower abundance of simple sugars for microorganisms
365 (Tiwari et al., 2019; Xiao-Chang and Qin, 2006). However, the V_{max} of LAP slightly increased with
366 soil depth, especially in unfertilized plots, indicating a nitrogen acquisition strategy (Sinsabaugh and
367 Moorhead, 1994).
368 The efficiency of enzymes' decomposition of substrates at low concentrations is directly related
369 to their K_m (Davidson et al., 2006; Marx et al., 2005). K_m values are negatively related to the
370 endurance of enzyme-substrate complexes (Kujur and Kumar Patel, 2014). Thus, the higher K_m
371 values of enzymes in fertilized plots (Table 3) could be due to more diverse active microorganisms
372 producing enzymes with lower substrate affinities than those in unfertilized plots (Blagodatskaya et
373 al., 2009; Blagodatskaya and Kuzyakov, 2013). In addition, the higher K_m values of AP in the deeper
374 layer could be due to a combination of multiple enzyme systems catalysing acid dephosphorylation
375 reactions and reductions in enzyme-substrate affinities through immobilization of the enzymes by
376 soil constituents (e.g., organic matter and clay) (Ferreira et al., 2016).
377 A negative correlation between the active biomass and lag-period confirmed that faster growth is
378 not solely associated with higher microbial specific growth rates (Blagodatskaya et al., 2014). The
379 higher GMB and lower T_{lag} in the topsoil of fertilized plots, especially under the MT treatment,
380 suggest that nitrogen fertilization increased both microbial populations and activities. However, these
381 effects were confined to the top layer, probably because aeration and nutrient availability are likely
382 to be highest close to the surface. Plate counts of microorganisms in the same experimental plots have
383 shown that the GMB of fungi and bacteria is higher under MT than under CT and NT treatments (De
384 Mastro et al., 2020). The longest lag-time in the deepest soil layer was probably due to the higher

fraction of dormant microorganisms under substrate and nutrient limitations, which extended the time required for a switch to growth.

C/N and C/P enzymatic ratios were significantly lower under MT than under both the CT and NT treatments, possibly because GMB, and thus microbial nutrient requirements, were highest under MT. The higher abundance of rapidly growing organisms under MT may also have contributed, as high amounts of N and P are needed to maintain high microbial turnover rates (Elser et al., 2003; Sterner and Elser, 2002). Although the GMB was higher in fertilized plots than in corresponding unfertilized plots, we detected no differences in eco-stoichiometric ratios between their top layers (except under CT). The increased disturbance under the CT treatment eliminated correlations between enzymatic and soil stoichiometric ratios. This demonstrates that correlation analysis did not distinguish between cause and effect and confirms that enzymatic activities are not solely affected by nutrient limitations but also by much broader interactions between microorganisms and their environments (Prosser, 2020).

Inverse correlations detected between the enzymatic C/N ratio and both NAG and LAP turnover times under MT could be related to the higher GMB causing a decrease in the turnover rate by promoting the accumulation of newly formed microbial necromass, and thus soil organic matter (Prommer et al., 2019). The finding that substrates turnover times were longest in the deepest soil layer also suggested an accumulation of soil organic matter due to lower microbial activity.

5. CONCLUSIONS

The results of our study confirmed the hypotheses relating to a greater amount of microorganisms (total and active microbial biomass) with less invasive agronomic management such as minimum tillage compared to the conventional one, a reduced enzymatic activity in fertilized plots compared to non-fertilized ones and a lesser amount of microorganisms in the deeper layers of the soil (Fig. 7). The fraction of growing microorganisms and the activity of the enzymes that acquire N and P were greater during the treatments that cause less disturbance of the soil compared to conventional tillage.

Fertilization favoured the rapid growth of microorganisms, especially in the topsoil, reducing the transition times from dormancy to growth and facilitating the enzymatic turnover of organic compounds. Resource depletion with depth strongly promoted activities of nutrient-acquiring enzymes and delayed decomposition of plant and microbial residues. With the results obtained, it was possible to test the applicability of the stoichiometric eco-enzymatic approach in Mediterranean agroecosystems. However, the lack of correlation between the stoichiometric ratios of soil and those of enzymes indicated that the aforementioned approach was not fully applicable, at least without considering other factors, under field conditions. It should be noted that our results only provide a snapshot of these phenomena at the start of a growing season. Further information is needed on the seasonal and annual dynamics of the functional traits of microbial communities under different agricultural practices and on their ecological consequences (especially in terms of greenhouse gas emissions).

