agronomy

Article

Effects of Earthworms and Phosphate-Solubilizing Bacteria on Carbon Sequestration in Soils Amended with Manure and Slurry: A 4-Year Field Study

Zhen Wang [1,2], Yifang An [1], Huayi Chen [1], Jiaxin Zhang [1], Haichun Zhang [1], Genfa Zhu [2], Jinfeng Chen [2], Wenyan Li [1], Jinjin Wang [1], Hui-Juan Xu [1], Yongtao Li [1] and Yulong Zhang [1,*]

[1] College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, China
[2] Guangdong Key Laboratory of Ornamental Plant Germplasm Innovation and Utilization, Environmental Horticulture Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China
* Correspondence: yulongzhang@scau.edu.cn; Tel.: +86-1356-034-5598

Abstract: The application of organisms as part of soil remediation can accelerate the decomposition of organic matter and the carbon cycle. To explore the synergistic effects of earthworms and phosphate-solubilizing bacteria on C accumulation in artificially improved soils of manure and in slurry-amended soil, a dry slope of land was established on the hillside of a large pig farm. Experiments involving six treatments were performed, including control (CK), pig manure (Pm), and pig manure + slurry (Pm + S) treatments, as well as manure + slurry + earthworms (Te), manure + slurry + phosphate-solubilizing bacteria (Tb), and manure + slurry + earthworms + bacteria (T(e + b)). Compared with the CK, both the Pm + S and T(e + b) treatments significantly increased the SOC content. In particular, the T(e + b) treatment increased the SOC by 196%. The synergistic effect of T(e + b) on the increase in organic carbon was consistent with the results of soil-carbon sequestration. After comprehensive fertilization, soil-carbon sequestration reached 2.87 Mg C hm^{-2}, while stable organic carbon increased to 1.88 Mg C hm^{-2}. It was also consistent with the result of PCA analysis in which applying earthworms promoted an increase in insoluble organic carbon. Therefore, in the future, earthworms and organic fertilizers can be applied to promote organic carbon sequestration on dry sloping land.

Keywords: soil; pig manure; slurry; earthworm; bacterial fertilizer; organic carbon components

Citation: Wang, Z.; An, Y.; Chen, H.; Zhang, J.; Zhang, H.; Zhu, G.; Chen, J.; Li, W.; Wang, J.; Xu, H.-J.; et al. Effects of Earthworms and Phosphate-Solubilizing Bacteria on Carbon Sequestration in Soils Amended with Manure and Slurry: A 4-Year Field Study. *Agronomy* **2022**, *12*, 2064. https://doi.org/10.3390/agronomy12092064

Academic Editor: Carlo Leifert

Received: 28 July 2022
Accepted: 24 August 2022
Published: 29 August 2022

1. Introduction

With the rapid development of animal husbandry, the large-scale generation of livestock manure has occurred during the past few decades. Livestock manure is an organic alternative to chemical fertilizers because it contains many beneficial nutrients that promote agricultural crop production [1,2]. Arid hillside land accounts for approximately 24% of the total cultivated area in southern China, and strong acidity, low fertility, and low organic carbon (OC) content impede soil tillage and cultivation practice [3]. Pig manure (Pm) is rich in organic matter and nutrients and can be converted into organic fertilizer after being composted [4]. Therefore, the fertility of soils of arid hillside lands could be improved by the application of Pm.

Soil organic carbon (SOC) in croplands is important with respect to soil fertility, crop production, and food security, and increasing SOC content is considered a vital measure to offset carbon dioxide (CO_2) emissions [5,6]. There are many factors that affect the relationship between carbon input and SOC content changes, such as the climate, soil properties, farming practices, fertilization practices, and crop type [7–10]; one study found that the application of organic materials can directly increase the input of farmland soil carbon, thereby improving the fixation of farmland SOC [7,11]. Organic matter enters the soil through the rhizosphere, straw return, or organic fertilizer and is then converted into SOC via biochemical activity, which involves a long-term process [12,13]. The new SOC slows the decomposition of the original soil organic matter (SOM) [6,14], and studies have found that when nutrients such as N and P are at sufficient levels, the decomposition of SOM is inhibited [15,16]. Phosphate-solubilizing bacteria can enhance the release of available phosphorus in the soil, and earthworms can increase the mineralization rate of the soil and fresh organic matter in a short period of time [17,18]. Cheng et al. [18] revealed that earthworms and bacteria accelerate soil nitrogen cycling by stimulating soil mineralization (ammonification and nitrification) and nitrifier denitrification, increasing the amount of nitrogen available to plants.

Water-soluble organic carbon (WSOC) refers to polymer-like substances that can be extracted with water or other extraction solutions and that can pass through a 0.45 μm filter [19,20]. WSOC represents the soil organic carbon in the soil solution and can often be directly taken up by plants and microorganisms [21]. In terms of chemical properties, WSOC can be divided into humus (HS) and nonhumus (NHS) components: humus components include humic acid (HA) and fulvic acid (FA), while nonhumus components include amino sugars, carbohydrates, organic acids, proteins, phenols, etc. [22,23]. In addition, organic compounds in the soil can be divided into two types: active OC compounds, which are unstable and easy to mineralize and assimilate, and inactive OC compounds such as humus and insoluble OM, which have complex structures and can resist microbial degradation and mineralization [24]. Methods including water dissolution, acid dissolution, and the use of humus are widely used to distinguish between active and recalcitrant OC pools. The relative composition of the two OC pools reflects the quantity and quality of SOM accumulation in a soil sample [3].

Reducing carbon sources and increasing carbon sinks represent the most basic method of achieving carbon neutrality, and soil-carbon sequestration is an essential method for carbon sinks [12]. Studies have demonstrated that if the carbon content in the soil at a depth of one meter increases by 4‰, CO_2 concentrations will not increase worldwide [25]. Returning straw or applying organic fertilizer to the field can enhance the SOC content, and Chen et al., found that the annual amount of organic matter mineralization increased to 4.4 t hm^{-2} after the application of animal manure; however, because the input amount was higher than the output amount, the SOC content of the cultivated paddy field after 60 years increased to 48.6 g kg^{-1} [26]. Therefore, the application of exogenous organic matter is one of the vital ways to achieve carbon neutrality. In this study, we chose a dry slope of land in a field on a large pig farm for our experimental, and field trials were conducted for 4 years to explore the mechanism through which OC accumulates in response to treatment with Pm, biogas slurry, earthworms, phosphorus-solubilizing bacteria, or their combination, the results of which could provide new insights into carbon neutrality.

2. Materials and Methods

2.1. Site Description

A typical dry slope of land southwest of Yunfu city, Guangdong Province, in southern China was selected as the study site (Figure 1). The soil parent material in this area is weathered granite (Table 1). This area belongs to the subtropical monsoon climate zone; the average annual precipitation ranges from 1700 to 3100 mm, and the temperature ranges from 9.7 °C to 39 °C. The rainy season from April to September contributes more than 70% of the total annual precipitation, with the most rainfall occurring in August.

