Temporal Variation of Nitrogen and Sulfur Species of Food Waste and Sludge during Anaerobic Co-Digestion

Pengzhou Kang 1,2, Yuxiu Zhang 1,*, Xiaopeng Ge 2,*, and Zhi Qian 3

1 School of Chemical and Environmental Engineering, China University of Mining & Technology (Beijing), Beijing 100083, China; bqt1900302021@student.cumtb.edu.cn
2 Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China
3 College of Resources and Environment, University of Chinese Academy of Sciences, Beijing 100049, China; qianz@ucas.ac.cn
* Correspondence: zhangyuxiu@cumtb.edu.cn (Y.Z.); xpge@rcees.ac.cn (X.G.); Tel.: +86-10-6233-1792 (Y.Z.); +86-10-62849156 (X.G.)

Abstract: Anaerobic co-digestion (AcoD) has been a widely accepted method to treat food waste (FW) and sewage sludge (SS). However, there is a knowledge gap regarding the key speciation transformation of nitrogen and sulfur in AcoD. Here, we explored the changes of nitrogen (N) and sulfur (S) compounds in liquid digestion and biogas, as well as the composition of microbial community structure and related metabolic functions. The results showed that H2S in the biogas was the main form of S in the early stage, and then, it was converted into SO3^{2−} and SO4^{2−}, while NH3 and NH4^{+} were the main forms of N during the AcoD. In addition, bacterial diversity was associated with N and S compounds; Syntrophomonas and Aminobacterium were positively correlated to H2S, NH3, NH4^{+} and SO4^{2−}, and Saccharibacteria_genera_incertae_sedis, Candidatus_Cloacamonas and Thermomonas were positively correlated to SO4^{2−} and NO3−. Additionally, the FAPROTAX prediction showed that the functional composition related to N and S metabolism was different from SS and inoculum after the AcoD. This study provides detailed information of conversion of N and S of the AcoD, which could lay a foundation for the subsequent regulation of the mechanism of nitrogen and sulfur compounds in the methanogenic process.

Keywords: food waste; sewage sludge; anaerobic co-digestion; nitrogen; sulfur

1. Introduction

Food waste (FW) has become a major source of pollution and is ever-increasing in the world. Due to the rapid growth of population and urbanization, more than 100 million tons of food waste will be processed annually in China [1]. If not being properly disposed of, it will cause a detrimental impact on the environment. Additionally, rapid industrialization and urbanization has led to an increase in municipal wastewater treatment plants, and sewage sludge (SS) production has also increased sharply [2]. Researchers have conducted many studies into the effective treatment of sludge. Pyrolysis is one of the solutions for volume reduction of SS, which generates by-products that could promote soil fertility. In particular, char from SS pyrolysis treated with alkaline hydroxide has a good ability to adsorb CO2 [3,4]. Therefore, it is worth studying the combined treatment technology of FW and SS, because large quantities of these wastes are generated at the same locations [5]. Among the existing recycling methods, anaerobic co-digestion (AcoD) has been increasingly used, which can generate biogas and achieve reduction principles for solid waste treatment. Furthermore, compared with mono-digestion of FW or SS, co-digestion could significantly improve the efficiency of methane production. However, a series of inhibitory intermediates are produced in the process of AcoD, which greatly reduces the efficiency of anaerobic digestion. The most significant inhibitors are volatile fatty acids (VFAs), ammonia, sulfate,
heavy metals, etc. Therefore, understanding the source, change and toxicity of these inhibitors is very important for the success of anaerobic treatment.

Microorganisms participating in nitrogen (N) transformation produced a series of intermediate products, such as nitrate (NO$_3^-$), nitrite (NO$_2^-$), ammonium (NH$_4^+$) and ammonia (NH$_3$). NH$_4^+$ and NH$_3$ were the main metabolic products inhibiting anaerobic digestion in the process of degradation of FW. At a certain concentration, NH$_4^+$ and NH$_3$ can penetrate through the bacterial cell membrane and interfere with methanogenic bacteria, resulting in ions imbalance in microbial cells. When the concentration of NO$_3^-$ was lower than 750 mg/L, NH$_3$ could be transformed into NO$_3^-$ by nitrification. Therefore, the inhibitory of NH$_3$ could be effectively reduced [6]. However, the impact of sulfur (S) compounds in FW and SS is usually overlooked in the AcoD. As we all know, H$_2$S is a corrosive, odorless and toxic substance in the biogas [7]. H$_2$S is mainly produced by the hydrolysis of organic sulfur of bacteria, the reduction reaction of sulfate (SO$_4^{2-}$) and the direct conversion of sulfide (S$^{2-}$) during anaerobic digestion [8]. The production of H$_2$S prolonged the start-up phase of anaerobic digestion and eventually caused a decrease of methanogenesis. Rusin et al. reported that the concentration of H$_2$S produced by anaerobic digestion of FW was around 800 ppm, which is much higher than the limit of 500 ppm [9].

