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Repeated Solid Digestate Amendment Increases Denitrifying Enzyme Activity in an Acid Clayey Soil

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Abstract: The use of organic fertilizers to replace chemically synthesized fertilizers has assumed an important role in managing plant nutrition and soil fertility. The various organic matrices currently available as organic byproducts and digestates are relatively abundant and have shown promising effects in terms of plant-available nutrients. However, like mineral fertilizers, organic fertilizers must be carefully managed to avoid negative effects on the environment, especially when they are repeatedly applied over time. The aim of the present study was to assess the effect of the single (DIG) and repeated application (DIGP) of solid anaerobic digestates compared to an unamended control (CTR) on the denitrifying enzymatic activity (DEA), which is responsible for nitrous oxide emissions into the atmosphere, and some related soil properties, such as total soluble nitrogen (TSN), nitrate (NO_3^--N), extractable carbon (C_{extr}), microbial biomass carbon (MBC), and basal respiration (R_{bas}), for a period of ~3 months after application. The application of solid anaerobic digestates progressively boosts N and C concentrations in the soil, with the degree of enhancement directly correlated with the frequency of application over the sampling period. Depending on the textural properties of soils, there was a notable rise in denitrification enzyme activity (DEA), particularly during the DIGP treatment, suggesting that clay soils are highly susceptible to denitrification under suitable conditions. The results of this study recommend the careful management of soils subjected to repeated digestate amendment to prevent the occurrence of conditions conducive to denitrification and the promotion of N_2O emissions.

Keywords: denitrification; digestate legacy; amendment; organic fertilization; nitrous oxide (N_2O); conservation agriculture

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1. Introduction

Growing concerns related to the topics of global warming and climate change are prompting farmers to revise their approaches to agricultural production toward systems that can reduce greenhouse gas (GHGs) emissions. These changes are also stimulated by European and global policies, such as the farm-to-fork [1] and four per thousand [2] strategies, which aim to transition agriculture toward so-called climate-smart management [3]. Among the measures commonly used in agriculture, synthetic fertilizers are characterized as having a significant environmental impact, especially when not used wisely, posing a risk to water and air [4–6]. In fact, fertilizers are among the main contributors to processes such as the eutrophication of surface water and emission of harmful gases into the atmosphere. In addition, their emission footprint related to production and transportation should not be underestimated [7]. For the reasons stated above, their consumption is being replaced by the use of organic fertilizers with the aim of increasing the integral fertility of soils and limiting the environmental impact of plant nutrition management. Numerous organic fertilizers have been discovered or rediscovered in recent times, such as manure [8–10], compost [11,12], and digestates [13–15]. They are characterized as

supplying a good amount of both stable and labile organic matter and nutrients such as nitrogen and phosphorus in both organic and mineral forms that are readily available to crops [16–18]. However, the use of these organic materials could have several side effects related to their release of huge amounts of soluble organic compounds and mineral forms, especially nitrogenous ones [9,12,19–21]. Indeed, for example, phenomena such as the priming of soil organic matter degradation processes, the eutrophication of surface waters, and the uncontrolled release of GHGs can offset the benefits intended from the use of these matrices or, even, be worse for the environment than mineral fertilizers [22–28]. Therefore, a good understanding of the behavior of organic fertilizers is of paramount importance for their careful, agroecosystem-friendly use. Among the aforementioned matrices, digestates are increasingly being used in agriculture, coping with the increase in the number of anaerobic digestion plants, and farmers are using them widely in herbaceous and tree crop management [29,30]. Given the bulky nature of this by-product, which entails high costs for transport to the spreading sites, its use often becomes recurrent in areas in close proximity to digestion plants, making these areas potentially risky for the environment. In this regard, if several studies were published after one digestate application, little published data would be available on the accumulated residual effects on soil properties following repeated application [31]. The objective of the present study was to evaluate the short-term (within three months) effects of the repeated application of solid digestate on the denitrifying enzymatic activity (DEA) of the soil, which controls nitrous oxide (N_2O) emissions into the atmosphere, as well as on some related chemical and biochemical properties, such as total soluble nitrogen (TSN), nitrate (NO_3^--N), extractable carbon (C_{extr}), and microbial biomass carbon contents (MBC) and basal respiration (R_{bas}).