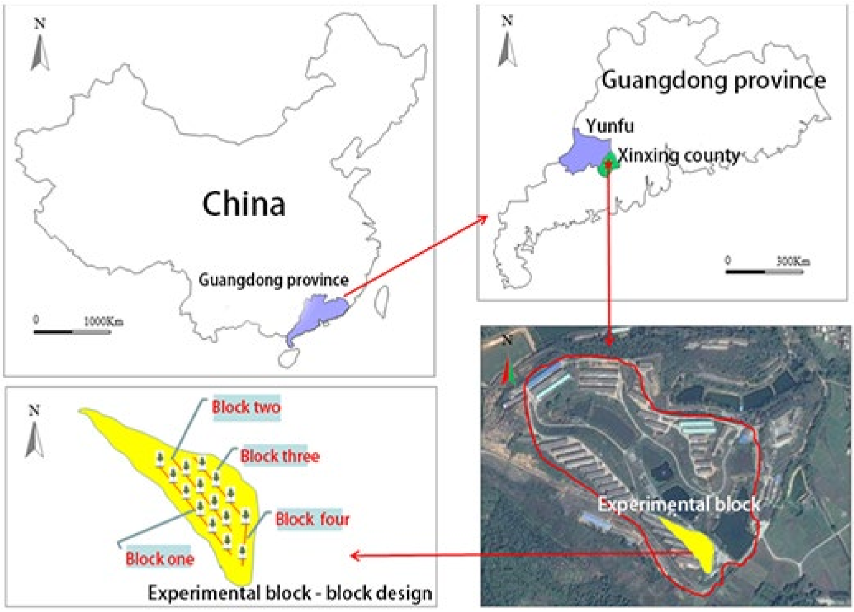

Figure 1. Location of experiment.

Table 1. Physicochemical properties of soil.

pH	SOC ($g{\cdot}kg^{-1}$)	TN ($g{\cdot}kg^{-1}$)	TP ($g{\cdot}kg^{-1}$)	TK ($g{\cdot}kg^{-1}$)	AP ($mg{\cdot}kg^{-1}$)	AK ($mg{\cdot}kg^{-1}$)
4.56 ± 0.22	10.44 ± 0.25	0.88 ± 0.03	0.29 ± 0.02	1.71 ± 0.04	21.95 ± 0.51	33.67 ± 0.85

2.2. Materials and Characteristics

Manure samples were taken from a Luochen pig farm. The physical and chemical characteristics of the Pm are as follows: pH: 7.45; OC: 37.66 $g{\cdot}kg^{-1}$; total N: 18.36 $g{\cdot}kg^{-1}$; and available N: 430.58 mg kg^{-1}. Two ecological earthworm species (the epigeic *Eisenia foetida* and the endogeic *Amynthas robustus*) were used. Both of the earthworm species adapted to the new environments well and reproduced quickly. Earthworms tend to preferentially consume livestock manure, which can accelerate the decomposition of the manure. Two phosphate-solubilizing bacterial strains (*Bacillus subtilis* HL-1 and *Bacillus cereus* NC-1) were applied as additives. Both strains have been deposited in the China General Microbiological Culture Collection Center (CGMCC), registered under CGMCCNO.5175 and CGMCCNO.6127. These strains are widespread in nature, grow rapidly, are not toxic, and are harmless to the environment and humans. *Bacillus subtilis* HL-1 can increase the availability of phosphate and the efficiency of phosphate availability, increase soil microbial diversity, and promote the growth of plants [25]. *Bacillus cereus* NC-1 can increase the availability of P and N, improve soil microbial community composition, improve soil fertility, and promote the growth of plants [26]. Information concerning the isolation, culture, purification, and identification, as well as a more detailed description, of *Bacillus subtilis* HL-1 and *Bacillus cereus* NC-1 can be found in Chinese patent Nos. CN102399713A [27] and CN103173380A [28], respectively. The copyrights of the related patents belong to the South China Agricultural University.

2.3. Experimental Design

The experimental design is shown in Table 2. Field research was conducted from August 2013 to August 2017 to study the long-term effects of Pm on soil nitrogen and the regulatory mechanisms of engineered organisms (earthworms and bacterial fertilizers) underlying these effects. The pig farm (the area inside the red line in the figure) and study plots (shown in yellow in Figure 1) are located in Xinxing County, west of central Guangdong Province, adjacent to the Pearl River Delta (111°57′37″ to 22°22′46″ East longitude, 22°22′46″ to 22°50′36″ North latitude). There were six treatments in total: a control without fertilizer or engineered organisms (CK), pig manure (Pm), pig manure + biogas slurry (Pm + S), pig manure + biogas slurry + earthworms (Te), pig manure + biogas slurry + bacterial fertilizer (Tb), and pig manure + biogas slurry + earthworms + bacterial fertilizer (Te + b). All six treatments were arranged randomly in each of the four blocks. The hillside slope angle was approximately 40 degrees, and the experimental plots covered an area of 3000 m^2. Based on different research purposes, the six treatments were divided into two groups. Group 1 involved three treatments, i.e., CK, Pm, and Pm + S, to evaluate the influences of the application of pig manure and biogas slurry on soil nitrogen pools and nitrogen cycling. Group 2 involved four treatments, i.e., Pm + S (T), Te, Tb, and Te + b, to investigate the regulatory mechanisms of engineered organisms (earthworms and bacterial fertilizer) on soil nitrogen pools, and nitrogen cycling in soils amended with manure and slurry. Notably, all the applications were concentrated around trees (1 m^2), rather than being spread evenly over whole plots (120 m^2). Four *Neolamarckia cadamba* saplings were planted in accordance with a spacing of 5.0 m by 6.0 m in each block. The manure was composted for a month before application and was applied to the trees at 10 kg $tree^{-1}$ and 5 kg $tree^{-1}$ from August 2013 to August 2017, respectively. The trees were irrigated four times a month from April to September and 10 times a month from October to March via a drip irrigation system at a volume of 15 L slurry $tree^{-1}$ each time. The treatments (CK and M) without biogas slurry were applied through irrigation with an equal volume of water. The irrigation amount each time was slightly adjusted depending on the precipitation amounts. Each time, 150 *Eisenia foetida* (70 mm length, 0.25 ± 0.05 g weight) and 30 *Amynthas robustus* (96–150 mm length, 0.7 ± 0.15 g weight) were placed on each tree every year (August 2013, August 2014, August 2015, August 2016, and August 2017). The survival rate of the earthworms was determined every 3 months via random sampling from three plots of each treatment. A solution of 125 mL of fermented liquid with 109 CFU mL^{-1} living bacteria was diluted to 500 mL and applied to each tree every year.

Table 2. Design of experiment.

Dispose	Pig Manure (kg·Strain $^{-1}$·Time $^{-1}$)	Biogas Slurry (L·Strain^{-1} ·Time $^{-1}$)	Earthworm (Strain $^{-1}$·Time^{-1})	Bacterial Fertilizer (L Strain $^{-1}$·Time $^{-1}$)
CK	—	—	—	—
Pm	10	—	—	—
P(m + S)/T	10	15	—	—
Te	10	15	200	—
Tb	10	15	—	1
T(e + b)	10	15	200	1

Pig manure, earthworms, and fungus fertilizer were added in each layout test, and biogas slurry irrigation was carried out according to the actual situation. The specific numbers are described in the test layout process. ″—″indicates that no substance has been added.

