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Effects of digestate on soil chemical and microbiological properties: A comparative study with compost and vermicompost

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HIGHLIGHTS

- Digestate led to a higher soil nitrification rate than manure in the short-term.
- *Escherichia coli* CFUs were not detected in any of the amended soils after 60 days.
- The lowest *Clostridium perfringens* CFUs were found in digestate treatment after 60 days.
- DGGE patterns showed that the date of sampling had an influence on soil AOB community.

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ABSTRACT

Anaerobic digestion has become increasingly popular as an alternative for recycling wastes from different origins. Consequently, biogas residues, most of them with unknown chemical and biological composition, accrue in large quantities and their application into soil has become a widespread agricultural practise. The aim of this study was to evaluate the effects of digestate application on the chemical and microbiological properties of an arable soil in comparison with untreated manure, compost and vermicompost. Once in the soil matrix either the addition of compost or digestate led to an increased nitrification rate, relative to unamended and manure-treated soil, after 15 and 60 days of incubation. Faecal coliform and *E. coli* colony forming units (CFUs) were not detected in any of the amended soils after 60 days. The highest number of *Clostridium perfringens* CFUs was recorded in manure-amended soil at the beginning of the experiment and after 15 days; whilst after 60 days the lowest CFU number was registered in digestate-treated soil. Denaturing gradient gel electrophoresis patterns also showed that besides the treatment the date of sampling could have contributed to modifications in the soil ammonia-oxidising bacteria community, thereby indicating that the soil itself may influence the community diversity more strongly than the treatments.

1. Introduction

The development and introduction of appropriate technologies represents a crucial asset in terms of waste resource efficiency and management. Biological waste treatment processes like composting and vermicomposting have been used to process a great variety of solid organic waste materials under aerobic conditions [1]. Anaerobic digestion (AD) has also become increasingly popular as an alternative for recycling wastes from different origins, such as farm and agroindustrial residues as well as domestic organic wastes [2–7]. This relies on the fact that on the one hand the available fossil fuel reserves are decreasing and, on the other hand the biogas produced by AD is utilised as an eco-friendly energy source [8].

The proper use of the digested material resulting from AD contributes to the sustainability of the production of biogas. In this sense, the use of digestate as an organic amendment in agriculture can therefore represent a suitable option, as it can act as an important source of nutrients for plants as recently summarised by Insam et al. [4]. Several factors including the soil type, the climate, the frequency of application and the properties of the digestate are important drivers affecting the magnitude of nutrient accumulation and its distribution in the soil profile [6,9–11]. Previous studies have found that amending the soil with digestate led to an improvement of soil aggregation, facilitating the maintenance of the structure, drainage and aeration of the soil thanks to the addition of organic matter [12–14]. From a microbiological viewpoint, an increase in microbial biomass and activity of several enzymes

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Table 1
Main properties of the four different substrates (cattle manure, compost, vermicompost and digestate) used in the present study. Values are means ± SE.

	Manure	Compost	Vermicompost	Digestate
pH	8.99 ± 0.02	8.73 ± 0.01	7.18 ± 0.02	8.01 ± 0.01
EC (mS cm^{-1})	2.15 ± 0.05	2.56 ± 0.05	0.28 ± 0.01	15.73 ± 0.03
Total carbon (%)	39.71 ± 1.21	35.73 ± 2.79	30.44 ± 1.71	38.06 ± 0.43
Total nitrogen (%)	2.95 ± 0.12	2.91 ± 0.28	2.16 ± 0.17	2.30 ± 0.04
C to N ratio	13.51 ± 0.43	12.55 ± 1.34	14.12 ± 0.17	16.58 ± 0.58
NH_4^+ (g kg^{-1} dw)	2.47 ± 0.14	5.58 ± 0.54	1.61 ± 0.03	15.91 ± 1.49
NO_3^- (g kg^{-1} dw)	0.13 ± 0.001	0.33 ± 0.04	0.23 ± 0.01	0.82 ± 0.08
Faecal coliforms (CFUs g^{-1} dw)	1700 ± 173	450 ± 38	1103 ± 119	1340 ± 265
E. coli (CFUs g^{-1} dw)	1180 ± 135	207 ± 20	960 ± 85	880 ± 63
C. perfringens (CFUs g^{-1} dw)	1667 ± 88	2350 ± 120	1900 ± 153	2167 ± 67

EC—electrical conductivity.
dw—dry weight.
SE—standard error.

involved in C and N cycles following the addition of digestate into soil has been observed [2,15–16]. AD processes have also been accompanied by a depletion of the amount of organic C [17] and in turn, less organic C is available for the growth and activity of soil microbial communities [12], which may affect the turnover of mineral N after the application of digestates into the soil-plant system. In spite of this fact, AD appears not to negatively affect the soil organic C status in the long-term, even though a post-treatment of digestates has been suggested by several authors as a means of improving their C sequestration [4].