2. Materials and Methods

2.1. Experimental Site

The field experiment was established under rainfed conditions during the 2017/18 growing season in an olive orchard (*Olea europaea* L. cv. Carolea) in the Calabria region (38°58′ N, 16°18′ E, 81 m a.s.l., Lamezia Terme, Catanzaro, Italy) (Figure 1).

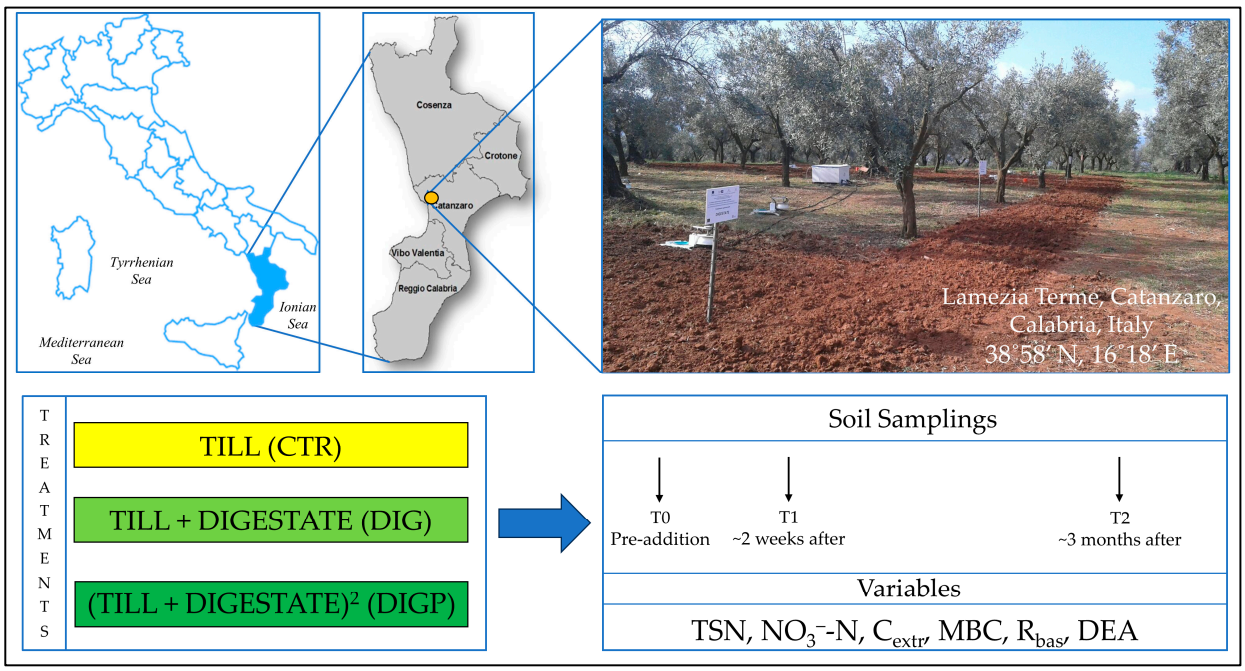

Figure 1. An overview of the experimental site (above) and a description of the experimental design (below). Specifically, these elements are stated at the bottom left of the tested treatments: TILL (CTR) is in yellow, TILL + DIGESTATE (DIG) is in light green and (TILL + DIGESTATE)^2 (DIGP) is in dark green; at the bottom right, the soil sampling epochs and the measured variables are stated.

