applied sciences

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Effects of Anaerobic Digestates and Biochar Amendments on Soil Health, Greenhouse Gas Emissions, and Microbial Communities: A Mesocosm Study

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Featured Application: The research outcomes significantly advance sustainable agricultural practices by providing insights into the utilization of organic materials such as digestate and biochar to improve soil fertility, production, mitigate greenhouse gas emissions and pathogen spreading.

Abstract: This study addresses the need for a comprehensive understanding of digestate and biochar in mitigating climate change and improving soil health, crucial for sustainable agriculture within the circular bioeconomy framework. Through a mesocosm experiment, soil was amended with digestates from pilot-scale reactors and two concentrations of biochar produced by pyrolysis of digested sewage sludge and waste wood. The Germination Index (GI) assay assessed phytotoxicity on *Lactuca sativa* and *Triticum aestivum* seeds. Greenhouse gas emissions (CO_2, CH_4, N_2O) measurements, soil characteristics analyses, and the study of microbial community structure enriched the study's depth. The GI assay revealed diverse responses among by-products, dilution rates, and plant types, highlighting the potential phyto-stimulatory effects of digestate and biochar water-extracts. While digestate proved to be effective as fertilizer, concerns arose regarding microbial contamination. Biochar application reduced Clostridiaceae presence in soil but unexpectedly increased N_2O emissions at higher concentrations, emphasizing the need for further research on biochar's role in mitigating microbial impacts. CO_2 emissions increased with digestate application but decreased with a 10% biochar concentration, aligning with control levels. CH_4 uptake decreased with digestate and high biochar concentrations. The study underscores the importance of tailored approaches considering biochar composition and dosage to optimize soil greenhouse gas fluxes and microbial communities.

Keywords: digestate; biochar; soil nutrient; greenhouse gas emission; microorganisms; Clostridiaceae

Citation: Pastorelli, R.; Casagli, A.; Rocchi, F.; Tampio, E.; Laaksonen, I.; Becagli, C.; Lagomarsino, A. Effects of Anaerobic Digestates and Biochar Amendments on Soil Health, Greenhouse Gas Emissions, and Microbial Communities: A Mesocosm Study. *Appl. Sci.* **2024**, *14*, 1917. https://doi.org/10.3390/app14051917

Academic Editor: Gerald Sims

Received: 7 February 2024
Revised: 21 February 2024
Accepted: 23 February 2024
Published: 26 February 2024

1. Introduction

From a circular bioeconomy perspective, the adoption of sustainable management techniques that enhance organic inputs into the soil while minimizing losses offers significant potential for mitigating climate change. This is achieved by reducing the emission of greenhouse gases (CO_2, CH_4, N_2O) and valorizing by-products traditionally considered as wastes [1–3].

Anaerobic digestion (AD) plays a crucial role in the circular economy by converting various biomass and waste into renewable biogas, offering a sustainable alternative to manage farmyard manure and/or industrial organic waste by producing green energy [4].

Additionally, AD results in the production of a valuable by-product known as digestate, which is rich in essential nutrients. This digestate can serve as a sustainable resource for enhancing soil quality and fertility in food production systems by being utilized as either soil amendments or fertilizer [4,5]. Throughout AD, a portion of organic nitrogen (N) is transformed into ammonium (NH_4^+-N), while phosphorus (P) is partially converted into orthophosphate. Both these nutrient compounds become readily available for plant uptake [6,7]. Furthermore, digestate contains a significant amount of carbon (C), contributing to the organic C supply in the soil [5] and improving its physical properties [6]. The application of digestate directly to soil has the potential to significantly increase environmental and economic values by reducing the use of mineral fertilizer, saving synthetic fertilizer costs, and restoring soil organic stocks [6,8].

Biochar, a C-rich by-product, is produced from organic materials like agricultural residues and sewage sludge through processes such as pyrolysis or bio-gasification conducted under low oxygen conditions [9–11]. Biochar serves as a valuable soil amendment and for long-term C sequestration [1,10]. It possesses a large specific surface area and porosity, making it an effective biosorbent, and contains trace minerals [9]. Biochar has been observed to reduce nitrous oxide (N_2O) and methane (CH_4) emissions from soil [12–14], as well as adsorb NH4+-N through chemisorption and physical adsorption [5]. Moreover, studies suggest that the combination of digestate with high rates of biochar can enhance soil organic carbon (SOC) in the <20 µm fraction by up to 20% [15] contributing to improve soil structure, particle aggregation, and water retention [16]. Given its agronomic and environmental benefits in water and nutrient retention, as well climate change mitigation, the application of biochar to soil has recently attracted much attention becoming a widely accepted practice [11,14,16].

Both digestate and biochar contribute to the circular bioeconomy principles by closing the nutrient loop [4]. However, it is important to note that their application to soil has the potential to alter microbial habitats, thereby impacting the composition and activity of soil bacterial communities [6,17,18]. Due to its porous nature, biochar can create an environmental niche conductive to sheltered microbial growth in the soil [19]. The application of digestate and/or biochar has the potential to enhance microbial processes, such as nitrogen mineralization and ammonia (NH_4^+-N) oxidation [20].

Microorganisms serve as key actors in soil ecological processes, influencing nutrient cycling, plant growth, and disease suppression [18]. Preserving the functionality and diversity of soil microbial community is crucial for fostering sustainable agricultural production [8]. However, predicting the consistent response of the soil microbial community to digestate and/or biochar application is challenging due to the inherent heterogeneity of soil and C amendments across studies. Some studies demonstrated a positive impact of digestate application on soil nutrient availability [8], microbial community composition [8,21], respiration, and enzyme activities [22], while others reported minor changes or no significant effects [23]. Digestate can stimulate soil bacterial growth as well as increase the development of arbuscular mycorrhizal hyphae [8,24]. The rhizosphere bacterial community responds to digestate by showing phylum-level shifts, influenced by nutrient dynamics [8]. Biochar, resistant to microbial consumption, may indirectly alter soil microbial communities [25]. Significant increases in soil microbial biomass and growth rates were observed after biochar application, attributed to enhanced soil moisture [26,27]. Conversely, other findings revealed minimal effects on microbial abundances [28,29]. Recent research described shifts in soil microbial community structure towards bacterial dominance and higher fungal-to-bacterial ratios in biochar amended soils [25,30].

Furthermore, when applied to the soil, digestate can raise concerns related to potential hygienic issues, such as the presence of pathogens [16,31], and environmental hazards, including antibiotics, pollutants, and metals [1,16,32]. Farmers currently express apprehensions about the use of digestate in cultivating forage crops, in particular for downstream processes such as cheese manufacturing [33]. Digestate may harbor the presence of *Clostridium* spp., like *Clostridium tyrobutyricum*, *Clostridium butyricum*, and *Clostridium sporogenes*.

These bacteria are known for their ability to produce metabolites, some of which can detrimentally affect the quality of forage crops and cheese during the aging phase. The distribution of digestate in fields raises concerns about the potential spread of these spore forming bacteria from one farm to another, leading to soil and crop contamination [33].