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657 **FIGURE CAPTIONS**

658 **Figure 1.** Maximum enzyme activities (V_{max}, nmol $h^{-1}g^{-1}$) at indicated soil depths under indicated
659 tillage and fertilization levels. NT, MT and CT refer to no, minimal and conventional
660 tillage without fertilization, respectively. NTF, MTF and CTF refer to these treatments
661 with fertilization. Different lowercase letters and uppercase letters within panels indicate

significant ($p < 0.05$) depth and tillage effects, respectively. The uppercase letter placed in the center of the plot means that it refers to all the data of the plot.

Figure 2. Michaelis constants (K_m, μmol g^{-1}soil) of indicated enzymes at indicated soil depths under indicated tillage and fertilization levels. NT, MT and CTF refer to no, minimal and conventional tillage without fertilization, respectively. NTF, MTF and CT refer to these treatments with fertilization. Different lowercase letters and uppercase letters within panels indicate significant ($p < 0.05$) depth and tillage effects, respectively. The uppercase letter placed in the center of the plot means that it refers to all the data of the plot.

Figure 3. Differences in glucose-induced CO_2 evolution rates of samples of soil of indicated depths from fertilized (NTF) and unfertilized (NT) plots.

Figure 4. Growing microbial biomass (GMB), total microbial biomass (TMB), and lag period (T-Lag) of microbial growth under indicated tillage and fertilization levels. NT, MT and CT refer to no, minimal and conventional tillage without fertilization, respectively. NTF, MTF and CTF refer to these treatments with fertilization. Different lowercase letters and uppercase letters within panels indicate significant ($p < 0.05$) fertilization and tillage effects, respectively. The uppercase letter placed in the center of the plot means that it refers to all the data of the plot. The asterisk highlights that the layer to which it refers is significantly different from the other two layers.

Figure 5. Spider diagrams of eco-enzymatic ratios of indicated soil layers under indicated tillage and fertilization levels. NT, MT and CT refer to no, minimal and conventional tillage without fertilization, respectively. NTF, MTF and CTF refer to these treatments with fertilization.

Figure 6. Turnover times of the four analysed enzymes in indicated soil layers under indicated tillage and fertilization levels. NT, MT and CT refer to no, minimal and conventional tillage without fertilization, respectively. NTF, MTF and CTF refer to these treatments with fertilization. Different letters above bars indicate significant differences, ns means not significant.

Figure 7. Directions of changes in microbial functional traits in soil with increasing nitrogen richness (from no-fertilization to fertilization), soil tillage (from no tillage to conventional tillage), and resource scarcity (from the lowest to top soil layer). The size of the arrows reflects the relative contribution of each factor (fertilization, tillage, and depth) to the variables' responses. Absence of arrows indicates that corresponding factors have negligible contributions (< 1%).