The local climate is mild and rainy in winter and warm and dry in summer, with a mean annual rainfall of 1094 mm and a mean air temperature of +14.3 °C (1985–2015 average). Data related to the weather condition during the experiment are presented in the Figure S1. The soil is acid clayey in nature, classified according to USDA as a Typic Hapludalf fine group soil and mixed thermic in nature [32], as well as a Cutanic Profondic Luvisol soil according to IUSS [33]. The main soil properties were as follows: 18.9% sand, 36.1% silt, 45.0% clay, clay texture (USDA), pH 5.44 (1:2.5 H_2O), EC 0.170 dS m^{-1}, CEC 51.9 $cmol_+$ kg^{-1}, total carbonates 0.0 g kg^{-1}, total organic C 21.30 g kg^{-1}, total N 2.03 g kg^{-1}, and Olsen-P 22.9 mg kg^{-1} (Table S1). The field had been continuously cultivated since the mid-1950s, with olive trees spaced at a distance of 6 × 6 m and periodically tilled. Further information regarding the soil is available in Badagliacca et al. [34,35].

2.2. Solid Anaerobic Digestate

An anaerobic continuous mesophilic biogas plant, situated near the experimental field, produced the solid digestate applied in the present experiment. This digestate was derived from a mixture of husbandry effluents (sourced from dairy cows and poultry farming), crop residues (pruning materials and cereal straws), and waste by-products from the agri-food industry (including citrus pomace, olive mill wastewater, and dairy wastewater). The output from the digester was divided in two fractions: the liquid, which was discarded, and the solid, which was utilized in the present experiment. The key characteristics of the solid digestate were a dry matter content of 18.0%, an ash content of 14.4%, a pH of 8.77, an electrical conductivity (EC) of 2.14 dS m^{-1}, a total carbon (C) content of 39.0%, a total nitrogen (N) content of 1.6%, an NH_4^+-N content of 5.59 g kg^{-1}, and an NO_3^--N content of 0.034 g kg^{-1}. For a more detailed description of the solid anaerobic digestate used, refer to Pathan et al. [36] and Badagliacca et al. [34].

2.3. Experimental Design and Soil Management

The trial commenced at the end of March 2017 in field plots measuring 20 m × 12 m each, arranged in a randomized complete block design (RCBD) with four replications (Figure S2). Three different treatments were evaluated, including the following ones: (1) an unamended control (CTR), subjected to inter-row harrowing at a depth of approximately 20 cm; (2) the application of solid digestate (DIG) at a rate of 30 t ha^{-1} (consistent with the common dose used by local farmers and in accordance with Barra Caracciolo et al. [37] and Badagliacca et al. [34]), which was incorporated into the soil through harrowing; (3) repeated solid digestate application (DIGP), where the digestate was applied at the same rate and using the procedure employed in DIG for two consecutive years (the previous cropping season 2016/17, i.e., 8 April 2016, and the experimental year 2017/18, i.e., April 2017) (Figure 1 and Figure S2).

2.4. Soil Sampling

During the 2017/18 olive growing season, soil samples were gathered at three different times: before (T0, end of March 2017), approximately two weeks after (T1, early April 2017), and around three months after (T2, late June 2017) the implementation of the treatments. Within each experimental plot, nine soil samples were collected from the 0–20 cm soil layer in the inter-row space between the plants. These samples were then combined to create a single representative sample per replication. Consequently, four composite samples were obtained per treatment, totaling twelve samples at each sampling time and thirty-six samples in total for the whole experiment.

