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Biogas Slurry as an Alternative to Chemical Fertilizer: Changes in Soil Properties and Microbial Communities of Fluvo-Aquic Soil in the North China Plain

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Abstract: Biogas slurry application offers an alternative to chemical fertilizer in realizing ecologically recycling agriculture. However, the responses of soil fertility and microbial communities to long term use of biogas slurry need to be explored in different soil types and regions. We investigated the effects of repeated applications over six years on the soil properties and microbial characteristics of a fluvo-aquic soil in the North China Plain. The experiment, with equivalent nitrogen inputs, comprised: biogas slurry (BS), chemical fertilizer (CF) or substitution of half the chemical fertilizer with biogas slurry (BSCF); a control treatment had no fertilizer addition. Soil samples, at a depth of 0–20 cm, were collected for their physicochemical properties. Microbial community diversity and composition was investigated using high-throughput sequencing. Biogas slurry application treatments tended to lower the soil bulk density while increasing the water-holding capacity and the water-stable aggregate mean weight diameter. Organic carbon and available nutrient concentrations (nitrogen, potassium and phosphorus) were enhanced in all fertilization treatments relative to the control, especially in the BSCF treatment. Significant differences in microbial community composition were detected between the control and all of the fertilization treatments. BSCF resulted in the greatest diversity and most evenly balanced assemblages of both bacteria and fungi at the phylum level. There were clear associations between microbial composition and changes in soil environmental variables caused by the fertilization treatments. Bacterial community composition and alpha diversity were associated particularly with differences in soil total nitrogen, pH, and available potassium, whereas fungal communities were more related to available potassium. Half substitution of the chemical fertilizer by biogas slurry gave the greatest improvement in soil structure and nutrient availability and this was associated with greater microbial diversity and better balanced microbial communities. Our results suggest that partial substitution with biogas slurry is an alternative to complete chemical fertilizer and that it offers clear benefits for the topsoil structure and fertility in fluvo-aquic soils. It also represents a promising approach to a biogas-linked agroecosystem that restores sustainable coordination between cropping and animal husbandry under an intensive production regime.

Keywords: biogas slurry; physiochemical properties; microbial community; fluvo-aquic soil; North China Plain

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1. Introduction

The rapid expansion of the livestock and poultry industries has met the rising demand for meat, eggs, and milk, however, it has created a thorny problem of how to dispose of the large amounts of livestock and poultry wastes generated [1]. In China, more than 3.9 billion tons of livestock and poultry wastes are produced annually. Furthermore, larger and more specialized production units have increasingly emerged over the last decade, weakening the links between livestock and crop production and, in the absence of appropriate waste disposal, raising environmental concerns [1–3]. Anaerobic digestion has gained popularity around the world as an environmentally friendly process for the disposal of agricultural wastes [4]. In addition to producing biogas, a renewable clean energy source, the process generates digestates (biogas residue and biogas slurry) that can be utilized in agricultural production [5–7]. Nevertheless, the massive volume and fluid properties of biogas slurry can incur extensive storage requirements and transportation costs; this constraint on its rational and effective utilization is the most serious bottleneck impeding sustainable development of middle- and large-scale animal husbandry [8].

Biogas slurry is regarded as a liquid organic fertilizer with readily available nutrients and bioactive substances that can be easily utilized by plants [3]. Numerous studies have shown that its application can improve soil structure, supplement soil nutrients, suppress soilborne diseases, and ultimately increase productivity [9–12]. In comparison with conventional chemical fertilizer alone, biogas slurry has demonstrated roles in regulating soil acidity, promoting soil aggregation and increasing organic carbon stock, all of which are critical for agroecosystem sustainability [3,13]. Thus, its application is an alternative fertilization regime that, potentially, can not only decrease chemical fertilizer consumption and other agricultural inputs, but can also save the high cost of traditional waste disposal. It has become a major approach for efficient resource utilization in more agriculturally developed and developing countries [14].

Microorganisms, the driving force of material transformations and energy metabolism in soil ecosystems, are extremely sensitive to the changes in environmental conditions associated with fertilization [9,15]. Topsoil is the main site of microbial activity and, therefore, its microbial community composition and diversity are seen as indicators of soil fertility and potential crop productivity [12]. Microbes occupy varied ecological niches and perform different ecological roles. Decreasing microbial diversity can result in a loss of ecological function and jeopardize stability. There is evidence that applying biogas slurry to farmland stimulated microbial activity, thus improving the rhizosphere environment, and providing a basis for increased crop yield and quality in different cropping systems and regions [12,13].

The North China Plain (NCP) is an important crop production region, covering 3.0×10^5 km^2 or 3.1% of the total land area of China. The deep fluvo-aquic soils, gently sloping topography and temperate climate are ideal for intensive agricultural production, with an annual wheat and maize rotation [7]. Furthermore, since 2010, animal husbandry, particularly medium- to large-scale pig production plants, has been rapidly developed in this region due to national policy-oriented support. Biogas engineering has been chosen by the majority of pig breeding operations to dispose of the resulting manure [8]. Using pressurized piping, biogas slurry can be applied to farmland within a radius of 2.5–4.5 km surrounding pig-producing units throughout the growing season. Its effects on crop yield and quality, soil properties with different soil types, crop species and rotation systems are thus of considerable interest. However, most previous work has focused on biogas slurry derived from small-scale pig breeding operations, with fluctuating properties arising from market demand fluctuations and non-standardized production methods [16]. These results do not allow for sufficient generalization and, consequently, practical adoption has been limited. Therefore, more generally representative information is needed on the longer-term effects of biogas slurry with relatively consistent composition, especially by probing changes of soil properties and the roles of soil microbes [13,17,18].

The aim of this research was to evaluate the effects of repeated application of biogas slurry from a large-scale standardized pig production plant over six years, as an alternative to chemical fertilizer, in fluvo-aquic soil of NCP. The specific objectives were: (1) to assess the effects of biogas slurry application on the physical and chemical properties of basic soil; (2) to investigate the composition and diversity of bacterial and fungal communities in response to biogas slurry application; and (3) to explore the relationships between soil properties and microbial communities. More broadly, we sought to inform changes to conventional fertilization modes and identify a suitable regime that provides synergy between resource utilization and efficient waste disposal to support sustainable agricultural development.