Additionally, there is a need to determine the appropriate doses of digestate that will achieve the desired fertilization effects while ensuring the safety of agroecosystems. In this regard, the evaluation of phytotoxicity also needs to be conducted, serving as a valuable parameter for assessing the actual impact of digestate spreading on crops and functioning as an indicator of its overall ecotoxicological effects [6]. The Germination Index (GI) assay is commonly used as an indirect test to assess the impact of external organic matter (EOM) on seed germination and plant growth, due to its simplicity, short time requirement, and sensitivity [6,34]. This assay employed water-extracts of the EOM, and its results are strongly dependent on the assumed solid-to-liquid ratio [6]. Di Maria et al. [35] documented residual phytotoxicity in digestate making it unsuitable for agricultural use, attributed to high organic N and C concentrations. Pastorelli et al. [36] revealed that highly concentrated digestate water extracts inhibited seed germination, while concentrations below <50% showed phyto-stimulation. Hence, application of digestate should follow appropriate rates and timings to prevent direct contact with seeds, as also noted by Alburquerque et al. [20].

Despite these concerns, there is a lack of comprehensive understanding regarding the impact of digestate and/or biochar on soil organic matter formation/decomposition, soil microbial community activity, and crop yield. Limited information is available on the combined applications of digestate and biochar, particularly concerning the microorganisms involved in the N transformations [4].

Our research was designed to better define the potential benefits and risks associated with the land spreading of digestate and biochar in agriculture. It aligns with the principles of sustainability and circular bioeconomy by exploring environmentally friendly practices aimed at minimizing waste and enhancing resource efficiency. The novelty lies in its focus on investigating digestate from two pilot AD reactors and biochar, specifically examining the soil microbial functional potential and phytotoxicity with macrophytes, filling a gap existing literature regarding the effects of their applications on soil health. Additionally, the study aims to explore the combined application of digestate and biochar, as well as their individual impacts on soil nutrients retention and GHG emissions. To achieve these objectives, we utilized soil mesocosms. The GI assay helped to assess phytotoxicity of digestate, biochar, and feedstocks on *Lactuca sativa* and *Triticum aestivum* seeds. We measured GHG (CO_2, CH_4, N_2O) emissions from soil using a gas spectroscopy analyzer and investigated the soil microbial community structure using real-time polymerase chain reaction (PCR).

We hypothesized that: (i) the application of digestate and biochar, ether individually or in combination, will positively influence soil nutrient retention by enhancing organic matter formation and decomposition; (ii) the addition of digestate and biochar to soil will alter the composition and functional potential of soil microbial communities, thereby affecting soil health and possibly GHG emissions. These hypotheses propose that the application of digestate and biochar will have beneficial effects on soil properties and microbial communities, leading to improved soil fertility and reduced GHG emissions.

2. Materials and Methods

2.1. External Organic Materials

In this study, five different EOMs were studied, (i) solid cattle manure, (ii) and (iii) two manure-based digestates, (iv) a mixture of digested sewage sludge and waste wood, and (v) biochar produced from the sludge mixture. The cattle manure originated from a dairy cattle barn (Jokioinen, Finland), which uses peat and straw as a bedding material. The manure-based digestates (R1 and R2) were obtained from parallel 1 m^3 batch-type leach-bed reactors, where the leachate liquid circulated through the solid substrate batch. Solid cattle manure and wheat straw in 84%/16% mass ratio, respectively, were used as feedstock.

A percolate liquid from another biogas plant was used as an inoculum with added water to facilitate percolate circulation. A detailed description of the pilot experiments is presented in Tampio et al. [37].

The mixture of digested sewage sludge and waste wood (80%/20% mass ratio, respectively) originated from the Helsinki Metropolitan was used to produce biochar in a pilot pyrolysis plant by Helsinki Region Environmental Services (HSY) at temperature of 565 °C and treatment time of 75 min.

The chemical characterization of the EOMs is detailed in Table 1. Analysis and results for the mixture of digestate sludge and wood waste are currently not available. Prior to elemental composition and mesocosm experiment, the EOM samples were dried at 37 °C. The elemental composition was determined using a Leco 2000 elemental analyzer (Leco Corporation). Total and volatile solids, nutrients, and trace elements were analyzed as described by Tampio et al. [37].

Table 1. Chemical characteristics of cattle manure, digestates [37] and biochar (n.d. = not determined).

		Cattle Manure	Digestate R1	Digestate R2	Biochar
Total solid (TS)	%	23.9	12.5	12.9	98.3
Volatile solid (VS)	% (TS)	91.3	83.5	85.4	29.8
Carbon (C)	% (TS)	48.0	46.0	46.1	29.8
Hydrogen (H)	% (TS)	5.8	4.8	5.3	0.3
Nitrogen (N)	% (TS)	2.2	2.0	2.0	2.4
Sulfur (S)	% (TS)	0.3	0.3	0.3	1.6
Oxygen (O)	% (TS)	34.9	30.3	31.6	−2.2 [1]
Ash	% (TS)	8.7	16.5	14.6	68.1
Phosphorous (P)	g/kg TS	3.6	3.1	2.7	53.7
Potassium (K)	g/kg TS	15.5	38.7	38.7	2.2
Calcium (Ca)	mg/kg TS	6.5	7.7	8.5	31.3
Iron (Fe)	mg/kg TS	0.8	1.5	2.4	246.0
Magnesium (Mg)	mg/kg TS	4.7	5.3	5.3	3.6
Sodium (Na)	mg/kg TS	2.3	1.9	1.7	1.1
Ammonium nitrogen (NH_4-N)	g/kg TS	23.0	8.0	6.20	0.4
Total Kjeldahl nitrogen (TNK)	g/kg TS	n.d.	23.9	21.69	n.d.

[1] negative value indicates incomplete combustion (550 °C, 2 h) in the analysis.

2.2. Germination Index

The potential phytotoxicity of EOMs was assessed by determining the Germination Index (GI) through water-soluble extracts using *Lactuca sativa* (dicotyledon) and *Triticum aestivum* (monocotyledon) seeds. Each EOM (20 g dry weight) was mixed with 240 mL of sterile deionized water using an orbital lab-shaker (Adolf Kühner AG, Switzerland) at 125 rpm for 24 h. Subsequently, the extracts were centrifuged at 5000× *g* for 10 min and the supernatants were used to prepare test solutions at concentrations of 100% (pure EOM aqueous extract; only for biochar and sludge), 50%, 25%, 12.5%, 6.25%, and 0% (distilled water as negative control). The 100% aqueous extracts of digestate and manure were excluded due to their high-water adsorption capacity, making it challenging to obtain sufficient aqueous extract for testing at 100% concentration and preparing subsequent dilutions.

Petri dishes (9 cm diameter) were prepared, each containing twenty *L. sativa* or *T. aestivum* seeds placed upon two sheets of Whatman N. 1 filter paper pre-treated with 10 mL of the test solution. Five replicates were prepared for a total of 100 seeds for each treatment. The dishes were transferred to a germination chamber under controlled temperature (20 °C) in the dark. The humidity within each Petri dish was regulated by the 10 mL of test solution added to moisten the Whatman sheets and no water was added during the incubation period to avoid diluting the test solution. After three days and one week of incubation, the number of germinated seeds in each Petri dish was counted. Seedling root elongation was measured after one week. The Germination Index (GI) was calculated by comparing the germination rate and main root length between the specific treatment and the negative control (water) [38,39].

