fermentation

MDPI

Article

Inhibitory Effects of Ammonia on Archaeal 16S rRNA Transcripts in Thermophilic Anaerobic Digester Sludge

Shenghui Wang [1], Caihong Song [1], Jie Li [1], Chen Zhang [2,*] and Peng Li [2,3]

[1] College of Life Science, Liaocheng University, Liaocheng 252059, China; wangshenghui@lcu.edu.cn (S.W.); songcaihong@lcu.edu.cn (C.S.); lijie@lcu.edu.cn (J.L.)
[2] State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China
[3] School of Entrepreneurship, Wuhan University of Technology, Wuhan 430070, China; cyxylp@whut.edu.cn
* Correspondence: chenzhang@ipe.ac.cn; Tel.: +86-10-82545039

Abstract: High temperatures exacerbate the ammonia inhibition of anaerobic digestion coupled with methanogenesis. The inhibition of methane production by ammonia has been observed in other studies. However, the underlying mechanism is not well understood and requires further investigation. This study explored the effect of ammonia stress on archaeal 16S rRNA transcripts in thermophilic anaerobic digester sludge. Different ammonium concentrations were checked for their influence on the methanogenic rate and hydrogen accumulation. Quantitative PCR was used to compare the changes in total archaeal 16S rRNA expression. A Monte Carlo permutation test within redundancy analysis (RDA) was adopted for exploring the relationship between environmental variables and archaeal 16S rRNA and their transcripts. The results showed that with the increase in ammonium concentration, the methanogenic rate decreased and hydrogen accumulation occurred. The total archaeal 16S rRNA genes and transcripts copy numbers decreased significantly in treatments with higher ammonium concentrations (7 and 10 g NH_4^+-N/L), but did not change much at lower ammonia concentrations (3 g NH_4^+-N/L) compared with the 0 g NH_4^+-N/L treatment. The RDA analysis further revealed that most environmental variables, including ammonia and methane, except for formate, were significantly correlated with the community structure activity of archaeal 16S rRNA transcripts rather than the community structure of their genes. The composition of archaeal 16S rRNA transcripts showed that the hydrogenotrophic methanogen *Methanothermobacter* dominated the methanogenic community activity in all incubations. It exhibited sensitivity to ammonia stress and should be responsible for the methanogenic inhibition under thermophilic conditions. Our findings suggested that archaeal 16S rRNA transcripts, rather than 16S rRNA genes, are key indicators of ammonia stress and methanogenic activity.

Keywords: ammonia stress; thermophilic; anaerobic digester; methanogens

Citation: Wang, S.; Song, C.; Li, J.; Zhang, C; Li, P. Inhibitory Effects of Ammonia on Archaeal 16S rRNA Transcripts in Thermophilic Anaerobic Digester Sludge. *Fermentation* **2023**, *9*, 728. https://doi.org/10.3390/fermentation9080728

Academic Editors: Hui Yun and Yutuo Wei

Received: 25 June 2023
Revised: 27 July 2023
Accepted: 31 July 2023
Published: 4 August 2023

1. Introduction

Anaerobic digestion (AD) is a widely practiced technology used for the treatment of high organic content waste and the recovery of renewable energy via biogas production [1,2]. In particular, thermophilic AD (approximately 55 °C) has more advantages, such as a higher organic digestion rate, biogas production rate and pathogen removal rate than mesophilic AD (approximately 35 °C) [3,4]. However, high temperatures will accelerate protein metabolism and ammonium accumulation in thermophilic AD [5]. A high ammonium concentration has a negative influence on methane production in anaerobic digesters [6–8]. Generally, inorganic ammonia nitrogen exists in aqueous solution in the equilibrium form of free ammonia (NH_3) and ammonium ions (NH_4^+). Increasing temperature shifts the equilibrium position from NH_4^+ to NH_3 in thermophilic AD [9]. NH_3 easily penetrates the cell membrane and then ionizes to form NH_4^+, which may inhibit methane-synthesizing

enzymes directly [10–12]. Therefore, an elevated temperature will aggravate the inhibition effects of ammonia on methane production in anaerobic digesters [13–15].

Methanogens, including aceticlastic methanogens (AM) and hydrogenotrophic methanogens (HM), are more vulnerable to ammonia stress. Ammonia could inhibit methanogenic activity, leading to the accumulation of volatile fatty acids (VFA), especially acetate, and a reduction in methane production. Methanogenic pathways of acetate include the acetoclastic methanogenesis pathway (AMP) mediated by aceticlastic methanogens and the syntrophic acetate oxidation methanogenesis pathway (SAOMP) involving syntrophic acetate oxidation bacteria (SAOB) and hydrogenotrophic methanogens [16]. Generally, AM are more sensitive to ammonia stress than HM [14,17,18]. Therefore, high ammonia levels would induce a shift of acetate methanogenesis from AMP to SAOMP [19–21]. Our previous study revealed that AMP was markedly inhibited at 7 g NH_4^+-N/L under mesophilic conditions [22], whereas the inhibition effects of ammonia on methanogens under thermophilic conditions need to be further studied.

The archaeal 16S rRNA gene is usually used as a biomarker for phylogenetic analysis to study the composition and succession of archaeal community structures in anaerobic reactors [23]. Few studies have focused on the activity of archaeal communities indicated by archaeal 16S rRNA transcripts. We found that the functional gene *mcrA* transcripts of methanogens reacted strongly to ammonium stress, rather than the change in the community structure [22]. However, the response of archaeal 16S rRNA genes and transcripts to ammonia stress in AD under thermophilic conditions is not clear. Comparative analysis of the response of archaeal 16S rRNA genes and transcripts to ammonia stress is helpful to elucidate the mechanism of ammonium stress inhibiting methane production in AD.

This study aims to observe the inhibitory effects of ammonia on archaeal 16S rRNA genes and transcripts in thermophilic anaerobic sludge, and the sludge samples were analyzed with different ammonium treatments in the laboratory under thermophilic anaerobic conditions. The study (1) determined the rate of CH_4 and CO_2 production; (2) detected the accumulation of H_2 and acetate; (3) obtained the copy number of archaeal 16S rRNA genes and transcripts; and (4) analyzed the archaeal composition and dynamic changes based on archaeal 16S rRNA genes and transcripts with different ammonia treatments.