2.5. Soil Chemical and Biochemical Variables Determination

Upon reaching the laboratory, the samples were divided into two sub-samples: one portion of moist field soil underwent processing within 24 h for biochemical analysis, while the remaining section was air-dried, sieved using a 2-millimeter sieve, and stored. The concentration of soil NO_3^--N was determined by extracting samples with 2 mol dm^{-3} KCl

(1:10, *w*/*v*) and analyzing the extracts using the Berthelot reaction using a Flow Injection Analysis System (FIAS 400, PerkinElmer, Inc., Shelton, CT, USA). Total soluble N (TSN) in 2 mol dm^{-3} KCl extracts was determined using an elemental analyzer TOC-LCSH (Shimadzu Corporation, Tokyo, Japan). Microbial biomass C (MBC) was measured using the chloroform fumigation–extraction method [38], with C in soil 0.5 mol dm^{-3} K_2SO_4 extracts quantified using the Shimadzu TOC-LCSH elemental analyzer. Organic C in non-fumigated soil extracts served as proxies for available C (C_{extr}) [39]. Soil basal respiration (R_{bas}) was measured by accounting for the total CO_2-C emissions during a 28-day incubation period, following the method outlined by Öhlinger [40] and using the Shimadzu TOC-LCSH elemental analyzer. Denitrifying enzymatic activity (DEA) was assessed using the acetylene inhibition method, as per the anaerobic slurry technique described by Šimek et al. [41] and Monti et al. [25]. In brief, soil samples at 50% water holding capacity (WHC) were incubated with a solution of glucose, KNO_3, and chloramphenicol under anaerobic conditions, replacing part of the headspace (25% *v*/*v*) with acetylene (C_2H_2) to inhibit the conversion of N_2O into N_2. Gas samples from the headspace were collected at 30 and 60 min after the addition of C_2H_2, and the evolved N_2O was analyzed using a gas chromatograph (TRACE-GC, Thermo Fisher Scientific, Milano, Italy) equipped with a ^{63}Ni electron-capture detector. The denitrification rate was calculated based on the N_2O increase between the 30- and 60-minute measurements.

2.6. Statistics

Statistical analyses were conducted using R within the R Studio environment (Version 2023.09.1 + 494, RStudio, Inc., Boston, MA, USA) [42]. A two-way analysis of variance (ANOVA) approach with repeated measures (Treatment × Time) was employed to evaluate the impacts of treatments, sampling time, and their interaction on the measured soil variables. For pairwise multiple comparisons of treatment means at each sampling time, Tukey's HSD (Honestly Significant Difference) test at a significance level of $p < 0.05$ was utilized. To explore the relationships between the investigated soil variables, Pearson's correlation coefficients were computed using the "cor" command, and the corresponding results were visually represented using the Corrplot library.

3. Results and Discussion

The application of agricultural solid digestates in Mediterranean cropping systems may represent a valuable tool for improving soil fertility, replacing mineral fertilizers, and globally reducing the carbon footprint of food production to encourage mitigation and adaptation from a climate change perspective [7,18,43]. However, the use of organic fertilizer and amendments, like synthetic chemical fertilizers, must be judicious in order to achieve the efficient utilization and uptake of their nutrients and avoid harmful dispersion into the environment in different forms and due to different processes, like leaching, volatilization, and denitrification. With this in mind, it is necessary to take into account the significant effects on the physical, chemical, and biochemical soil properties that derive from their use [19,44,45].

Treatments, sampling times, and their interactions significantly affected all the soil chemical properties investigated (*p*-values = Trt: < 0.0001, T: < 0.0001, Trt × T: < 0.05; Tables 1 and 2). The previous amendment increased TSN values in DIGP already at T0 sampling by +51% compared with CTR and DIG. Then, the second incorporation of solid anaerobic digestates into the soil further increased TSN in both the amended treatments (DIG and DIGP) compared to CTR by +155% at T1 and +121% at T2 for DIG and by +265% at T1 and +191% at T2 for DIGP, respectively (Table 1). Similarly, the soil NO_3^--N increased due to the amendment reaching the highest values at T2 with concentrations of 18.6 mg kg^{-1} for DIG and 26.9 mg kg^{-1} for DIGP treatment. This difference between DIG and DIGP treatments can be derived from the NO_3^--N concentration at the time of the amendment (T0) which was about 2.3 times higher in DIGP plots than in CTR and DIG plots (Table 1).

