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Digestate Not Only Affects Nutrient Availability but Also Soil Quality Indicators

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Abstract: Digestate contains many essential nutrients for crops, including nitrogen (N) and phosphorus (P), and it can alter the biogeochemical cycle of nutrients and soil functionality. This work aimed to assess the fertilizing effects of digestate on chemical and biological soil properties in a field experiment in eastern Portugal with two horticultural crops involving nine treatments: control without fertilization; mineral N fertilization with 85 kg ha^{-1}; fertilization with digestate (DG) with increasing N rates (85, 170, 255, or 340 kg N ha^{-1}); and fertilization with different combinations of digestate plus mineral N (DG at 85 or 170 kg N plus 60 kg mineral N ha^{-1} or DG at 170 kg N plus 25 kg mineral N ha^{-1}). In addition to N, digestate supplied significant amounts of P, Ca, K, and Mg and significantly increased soil Olsen P, mineral N, and organic C. At high doses, it decreased phosphatase and β-glucosidase activities, as well as fungi and bacterial biomass, compared to the control or mineral N fertilization, and it also negatively affected soil P and C cycling capacity and microbial biomass. The organic to total N ratio and the N to P ratio in digestate are crucial properties for evaluating its agronomic management as fertilizer.

Keywords: phosphorus; nitrogen; enzymatic activity; organic amendment; soil microbial biomass

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1. Introduction

Facing the needs of a growing population requires increasing use of nitrogen (N) and phosphorus (P) fertilizers, particularly in developing countries, where crop yields are constrained by low fertilizer rates [1]. However, N and P can be considered non-renewable resources; N fertilizer production relies on high energy consumption, and P fertilizer production depends on mine resources, whose production is expected to peak in the present century [2,3]. Agricultural production in many regions of the world, such as South America and Europe, is highly dependent on imported P [4]. Thus, N and P resources in agriculture may represent a major constraint on world food security in the near future. The P crisis in 2008 [5,6] and the fertilizer price crisis in 2022 are evidence of the volatility of the global fertilizer market and an expected price rise in the near future. Thus, reducing the amount of mineral N and P fertilizers applied while avoiding deficiencies in both nutrients is currently one of the main challenges in crop management to ensure more sustainable agricultural production and global food security [7,8]. Consequently, recycling any nutrient source to replace mineral fertilizers has become crucial for the sustainability of agricultural systems [9]. The by-product of the production of biogas with

organic agricultural residues, the so-called anaerobic digestates, can be used as fertilizer [10]. However, adequate management of digestates is required to reduce negative agricultural and environmental impacts and to fully meet a circular economy approach. Currently, many studies have focused on the effects of digestates on crop and vegetable yields and quality [10–13]. Since digestates are usually used as fertilizers, they may contribute to the sustainable use of N and P resources in agriculture. However, the fertilizer value of digestates on soil is affected by the soil nutrient status, nutrient requirements of the crops, soil organic carbon (SOC), and the potential negative effects on crops due to phytotoxic compounds [14]. These potential negative effects can be decreased through composting, but this implies an additional cost [15]. Soil enzymatic activities are an important indicator of microbial activity, which is frequently altered by applications of fertilizers or organic amendments [16,17]. Digestate application contributes to increased soil organic C content. However, it may also promote the mineralization of native SOC through increased microbial activity ascribed to a "priming effect" [18,19]. This may have an impact on extracellular enzyme activities [19–23]. Increased hydrolytic enzyme activity resulting from organic amendments, such as phosphatases, may increase the mineralization of nutrients in organic form, enhancing nutrient cycling applied to soil as organic wastes or by-products [24].

In this context, to improve soil fertility and quality with digestate application, deeper knowledge about the effects of digestate on the availability and dynamics of nutrients and on the intrinsic nutrient cycling capacity of soil is needed. Previous research has shown that the combined use of organic and mineral fertilizers can maintain soil fertility while enhancing its functionality by increasing microbial biomass and activity [16,25]. This combined use of different nutrient sources contributes to a decrease in the use of non-renewable resources in agriculture. Therefore, the integrated management of nutrients and organic C by combining mineral and organic fertilizers is a recommended practice for more sustainable management of soil fertility [26]. In addition, the joint application of digestates with mineral fertilizer can reduce P adsorption and precipitation, thus increasing the recovery of applied P by crops [27,28]. Furthermore, this joint application of digestate and N fertilizer can, in the short term, help overcome the immobilization of N in the soil due to the temporary immobilization of N by soil microorganisms in treatments with digestates.

All of this reveals the need for an integrated approach to the study of the effect of digestates on soil fertility and quality, with particular emphasis on their effects on N and P available pools and on the nutrient cycling capacity in soils. Thus, the present study investigated the effect of digestate application, alone or in combination with mineral fertilizers at different rates, on soil properties and functionality, such as soil pH, N and P availability, enzymatic activities, total microbial biomass (TMB), and microbial community. These effects were evaluated in a field experiment with two consecutive crops (lettuce and kale).

2. Materials and Methods

The study was carried out in an experimental field of the Castelo Branco Polytechnic Institute of Castelo Branco, eastern Portugal (39.823655, −7.451606). The field soil was an Inceptisol [29]. The main physiochemical properties are presented in Table 1. At the experimental site, average temperatures ranged from 7.1–24.6 °C, with a mean annual value of 15 °C. The annual precipitation was 735 mm, with very dry summers [30]. The climate conditions during both experiments are shown in Figure 1.

Table 1. Soil properties.

pH	EC	SOM	C/N	CEC	Ca^{2+}	Mg^{2+}	Na^{2+}	K^{+}	Total N	Olsen P	Pi	Po
	dS m^{-1}	%				$cmol_{(+)}$ kg^{-1}			g kg^{-1}		mg kg^{-1}	
6.4	0.10	5.4	8.3	36.9	7.30	0.90	0.02	1.37	2.43	149	663	373

± sampling in February 2020. SOM, soil organic matter; CEC, cation exchange capacity; Pi, inorganic phosphorus; Po, organic phosphorus.