2. Materials and Methods

2.1. Operation of Sludge Anaerobic Incubation

Sludge samples were collected from a mesophilic digester treating swine manure located in Shunyi District, Beijing. The activated sludge was pre-cultured at 55 °C for 3 months in the lab before use. Anaerobic incubation was initiated by adding 30 mL HEPES buffer (50 mM) into serum bottles containing 1 g swine manure and 10 g sludge under nitrogen blowing, as described previously [22]. Then, NH_4Cl was added to these bottles to obtain the different concentrations of 0 (named 0-N), 3(3-N), 7(7-N) and 10 (10-N) g NH_4^+-N/L. Three parallel treatments were set at each ammonia concentration. The pH of the mixed buffer was adjusted to 7.0 before sealing with butyl rubber stoppers. After flushing with N_2, these bottles were incubated statically in the dark at 55 °C.

2.2. Physicochemical Analyses

Samples for gaseous CO_2, CH_4 and H_2 were regularly collected and detected with GC-7890A [22]. Samples for liquid acetate were also collected and stored. After centrifugation and membrane filtration, the filtrate was analyzed using high-performance liquid chromatography (HPLC) [24]. The pH of all samples was measured using a pH meter. The concentration of NH_4^+-N was measured colorimetrically, as described previously [2]. The free ammonia (NH_3-N) was estimated using the equilibrium of NH_4^+/NH_3 [5].

2.3. Archaeal 16S rRNA Gene Amplification and Reverse Transcription PCR

The samples were collected for the extraction of total DNA and RNA according to the methods used previously [25,26]. After removing DNA, RNA samples were purified and

then used as a template to amplify the 16S rRNA genes with the universal archaea primers 109f and 934r to confirm the complete removal of DNA [24]. Finally, the synthesis of cDNA was performed via reverse transcription PCR (RT-PCR), as previously described [22].

2.4. High-Throughput Sequencing and Data Analysis

The archaeal community was analyzed based on Illumina high-throughput sequencing with primers of Ar344f (5′-ACGGGGYGCAGCAGGCGCGA-3′) and Ar806r (5′-GGACTACVSGGGTATCTAAT-3′), which were used to amplified the hyper variable V3–V4 region of the archaeal 16S rRNA genes [24]. The high-throughput amplicon sequencing data were compared with the Silva 16S rRNA gene database and then analyzed with the QIIME program and RDP Classifier. The GenBank accession numbers of archaeal 16S rRNA genes and transcripts sequences are PRJNA830298 and PRJNA830311, respectively.

2.5. Genes Quantification by qPCR

Archaeal 16S rRNA genes and transcripts were quantified using quantitative (real-time) PCR with the primer pair Ar364f/934r [27], according to the research [26]. Plasmids containing the archaeal 16S rRNA genes obtained from the positive clones were used as the standard plasmids. The concentrations of the standard plasmids ranged from 1.2×10^8 to 1.2×10^2 copies/μL. Data processing followed previous research [2].

3. Results

3.1. Effect of Ammonia on Methanogenesis

The pH fluctuated at 7.40 and 6.78 during two months of incubation in all ammonia treatments (Figure 1a). The pH of 0-N and 3-N treatments increased slightly, varying between 7.0 and 7.3. The pH of 7-N and 10-N treatments continuously decreased to 6.78 and 6.9 on day 56, respectively. The lower pH value in 7-N and 10-N treatments was related to the continuous accumulation and high concentration of acetate (Figure 1e). These stable pH values can maintain the chemical equilibrium between ammonium and ammonia, and these values do not affect the activity of methanogens [22]. The concentration of NH_4^+-N remained stable throughout the incubation in all ammonia treatments (Figure 1b). The concentration of free ammonia was below 2.2% of total NH_4^+-N according to the equilibrium of NH_4^+/NH_3 at 55 °C [28,29]. The ammonia concentrations (approximately 6 to 127 mg NH_3-N/L) in this study covered the critical range (10–30 mg NH_3-N/L) for ammonia effects on methanogens [30,31].

The hydrogen partial pressure increased first and then decreased in all ammonia treatments, and the average H_2 partial pressure gradually increased with increasing ammonia concentration during the incubation (Figure 1c). In the 0-N treatment, the H_2 partial pressure showed a transient increase and reached a maximum value of 40 Pa on day 4. After that, it dropped to about 6 Pa on day 30 and then rose to 11 Pa at the end of incubation. The maximum H_2 partial pressure was 25 Pa (day 17), 35 Pa (day 17) and 47 Pa (day 21) in the 3-N, 7-N and 10-N treatments, respectively. Then, the H_2 partial pressure decreased significantly and fluctuated at 11 Pa, 18 Pa and 31 Pa on day 86 in the 3-N, 7-N and 10-N treatments, respectively. Acetate increased rapidly to about 43 mM on day 14 in both the 0-N and 3-N treatments and then dropped to 1.7 mM on day 30 (0-N treatment) and 0.6 mM on day 56 (3-N treatment), respectively (Figure 1e). In the 7-N treatment, the acetate concentration reached 44 mM on day 56. Subsequently, it fell to 33 mM on day 86. The concentration of acetic acid kept accumulating in 10-N treatment, reaching 43 mM at the end of the experiment. High acetate accumulation was consistent with low pH in 7-N and 10-N treatments (Figure 1a,e).

Methane production showed an 8-day (0-N treatment) and 23-day (3-N treatment) lag phase at the beginning of incubation. Then, the methane production rate increased rapidly and finally reached 3.6 mM (0-N treatment) and 3.1 mM (3-N treatment), respectively (Figure 1f). The CH_4 production rate in the 3-N treatment was 23.4% lower than that in

the 0-N treatment. Methanogenesis was markedly inhibited and about 1 mM methane accumulated at the end of incubation in the 7-N treatment. Methanogenesis was absolutely inhibited during the whole incubation in the 10-N treatment. CO_2 rapidly increased to more than 0.5 mM on the first day in all treatments, and then the CO_2 production rate decreased in all treatments (Figure 1d). In the 0-N treatment, the concentration of CO_2 was up to 1.86 mM by the end of the experiment. The production rate and accumulation of CO_2 decreased with the increasing ammonia concentration.