Zan et al. discovered that the presence of SO$_4^{2-}$ could accelerate the methane production by improving the hydrolysis rate at the concentrations of 50–400 mg/L [10]. However, when the concentration of SO$_4^{2-}$ is higher than 500 mg/L, the microorganism participated in the AcoD would be inhibited [11]. Interestingly, when occurring simultaneously with other inhibitory conditions or components such as NH$_3$, high H$_2$S concentration hinders the formation of methane generation [12]. In addition, the coupling reactions between N and S compounds could happen with the actions of microorganisms. It has been reported that SO$_4^{2-}$ and NH$_3$ can be used as an electron donor and acceptor to remove N and S simultaneously under anaerobic conditions, to which nitrate-reducing and sulfide-oxidizing bacteria contribute greatly. Wu found that 0.1 mM NO$_2^-$ and 0.4 mM NO$_3^-$ could promote generation of H$_2$S by Shewanella oneidensis in the presence of 0.8 mM SO$_3^{2-}$. In addition to promoting biomass, NO$_2^-$ and NO$_3^-$ were the two preferred electron acceptors in sulfite respiration [13].

Nevertheless, the conversion of N and S compounds in the AcoD of FW and SS is not clear. Understanding the regulation of N and S compounds in the anaerobic digester can be a potential strategy to optimize methane production. In this study, different ratios of SS and FW were mixed as variations to investigate the characteristics and mechanism of the temporal variability of the inorganic N-forms (NO$_3^-$, NO$_2^-$, NH$_4^+$ and NH$_3$) and inorganic S-forms (SO$_4^{2-}$, SO$_3^{2-}$, S$^{2-}$ and H$_2$S) in an AcoD batch process, and the compositions of microorganisms were investigated. The results will help improve the efficiency of methane production and provide opinions for an in situ strategy to regulate nitrogen and sulfur compounds suppression in AcoD of FW with SS in the future.

2. Materials and Methods
2.1. Substrates and Inoculum

The FW was collected in seven consecutive days (a week) from the same restaurant in Erdos City. After classification and weighing, FW were mixed using a grinder to ensure the homogeneity and then stored at 4 °C before use. The FW contained rice, noodles, vegetables and meat with the following composition (terms of wet weight): noodles 10.22%, rice 22.27%, vegetables 44.59%, meat 20% and tofu 2.4%. Sewage sludge used for AcoD was collected from the Beijiao sewage treatment plant of Erdos City and then filtered through a 2 mm sieve. The inoculum was obtained from a reactor running for half a year at 37 °C.

2.2. AcoD Experiments

The batch methane tests were carried out in 1000 mL glass serum bottles with a working volume of 800 mL and at 37 ± 0.2 °C. Different ratios of SS:FW were carried out (based on volatile solids) as follows:10.0:0.0 (R-1), 8.0:2.0 (R-2), 7.0:3.0 (R-3), 5.0:5.0 (R-4),
3.0:7.0 (R-5), 2.0:8.0 (R-6) and 0.0:10.0 (R-7). R-1 and R-7 were used as the control groups for mono-digestion of SS and FW, respectively. The ratio of inoculum to substrates (SS + FW) was 1.0:1.0. The pH value was adjusted by addition of aqueous solutions of 3 M HCl and NaOH to 7.2. Finally, N\textsubscript{2} was bubbled into the mixture for 5 min and immediately sealed to create the anaerobic environment.