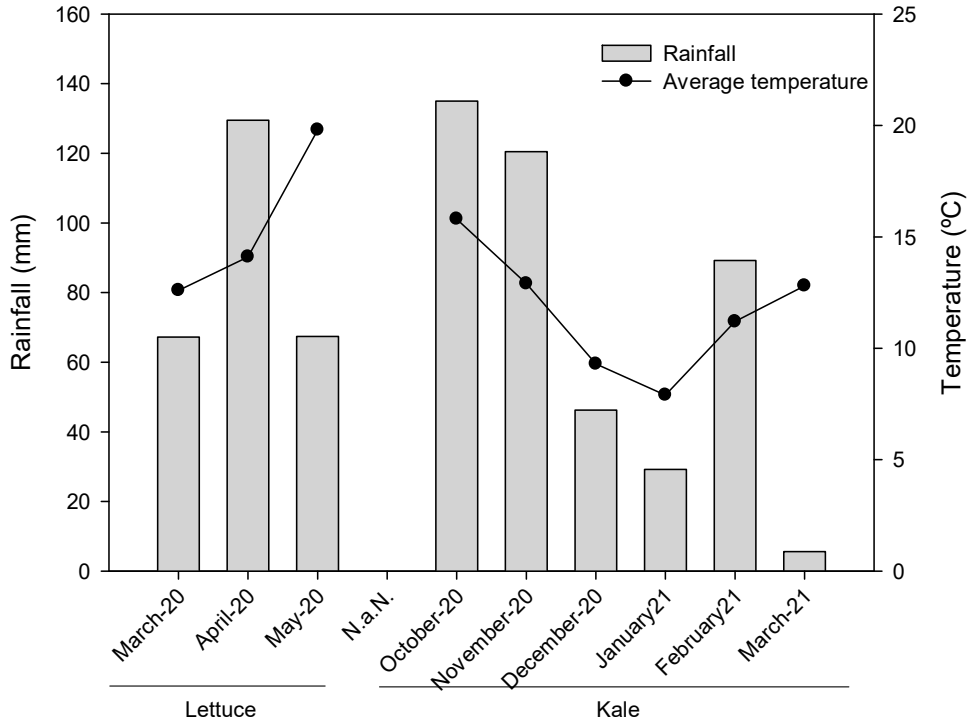

Figure 1. Climate conditions during the experiments.

2.1. Experimental Design

An experiment with a fully randomized design and four replications was performed. Each replication corresponded to a plot of 1.08 m^2 (1.20 × 0.9 m). The experiment was the same as reported by [31], and it involved nine treatments (Table 2): (i) no fertilization (control); (ii) mineral N fertilization at a rate of 85 kg ha^{-1} (Nm85) split in three applications (35 kg N ha^{-1} at preplant and two topdressings of 25 kg N ha^{-1}); (iii) application of digestate (DG) equivalent to 85 kg N ha^{-1} (DG-N85); (iv) application of digestate equivalent to 170 kg N ha^{-1} (DG-N170); (v) application of digestate equivalent to 255 kg N ha^{-1} (DG-N255); (vi) application of digestate equivalent to 340 kg N ha^{-1} (DG-N340); (vii) application of digestate equivalent to 85 kg N ha^{-1} plus mineral nitrogen split in two applications (35 kg N ha^{-1} at preplant and 25 kg N ha^{-1} at topdressing (DG-N85 + Nm60)); (viii) application of digestate equivalent to 170 kg N ha^{-}1 plus mineral nitrogen split in two applications (35 kg N ha^{-1} at preplant and 25 kg N ha^{-1} at topdressing (DG-N170 + Nm60)); (ix) application of digestate equivalent to 170 N ha^{-1} plus mineral nitrogen at 25 kg ha^{-1} at topdressing (DG-N170 + Nm25). Digestate was always applied at preplant. The mineral N at a rate of N of 85 kg ha^{-1} is the recommended N fertilizer rate for both crops [32]. The treatments were the same for the two crops.

Both crops, lettuce and kale, were grown during two consecutive growing seasons (2020 and 2021). The digestate was manually incorporated into the soil 11 days before planting each crop. Then, plots were watered to maintain soil humidity at 80% field capacity, which is an adequate level for the correct growth of both crops [33]. Subsequently, two days before planting, preplant mineral N fertilizer was manually applied and incorporated into the soil. The lettuce had a crop cycle of 62 days (planted on 6 March 2020 and harvested on 7 May 2020). Topdressing N fertilization with mineral fertilizer was performed for the two crops at 22 and 49 days after planting or at the first date if only one topdressing was applied. The kale crop had a crop cycle of 152 days (planted on 15 October 2020 and harvested on 16 March 2021). Ammonium sulphate was used at preplant to reduce the risk of N leaching. The different mineral fertilizers and concentrations used in the experiment are described in Table 2. The soil for the experiment had a high level of available P (Olsen P = 149 mg kg^{-1})

and potassium (K = 530 mg kg^{-1}). Therefore, there was not an expectable response to the application of P and K fertilizers, and only N fertilization was performed.

Table 2. Treatments and fertilizations performed during field experiments with lettuce (2020) and kale (2020–2021).

Treatments	**1st Crop—Lettuce**			**2nd Crop—Kale**		
	Preplant February 2020	1st topdressing March 2020	2nd topdressing April 2020	Preplant October 2020	1st topdressing December 2020	2nd topdressing February 2021
			kg N ha^{-1}			
Control	-	-	-	-	-	-
Ni85	Ni35 (CAN)	Ni25 (CAN)	Ni25 (CN)	Ni35 (AS)	Ni25 (AS)	Ni25 (AS)
DG-N85	DG-85	-	-	DG-85	-	-
DG-N170	DG-170	-	-	DG-170	-	-
DG-N255	DG-255	-	-	DG-255	-	-
DG-N340	DG-340	-	-	DG-340	-	-
DG-N85 + Ni60	DG-85 + Ni35 (CAN)	Ni25 (CAN)	-	DG-85 + Ni35(AS)	Ni25 (AS)	-
DG-N170 + Ni60	DG-170 + Ni35 (CAN)	Ni25 (CAN)	-	DG-170 + Ni35(AS)	Ni25 (AS)	-
DG-N170 + Ni25	DG-170	Ni25 (CAN)	-	DG-170	Ni25 (AS)	-

Ni, mineral N, DG, digestate; CAN, calcium ammonium nitrate (27% Ni); CN, calcium nitrate (15.5% Ni); AS, ammonium sulphate (20.5% Ni). Source: Horta, C. and Carneiro, J.P. Use of Digestate as Organic Amendment and Source of Nitrogen to Vegetable Crops. Appl. Sci. **2022**, 12, 248. https://doi.org/10.3390/app12010248, Ref. [31].