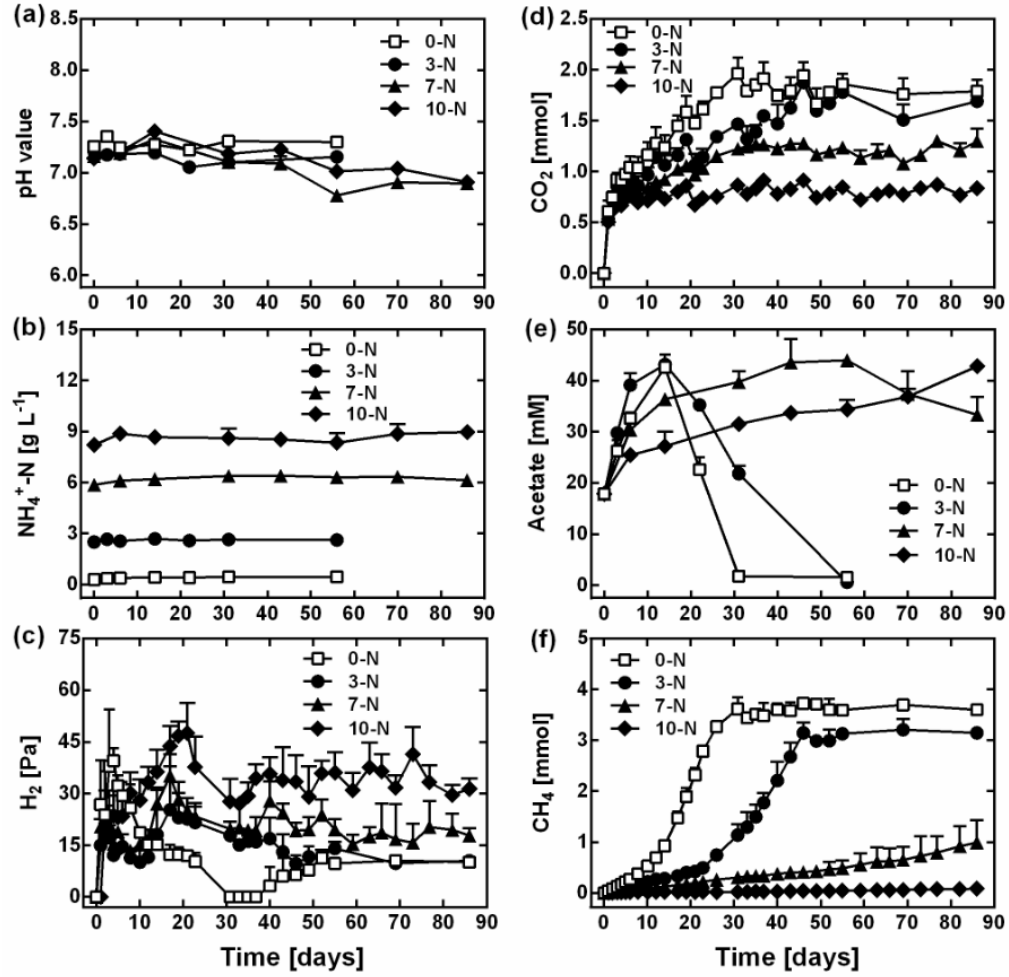

Figure 1. Effects of different ammonia concentrations on pH (**a**), NH_4^+-N (**b**), H_2 (**c**), CO_2 (**d**), acetate (**e**) and CH_4 (**f**) in thermophilic anaerobic digester sludge.

3.2. Methanogenic Archaea Dynamics

High-throughput sequencing of archaeal 16S rRNA genes and transcripts was used to analyze the archaeal community structure and activity in sludge incubations at different time points (Figure 2). The composition of 16S rRNA genes revealed that the community structure of archaea contained *Methanothermobacter*, *Methanosaeta*, *Methanospirillum*, *Methanosarcina*, *Methanobacterium*, *Methanoculleus* and *Methanobrevibacter* at the genus level (Figure 2a–d). *Methanothermobacter* had the highest average relative abundance in all ammonia treatments. *Methanothermobacter* is a representative thermophilic hydrogenotrophic methanogen capable of reducing CO_2 with H_2 to produce methane [32], and is usually founded in thermophilic conditions [33]. In the 0-N treatment, the relative abundance of *Methanothermobacter* increased from 44% on day 1 to 70% on day 31, then decreased significantly to 29% on day 56. The dynamic trends of *Methanothermobacter* relative abundance in the 3-N, 7-N and 10-N treatments were similar to that in the 0-N treatment. However, the relative abundance of *Methanothermobacter* in the 10-N treatment was only

19% on day 56, about 30% lower than that in 0-N, 3-N and 7-N treatments. The inhibition effects of ammonia on the relative abundance of *Methanothermobacter* could only be seen in the 10-N treatment after 31 days' incubation. Although the 16S rRNA genes analysis showed that *Methanothermobacter* had a high relative abundance, the dynamics of the *Methanothermobacter* relative abundance could not reflect the inhibitory effect of ammonia on methanogenesis during the 31 days' incubation. The genus *Methanosaeta* was a strictly aceticlastic methanogen and has been recognized as the most sensitive methanogen to ammonia [18,34]. However, the relative abundance of *Methanosaeta* increased from 30% (0-N) to 39% (10-N) on day 56 in this study. In addition, the relative abundance of hydrogenotrophic methanogen *Methanospirillum* increased from 18% (0-N) to 22% (10-N) on day 56 in this study.