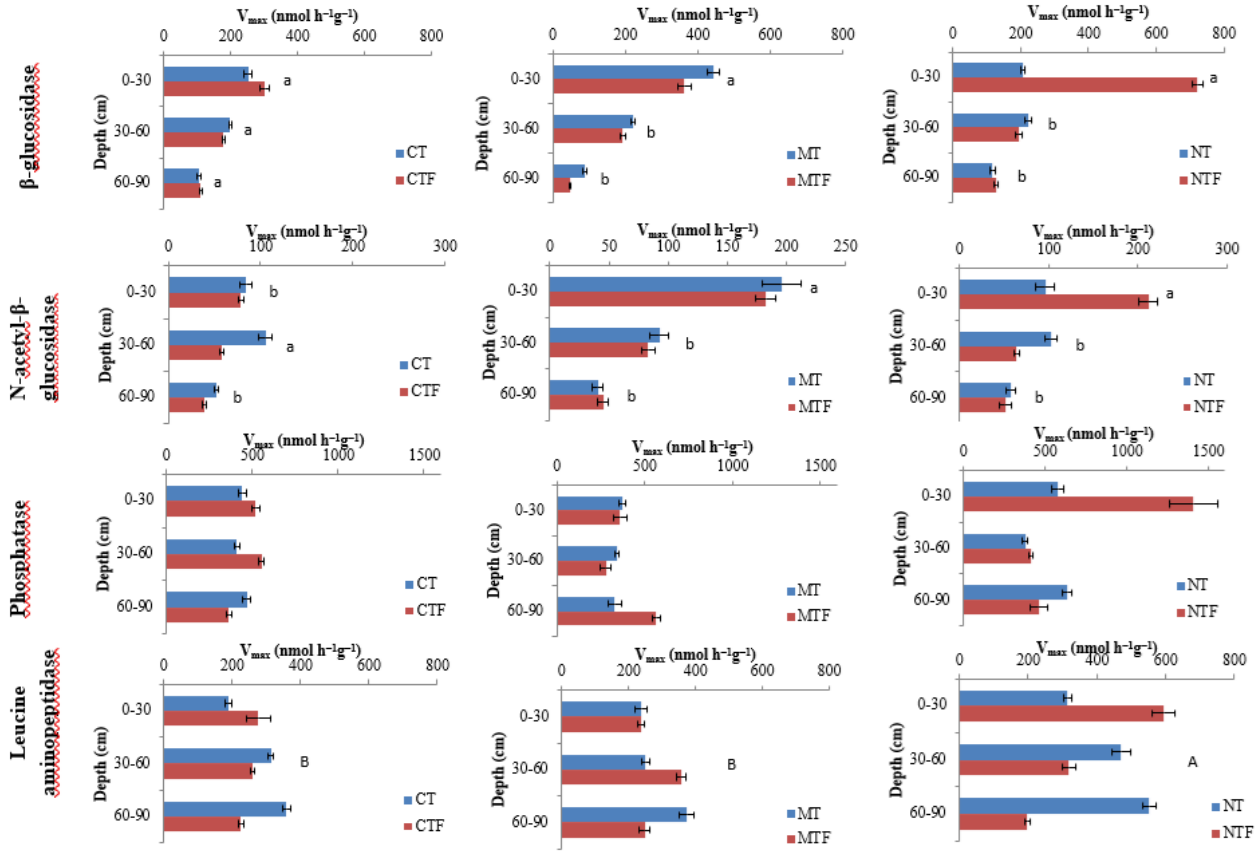
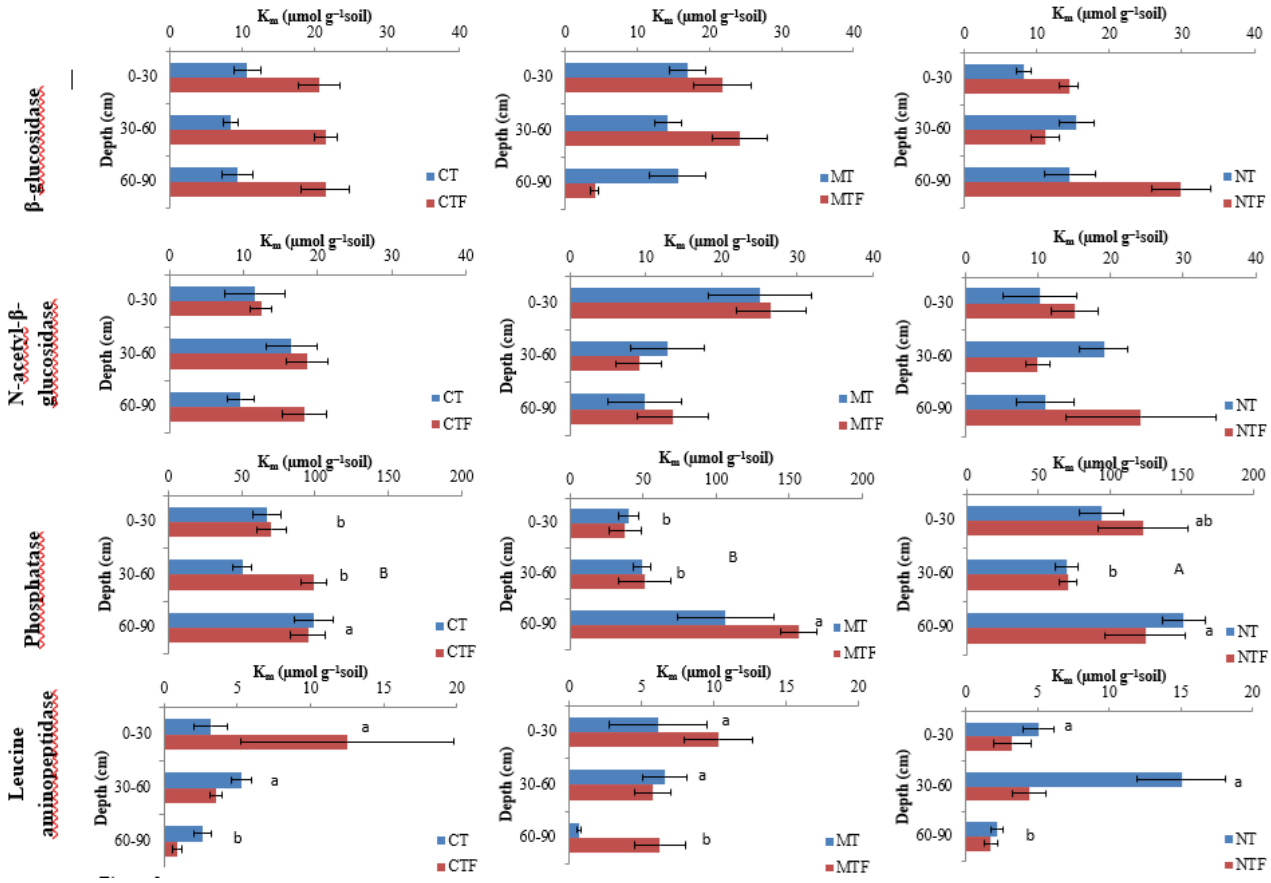