Table 1. Effects of CTR, DIG, and DIGP treatments on soil TSN and NO_3^--N concentrations (mean, $n = 4$) at T0 (before treatment application), T1 (~2 weeks after treatment application), and T2 (~3 months after treatment application) sampling epochs. Different letters, within each sampling epoch, indicate significant differences between treatments (Tukey's HSD test at $p < 0.05$).

	TSN [mg N kg^{-1}]			**NO_3^--N [mg N kg^{-1}]**		
	CTR	DIG	DIGP	CTR	DIG	DIGP
T0	31.0 b	26.8 b	43.8 a	2.84 b	2.70 b	5.05 a
T1	39.2 c	73.5 b	105.3 a	2.86 c	4.62 b	11.69 a
T2	28.6 c	63.7 b	83.9 a	4.11 c	18.56 b	26.9 a
			p-values			
Trt		<0.0001			<0.0001	
Time		<0.0001			<0.0001	
Trt × Time		<0.0001			<0.0001	

Compared to the control plots, the application of solid digestates increased TSN and NO_3^--N as a result of the high nitrogen content in this matrix in various organic and mineral forms, such as NH_4^+-N and NO_3^--N, according to Eickenscheidt et al. [46], Martin et al. [47], Cucina et al. [48], and Slepetiene et al. [15]. The increases in the concentration of both analyzed nitrogen forms over time resulted from the decomposition of organic matter and its mineralization, with conversion first to the NH_4^+-N form and then to the NO_3^--N form caused by the soil microbial community, with effects observed even one year after application, as retrieved via the DIGP treatment. This evidence confirms the long-lasting release of nutrients from solid digestates, as observed by Badagliacca et al. [35] and Walsh et al. [49].

The concentration of C_{extr} in the soil at T0 was significantly higher (+59%) in the DIGP plots than in the CTR and DIGP plots not already amended. The amendment with solid digestates had a positive and progressive effect on this variable over the sampling time that was proportional to the number of additions. In particular, at T1, the digestates increased C_{extr} concentrations by +82% in DIG and +153% in DIGP with respect to CTR; this effect was even higher in T2, with increments equal to +84% in DIG and +176% in DIGP compared to CTR (Table 1). As observed for the other chemical variables, microbial biomass C at T0 was higher in DIGP (+56%) than in the other treatments (CTR and DIG). After the application of treatments, MBC increased in the amended treatments according to the trend CTR < DIG < DIGP, with increments, respectively, for DIG and DIGP, compared to CTR, of +54% and +123% in T1 and +47% and +122% in T2, where the highest values were recorded as being equal to 893.4 mg C kg^{-1} (Table 2).

As a result, the increase in soluble N and C substrates boosted the soil microbial community, as highlighted by MBC concentration values, in agreement with what has been observed in other studies (i.e., [37,50,51]). In turn, the growth of the microbial community promoted the decomposition processes of organic matter and the release of additional nutrients in support of microbial growth. Also, for C_{extr} and MBC, the effect of digestate use was long-lasting, resulting in a cumulative effect over time and across applications.

The R_{bas} behavior across the sampling periods closely mirrored the pattern observed in microbial biomass C. At T0, the DIGP plots, on average, showed higher values (+88%) than CTR and DIG. At T1, amendment increased soil basal respiration according to this trend: CTR < DIG (+277%) < DIGP (+57%), reaching the highest values equal to 837.6 for DIG and 1313.9 µg CO_2-C g^{-1}(28d)$^{-1}$ for DIGP. Later, in T2 R_{bas}, the values experienced a reduction but continued to express the same pattern between treatments, with +306% for DIG and +625% for DIGP compared to the CTR treatment (Table 2). Thus, amendment with solid digestates not only increased the size of the soil microbial community but also increased its metabolic activity, as represented by their respiration. In particular, with the increase in the size of the microbial community, basal respiration increased to a greater

extent, indicating a lower efficiency in terms of the utilization of the available substrates by the soil microbiota [52,53]. However, this phenomenon seems to have attenuated in the last sampling period, where lower R_{bas} values were observed, suggesting the beginning of the use of more complex organic matter and/or the increased C use efficiency of the soil microbial community. However, analyses show that although the soil has a high clay content (45%), the high presence of mineral colloids is not sufficient to protect such a high amount of organic matter from microbial attack.