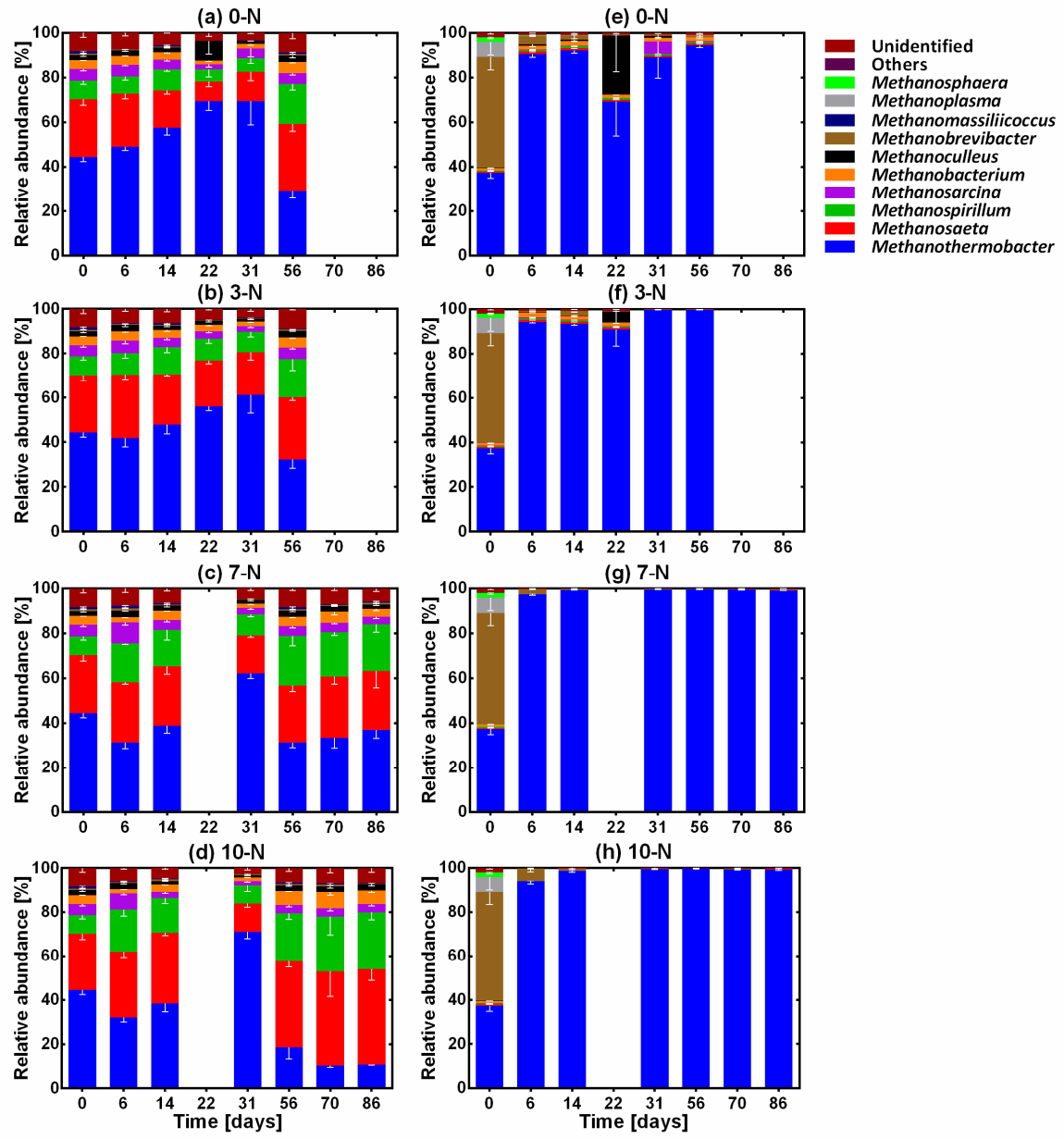

Figure 2. Relative abundance of archaea estimated with Illumina high-throughput sequencing analyses of archaeal 16S rRNA genes (**a**–**d**) and 16S rDNA transcripts (**e**–**h**) in the sludge with different ammonia concentrations.

The relative abundance of archaeal 16S rRNA transcripts showed that *Methanothermobacter* was the main active methanogenic archaea in all ammonium treatments during the whole incubation. Although the relative abundance of *Methanobrevibacter* was 50% on day 0, it decreased significantly to less than 6% during the incubation in all ammonium treatments. In the 0-N and 3-N treatments, *Methanobacterium*, *Methanosaeta* and *Methanospirillum* showed a low relative abundance of transcripts, while *Methanoculleus* increased briefly to 30% and 6% at day 22 in the 0-N and 3-N treatments, respectively. In the 7-N and 10-N treatments, the relative abundance of *Methanothermobacter* was more than 95%, indicating that *Methanothermobacter* dominated the methanogenesis with the increasing ammonium concentration. It appears that the composition of the archaeal 16S rRNA transcripts became less diverse with the increase in ammonium.

3.3. Correlation Analysis

Environmental variables of the AD process could really reflect the metabolic activity of microbial community. RDA was performed in order to determine the relationships between themajor environmental variables of methanogenesis and the relative abundance of archaeal 16S rRNA genes (Figure 3a) and their transcripts (Figure 3b). The first and second RDA components (RDA1 and RDA2) explained 59% and 77% of the total variations in archaeal 16S rRNA genes and transcripts, respectively. A Monte Carlo permutation test within RDA further showed significant correlations between environmental variables and archaeal community structure and activity (Table 1). As illustrated by the relatively close clustering, Formate (Pseudo-F = 41.6, $p < 0.01$) and Time (Pseudo-F = 24.4, $p < 0.01$) among the environmental variables were significantly correlated with the change in archaeal 16S rRNA genes abundance (Table 1). These results indicated that Formate and Time were the main environmental factors that reflected or led to the change in archaeal 16S rRNA genes abundance. However, except for Formate and Time, other environmental variables, including CO_2, NH_4^+, H_2, CH_4 and pH, were significantly correlated with the abundance of archaeal 16S rRNA transcripts with the p value < 0.01 (Table 1). These results indicated that the community activity reflected by archaeal 16S rRNA transcripts was significantly correlated with the metabolic compounds related to methanogenesis. However, the environmental factor Time mainly affected the community structure reflected by archaeal 16S rRNA genes. It suggested that compared with the archaeal 16S rRNA genes, the 16S rRNA transcripts were a key biomarker for studying the function and metabolic activity of the flora.

Table 1. The Monte Carlo permutation test within the redundancy analysis of the relationships between the major environmental variables and the relative abundance of archaeal 16S rRNA genes and their transcripts.

	Archaeal 16S rRNA Genes				Archaeal 16S rRNA Transcripts			
	Explanation [%]	**Contribution [%]**	**Pseudo-F**	***p***	**Explanation [%]**	**Contribution [%]**	**Pseudo-F**	***p***
Formate	38.3	63.9	41.6	0.002 **	0.2	0.3	0.6	0.528
Time	16.7	27.9	24.4	0.002 **	1.8	2.4	4.7	0.016 *
Acetate	1.3	2.1	1.9	0.158	2.2	2.8	5.4	0.008 **
CO_2	1.8	3.1	2.8	0.086	31.3	40.0	43.0	0.002 **
NH_4^+	1.0	1.7	1.5	0.21	8.1	10.4	18.4	0.002 **
H_2	0.4	0.6	0.6	0.426	20.5	26.2	17.3	0.002 **
CH_4	0.2	0.3	0.3	0.688	11.6	14.9	20.7	0.002 **
pH	0.2	0.3	0.3	0.69	2.3	3.0	6.5	0.002 **

* $p < 0.05$, ** $p < 0.01$.