GI value equal to 100% indicates no differences in average seedling length and germination rate between a specific treatment and control. GI < 80% or >120% suggest inhibitory or bio-stimulatory effects of the specific treatment, respectively [6,38].

2.3. Soil Collection and Characteristics

The soil used in this mesocosm laboratory experiment was collected in April 2023 from the 0–20 cm plow layer of an agricultural field in Fagna experimental farm (Scarperia, Firenze, Italy; 43°59′03″ N, 11°20′34″ E) managed by the Research Center for Agriculture and Environment (CREA-AA, Firenze, Italy). The soil, classified as *Fluventic Eutrudept* [40], has a loam texture (sand = 30%, silt = 44%, clay = 26%). After air-drying, the soil was sieved through a 2 mm sieve to remove larger debris and coarse materials, preparing it for the mesocosm experiment.

2.4. Mesocosm Experiment Setup

The mesocosm experiment utilized 1000 cm^3 polyethylene jars (80 cm^2 surface area; 12.5 cm height), each filled with 0.5 Kg of dry soil and supplemented with the digestate and/or biochar. The jars were equipped with a gas-tight cap with two valves (in-out). A total of 27 mesocosms were prepared with three replicates for each treatment (details in Table 2). The digestate addition aimed to simulate an N application of 150 Kg N ha^{-1}, mirroring typical arable farm soil management. For shipping convenience, the digestate was dried (37 °C) and consequently hydrated for mesocosm setup by adding 10 mL of water (Table 2). Soil water content was maintained at around 35% of field capacity by watering the jars after each GHG measurement to achieve specific target weight.

Jars were incubated in darkness at a controlled temperature of 20 °C for 3 weeks.

Table 2. Mesocosms set up: soil, digestate and biochar combinations. Biochar was added at concentration of 2 or 10 g kg^{-1} of dry soil. Digestate was added in order to obtain an equivalent of 150 Kg N ha^{-1}. The water added at the beginning of the mesocosms set up was to keep the soil water content at around 35% of field capacity and to hydrate digestate. Bio, biochar; Dig1, digestate from reactor 1; Dig2, digestate from reactor 2.

Treatment		Soil	Digestate R1	Digestate R2	Biochar	Initial Water
Control soil	Control	0.5 kg				105 mL
Soil + 2% biochar	Bio2%	0.5 kg			2 g kg^{-1}	105 mL
Soil + 10% Biochar	Bio10%	0.5 kg			10 g kg^{-1}	105 mL
Soil + digestate from pilot R1	Dig1	0.5 kg	3.8 g kg^{-1}			115 mL
Soil + digestate from pilot R1 + 2% biochar	Dig1+bio2%	0.5 kg	3.8 g kg^{-1}		2 g kg^{-1}	115 mL
Soil + digestate from pilot R1 + 10% biochar	Dig1+bio10%	0.5 kg	3.8 g kg^{-1}		10 g kg^{-1}	115 mL
Soil + digestate from pilot R2	Dig2	0.5 kg		4.1 g kg^{-1}		115 mL
Soil + digestate from pilot R2 + 2% biochar	Dig2+bio2%	0.5 kg		4.1 g·kg^{-1}	2 g kg^{-1}	115 mL
Soil + digestate from pilot R2 + 10% biochar	Dig2+bio10%	0.5 kg		4.1 g·kg^{-1}	10 g kg^{-1}	115 mL

2.5. GHG Emissions and Soil Analysis

The production of GHGs (CO_2, N_2O, CH_4) was measured after 1, 2, 4, 6, 8, 11, 15, and 22 days of incubation using a portable DX4040 Fourier Transformed Infrared (IR) Spectroscopy (FTIR)-Gas Analyzer (Gasmet Technology Oy, Helsinki, Finland). The detected signal is digitized, and Fourier transformed resulting in an IR spectrum of the sample gas. Background calibration with pure N (99.999%) was performed before each measurement. The instrument is equipped with Teflon tubes with quick-connect fittings and an on-board sample pump, which has a neoprene diaphragm membrane, maximum pressure is 1.0 bar, and maximum flow is 2.0 L min^{-1}. To measure the flux for each gas, consecutive measurements over time were performed for 10 min, reading gas concentrations every minute, for a total of 10 readings every measurement time. At each incubation time, blank controls concentration was subtracted from the sample concentrations, to remove background noise of atmospheric concentrations of CO_2, N_2O, and CH_4. Gas concentrations (ppm) were

converted to mass per volume units using the Ideal Gas Law, factoring in air temperatures and volumes, and expressed in mmol g^{-1} considering the headspace of each flask and the weight of each soil sample [41]. The cumulative curve was calculated by summing concentrations at each incubation time.

After incubation moisture content (%) was determined gravimetrically and total nitrogen (TN), total carbon (TC), and total organic carbon (TOC), were measured by dry combustion on a Thermo Flash 2000 NC soil analyzer (Fisher Scientific, Waltham, MA, USA) [28]. To this aim, 10 to 20 mg samples were weighed into Ag-foil capsules and pre-treated with 10% HCl until complete removal of carbonates. Soil pH and electrical conductivity (EC) were measured using a digital calibrated pH meter (Hanna-pH211, Padova, Italy) on aqueous extracts (1:20 soil/water ratio).

2.6. DNA Extraction and Microbial Community Structure

At the end of incubation period, DNA extraction from 0.5 g of soil was carried out using the FAST DNA SPIN kit for SOIL (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions. The concentration and purity of DNA extracts were determined using a NanoDrop Lite Plus spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA).

To assess the impact of the different EOMs and their combinations on soil bacterial community structure, real-time (quantitative) PCR was conducted using taxonomic group-specific primers (Table 3) for 16S ribosomal DNA (bacteria and Clostridiaceae) or primers targeting marker genes specific for functional microbial groups involved in C and N cycles (*mcrA* for methanogens; *pmoA* for methanotrophs; *amoA* for nitrifiers; *nirK*, *nirS*, and *nosZ* for denitrifiers). PCR reactions were carried out on an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Blas Taq qPCR MasterMix (Applied Biological Materials Inc., Richmond, BC, Canada). The cycling conditions were as prescribed by the manufacturer and the annealing-extension temperatures of each primer set are detailed in Table 3. A dissociation protocol ranging from 60 to 95 °C was applied. Each plate included triplicate samples, negative controls, and were necessary, the appropriate standard curve.

Table 3. Primer pairs used for real time PCR absolute quantification of the different microbial groups assessed in soil of the different mesocosms.