2.4. Soil Sampling

Twenty-four soil samples (0–20 cm depth) from the six treatments were collected using a soil auger to minimize the impact of soil fertility differences at different sampling points in August 2013, August 2014, August 2015, August 2016, and August 2017. Each sample was a composite of 24 subsamples, with six subsamples collected approximately 20 cm from the trunk of four trees in the same block. Sampling was conducted before the addition of manure, earthworms, and bacteria. After the samples were taken back to the laboratory, the samples were sieved using a 2 mm mesh. A portion of each sample was stored at 4 degrees for analyses of microbial biomass and soil enzyme activity, and the remaining portion was air dried at ambient temperature for the determination of soil chemical properties. Then, some soil samples were ground and subsequently passed through a 0.149 mm sieve for determination of the total organic matter.

2.5. Soil Chemical and Microbial Analyses

The OC content was determined by the potassium dichromate external heating method [29], and the acid-extractable OC components were determined according to previous methods. Similarly, WSOC and HWSOC were determined according to previous methods [28]. The reference for the determination of organic carbon components in acid extraction was from [27]. The composition of humus was determined using the potassium dichromate oxidation method (LY/T1238-1999), and microbial biomass carbon was determined using chloroform fumigation [30].

2.6. Statistical Analysis

Excel 2010 software and SPSS 12.0 software (SPSS Corporation, located in Chicago, IL, USA) were used for statistical analysis of the experimental data, and significance was determined using one-way ANOVA and Duncan's new complex range method for multiple comparisons between pairs ($p < 0.05$). ADE-4 software was used for statistical analysis of the correlations between variables.

3. Results

3.1. Effects of Different Fertilizer Treatments on the Soil Carbon Pool

3.1.1. Changes in SOC Content

The effects of applying Pm and Pm + S on the SOC content are shown in Figure 2A. Compared with those of the CK, the SOC content of the Pm and Pm + S treatments increased, especially in the second year when the difference was significant ($p < 0.05$). In addition, with increasing fertilization time, the SOC content gradually increased, peaked in the third year, reached 19.96 g·kg^{-1}, and then, decreased.

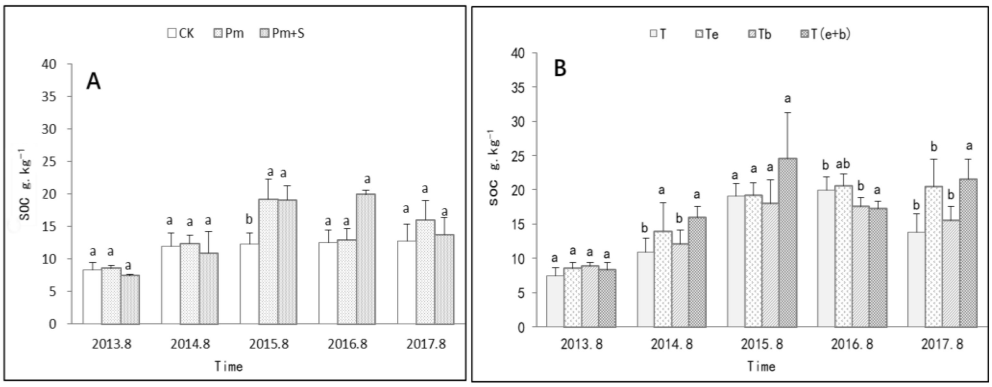

Figure 2. Dynamic change of soil organic carbon content. Bars represent standard errors. Lower case letters above each panel show statistical significance at $p < 0.05$. CK pig manure, S slurry, e earthworms, b bacteria, T Pm + S, Te T + earthworms, Tb T + bacteria, T(e + b) T + earthworms + bacteria. (**A**) Pig manure, biogas slurry agricultural use (**B**) Biological control.

Figure 2B indicates that biological synergy could influence the SOC content. Compared to the T treatment, the Te treatment obviously increased the SOC content in the first year, and simultaneously adding earthworms and phosphate-solubilizing bacteria also enhanced the SOC content, particularly in the first and fourth years when the differences were significant.

3.1.2. WSOC Content in the Soil

The effects of applying Pm and Pm + S on the WSOC content are shown in Figure 3A. With the exception of 2015, the WSOC content during each year after the application of Pm decreased, but the difference was not significant. However, Pm increased the WSOC content in the second and third years after treatment. In addition, the application of Pm alone gradually increased the WSOC content before 2015, but then, the WSOC tended to decrease after two years. Pm + S gradually enhanced the WSOC in the first three years, after which it decreased.

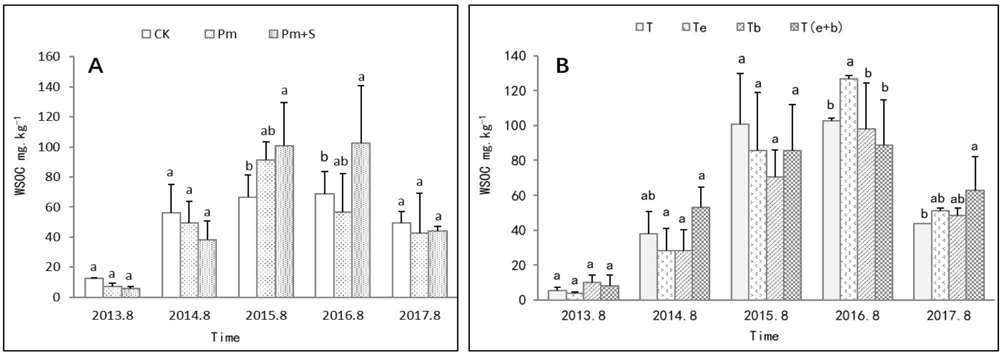

Figure 3. Dynamic change of soil water-soluble organic carbon content. Bars represent standard errors. Lower case letters above each panel show statistical significance at $p < 0.05$. CK pig manure, S slurry, e earthworms, b bacteria, T Pm + S, Te T + earthworms, Tb T + bacteria, T(e + b) T + earthworms + bacteria (**A**) Pig manure, biogas slurry agricultural use (**B**) Biological control.

The influence of biological synergy on the WSOC content is demonstrated in Figure 3B. The WSOC content showed an increasing trend in the first three years compared with 2013, and then, decreased in the fourth year. Furthermore, the application of earthworms reduced the WSOC in the first two years, while it increased the WSOC in the last two years, which peaked at 126.60 mg·kg^{-1} in 2016.

3.1.3. HWSOC Content in the Soil

The impact of Pm and Pm + S on the HWSOC content of the soil is shown in Figure 4A. With the exception of the first year after treatment, compared with the CK treatment, the Pm treatment increased the content of HWSOC in the soil each year, which reached a significant level in the second year, and the difference was not significant in the other years. Pm + S also promoted HWSOC, however, and the difference reached a significant level in the second year after the treatment.