2.2. Digestate

The digestate was produced as a by-product of the anaerobic digestion of a mixture of pig slurry and cereal straw. This digestion was performed during 30 days of retention time in mesophilic reactors with a capacity of 3000 m^3. The digestate slurry was pressed to separate the solid and liquid phases. Only the solid fraction of the digestate was used in the present work. The chemical composition and biological properties of the solid digestate are shown in Table 3. The methods used in the characterization of digestates can be referenced in [31]. The total microbial biomass (TMB) of the digestates used in this experiment, estimated from the lipid acid profile, had a value of 552.67 nmol g^{-1} and was composed mainly of bacteria (135.49 nmol g^{-1}). The ratio of bacteria:fungi was 17.11, and the ratio between Gram+ and Gram− bacteria was 1.95.

Table 3. Chemical properties of the digestate (DG).

	DG Lettuce	**DG Kale**
DM (g kg^{-1})	282	248
OM (g kg^{-1})	637	761
pH (H_2O)	7.7	8.3
EC (dS m^{-1})	1.46	1.25
N-total (g kg^{-1})	29.7	29.7
N-org (g kg^{-1})	19.3	24.1
C/N	12	15
PT (g kg^{-1})	4.8	7.1
Pi (g kg^{-1})	4.5	6.8
Po (g kg^{-1})	0.3	0.3
K (g kg^{-1})	17	20.3

DM, dry matter; OM, organic matter; EC, electrical conductivity; N-org, organic nitrogen; PT, total phosphorus; Pi, inorganic phosphorus; Po, organic phosphorus.

2.3. Soil Analysis

At the end of the experiments in May 2020 and March 2021, soil samples were taken for each repetition (0–20 cm depth) at 10 different locations and mixed to obtain a representative and unique sample. Each soil sample was divided into two fractions, one of which was immediately sieved to <2 mm and kept at 4 °C for biological and biochemical analyses, while the other fraction was air-dried for chemical analyses.

The pH was measured at a 1:2.5 soil:solution ratio, and the EC was measured with a conductivity meter (WTW, Weilheim, Germany) at a soil:water suspension ratio of 1:2. According to the procedure described by Walkley and Black, the organic matter was analyzed using a potentiometric titration method [34]. P was determined according to [35], and P in the solution was measured according to [36]. Total P (PT) was determined after ignition at 550 °C for 3 h and extracted with H_2SO_4 0.5 M, and inorganic P (Pi) was extracted with H_2SO_4 0.5 M and then quantified by molecular absorption spectrophotometry. Organic P (Po) was calculated as the difference between PT and Pi. All the P in the extracts was centrifugated at 3000 rpm, and P in the supernatant was quantified using the method of Murphy and Riley [36]. The content of Nt was determined after the wet digestion process with sulfuric acid (H_2SO_4) using the Kjeldahl method. Nitrate and ammonium were determined by ISO 14256-1 with 1 mol L^{-1} potassium chloride solution. For nitrate, all samples were below the detection limit according to this method. The methods used in the chemical analyses of the soil can be referenced in [31]. Enzymatic activities were determined after harvesting at the end of the second crop. β-glucosidase (Bglu) activity was determined according to the methods described in [37]. The activity of acid and alkaline phosphatase (AcPh and AlPh) was determined by measuring the amount of p-nitrophenol (PNP) released from the addition of 5 mM p-nitrophenylphosphate at pH 6.5 for AcPh and at pH 11 for AlPh, according to [38].

Total microbial biomass (TMB) was determined according to ester-linked fatty acids (ELFAs) after extraction in mild alkaline methanol, which is known to extract ester-linked fatty acids but not free fatty acids. First, 15 mL of 0.2 M KOH in methanol was added to 3 g of soil and incubated at 37 °C for 1 h. The tubes were vortexed every 10 min during incubation. Then, the pH of the suspension was neutralized by adding 3 mL of 1 M acetic acid. ELFAs were separated into an organic phase by adding 10 mL of hexane, followed by centrifugation at 480 g for 10 min. After that, the hexane layer was transferred to a clean glass test tube, and the hexane evaporated with a rotavapor (Eppendorf AG concentrador plus, Hamburg, Germany) at 30 °C for 20 min. Finally, ELFAs were dissolved in 0.5 mL of 1:1 hexane:methyl tert-butyl ether and transferred to a gas chromatograph (6890 N Network GC System, Agilent Technologies Wilmington, EEUU) vial for analysis.

Four near infrared reflectance (NIR) spectra (one for each soil sample of each repetition) (MPA Bruker, Hetlingen, Germany) were obtained using reflectance light in a macrosample rotating integrated sphere. Samples were measured with a spectral resolution of 8 cm^{-1}, and the final spectra were obtained as the average of 64 scans from 4000 to 12,000 cm^{-1}.

2.4. Data Analysis

A heat map was carried out with the analytical data (chemical, enzymatic, and microbiological), aiming to have an overall evaluation of the influence of the different soil treatments. In this work, the classification tree was used to understand which variables were more representative to differentiate soil treatments. At each classification tree step, the most informative parameters were selected as the source of the (sub)tree, and the current training set was split into subsets according to the values of the selected attribute. The selected parameter was considered a good discriminator if the branches separated all the measurements observed for each sample group.

To identify the effects of the treatments, ANOVA was performed. Normality was checked using the Kolmogorov–Smirnov test and homoscedasticity of variance with the Levene test [39]. The data were transformed (power or logarithmically) when one or both tests were not passed. Means were compared using Tukey's test at $p < 0.05$.

