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No-Till and Solid Digestate Amendment Selectively Affect the Potential Denitrification Activity in Two Mediterranean Orchard Soils

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Abstract: Improved soil managements that include reduced soil disturbance and organic amendment incorporation represent valuable strategies to counteract soil degradation processes that affect Mediterranean tree cultivations. However, changes induced by these practices can promote soil N loss through denitrification. Our research aimed to investigate the short-term effects of no-tillage and organic amendment with solid anaerobic digestate on the potential denitrification in two Mediterranean orchard soils showing contrasting properties in terms of texture and pH. Denitrifying enzyme activity (DEA) and selected soil variables (available C and N, microbial biomass C, basal respiration) were monitored in olive and orange tree orchard soils over a five-month period. Our results showed that the application of both practices increased soil DEA, with dynamics that varied according to the soil type. Increased bulk density, lowered soil aeration, and a promoting effect on soil microbial community growth were the main DEA triggers under no-tillage. Conversely, addition of digestate promoted DEA by increasing readily available C and N with a shorter effect in the olive grove soil, due to greater sorption and higher microbial efficiency, and a long-lasting consequence in the orange orchard soil related to a larger release of soluble substrates and their lower microbial use efficiency.

Keywords: conservative agriculture; denitrification; no-tillage; solid anaerobic digestate; DEA

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1. Introduction

Common tree cultivations in the Mediterranean areas often have to face several degradation processes induced by inappropriate management activities and/or environmental causes, such as soil erosion, soil organic matter depletion, loss of biodiversity, a general reduction in fertility, and diffuse pollution, which lead to limited ecosystem service benefits and increased desertification [1,2]. These processes, in turn, negatively affect their production and, subsequently, farmers' income. Moreover, in these contexts, conventional managements do not help to counteract soil fertility depletion, or they even stimulate degradation [2,3]. Therefore, correct implementation of sustainable crop management practices is needed to mitigate the effects of climate change and address the expectations of European citizens achieving social, economic, and environmental sustainability [4,5]. With this aim, management practices that contemplate reduced soil disturbance and organic amendment incorporation have been successfully proposed to prevent and hinder soil degradation in tree crops [1,6]. As postulated by several authors, no-tillage can be considered an environmentally friendly soil management technique due to its positive role in reducing soil erosion, increasing carbon (C) sequestration and soil biodiversity [7,8], and enhancing soil water holding capacity, with beneficial effects on crop growth and yield [9–11]. In addition, solid digestate has raised growing interest from farmers for use as soil conditioner and partial substitute of synthetic fertilizers thanks to its organic matter content (~40%) and relative abundance of nutrients (i.e., ammonium nitrogen (N), phosphorus

(P), and potassium (K)) [12,13]. Indeed, the application of this organic matrix to the soil is capable of raising the organic pool with related beneficial effects on soil structure, microbial efficiency, and long-lasting release of soluble C and N forms available for microbial and plant nutrition [14–16]. However, both of these improved soil managements could have unforeseen negative effects related to their influence on soil properties that control specific processes such as denitrification. Denitrification represents an important process that can affect soil N balance, agronomical N use efficiency and, even more seriously, the release of N_2O in the atmosphere, thus playing a critical role in global warming, ozone layer depletion, and climate change. Biological denitrification is a process of dissimilation implemented by facultative anaerobic bacteria that use nitrate-N (NO_3^--N), instead of oxygen, as the terminal electron acceptor during respiration. According to Wrage et al. [17], denitrification is driven by specific enzymes (NO_3^--reductase (NAR), NO_2^--reductase (NIR), NO-reductase (NOR), and N_2O-reductase (N_2OR) [17,18]. Generally, it occurs under waterlogged soil conditions or in unsaturated soils within aggregates, where oxygen diffusion is reduced and the soil redox potential is low [19,20]. Availability of NO_3^--N and soluble C substrates, together with mesophilic soil conditions, are also needed [21]. No-tillage could promote denitrification by inducing changes in soil properties, such as the increase in bulk density, which could affect soil gas diffusion and water drainage (varying the incidence of soil anaerobic microsites and the percentage of pore filled by water). Altered soil organic matter stratification, in turn, could affect detritusphere processes in the soil surface and increase the availability of C substrates for microorganisms [22,23]. Application of anaerobic digestate to soil determines the release of a large amount of ammonium-N (NH_4^+-N), which can stimulate the nitrification process with an uncontrolled spread of the NO_3^--N. This, in conjunction with a large amount of soluble C substrates entering the soil, can promote microbial growth and, in turn, upset microbial respiration, thus increasing soil CO_2 emissions and the formation of anaerobic microsites [24–28]. However, the response of soil microbiota to conservative management practices, such as no-tillage or amendment with anaerobic digestate, has not always been unique and strongly changes among the soils and their principal properties [29,30].

Given these premises, our research aimed to investigate the short-term effects of two management practices (no-tillage and/or organic amendment with solid anaerobic digestate) in comparison with conventional tillage on the denitrifying activity in two Mediterranean orchard soils with different properties in terms of texture, carbonate content, and pH. To this aim, soil denitrifying enzyme activity (DEA) was measured following the application of the treatment over a five-month study period in olive and orange orchard soils. In order to assess the factors controlling DEA in the soil, total organic C (C_{org}), available C and N (C_{ext} and NO_3^--N), microbial biomass C (MBC), and basal respiration (R_{bas}) were monitored during the experimental period.

2. Materials and Methods

2.1. Solid Anaerobic Digestate

Digestate from the anaerobic digestion process of dairy cattle slurry, as the major component, mixed with various agro-industrial by-products (i.e., solid residues from olive processing plants, pruning materials, crop residues) was provided by a local medium-scale biogas producing plant (<1 MW) operating under mesophilic conditions (T ~40 °C). The resulting digestate was mechanically separated into the aqueous fraction, which was discarded, and the solid fraction, which was collected and fully characterized (Table 1) according to official analytical methods [31,32].