2. Material and Methods

2.1. Experimental Area Description

The research was conducted in Gubei village, Fugou County, Henan Province of China (32°94′ N, 114°32′ E, 55 m a.s.l), in the hinterland of the North China Plain. The climate is warm-temperate continental with an average annual temperature of 14.5 °C and average annual precipitation of 611.5 mm (1980–2020). Rainfall falls mainly (>70%) between June and September and there are approximately 215 frost-free days per year. The terrain is relatively flat, with alluvial sediments accumulating over time to form deep soils, which favors agricultural mechanization and construction of biogas slurry transportation pipelines. Winter wheat (*Triticum aestivum*) and summer maize (*Zea mays*) have been cultivated in long-term annual rotation. The soil is fluvo-aquic, according to the soil taxonomy system of China (an Inceptisol in the soil taxonomy of the USDA), with the following characteristics before the experiment: pH 7.30, organic carbon of 10.08 g kg^{-1}, total nitrogen content of 1.16 g kg^{-1}, alkaline nitrogen content of 80.55 mg kg^{-1}, available phosphorus and potassium content of 25.61 mg kg^{-1} and 242.00 mg kg^{-1}.

The biogas slurry was provided by a large-scale pig production plant with an annual production capacity of 3.0×10^4, which began operation in March 2015. Pig manure and pigsty cleansing water were the main raw materials for anaerobic digestion. This digestion process was set at temperatures of 30–40 °C with a retention time of 7–10 days, according to the season. Solid-liquid filter separators were employed to separate the ensuing digestate and to acquire biogas residue, and the leftover liquid fraction (biogas slurry) was put into a storage pool covered with black high-polyester film to prevent disagreeable gas volatilization and secondary fermentation for at least a half year. Consistent slurry composition over the experimental period was regulated by standardized production schedules and processing. The main properties of biogas slurry were: dry matter of 1.5–2.4%, pH of 7.36–7.50 with average total nitrogen contents (TN), ammonia-N (AN), total phosphorus (TP) and total potassium (AK) contents of 1.50 g L^{-1}, 1.08 g L^{-1}, 0.30 g L^{-1}, 0.49 g L^{-1}, respectively.

2.2. Experimental Design and Agricultural Managements

A field experiment with four treatments in a fully randomized design was conducted over 6 years, beginning in October 2015. The treatments were: (1) control, no amendment (CK); (2) chemical fertilizer application (CF); (3) biogas slurry application (BS); and (4) 50% substitution by biogas slurry for chemical fertilizer (BSCF). With the exception of CK, all fertilization treatments received an equal amount of nitrogen; 50% of nitrogen was from the biogas slurry and the rest was from a chemical fertilizer for the BSCF treatment. Each treatment had 5 replicate plots of 134 m^2 (20 m $\times$ 6.67 m), giving 20 plots in total. Nutrient inputs for the wheat and maize seasons were equal (N 180.0 kg ha^{-1}, P 39.3 kg ha^{-1}, and K 74.7 kg ha^{-1}) and corresponded to the local recommendations for crop cultivation. Chemical nitrogen, phosphorus, and potassium were obtained from urea, heavy superphosphate ($Ca(H_2PO_4)_2 \bullet CaHPO_4$) and potassium sulfate (K_2SO_4), respectively. Each year, 70% of the urea was applied as a base fertilizer for maize in early June and for wheat in early October, with the remaining was applied as a top-dressing at the elongation stage for maize in mid-July and for wheat in the following March, respectively. Both heavy superphosphate and potassium sulfate were applied as a base fertilizer. A modified micro-spraying hose was used to apply biogas slurry, which was connected to a pre-existing biogas slurry outlet at the end of transmission network (Figure 1). The volume of biogas slurry was calculated based on its nitrogen content and quantified by an electromagnetic flowmeter. With the help of a booster pump, two hoses connected to the same biogas slurry outflow were placed in tandem to cover an area of 134 m^2 (20 m long × 6.67 m wide). Biogas slurry permeated the soil by gravity. Supplementary heavy superphosphate and potassium sulfate were added after allowing for the nutrients in the biogas slurry to equalize phosphorus and potassium concentrations in all fertilization treatments. The amounts of total nitrogen, phosphorus and potassium delivered by chemical fertilizer and biogas slurry are shown in Table 1.

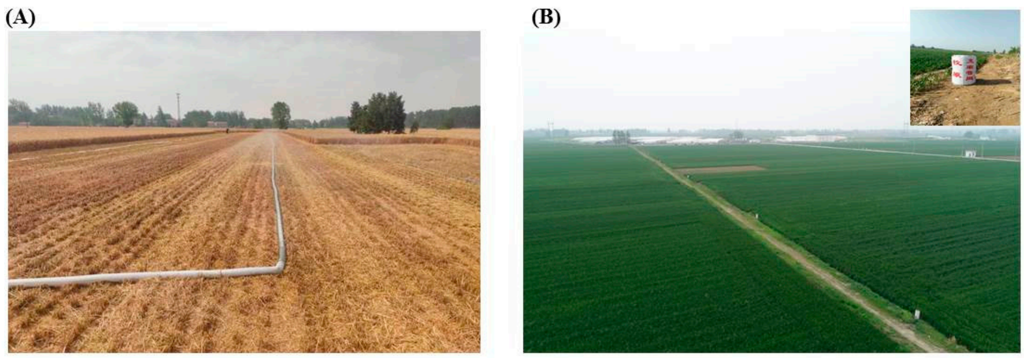

Figure 1. Biogas slurry application by a modified micro-spraying hose with no tillage and straw incorporation after wheat harvest (**A**) and the aerial view of the whole experiment area and established biogas slurry outlets (**B**).

Table 1. Amounts of nitrogen, phosphorus and potassium provided by chemical fertilizer and biogas slurry in experimental treatments.