To perform PCA with the spectral data collected with NIR, different mathematical preprocesses were used, namely the Savitzky–Golay first and second derivative, standard normal variate (SNV) transformation, multiplicative scatter correction (MSC), and different combinations of these treatments.

Heat map and classification tree analyses were performed using Statistics® version 7.0.0, the ANOVA with the statistical package Statgraphics Centurion XVI [40], and the spectral data analyses with the Unscrambler® X software, version 10.5.46461.632 (CAMO Software AS, Oslo, Norway).

3. Results

3.1. Effect of Digestates on Soil Chemical Properties

The ratio of organic N to total N (No:NT) was 0.65 for the digestates applied to the lettuce crop and 0.81 for the digestates applied to the kale crop, which means that most of the N was in organic forms. On the contrary, most of the P was in inorganic forms, with a ratio between inorganic P and total P (Pi:PT) of 0.94 for the digestates used in both seasons. (Table 3). The applied digestate provided P rates ranging from 13.7 to 54.9 kg P ha^{-1}. In addition, K, Ca, and Mg were supplied by the digestates in significant amounts (Table 3). The total N in the soil increased significantly with the application of digestates in both crops. After both crops, the highest soil total N was achieved with the treatments providing the highest total N rate (DG-N340 and DG-N255, 3.5 and 3.97 g kg^{-1}, respectively). The average NH_4^+-N was 42% higher after the lettuce crop (0.88 g kg^{-1}) than the kale crop (0.50 g kg^{-1}) (Tables 4 and 5). This is explained in part by the high initial NH_4^+-N concentration or release from organic N mineralization in the initial soil, as can be seen from the control plots after the lettuce crop (Table 4). The high content of organic matter produces a supply of N to the soil due to its mineralization of approximately 85 kg N ha^{-1}, estimated with the mineral N content of the soil, mineral N at the end of the crop, and total N uptake by the plants in the control plots.

Table 4. Soil properties after lettuce crop.

Treatment	Nt	Nam	Olsen P	Po	Pi	SOM	pH	EC
	g kg^{-1}			mg kg^{-1}		%		dS m^{-1}
DG-N85	2.46 cd [a)]	0.88 b	100.73 cd	253	768 ab	4.6	6.15 ab	0.11 c
DG-N170	2.63 bc	0.86 b	111.83 bc	275	827 a	7.87	6.28 a	0.12 bc
DG-N255	2.94 ab	0.89 b	132.1 a	272	852 a	6.70	6.31 a	0.15 ab
DG-N340	3.08 a	1.06 ab	123.76 ab	267	835 a	6.96	6.31 a	0.15 ab
DG-N85 + Ni60	2.82 ab	0.81 b	96.4 cd	293	734 ab	6.42	5.96 c	0.11 c
DG-N170 + Ni60	2.88 ab	1.21 a	103.15 cd	336	794 ab	7.47	6.08 bc	0.12 bc
DG-N170 + Ni25	2.8 ab	0.90 b	102.14 cd	254	745 ab	5.48	6.24 ab	0.12 bc
Ni85	2.36 cd	1.28 a	94.17 cd	302	666 b	4.31	5.72 d	0.19 a
Control	2.27 d	1.04 ab	88.88 d	300	655 b	5.11	6.08 bc	0.11 c
ANOVA	*	**	***	NS	***	NS	***	***

Nt, total nitrogen; Nam, ammonium nitrogen; Po, organic phosphorus; Pi, inorganic phosphorus; SOM, soil organic matter; EC, electrical conductivity. *, **, and *** Significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, and NS-not significant. [a)] Different letters in the same column indicate that the means ($n = 4$) were significantly different according to Tukey's test at a probability level of 0.05 for the treatments.

Organic P did not show significant differences between treatments after lettuce, with values in the range of 253–336 mg kg^{-1}. On the contrary, after the kale crop, the organic P content in the soil amended with digestates at all rates was higher (on average 38%) than that with mineral fertilizer and that in the nonfertilized control (on average 48%, Table 5). The inorganic P measured in the soil after the lettuce crop increased with digestate application compared to Nm fertilizer treatment and the nonfertilized control (Table 4).

Table 5. Soil properties after kale crop.

Treatment	Nt	Nam	Olsen P	Pi	Po	SOM	pH	EC
	g kg^{-1}			mg kg^{-1}		%		dS m^{-1}
DG-N85	2.93 ab [a)]	0.64 ab	77.27 cde	596 cd	269 abc	7.61 abc	6.38 c	0.08 b
DG-N170	2.85 ab	0.66 ab	83.51 bcde	692 bc	443 a	7.87 abc	6.53 b	0.09 b
DG-N255	3.97 a	0.78 a	109.91 a	806 a	376 ab	8.22 a	6.66 ab	0.1 ab
DG-N340	3.50 ab	0.41 ab	107.98 a	759 ab	372 abc	8.26 a	6.70 a	0.12 a
DG-N85 + Ni60	3.08 ab	0.67 a	85.46 bcd	685 bc	451 a	8.01 ab	6.08 d	0.07 b
DG-N170 + Ni60	3.67 ab	0.60 ab	96.10 ab	729 ab	352 abc	7.85 abc	6.11 d	0.09 b
DG-N170 + Ni25	2.83 ab	0.39 ab	91.06 bc	680 bc	237 bc	7.29 abc	6.27 c	0.08 b
Ni85	2.36 b	0.31 ab	73.33 de	539 d	219 bc	6.83 c	5.91 e	0.07 b
Control	2.59 ab	0.13 b	66.98 e	545 d	185 c	7.10 bc	6.27 c	0.08 b
ANOVA	*	**	***	***	***	**	***	***

Nt, total nitrogen; Nam, ammonium nitrogen; Po, organic phosphorus; Pi, inorganic phosphorus; SOM, soil organic matter; EC, electrical conductivity. *, **, and *** Significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. [a)] Different letters in the same column indicate that the means ($n = 4$) were significantly different according to Tukey's test at a probability level of 0.05 for the treatments.