With regard to the pathogenic load, Franke-Whittle and Insam [18] summarised that AD generally reduces the pathogen risk in comparison with untreated substrates, with the exception of prions and spore-forming bacteria. In fact, pathogenic bacteria with a spore-forming capacity like *Clostridium* and *Bacillus* species have been found to survive the process of AD and persist in the digestate in particular for mesophilic reactors [4,19,20]. The sanitation of the digestate will mainly depend on the conditions of the reactor and the type of substrate fed into it [21,22]. In this context, *Clostridium* species such as *Clostridium perfringens* are known to inhabit the gastrointestinal system of warm-blooded animals, which highlights the fact that a major risk could exist when digestate produced from the AD of animal manures is applied to soil as an organic amendment [19,23]. The fact that some pathogens can exhibit a regrowth after the deposition to soil also increases the risk for the environment derived from the application of this type of amendments [24].

The aim of the present study was therefore to investigate the effects of digestate application on the chemical and microbiological properties of an arable soil in comparison with its ingestate (i.e. untreated manure) and two other widely-known organic amendments, compost and vermicompost. For this purpose we determined the changes in (i) mineral N concentration (NH_4^+ and NO_3^-) and rate of nitrification; (ii) levels of microbial biomass and activity; and (iii) survival of selected pathogens (*E. coli*, faecal coliforms and *Clostridium perfringens*) in all the treated soils. Moreover, we also evaluated whether the application of digestate affected the community structure of ammonia-oxidising bacteria (AOB), which are responsible for ammonia oxidation, the first and rate limiting step in the process of nitrification. The quality of the digestate, which is related to the efficiency with which the process of AD took place, together with the characteristics of the soil native microbiota are expected to be important factors in determining the effects of the digestate on soil properties.

2. Material and methods

2.1. Soil and organic substrates

Soil samples were taken from an arable field located near the agricultural school in Rotholz (Austria, 47°23′27″N 11°47′52″O) in April, 2011. Soil samples were homogenised and sieved (<2 mm) before the experiment started. The main soil properties were as follows: loamy-silty texture, pH ($CaCl_2$) 7.11 ± 0.001, electrical conductivity (EC) 0.15 ± 0.002 mS cm^{-1}, total C 5.60 ± 0.07%, total N 0.48 ± 0.008%, NH_4^+ content 0.64 ± 0.04 mg kg^{-1} dw, and NO_3^- content 4.55 ± 0.30 mg kg^{-1} dw.

The main chemical properties of the different substrates used in the present study are shown in Table 1. The cattle manure and the digestate were sampled from the BIO4GAS® plant located in Garmisch (Tirol, Austria). This biogas plant is composed of a two chamber reactor, which is characteristic of the BIO4GAS®-technology, with a filling volume of 400 m^3. The process temperature of the mesophilic digester tank amounted to 48 °C; and 4–5 m^3 of cattle manure were filled into the tank per day, representing a hydraulic retention time of 40 days. Both the manure and the digestate were collected from the biogas plant no later than three days prior to the start of the experimental set up and stored at 4 °C until use. Compost and vermicompost were obtained from the vermicomposting company Ecocelta (Galicia, NW Spain). Briefly, for compost production cattle manure was subjected to a thermophilic phase in trenches with approximate dimensions of 50 m long, 2 m wide and 2.5 m high for two weeks. Throughout this phase the trenches were turned daily for aeration in order to avoid substrate compaction as well as to homogenise the composting mass, in which the temperature reached and kept the value of 68 °C during 4 days. Once the thermophilic phase had finished, the material was left undisturbed for a further 3-month maturation period, and the finished by-product was collected to analyse. The vermicomposting process was carried out in vertical continuous feeding reactors (45 × 22 × 20 cm), in which new modules with cattle manure were added sequentially according to the feeding activity of the earthworm population (*Eisenia andrei*). After a 3 month-period the vermicompost from the lowermost module, in which there was no earthworm presence, was used for the present study.

2.2. Experimental set-up

The experiment was performed in Perspex columns (11 cm diameter, 20 cm depth) with a tight mesh (0.5 mm pore size) in the bottom of each column, and filled with 2000 g soil each (fresh weight). The studied amendments (cattle manure, compost, vermicompost and digestate) were mixed with the soil columns by turning at a rate equivalent to 80 kg N ha^{-1}, considering the soil bulk density of 1 g cm^{-3} and a plough depth of 20 cm. A control treatment that consisted of soil without the addition of any of the amendments was also included. Soil water content was adjusted to 50% water-holding capacity (100% WHC = 0.65 mL H_2O g^{-1} soil) in all of the differently treated soils with distiled water. A total of 45 experimental units (5 amendment levels × 3 incubation times × 3

Table 2
Overview of the physico-chemical properties in the differently treated soils at the 3 incubation times (0, 15 and 60 days). Values are given for $n = 3$ (standard error in brackets).