Treatment	Biogas Slurry				Chemical Fertilizer		
	Application Amount	N	P	K	N	P	K
	m^3 hm^{-2}	kg hm^{-2}	kg hm^{-2}	kg hm^{-2}	kg hm^{-2}	kg hm^{-2}	kg hm^{-2}
CK	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CF	0.00	0.00	0.00	0.00	180.00	39.30	74.70
BS	120.00	180.00	36.00	58.80	0.00	3.30	15.90
BSCF	60.00	90.00	18.00	29.40	90.00	21.30	45.30

The Bainong 207 wheat variety was sown at 225 kg seeds ha^{-1} in mid-October. Following the wheat harvest, 75,000 plants ha^{-1} of Yuyu 30 maize variety were sown in early June. All of the maize stalks in each treatment were crushed to 3 cm and mixed to a depth of 15 cm by rotary tillage using an offset disc harrow before the wheat season. The biogas slurry was then applied in two stages: 70% of the biogas slurry was applied immediately, and the remainder was applied in the overwintering period for wheat. Chemical fertilizer was administered at the same time as seeding. There was no tillage before the maize season, and all of the crushed wheat straw in each treatment was left on the soil surface. Grain drills were used to apply base fertilizer along with maize seeds, in rows between the harvested wheat rows. The biogas slurry was applied at least twice before tasseling, with a 7 day gap between applications, and depending on the weather conditions. Conventional crop managements including herbicide and insecticide application were consistently performed during the whole experimental period.

2.3. Soil Sampling and Physicochemical Analysis

After the maize harvest, at the end of September 2021, soil samples were taken at a depth of 0–20 cm. In each plot, 5 soil cores were obtained by spade and combined after manually removing visible crop litter in the soil surface. The collected soil samples were separated into three parts. The first was kept and transferred onto ice in a sterile polyvinyl chloride pipe for micro high-throughput sequencing. The second was placed in a plastic ziplock bag for determination of alkaline nitrogen concentration. The third was air dried and passed through a 2-mm sieve to remove debris and litter for chemical analysis. Further, undisturbed soil bulk samples, with volumes of approximately 1000 cm^3 (150 mm × 80 mm × 80 mm) were obtained and similarly combined from the same sample points. These were transported in rigid plastic boxes to prevent extrusion. Before air drying, visible roots and crop residues were removed and soil clods were broken into pieces less than 10 mm in diameter following natural cracks for soil aggregate fractionation. Meanwhile, 10 core samples were also taken from each experimental plot using stainless-steel rings (50 mm height × 50 mm diameter) for determination of soil bulk density (BD) and water holding capacity (WHC).

BD and WHC were obtained using the cutting ring, as described by Lu [19]. Soil pH and electrical conductivity (EC) were measured in a soil-water aqueous extract (1:2.5 by mass) after 30 min shaking at low speed (Orion Star 310p, Thermo, Waltham, MA, USA). Soil organic carbon was determined by the potassium dichromate oxidation method [20]. The total N was estimated by titration of distillations after Kjeldahl digestion. Alkaline nitrogen was determined by alkaline hydrolysis diffusion. Soil available phosphorus (AP) and potassium (AK) were measured using the Olsen and Dean methods and flame atomic absorption spectrophotometry (AA-6300, Shimadzu, Kyoto, Japan), respectively.

Aggregate fractionations were achieved by the dry and wet sieving methods. Soil samples that passed a 10 mm mesh were firstly fractionated by dry sieving. Then, 100 g air-dried bulk soil was distributed evenly on the top of a 5 mm sieve and over a series of sieves of 2, 0.5, 0.25 and 0.053 mm mesh, spaced 5 cm apart on an electric shaker, and shaken for 5 min at 30 times per min^{-1}. Next, 50 g soil, proportionally constituted from each dry sieving fraction, was wet-sieved using the same sized sieves. The soil in the topmost sieve was submerged with deionized water. A soil aggregate analyzer (TTF-100, Shunlong Instrument, Shaoxing, China) was programmed with a 3 cm vertical oscillation, once per second, for 2 min. Soil particles that were retained on each sieve were collected and weighed after drying at 40 °C for 48 h. Sedimentation was required for the size fraction with a diameter less than 0.053 mm, and all sediments were decanted and dried at the same temperature and duration. The recovery ratio of aggregates after wet-sieving was 90–99%. Finally, the mean weight diameter (MWD) of the water-stable aggregates was calculated [21].

2.4. Soil DNA Extraction and Microbial High-Throughput Sequencing

Soil DNA samples were extracted using the OMEGA Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) and kept at −20 °C, according to the manufacturer's instructions. Extracted DNAs were quantified and qualified using a spectrophotometer (NanoDrop NC2000, Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis. All samples were stored at −80 °C before PCR amplification and sequencing. Primers 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT) targeting the bacterial V3–V4 region of 16S rRNA were used for PCR amplification, whereas primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS2 (GCTGCGTTCTTCATCGATGC), targeting the fungus ITS-V1 region, were used for PCR amplification. After the PCR products were purified, they were adjusted to equal quantities, and paired-end 2 × 300 base pair (bp) sequencing was performed on Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

Microbiome bioinformatics were carried out with QIIME 2 2019.4, with minor changes made according to the official tutorials (https://docs.qiime2.org/2019.4/tutorials/, accessed on 10 October 2021). The raw sequence data were de-multiplexed using the demux plugin, followed by primer cutting with the cutadapt plugin. Sequences were quality-filtered, denoised, merged and chimeras were removed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin. DADA2 deduces the sequences and produces amplicon sequence variations that are identical (ASVs). DADA2 no longer clustered based on similarity; instead, only de-replication or grouping based on 100% similarity was performed (Callahan et al., 2016) The classify-sklearn naïve Bayes taxonomy classifier in the feature-classifier plugin was used to assign taxonomy to ASVs against the SILVA Release 132 (http://www.arb-silva.de/, accessed on 10 October 2021) and UNITE Release 8.0 (https://unite.ut.ee/, accessed on 10 October 2021) Databases. All raw sequences files were deposited in the NCBI Sequence Read Archive under accession number PRJNA885974.

2.5. Statistical Analysis

The microbial alpha-diversity parameters, Chao1 richness, Observed species, Shannon diversity index and Simpson index were calculated using the ASV table in QIIME 2. Using principal coordinate analysis (PCoA) based on Bray-Curtis distances, beta-diversity was estimated to assess the key similarity and variance components of the bacterial and fungal community compositions among all of the soil samples. Tukey's Honestly Significant Difference (Tukey's HSD), at a 5% threshold of significance, was used to evaluate soil physiochemical parameters and microbiological features for significant differences. Redundancy analysis (RDA) was used to investigate the correlations between physiochemical variables and microbiological characteristics using Canoco 5.0 software. Other statistical analyses were performed, and graphs were visualized with Origin Pro 2019b (Origin Lab Corp., Northampton, MA, USA).