Table 2. Effects of CTR, DIG, and DIGP treatments on soil C_{extr}, MBC, and R_{bas} values (mean, $n = 4$) at T0 (before repeated digestate application), T1 (~2 weeks after digestate application), and T2 (~3 months after digestate application) sampling epochs. Different letters, within each sampling epoch, indicate significant differences between treatments (Tukey's HSD test at $p < 0.05$).

	C_{extr} [mg C kg^{-1}]			**MBC [mg C kg^{-1}]**			**R_{bas} [mg CO_2-C kg^{-1} $(28d)^{-1}$]**		
	CTR	DIG	DIGP	CTR	DIG	DIGP	CTR	DIG	DIGP
T0	104.1 b	81.6 b	147.9 a	236.7 b	185.5 b	329.0 a	157.2 b	157.7 b	296.0 a
T1	130.3 c	237.1 b	329.7 a	296.1 c	455.8 b	659.5 a	222.0 c	837.6 b	1313.9 a
T2	168.2 c	309.6 b	464.7 a	403.1 c	594.2 b	893.4 a	139.0 c	563.8 b	1007.4 a
					p-values				
Trt		<0.0001			<0.0001			<0.0001	
Time		<0.0001			<0.0001			<0.0001	
Trt × Time		<0.0001			<0.0001			<0.0001	

Soil denitrification is an alternative respiration process that permits the denitrifying microorganisms to use N oxides, as an alternative to O_2, as an electron acceptor in the process of energy transfer and the generation of ATP [54,55]. This process is controlled by a series of enzymes that control specific steps in the denitrification chain present in numerous microorganisms and make denitrification a redundant function in the soil [56]; therefore, changes in the enzymatic activity in response to different treatments can represent an effective approach to study this process in soil. DEA is a laboratory analysis method that quantifies the potential cumulative and overall activity of the denitrifying enzymes arising from the soil conditions at the sampling moment [57,58]. Moreover, it is important to take into account the fact that the incubation under C_2H_2, a fundamental step involved in this enzyme assay, inhibits the conversion of N_2O into N_2, permitting us to assess the total potential gaseous losses from the soil due to denitrification, both in the form of N_2O and N_2, and, thus, their environmental and economic impacts [59–61]. The amendment with digestates in the previous cropping year led to higher DEA values at T0 in DIGP (+50%) than the other treatments. Then, after digestate application in T1, compared to the CTR treatment, DIG increased DEA values by +24%, while DIGP values were increased by 107%. In T2, the amendment effect was greater, with larger differences between treatments (+77% for DIG and +176% for DIGP) (Figure 2).

During the experiment, the field data related to the control plots showed a significant effect on the climatic trend caused by the tillage operation. Indeed, during the observation period, a slight increase in NO_3^--N and C_{extr} contents promoted the increase in MBC and DEA as a consequence of the ordinary processes of the mineralization of organic matter in the soil.