Soil treatments	pH			EC (μS cm^{-1})			Total C (%)			Total N (%)		
	Incubation time (days)											
	0	15	60	0	15	60	0	15	60	0	15	60
Unamended	6.9 (0.3)	6.6 (0.03)	6.8 (0.03)	153.5 (0.3)	178.3 (4.3)	251.3 (4.9)	4.6 (0.04)	4.5 (0.45)	4.3 (0.2)	0.4 (0.02)	0.3 (0.02)	0.3 (0.02)
Manure	7.1 (0.01)	7.2 (0.01)	6.7 (0.02)	310.0 (0.02)	250.7 (24.1)	372.7 (4.9)	4.7 (0.06)	4.8 (0.1)	4.5 (0.3)	0.4 (0.001)	0.4 (0.001)	0.3 (0.03)
Compost	7.3 (0.02)	7.3 (0.01)	6.8 (0.01)	823.7 (0.01)	971.7 (30.2)	1164.7 (30.1)	5.0 (0.03)	5.0 (0.11)	4.9 (0.3)	0.4 (0.03)	0.4 (0.01)	0.5 (0.06)
Vermicompost	7.0 (0.1)	7.1 (0.01)	6.8 (0.01)	248.0 (0.01)	220.0 (8.7)	295.7 (8.3)	5.1 (0.2)	5.1 (0.1)	4.9 (0.02)	0.3 (0.03)	0.3 (0.02)	0.3 (0.001)
Digestate	7.0 (0.03)	7.1 (0.02)	6.7 (0.02)	297.3 (0.02)	351.3 (4.2)	433.3 (17.1)	1.5 (0.1)	1.8 (0.09)	1.7 (0.05)	0.1 (0.02)	0.1 (0.01)	0.09 (0.01)

EC—electrical conductivity.

Table 3
Two-way ANOVA for the chemical and microbiological parameters analysed in this study.

Parameters	Amendment treatment		Time		Amendment treatment × time	
	F	*P*	*F*	*P*	*F*	*P*
pH	2.3	0.07	16.7	0.0001	0.9	0.15
EC (μS cm^{-1})	287.4	$P < 0.0001$	24.2	$P < 0.0001$	3.6	0.005
Total C (%)	8.8	$P < 0.0001$	1.2	0.31	0.5	0.84
Total N (%)	12.1	$P < 0.0001$	2.6	0.12	1.7	0.55
NH_4^+ (mg kg^{-1} dw)	27.3	$P < 0.0001$	92.1	$P < 0.0001$	25.2	$P < 0.0001$
NO_3^- (mg kg^{-1} dw)	132.3	$P < 0.0001$	75.3	$P < 0.0001$	9.9	$P < 0.0001$
Nitrification rate (mg kg^{-1} dw 24 h^{-1})	10.6	$P < 0.0001$	24.8	$P < 0.0001$	2.2	0.06
Basal respiration (mg C kg^{-1} dw h^{-1})	193.6	$P < 0.0001$	252.6	$P < 0.0001$	131.6	$P < 0.0001$
SIR (mg C—CO_2 kg^{-1} dw h^{-1})	68.5	$P < 0.0001$	80.1	$P < 0.0001$	8.9	$P < 0.0001$
C. perfringens (CFUs g^{-1} dw)	12.2	$P < 0.0001$	29.7	$P < 0.0001$	9.1	$P < 0.0001$

EC—electrical conductivity.
SIR—substrate-induced respiration.

replicates) were set up in the present study. Three extra columns containing soil were also incubated throughout the whole period for humidity control, which was adjusted as needed in all of the columns. After an equilibration period of 4 days at 4 °C, 15 columns were dismantled and the sample material was collected to analyse (referred as the start of the experiment, i.e. incubation time 0 months). The remaining thirty columns were then maintained at 22 °C, which is the average temperature of the hottest and wettest month in the sampling area and it was expected to be the most suitable for the survival of pathogens [25]. These columns were destructively sampled after 15 and 60 days in order to determine the effects of the different amendments on the following parameters, as detailed below.

2.3. Methods

Water holding capacity (WHC) was estimated gravimetrically. To estimate the soil moisture at 100% WHC, glass tubes were filled with soil, saturated with distiled water and allowed to drain for 3 h on silica sand [26]. Soil samples (10 g, fresh weight) were placed into a Petri dish and oven-dried (105 °C) for at least 24 h. After this time, Petri dishes with dried soil samples were weighed and total solids were determined. The organic matter (OM) content was determined from the weight loss following ignition in a muffle furnace (Carbolite, CWF 1000) at 550 °C for 5 h. Total C and N contents were analysed in dried samples, using a CN analyser (TruSpec CHN; LECO, Michigan, U.S.A.). EC and pH were determined in distiled water and 0.01 M $CaCl_2$ extracts (10:25, *w*/*v*), respectively. Inorganic nitrogen (NH_4^+ and NO_3^-) was determined in 0.0125 M $CaCl_2$ extracts, as described by Kandeler [27,28]. The actual nitrification rate was determined following the method proposed by Berg and Rosswall [29], as modified by Kandeler [30]. Basal respiration was measured as CO_2 evolution from soil samples (70 g, fw) at 22 °C, using a continuous flow infrared gas analyzer system (IRGA) [31]. Substrate-induced respiration (SIR) was also determined by using the IRGA. Soil samples were mixed with 1% (w/w) glucose, and the substrate-induced CO_2 production rate was determined 6 h after the glucose amendment [31]. Soil suspensions for pathogen cultivation (1:10 soil: 0.95% NaCl) were prepared and shaken at 200 rpm for 15 min. After letting them settle for 1 h, 1/10 serial dilutions from the supernatant down to 10^{-3} were prepared in 0.95% NaCl. Faecal coliforms colony forming units (CFUs) were determined after incubation at 44 °C for 24 h in a violet read bile agar [32]. *E. coli* CFUs were determined by incubating the samples in Trypton Bile X-lucuronide and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide at 37 °C for 3 h, and then at 44 °C for 18–24 h [33]. *C. perfringens* CFUs were determined after incubation at 44.5 °C for 24 h in anaerobic conditions [34].