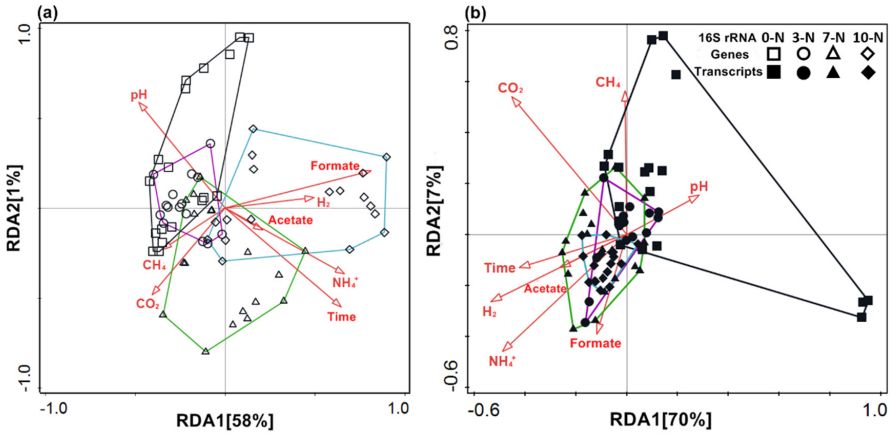

Figure 3. Redundancy analysis (RDA) of archaeal 16S rRNA genes (**a**) and 16S rDNA transcripts (**b**) amplicon sequencingprofiles with NH_4^+, pH, formate, acetate, H_2, CH_4, CO_2 and time as environmental variables. The first two axes explained 58% and 1% of the total inertia at 16S rDNA level and 70% and 7% of the total inertia at 16S rRNA level, respectively.

3.4. Archaeal 16S rRNA Gene and Transcript Dynamics

The copy numbers of the archaeal 16S rRNA gene and transcript were estimated using quantitative PCR (Figure 4). Compared with the 0-N treatment, ammonium significantly inhibited both archaeal 16S rRNA genes (Figure 4a,c) and their transcripts in 7-N and 10-N treatments (Figure 4b,d). The maximum value of 16S rRNA gene copy numbers were 2.07×10^{14}, 6.11×10^{13}, 5.30×10^{13} and 4.91×10^{13} copies/g sludge (dw) in 0-N, 3-N, 7-N and 10-N treatments, respectively (Figure 4a). The maximum values of 16S rRNA transcript copy numbers were 1.36×10^{14}, 4.09×10^{13}, 1.34×10^{13} and 5.59×10^{12} copies/g sludge (dw) in 0-N, 3-N, 7-N and 10-N treatments, respectively (Figure 4b). The effect of ammonium concentration on the maximum of archaeal 16S rRNA transcript copy numbers was more significant than that of archaeal 16S rRNA gene copy numbers. These results showed that the difference in maximum copy numbers of 16S rRNA transcripts could indicate the effect of ammonia on the community activity in the ammonium treatments.

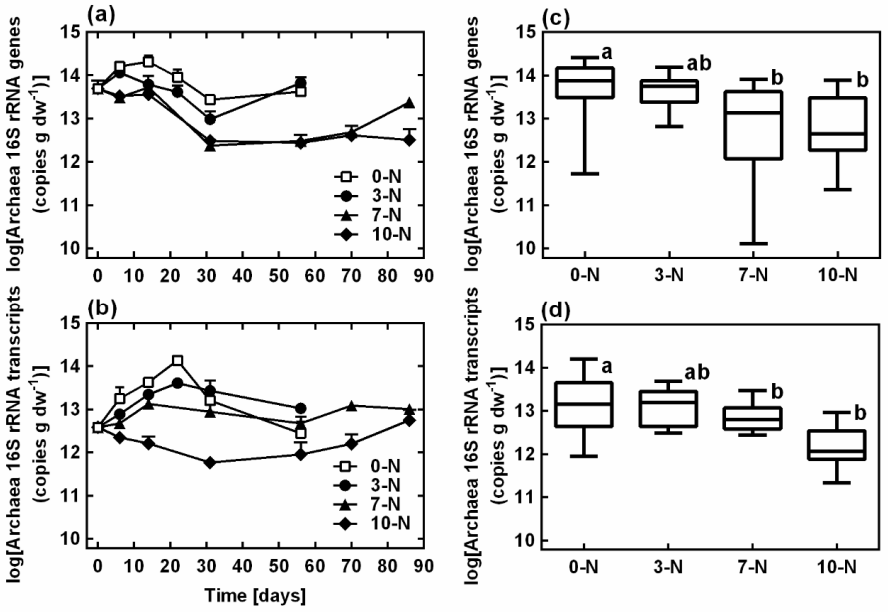

Figure 4. Quantitative PCR (**a**,**b**) and Box-chart (**c**,**d**) analysis of the abundance and activity based on archaeal 16S rDNA copy numbers and transcripts in the sludge with different ammonia concentrations. Different letters above the boxes indicate a significant difference ($p < 0.01$).

4. Discussion

In this study, our results showed that elevated concentrations of ammonium could inhibit methanogenesis in thermophilic anaerobic sludge, consistent with other studies [10,14,15,29,35]. The 0-N treatment exhibited a clear accumulation of hydrogen and acetate in the first 5 days and first 20 days during the incubation, respectively, and then degraded rapidly. The accumulation and degradation of acetate and hydrogen were accompanied by a 10-day lag and subsequent rapid methane production. Compared with the 0-N treatment, the acetate and hydrogen metabolisms were slower in the 3-N treatment, accompanied by a longer methane production lag phase and a slower methane production rate. In the 7-N treatment, acetate and hydrogen accumulated continuously and methane production was significantly inhibited during 56 days of incubation. Then, the concentration of acetate decreased and methane production was slightly restored. There was a longer lag phase and a lower rate of methane production at 55 °C than at 35 °C in 7-N treatment [22]. The increased concentration of NH_3 with increasing temperature may aggravate the ammonium stress on methanogenesis [6,9,15]. Methane production was completely inhibited, and acetate and hydrogen continued to accumulate during the whole incubation in the 10-N treatment. The metabolism of hydrogen and acetate corresponded well to methane production in this study. Although a high temperature can enhance the inhibition of ammonium on methanogenesis, the methanogenic adaptation to ammonium stress can occur at 55 °C if the concentration of ammonium is below 7 g NH_4^+-N/L.

The community of methanogen based on archaeal 16S rRNA genes sequencing has high diversity. In 0-N and 3-N treatments, the hydrogenotrophic methanogens, such as *Methanothermobacter* and *Methanospirillum*, dominated the methanogen community (relative abundance above 55%) on day 0 and the relative abundance gradually increased to more than 70% on day 31. However, the abundance of the aceticlastic methanogens, including *Methanosaeta* and *Methanosarcina*, decreased from 30% on day 0 to 20% on day 31 (Figure 2a,b). These results indicated that a hydrogenotrophic pathway might dominate during the methane production at 55 °C. Previous studies also found that methanogenesis shifted from the aceticlastic pathway in the mesophilic condition to the hydrogenotrophic pathway in the thermophilic condition [16,36,37]. In addition, the lower relative abundance of *Methanothermobacter* (about 10% at day 70) in the 10-N treatment was consistent with the previous studies, which found that the growth of *Methanothermobacter* decreased markedly after being exposed to 7 g NH_4^+-N/L [38].