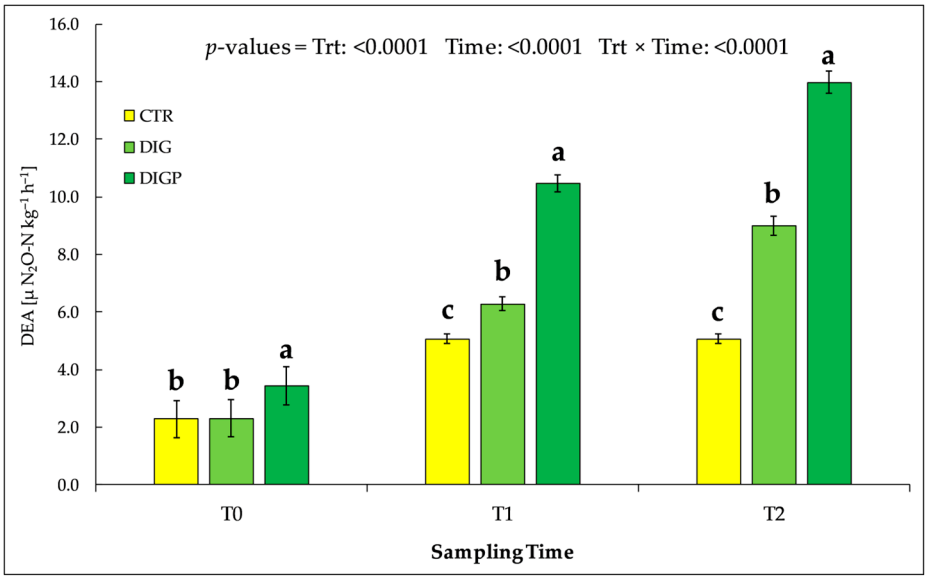

Figure 2. Effects of CTR, DIG, and DIGP treatments on soil DEA values (mean, $n = 4$) at T0 (before repeated digestate application), T1 (~2 weeks after repeated digestate application), and T2 (~3 months after repeated digestate application) sampling epochs. Different letters, within each sampling epoch, indicate significant differences between treatments (Tukey's HSD test at $p < 0.05$).

As outlined by Pearson's correlation analysis, all the soil variables investigated showed significant correlations ($p < 0.05$) with each other (Figure 3). With regard to the DEA, the strongest correlations were observed with NO_3^--N ($r = 0.92$), C_{extr} ($r = 0.93$), and MBC ($r = 0.93$).

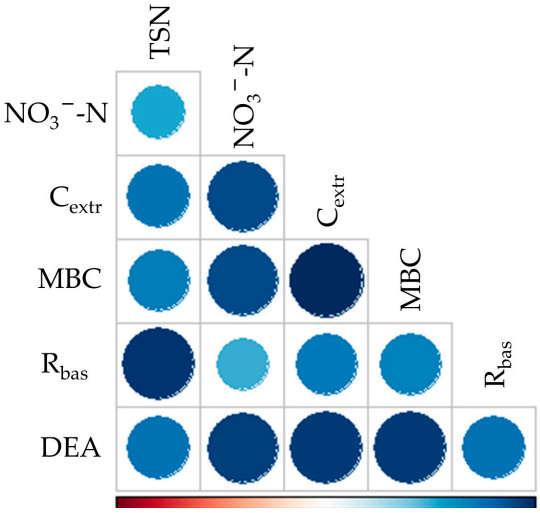

Figure 3. Pairwise Pearson's correlation analysis. The circle indicates the significant correlation between the considered variables ($p < 0.05$) described in the M&M section. Blue and red colors indicate positive and negative correlations, respectively, while the circle size reflects the intensity of the correlation (r-value).

The increase in soil denitrification activity after the application of digestates is in agreement with what has been observed by other authors [19,62,63]. As highlighted by Pearson's correlations, the increase in DEA is linked to the augmented N and C substrate, which positively affected the MBC and, consequentially, aerobic (R_{bas}) and anaerobic (DEA) metabolisms [19,64–68]. In particular, with regard to denitrification, if NO_3^--N represents the principal input, the C substrates are also fundamental inputs as substrates for microbial growth and electron providers [67–69]. Therefore, it is not surprising that the use of an