Total DNA was extracted in triplicate from 0.25 g of soil samples taken on days 0 (t_0) and 60 (t_{60}) using the PowerSoil DNA Isolation Kit (MO Bio Laboratories Inc., Carlsbad, USA) according to the manufacturer's protocol, with some minor modifications as shown in Fekadu et al. [35]. DNA yield and quality were assessed as indicated in Fekadu et al. [35]. The amplification of AOB communities was performed by using the two forward CTO primers 189f A/B and 189f C in a ratio of 2:1, and the reverse primer CTO654r, by following the cycling conditions described in Kowalchuk et al. [36]. The denaturing gradient gel electrophoresis (DGGE) was performed by loading 60 ng of PCR products in a 7% (*w*/*v*) polyacrylamide gel in 1xTAE (20 mM Tris–Cl, 10 mM acetate, 0.5 mM Na_2EDTA) containing a denaturing gradient of 40–60% (100% denaturants consisting of 7 mol/L urea and 40% formamide). Gels were run and stained as shown in Fekadu et al. [35]. The comparison of DGGE patterns were assessed with the GelCompar II software package (Applied Maths, Kortrijk, Belgium). A cluster analysis was performed using the Ochiai correlation coefficient and the unweight-pair-group method with the arithmetic averages (UPGMA) clustering algorithm. The programme settings were at 1.0% optimisation and 1.0% position tolerance.