Digestate application in the lettuce crop at rates of 170 kg N ha^{-1} or higher showed the highest inorganic P values (827–852 mg kg^{-1}) (Table 4); on average, these three DG treatments showed an inorganic P concentration 28% higher than the treatment in the control without fertilizer and 26% higher than mineral N fertilizer treatment. The inorganic P content after the lettuce crop showed a mean value (764 mg kg^{-1}) higher than that after the second crop with kale (670 mg kg^{-1}) (Tables 4 and 5). After the second year with kale, DG-N255 showed the highest content of inorganic P in the soil, with a mean value of 806 mg kg^{-1}, which was 48% higher than the nonfertilized control and 50% higher than with mineral N fertilizer (Table 5).

The P available in the soil measured as Olsen P showed a similar trend to inorganic P (Tables 4 and 5). Olsen P after the first crop was significantly increased with the application of the two highest digestate rates relative to mineral or not fertilized treatments, with the highest value obtained with DG-N255 (132.10 mg kg^{-1}; Table 4). A similar trend was observed after the second crop, with DG-N340 and DG-N255 being the treatments with the highest Olsen P (108.94 mg kg^{-1}) (Table 5).

Digestates applied alone significantly increased pH values relative to the nonfertilized control or treatments involving the application of mineral N. The highest digestate rate increased the pH to 6.3 and 6.75 after lettuce and kale, respectively. Overall, pH increased with increased digestate rate, while the lowest pH values were obtained with mineral N fertilizer (Nm 85), at 5.72 and 5.91 after lettuce and kale, respectively (Tables 4 and 5).

As expected, the soil organic matter (SOM) content in soil increased with increased digestate rates, but the differences were only significant after the kale crop. After the lettuce crop, SOM varied in the range of 4.31–7.87% SOM, which were lower values than after the kale crop (6.95–8.30%) (Tables 4 and 5). At the end of the two crop cycles, DG-N255 and DG-N340 increased SOM by 17% relative to the nonfertilized control (Table 5).

3.2. *Effect of Digestates on Soil Biochemical and Biological Properties*

The digestates at the highest rate decreased acid phosphatase and β-glucosidase activities compared to the nonfertilized control, and the three highest digestate rates decreased alkaline phosphatase relative to the mineral N fertilizer treatment (Table 6). Alkaline phosphatase activity was negatively correlated with inorganic P in soil ($r = -0.5$; $p < 0.01$) and positively correlated with total microbial biomass (TMB; $r = 0.55$; $p < 0.001$). Overall, digestate application decreased TMB as well as fungi and bacteria populations relative to the mineral N fertilizer treatment and the nonfertilized control (Table 6).

Table 6. Total microbial biomass and microbial communities in soil after two consecutive crops.

Treatment	AcPh	AlPh	B-glu	TMB	Bacteria	Fungi	BacG+	BacG-	Mycorrhizae
	mg pnp kg^{-1} h^{-1}			nmol g^{-1}					
DG-N85	495 ab [a)]	179 ab	164 ab	262 ab	53.7 ab	19.0 ab	24.0 abc	20.9 ab	11.2 ab
DG-N170	455 bc	144 bc	140 b	225 c	48.1 b	17.2 ab	21.6 bc	20.8 ab	7.9 ab
DG-N255	480 ab	153 bc	159 ab	224 c	46.8 b	14.2 b	20.1 c	21.3 ab	6.6 ab
DG-N340	415 c	125 c	124 b	230 bc	48.4 b	15.0 b	21.7 bc	18.0 b	8.4 b
DG-N85 + Ni60	461 abc	144 bc	141 b	218 c	46.3 b	15.8 b	21.0 bc	19.9 b	8.2 ab
DG-N170 + Ni60	520 a	170 ab	171 ab	231 bc	50.1 ab	15.1 ab	21.4 bc	18.8 ab	8.1 ab
DG-N170 + Ni25	487 ab	130 c	170 ab	241 bc	48.2 b	17.8 ab	20.9 bc	19.7 ab	8.8 ab
Ni85	462 abc	201 a	204 a	281 a	57.6 a	20.9 a	26.7 a	21.2 ab	10.4 ab
Control	484 ab	160 bc	197 a	295 a	57.0 a	20.6 a	24.8 ab	25.0 a	11.5 a
ANOVA	**	***	***	***	***	**	***	*	

AcPh, acid phosphatase; AlPh, alkaline phosphatase; TMB, total microbial biomass; BacG+, bacteria Gram+; BacG−, bacteria Gram−. *, **, and *** Significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. [a)] Different letters in the same column indicate that the means ($n = 4$) were significantly different according to Tukey's test at a probability level of 0.05 for the treatments.

However, the highest digestate rate did not lead to a lower mycorrhizae population compared to the nonfertilized control (Table 6).

3.3. Clustering the Effect of Fertilizer Treatments on Soil

The heat map (Figure 2) showed that the treatments can be organized into two big clusters (Cluster 1 group 2) with different behaviors regarding their correlations with the chemical or enzymatic and microbiological soil properties. Concerning the treatments, the first group was composed of the control, Ni85, DG-N85, and DG-N170 + Nm25, which showed overall positive correlations between enzymatic and microbiological properties and negative correlations with soil chemical properties. The second group was formed by all the other treatments (DG-N170, DG-N170 + Nm60, DG-N85 + Nm60, DG-N255, and DG-N340) and was shown to have correlations with opposite trends.

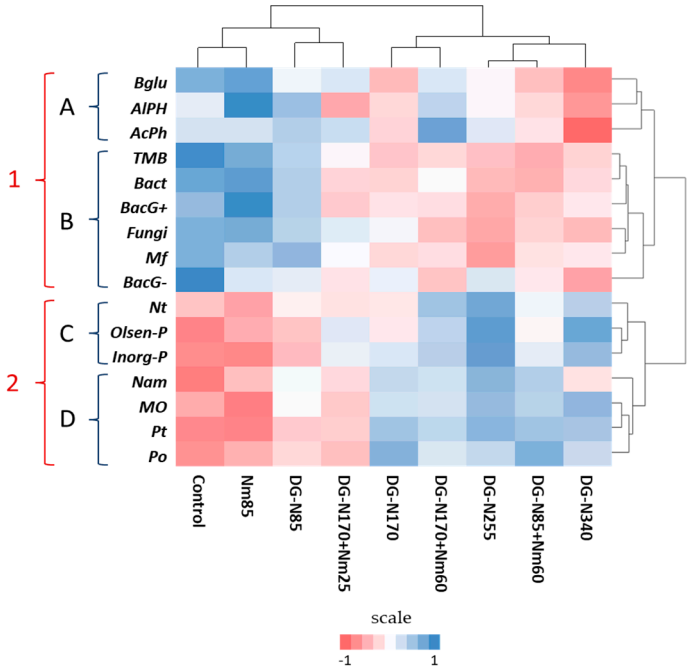

Figure 2. Heatmap of the correlations and tendencies between chemical, enzymatic, and microbiological data and soil treatments.