Target Group	Target Gene	Primers	Sequences	Annealing Temperature	Reference
Bacteria	16S rRNA	Bac341F Bac805R	5′-CCTACGGGAGGCAGCAG-3′ 5′-GGACTACHVGGGTWTCTAA-3′	60 °C	[42] [43]
Clostridiaceae cluster I and II	16S rRNA	CHIS150F CLOST1R	5′-AAAGGRAGATTAATACCGCATAA-3′ 5′-5′TTCTTCCTAATCTCTACGCA-3′	55 °C	[44] [45]
Clostridiaceae cluster IV	16S rRNA	CLEPF CLEPR	5′-GCACAAGCAGTGGAGT-3′ 5′-TCCCGTAGAGTCTGG-3′	60 °C	[46]
Methanogenic archaea	*mcrA*	qmcrAF qmcrAR	5′-TTCGGTGGATCDCARAGRGC-3′ 5′-GBARRTCGWAWCCGTAGAAWCC-3′	60 °C	[47]
Methanotrophic bacteria	*pmoA*	A189F Mb661R	5′-GGNGACTGGGACTTCTGG-3′ 5′-GCCGGMGCAACGTCYTTACC-3′	60 °C	[48]
Nitrifying bacteria	*amoA*	AmoA1F AmoA1R	5′-GGGGTTTCTACTGGTGGT-3′ 5′-CCCCTCKGSAAAGCCTTCTTC-3′	57 °C	[49]
Nitrifying archaea	*amoA*	archamoAF archamoAR	5′-STAATGGTCTGGCTTAGACG-3′ 5′-GCGGCCATCCATCTGTATGT-3′	55 °C	[49]
Denitrifying bacteria	*nirK*	F1ACu R3Cu	5′-ATCATGGTSCTGCCGCG-3′ 5′-GCCTCGATCAGRTTGTGGTT-3′	57 °C	[50]
Denitrifying bacteria	*nirS*	Cd3aF1 R3Cd	5′-GTSAACGTSAAGGARACSGG-3′ 5′-GASTTCGGRTGSGTCTTGA-3′	52 °C	[50]
Denitrifying bacteria	*nosZ*	nosZF nosZ1733R	5′-CGYTGTTCHTCGACAGCCAG-3′ 5′-ATRTCGATCARCTGBTCGTT-3′	55 °C	[50]

The absolute quantity of soil bacteria was estimated using a standard curve generated from known amounts of bacterial 16S rRNA gene fragment as template. The relative amounts of each microbial group, compared to bacterial rDNA, were determined using the $2^{-\Delta\Delta CT}$ method [51]. Control soils served as calibrators, with all calculated values of the marker genes relative amounts related to the calibrator value, approximated as 1.

2.7. Statistical Analysis

Chemical analysis and DNA amplification were performed on individual replicates, and the results were presented as the mean and standard error of the replicates. Statistical significance was determined using analysis of variance (ANOVA) followed by Fisher's least Significant Difference, LSD post hoc test ($p < 0.05$) with Statistica 7 software (StatSoft, Palo Alto, CA, USA). Normality and the variance homogeneity of the data were tested before ANOVA.

3. Results

3.1. Seed Germination Index

The GI calculated using lettuce and wheat seeds exhibited significant variation based on the EOM and/or the dilution rate applied (Table 4; Figure 1). For lettuce seeds, the biochar feedstock mixture (digested sewage sludge + waste wood) expressed the lowest GI, indicating phytotoxicity in the more concentrated test solutions (100%, 50%, and 25%). As the dilution ratio increased, GI values approached 100%, suggesting a reduction in inhibitory factors due to dilution. A different trend was observed for biochar, digestates, and cattle manure. The most concentrated test solutions exhibited the highest GI, which gradually decreased with increasing dilution rates, reaching values close to 100%, suggesting the presence of phyto-stimulatory factors in the more concentrated water extracts.

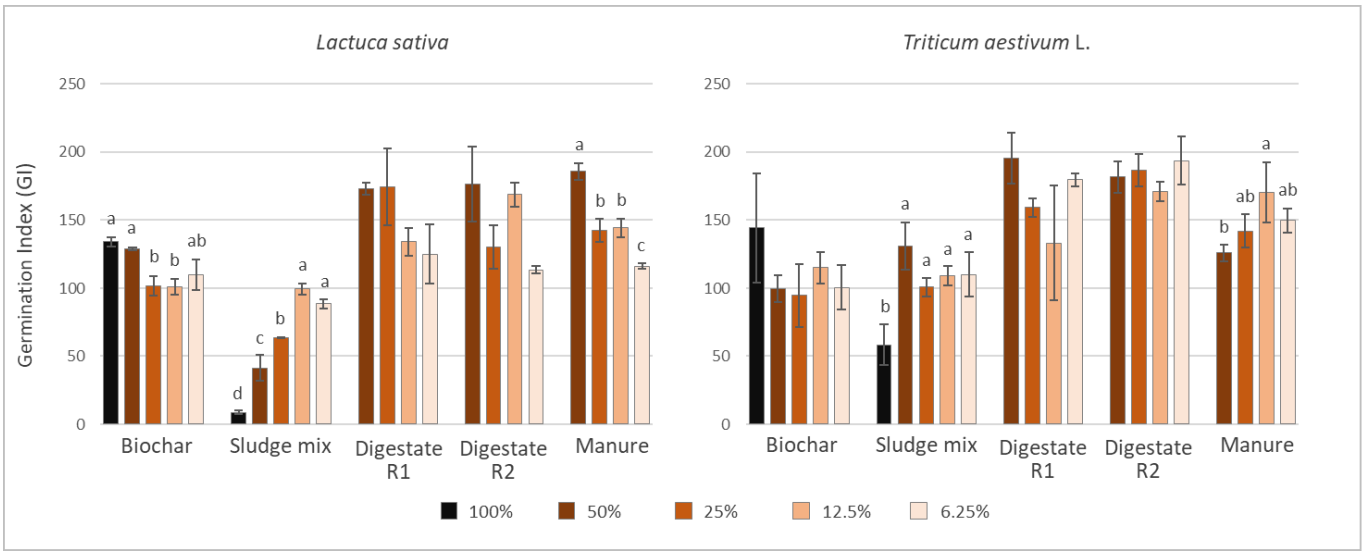

Figure 1. Germination index (GI) of different dilution water-soluble extracts obtained by biochar, sludge mixture (digested sewage sludge and waste wood), digestates (from R1 and R2 reactors), and cattle manure. Error bars calculated as standard errors in replicate petri dishes for each treatment and dilution. Different letters indicate significant differences at $p < 0.05$ (Fisher's least Significant Difference, LSD). The 100% aqueous extract concentration was excluded for digestates and manure due to challenges associated with their high-water adsorption capacity (refer to Section 2.2).

Table 4. Germination index two-way ANOVA results for *L. sativa* (lettuce) and *T. aestivum* (wheat) seeds according to the different EOMs added to soil (treatment), dilution ratio (dilution), or their interaction (treatment × dilution); n.s. = not statistically significant.

	Lactuca sativa		*Triticum aestivum*	
Factor	**F Value**	***p*<**	**F Value**	***p*<**
Treatment	54.9	0.001	18.1	0.001
Dilution	6.0	0.01	0.5	n.s.
treatment × dilution	5.8	0.001	1.2	n.s.

Similar trends were observed in wheat seeds treated with biochar or digestates. Wheat seeds treated with the mixture of digested sewage sludge and waste wood did not show phytotoxicity in water extracts diluted more than 50%. Wheat seeds treated with cattle manure exhibited an opposing trend compared to lettuce seeds with manure 50% test solution having the lowest GI, suggesting the presence of phytotoxic precursors that decreased with increasing dilution.

3.2. Changes in Soil Properties

Following the incubation period, an upward trend was noted for almost all measured chemical parameters with the addition of 2% and 10% biochar to the soil (Table 5). Soil TC, TOC, TN, and EC significantly increased in soil amended with 10% biochar, regardless of the addition of digestate. The increase was approximately three times greater than the control for TC and TN, six times for TOC, and two times for EC. No significant differences were observed between the control, digestate, and +2% biochar treatments. Soil pH values across the treatments showed minimal variations, ranging from 7.8 to 8.0, with a decreasing trend attributed to the addition of biochar.