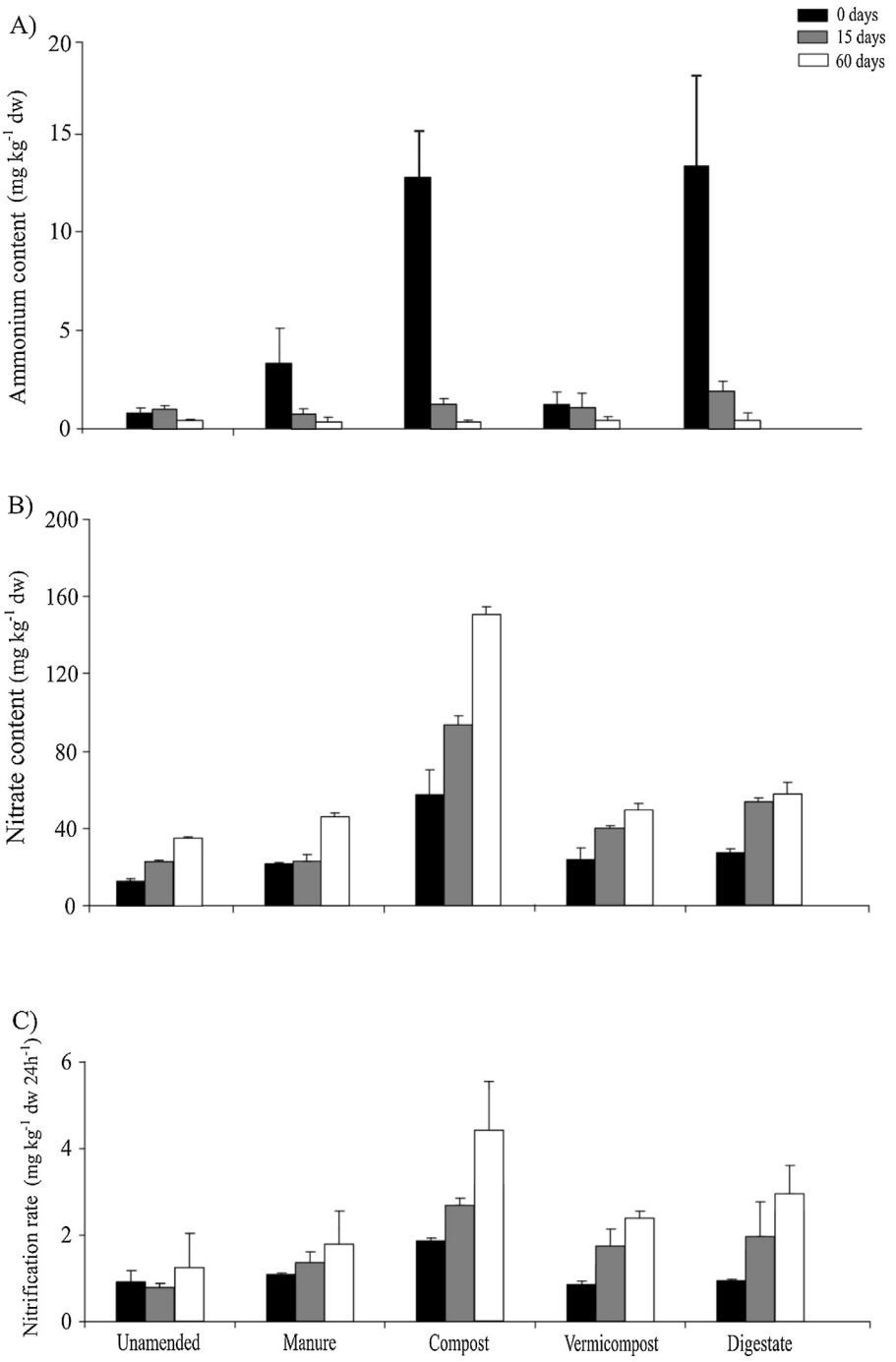

Fig. 1. Changes in (a) NH_4^+ content, (b) NO_3^- content, and (c) the rate of nitrification in the control soil (unamended) and the soil amended with manure, compost, vermicompost and digestate at the 3 incubation times (0, 15 and 60 days). Values are means ± SE.

2.4. Statistical analysis

Significant differences amongst the differently treated soil samples regarding the chemical and microbiological parameters were determined by a two-way analysis of variance (ANOVA), in which two factors were fixed: type of amendment (none, cattle manure, compost, vermicompost and digestate) and incubation time (0, 15 and 60 days). Significant differences in the main effects were further analysed by paired comparisons with the Tukey HSD test. The normality and the variance homogeneity of the data were tested prior to ANOVA. All the analyses were performed with the Statistica software program v9.

3. Results and discussion

Bioenergy from renewable organic materials is currently promoted in many countries. Consequently, biogas residues, most of them with unknown chemical and biological composition, accrue in large quantities and their application into soil has become a widespread agricultural practise [4]. The evaluation of the soil chemical properties is an important aspect to bear in mind, as variations in either soil pH or electrical conductivity might affect the nutrient availability and uptake, as well as the biomass, activity and composition of the soil microbial community [37]. In the present study, all of the amended soils had a pH close to the control

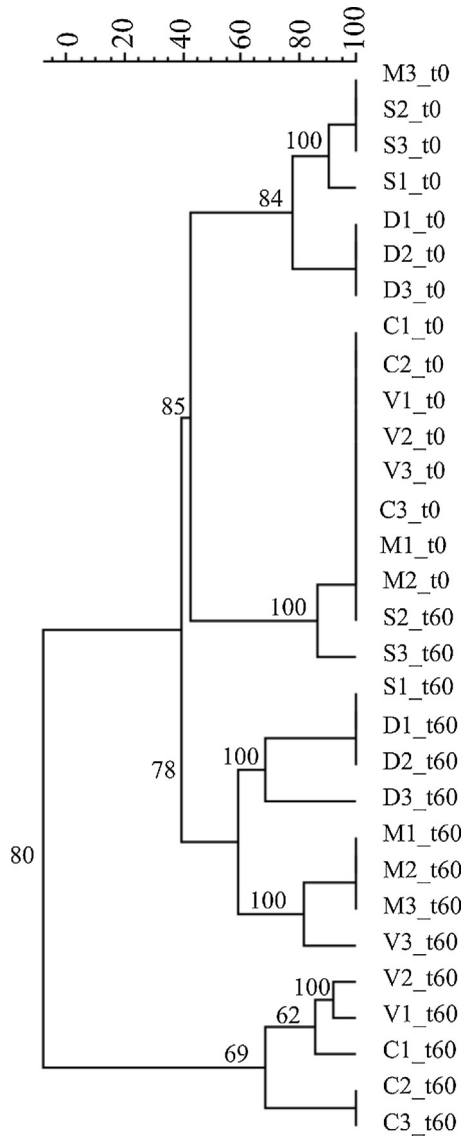

Fig. 2. Cluster analysis of ammonia-oxidising bacterial (AOB) fingerprints based on 16S rRNA gene obtained from the control soil (S) and the soil amended with manure (M), compost (C), vermicompost (V) and digestate (D) at the starting of the incubation period (t_0) and after 60 days incubation (t_{60}). Values at the branches of the dendrograms correspond to the percentage of similarity, based on the Ochiai correlation coefficient.