Figure 1



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699 Figure 2

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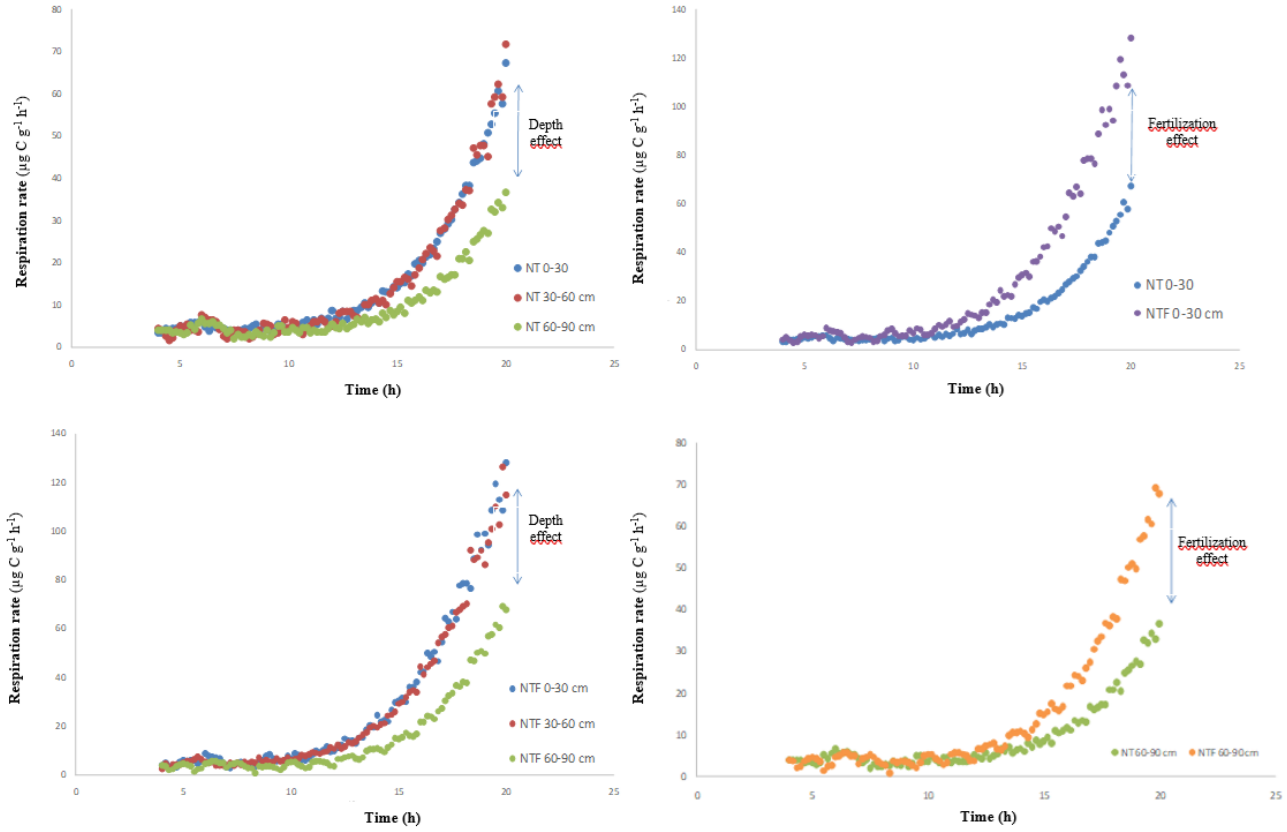