Regarding the classification tree (Figure 3), acid phosphatase discriminates DG-N340 from all the other treatments. Then, the level of soil total P discriminates two groups: the first group was composed of DG-N85, DG-N170 + Nm25, Ni85, and the control; and the second group was composed of DG-N170, DG-N255, DG-N85 + Nm60, and DG-N170 + Nm60. Next, node 4 with Gram+ bacteria discriminate DG-N170 + Nm25, and Olsen P discriminates DG-N85, Nm85, and the control. Node 5, using the soil Olsen P level, discriminates treatments DG-N170, DG-N85 + Nm60, DG-N170 + Nm60, and DG-N255. Node 7 discriminates treatments though the soil total P into DGN85 and another group consisting of Nm85 and the control. Node 10 discriminates these two last treatments through AlPh. Node 8 also used alkaline phosphatase activity to discriminate between DG-N170 and DG-N85 + Nm60 from DG-N170 + Nm60. Note that the treatments DG-N170 and DG-N85 + Nm60 cannot be separated by any soil property, suggesting that they have a similar effect on soil.

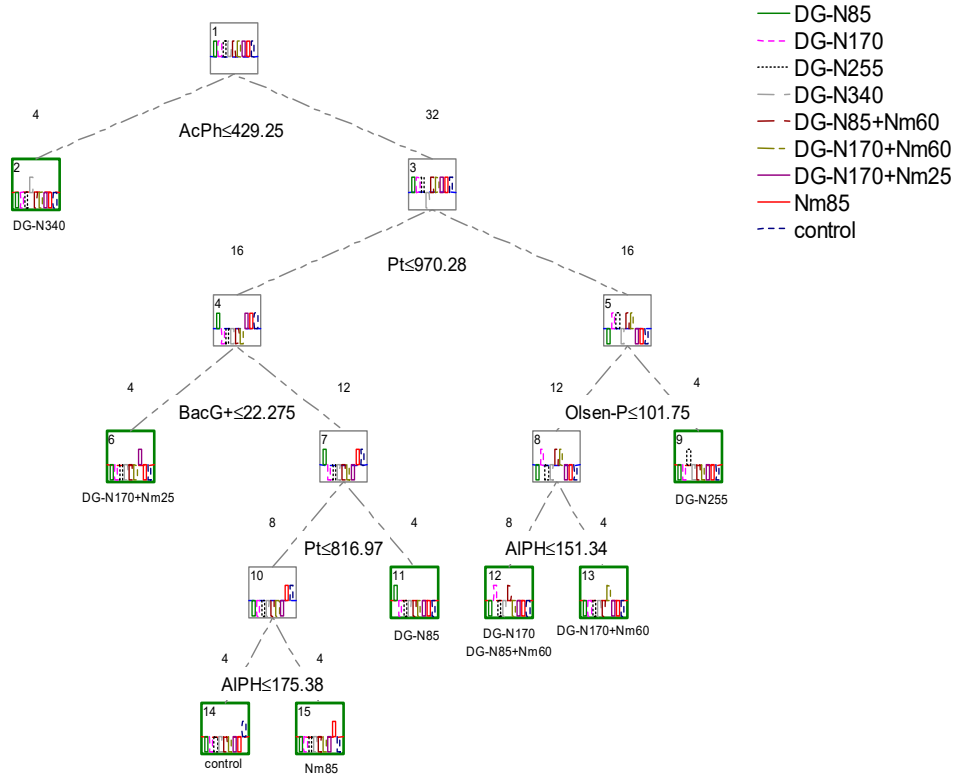

Figure 3. Classification tree results for the chemical, enzymatic, and microbiological data with soil properties using Gini methodology.

3.4. Effect of Digestate on Soil Spectral Traits

The NIR spectra of all the samples that were analyzed had similar profiles, with the main typical and important absorbance bands of soil at 4512, 5200, and 7054 cm^{-1} [41]. The spectral peaks around 5200 cm^{-1} and 7054 cm^{-1} correspond to the water absorption bands and can be assigned to the first and second overtones of -OH stretching. These two peaks were strongly affected by soil moisture; however, prior analysis of the soil it was dried until constant moisture. These bands can also be associated with organic matter, clay minerals, and the carbonyl group (C=O) in organic matter [42,43].

The peak around 4530 cm^{-1} could be associated with organic functional groups of organic matter in the soil (C-H bonds of aliphatic compounds), OH-, and Al-OH bonds of clay minerals [44,45]. The same NIR region was used by [46] to assess soil quality and classify sites according to their global degradation status and by [47] to predict total C, organic C, total N, and moisture content of soils from different geographic regions.

The correlation between soil samples obtained with PCA was quite similar to the results obtained with the heat map, but there were some differences, because with NIR we have information about all soil constituents and not only on the parameters measured in this work. Nevertheless, it is possible to plot that the most relevant regions for distinguishing the samples analyzed were from 4000 cm^{-1} to 5450 cm^{-1} and from 6970 cm^{-1} to 7347 cm^{-1} (Figure 4). PCA analysis showed that component 1 explained 74% of the total variability. This component clearly distinguishes the control group and N85 (the treatments without any fertilization or without DG) from the other groups with higher levels of DG application (DG-N255 and DG-N340). The other samples showed intermediate behavior. Component 2, which explains 30% of the total variation, distinguishes the control treatment from N85. This component is also responsible for the discrimination of DGN85 from DG-N170 + Nm60 (Figure 4).