soil throughout the whole incubation period (Table 2). Electrical conductivity was influenced by the type of amendment (ANOVA $F_{4,30} = 287.4$, $P < 0.0001$; Table 3), and the highest EC, relative to the control, was found in the compost-amended soil at the beginning of the trial and after either 15 or 60 days (around 4–5 times higher; Table 2). Higher EC levels than that in the control (around 1.5–2 times higher) were also registered for the remaining treatments (Table 2); with higher EC values after 60 days incubation (ANOVA $F_{2,30} = 24.2$, $P < 0.0001$; Table 3). Nonetheless, in any of the amended treatments, soil conductivity did not exceed the threshold level of 2 dS m^{-1} proposed by Herrero and Pérez-Coveta [38] for reasons of plant health. The amount of total C was affected by the nature of the organic waste (ANOVA $F_{4,30} = 8.76$, $P < 0.0001$), being around 3 times lower in digestate-amended soil compared to the control and the other treated soils (Table 2). However, no significant changes were found in this parameter over time (Table 2), regardless of the type of amendment (amendment treatment × time, $F_{8,30} = 0.51$, $P = 0.84$; Table 3). As occurred with total C, the total N content was also significantly lower in digestate-amended soil (around 3–4 times lower; ANOVA $F_{4,30} = 12.1$, $P < 0.0001$) irrespective of the sampling time (Table 3). It is widely known that during the AD process the organic N compounds of the substrate fed into the reactor are mineralised, resulting in increased levels of the soluble forms of inorganic N, mainly ammonia [39]. Accordingly, in the current study the initial digestate had a level of NH_4^+ higher than that in the manure (Table 1). Once applied to the soil, the highest NH_4^+ concentration (close to 15 mg kg dw^{-1}) was found in the treatment amended with digestate (Fig. 1A), as well as in compost-amended soil at the start of the experiment (ANOVA $F_{4,30} = 27.3$, $P < 0.0001$; Table 3). However, after 15 days of incubation there was a remarkable drop in NH_4^+ concentration, relative to time zero, in both treatments (Fig. 1A); thereby reaching a value (around 0.5–1 mg kg dw^{-1}) similar to that observed in the unamended soil and those soils amended with manure and vermicompost (amendment treatment × time $F_{8,30} = 25.2$, $P < 0.0001$; Fig. 1A, Table 3). This might have occurred most likely through nitrification during incubation since, as previously reported by Goberna et al. [25], losses of NH_4^+ as a consequence of volatilisation seem more unlikely because the amendments were mixed with the soil and not spread on the surface. Accordingly, an increase in NO_3^- content was found in the amended soils over time (ANOVA $F_{2,30} = 75.3$, $P < 0.0001$, Fig. 1B, Table 3) in comparison with the beginning of the experiment. Higher nitrification rates were also recorded over time (ANOVA $F_{2,30} = 24.8$, $P < 0.0001$; Fig. 1C, Table 3), being the values in compost- and digestate-amended soils greater than those recorded in either unamended or manure-treated soils after 15 and 60 days of incubation (ANOVA $F_{4,30} = 10.6$, $P < 0.0001$; Fig. 1C, Table 3). These results indicated that the processing of manure through either aerobic or anaerobic pathways contributed to soil nitrification, as once in the soil matrix the final products of these processes (composts/digestates) led to an increased nitrification rate in the short-term. Along these lines, Johansen et al. [40] also observed, at a microcosm level, that amending soil with two anaerobically digested materials (cattle slurry/maize and cattle slurry/grass clover) increased the soil concentration of NO_3^- circa 30–40% compared to raw cattle slurry. All in all it points out that the application of digestate into soil might reduce the needs for supplemental mineral N fertilisation.

Nonetheless, the use of digestate as an organic soil amendment should match the crop demand in order to avoid a risk of N leaching, as inorganic N surface run-off or leaching through the soil profile occurs mainly in the form of nitrate [25,41]. These latter authors found that amending an arable soil with digestate resulted in N losses (around 45% of the total N contained in the soil) in the form of nitrate during an incubation period of 100 days. Nyberg et al. [42] also found that depending on the dose, the digestate could contain compounds inhibitory to ammonia-oxidising activity, which affected negatively the rate of potential ammonia oxidation in the soil after 12 weeks of incubation. In our study, DGGE patterns showed that the AOB communities clustered into two main groups with 80% similarity (Fig. 2). On the one hand, the first group was divided into two main subclusters with regard to the incubation time: one of which was comprised of the unamended soil and all of the treated soils from day 0 (85% similarity, Fig. 2), with the exception of the presence of two replicates belonging to control soil from day 60 (S_2 and $S_3\_t_{60}$); and the other subcluster was represented by digestate- and manure-amended soils from day 60 (78% similarity, Fig. 2). On the other hand, the second group was comprised of vermicompost- and compost-amended soils from day 60 (except for the replicate $V_3\_t_{60}$) (69% similarity, Fig. 2). All in all suggested that besides the different amendment treatments the date of sampling could have contributed to modifications in the soil AOB community. Accordingly, Calbrix et al. [43] also found that