3. Results

3.1. Soil Physicochemical Properties

There were small differences among the treatments in soil bulk density (BD) and pH, but much larger differences were observed in the water holding capacity (WHC), water-stable aggregate mean weight diameter (wMWD) and electrical conductivity (EC) (Table 2). Biogas slurry application treatments (BS and BSCF) significantly reduced soil bulk density, but increased the water holding capacity, relative to the CK. The BD in BSCF was 4.4% lower, while WHC was 29.3% higher than CK, respectively. However, there was no significant difference between CK and CF. All of the fertilization treatments significantly increased the values of wMWD. The maximum of wMWD, at1.5 mm, was obtained in BSCF, which is 2.6-fold greater than CK, followed by BS and CF. Both biogas slurry and chemical fertilizer application alone decreased soil pH compared with CK, whereas the combination BSCF ameliorated this effect. Similarly, all fertilizer treatments increased electrical conductivity, but those containing biogas slurry increased it the most.

Table 2. Main physicochemical properties of fluvo-aquic soil in response to biogas slurry and chemical fertilizer application.

Treatments	BD	WHC	wMWD	pH	EC
	g cm^{-3}	%	mm		µs cm^{-1}
CK	1.37 ± 0.04 a	17.18 ± 0.79 c	0.42 ± 0.04 d	7.90 ± 0.06 a	247.80 ± 32.32 d
CF	1.35 ± 0.02 ab	18.98 ± 0.87 bc	0.84 ± 0.16 c	7.36 ± 0.17 c	401.60 ± 25.13 c
BS	1.30 ± 0.02 b	19.93 ± 0.86 b	1.08 ± 0.15 b	7.38 ± 0.11 c	696.00 ± 16.70 a
BSCF	1.31 ± 0.03 b	22.21 ± 1.50 a	1.53 ± 0.04 a	7.67 ± 0.04 b	574.40 ± 19.84 b

BD, bulk density; WHC, water holding capacity; wMWD, mean weight diameter of water-stable aggregates; EC, electrical conductivity. Values sharing same lowercase letters in the same column are not significantly different at $p < 0.05$.

Similar changes were seen in the soil organic carbon and total nitrogen levels (Figure 2A,B). All of the fertilization treatments significantly increased soil organic carbon and total nitrogen content in comparison with CK; both were increased by either CF or BS, but were increased most by the combined treatment of BSCF (17.3 g kg^{-1} of SOC and 1.7 g kg^{-1} of TN). The chemical fertilizer application treatments (CF and BSCF) significantly enhanced the soil alkaline nitrogen content, relative to CK and BS (Figure 2C). The available phosphorus was significantly increased by all fertilization treatments (Figure 2D). In particular, BS had the highest available phosphorus level, reaching 54.8 mg kg^{-1}. Both of the treatments involving biogas slurry application (BS and BSCF) were extremely effective in maintaining higher available potassium, relative to the control and chemical fertilizer alone (Figure 2E). Available potassium in BS and BSCF was 1.7- and 1.7-fold greater, respectively, than in CK.

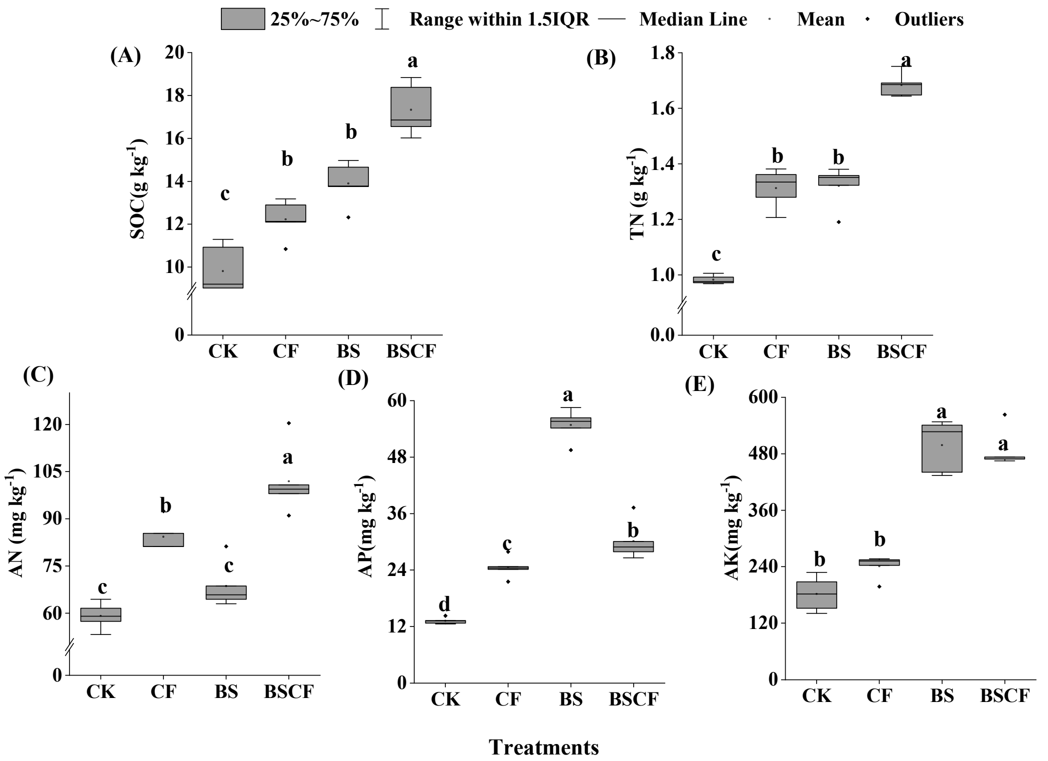

Figure 2. Nutrients of fluvo-aquic soil in response to biogas slurry and chemical fertilizer application: (**A**) Soil organic carbon (SOC); (**B**) Total nitrogen (TN); (**C**) Alkaline nitrogen (AN); (**D**) Available phosphorus (AP); (**E**) Available potassium (AK). Plots sharing lower case letters are not significantly different at $p < 0.05$.