2.3. Analytical Methods

During the AcoD process on days 0, 1, 3, 5, 11, 21, 31 and 41, 2 mL of digestate were taken for inorganic ions analysis. The pH was analyzed with the pH meter (PHS-3C, INESA, Shanghai, China). SCOD\textsubscript{a} and S\textsubscript{2}\textsuperscript{−} were measured according to standard methods [14]. Methylene blue method described in the Standard Methods according to the manufacturer’s instructions. Briefly, 0.5 mL of Methylene Blue Kit 1 and 2 were added to the 10 mL samples in turn and shaken. After 5 min, samples were quantified by using a portable spectrophotometer (DR1900, HACH, Loveland, CO, USA) at a wavelength of 665 nm. SO\textsubscript{4}\textsuperscript{2−}, SO\textsubscript{3}\textsuperscript{2−}, NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{3}− and NO\textsubscript{2}− were measured by using ion chromatography (ICS-600, Thermo Scientific, Waltham, MA, USA). Anion analysis was equipped with the SH-AC-18 (250 × 4.6 mm, SHINE, Shanghai, China) analytical column. The eluent of ionic consisted of 2.0 mM Na\textsubscript{2}CO\textsubscript{3} and 10.0 mM NaHCO\textsubscript{3}. Cation Dionex IonPac\textsuperscript{TM} CS 12A RFIC\textsuperscript{TM} (250 × 4 mm, Thermo Fisher, Waltham, MA, USA) was used to analyze the NH\textsubscript{4}+, and the corresponding eluent of ionic consisted of 20 mM CH\textsubscript{3}O\textsubscript{3}S. The flow rate was set at 1.0 mL/min. The concentration of methane was analyzed by Gas chromatography (FuLi, GC9790II, Taizhou, China) equipped with thermal conductivity (TCD) and flame ionization detector (FID). The column of Gas chromatography was KB-Wax (30 m × 0.32 mm × 0.50 µm, Kromat Corporation, Bordentown, NJ, USA). The temperatures of the TCD, column and the inject port were 100, 80 and 200 °C, respectively. N\textsubscript{2} was used as the carrier gas. H\textsubscript{2}S and NH\textsubscript{3} were measured by a Portable gas meter (Dräger X-am, 5600, Dräger, Lübeck, Germany).

2.4. Reagent

Standard materials used for the analysis of inorganic nitrogen and sulfur including an anion mix standard solution of SO\textsubscript{4}\textsuperscript{2−}, SO\textsubscript{3}\textsuperscript{2−}, NO\textsubscript{3}− and NO\textsubscript{2}− (1000 mg/L) and a standard solution of NH\textsubscript{4}+ (1000 mg/L) were obtained from Beijing North Weiye Institute of Measuring and Testing Technology, China. Methylene Blue Kit 1 and 2 used for analysis of S\textsuperscript{2−} were purchased from HACH, China. NaOH, K\textsubscript{2}CrO\textsubscript{7}, (NH\textsubscript{4})\textsubscript{2}Fe(SO\textsubscript{4})\textsubscript{2}•6H\textsubscript{2}O, Ag\textsubscript{2}SO\textsubscript{4} and HgSO\textsubscript{4} were obtained from MACKLIN (AR), China. HCl and H\textsubscript{2}SO\textsubscript{4} were obtained from DM (aq, AR), China. N\textsubscript{2} (99.999%) for gas chromatography and for the generation of anaerobic conditions was provided by Kylingas, China.

2.5. Statistical and Kinetic Analysis of Biogas Generation

When using complex solid organic materials as the matrix, the cumulative gas production curve could form a smooth curve. Therefore, the cumulative methane production, the methane productivity constants and the lag phase (\(\lambda\)) are important factors reflecting the efficiency of anaerobic digestion. The modified Gompertz method could be used to estimate the above parameters in the experiment as follows:

\[
CMY(t) = P \times \exp \left\{- \exp \left[ R_{m} \times e \times (\lambda - t)/P + 1\right] \right\}
\]

(1)

where CMY(t) is the actual cumulative methane generation obtained for duration of \(t\); P is the predicted cumulative methane generation of the time \(t\); \(R_m\) is the maximum biogas generation; \(\lambda\) is the lag phase period; \(t\) indicates the days of experiments, and \(e\) is Euler’s number (\(\approx 2.71828\)).

2.6. Microbial Community Analysis

When the production of methane stopped, the samples were collected for the analysis of bacterial and archaeal community composition. DNA for microbial analysis was ex-
tracted from suspending mixtures using the E.Z.N.A™ Mag-Bind Soil DNA Kit (OMEGA, USA). Communities of bacteria and archaea were evaluated by PCR amplification of V3-V4 region of 16S rRNA genes. The primers of bacteria were 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC). The primers of archaea were Arch 340F (CCCTAYGGGGYGCASCAG) and 1000R (GGCCATGCACYWCYTCTC), and Arch 349F (GYGCASCAGKCGMGAAW) and 806R (GGACTACVSGGGTATCTAAT). The main PCR amplification of bacteria and archaea were set up as follows: 2 µL of microbial DNA, 1 µL of forward primer, 1 µL of reverse primer, and 2 × Hieff® Robust PCR Master Mix. The thermal cycles protocol was performed as follows: 1 cycle of denaturing at 94 °C for 3 min followed by 5 cycles at 94 °C for 30 s, annealing at 55 °C for 20 s, and elongating at 72 °C for 30 s. Finally, the extension occurred at 72 °C for 5 min. AMPure XP beads were used to purify the free primers and primer dimer species in the application products. The DNA concentrations of PCR products were determined by using Qubit® 4.0 Green double-stranded DNA assay, and they were quality controlled by using a bioanalyzer (Agilent 2100, Santa Clara, CA, USA). The details of the richness and diversity indices can be found in the Supplementary Materials. Redundancy analysis (RDA) was used to discuss the relationships among bacterial community, inorganic nitrogen and sulfur compounds by running the CANOCO 5.0 Program.