Table 1. Chemical properties of the solid fraction of the anaerobic digestate. Values are means ± SD (n = 3) expressed on a dry matter basis.

Variable	Value
Chemical analyses	
pH	8.77 ± 0.01
EC (dS m−1 at 25 °C)	2.14 ± 0.01
Dry matter (% fresh weight)	18.0 ± 0.49
Ash (%)	14.4 ± 0.16
Volatile solids (%)	85.6 ± 0.16
Tot-C (g kg−1)	389.6 ± 0.8
Tot-N (g kg−1)	16.02 ± 0.70
C/N	24.3 ± 1.5
NH4+-N (g kg−1)	5.59 ± 0.47
NH4+-N (% Tot-N)	34.9
NO3−-N (g kg−1)	0.034 ± 0.002
P (g kg−1)	1.24
K (g kg−1)	2.25
Tot-polyphenols (mg g−1)	1.62 ± 0.05

2.2. Study Sites

The field experiment was established during the 2016 growing season in two different agricultural sites located within the Calabria region, in Southern Italy (Figure 1).

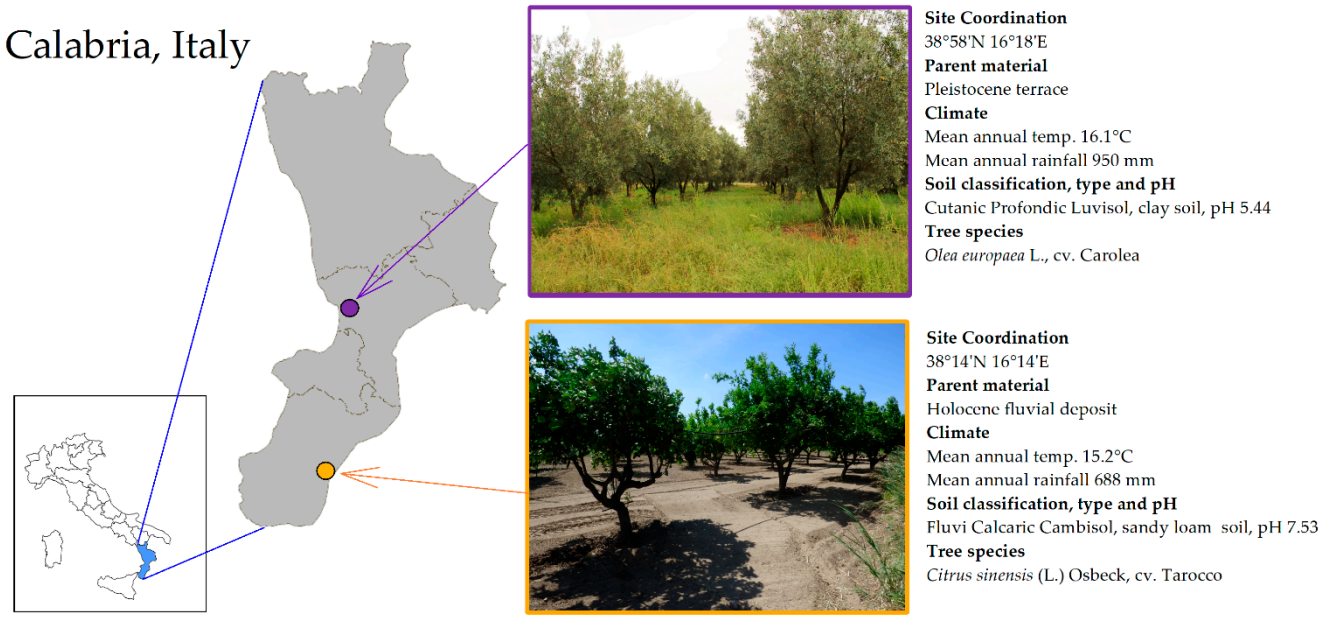

Figure 1. Overview of the two experimental sites: Lamezia Terme (olive orchard) and Locri (orange orchard). Inset text includes mean annual temperature and rainfall, crop species, site coordination, and major soil information.

One site is represented by an olive orchard (*Olea europaea* L. cv. Carolea), with a 6 × 6 m planting distance, located in the area nearby Lamezia Terme (Catanzaro, 38°58′ N, 16°18′ E, 81 m above sea level). According to the data from the weather station located nearby, mean annual rainfall and air temperature are, respectively, 1094 mm and +14.3 °C (averages over the 1985–2015 period), with mild and rainy winters and relatively warm and dry summers. The coldest month is January (lowest mean monthly temperature +9.7 °C) and the hottest one is August (mean temperature +23.0 °C). Soil thermal and moisture regimes are thermic and udic (first 150 cm), respectively [33]. The soil is classified as Typic Hapludalf fine, mixed thermic [34], or Cutanic Profondic Luvisol [35]. The soil has

evolved over ancient conoids forming a terrace plane constituted of Pleistocene sands and brown-reddish conglomerates of metamorphic origin. The slope is less than 10% facing a W exposure. Soil depth is >180 cm, and the available water-holding capacity (AWC, available moisture between the field capacity and the wilting point) equals 180 mm/m. Drainage is good, and permeability is moderately slow. The soil is an acid clayey soil (Table 2) and has been kept continuously cultivated with olive trees since the mid-1950s and since then has been periodically ploughed (till layer 0–20 cm).

Table 2. Main physical and chemical properties of tested soils from the two study sites. Values are means (±SD, n = 4) expressed on a dry matter basis.