3.2. Changes in Composition and Diversity of Microbial Communities

The soil microbial community composition was very different across the experimental treatments (Figure 3). The bacteria at phylum level were mainly dominated by *Proteobacteria*, *Chloroflexi*, and *Actinobacteria* (Figure 3A). Both the chemical fertilizer and biogas slurry alone treatment significantly reduced the mean relative abundances of *Chloroflexi*, while dramatically increasing *Actinobacteria*, relative to the CK. There was no significant change between these two treatments in the mean relative abundances of *Chloroflexi* and *Actinobacteria*. The half substitution of biogas slurry for chemical fertilizer produced the highest value of *Actinobacteria* and the lowest value of *Proteobacteria*. The biogas slurry application treatments (BS and BSCF) resulted in a significantly lower representation of *Acidobacteria* in comparison with the CK. However, no statistically significant changes were seen between CF and either CK or BS. All of the fertilization treatments also significantly decreased the proportions of *Bacteroidetes*, *Gemmatimonadetes* and *Firmicutes*, with these phyla having mean relative abundance exceeding 2.0%. The BSCF, BS and CF treatments resulted in the lowest values of *Bacteroidetes*, *Gemmatimonadetes* and *Firmicutes* observed in the experiment.

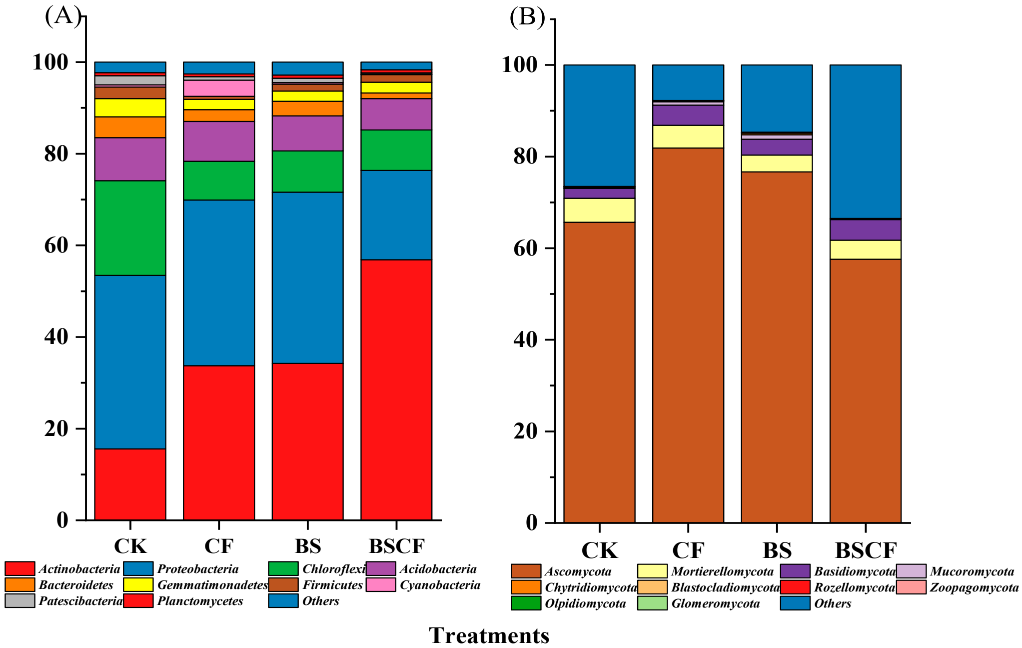

Figure 3. Mean relative abundances of bacterial (**A**) and fungal (**B**) communities at phylum level in response to biogas slurry and chemical fertilizer application in fluvo-aquic soil.

In the fungal community, the dominant phyla were *Ascomycota*, *Mortierellomycota* and *Basidiomycota* (Figure 3B). Both the chemical fertilizer and the application of biogas slurry alone significantly changed the mean relative abundances of *Ascomycota* in comparison with CK. The values of *Ascomycota* in BSCF were 29.7% and 24.9% lower than in CF and BS. The biogas slurry application treatments (BS and BSCF) yielded significantly lower values of *Mortierellomycota*, although there was no significant difference between BS and BSCF. The mean relative abundances of *Basidiomycota* increased significantly in all of the fertilization treatments, reaching a maximum of 4.5% in the treatment of BSCF.

Although all of the fertilization treatments tended to have positive effects on diversity, there were differences between the effects on bacterial and fungal alpha diversity (Table 3). For bacteria, the Chao1, Observed-species and Shannon indices all exhibited the similar trend, increasing CK < CF < BS < BSCF, but this trend was not evident in the Simpson index (Figure 4D). For fungi, the diversity indexes were significantly higher in the biogas slurry treatments (BS and BSCF) than in CK and CF. The Chao1 index was 55.6% higher than in the CK and 51.8% higher than in the CF, whereas the Observed-species index in BS was 56.1% higher than in CK and 52.1% higher than in CF. The Shannon and Simpson indices, on the other hand, were similar across all treatments.

Table 3. Microbial alpha diversity indexes in response to biogas slurry and chemical fertilizer application in fluvo-aquic soil.

Treatments	Chao1	Observed-Species	Shannon Index	Simpson Index
Bacteria				
CK	5211.55 ± 108.81 d	4670.46 ± 92.13 d	10.66 ± 0.05 d	0.998 ± 0.002 b
CF	6718.14 ± 134.41 c	5868.18 ± 114.77 c	11.00 ± 0.10 c	0.997 ± 0.007 b
BS	7162.74 ± 118.52 b	6274.82 ± 90.13 b	11.37 ± 0.05 b	0.999 ± 0.002 ab
BSCF	8080.80 ± 36.00 a	7628.96 ± 68.62 a	11.74 ± 0.02 a	0.999 ± 0.002 a
Fungi				
CK	403.23 ± 31.07 b	399.06 ± 30.79 b	6.30 ± 0.14 a	0.966 ± 0.004 a
CF	413.30 ± 14.42 b	409.60 ± 14.49 b	6.33 ± 0.10 a	0.969 ± 0.003 a
BS	628.41 ± 40.99 a	622.96. ± 40.87 a	6.83 ± 0.07 a	0.975 ± 0.003 a
BSCF	590.05 ± 32.85 a	584.88 ± 33.24 a	6.51 ± 0.21 a	0.967 ± 0.007 a

Values sharing same lowercase letters in the same column are not significantly different at $p < 0.05$.