organic matrix such as digestates, rich in degraded C substrates derived from anaerobic digestion and with an abundant content of NH_4^+-N, which can be rapidly transformed into nitrate, results in a strong increase in the DEA [25,70]. The N and C substrates' availability, thus, determined the DEA trend along the sampling time and the differences between the amended treatments (DIG vs. DIGP). Indeed, the higher availability of substrates in DIGP, related to the legacy effect of the previous year's application, resulted in higher DEA values than DIG. Then, it is necessary to take into account the fact that the triggered microbial biomass and respiration conditions, resulting from the solid digestate application, can determine a reduction in the O_2 concentration in the soil and, thus, further stimulate the selection of denitrifying microorganisms [68,71,72]. This circumstance in the studied soil could be exacerbated by its clayey texture, which leads to a lower gas diffusivity and a higher incidence of anoxic microsites [73,74].

As shown in the present study and according to other authors, as stated above, the organic fertilization with digestates promotes denitrification in soils and increases $N_2 + N_2O$ emissions. Although this information, in terms of its absolute values, may worry and discourage the use of this organic matrix by farmers, it should be interpreted in a relative way by comparing it to the effects of applying other type of fertilizers, especially synthetic ones. In this regard, Tatti et al. [75] observed lower denitrification in soils fertilized with an organic fertilizer than with mineral N. The same outcome was retrieved by Vallejo et al. [76] and Tao et al. [77] in a comparison between different organic matrices and fertilization with urea and NPK. Other studies [78,79] found similar emissions between the two types of fertilizers. Finally, a meta-analysis conducted by Wang et al. [80], considering 353 different studies, identified lower denitrification after organic fertilization than mineral fertilization, attributable to the lower and slower release of NO_3^--N into the soil compared to the more sudden effect of chemical fertilizers. A more complete evaluation of soil amendment with solid digestates must also consider the substantial benefits that it produces in terms of improving the chemical, biochemical, and microbiological parameters of the soil and its integral fertility compared to chemical fertilizers, even with the same denitrifying activity.

4. Conclusions

The transition of agriculture toward a more environmentally friendly management system that prioritizes the circular economy and the reuse of resources is a topical issue for overcoming current and future challenges. The use of digestates is part of these strategies for sustainable crop nutrition and food production. However, the studies related to the application of this matrix often are short and do not consider long-term effects or those derived from repeated applications. In response, the present study assessed the effects of repeated applications of solid anaerobic digestate on DEA and some related soil properties. Based on the results retrieved, the application of solid anaerobic digestate increases both N and C pools in the soil during the sampling period, both due to matrix decomposition and in proportion to the number of applications. This consequently led to an increase in the size of the microbial community and its metabolic activity under both aerobic and anaerobic conditions. In particular, the use of digestates caused a significant increase in DEA, with a greater effect in DIGP treatment indicating a greater susceptibility to denitrification when placed under conditions suitable for this process (anoxia or partial anoxia), such as in fine textured soils. Finally, the results obtained from this enzyme assay should be complemented by a field measurement campaign assessing N and N_2O emissions in order to further study the denitrification process under field soil and climate conditions.

Supplementary Materials: The following supporting information can be downloaded via this link: https://www.mdpi.com/article/10.3390/soilsystems8010014/s1, Figure S1: Daily rainfall (mm), mean temperatures (°C) at the experimental site during the growing season; Figure S2: Layout of the field experiment; Table S1: Main physical and chemical properties of the olive orchard soil (0–20 cm soil layer). References [81–89] are cited in the Supplementary Materials.

Author Contributions: Conceptualization, M.M. and A.G.; methodology, G.B. and E.L.P.; investigation, G.B.; data curation, G.B. and E.L.P.; writing—original draft preparation, G.B. and M.M.; writing—review and editing, G.B., M.M., E.L.P. and A.G.; supervision, A.G.; project administration, M.M. and A.G.; funding acquisition, M.M. and A.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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