Soil Variable	Study Site	
	Olive Orchard	Orange Orchard
Coarse sand (%)	6.6 ± 0.1	23.7 ± 0.7
Fine sand (%)	12.3 ± 0.3	34.0 ± 0.8
Coarse silt (%)	13.6 ± 0.3	17.3 ± 0.3
Fine silt (%)	22.5 ± 0.3	12.5 ± 0.3
Clay (%)	45.0 ± 0.8	12.5 ± 0.6
Texture (according to USDA)	Clay	Sandy loam
Bulk density (g cm^{-3})	1.24 ± 0.02	1.22 ± 0.14
Structural stability index (%)	73.9 ± 7.5	66.9 ± 1.1
pH_{CaCl2}	5.44 ± 0.11	7.46 ± 0.12
$EC_{1:2}$ (dS m^{-1})	0.170 ± 0.013	0.210 ± 0.087
Total $CaCO_3$ (g kg^{-1})	0	22.5 ± 3.0
Active $CaCO_3$ (g kg^{-1})	0	6.9 ± 0.1
CEC ($cmol_+$ kg^{-1})	51.9 ± 2.4	36.1 ± 1.2
C_{org} (g kg^{-1})	21.30 ± 3.24	13.74 ± 0.15
N_t (g kg^{-1})	2.03 ± 0.29	1.03 ± 0.05
C/N	10.51 ± 0.35	13.34 ± 0.66
Exchangeable NH_4^+-N (mg kg^{-1})	3.2 ± 0.2	5.1 ± 1.0
NO_3^--N (mg kg^{-1})	2.8 ± 2.0	2.2 ± 1.3
Olsen-P (mg kg^{-1})	22.9 ± 2.2	20.4 ± 2.1

The second site is represented by an orange orchard (*Citrus sinensis* (L.) Osbeck cv. Tarocco), with a 4 × 4 planting distance, located near Locri (Reggio Calabria, 38°14′ N, 16°14′ E, 12 m above sea level). Mean annual rainfall and air temperature are, respectively, 792 mm and +18.3 °C (averages over the 1988–2015 period), with mild, rainy winters and arid and warm summers. The coldest month is January (lowest mean monthly temperature +8.8 °C) and the hottest one is July (mean temperature +23.1 °C). Soil thermal and moisture regimes are thermic and xeric (first 150 cm), respectively [33]. The soil is classified as Typic Xerofluvent [34] or Fluvi Calcaric Cambisol [35]. The soil has evolved over Holocene alluvial deposits from the nearest river, Gerace. Soil depth is >180 cm, and the available water-holding capacity (AWC) equals 170 mm/m. Drainage is good, and permeability is moderately high. The soil is a slightly calcareous sandy loam soil (Table 2) and has been cultivated with orange trees for the past 30 years and conventionally tilled to the depth of 20 cm.

2.3. Experimental Design and Soil Treatments

Before the establishment of the experiment, the areas had been cropped with olive and orange trees, respectively, for about 70 and 30 years, receiving the same management and inputs; therefore, the soil was considerably uniform in terms of fertility. At each site, the experimental set up consisted of twelve field plots covering a surface of 1350 m^{-2} (75 m × 18 m) each arranged in a randomized complete block design (RCBD), with four replications, in order to compare the following three treatments: no-till (NT), where weeds were controlled by mechanical mowing, and their biomasses were left on the soil surface; conventional tillage (TILL), which consisted of an interrow harrowing (~20 cm) followed

by a slight rolling; amendment with solid anaerobic digestate at a rate of 30 t ha^{-1} digestate (DIG) in soil prepared the same as in TILL. The solid anaerobic digestate dose was established considering the dosages commonly used in agriculture and was also similar to that used by other authors in C and N mineralization field experiments using organic conditioners [14,36]. According to traditional practices, field plots were fertilized with 400 kg of a complex 20N-10P_2O_5-10K_2O chemical fertilizer supplying 80 kg N ha^{-1}, 18 kg P ha^{-1}, and 34 kg K ha^{-1}. Since the annual rainfall was markedly different between the two areas, the olive orchard was managed under rainfed conditions; whereas the orange orchard was irrigated by drip irrigation every 15 days, from June to September, in order to meet the irrigation requirements for orange trees, which are estimated at 900–1000 mm per year.

2.4. Soil Sampling

Soil samples were collected at the following stages during the 2016 growing season: immediately before (T0, early May) and then two days (T1, May), one month (T2, late June), and five months (T3, mid-September) after the beginning of the experiment. In each sampling time, four composite soil cores per treatment (each resulting from nine soil samples per plot pooled together) were collected from the Ap horizon (0–20 cm soil depth). On return to the laboratory, field moist samples were store at 4 °C before being split into two aliquots: a representative amount (500 g) was promptly (within 24 h) processed for biochemical analyses, while the remaining aliquot (500 g) was air-dried, sieved to pass through a 2 mm sieve, and then stored at room temperature before chemical characterization. Twelve composite samples were collected at each sampling time (3 treatments × 4 replicates) for each of the two study sites, thus producing an overall number of 96 soil samples (12 samples × 4 sampling times × 2 sites).

2.5. Soil Chemical and Biochemical Analysis

Soil chemical (C_{org}, C_{ext}, NO_3^--N) and biochemical variables (MBC, R_{bas}, DEA) were determined using the standard methods recommended by the Soil Science Society of America (Sparks et al. [37] and Bottomley et al. [38], respectively). Briefly, total soil organic C (C_{org}) was determined by means of the automatic elemental analyzer LECO CN628 (LECO Corporation, St. Joseph, MI, USA). The NO_3^--N concentration in 2 M KCl soil extracts (1:10, w/v) was determined calorimetrically by the Griess–Ilosvay reaction using a Flow Injection Analysis System (FIAS 400 PerkinElmer Inc., Shelton, CT, USA) coupled with an AS90 (PerkinElmer) autosampler and connected to a UV/Vis spectrophotometer Lambda 25 (PerkinElmer). Soil microbial biomass C (MBC) was determined according to the $CHCl_3$ fumigation-K_2SO_4 extraction (CFE) method [39] by using a conversion factor of K_{EC} = 0.45 [40]. K_2SO_4-extractable C determined in non-fumigated soil samples was considered as C soluble pool (C_{ext}). Soil basal respiration (R_{bas}) was determined by measuring the total CO_2 evolved during a 28 day incubation period (after 1, 4, 7, 14, 21, and 28 days of incubation) at 25 ± 1 °C and trapped in NaOH vials. Total organic C concentration in soil extracts (MBC and C_{ext}) and total CO_2-C in NaOH traps were measured by using an elemental analyzer TOC-L CSH Shimadzu (Shimadzu Corporation, Tokyo, Japan) equipped with an ASI-L autosampler unit (Shimadzu Corporation). The denitrifying enzymatic activity (DEA) was measured using the acetylene inhibition method according to the anaerobic slurry technique described by Šimek et al. [41]. Briefly, an amount of 3.2 g soil brought to 50% WHC was placed in a 20 mL vial, and then 3.2 mL of a 1 mM glucose, 1 mM KNO_3 and 1 g L^{-1} chloramphenicol aqueous solution was added. Vials were sealed with butyl rubber septa, and the headspace was sequentially flushed (four times, each evacuation and flushing lasted for 2 min) with 99.999% He to create an anaerobic environment. The incubation started when 25% (v/v) of the headspace He was replaced with pure acetylene (C_2H_2) in order to inhibit the conversion of N_2O to N_2. Vials were then horizontally shaken (70 rpm, room temperature) to evenly distribute the C_2H_2 throughout the soil slurry. Headspace gas samples (1 mL) were taken at 30 and 60 min after the addition of C_2H_2, and evolved N_2O was analyzed by a gas chromatograph (TRACE-GC, Thermo Fisher Scientific, Milano,