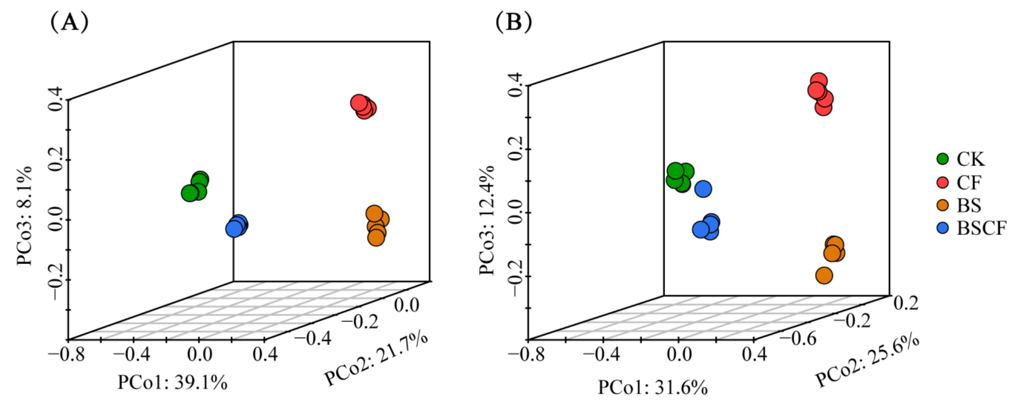

Figure 4. Structure of soil microbial communities ordinated by the first three axes of principal coordinate analysis (PCoA): (**A**) bacteria and (**B**) fungi.

Beta diversity was displayed by the Principal Coordinates Analysis (PCoA). The first three axes of variation explained 68.9% of the total variation in the composition of the bacterial communities, with PCo axis 1, PCo axis 2 and PCo3 accounting for 39.1%, 21.7% and 8.1%, respectively (Figure 4A). Similarly, the first three components accounted for 31.6%, 25.6% and 12.4% of total fungal variation, respectively (Figure 4B). The similarity between community compositions was reflected in proximity in these ordinations. The replicate plots consistently grouped closely together. For bacteria and fungi, CK was strongly differentiated from all of the fertilizer treatments on Axis 1. On the other hand, the fertilizer treatments themselves were differentiated on Axis 2, with BSCF clearly separated from CF and BS, which grouped quite closely together in both cases. CF and BS were themselves differentiated on Axis 3 for both bacteria and fungi.

3.3. Relationships between Microbial Characteristics and Soil Properties

Redundancy Analysis (RDA) revealed that TN (F = 47.1, p = 0.002), pH (F = 7.3, p = 0.002) and AK (F = 5.3, p = 0.022) were significantly correlated with soil bacterial community composition and its alpha diversity parameters at phylum level, explaining 77.2%, 8.9% and 5.1% of the total community variability, respectively (Figure 5A). The coordinates of the first and second ordination axes explained 76.7% and 10.8% of the total variance, respectively. The abundance of *Proteobacteria* and *Chloroflexi* were strongly associated with BD, while the abundance of *Actinobacteria*, Shannon, Chao1, and Observed-species indexes were highly correlated with TN.

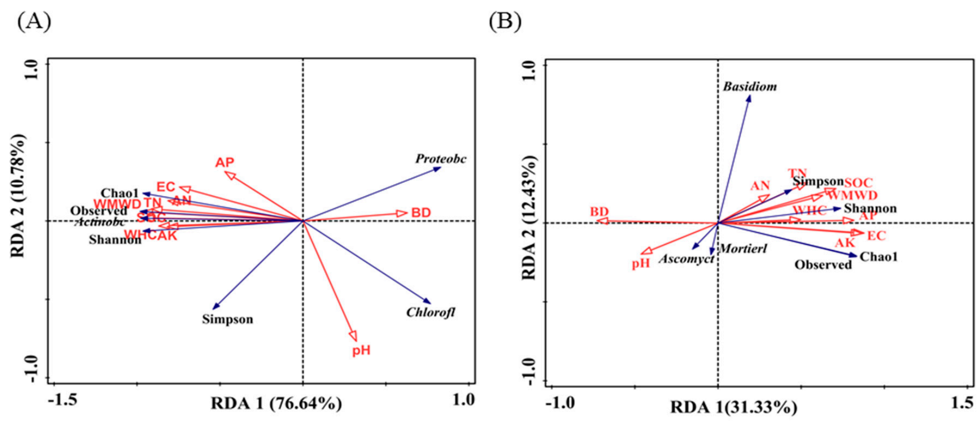

Figure 5. Redundancy analysis (RDA) of the relationships between soil microbial community composition, alpha diversity indexes at the phylum level and soil physiochemical properties. (**A**) bacteria and (**B**) fungi.

The first two RDA components could explain 43.8% of the overall variation in the fungal community, while the first and second axes explained 31.3% and 12.4%, respectively (Figure 5B). There were significant correlations between AK and soil fungal community composition and alpha diversity characteristics at phylum level, explaining 40.6% of the total community variability. The abundances of *Mortierellomycota* and *Ascomycota* were strongly linked with soil pH, while AK and AN were positively correlated with all alpha diversity indexes and *Basidiomycota*.