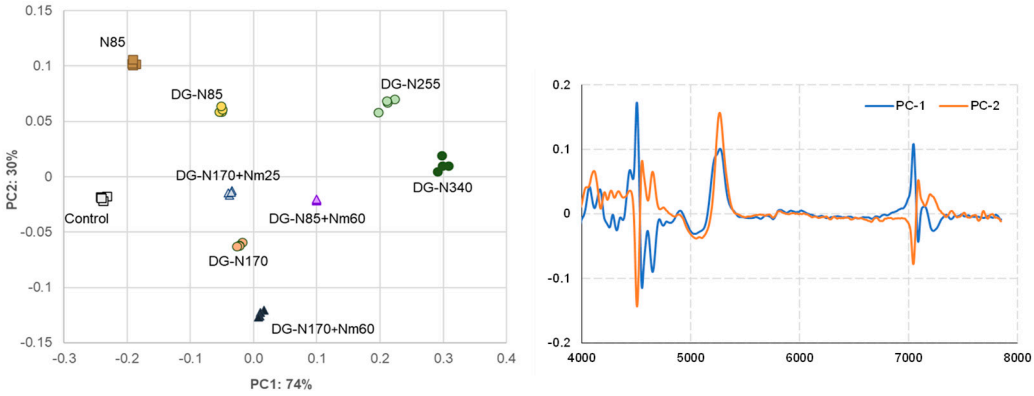

Figure 4. Principal component analysis performed with the NIR spectra applying Savitzky–Golay 1st derivative with 17 smoothing points as a spectral pre-process and spectral loading for the first two components.

4. Discussion

4.1. Effect of Digestate on Soil Nutrient Content and Chemical Properties

Digestates applied alone or combined with mineral N fertilizer increased the total content of N and P and the availability of P in the soil. After the first crop, digestate increased total N but not ammonium in the soil, revealing that a significant portion of supplied N was in organic form and was not mineralized along the crop cycle (Table 4). In fact, the highest digestate rate led to less ammonium than the mineral fertilizer treatment at a much lower N rate. After the second crop, total N and ammonium increased, likely revealing a cumulative effect of N mineralization after two growing seasons (Table 5). Most of the mineral N was present as ammonium, since nitrate was not present in detectable amounts. This reveals an accumulation of ammonium from SOM mineralization and fertilization. Low nitrate contents can be explained by leaching and crop extraction. However, with the acidic pH of the soil, nitrification but not mineralization of SOM can be constrained. In fact, the C to N ratio of SOM at the beginning of the experiment did not reveal limited decomposition or mineralization of SOM. Increased ammonium concentration due to digestates, which mostly supply N in organic form, after the kale crop can also reveal mineralization of the product and subsequent accumulation of mineral N in the form of ammonium with limited nitrification. In soil with high levels of total and mineral N, the accumulation of ammonium instead of nitrate is positive in terms of reducing leaching, but it may have potential phytotoxic effects depending on the crop. All this indicates that the supply of readily available N (mineral) from digestate is limited. In fact, this agrees with the results in [31], testing the same products in which, to achieve the same yield as with the application of 85 kg N ha^{-1} of mineral N, it was necessary to apply 340 kg of N ha^{-1} as digestate. Thus, apparent N recoveries by crops are much lower from digestate than from mineral fertilizer, which is consistent with the high ratio of organic to mineral N in

the product and with the enrichment in total and ammonium N in the soil (Tables 4 and 5, also increasing due to mineralization of the OM in the soil). However, the proportion of organic N in the digestates, a highly stable organic matter in these products, may lead to a low mineralization rate, reducing the mineral N released into soil. In fact, changes in the NIR spectra can be ascribed not only to different SOM and organic N content of soil but also to changes in composition, leading to different mineralization rates, particularly in bands in the range from 4000 to 5800 cm^{-1} [41,45]. Bands observed in this range were crucial for distinguishing the effects of the different treatments according to the PCA of spectral traits (Figure 4). An additional effect contributes to explaining the lack of effect of digestates on ammonium after lettuce that cannot be explained by an increased crop uptake according to [31], since apparent recoveries of mineral N were lower with DG than with mineral fertilizer. This may perhaps be an effect of increased nitrification with DG, since this product increases pH, which may enhance nitrate loss through leaching. In the second crop, the effect of increased pH was more limited, since the initial values in plots receiving digestates were higher than before the lettuce was sown.

According to [31], a combination of 170 kg N ha^{-1} as digestate and 25 kg N ha^{-1} as mineral fertilizer may not provide significantly different results from those obtained with 85 kg N ha^{-1} of mineral fertilizer. This means that digestates can be used as N fertilizers efficiently when combined with small rates of mineral fertilizer. In the long term, further analysis is required to assess the effect of organic N mineralization. However, this slow release of mineral N from applied digestates through mineralization may reduce leaching losses [48], thus providing environmental benefits.

Digestates have the significant effect of increasing total and available P in the soil, in agreement with [11]. Digestate has a significant P concentration, but most of the P applied with the digestate was in inorganic form. However, digestate not only increased inorganic P but also organic P after the second crop. This means that these digestates can alter the biogeochemistry of this nutrient in soil [49]. The results suggest that the accumulation of organic P must be attributed to the microbial biomass that accumulated P from inorganic forms previously obtained from the available P supplied by the digestates [50]. It should be noted that the increase in Olsen P was roughly equivalent to the amounts of P supplied with digestates when considering the sampling soil depth of 20 cm and an average bulk density for soil texture of 1.4 Mg m^{-3} (about 20 mg kg^{-1} for the highest P digestate rate in a year). Therefore, most of the P applied with the digestate remained available to the plants. This is not expected when P is applied as a mineral fertilizer due to adsorption and precipitation reactions in the soil [51]. Another reason for the increase in inorganic and bioavailable P may be the role of organic matter provided by digestates in reducing P adsorption and precipitation in soils [27,28]. In addition, the initial Olsen P values in the soil were high, thus revealing a high degree of sorbent surface saturation. This will contribute to explaining the increased availability of applied P. An increased mineralization rate of organic P and, consequently, an increase in the values of bioavailable inorganic P in the soil may be favored by microbial proliferation [52]. However, the latter alternative seems less likely, as phosphatase activity was decreased in the treatments, promoting the highest available P.