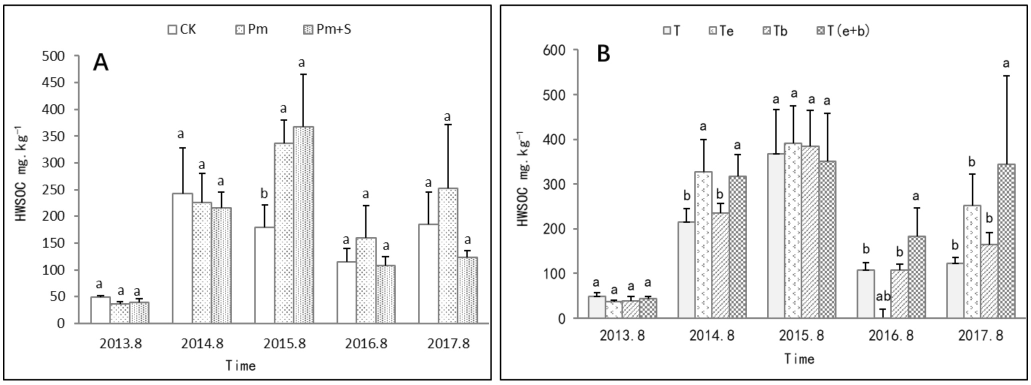

Figure 4. Dynamic change of soil hot water-soluble organic carbon content. Bars represent standard errors. Lower case letters above each panel show statistical significance at $p < 0.05$. CK pig manure, S slurry, e earthworms, b bacteria, T Pm + S, Te T + earthworms, Tb T + bacteria, T(e + b) T + earthworms + bacteria (**A**) Pig manure, biogas slurry agricultural use (**B**) Biological control.

Compared with the T treatment, the Te, Tb, and T(e + b) treatments increased the content of HWSOC in different years. In addition, the HWSOC of each treatment peaked in the first two years (2015). These results suggest that biological synergy had a positive influence on the WSOC content (Figure 4B).

3.1.4. Weak Acid-Extractable SOC (Active Component I) Content

The impact of Pm treatment on weak acid-extractable SOC (active component I) is shown in Figure 5A. The data show that the active component I of weak acid-extractable SOC decreased in both the first and the third years after the application of Pm and Pm + S but increased in the second and fourth years; however, the difference was not significant.

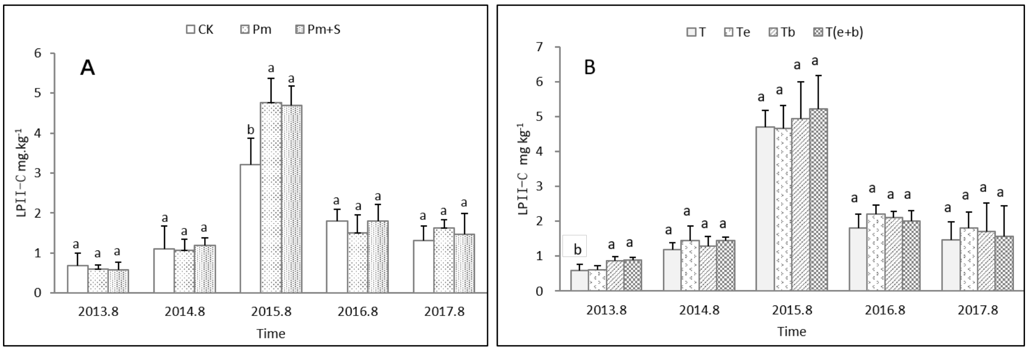

Figure 5. Dynamic change of soil organic carbon content (active component I) extracted by weak acid. Bars represent standard errors. Lower case letters above each panel show statistical signif-icance at $p < 0.05$. CK pig manure, S slurry, e earthworms, b bacteria, T Pm + S, Te T + earthworms, Tb T + bacteria, T(e + b) T + earthworms + bacteria (**A**) Pig manure, biogas slurry agricultural use (**B**) Biological control.

Figure 5B shows the effects of biological synergy on weak acid-extractable SOC (active component I) content. Compared to the control (T) treatment, the biological synergy treatment increased the active component I content of the weak acid-extractable SOC, but the difference was not significant. Furthermore, the weak acid-extractable SOC (active component I) increased in all the tested years, except 2013, after the biological synergy treatment was applied.

3.1.5. Strong Acid-Extractable SOC (Active Component II) Content

Compared to the CK treatment, the other two tested treatments significantly enhanced the content of strong acid-extractable SOC (active component II) in the second year (Figure 6A), and there was no obvious difference in the results across the other years. In addition, active component II of the strong acid-extractable SOC exhibited a gradual increase in the first two years, followed by a decrease in the last two years.

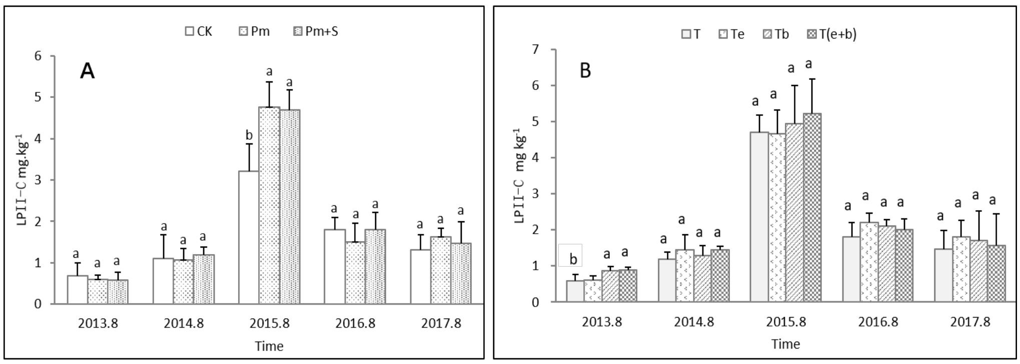

Figure 6. Dynamic change of organic carbon (active component II) content in soil by strong acid leaching. Bars represent standard errors. Lower case letters above each panel show statistical significance at $p < 0.05$. CK pig manure, S slurry, e earthworms, b bacteria, T Pm + S, Te T + earthworms, Tb T + bacteria, T(e + b) T + earthworms + bacteria (**A**) Pig manure, biogas slurry agricultural use (**B**) Biological control.

Compared to the control (T) treatment, all of the biological synergy treatments increased the SOC (Figure 6B), but the difference was not obvious. Furthermore, the results showed a gradual increase in the first two years, after which the SOC peaked in 2015, but it decreased in the last two years.

3.1.6. Soil Microbial Biomass Carbon Content

The effects of the different treatments on the soil microbial biomass carbon content are shown in Figure 7A. With increasing time, compared with the CK, Pm gradually increased the microbial biomass carbon, the content of which peaked in 2015. Then, it decreased in 2016 but increased again in 2017. Pm + S applied to the soil increased the microbial biomass carbon content, and then, reduced it annually thereafter, and its content peaked in 2014 but was lowest in 2017. However, compared with that of Pm, the increased application of biogas slurry (Pm + S) had no significant effect on the microbial biomass carbon content.