Archaeal 16S rRNA transcript sequencing analysis revealed distinct patterns from 16S rRNA genes fingerprinting (Figure 2e–h). There was a clear correlation between the response of methanogen 16S rRNA transcripts and methanogenic activities, and it avoided the potential bias of DNA detection from dormant or dead cells [39]. The inactive organisms could not be washed out in batch incubation, and hence both active and inactive archaea were counted in the analyses. In fact, the community structure represented by 16S rRNA genes included a pool of all existing archaea, but only a small portion of the archaeal community in the pool might be active and react to environment changes [40]. The relative abundance of hydrogenotrophic methanogens including *Methanothermobacter*, *Methanobrevibacter* and *Methanoculleus* was about 90% during the incubation under thermophilic conditions. In particular, *Methanothermobacter* dominated in archaeal 16S rRNA transcripts fingerprinting during the whole incubation in all ammonium treatments. This means that *Methanothermobacter* was capable of rapid adaptation to the thermophilic condition and ammonia stress during the anaerobic digestion process. However, due to the heat sensitivity of most strains in *Methanosaeta* [41], *Methanospirillum* [42], and *Methanobrevibacter* [43], their growth and activity were significantly inhibited under thermophilic conditions. The metabolic activity of aceticlastic methanogen *Methanosaeta* was severely inhibited, and its abundance decreased from the initial 2% to 0.02% with increasing ammonium concentration in 7- and 10-N treatments. Previous studies have shown that the growth of *Methanosaeta* spp. was most sensitive to ammonium stress and it reduced sharply

at 1.9 g NH_4^+-N/L [44–46]. Meanwhile, CH_4 production in *Methanospirillum hungatei* was completely inhibited at 7.2 g NH_4^+-N/L [47].

Compared with *Methanosaeta* spp. and *Methanospirillum*, *Methanothermobacter* showed tolerance to ammonia stress. It was reported that the growth and methane production of *Methanothermobacter* spp. decreased with the rise in ammonia concentration, from 0.26 to 7 g NH_4^+-N/L [28,38]. Ammonium significantly inhibited both archaeal abundance (reduced 16S rRNA gene copy number, Figure 4a,c) and activity (reduced transcripts, Figure 4b,d) in 7-N and 10-N treatments. This means that *Methanothermobacter*, the main active methanogen in all incubations, was significantly inhibited by higher ammonium concentrations. Ultimately, the total methane production was inhibited. In addition, the copy numbers of total methanogenic archaea 16S rRNA transcripts, rather than the relative abundance of methanogenic communities, was a more direct indicator of the methane production. These results suggested that the methanogenic inhibition of digester sludge by ammonia can mainly be attributed to the inhibition of *Methanothermobacter* activity under thermophilic conditions. We found that aceticlastic methanogens were significantly inhibited, but hydrogenotrophic methanogens were tolerant to ammonium stress at 7g NH_4^+-N/L under thermophilic conditions.

The archaeal community structure and activity reflected by archaeal 16S rRNA genes and transcripts interacted with environmental factors. RDA and the Monte Carlo permutation test were used to determine the correlations between environmental variables and sequencing patterns of archaeal 16S rRNA genes and transcripts. Interestingly, the sequencing patterns of archaeal 16S rRNA transcripts were significantly correlated with environmental variables, such as NH_4^+, Acetate, H_2, CH_4, CO_2, pH and Time. In addition, the copy numbers of 16S rRNA transcripts were significantly inhibited in 7-N and 10-N treatments. These results indicated that the archaeal 16S rRNA transcripts were closely related to changes in environmental factors. Ammonium could significantly affect the sequencing patterns and total expression of 16S rRNA transcripts. However, the sequencing patterns of archaeal 16S rRNA genes were only significantly correlated with environmental factors such as Time and Formate. These results showed that sequencing patterns of 16S rRNA transcripts, rather than genes, corresponded significantly to ammonium stress. Generally, the microbial community structure at the DNA level could not reflect the activity and function of microorganisms [48,49]. The transcriptional analysis of 16S rRNA and mRNA could reflect the active state and function of microorganisms, and avoid the potential bias of DNA detection from dormant or dead cells [6,39]. These results suggested that the archaeal 16S rRNA transcripts could be used to analyze the effects of environmental factors including ammonium on methanogenic active microorganisms in anaerobic reactor sludge.

5. Conclusions

In this study, we estimated the inhibitory effects of ammonia on archaeal 16S rRNA transcripts in thermophilic AD. It was found that with the increase in ammonium concentration, the methanogenic rate decreased and hydrogen accumulation occurred. The total archaeal 16S rRNA genes and transcripts copy numbers decreased significantly in treatments with higher ammonium concentrations (7 and 10 g NH_4^+-N/L), but did not change much in the lower ammonia concentration (3 g NH_4^+-N/L). The hydrogenotrophic methanogen *Methanothermobacter* dominated the methanogenic community activity in all incubations, and it exhibited sensitivity to ammonia stress and should be responsible for methanogenic inhibition under thermophilic conditions. The composition and quantity of 16S rRNA transcripts rather than community structures were significantly correlated with ammonium and environmental factors of the methanogenic process. The archaeal 16S rRNA transcripts, rather than archaeal 16S rRNA genes, might better reflect the inhibition of ammonium on methanogenesis in thermophilic anaerobic digester sludge. Further validation experiments will be conducted in a future study.

Author Contributions: Conceptualization, S.W.; investigation, J.L.;data curation, C.S.; writing—original draft, S.W.; writing—review and editing, P.L. and C.Z.; supervision, C.Z.; funding acquisition, S.W. and C.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 (no. 31870802, no. 41501251 and no. 41401288), the Natural Science Foundation of Shandong Province (no. ZR2020MD103), the Open Project of Liaocheng University Animal Husbandry Discipline, no. 319312101-19 and the Science and Technology and Culture Innovation Fund (no. CXCY2022055, no. CXCY2023335 and no. CXCY2023339) for College Students of Liaocheng University.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We are grateful to Zhe Lyu (NC State University, Plant and Microbial Biology) for his insightful discussion and review during the manuscript preparation and revision.