3. Results

3.1. Cumulative Methane Production

Different nutritional compositions of substrates could lead to different methane production trends. Cumulative methane yield (CMY) for AcoD of SS with FW at different ratios are displayed in Figure 1. With the increase of FW ratio, the cumulative yield of methane also gradually increased, and there was almost no obvious lag phase. These results demonstrated that the activity of the inoculum would not cause severe acidification inhibition during the AcoD. Gu et al. found that sufficient microbial biomass could reduce acid inhibition in the early stage of AcoD, while the inoculum to substrate was 1:1 (based on VS) [15]. The order of CMY showed at the end of the AcoD as follows: 5.95 L(R-1) < 6.75 L(R-2) < 10.73 L(R-3) < 12.84 L(R-4) < 14.81 L(R-5) < 15.12 L(R-6) < 19.57 L(R-7). The order indicated that the mono-degradation of FW generated more methane than the mono-degradation of SS. Therefore, the organic matter of AcoD to produce methane was mainly coming from FW. Additionally, SS could be better utilized together with FW in the increasing ratio of FW. It was worth noting that at the ratio of 2:8, the maximum methane production is achieved faster than 0:10, indicating that a slightly synergistic effect might occur. Liu et al. and Heo et al. also reported that the CMY increased with increasing proportion of FW on anaerobic co-digestion of FW and SS [16,17].

3.2. Kinetic Features

The kinetic parameters could be assessments and predictions of functions of anaerobic digestion properties of FW and SS [15]. However, it is still necessary to select the optimum conditions among different substrate ratios, representing the best performing co-digestion system. In this study, the methane production results were fitted by using a modified Gompertz model in order to obtain these kinetic parameters at different ratios of substrate. From Table 1, the modified Gompertz model provides a high accuracy on fitting data from anaerobic mono- or co-digestion processes ($R^2 \geq 0.96$). The predicted maximum methane potential increases with the rise of FW proportion in raw materials. $R_m$ and $\lambda$ are important parameters indicating methane productivity. Higher $R_m$ and lower $\lambda$ values are more favorable for methane production efficiency. The $R_m$ of 2:8 ratio was 1.30 ± 0.11, which increased by 4.82 and 1.25 folds compared with the SS and FW mono-digestion, respectively. Although the $\lambda$ of 8:2 ratio was the lowest, which was shortened 1.91 times more than that of 2:8 ratio, its CMY was less than half that of 2:8 ratio. The result is in line with the actual methane yield before (Section 3.1).
concentrations of SCOD were
would form H\textsubscript{2}S with H\textsuperscript{+} later. As soon as H\textsubscript{2}S was formed, it hindered the activity and growth of methanogenic archaea and ultimately brought about system instability. As shown in Figure 3a, the H\textsubscript{2}S concentrations for AcoD exhibited a short increasing trend in the initial stage. On Days 1 to 3, the peak values of 258, 172, 126, 121, 22, 19 and 9 ppm
corresponded to an SS:FW ratio of 0:10, 2:8, 3:7, 5:5, 7:3, 8:2 and 10:0, respectively. The results indicated that the main source of H$_2$S was from FW. After the short peaks, H$_2$S concentrations declined sharply and remained at a low level from day 5. Previous studies had reported H$_2$S concentrations within full-scale AD at 600–1500 ppm [18], while other researchers observed the corresponding H$_2$S emission in the range of 200–270 ppm during the methane production stage in a two-phase AD experiment [19]. Furthermore, the generating conditions of H$_2$S are closely related to the acidification stage of anaerobic digestion. Tian et al. found that most of the H$_2$S initially produced could be attributed to the high concentration of supernatant sulfide, which was produced by systematic acidification and rapid biogas production [20].

Figure 2. Stability of AcoD: (a) variations of pH during AcoD process; (b) variations of SCOD during AcoD process.