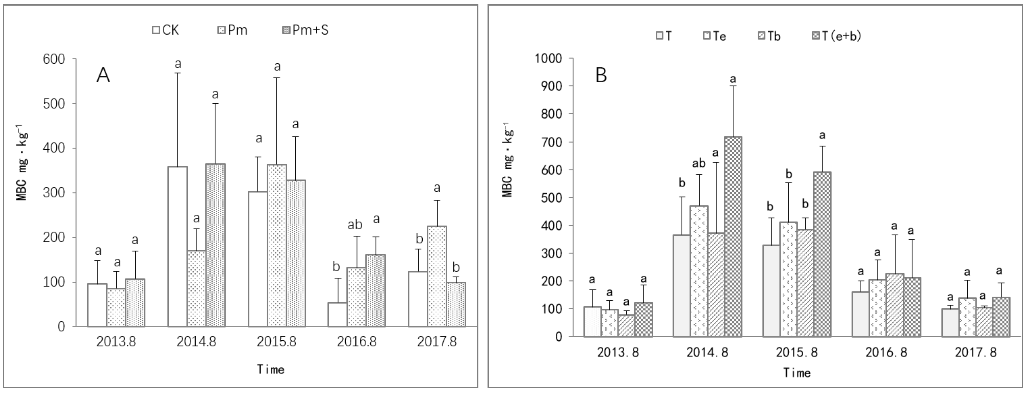

Figure 7. Dynamic change of soil microbial biomass carbon content. Bars represent standard errors. Lower case letters above each panel show statistical signif-icance at $p < 0.05$. CK pig manure, S slurry, e earthworms, b bacteria, T Pm + S, Te T + earthworms, Tb T + bacteria, T(e + b) T + earthworms + bacteria (**A**) Pig manure, biogas slurry agricultural use (**B**) Biological control.

The data in Figure 7B demonstrate the variation in microbial biomass carbon content caused by biological synergy. Compared with that under the CK, the microbial biomass carbon content under Te and T(e + b) exhibited similar changes; that is, it increased, peaking in 2014, and then decreased annually thereafter. The microbial biomass carbon content under the Tb treatment showed an increase in the first three years, reached a maximum in 2015, and gradually decreased in the next two years. These results suggest that biological synergy can rapidly increase the soil microbial biomass carbon content in the short term (2 years), while after an increased amount of time (2 years later), the promotive effect weakened.

3.2. Variation in Soil Humus Content under Different Treatments

Soil humus is mainly composed of HA, FA, and humin. As shown in Figure 8A, compared with the CK treatment, the Pm treatment increased the content of HA in the soil, except in 2016, and the same was true for the Pm + S treatment, except in 2013. Compared with CK, Pm and Pm + S enhanced the FA quantity from 2013 to 2017 (Figure 8C). However, except in 2014, compared with the CK treatment, the Pm and Pm + S treatments also increased the amount of humin (Figure 8E). In addition, the maximum content of HA was detected in the first year rather than in the other years under the Pm and Pm + S treatments (Figure 8E).

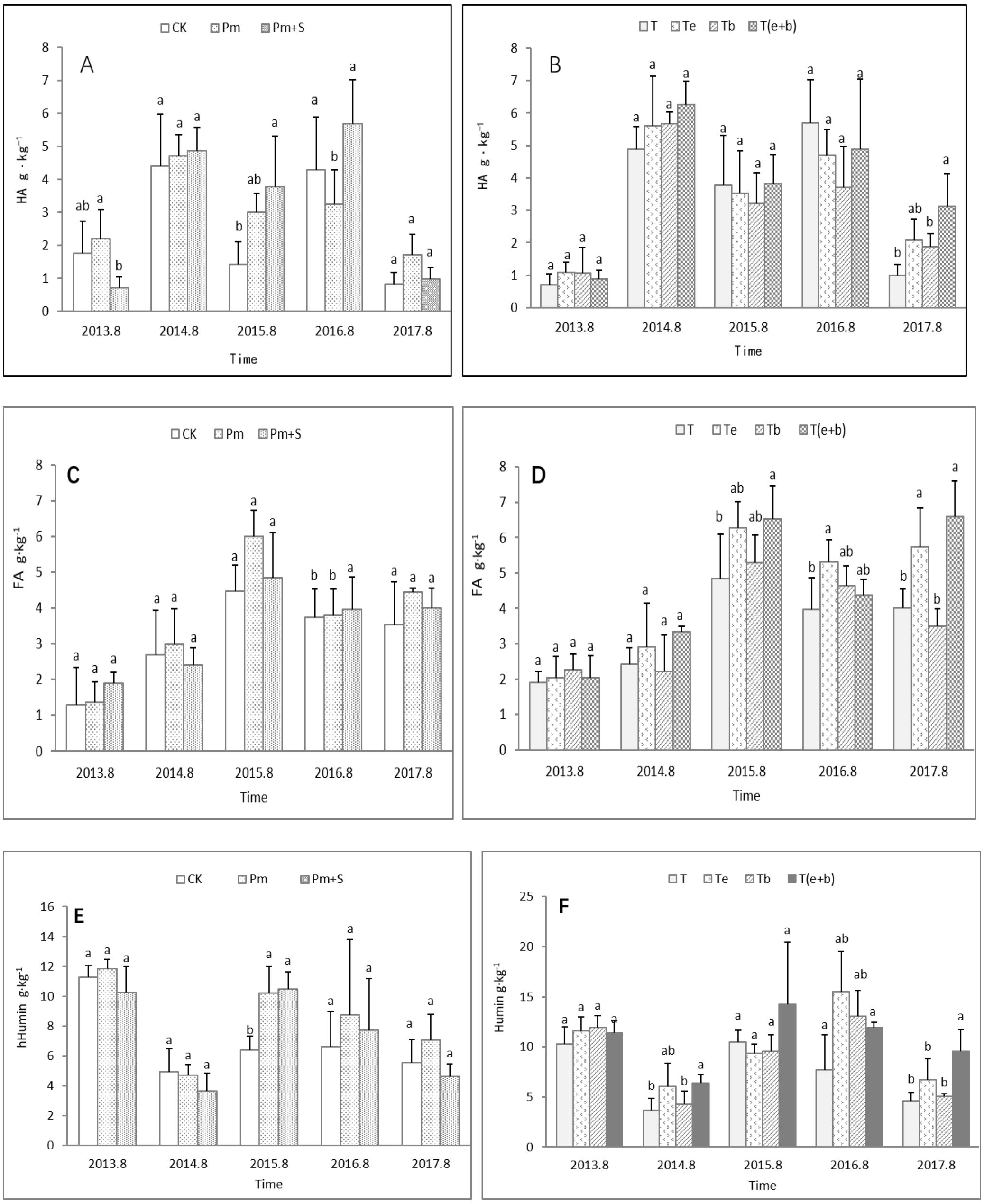

Figure 8. Dynamic changes of soil humus content. Bars represent standard errors. Lower case letters above each panel show statistical signif-icance at $p < 0.05$. CK pig manure, S slurry, e earthworms, b bacteria, T Pm + S, Te T + earthworms, Tb T + bacteria, T(e + b) T + earthworms + bacteria (**A**) Pig manure, biogas slurry agricultural use (**B**) Biological control (**C**) Pig manure, biogas slurry agricultural use (**D**) Biological control (**E**) Pig manure, biogas slurry agricultural use (**F**) Biological control.