Table 5. Chemical properties of soil at the end of mesocosm experiments (standard errors in parenthesis). Different letters indicate significant differences at $p < 0.05$ (Fisher's least Significant Difference, LSD). Bio, biochar; Dig1, digestate from reactor 1; Dig2, digestate from reactor 2.

Treatment	TC (%)	TOC (%)	TN (%)	C/N	pH	EC (dS/m)
Control	1.45 (0.02) b	0.69 (0.01) d	0.11 (0.002) e	13.4 (0.3) a	7.91 (0.04) ab	30.9 (2.8) c
Dig1	1.50 (0.05) b	0.78 (0.01) d	0.13 (0.002) de	11.9 (0.2) bc	8.00 (0.02) a	28.5 (0.8) c
Dig2	1.59 (0.06) b	0.79 (0.02) cd	0.14 (0.001) cde	11.5 (0.5) c	7.93 (0.03) ab	26.9 (1.6) c
Bio2%	1.90 (0.01) b	1.31 (0.01) bcd	0.15 (0.003) bcd	12.3 (0.3) abc	7.87 (0.03) bc	27.1 (1.3) d
Bio10%	4.89 (0.66) a	4.24 (0.51) a	0.38 (0.035) a	12.9 (0.7) ab	7.81 (0.01) cd	49.3 (4.9) a
Dig1+bio2%	2.15 (0.12) b	1.47 (0.17) bc	0.17 (0.010) bc	12.4 (0.1) abc	7.93 (0.02) ab	29.6 (1.7) c
Dig2+bio2%	2.15 (0.13) b	1.63 (0.24) b	0.18 (0.016) b	11.7 (0.4) c	7.94 (0.03) ab	34.1 (2.5) c
Dig1+bio10%	4.31 (0.20) a	3.68 (0.21) a	0.34 (0.015) a	12.7 (0.1) abc	7.78 (0.05) d	61.6 (3.4) b
Dig2+bio10%	4.46 (0.38) a	3.92 (0.29) a	0.34 (0.013) a	13.3 (0.7) a	7.82 (0.02) cd	61.7 (3.4) b

3.3. GHG Emissions

The addition of digestates to soil led to a significant increase in CO_2 emissions (Figure 2, top), without differences between the two tested samples. Biochar showed a contrasting, dose dependent pattern: a significant increase at the lower concentration of 2% and a reduction with 10%, which lowered the CO_2 production to the level of control soil (Table 6).

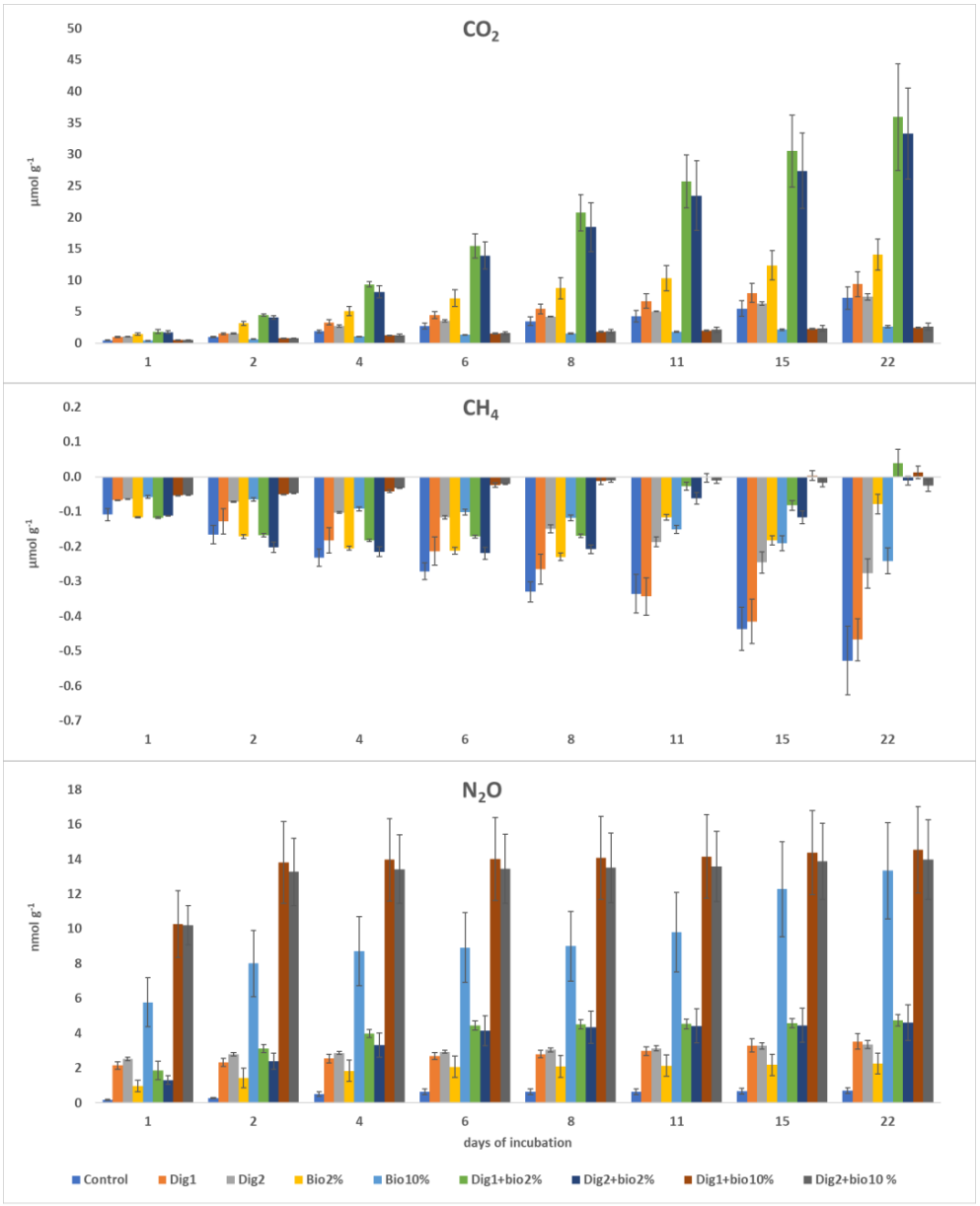

Figure 2. Potential production/consumption of CO_2 (**top**), CH_4 (**middle**) and N_2O (**bottom**) in control soils, and after addition of digestate R1 and R2 (Dig1 and Dig2) and/or 2% and 10% of biochar (Dig1+bio2%, Dig2+bio2%, Dig1+bio10%, Dig2+bio10%) during the 22 days of incubation. Error bars calculated as standard errors in replicate mesocosms.

Overall, CH_4 uptake was prevalent throughout the incubation period (Figure 2, middle), with values around 0 after 2 weeks in samples with digestate + biochar. Digestate addition reduced CH_4 uptake, likewise the biochar at the highest concentration (10%). Biochar at 2% concentration did not differ from control soils (Table 6).

N_2O production (Figure 2, bottom) was minimum for control soils and increased significantly with digestate addition and with biochar at the highest concentration of 10% (Table 6). The combination of digestate + biochar showed contrasting results depending on biochar concentration, with a decrease to control values with 2% and a sharp increase (around 3 times higher that digestate alone) with 10% of biochar.