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

References

1. McKendry, P. Energy production from biomass (Part 1): Overview of biomass. *Bioresour. Technol.* **2002**, *83*, 37–46. [CrossRef] [PubMed]
2. Zhang, C.; Yuan, Q.; Lu, Y.H. Inhibitory effects of ammonia on syntrophic propionate oxidation in anaerobic digester sludge. *Water Res.* **2018**, *146*, 275–287. [CrossRef]
3. Guo, X.H.; Wang, C.; Sun, F.Q.; Zhu, W.J.; Wu, W.X. A comparison of microbial characteristics between the thermophilic and mesophilic anaerobic digesters exposed to elevated food waste loadings. *Bioresour. Technol.* **2014**, *152*, 420–428. [CrossRef]
4. Shi, X.C.; Guo, X.L.; Zuo, J.N.; Wang, Y.J.; Zhang, M.Y. A comparative study of thermophilic and mesophilic anaerobic codigestion of food waste and wheat straw: Process stability and microbial community structure shifts. *Waste Manag.* **2018**, *75*, 261–269. [CrossRef] [PubMed]
5. Rajagopal, R.; Masse, D.I.; Singh, G. A critical review on inhibition of anaerobic digestion process by excess ammonia. *Bioresour. Technol.* **2013**, *143*, 632–641. [CrossRef] [PubMed]
6. Wu, Z.L.; Lin, Z.; Sun, Z.Y.; Gou, M.; Xia, Z.Y.; Tang, Y.Q. A comparative study of mesophilic and thermophilic anaerobic digestion of municipal sludge with high-solids content: Reactor performance and microbial community. *Bioresour. Technol.* **2020**, *302*, 122851. [CrossRef] [PubMed]
7. Duan, H.W.; He, P.J.; Zhang, H.; Shao, L.M.; Lu, F. Metabolic regulation of mesophilic *Methanosarcina barkeri* to ammonium inhibition. *Environ. Sci. Technol.* **2022**, *56*, 8897–8907. [CrossRef]
8. Jo, Y.; Cayetano, R.D.A.; Kim, G.B.; Park, J.; Kim, S.H. The effects of ammonia acclimation on biogas recovery and the microbial population in continuous anaerobic digestion of swine manure. *Environ. Res.* **2022**, *212*, 113483. [CrossRef]
9. Garcia, M.L.; Angenent, L.T. Interaction between temperature and ammonia in mesophilic digesters for animal waste treatment. *Water Res.* **2009**, *43*, 2373–2382. [CrossRef]
10. Barber, W.P.F. Thermal hydrolysis for sewage treatment: A critical review. *Water Res.* **2016**, *104*, 53–71. [CrossRef]
11. Yu, D.W.; Zhang, J.Y.; Chulu, B.H.; Yang, M.; Nopens, I.; Wei, Y.S. Ammonia stress decreased biomarker genes of acetoclastic methanogenesis and second peak of production rates during anaerobic digestion of swine manure. *Bioresour. Technol.* **2020**, *317*, 124012. [CrossRef] [PubMed]
12. Wijesinghe, D.T.N.; Suter, H.C.; Scales, P.J.; Chen, D.L. Lignite addition during anaerobic digestion of ammonium rich swine manure enhances biogas production. *J. Environ. Chem. Eng.* **2021**, *9*, 104669. [CrossRef]
13. Wilson, C.A.; Novak, J.; Takacs, I.; Wett, B.; Murthy, S. The kinetics of process dependent ammonia inhibition of methanogenesis from acetic acid. *Water Res.* **2012**, *46*, 6247–6256. [CrossRef] [PubMed]
14. Kato, S.; Sasaki, K.; Watanabe, K.; Yumoto, I.; Kamagata, Y. Physiological and transcriptomic analyses of the thermophilic, aceticlastic methanogen *Methanosaeta thermophila* responding to ammonia stress. *Microbes Environ.* **2014**, *29*, 162–167. [CrossRef] [PubMed]
15. Cerrillo, M.; Viñas, M.; Bonmatí, A. Overcoming organic and nitrogen overload in thermophilic anaerobic digestion of pig slurry by coupling a microbial electrolysis cell. *Bioresour. Technol.* **2016**, *216*, 362–372. [CrossRef]
16. Yin, D.M.; Westerholm, M.; Qiao, W.; Bi, S.J.; Wandera, S.M.; Fan, R.; Jiang, M.M.; Dong, R.J. An explanation of the methanogenic pathway for methane production in anaerobic digestion of nitrogen-rich materials under mesophilic and thermophilic conditions. *Bioresour. Technol.* **2018**, *264*, 42–50. [CrossRef]
17. Hagen, L.H.; Frank, J.A.; Zamanzadeh, M.; Eijsink, V.G.H.; Pope, P.B.; Horn, S.J.; Arntzen, M.Ø. Quantitative metaproteomics highlight the metabolic contributions of uncultured phylotypes in a thermophilic anaerobic digester. *Appl. Environ. Microb.* **2017**, *83*, e01955-16. [CrossRef]