Italy) equipped with a ^{63}Ni electron-capture detector and an 80–100 mesh stainless-steel column packed with Porapak Q (Supelco, Bellefonte, PA, USA). Operating chromatograph conditions were as follows: injector temperature 50 °C; base and detector temperature, 300 and 350 °C, respectively; pure He (99.999%) was used as carrier gas at 5 mL min^{-1}, while pure N_2 (99.999%), directly introduced into the detector, was used as a make-up gas at 40 mL min^{-1}. Instrumental detection limit was <20 ppb. The denitrification rate was calculated as the N_2O increase between the 30 and 60 min measurements.

2.6. Statistics

Soil chemical and biochemical data, reported as mean values (n = 4), were expressed on a dry weight (dw) basis (105 °C, 24 h). Data were first tested for deviation from normality (Kolmogorov–Smirnov test) and homogeneity of within-group variances (Levene's test). Three-way analysis of variance (ANOVA) (time × soil × management) was performed to assess the effects of the treatments on the two experimental sites during the experimental period. Moreover, individually for each soil, in order to highlight the effects of treatments along the sampling times, a two-way ANOVA (time × management) analysis with repeated measures was run, and treatment means were compared by multiple pairwise comparisons of means by Tukey's HSD (honestly significant difference) test at $p < 0.05$ level of significance. Statistical analysis was performed by using SAS 9.3 software (SAS Institute, Cary, NC, USA), while all graphs were drawn by using SigmaPlot v10 software (Systat Software Inc., San Jose, CA, USA).

3. Results

Soil C_{org} concentration showed a similar trend, although with a different magnitude, in both tested soils (Trt: $p < 0.001$; Figure 2). Indeed, both soil management applied (NT and TILL) did not substantially alter the soil C_{org} level, while digestate amendment (DIG) increased it markedly, on average, by +25% (5.4 g C kg^{-1} soil) in the olive and by +12% (1.7 g C kg^{-1} soil) in the orange grove soil, respectively.

Soil C_{ext} showed a different trend in the two tested soils during the experiment (T × S × Trt; $p < 0.001$; Figure 2). NT plots showed a constant level of C_{ext} across sampling times, while both tilled treatments, with (DIG) and without (TILL) amendment, had a fluctuating trend. In particular, in conventionally tilled olive soil (TILL) soil C_{ext} immediately decreased (−21%, respect to NT) after the start of the trial (T1, 48 h), it reached the highest level in T2 (+45% more than NT and with a similar level to DIG), and then decreased to the initial pre-treatment values at the latest stage (T3). Digestate amendment increased C_{ext} (+34%, respect to NT, in T1), which reached the highest level in T2 (+59%, with respect to NT), before declining sharply in T3. On the other hand, in the orange grove soil, C_{ext} showed a slightly increasing trend from T1 to T3 in NT but with lower values compared with TILL and DIG (+36%, on average). TILL treatment had a similar C_{ext} concentration to NT at T1, while in T2 and T3 it was increased by +49% and +25%, showing a similar level retrieved in DIG treatment.

Soil NO_3^--N was affected by the interaction among all experimental factors (T × S × Trt; $p < 0.001$; Figure 2). In the olive grove, DIG increased soil NO_3^--N concentration by +518% and +186%, compared to the average of the other two treatments, at T1 and T2, respectively; whereas at the last sampling, differences among treatments were not significant. A similar trend was also observed in the orange orchard soil (DIG +424% at T1 and +360% at T2, compared to the average of NT and TILL). However, differences between treatments were observed at the last sampling time (DIG +137% at T3, compared to the average of the other two treatments). Moreover, in the orange orchard, soil management also showed a trend TILL > NT (+105%) at T2.