Respiration rate (µg C g-1 h-1)
Time (h)
Depth effect
NT 0-30
NT 30-60 cm
NT 60-90 cm
Fertilization effect
NT 0-30
NTF 0-30 cm
NTF 0-30 cm
NTF 30-60 cm
NTF 60-90 cm
NT 60-90 cm
NTF 60-90cm

Figure 3

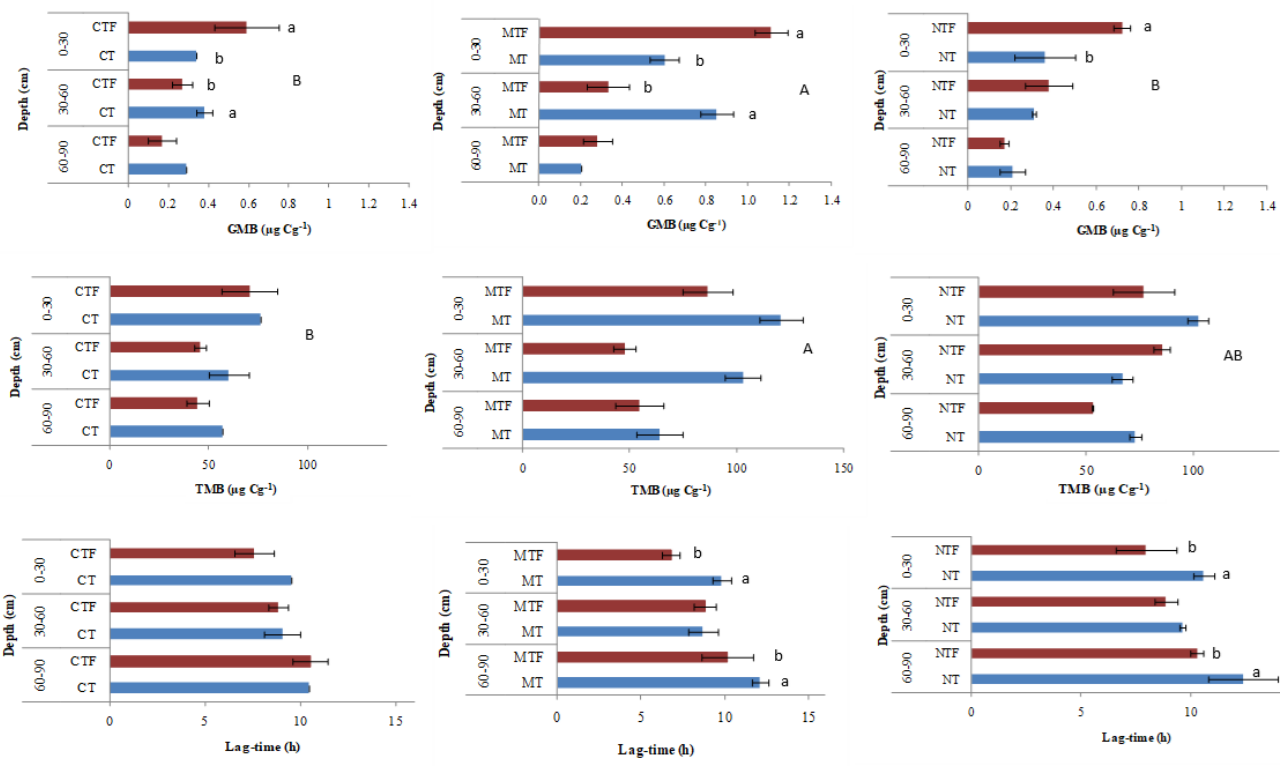
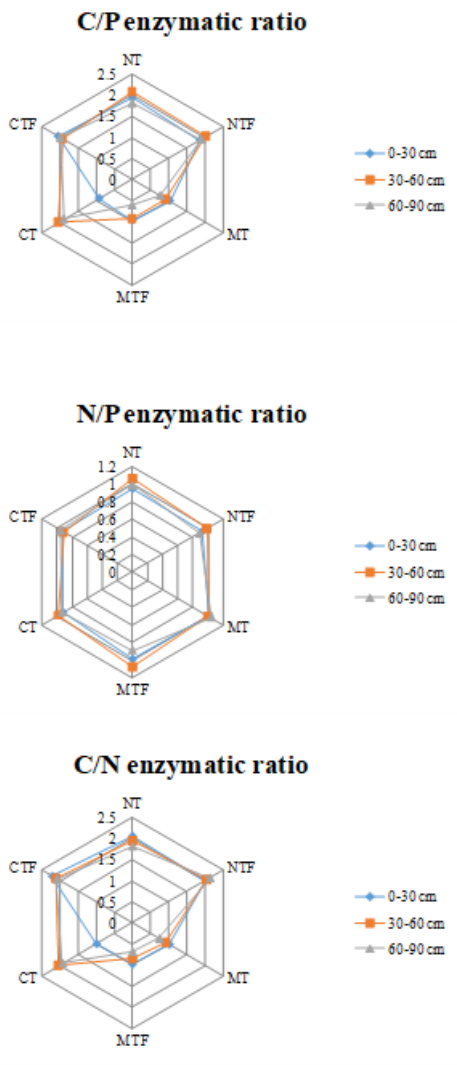