18. Lv, Z.P.; Leite, A.F.; Harms, H.; Glaser, K.; Liebetrau, J.; Kleinsteuber, S.; Nikolausz, M. Microbial community shifts in biogas reactors upon complete or partial ammonia inhibition. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 519–533. [CrossRef]
19. Lv, Z.P.; Hu, M.; Harms, H.; Richnow, H.H.; Liebetrau, J.; Nikolausz, M. Stable isotope composition of biogas allows early warning of complete process failure as a result of ammonia inhibition in anaerobic digesters. *Bioresour. Technol.* **2014**, *167*, 251–259. [CrossRef]
20. Vigneron, A.; Cruaud, P.; Pignet, P.; Caprais, J.C.; Gayet, N.; Cambon-Bonavita, M.A.; Godfroy, A.; Toffin, L. Bacterial communities and syntrophic associations involved in anaerobic oxidation of methane process of the sonora margin cold seeps, guaymas basin. *Environ. Microbiol.* **2014**, *16*, 2777–2790. [CrossRef]
21. Frank, J.A.; Arntzen, M.Ø.; Sun, L.; Hagen, L.H.; Mchardy, A.C.; Horn, S.J. Novel syntrophic populations dominate an ammonia-tolerant methanogenic microbiome. *Msystems* **2016**, *1*, e00092-16. [CrossRef] [PubMed]
22. Zhang, C.; Yuan, Q.; Lu, Y.H. Inhibitory effects of ammonia on methanogen *mcrA* transcripts in anaerobic digester sludge. *FEMS Microbiol. Ecol.* **2014**, *87*, 368–377. [CrossRef] [PubMed]
23. Weisburg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **1991**, *173*, 697–703. [CrossRef] [PubMed]
24. Rui, J.P.; Peng, J.J.; Lu, Y.H. Succession of bacterial populations during plant residue decomposition in rice field soil. *Appl. Environ. Microb.* **2009**, *75*, 4879–4886. [CrossRef] [PubMed]
25. Noll, M.; Matthies, D.; Frenzel, P.; Derakshani, M.; Liesack, W. Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* **2005**, *7*, 382–395. [CrossRef]
26. Ma, K.; Conrad, R.; Lu, Y. Responses of methanogen *mcrA* genes and their transcripts to an alternate dry/wet cycle of paddy field soil. *Appl. Environ. Microb.* **2012**, *78*, 445–454. [CrossRef]
27. Kemnitz, D.; Kolb, S.; Conrad, R. Phenotypic characterization of Rice Cluster III archaea without prior isolation by applying quantitative polymerase chain reaction to an enrichment culture. *Environ. Microbiol.* **2005**, *7*, 553–565. [CrossRef]
28. Wang, H.; Fotidis, I.A.; Angelidaki, I. Ammonia effect on hydrogenotrophic methanogens and syntrophic acetate oxidizing bacteria. *FEMS Microbiol. Ecol.* **2015**, *91*, fiv130. [CrossRef]
29. Wang, H.; Zhang, Y.F.; Angelidaki, I. Ammonia inhibition on hydrogen enriched anaerobic digestion of manure under mesophilic and thermophilic conditions. *Water Res.* **2016**, *105*, 314–319. [CrossRef]
30. Sawayama, S.; Tada, C.; Tsukahara, K.; Yagishita, T. Effect of ammonium addition on methanogenic community in a fluidized bed anaerobic digestion. *J. Biosci. Bioeng.* **2004**, *97*, 65–70. [CrossRef]
31. Schnurer, A.; Nordberg, A. Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. *Water Sci. Technol.* **2008**, *57*, 735–740. [CrossRef] [PubMed]
32. Prathiviraj, R.; Chellapandi, P. Comparative genomic analysis reveals starvation survival systems in *Methanothermobacter thermautotrophicus* ΔH. *Anaerobe* **2020**, *64*, 102216. [CrossRef] [PubMed]
33. Wasserfallen, A.; Nölling, J.; Pfister, P.; Reeve, J.; de Macario, E.C. Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov. *Int. J. Syst. Evol. Micr.* **2000**, *50 Pt 1*, 43–53.
34. Capson-Tojo, G.; Moscoviz, R.; Astals, S.; Robles, A.; Steyer, J.P. Unraveling the literature chaos around free ammonia inhibition in anaerobic digestion. *Renew. Sust. Energy Rev.* **2020**, *117*, 109487. [CrossRef]
35. Yi, Y.; Dolfing, J.; Jin, G.; Fang, X.Y.; Han, W.H.; Liu, L.Y.; Tang, Y.Q.; Cheng, L. Thermodynamic restrictions determine ammonia tolerance of methanogenic pathways in *Methanosarcina barkeri*. *Water Res.* **2023**, *232*, 119664. [CrossRef]
36. Cerrillo, M.; Viñas, M.; Bonmatí, A. Unravelling the active microbial community in a thermophilic anaerobic digester-microbial electrolysis cell coupled system under different conditions. *Water Res.* **2017**, *110*, 192–201. [CrossRef]
37. Ao, T.J.; Xie, Z.J.; Zhou, P.; Liu, X.F.; Wan, L.P.; Li, D. Comparison of microbial community structures between mesophilic and thermosphilic anaerobic digestion of vegetable waste. *Bioproc. Biosyst. Eng.* **2021**, *44*, 1201–1214. [CrossRef]
38. Kato, S.; Kosaka, T.; Watanabe, K. Comparative transcriptome analysis of responses of *Methanothermobacter thermautotrophicus* to different environmental stimuli. *Environ. Microbiol.* **2008**, *10*, 893–905. [CrossRef]
39. Munk, B.; Bauer, C.; Gronauer, A.; Lebuhn, M. A metabolic quotient for methanogenic archaea. *Water Sci. Technol.* **2012**, *66*, 2311–2317. [CrossRef]
40. Yuan, Y.; Conrad, R.; Lu, Y. Transcriptional response of methanogen *mcrA* genes to oxygen exposure of rice field soil. *Environ. Microbiol. Rep.* **2011**, *3*, 320–328. [CrossRef]
41. Smith, K.S.; Ingram-Smith, C. *Methanosaeta*, the forgotten methanogen? *Trends Microbiol.* **2007**, *15*, 150–155. [CrossRef]
42. Iino, T.; Mor, K.; Suzuki, K.I. *Methanospirillum lacunae* sp. nov., a methane-producing archaeon isolated from a puddly soil, and emended descriptions of the genus *Methanospirillum* and *Methanospirillum hungatei*. *Int. J. Syst. Evol. Microbiol.* **2010**, *60 Pt 11*, 2563–2566. [CrossRef] [PubMed]
43. Misiukiewicz, A.; Gao, M.; Filipiak, W.; Cieslak, A.; Patra, A.K.; Szumacher-Strabel, M. Review: Methanogens and methane production in the digestive systems of nonruminant farm animals. *Animal* **2021**, *15*, 100060. [CrossRef] [PubMed]
44. Karakashev, D.; Batstone, D.J.; Angelidaki, I. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Appl. Environ. Microb.* **2005**, *71*, 331–338. [CrossRef]

45. Steinhaus, B.; Garcia, M.L.; Shen, A.Q.; Angenent, L.T. A portable anaerobic microbioreactor reveals optimum growth conditions for the methanogen *Methanosaeta concilii*. *Appl. Environ. Microb.* **2007**, *73*, 1653–1658. [CrossRef]
46. Nettmann, E.; Bergmann, I.; Pramschufer, S.; Mundt, K.; Plogsties, V.; Herrmann, C.; Klocke, M. Polyphasic analyses of methanogenic archaeal communities in agricultural biogas plants. *Appl. Environ. Microb.* **2010**, *76*, 2540–2548. [CrossRef]
47. Sprott, G.D.; Patel, G.B. Ammonia toxicity in pure cultures of methanogenic bacteria. *Syst. Appl. Microbiol.* **1986**, *7*, 358–363. [CrossRef]
48. Lu, Y.; Liaquat, R.; Astals, S.; Jensen, P.D.; Batstone, D.J.; Tait, S. Relationship between microbial community, operational factors and ammonia inhibition resilience in anaerobic digesters at low and moderate ammonia background concentrations. *New Biotechnol.* **2018**, *44*, 23–30. [CrossRef]
49. De Vrieze, J.; Verstraete, W. Perspectives for microbial community composition in anaerobic digestion: From abundance and activity to connectivity. *Environ. Microbiol.* **2016**, *18*, 2797–2809. [CrossRef] [PubMed]