4. Discussion

4.1. Impacts on Soil Physicochemical Properties

Fertilization practices are critical, not just for maintaining and increasing crop yield, but also for the formation and development of sustainable agricultural systems [11,22]. This field experiment, conducted over six consecutive years, demonstrated that all of the fertilizer treatments had a significant impact on soil physicochemical parameters. Compared with the control, the application of fertilizer, no matter which chemical or biogas slurry, could effectively lower soil bulk density whilst increasing water holding capacity (WHC), potentially improving water supply, aeration and nutrient availability. Soil aggregation, particularly water-stable aggregates, played an important part in soil structure improvement and soil conservation [5,23]. This is exemplified by changes in mean weight diameter (MWD), a stability index representing the aggregate size distribution and mass proportion [21]. Although the benefits of applying biogas slurry alone were not significantly better than those of chemical fertilizer alone, there were substantially greater differences when biogas slurry was combined with chemical fertilizer (BSCF), especially for WHC and wMWD. These differences may be primarily due the fluidity of biogas slurry, compared with other organic amendments (manure, compost and biochar), allowing it to spread more quickly over the soil particle surfaces, thus improving aggregate stability [4]. These results were consistent with findings that long-term application of dairy liquid manure (DLM) improved soil macro-porosity and aggregate MWD, with concomitant reduction in bulk density in a clayey Ferralsol of southern Brazil [1]. Similarly, Li et al. (2012) found that biogas slurry application enhanced soil water conservation capacity and reduced its compaction in a greenhouse-soil ecosystem in China [14]. In general, lower BD has been linked to increased soil aggregation [24].

Long-term significant amounts of mineral fertilizer application, coupled with base cation uptake and removal by plants, can easily lead to soil acidification, whereas manure application may cause salinization [25,26]. In this experiment, biogas slurry application did not modify soil pH considerably, compared with chemical fertilizer application, but all of the fertilizer treatments increased salt accumulation in topsoil. Biogas slurry application caused the greatest increase in soil EC, as reported previously [27]. This is likely due to its high soluble salt content, which stems from high salt concentrations in pig excrements prior to anaerobic digestion [28]. Hence, the slurry contains large quantities of exchangeable base cations, particularly Na^+, K^+ and Mg^{2+} [4,5,16]. Despite the fact that some of these bases are plant nutrients, biogas slurry application is more likely to create salt buildup at the soil surface, increasing the risk of secondary salinization. Plant growth may be hindered at soil EC values above a threshold of 2 ds m^{-1}, therefore decreasing crop yield [27]. Furthermore, exchangeable cations could affect soil aggregation, facilitating swelling or dispersion of clay minerals by sodium (Na^+), as well as slaking of unstable aggregates [5,22]. Our results also indicated that the combination of biogas slurry with chemical fertilizer appeared to mitigate such adverse side effects. However, future research should explore the potential risks of salinization from longer-term biogas slurry application.

Biogas slurry contains more easily degradable dissolved carbon than chemical fertilizers and most other conventional organic fertilizers, potentially promoting biological activity and influencing soil organic carbon accumulation [9,22]. We found similar organic carbon contents in chemical fertilizer and biogas slurry treatments, whereas their combination effectively increased the value in topsoil after six years of application. Terhoeven-Urselmans et al. (2009) reported that 27% of the organic matter from biogas slurry was mineralized and released into the atmosphere as CO_2 within 50 days after application [2]. Other reports have suggested that increased organic matter in the topsoil after biogas slurry application was transient [5,11]. It has also been suggested that biogas slurry application might suppress soil organic carbon sequestration in topsoil by promoting microbial metabolism due to its relatively high nitrogen content [15,29]. In addition, other studies have found that biogas slurry had little or no effect on soil organic carbon content, depending on the original carbon content and stability [9,30]. Our field experiment showed that long-term biogas slurry application, particularly in combination with chemical fertilizer, had the potential for carbon accumulation, which would be beneficial for mitigating greenhouse effects and agricultural sustainable development.

Biogas slurry application increased the total nitrogen in the fluvo-aquic soil, similarly to chemical fertilizer, but the combination of the two increased it even more. Similarly, the alkaline nitrogen content was also greatest in BSCF. In animal production, less than 45% of the nitrogen from plant protein was transformed into animal protein and the rest was excreted as organically bound nitrogen. Most of this organic N is rapidly hydrolyzed to ammonium (NH_4^+) [31,32]. Approximately 70% of the nitrogen was retained in the biogas slurry after anaerobic digestion, predominantly as NH_4^+ [33,34]. Phosphorus is another important nutrient in animal feed, and approximately 50–60% of it is discharged in feces and urine [16]. Orthophosphate comprises more than 80% of dissolved phosphorus, but only 30% of it was retained in the biogas slurry [4,33]. Consequently, it was of interest that the available phosphorus was significantly higher in the biogas slurry treatment than in any of the others, which is in agreement with previous reports that phosphorus availability was enhanced by labile phosphorus from biogas slurry [7,14]. Moreover, there was a suggestion that biogas slurry may stimulate the release of native soil phosphorus [6]. The potassium concentrations were similar in the BS and BSCF treatments, and were substantially higher than in the CF. This might be explained by high concentrations of potassium in biogas slurry, which range from 200 to 600 mg L^{-1} [8]. Despite being fertilized with the same amounts of nitrogen, phosphorus and potassium, the soil available nutrients subsequently displayed significant differences. These results might be attributed to the greater accumulation of accessible phosphorus and potassium after biogas slurry application and, therefore, it is possible that long-term application with biogas slurry might trigger soil nutrient imbalances. Consequently, fertilizer amounts should be continually adjusted to match crop demands, according to the soil nutrient status, to minimize nutrient loss by surface run-off or leaching.

4.2. Impacts on Soil Microbial Community Composition and Diversity

Microorganisms are involved in a wide range of soil processes, including organic matter decomposition and formation, as well as nutrient mineralization, absorption, and usage by plants [10,25]. Microbial communities are sensitive to soil structure and nutrient status and, thus, to different fertilization and management measures [12]. The finding that fertilization treatments in the fluvo-aquic soil produced significant differentiation of microbial community composition was therefore important. Whilst the effects of CF and BS were broadly similar, their combination had distinctly different effects. *Proteobacteria*, *Chlorobacteria* and *Actinomycetes* are common dominant bacterial phyla in farmland [35]. *Proteobacterial* species are facultative or anaerobic heterotrophs and most of them colonize nutrient-rich habitats [36]. A remarkable reduction in the relative abundance of *Proteobacteria*, after the combined application of biogas slurry and chemical fertilizer, indicated its reproductive inhibition. Previous evidence has suggested that biogas slurry has a positive effect on *Proteobacteria,* as high carbon availability in environments favor their fast growth and reproduction [13,37]. Discrepancies may be due to different soil texture, crop species and rotation systems, as well as sampling time in varied research regions. Most *Chlorobacteria* are facultative anaerobes and could produce toxic degradation substances [37]. The relative abundance of *Chlorobacteria* seen in all of the fertilization treatments was less than 50% of that in the control. *Actinomycetes* are involved with organic matter decomposition and organic carbon mineralization [36]. Both biogas slurry and chemical fertilizer applications can provide microorganisms with abundant nutrients and energy resource, either directly or indirectly [8,30]. Hence, two- or three-fold increases in the relative abundance of *Actinomycetes* were seen in these treatments.