The effects of biological synergy on the humus content are demonstrated in Figure 8B,D,F. As time increased, the HA content significantly increased compared to that in 2013 (Figure 8B). Compared with the T treatment, the Te, Tb, and T(e + b) treatments increased the FA and humin content (Figure 8D,F). Furthermore, the maximum FA content in each treatment was recorded in 2015 (Figure 8D), while the peak humin content in Te and Tb was recorded in 2016; the peak humin content in T and T(e + b) was recorded in 2015 (Figure 8F).

3.3. Principal Component Analysis (PCA) of Soil Chemical and Microbiological Properties

PCA of SOC and Humus Components

The PCA results of the SOC components and soil humus components are shown in Figure 9A. The first principal component (PC1) contributed to 53.6% of the variance, the contribution of which was dominated by total organic carbon (TOC), humin, HA, FA, LP I, and LPII content, and the second principal component (PC2) contributed to 14.1% of the variance, the contribution of which was dominated by LPC, RP-C and HWSOC content. Both PC1 and PC2 cumulatively explained 67.7% of the variance. The variables of PC1 represented the relatively refractory OM, including the OM fraction initially preserved in the soil and the fraction stabilized by earthworms. In addition, the results also suggest that the SOC content was largely dependent on stable C pools. In contrast, PC2 reflected the labile C pool in the soil.

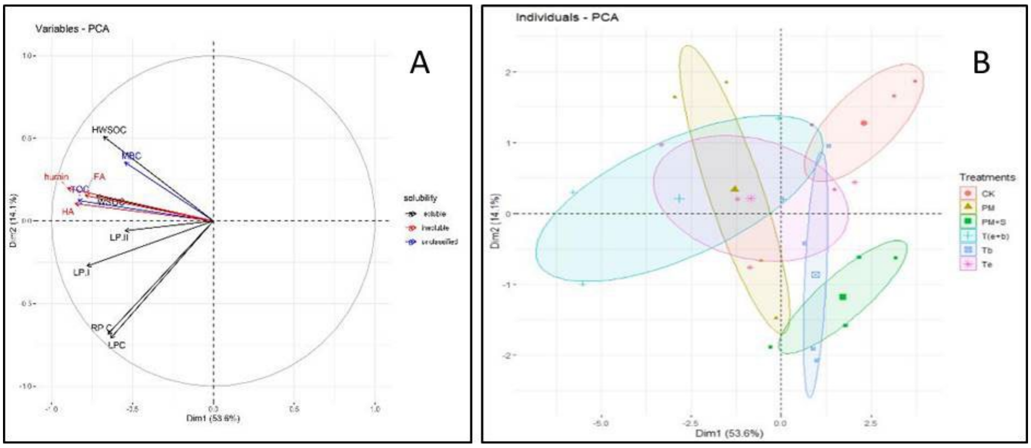

Figure 9. PCA loading values, CK—control; Pm—pig manure; S—slurry; e—earthworms; b—bacteria; T—Pm + S; Te—T + earthworms; Tb—T + bacteria; T(e + b)—T + earthworms + bacteria. PC2 is mainly related to the microbial biomass carbon content. (**A**) Corresponding scores (**B**) The total, labile, and recalcitrant OC data from the respective treatments after 5 years.

Figure 9B shows that the treatment with the bacterial agent mainly increased the content of active carbon components such as HWSOC, MBC, and LPC. On the contrary, the treatment with earthworms mainly increased the content of humus components, RP-C, and other insoluble organic carbon components, which indicates that earthworms could increase the soil-carbon sequestration ability of dry sloping land.

3.4. Implication for C Sequestration in the Soil

We calculated the SOC stocks, expressed as megagrams per hectare, by multiplying the SOC content (g kg^{-1}) by the soil bulk density (2.65 Mg m^{-3}) and depth (20 cm). It was assumed that soil C predominantly accumulated in the fertilized area (1 m^2) of each plot (30 m^2). Therefore, the SOC increase in each plot after 1 year should be 1/30 of that in the fertilized area. The amount of C sequestered and stabilized in the soils after 4 years of application was calculated via the following equation [28]:

$$\text{C sequestered (Mg C ha}^{-1}\text{ soil)} = SOCS_{current} - SOCS_{initial} \tag{1}$$

$$C_{stabilized}\text{ (Mg C ha}^{-1}\text{ soil)} = \text{C sequestered } {*}f_{RP\text{-}C} \tag{2}$$

where $SOCS_{current}$ and $SOCS_{initial}$ represent the SOC stocks before and after, respectively, the 5 years of applications, and $f_{RP\text{-}C}$ is the mass fraction of RP-C in SOC. RP-C is one of the most refractory constituents of SOM. Thus, it was used to calculate the stabilized C stocks in this study. The estimated C stocks are shown in Table 3. The 5-year treatments considerably increased the SOC stocks, which ranged from 0.70 to 2.88 Mg C ha^{-1} soil. The sequestered C was the highest in T (e + b), followed by Pm + S (2.06 Mg C ha^{-1} soil) and Te

(1.88 Mg C ha^{-1} soil). The annual C sequestration in T (e + b) was markedly higher than that in other treatments under long-term fertilization [28,30], although the C sequestration in response to manure application (5 t ha^{-1}) in this study was quantitatively lower.

Table 3. Effects of the treatments on sequestrated and stabilized C stocks in soil after 4 years.

Amount	CK	Pm	Pm + S	Te	Tb	T(e + b)
C sequestrated	0.70	1.87	2.06	1.88	1.62	2.88
C stabilized	0.37	1.15	1.28	1.18	1.01	1.87

Mg C ha^{-1} soil. CK—control; Pm—pig manure; S—slurry; e—earthworms; b—bacteria; T—Pm + S; Te—T + earthworms; Tb—T + bacteria; T(e + b)—T + earthworms + bacteria.

The mass fraction of the stabilized C in SOC increased by 20%. The above phenomena suggest that the T(e + b) treatment was an effective way to sequester and stabilize manure-based OM in arid hillside soils. In addition, by accelerating tree growth, the T(e + b) treatment was effective for C fixation in the aboveground parts of trees [31].

4. Discussion

Soil is the largest and most active ecosystem carbon pool on Earth's surface, and its small change can have a huge impact on atmospheric CO_2 concentration. Our results show that manure can increase SOC content, especially in the second year (Figure 2A), possibly due to the preponderance of organic matter in organic manure [32]. In addition, we found that soil organic carbon content did not change significantly after the application of earthworms or phosphorus-solubilizing bacteria on top of fertilizer (Figure 2B). These results indicate that increasing soil-carbon storage with organic fertilizers is an important way to achieve carbon neutrality. HWSOC, which can be used to estimate the content of easily decomposed organic matter, is the active component of SOC, and its content is usually higher than the content of SOC, which can be extracted using cold water. However, HWSOC has a low stability [33,34]. Our results showed that fertilizer could increase WSOC and HWSOC content in the soil, especially in the second year (Figures 3A and 4A), possibly due to increased microbial biomass and enzyme activities [35]. T(E + B) treatment had little effect on WSOC content, and there was only a significant difference in last year. However, this treatment significantly increased HWSOC content, which occurred in the first, third, and fourth years (Figures 3B and 4B). This may be because the activities of earthworms and phosphate-solubilizing bacteria increased the number of soil aggregates, as well as the phosphorus content and soil microbial activity [36].