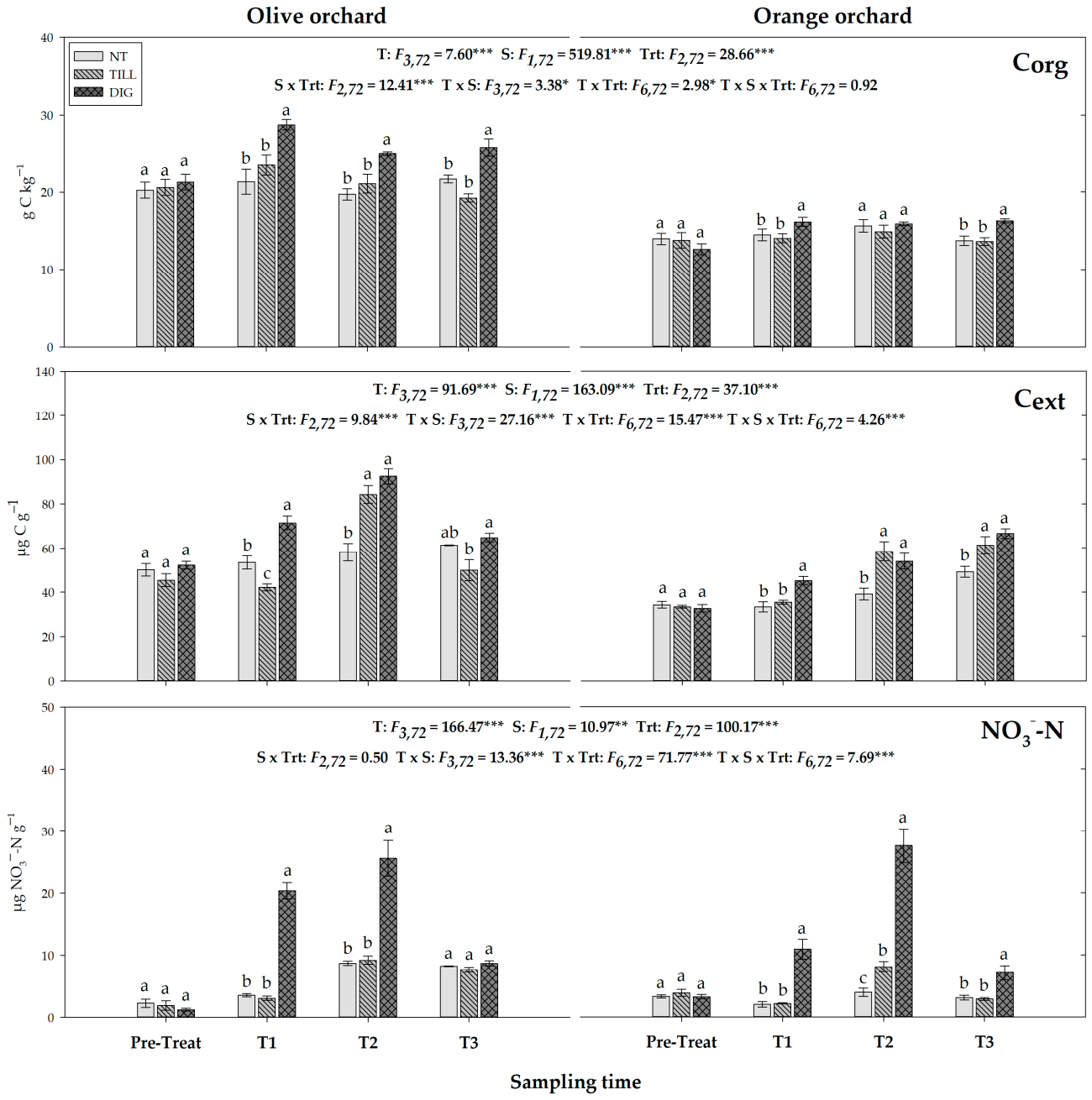

Figure 2. Changes in total soil organic C (C_{org}), K_2SO_4-extractable C (C_{ext}), and nitrate N (NO_3^--N) (mean ± SD, n = 4) in the field plots arranged in an olive and an orange orchard under different treatments—no-tillage (NT), conventional tillage (TILL), solid anaerobic digestate amendment (DIG)—at four sampling times (from May to September) during the 2016 growing season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $p < 0.05$). Significant effects due to time (T), soil type (S), soil management (Trt), and their interactions are presented as F-values, and level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was estimated by a three-way ANOVA (time × soil × management).

The MBC showed a rather contrasting trend depending on the type of the recipient soil (interaction T × S × Trt; $p < 0.01$; Figure 3).