Depth (cm)
0-30
30-60
60-90
CTF
CT
MTF
MT
NTF
NT
a
b
B
A
AB
GMB (µg Cg-1)
TMB (µg Cg-1)
Lag-time (h)

Figure 4



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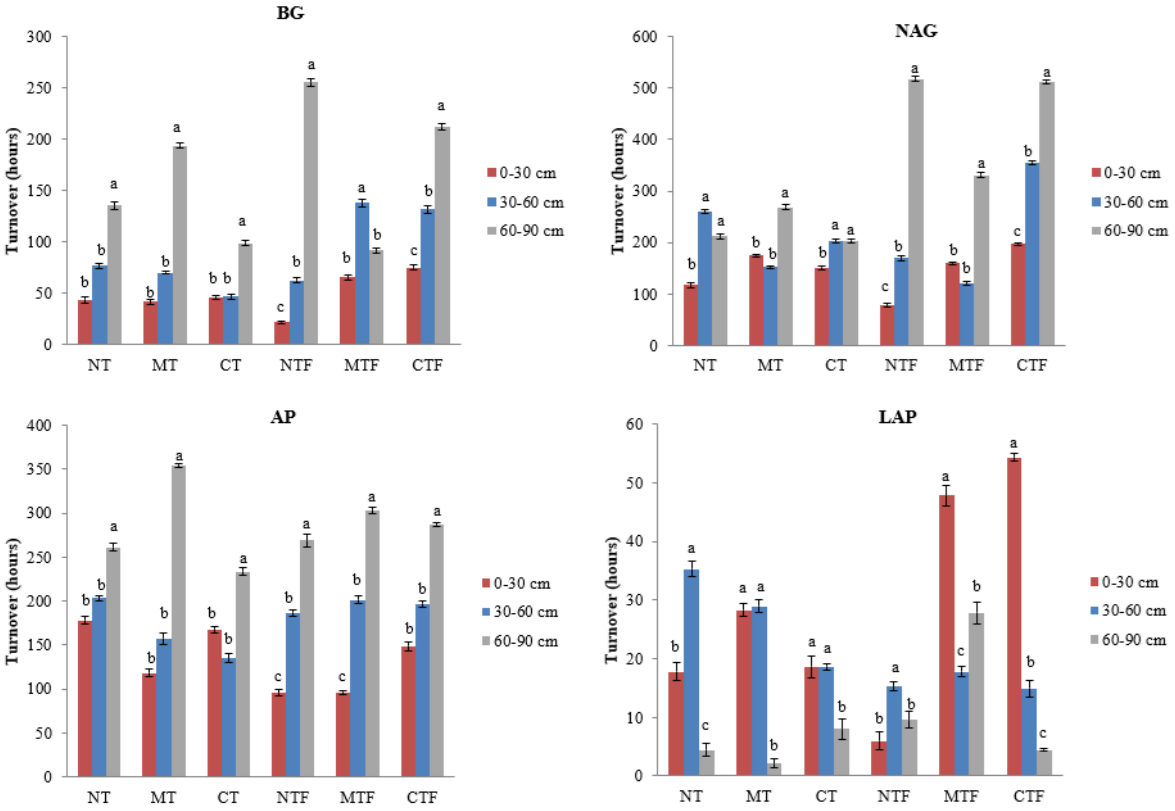
710 Figure 5