In soil ecosystems, the three most common fungal phyla are *Ascomycota*, *Mortierellomycota*, and *Basidiomycota*. *Ascomycota* are major saprotrophic decomposers of organic substrates. They can respond rapidly to the addition of carbon sources by degrading cellulose to meet their own demands, thus reproducing quickly enough to become dominant [38]. More than half of the numbers in the fungal community at the phylum level were represented by *Ascomycota* in all treatments, with the highest and lowest values obtained in the CF and BSCF treatments, respectively. Chemical fertilizer application tended to favor mono-dominant community formation, however, combining biogas slurry with chemical fertilizer appeared to regulate fungal community composition and allow additional fungal species to cohabit. *Mortierellomycota* can promote the absorption and utilization of phosphorus and potassium by secreting organic acids [39]. We found that the biogas slurry application inhibited *Mortierellomycota*, relative to the control and CF, similarly to biogas slurry combined with chemical fertilizer in a paddy field [40]. This change may be due to higher contents of phosphorus and potassium in the treatment of BS and BSCF. *Basidiomycota* not only include species of fungi that have ability to degrade plant residues, but also some harmful species that cause crop disease [41]. Chemical fertilizer treatments significantly enhanced *Basidiomycota* abundance but biogas slurry application alone suppressed its abundance, which suggested there was a higher potential risk of soilborne diseases with intensive agriculture that relies on entirely chemical fertilizers [10].

Microbial biodiversity is the basis for maintaining soil ecological functions [4,38]. Higher diversity is thought to contribute to a more stable soil ecosystem. Biogas slurry application significantly promoted bacterial richness (Chao1 and Observed-species) and Shannon indexes. Similar results were obtained by Xu et al. for biogas slurry application in a rice-rape rotation on yellow soil in Southwest China [13]. Although biogas slurry treatment increased fungal richness significantly, there was no effect on their diversity indexes. Significant connections were found between the phylum and alpha diversity indexes of bacteria, as well as TN, pH, and AK. Chen et al. and Xu et al. found that soil structure, nutrient availability, and microorganisms interact in Shajiang black soil and a rice-rape rotation of yellow soil, respectively, and soil nutrient availability was the primary determinant of microbial community diversity, which was in accordance with our findings [13,25]. PCoA also corroborated that biogas slurry application had a remarkable influence on microbial beta diversity. This might be explained using the species redundancy hypothesis [42]. Some microbial species are eliminated as a result of their low adaptability to soil microhabitats, but their niches are swiftly filled by new species that perform the same ecological function [43]. Furthermore, the composition and diversity of microbial communities influence interspecific interactions, modifying and regulating ecological processes [10]. More research is needed to fully understand these interspecific connections between soil bacteria and fungus species after biogas slurry application.

4.3. Implications for Development of Sustainable Crop Production and Animal Husbandry

In recent years, soaring meat demands have created many large-scale intensive livestock production units in China, concomitant with a significant increase in biogas slurry production [16]. This has in turn increased the risk of environmental pollution resulting from inappropriate biogas slurry disposal and utilization. Biogas-linked agro-ecosystems are considered an effective link between livestock and plant production to mitigate this bottleneck in the sustainable development of animal husbandry [8]. Using biogas slurry as an alternative to chemical fertilizer not only recycles agricultural wastes, but also has beneficial influences on soil physical structure and chemical properties, as well as microbial community composition and diversity, both of which are consistent with established views of agricultural recycling [3,25]. The NCP is an important production base for agriculture and animal husbandry in China. Large areas of farmland with a wheat and maize rotation require large nutrient inputs and replenishments of organic matter, as well as irrigation water, making them ideal recipients of large amounts of biogas slurry from animal husbandry. Furthermore, the flat terrain is convenient for cultivation and a biogas slurry transmission network layout at the minimal cost. Medium- and large-scale animal production facilities have been established around the farmland and most of the biogas slurry is now available free of charge for local farmers. According to our investigation, there are approximately 615 pig farms of similar size scattered throughout the central region of NCP, producing at least 58.2 million m^3 of biogas slurry every year. The biogas slurry management procedures in this experiment would be replicable and provide relevant insights for coordinated regional agriculture and animal husbandry. However, from the perspective of environmental protection, some potential disadvantages, such as heavy metal pollution, ammonia volatilization and residual antibiotics accumulating in soils should not be ignored from the perspective of environmental protection [29,32,44]. Further experiments are needed for comprehensive assessment of biogas slurry application over a longer time.

5. Conclusions

The use of biogas slurry as a partial alternative to chemical fertilizer on fluvo-aquic soil is a promising and practical tool to regulate soil fertility and microbial characteristics in the North China Plain. Our six year field experiment demonstrated that long-term consecutive biogas slurry application improved topsoil physical characteristics by reducing soil bulk density and enhancing water-holding capacity and water-stable aggregate mean weight diameter. Biogas slurry application treatments influenced chemical properties, increasing topsoil organic carbon and total nitrogen, as well as improving soil NPK availability. Furthermore, biogas slurry application significantly altered the composition and diversity of soil bacterial and fungal communities at the phylum level. Total nitrogen, pH, and available potassium were the most important features correlated with soil bacterial community composition and alpha diversity, whereas soil fungal communities were predominantly correlated with available potassium. Substituting half of the chemical fertilizer with biogas slurry resulted in the lowest soil bulk density and the highest soil aggregate stability, soil organic carbon, total nitrogen and available nitrogen and potassium. It also contributed to maintaining well-proportioned microbial community composition and higher microbial diversity. It is likely that this represents an effective practice to promote the development of biogas-linked agro-ecosystems.

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