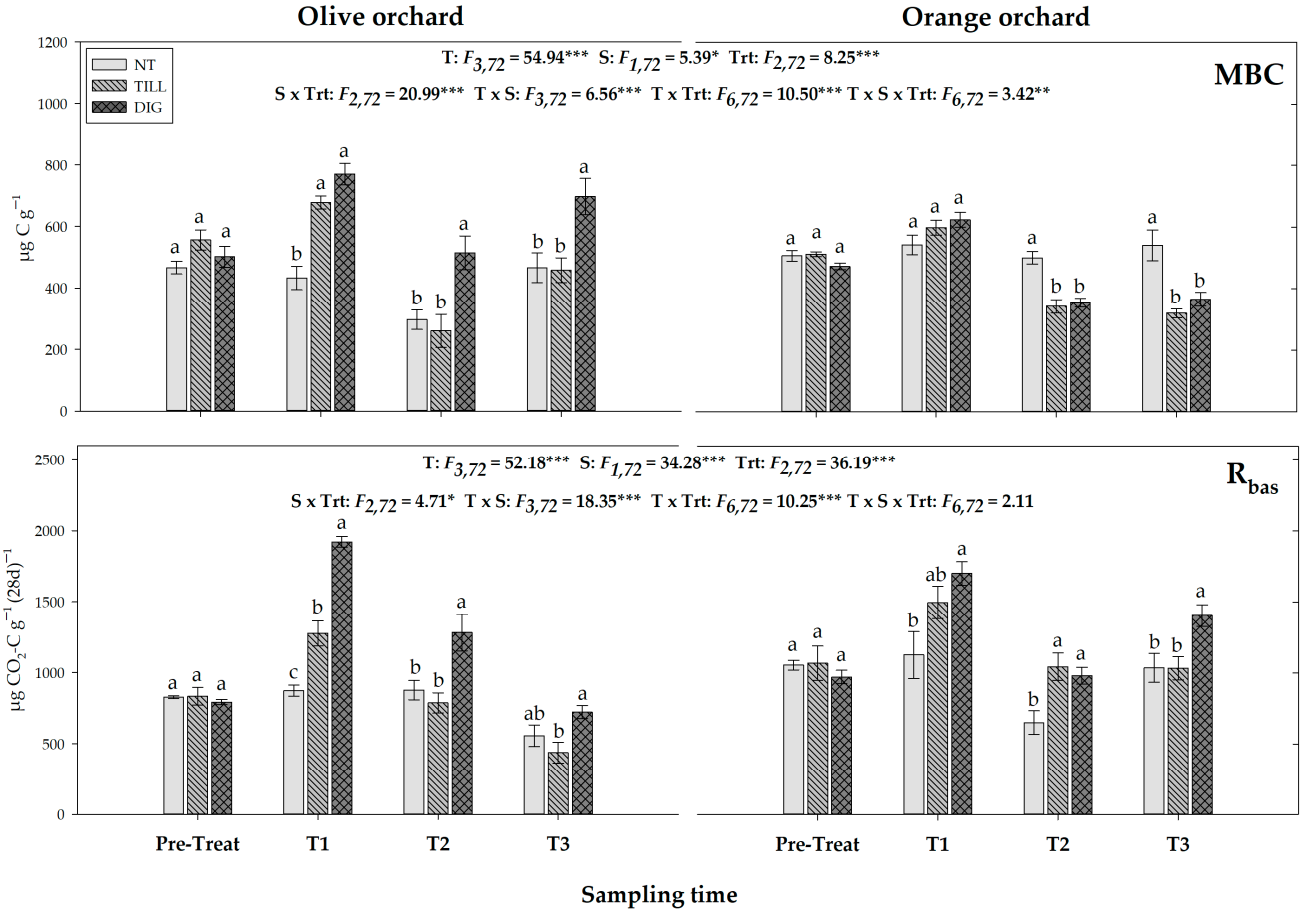

Figure 3. Changes in microbial biomass carbon (MBC) and soil basal respiration (R_{bas}) (mean ± SD, n = 4) in the field plots arranged in an olive and an orange orchard under different treatments—no-tillage (NT), conventional tillage (TILL), solid anaerobic digestate amendment (DIG)—at four sampling times (from May to September) during the 2016 growing season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $p < 0.05$). Significant effects due to time (T), soil type (S), soil management (Trt), and their interactions are presented as F-values, and level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was estimated by a three-way ANOVA (time × soil × management).

In the olive orchard, DIG treatment showed an MBC concentration higher than that of the other treatment from T1 to T3 (+66% on average, with respect to NT). On the contrary, NT treatment had a lower MBC for the entire experimental period. TILL showed a sudden increase in MBC after the treatment application (T1, +57% with respect to NT); at the later stages (T2 and T3) MBC levels were similar to NT. In the orange grove soil, the treatment effect was clearly observed only at T2 and T3. In particular, NT showed a higher MBC compared to tilled treatments (TILL and DIG), despite the digestate amendment (521 vs. 345 μg C g^{-1}, +51%, on average).

The ANOVA proved that R_{bas} was affected by the interactions S × Trt and T × Trt ($p < 0.05$; Figure 3). In the olive orchard, R_{bas} was higher in DIG than in NT and TILL (+63%, on average). Moreover, with the exception of T1 where a trend TILL > NT (TILL +46%) was observed, no significant differences between these two soil managements were found. Similarly, in the orange grove soil, the highest levels of Rbas were observed in DIG, while the lowest were retrieved in NT. Lastly, TILL treatment had a variable trend among samplings, showing an increasing R_{bas} from T1 to T2 and lower and similar values to NT at T3.

Similar to the other soil variables here monitored, DEA was affected by the interaction T × S × Trt ($p < 0.001$; Figure 4). In the olive orchard, the treatment trend was not constant across sampling times. In particular, at T1 DEA was higher in DIG than in both TILL and NT, whereas at the other stages the three treatments were well differentiated from each

other (NT > DIG > TILL). NT determined a higher DEA, by +80% at T2 and +125% at T3, compared to TILL, while the increase in the amended thesis (DIG) was more reduced (+63% in T2 and +40% at T3, compared to TILL). In the orange grove, a significant treatment effect was observed only at T2 and T3, with a unique trend, DIG (+58%) > NT (+119%) > TILL.

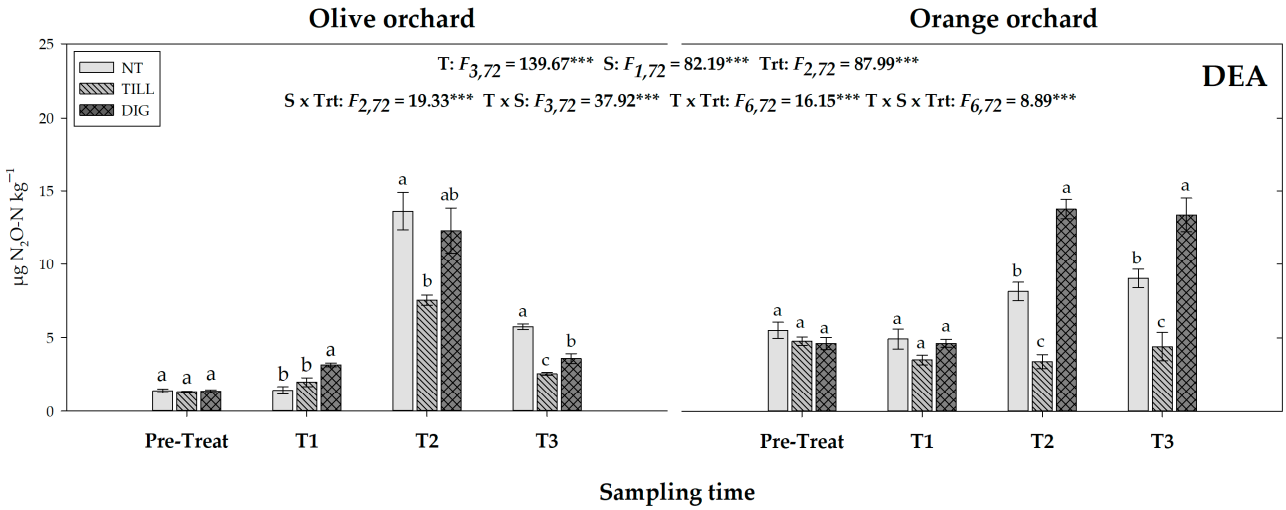

Figure 4. Changes in soil denitrifying enzymatic activity (DEA) (mean ± SD, n = 4) in the field plots arranged in an olive and an orange orchard under different treatments—no-tillage (NT), conventional tillage (TILL), solid anaerobic digestate amendment (DIG)—at four sampling times (from May to September) during the 2016 growing season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $p < 0.05$). Significant effects due to time (T), soil type (S), soil management (Trt), and their interactions are presented as F-values, and level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was estimated by a three-way ANOVA (time × soil × management).