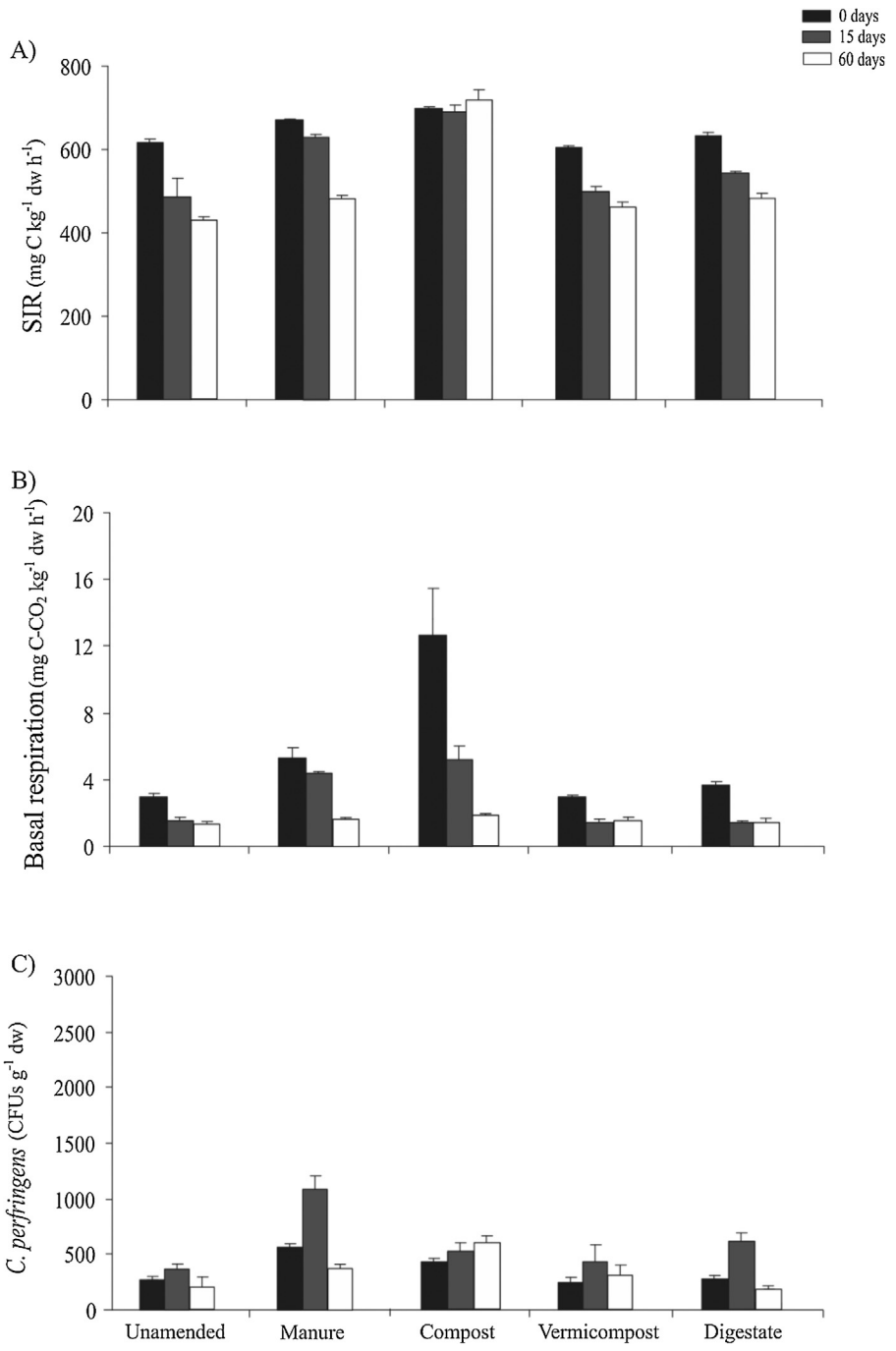

Fig. 3. Changes in (a) microbial activity, measured as basal respiration; (b) microbial biomass, assessed by substrate-induced respiration (SIR); and (c) *Clostridium perfringens* CFUs in the control soil (unamended) and the soil amended with manure, compost, vermicompost and digestate at the three incubation times (0, 15 and 60 days). Values are means ± SE.

the date of sampling largely influenced on the bacterial community structure of an agricultural soil in a fertiliser experiment.

The supply of readily metabolizable C in organic waste is considered one of the most influential factors affecting both soil microbial biomass and activity. Odlare et al. [44] established that the C in biogas residues is more easily degradable than that of composts mainly due to the fact that the mineralisation is less efficient under anaerobic conditions; as such, when biogas residues are applied to soil, their C content is expected to be more rapidly metabolised and thus is likely to enhance soil microbial biomass in the short-term. However, in our study we found that on the one hand the addition of digestate did not induce a significant increase in soil microbial biomass and activity, relative to the unamended soil and raw manure, irrespective of the sampling time (Fig. 3A and B). Similar results were reported by Johansen et al. [40]. And, on the other hand, the highest level of microbial biomass was recorded following compost addition after 15 and 60 days of incubation (amendment treatment × time $F_{8,30} = 8.9$, $P < 0.0001$; Table 3). A higher microbial activity (13.70 ± 1.5 mg C—CO_2 kg^{-1} dw h^{-1}) was also recorded in compost-amended soil at the beginning of the incubation (ANOVA $F_{4,30} = 193.6$, $P < 0.0001$; Fig. 3B, Table 3); whilst at the end of the experiment (60 days) all of the differently treated soils reached a level of microbial activity close to that in the control (1.32 ± 0.02 mg CO_2—C kg^{-1} dw h^{-1}; Fig. 3B). A direct effect of exogenous microor-

ganisms' compost could have contributed to the initial increase in microbial biomass and activity, although the ability of the native soil microbiota to outcompete the allochthonous microorganisms applied might minimise such effect. This fact has been previously suggested for compost amendments concerning fungal denaturing gradient gel electrophoresis profiles [45].