Table 6. Fisher's LSD post hoc test ($p < 0.05$) of cumulative emissions of CO_2, CH_4 and N_2O. Mean values of each flux are reported (standard error in parenthesis). Different letters indicate significant differences among treatments.

Treatment	CO_2	CH_4	N_2O
Control	3.30 (0.7) c	−0.30 (0.02) c	0.53 (0.12) d
Dig1	4.97 (0.8) bc	−0.26 (0.04) c	2.79 (0.27) cd
Dig2	3.97 (0.1) bc	−0.15 (0.01) b	2.99 (0.10) cd
Bio2%	7.79 (1.4) b	−0.16 (0.01) b	1.88 (0.57) cd
Bio10%	1.44 (0.1) c	−0.13 (0.01) b	9.48 (2.1) b
Dig1+bio2%	17.98 (2.8) a	−0.11 (0.01) b	3.97 (0.19) c
Dig2+bio2%	16.30 (3.2) a	−0.14 (0.01) b	3.63 (0.69) c
Dig1+bio10%	1.56 (0.1) c	−0.02 (0.01) a	13.65 (2.33) a
Dig2+bio10%	1.63 (0.3) c	−0.03 (0.01) a	13.16 (1.94) a

3.4. Microbial Community Structure

The abundance of bacterial 16S rRNA gene is reported Table 7 and ranged between 1.3×10^{10} and $7.6 \times 10^{9} \pm 7.2 \times 10^{8}$ copy dry soil g^{-1} (soil added with 10% biochar).

The relative abundance of nitrifying, denitrifying, and methanotrophic microbial groups showed significant differences due to treatment (Table 8), although a direct relationship between bacterial abundance and treatment is not clearly evident.

The addition of digestate significantly increased methanogenic archaeal abundance, with the mcrA gene copy number being 42–64 times higher in soils treated with digestate, with or without biochar, and approximately two times higher in soils treated with biochar only, compared to control soil. Similarly, the addition of digestate led to a 15–22 times increase in 16S rRNA gene copy number from cluster IV Clostridiaceae compared to control soil. Clostridiaceae from clusters I and II resulted 2.5–4 times higher in soil samples added with digestate only compared to control soil. Conversely, a drastic reduction in the specific 16S rRNA gene copy number was observed in soil samples where biochar was added at both 2% and 10% (Table 8).

Table 7. Absolute quantification of total bacteria expressed as 16S rDNA gene copy number g $soil^{-1}$ (standard errors in parenthesis). Different letters indicate significant differences at $p < 0.05$ (Fisher's least Significant Difference, LSD). Bio, biochar; Dig1, digestate from reactor 1; Dig2, digestate from reactor 2.

Treatment	Total Bacteria
Control	8.6×10^{9} (6.1×10^{8}) bc
Dig1	1.1×10^{10} (1.1×10^{9}) ab
Dig2	1.3×10^{10} (2.5×10^{8}) a
Bio2%	1.1×10^{10} (5.1×10^{8}) abc
Bio10%	7.6×10^{9} (7.2×10^{8}) c
Dig1+bio2%	1.3×10^{10} (1.6×10^{9}) a
Dig2+bio2%	1.1×10^{10} (1.1×10^{9}) ab
Dig1+bio10%	1.3×10^{10} (2.4×10^{9}) a
Dig2+bio10%	9.2×10^{9} (4.7×10^{8}) bc

Table 8. Relative abundance of the target microbial groups versus bacterial 16S rDNA (standard errors in parenthesis). Different letters indicate significant differences (Fisher's least Significant Difference, LSD). Bio, biochar; Dig1, digestate from reactor 1; Dig2, digestate from reactor 2.

Thesis	Nitrifiers		Methanotrophs	Methanogens	Denitrifiers			Costridiaceae	
	Bacterial *amoA*	Archaeal *amoA*	*pmoA*	*mcrA*	*nirK*	*nirS*	*nosZ*	Cluster I and II 16S rDNA	Cluster IV 16S rDNA
Control	1.0 (0.15) ab	1.1 (0.44)	1.0 (0.03) a	1.0 (0.10) b	1.0 (0.14) b	1.0 (0.08)	1.0 (0.11) ab	0.9 (0.05) bc	1.0 (0.16) d
Dig1	0.8 (0.09) b	2.0 (0.28)	0.9 (0.15) ab	54.1 (6.55) a	1.4 (0.07) ab	1.1 (0.04)	1.1 (0.03) ab	4.1 (1.06) a	21.3 (1.15) ab
Dig2	0.8 (0.25) b	2.0 (0.70)	0.8 (0.06) abc	42.0 (13.8) a	1.2 (0.14) ab	1.2 (0.08)	1.0 (0.03) ab	2.5 (1.68) ab	15.2 (1.57) bc
Bio2%	0.8 (0.15) b	2.5 (0.47)	0.6 (0.02) c	1.8 (0.09) b	1.2 (0.31) ab	1.1 (0.11)	1.0 (0.10) ab	0.2 (0.18) c	3.0 (1.25) d
Bio10%	1.2 (0.22) ab	1.2 (0.64)	1.0 (0.08) a	1.8 (0.30) b	0.8 (0.02) b	0.9 (0.03)	1.1 (0.05) ab	0.1 (0.03) c	2.4 (0.36) d
Dig1+bio2%	0.7 (0.08) b	2.3 (0.29)	0.8 (0.13) bc	62.6 (7.07) a	1.7 (0.74) ab	1.1 (0.23)	0.9 (0.08) b	0.6 (0.50) bc	18.1 (1.0) abc
Dig2+bio2%	1.5 (0.32) a	1.5 (0.32)	0.7 (0.04) bc	64.2 (5.87) a	2.2 (0.49) a	1.2 (0.18)	1.1 (0.06) ab	0.1 (0.01) c	22.4 (0.83) a
Dig1+bio10%	0.8 (0.12) b	2.6 (1.40)	0.7 (0.07) bc	50.2 (12.4) a	1.1 (0.15) b	0.9 (0.06)	1.0 (0.09) ab	0.0 (0.00) c	14.8 (3.79) c
Dig2+bio10%	1.0 (0.19) b	1.3 (0.15)	0.8 (0.06) abc	51.8 (11.6) a	1.4 (0.23) ab	1.1 (0.13)	1.2 (0.22) a	0.3 (0.13) c	14.4 (4.16) bc

4. Discussion

Overall, the GI assay results reveal intricate interactions among utilized bio-based fertilizing products, dilution rates, and plant types. The lower GI observed in concentrated digested sludge mixture's water-extracts suggests the presence of phytotoxic factors, with their impact diminishing as dilution ratio increases. Notably, lettuce seeds exhibit a higher sensitivity compared to wheat seeds when exposed to concentrated extracts of digested sludge mixture. This finding aligns with existing literature indicating a general lower sensitivity to toxicity in seeds from root crops, cereals, and legumes when compared to seeds of leafy plants, which is attributed to the higher food reserve content in the former [52,53].