4. Discussion

Agricultural soil managements, such as soil tillage and amendment application, could determine considerable influences on integral soil fertility in the short and long term, that affect structure, nutrients concentration, microbial community growth and activity, and the relationship between them, resulting in changes in different soil dynamics such as soil organic matter turnover, nutrient cycling, and related soil processes [1,42]. The present study was aimed to assess the effect of soil management (TILL and NT) and solid digestate amendment (DIG) on a specific edaphic process, the denitrification (and on some related chemical and biochemical variables), in two sites characterized by contrasting pedoclimatic conditions.

Denitrifying microorganisms are widely distributed in numerous taxonomic groupings; they are ubiquitous in soil and have various ecological niches [17,43]. Therefore, the redundancy of the denitrifying function among soil microorganisms leads one to consider this process as strongly controlled not by the presence or absence of specific microbes but, rather, from the ecological conditions that occur in the soil. According to Gardner and White [44] and Schipper et al. [45], by quantifying the overall denitrifying enzymatic activity in soil, DEA in-lab analysis, ensuring that the evolved N_2O is produced by active denitrifiers in situ, provides insight on soil microbiota at the time of sampling [46]. Therefore, DEA can be a reliable indicator of soil condition triggering the synthesis of denitrifying enzymes at the moment of sampling [47]. Regarding the role of denitrification in determining N_2O field emission, it is important to consider that a higher DEA may not necessarily result in a proportional increase in the N_2O emissions. Indeed, this process also depends on the total or partial conversion of N_2O to N_2, performed by the N_2OR encoded by the nosZ gene, that during the DEA assay is inhibited by C_2H_2, thus leading to N_2O accumulation [46,47].

With regard to our study, in the fine-textured olive grove soil, NT determined a significant and long-lasting increase in DEA, compared to TILL, in agreement with other studies [48,49] as also highlighted by a recent meta-analysis by Wang and Zou [50]. Moreover, since the increase in DEA did not appear to be related to NO_3^--N, MBC, or R_{bas}, and only partly with C_{ext}, we suppose that an NT-induced change in soil physical conditions altered the microbial metabolic activity (denitrification is a facultative process in many cases, e.g., nitrifier-denitrification), thus promoting the denitrification process [48]. In particular, the observed increase in bulk density due to the application of this technique (i.e., 1.46 g cm^{-3} under NT vs. 1.03 g cm^{-3} and 0.98 g cm^{-3} in TILL and DIG, respectively) could have determined a double promoting effect on soil water retention (higher under NT than in TILL and DIG, Supplementary Table S1) and on water-filled pore space, as argued by Deepagoda et al. [51] and by Wang and Zou [50] in an in-depth meta-analysis study and as observed, also, by Tellez-Rio et al. [23], Buchen-Tschiskale et al. [26], Liu et al. [52], and by Badagliacca et al. [49] in a soil with a clay texture (clay > 45%) like that of the olive grove of this experiment. Moreover, the no-till practice in the fine-textured soil (clay, olive) could have promoted beyond the increase in bulk density, as asserted by Reichert et al. [53] and Xu et al. [54], leading to limited gas diffusivity and reducing soil aeration with the formation of anoxic microsites where microbial denitrification was greatly enhanced. Conversely, DIG treatment had a limited influence on DEA along sampling times compared to NT, and our results agree with Abubaker et al. [42], Köster et al. [55], and Dietrich et al. [27] who observed an increase in denitrification activity due to the soil amendment with digestate. In particular, the increased availability of C and N substrates following digestate addition could have triggered soil microbial growth (MBC) and metabolic activity (R_{bas}) including denitrification [42,56]. In fact, although soil N concentration, especially in NO_3^--N form, represents the major substrate for denitrification, C availability greatly acts as a controlling factor of this process, providing electrons for the reaction through mineralization and substrates for microbial growth and metabolism [57–59]. Therefore, it was unsurprising that an organic matrix-like digestate, rich in NH_4^+-N and likely to be quickly transformed into NO_3^--N and light C compounds available to microorganisms, was able to determine a time-dependent variation and a sudden increase in DEA at T2, as also reported by Alburquerque et al. [60] and Askri et al. [57]. Subsequently, DEA varied over sampling time depending on the availably of N substrate, and the rapid decrease in NO_3^--N determined a drastic reduction in DEA. In other words, the rapid NH_4^+-N/NO_3^--N turnover was the main driver of the DEA fluctuations observed following the amendment. In addition, evidence suggests that after five months (last sampling date, T3) changes in the substrate availability (lower), likely related to the decomposition of more recalcitrant organic compounds, and in the C respiration efficiency (higher), capable of sustaining a larger microbial biomass (MBC) but with lower respiration (high C_{ext} but lower R_{bas}), were responsible for lowering the observed DEA. Therefore, it is possible to assume that lowering the microbially mediated CO_2 evolution/O_2 consumption ratio in soil porosity created less conducive conditions to denitrification, particularly important in fine-textured soils such as that of the olive grove.