The stoichiometry of digestates must be a fundamental parameter to determine its application in the field. The application of high rates of digestates with low N:P ratios leads to an accumulation of P in soil. Thus, the joint strategy of applying mineral N and digestates will contribute not only to better apparent N recoveries by crop but also to a reduction in the accumulation of P in soil.

Digestates combined or not with mineral N fertilizers increased soil pH and organic matter. Thus, digestates may be a source of organic matter for soil, which, even at the highest rate, did not contribute to increased microbial biomass. Thus, not only the quantity but also the quality of organic matter is relevant, explaining the effects on soil microbial communities. In acid soil, increased pH has a positive effect. In contrast, mineral fertilizer showed slight acidification of the soil, as expected, from the application of ammonium. Soil

properties, as well as soil microbial communities, can be affected by a slight decrease in soil pH [53], which affects its functionality.

4.2. Effect on Soil Biological Properties

Several studies have shown that digestate fertilization is beneficial for soil microbial activity, as it increases the activity of some enzymes, such as phosphatase [11,54]. Our study agrees with this, since AlPh was significantly and positively correlated with soil TMB ($r = 0.55$; $p < 0.001$). Unlike AlPh, AcPh activity did not show a significant relationship with microbial biomass. This may be explained by the fact that it is produced not only by soil [55,56] but also by plant roots [57]. However, in our study, digestates at the highest rate decreased AlPh activity relative to mineral fertilizers and AcPh and β-glucosidase relative to the control. This fact could be attributed to the supply of P available to plants with digestates, since these enzymes are very sensitive to the availability of P [58], as shown by the significant and negative correlation between inorganic P and AlPh ($r = -0.50$; $p < 0.01$). Enzymatic activities do not always show appreciable changes at the time of sampling, mainly because changes and relationships between soil microorganisms, nutrients incorporated into the soil, and said enzymatic activities are constantly changing [59]. The transformations of organic matter in soil are closely associated with soil microbial activities, which are a clear indicator of soil quality and fertility [60]. However, β-glucosidase activity does not always increase with increased organic matter [11,54]. In our case, the activity of β-glucosidase in the soil decreased with the increasing rates of digestates. All this reveals that the organic matter supplied by the digestate in our field experiment did not stimulate or even decreased the carbon biochemical activity of soil microorganisms. This is probably due to the stable and recalcitrant organic compounds (C) that remain after anaerobic digestion and are hardly used by soil microorganisms to cover their energy needs. Similarly, this can occur with most digestates derived from agricultural raw materials, such as animal manure [61,62]. Not only the quantity but also the composition (mainly the content of lignin and cellulose) and the C/N of the digestates [63] are relevant to explain microbial mineralization in the soil. In fact, this agrees with the observed changes in NIR spectra, where changes in spectral traits of soils ascribed to SOM were observed. Although the impacts of digestates on soil microbial communities have been investigated, there is a high degree of disagreement depending on soil type and the nature of the digestates. Although we found that microbial biomass was decreased by digestates at high rates, the ratio of fungi to bacteria or Gram+ to Gram− bacteria was not affected by digestate treatments (Table 6). It is also possible that the proliferation of soil microorganisms may be inhibited by toxic substances in digestates [64,65], such as volatile organic compounds [66,67]. Doyeni et al. (2021) [68] showed that the high content of potassium in pig digestate can negatively affect soil microorganisms. It can be concluded that these products affect the functioning and proliferation of soil microorganisms. In the case of phosphatases, it can be attributed in part to the increased availability of P in the soil, as soil microorganisms regulate enzyme synthesis based on resource availability [65].

4.3. Assessing the Overall Effects of Digestates on Soil

The soil properties more relevant for discriminating the treatments in the decision tree (Figure 3) allowed us to assess the soil properties that can be used to assess the real effects of digestates on soil processes and functions. These soil properties can be considered as soil use indicators, as they are the properties most affected by fertilizer treatments. The AcPh and AlPh activities, population of Gram+ bacteria, and total P and Olsen P contents were the soil properties that were more relevant for discriminating between treatments. The discrimination between treatments obtained with the NIR spectra analysis was slightly different from that obtained with the heat map. NIR spectra analyze every matrix, and these results suggest that more parameters could affect the discrimination between treatments than those we evaluated. Thus, additional studies will be necessary to obtain further knowledge on how digestate application affects soil properties.

Repeated application of digestates in the long-term fertilization trials described by [65] has been shown to have effects on soil microbial abundance and diversity. Since the current study was carried out over a relatively short period of two years, it was difficult to detect major changes in the microbial communities after digestate applications. Furthermore, only one soil was used in this field experiment (i.e., only one soil with high C and high bioavailable P). The results could likely be different with different soils and environmental conditions. Therefore, further research considering different environmental conditions in the long term is necessary for more conclusive results and recommendations.

5. Conclusions

The application of digestate significantly affected the chemical, biochemical, and biological properties. These properties influenced microbial activity in the soil, leading to a decrease in enzyme activities related to the P and C cycles, particularly at high digestate rates. The phosphatase activity, the amount of Gram+ bacteria, and the levels of total and available P were the more relevant variables for discriminating the effect of treatments on soils. The high proportion of inorganic to total P in the digestates led to an accumulation of P in the soil microbial biomass. Digestates showed an important fertilizer value for essential nutrients, with the effect on soil functionality being dependent on the application rate. The application of digestate together with mineral fertilization proved to be the most favorable practice for soil functionality. This joint application can thus contribute to the sustainability of agricultural systems by recycling N from digestates. NIR can be used to distinguish different soil treatments, with the advantage of being faster, nondestructive, and environmentally friendly compared with standard analyses. Future work with digestate application in soils with different properties will be needed for a deeper understanding of its effects on soil functionality.

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Data Availability Statement: The data that support the findings of this study are available upon reasonable request.

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