The long-term application of pig manure could significantly improve soil microbial biomass carbon content. In the short term, earthworms and bacterial agents had synergistic effects on soil microbial biomass carbon content, but the long-term effect disappeared.

Humus is an important component of organic matter which plays an important role in the recycling and conversion of soil organic carbon. HA is the most active component of soil humus, and is closely related to soil fertility. As can be seen from the data in Figure 8, the short-term application of pig manure and biogas slurry can improve the content of HA and FA in soil, but the long-term effect is not obvious. The long-term application of earthworms and bacterial agents showed obvious synergistic effect. One possible reason for the increase in FA content is that earthworm and phosphate lytic bacteria enhance the activity of microorganisms, accelerate the decomposition rate of Pm, and increase the conversion rate of humus [37]. As the most stable component of humus, the content of humin showed a downward trend, indicating that the stable humus component was transformed to the active humus component.

Rudrappa et al. [38] observed that balanced fertilization with NPK + manure was the most efficient strategy for C sequestration, with an annual amount of 0.73 Mg C ha^{-1} soil. Mandal et al. [39] applied NPK + manure to different cropping systems and reported an average annual C sequestration of 0.70 Mg C ha^{-1} soil. In addition to its quantity, the quality of C sequestered in soil is also notable for restricting global warming. The stabilized C accounted for half of the sequestered C in the treatments, ranging from 0.37 to 1.87 Mg C ha^{-1} soil (Table 3).

5. Conclusions

The soil C pools increased significantly in the short term (2 years) under manure treatment, and the integrated application of manure, slurry, earthworms, and bacteria significantly increased various C fractions, such as the content of SOC, HWSOC, humus, and microbial biomass carbon; this indicates the rapid and positive effects of earthworms and bacteria on C accumulation. In addition, the positive effects of earthworms and bacteria on OM stabilization were supported by increases in the recalcitrant C pools and the PCA results. Furthermore, C sequestration in response to the integrated application reached 2.87 Mg C ha^{-1} soil, while 1.88 Mg C ha^{-1} soil was stabilized.

Author Contributions: Conceptualization, Z.W., H.C. and Y.Z.; methodology, H.Z; software, J.Z., J.C.; validation, Y.A. and Z.W.; investigation, Z.W., H.-J.X. and G.Z.; resources, Y.L.; data curation, Z.W., J.Z., H.Z., W.L. and J.W.; writing—original draft preparation, Z.W., H.C. and Y.A.; writing—review and editing, Z.W., H.C. and Y.Z.; visualization, W.L. and J.W.; supervision, H.C.; project administration, H.-J.X.; funding acquisition, Y.L. and Y.Z.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (41877117), the Double First-class Discipline Promotion Project (2021B10564001), and the Key-Area Research and Development Program of Guangdong Province (2019B020218002).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: The study did not involve humans.

Data Availability Statement: Not applicable.

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

References

1. Bittman, S.; Forge, T.; Kowalenko, C. Responses of the bacterial and fungal biomass in a grassland soil to multi-year appli-cations of dairy manure slurry and fertilizer. *Soil Biol. Biochem.* **2005**, *37*, 613–623. [CrossRef]
2. Kumar, R.R.; Park, B.J.; Cho, J.Y. Application and environmental risks of livestock manure. *J. Korean Soc. Appl. Biol. Chem.* **2013**, *56*, 497–503. [CrossRef]
3. Zhang, Y.; Wang, L.; Li, W.; Xu, H.; Shi, Y.; Sun, Y.; Cheng, X.; Chen, X.; Li, Y. Earthworms and phosphate-solubilizing bacteria enhance carbon accumulation in manure-amended soils. *J. Soils Sediments* **2017**, *17*, 220–228. [CrossRef]
4. Huang, G.F.; Fang, M.; Wu, Q.T.; Zhou, L.X.; Liao, X.D.; Wong, J.C. Co-composting of pig manure with leaves. *Environ. Technol.* **2001**, *22*, 1203–1212. [CrossRef]
5. Lal, R. Soil carbon sequestration impacts on global climate change and food security. *Science* **2004**, *304*, 1623–1627. [CrossRef]
6. Zhao, Y.; Wang, M.; Hu, S.; Zhang, X.; Ouyang, Z.; Zhang, G.; Huang, B.; Zhao, S.; Wu, J.; Xie, D.; et al. Economics- and policy-driven organic carbon input enhancement dominates soil organic carbon accumulation in Chinese croplands. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 4045–4050. [CrossRef]
7. Maillard, É.; Angers, D.A. Animal manure application and soil organic carbon stocks: A meta-analysis. *Glob. Change Biol.* **2014**, *20*, 666–679. [CrossRef]
8. Tian, K.; Zhao, Y.; Xu, X.; Hai, N.; Huang, B.; Deng, W. Effects of long-term fertilization and residue management on soil organic carbon changes in paddy soils of China: A meta-analysis. *Agric. Ecosyst. Environ.* **2015**, *204*, 40–50. [CrossRef]
9. Liang, F.; Li, J.; Yang, X.; Huang, S.; Cai, Z.; Gao, H.; Ma, J.; Cui, X.; Xu, M. Three-decade long fertilization-induced soil organic carbon sequestration depends on edaphic characteristics in six typical croplands. *Sci. Rep.* **2016**, *6*, 30350. [CrossRef]
10. Malyan, S.K.; Kumar, S.S.; Fagodiya, R.K.; Ghosh, P.; Kumar, A.; Singh, R.; Singh, L. Biochar for environmental sustainability in the energy-water-agroecosystem nexus. *Renew. Sustain. Energy Rev.* **2021**, *149*, 111379. [CrossRef]
11. Gattinger, A.; Muller, A.; Haeni, M.; Skinner, C.; Fliessbach, A.; Buchmann, N.; Mäder, P.; Stolze, M.; Smith, P.; Scialabba, N.E.; et al. Enhanced top soil carbon stocks under organic farming. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 18226–18231. [CrossRef]
12. Zhu, Z.K.; Xiao, M.L.; Wei, L.; Wang, S.; Ding, J.N.; Chen, J.P.; Ge, T.D. The key biogeochemical processes of carbon se-questration in paddy soil and its countermeasures for carbon neutralization. *China J. Eco-Agric.* **2022**, *30*, 592–602.
13. Malyan, S.K.; Bhatia, A.; Kumar, A.; Gupta, D.K.; Singh, R.; Kumar, S.S.; Tomer, R.; Kumar, O.; Jain, N. Methane production, oxidation and mitigation: A mechanistic understanding and comprehensive evaluation of influencing factors. *Sci. Total Environ.* **2016**, *572*, 874–896. [CrossRef]