Cultivable forms of faecal coliforms and *E. coli* were isolated from all of the initial amendments (Table 1), although the levels of these pathogenic bacteria were around 3 and 5 times lower in compost, respectively (Table 1). The composting process, unlike vermicomposting, involved a four-day thermophilic phase, during which the substrate was subjected to a temperature of 70 °C, which is expected to reduce the pathogenic load in the composting mass. Moreover, it has been reported that the functioning of vertical continuous feeding vermireactors, like those used in the present study, can promote a reduced aeration derived from the accumulation of layers and compaction of the substrate, which could have favoured pathogen survival [46]. The aforementioned pathogenic bacteria were also detected in the digestate. As stated by Pepper et al. [21], nutrient availability is considered as one of the major factors influencing pathogen survival in biogas digesters. Therefore, one plausible explanation could be that feeding the reactor with 4–5 m^3 of cattle manure per day provided enough nutrients to maintain a population of the studied pathogens after 40-days of anaerobic digestion.

Once applied to soils, faecal coliform CFUs were recorded in all of the differently treated soils at the beginning of the incubation, with highest levels in manure-amended soil (1300 ± 58CFUs g^{-1} dw), followed by digestate and vermicompost treatments (1133 ± 56 and 900 ± 33CFUs g^{-1} dw, respectively); whilst the soil amended with compost had a lower number of faecal coliforms CFUs (400 ± 53CFUs g^{-1} dw). After 15 days faecal coliform CFUs were only found in manure- and digestate-treated soils (297 ± 24 and 183 ± 16CFUs g^{-1} dw, respectively); whereas no detection was recorded for any of the treatments after 60 days. *E. coli* CFUs were only detected in manure-amended soil reaching a value of 307 ± 25CFUs g^{-1} dw and 83 ± 3CFUs g^{-1} dw at the start of the experiment and after incubation for 15 days, respectively. The survival period of these pathogenic bacteria in soil is usually no longer than some days or weeks, despite previous studies have shown that *E. coli* can survive for periods of up to 40–68 days in soil following the application of pig manure [47]. Several environmental factors including temperature, soil moisture and pH may have a large influence on the survival time of faecal bacteria in soil [48]. The manure application method as well as the microbial competition with the native soil microbiota can also be determinants in governing the survival of potential pathogens in amended soils [47]. In this context, Goberna et al. [25] observed that the numbers of cultivable potential pathogens such as *Listeria* and *Salmonella* were more abundant in γ- than in non-irradiated arable soils, indicating that they proliferated better in soils lacking an autochthonous microbiota, probably due to the reduced competition for resources and large niche availability.

C. perfringens CFUs were detected in all of the initial amendments, with values ranging from 1600 to 2400CFUs g^{-1} dw (Table 1). This is not surprising as this bacterial species is in general heat-resistant in its spore form, and consequently it is expected not to be greatly reduced during the composting treatment [49]. Moreover, Aira et al. [46] found that there were no changes in the levels of vegetative and sporulated forms of *Clostridium* in an industrial-scale vermireactor fed on cattle manure, irrespective of the age of the vermicompost layers. Olsen and Larsen [20] also observed that the spores of *C. perfringens* were not inactivated in either mesophilic or thermophilic biogas digesters. Similar results were also observed by Chauret et al. [50] and Aitken et al. [51] in a reactor operating under mesophilic and thermophilic conditions, respectively. Once applied to soil, the highest number of *C. perfringens* CFUs, relative to the control, was registered in manure-amended soil at the beginning of the experiment and after 15 days of the incubation (560 ± 69CFUs g^{-1} dw and 1090 ± 90CFUs g^{-1} dw, respectively; Fig. 3C). After 60 days the lowest CFU number was recorded in digestate-amended soil (235 ± 48CFUs g^{-1} dw), and this value was closer to that in the control soil (amendment treatment × time $F_{8,30} = 9.1$, $P < 0.0001$; Fig. 3C, Table 3). Nevertheless, it should be noted that the digestate has a low solid content and in turn, any microorganism supplied with this substrate could have a higher mobility and more easily reach deeper soil layers.

Although the present findings should not be extrapolated to all soil types and field conditions, it is expected that the current work adds further evidence as to the potential value of AD and the impact of digestate into soil properties. Future studies carried out in a longer term and varying the source and application rate of digestates will contribute to shed light into the agronomic effects of biogas residues for agricultural purposes.

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