No-tillage increased DEA also in the sandy loam soil (orange) and, contrarily to what was retrieved in the clay (olive) soil, this activity was correlated with MBC. This observation suggests that within a general increase/subsistence of soil microbial biomass, the incidence of the microorganisms capable of denitrifying, when placed in the appropriate conditions, also was higher. Soil microbial biomass larger in NT, than in TILL and DIG plots, can be due to a lesser soil disturbance and lower stressing conditions for the microbial community (i.e., soil water content; Supplementary Table S1), as argued by Badagliacca et al. [6]. On the contrary, in DIG treatment, the increase in DEA was correlated with an augmented availability of C and N substrates (namely C_{ext} and NO_3^--N, as observed in the olive grove), which prompted R_{bas} but did not determine an increase in MBC. Therefore, in the orange orchard, due to its sandy loam texture and low organic matter concentration, tillage determined a critical stressing condition (i.e., aggregate disruption, rapid soil desiccation, soil compaction, reduced pore volume) to the native microbial community that was not

overcome five months (T3) after application. As a result, this circumstance determined a low efficient use of the abundant substrates [6], with a significant increase, alternatively, in soil respiration under aerobic and denitrification under anoxic conditions, similar to those assessed by DEA. This assumption also is supported by research from An et al. [61] who observed a more effective stimulation of microbial activity (and also denitrification) after C input in a low-fertility soil (lower C_{org} and C_{ext} at T0 compared to the olive orchard) than in a high-fertility soil, probably as a consequence of the starvation of the soil microbial community [62,63]. Moreover, as postulated above, a rise in soil respiration determining a transient reduction in O_2 concentration and the development of anaerobic microsites in the soil could further stimulate the selection of microorganisms capable of denitrifying, as argued by Pezzolla et al. [64] and retrieved by several other researchers [55,57,65,66].

Soil characteristics play an important role in inducing and controlling denitrification, but detailed knowledge about the effects of the interaction between edaphic properties and management practices on this process is still limited [28,56,67]. We found that, in both tested soils, the application of solid anaerobic digestate had a similar positive effect on DEA in terms of magnitude but determined a longer-lasting influence on the sandy loam (orange) than in clay (olive) orchard soil. This evidence agrees with Velthof et al. [68] who supposed that the application of large amounts of readily available C forms affects the denitrification activity more markedly in C_{org}-poor than in C_{org}-rich soils, as also reported by Eickenscheidt et al. [24] and Badagliacca et al. [62]. Moreover, as a consequence of contrasting soil texture, in the olive orchard a higher fixation of NH_4^+-N (related also to the higher CEC) cannot be excluded, to which can be attributed the reduction in NO_3^--N availability along the samplings times; on the contrary, in the orange orchard soil with a sandy loam texture, and thus higher porosity, nitrification can be higher and continued providing substrate for soil respiration as well as for denitrification [28,69]. In the same way, in the olive grove soil compared with orange grove soil, lower C_{ext} availability and R_{bas} levels could reflect higher labile C adsorption, linked to greater clay content and adversely affecting denitrification [69,70]. Therefore, both these aspects and the different behaviors among the two tested soils affected the substrate supply for the microbes, with repercussions on magnitude and duration of DIG effects on promoting DEA. Conversely, as argued above, soil texture affecting bulk density and porosity, gas diffusivity, and water retention were the significant concurrent factors determining microbial growth and DEA dynamics in NT, at both sites. However, among the two sites, the higher effect observed in olive than in orange orchard can be ascribed to the concomitant presence of a soil microbial community more susceptible to denitrify, which could be attributed to the clay texture capable to support a more compact structure, and a high C substrate availability [48,49]. Finally, no significant effect can be attributed to the different pH among the two tested soils.

Denitrifying enzymatic activity determines a potential enzymatic process, represents the susceptibility of soil to promote denitrification, and, thus, to lose mineral N due to the emission of partially reduced forms (N_2 and N_2O). Specifically, DEA measures the overall metabolically active enzymes at the time of sampling and expresses the ability of the site (soil and imposed treatments) to trigger denitrification. Moreover, close correlations between DEA and denitrification/N_2O emission measured in the field have been observed [29,47]. In the same way, several studies have shown the existence of relationships between DEA levels and the abundance of the microbial genes involved in denitrification such as nirS, nirK, and nosZ measured in the field [29,48,49,60]. Therefore, the evidence retrieved in the present study can provide a useful and realistic representation of microbially mediated N-related processes following the application of no-tillage or digestate amendment by a synthetic assessment of their effects on the potential denitrification.

5. Conclusions

Our study showed that the application of the improved management practices could lead to increase DEA into the soils with dynamics that vary according to the soil type. In particular, with regard to no-till use, our study suggests that higher bulk density,

lower porosity, and reduced soil aeration, in the olive grove, and a promoting effect on soil microbial growth, in the orange orchard, were the main factors that determined an increase in soil DEA, respectively. Conversely, the addition of solid anaerobic digestate in both experimental sites determined a sudden increase in DEA imputable to the increase in readily available C and N. This effect was shorter in the olive grove, which could be due to progressive sorption of C and N substrates by clay in conjunction with a higher microbial efficiency, and it was a long-lasting consequence in the orange orchard that could be attributable to a higher release of C and N over time and a lower substrate use efficiency that supported a higher respiration rate and anoxic microsite formation. Therefore, as revealed by the present research, our study suggests that the implementation of conservative agricultural practices should be modulated considering soil characteristics. Moreover, it is important to specify that DEA represents a potential measurement under laboratory-controlled conditions; therefore, although it provides useful information on the denitrifying ability of the soil microbial community, the evidence provided must be validated by further field surveys in order to measure undisturbed soil N_2 and N_2O emissions; this in order to have an overall assessment of the positive and negative effects of conservative agricultural practices. Finally, considering the importance of the soil microbial community and denitrifying chain enzymes in determining the denitrification process, future research should have the objective to deepen the knowledge about the changes induced by conservative management practices on the microbial communities responsible for this process and on their specific role in its different dissimilatory reduction phases.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/soilsystems5020031/s1, Table S1. Changes in soil water content (%) expressed on dry weight basis. (mean ± SD, n = 4) in the field plots arranged in an olive and orange orchard under different treatments [no-tillage (NT), conventional tillage (TILL), solid anaerobic digestate amendment (DIG)] at four sampling times (from May to September) during the 2016 growing season.

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