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712 Figure 6

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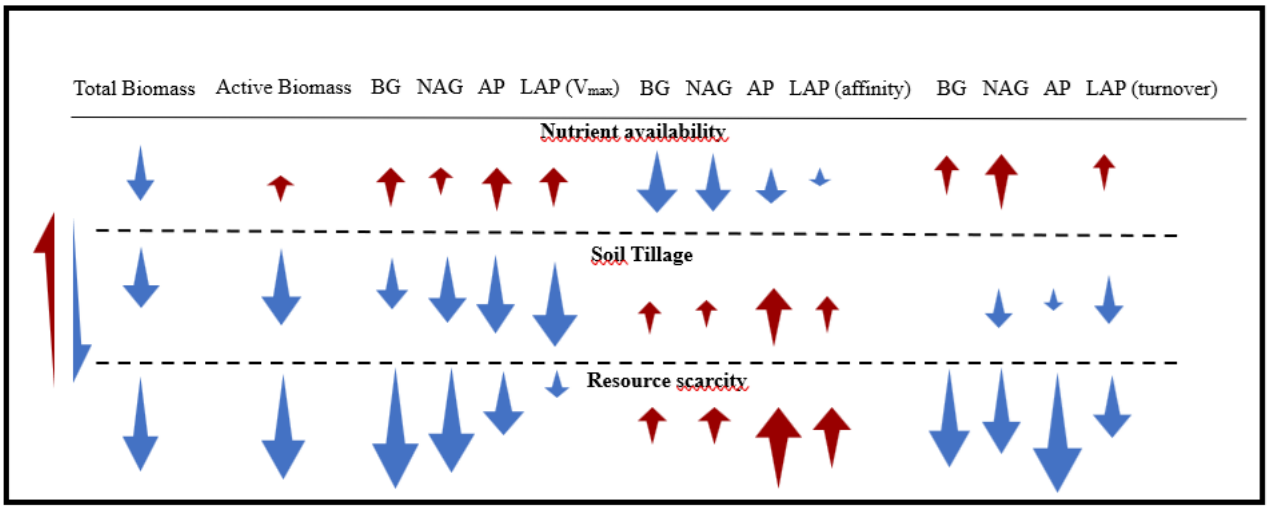

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715 Figure 7

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720 **Table 1.** Substrates for the estimation of enzyme activities

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Enzyme	Substrate	Buffer
C-cycle enzymes		
β-glucosidase	4-methylumbiliferyl-β-D-glucopyranoside	**MES**
N-cycle enzymes		
Chitinase	4-methylumbiliferyl-N-acetyl-glucosaminide	**MES**
Leucine aminopeptidase	L-leucien-7-amido-4-methylcoumarin	**TRIZMA**
P-cycle enzyme		
Acid phosphatase	4-methylumbiliferyl -phosphate	**MES**

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Table 2. Analysis of variance and mean values of chemical parameter subdivided by soil depth, tillage and fertilization

	pH	**EC**	**OC**	**TN**	**C/N**	**P_{ava}**	**K_{exc}**
	(KCl) (1 : 2.5)	($\mu S\ cm^{-1}$) (1 : 2)	($g\ kg^{-1}$)			($mg\ kg^{-1}$)	($cmolc\ kg^{-1}$)
Depth	***	***	***	***	**	**	**
Tillage	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
Fertilization	***	n.s.	n.s.	n.s.	n.s.	***	**
Depth							
0-30	7.5 c	326.1 b	15.6 a	1.7 a	9 a	7.3 a	0.6 b
30-60	7.6 b	450.1 b	9.7 b	1.2 b	7.9 ab	2.5 a	1.5 a
60-90	7.8 a	1162.1 a	6.1 c	0.8 c	7.1 b	1.3 b	1.1 a
Tillage							
NT	7.7 a	785.7 a	10.0 a	1.2 a	8.2 a	2.4 a	1.4 a
MT	7.6 a	624.3 a	9.6 a	1.2 a	7.5 a	1.8 a	1.0 ab
CT	7.6 a	578.3 a	11.7 a	1.4 a	8.3 a	3.3 a	0.8 b
Fertilization							
Control	7.6 b	791.5 a	10.4 a	1.2 a	1.8 a	0.8 b	1.3 a
90 kg/ha nitrogen	7.7 a	500.6 a	10.4 a	1.3 a	7.9 a	4.3 a	0.8 b

n.s.: not significant. The values in each column followed by a different letter are significantly different according to Tukey's test.

* Significant at the $P \leq 0.05$; ** Significant at the $P \leq 0.01$; *** Significant at the $P \leq 0.001$. EC: electrical conductivity, OC: organic carbon, TN: total nitrogen, P_{ava}: available phosphorous, K_{exc}: exchangeable potassium

Table 3. Analysis of variance and mean values of Michaelis constants (Km and Vmax) of different soil enzymes (β-glucosidase, N-acetyl-β-glucosidase, Phosphatase and Leucine aminopeptidase) subdivided by soil depth, tillage and fertilization.