In contrast, biochar and digestate water-extracts show an opposite trend, with higher GI in concentrated extracts gradually decreasing with increasing dilution rates, reaching values comparable to the control. This pattern suggests the presence of phyto-stimulatory factors in more concentrated water-extracts, positively influencing germination process and subsequent plant growth. Manure water-extracts also show phyto-stimulant effects on lettuce and wheat seeds, although a distinct sensitivity of these two plants is evident. Generally, phytotoxicity results from a combination of factors, including phytotoxins, salinity, high ammonium concentration, and heavy metals, which can delay seed germination and inhibit plant growth [6,54]. In addition, cytotoxic effects can also occur. In a study by Albero et al. [55] the same biochar sample's organic extracts show cytotoxic effect at concentration of 1%, while biochar and digestates water-extracts do not show toxic effects. Therefore, it is crucial to consider not only nutrient concentrations but also potential risks of metal accumulation, soil salinization, and cyto- and phyto-toxic effects, when applying EOMs to soil, comprehending their specific impacts on different types of crops.

In our mesocosm experiment, a higher biochar rate (10%) significantly increases soil salinity, C and N contents, potentially posing risks of phytotoxicity effects for more sensitive crops. On the positive side, the observed phyto-stimulatory effects of biochar and digestate water extracts highlight their potential benefits attributed to the presence of plant nutrients, growth stimulants, or even phytohormone-like substances [20,36].

Furthermore, downstream processing technologies could reduce the risks for phytotoxicity effects of EOMs. Our results clearly indicate the effectiveness of AD and pyrolysis in reducing inhibitory factors, making the digestate and biochar suitable for use as fertilizer and/or soil amendment. The stability of organic matter is crucial in preventing phyto-toxicity, with AD significantly contributing to the stabilization of organic material by partially degrading organic matter [37] and organic pollutants derived from feedstocks and reducing salinity [56]. The sewage sludge in this study's mixture is digested, but evidently it does not reach the same stability as cattle manure-based digestate likely due to its origin and presence, for example, of organic contaminants [55].

However, a critical consideration in utilizing digestate is ensuring that it does not harbor harmful microorganisms, particularly pathogens, which could pose a risk of infection spread [16,31]. We observe a significant increase in the relative abundance of Clostridiaceae in digestate-amended soil, which underscores the impact of AD by-products on the soil microbial community. This finding reflects the resilience and functional importance of the Clostridiaceae microbial group in AD systems, as indicated by various studies [57–60]. However, it also raises concerns about potential contamination risks. *Clostridium* bacteria are common in animal manure and naturally occur in soils at relatively high number. Yet, spores from pathogenic species like *Clostridium botulinum* and *Clostridium tetani* are already present in the soil, suggesting that fertilization with digestate is unlikely to increase the risk of diseases caused by these organisms [33,61]. Favorable growth conditions can emerge in aerobic soil through the formation of transient anaerobic microniches, which offer a suitable environment for these obligate anaerobic bacteria. For instance, increased respiratory activity by one or more aerobic bacteria can create anaerobic conditions allowing obligate anaerobic bacteria to survive and operate effectively [62]. Nevertheless, an ongoing debate surrounds the potential risks associated with the repeated use of digestate as fertilizer.

Interestingly, our observations indicate that the addition of biochar led to a substantial reduction in the presence of Clostridiaceae, particularly those belonging to Cluster I and II. Similarly, decreases in the relative abundances of Clostridiaceae due to biochar application were also observed in studies by Zhu et al. [63] and Tang et al. [17]. This suggests that biochar may be effective in mitigating the impact of Clostridiaceae resulting from the spread of digestate. While the mechanism by which biochar achieves this reduction is not fully clear, it could be related to an increase in soil aeration [64] that interferes with *Clostridium* survival in the soil, or to the ability of biochar to facilitate electron transfer in the surrounding environment, acting as an electron mediator of exoelectrogenic microorganisms as proposed by several authors [9,65,66]. Further research is needed to explore the potential of biochar as a valuable tool in minimizing the impact of Clostridiaceae in soil ecosystems influenced by digestate application.

In terms of GHG emissions, the dosage of biochar played a crucial role in CO_2 emissions, resulting in a divergent trend with a significant increase at lower concentrations (2%) and a substantial reduction at higher concentrations (10%). In lower concentrations, biochar may stimulate microbial activity by serving as a substrate for microbial growth, influencing nutrient availability, and promoting microbial metabolism [67]. Conversely, the strong reduction in CO_2 emissions at higher biochar concentrations suggests the suppression of microbial metabolic activities or carbon turnover rates [68]. However, this observed divergent pattern underscores the importance of considering biochar dosage as a crucial factor in its impact on soil processes.

In the soil, a consistent trend of CH_4 uptake is evident throughout the incubation period, indicating a prevailing microbial activity responsible for CH_4 consumption. This pattern is suggestive of the presence of methanotrophic microorganisms known for utilizing CH_4 as both C and energy source [48]. The biochar amendment, however, resulted in a general reduction in CH_4 uptake. Surprisingly, contrasting differences were noted in *pmoA* gene abundance, a marker associated with methane oxidation processes. These results suggest that biochar application may have influenced the activity of methanotrophic microbial community rather than its abundance and dynamic.

The observed pattern of CH_4 consumption appears to contradict the significant increase in *mcrA* gene copy number in soil amended with digestate. The *mcrA* gene is commonly used as an indicator of methane production potential [47]. However, the simultaneous prevalence of methanotrophs, aerobic microorganisms that oxidize CH_4, may have led to a net reduction in CH_4 emissions. This suggests that although digestate may increase CH_4-producing microorganisms, the application of biochar might aid in aerating the soil and promoting the activity of CH_4-consume microorganisms [14].

Furthermore, it is crucial to assess the persistence, encompassing both resistance and resilience, of the introduced microorganisms in the soil following digestate application. Current evidence suggests that, over time, the soil microbial community tends to recover and restore itself to levels comparable to the control group after digestate application [36,58]. This insight is valuable in comprehending the long-term impacts of digestate on soil microbiota.

The addition of biochar led to a significant increase in N_2O soil emissions, particularly at a 10% dose, contradicting the documented lower emissions and increased reduction to N_2 observed in various field and laboratory experiments [12–14]. This contrasts with the trend observed in CO_2 soil emissions. Nitrification is considered a crucial pathway for N_2O production in soil [69], is typically associated with an increase in CO_2 emissions, as well as N-mineralization process. Therefore, our findings suggest that, at high concentrations, the biochar used in this study inhibits both N mineralization and nitrification processes, while impacting the activity of denitrifying bacteria and leading to incomplete denitrification process with increased N_2O production. Furthermore, the addition of digestate to biochar enhances the maximum production of N_2O.

Several studies have reported conflicting effects of biochar on nitrification and N-mineralization [10]. The extensive variability in factors such as biochar type, application

rate, and soil type can account for the diverse literature reports on how biochar affects nitrification, N-mineralization, and denitrification processes influencing N_2O loss from biochar-amended soils [12]. For instance, variations in water-holding capacity and surface charge density among biochars could affect N-cycling processes in both negative and positive ways [5,70–73]. Biochar in soil directly influences the activity of ammonia oxidizers and nitrifiers by altering NH_4^+ availability. In fact, biochar can limit NH_4^+ availability to ammonium oxidizers by adsorbing NH_4^+, while favoring nitrification in soils receiving inorganic and organic N fertilizers or having higher organic C content. On the other hand, denitrification process is more significant in waterlogged soils [73]. N_2O reduction to N_2 is more prominent in fine-textured soils, where soil micropores play a role in its retention. Biochar-amended soils show a general trend of lower N_2O emissions and enhanced N_2O reduction to N_2, possibly due to the colonization of biochar micropores by denitrifiers or micropores acting as traps for N_2O [73]. Our biochar may not have the characteristics to favor denitrifiers' colonization or N_2O entrapment. Previously, it has been reported that sewage sludge-based chars differ in porosity, which is lower in sludge chars (<0.2 μm) compared to willow-based biochars (<0.6 mm) [74,75].