14. Wei, L.; Ge, T.; Zhu, Z.; Luo, Y.; Yang, Y.; Xiao, M.; Yan, Z.; Li, Y.; Wu, J.; Kuzyakov, Y. Comparing carbon and nitrogen stocks in paddy and upland soils: Accumulation, stabilization mechanisms, and environmental drivers. *Geoderma* **2021**, *398*, 115121. [CrossRef]
15. Zhu, Z.; Ge, T.; Liu, S.; Hu, Y.; Ye, R.; Xiao, M.; Tong, C.; Kuzyakov, Y.; Wu, J. Rice rhizodeposits affect organic matter priming in paddy soil: The role of N fertilization and plant growth for enzyme activities, CO_2 and CH_4 emissions. *Soil Biol. Biochem.* **2018**, *116*, 369–377. [CrossRef]
16. Fontaine, S.; Mariotti, A.; Abbadie, L. The priming effect of organic matter: A question of microbial competition. *Soil Biol. Biochem.* **2003**, *35*, 837–843. [CrossRef]
17. Wan, J.H.C.; Wong, M.H. Effects of earthworm activity and P-solubilizing bacteria on P availability in soil. *J. Plant Nutr. Soil Sci.* **2004**, *167*, 209–213. [CrossRef]
18. Cheng, X.; Zhang, Y.-L.; Li, W.-Y.; Wang, L.-Y.; Zhang, H.-C.; Lu, W.-S.; Chen, X.-Y.; Li, Y.-T.; Xu, H.-J. Earthworms and phosphate-solubilizing bacteria stimulate nitrogen storage and cycling in a manured arid soil. *Soil Sci. Soc. Am. J.* **2019**, *83*, 153–162. [CrossRef]
19. Zsolnay, Á. Dissolved organic matter: Artefacts, definitions, and functions. *Geoderma* **2003**, *113*, 187–209. [CrossRef]
20. Bożena, S.; Aleksandra, U. Dissolved organic matter: Biogeochemistry, dynamics, and environmental significance in soils. *Adv. Agron.* **2011**, *110*, 1–75.
21. Liang, X.; Chen, Q.; Rana, M.S.; Dong, Z.; Liu, X.; Hu, C.; Tan, Q.; Zhao, X.; Sun, X.; Wu, S. Effects of soil amendments on soil fertility and fruit yield through alterations in soil carbon fractions. *J. Soils Sediments* **2021**, *21*, 2628–2638. [CrossRef]
22. Wang, X.G.; Li, C.S.; Luo, Y.; Hua, K.K.; Zhou, M.H. The impact of nitrogen amendment and crop growth on dissolved organic carbon in soil solution. *J. Mt. Sci.* **2016**, *13*, 95–103. [CrossRef]
23. Smreczak, B.; Ukalska-Jaruga, A. Dissolved organic matter in agricultural soils. *Soil Sci. Annu.* **2021**, *72*, 132234. [CrossRef]
24. Lützow, M.V.; Kögel-Knabner, I.; Ekschmitt, K.; Matzner, E.; Guggenberger, G.; Marschner, B.; Flessa, H. Stabilization of organic matter in temperate soils: Mechanisms and their relevance under different soil conditions—A review. *Eur. J. Soil Sci.* **2006**, *57*, 426–445. [CrossRef]
25. Li, Y.T.; Cai, Y.F.; Sun, L.Y.; Wang, Y.J.; Zhao, S.Q.; Kong, D.Y.; Huang, J.; Chen, H. Bacillus subtiis HL-1 and Its Application in Solubilizing. CN102399713A, 22 September 2012.
26. Cai, Y.F.; Li, Y.T.; Sun, L.Y.; Huang, J.; Kong, D.Y.; Zhao, F.Y. One Bacillus Cereus of Soil Nutrient Activation and Its Application. Patent, 2013.
27. Rovira, P.; Vallejo, V.R. Labile and recalcitrant pools of carbon and nitrogen in organic matter decomposing at different depths in soil: An acid hydrolysis approach. *Geoderma* **2002**, *107*, 109–141. [CrossRef]
28. Ghani, A.; Dexter, M.; Perrott, K.W. Hot-water extractable carbon in soils: A sensitive measurement for determining impacts of fertilisation, grazing and cultivation. *Soil Biol. Biochem.* **2003**, *35*, 1231–1243. [CrossRef]
29. Bao, S.D. *Soil Agrochemical Analysis*; China Agriculture Press: Beijing, China, 2000.
30. Wu, J.; O'Donnell, A.G.; He, Z.L.; Syers, J.K. Fumigation-extraction method for the measurement of soil microbial biomass-S. *Soil Biol. Biochem.* **1994**, *26*, 117–125. [CrossRef]
31. Sun, Y. The effect of Combined Application of Pig Manure and Engineering Organisms on Soil Chemical and Microbial Properties. Ph.D. Dissertation, South China Agricultural University, Guangzhou, China, 2014.
32. Dong, W.; Zhang, X.; Wang, H.; Dai, X.; Sun, X.; Qiu, W.; Yang, F. Effect of different fertilizer application on the soil fertility of paddy soils in red soil region of southern China. *PLoS ONE* **2012**, *7*, e44504. [CrossRef]
33. Sparling, G.; Vojvodić-Vuković, M.; Schipper, L.A. Hot-water-soluble C as a simple measure of labile soil organic matter: The relationship with microbial biomass C. *Soil Biol. Biochem.* **1998**, *30*, 1469–1472. [CrossRef]
34. Jones, D.; Willett, V. Experimental evaluation of methods to quantify dissolved organic nitrogen (DON) and dissolved or-ganic carbon (DOC) in soil. *Soil Biol. Biochem.* **2006**, *38*, 991–999. [CrossRef]
35. Tian, S.Y.; Wang, M.W.; Cheng, Y.H.; Chen, X.Y.; Liu, M.Q. Long-term effects of chemical and organic amendments on red soil enzyme activities. *Acta Ecol. Sin.* **2017**, *37*, 4963–4972.
36. Wolters, V. Invertebrate control of soil organic matter stability. *Biol. Fertil. Soils* **2000**, *31*, 1–19. [CrossRef]
37. Li, X.; Liu, C.; Zhao, H.; Gao, F.; Ji, G.; Hu, F.; Li, H. Similar positive effects of beneficial bacteria, nematodes and earthworms on soil quality and productivity. *Appl. Soil Ecol.* **2018**, *130*, 202–208. [CrossRef]
38. Rudrappa, L.; Purakayastha, T.J.; Singh, D.; Bhadraray, S. Long-term manuring and fertilization effects on soil organic carbon pools in a Typic Haplustept of semi-arid sub-tropical India. *Soil Tillage Res.* **2006**, *88*, 180–192. [CrossRef]
39. Mandal, B.; Majumder, B.; Bandyopadhyay, P.K.; Hazra, G.C.; Gangopadhyay, A.; Samantaray, R.N.; Mishra, A.K.; Chaudhury, J.; Saha, M.N.; Kundu, S. The potential of cropping systems and soil amendments for carbon sequestration in soils under long-term experiments in subtropical India. *Glob. Change Biol.* **2007**, *13*, 357–369. [CrossRef]