	β-Glu		N-Ac		Phos		L-Leu	
	V max	**Km**	**V max**	**Km**	**V max**	**Km**	**V max**	**Km**
	(mmol $h^{-1}g^{-1}$)	**(µmol g^{-1})**	**(mmol $h^{-1}g^{-1}$)**	**(µmol g^{-1})**	**(mmol $h^{-1}g^{-1}$)**	**(µmol g^{-1})**	**(mmol $h^{-1}g^{-1}$)**	**(µmol g^{-1})**
Depth	**	n.s.	**	n.s.	n.s.	***	n.s.	*
Tillage	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	n.s.
Fertilization	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Depth								
0-30	394.38 a	17.68 a	133.47 a	14.57 a	626.25 a	75.42 b	304.48 a	6.05 a
30-60	201.37 b	15.82 a	77.94 b	12.21 a	396.32 a	65.02 b	328.22 a	6.77 a
60-90	100.68 b	14.33 a	47.58 b	14.43 a	479.77 a	132.66 a	326.46 a	2.40 b
Tillage								
NT	270.48 a	14.74 a	93.57 a	13.62 a	655.15 a	115.87 a	408.15 a	5.30 a
MT	226.47 a	16.13 a	100.18 a	13.62 a	387.24 a	76.92 b	284.71 b	5.97 a
CT	199.49 a	16.96 a	65.24 a	13.06 a	459.94 a	80.31 b	266.30 b	3.96 a
Fertilization								
Control	208.97 a	13.02 a	83.32 a	11.47 a	440.28 a	80.90 a	340.34 a	5.97 a
90 kg/ha nitrogen	255.32 a	18.86 a	89.34 a	16.00 a	561.28 a	101.17 a	299.10 a	4.94 a

n.s.: not significant. The values in each column followed by a different letter are significantly different according to Tukey's test.

* Significant at the $P \leq 0.05$; ** Significant at the $P \leq 0.01$; *** Significant at the $P \leq 0.001$.

Table 4. Analysis of variance and mean values of total microbial biomass (TMB), growing microbial biomass (GMB) and lag period (T-Lag) subdivided by soil tillage and fertilization of the first soil layer (0-30 cm).

	GMB	**TMB**	**T-Lag**
Tillage	*	*	n.s.
Fertilization	*	n.s.	*
Tillage			
NT	0.54 b	89.32 ab	9.26 a
MT	0.85 a	103.74 a	8.31a
CT	0.46 b	73.26 b	8.55 a
Fertilization			
Control	0.43 b	99.64 a	9.97 a
90 kg/ha nitrogen	0.80 a	77.91 a	7.44 b

n.s.: not significant. The values in each column followed by a different letter are significantly different according to Tukey's test.

* Significant at the $P \leq 0.05$; ** Significant at the $P \leq 0.01$; *** Significant at the $P \leq 0.001$.

Table 5. Analysis of variance and mean values of enzyme and soil ratios subdivided by soil depth, tillage and fertilization.

	N/P	C/N	C/P	N/P	C/N	C/P
	Enzyme ratio			Soil ratio		
Depth	n.s.	n.s.	n.s.	n.s.	**	**
Tillage	n.s.	***	***.	n.s.	n.s.	**
Fertilization	n.s.	n.s.	n.s	**	n.s.	**
Depth						
0-30	0.9 a	1.5 a	1.5 a	0.9 a	8.9 a	8.8 b
30-60	1.0 a	1.6 a	1.6 a	2.4 a	7.9 ab	19.5 a
60-90	0.9 a	1.5 a	1.5 a	2.8 a	7.1 b	20.4 a
Tillage						
CT	0.9 a	1.9 a	1.8 a	1.2 a	8.3 a	8.9 b
NT	1.0 a	2.0 a	1.9 a	3.0 a	8.2 a	23.8 a
MT	1.0 a	0.8 b	0.9 b	2.0 a	7.5 a	15.8 a
Fertilization						
Control	0.9 a	1.5 a	1.5 a	3.6 a	8.1 a	28.8 a
90 kg/ha nitrogen	0.9 a	1.7 a	1.6 a	0.5 b	7.9 a	3.6 b

n.s.: not significant. The values in each column followed by a different letter are significantly different according to Tukey's test.

* Significant at the $P \leq 0.05$; ** Significant at the $P \leq 0.01$; *** Significant at the $P \leq 0.001$.