Our unexpected results could be attributed to the chemical composition of the biochar used, characterized by a high N content and low C content compared to other biochars reported in the literature [9,67,76]. Thus, the low C content is characteristic for sewage sludge-based biochars [77]. As a consequence, the soil treated with 10% biochar exhibited a significantly higher TN content. Additionally, the application of higher rate of biochar might have contributed to the creation of aerobic microsites favoring coupled nitrification–denitrification pathway leading to increased N_2O emissions. The reduction of N_2O to N_2 is highly influenced by oxygen exposure, particularly affecting the *nosZ* enzyme responsible for this reaction [78].

The properties of biochar undergo significant variations depending on its source materials (e.g., manure, sludge, or wood) and the conditions under which it is produced. The diverse production temperatures contribute to differences in the type and abundance of nitrogen forms present in biochar. Specifically, higher pyrolysis temperatures tend to yield biochar with an increased content of NO_3^-, while lower temperatures favor the presence of NH_4^+. Moreover, biochar produced through fast pyrolysis exhibits a larger surface area compared to biochar produced through slow pyrolysis methods. This increased surface area in fast pyrolysis enhances its capacity to adsorb inorganic N compounds or facilitating microbial N-immobilization [73]. In simpler terms, biochar produced through fast pyrolysis may possess a greater ability to capture and retain N compounds, either through physical adsorption or by fostering microbial processes that immobilize N. All of these variations have implications for biochar's potential impact on N-cycling within soil ecosystems [73].

In our mesocosm experiment, the soil treated with 10% biochar exhibits the lowest abundance of bacteria compared to the other treatments. Nevertheless, no particular relationship between bacterial abundance and treatment is evident. Similarly, the abundance of soil nitrifiers and denitrifiers does not show a particular influence due to the application of digestate and/or biochar. This contrasts with the findings of Mickan et al. [5], who documented alterations in these microbial functional groups following the application of biochar and digestate. Specifically, they recorded those soils receiving biochar significantly suppressed the abundance of *amoA* gene, while soils amended with digestate show an increase in the abundance of denitrification (*narG*, *nrfA*, *nirK*, *nosZ*) and nitrification (*amoA*, *amoB*) genes. Contrasting results were obtained by Xu et al. [69] in a pot trial under greenhouse conditions, revealing no significant responses to biochar in bacterial-*amoA*, *nirS*, and *nirK* gene transcripts, while archaeal-*amoA* and *nosZ* gene transcription was significantly stimulated.

Our results could be explained by the short-incubation period of the mesocosm experiment, which does not provide sufficient time for significant changes in functional microbial groups due to the application of digestate and/or biochar. The additional N dose from both manure-based digestate and biochar, along with labile C substrates supplied

by digestate [5], appears to primarily affect the activity of microorganisms involved in the initial steps of denitrification process. This influence might not have enough time to exert substantial modifications on the overall microbial functional potential related to the N-cycle in the soil. Nevertheless, changes in the structure of the denitrifying community provide limited predictive power for N_2O emissions [79]. Denitrification is a facultative process that relies on specific environmental conditions, and key enzymes are synthesized accordingly. Thus, *nirK*, *nirS*, and *nosZ* abundances may not be significant factors. Understanding the physiological and ecological dynamics of N_2O-reducing communities is crucial for devising strategies to mitigate N_2O emissions. While our study sheds light on the initial impacts of digestate and biochar application on soil microbial communities, a longer incubation period may be necessary to observe more significant changes in microbial function and N-cycling dynamics.

5. Conclusions

In conclusion, the study underscores the critical role of biochar composition and dosage in influencing soil GHG fluxes, emphasizing the need for tailored approaches to optimize its impact on microbial communities and nutrient availability. The unexpected stimulation of N_2O emissions by high biochar concentrations challenges previous hypothesis, highlighting the complexity of biochar-soil interactions. Understanding the specific soil and biochar characteristics and origin is crucial for sustainable agricultural practices.

Further research may be needed to identify the phyto-stimulatory or phytotoxic components in by-product organic materials and comprehend implications for crop growth and environmental sustainability. This study offers new insights into the short-term effects of anaerobic digestates and biochar on soil microorganisms, emphasizing the complexity of microbial responses. Persistence and contrasting effects on microbial functional groups highlight the need for continued research, especially exploring biochar as a tool to mitigate the impact of Clostridiaceae in soil ecosystems influenced by digestate application.

As agriculture and environmental sectors seek sustainable practices, knowledge about the resilience and recovery of soil microbiota becomes instrumental for informed decision-making regarding the repeated use of digestate as a soil amendment. Ongoing research in this area is expected to contribute further to optimizing digestate application for both agricultural productivity and environmental sustainability. Field studies with repeated digestate applications over several years, focusing on microbial communities involved in biogeochemical cycles, are essential for a holistic understanding of digestate sustainability.

Limitations in the study, such as only determining the presence of functional genes without assessing their expression or quantifying enzyme activities, call for further research to establish a link between microbial functional genes and activities. Real-time PCR quantification, while suitable for characterizing soil microbial diversity, provides limited information on microbial functioning. Future research, employing metagenomic or metatranscriptomic approaches, can provide deeper insights into the mechanisms driving soil GHG emissions.

Future perspectives should aim to scale up research findings from small-scale experiments to field-level application and assess the feasibility, practicality, and economic viability of using digestate and biochar as soil amendments on a larger scale. Establishing field experiments employing integrated approaches that combine digestate and biochar with other soil amendments or management practices can help to explore the synergistic or antagonistic effects between different amendments in order to optimize soil health, crop productivity and environmental sustainability. Educating farmers, agronomists, and stakeholders about the benefits, challenges, and best practices associated with digestate and biochar application is crucial for promoting the adoption of sustainable soil management practices.

Author Contributions: Conceptualization, R.P. and A.L.; formal analysis, R.P., A.C., F.R., C.B. and A.L.; investigation, R.P., A.C., F.R., C.B. and A.L.; resources, E.T. and I.L.; data curation, R.P. and A.L.; writing—original draft preparation, R.P., A.L., E.T. and I.L. All authors have read and agreed to the published version of the manuscript.

Funding: This study was developed in the context of the EOM4SOIL project (External organic matters for climate mitigation and soil health), from the European Joint Program for SOIL 'Towards climate-smart sustainable management of agricultural soils' (EJP SOIL) consortium funded by the European Union Horizon 2020 research and innovation program (Grant Agreement No. 862695).

Data Availability Statement: Data that support the findings of this study are available on reasonable request from the corresponding author.

Acknowledgments: The authors wish to thank Helsinki Region Environmental Services (HSY) for providing samples for this study.

Conflicts of Interest: The authors declare no conflict of interest.

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