The change of SO$_4^{2–}$ concentrations over time could be generally divided into three phases shown in Figure 3b. During the first phase (Days 1–2), the SO$_4^{2–}$ concentrations in all reactors increased rapidly; the maximum reached 45 mg/L at a ratio of 0:10. However, in the second stage (Days 3–20), the peak of sulfate concentrations reached at 20 mg/L in the substrate with the highest proportion of sludge. It might be that FW was more prone to hydrolysis and the organic matter in the sludge was gradually released, as the AcoD process continued. Meanwhile, in the third stage, the SO$_4^{2–}$ concentrations gradually decreased. It has been demonstrated that the presence of SO$_4^{2–}$ could accelerate the methane production by improving the hydrolysis rate, at concentrations from 50 to 400 mg/L [10].

When sulfate-reducing bacteria exist in the anaerobic environment, SO$_4^{2–}$ could be easily reduced to SO$_3^{2–}$. As shown in Figure 3c, the SO$_3^{2–}$ concentrations got 7.0, 6.2, 5.5, 4.8, 3.2, 2.1 and 2.0 mg/L at ratios of 2:8, 5:5, 3:7, 0:10, 8:2, 7:3 and 10:0, respectively, on day 11. After that, there were no significant fluctuations until day 31. The results showed that FW is the main source of sulfite SO$_3^{2–}$. A previous study proved that SO$_3^{2–}$ concentration over 400 mg/L could depress the acetate oxidation to inorganic carbon, which inhibited the organic removal rate [21]. The SO$_3^{2–}$ concentration in this study was much lower than 400 mg/L, so there was no inhibitory effect on the methanogenesis process.

After H$_2$S was produced in aqueous solution, it could dissociate into hydrosulfide ions (HS$^–$) and then sulfide ion (S$^{2–}$). It was clearly found in Figure 3d that S$^{2–}$ concentrations decreased rapidly at the beginning of AcoD; then, all reactors fluctuated below 7 mg/L. It indicated that S$^{2–}$ was readily converted to other sulfur compounds under anaerobic conditions and mainly in gaseous H$_2$S accumulation, not in the aqueous solution.
The concentration of ammonia is considered to be an important indicator affecting the performance of anaerobic digestion. High ammonia inhibits the process of methanogenesis, while an appropriate concentration of ammonia could neutralize the low pH to promote the growth of methanogens [22]. Figure 4a showed a general increasing trend of the NH$_3$ concentrations for AcoD. The peaks of NH$_3$ were observed around day 3 at the first stage; then, they declined rapidly in the next two days. From day 5 to day 25, the maximum of NH$_3$ for the second stage was observed. In general, anaerobic digestion processes are enhanced when ammonia concentrations are below 200 mg/L because low ammonia inhibits the process of methanogenesis, high ammonia inhibits the process of methanogenesis, and the growth of methanogens [22]. These results are consistent with inhibition of methanogenesis by promoting the reaction between hydrochloric acid and amino acids as well as possibly increasing ammonium release.

A certain concentration of NH$_4^+$ could be conducive to inhibiting the reduction of pH caused by acidification, while excessive concentration of NH$_3$ would have a toxic effect on the activity of methanogen enzyme [24]. The NH$_4^+$ changing over time could be divided into two phases (Figure 4b). Protein or other organic matter was decomposed in the hydrolysis process in the early stage of anaerobic digestion, resulting in the concentration of NH$_4^+$ raised rapidly, while the second increase was due to the dissolution of gaseous NH$_3$. The ratio of FW:SS = 0:10 got the highest NH$_4^+$ at each stage, 3651.16 and 3446.26 ppm on days 5 and 25, respectively. Due to differences in substrate composition and experimental condi-

Figure 3. Dynamics of sulfur chemical forms: (a) variations of H$_2$S during AcoD process; (b) variations of SO$_4^{2−}$ during AcoD process; (c) variations of SO$_3^{2−}$ during AcoD process; (d) variations of S$^{2−}$ during AcoD process.

3.5. Dynamics of Nitrogen Chemical Forms

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tions, various ammonia inhibitory concentrations were obtained. For example, Benabdallah et al. reported that 215 mg/L of NH$_4^+$ could reduce methane production, whereas the threshold for inhibition of methane reported by Angelidaki et al. was 700 mg/L [25,26].

As shown in Figure 4c, the NO$_3^-$ increased from the beginning and reached a peak on Day 25, while the ratio of SS:FW = 0:10 reached 13.86 mg/L. The result was consistent with that group of I/S ratio = 1:0 by Zhang et al. [27]. From Day 20, the NO$_3^-$ increased for all ratios given the recovery of nitrification as evidenced by the declined of NH$_4^+$. The low molecular organics in the FW could be easily utilized by microorganisms, resulting in a faster denitrification rate. However, solid and macromolecular organics were firstly hydrolyzed into soluble organic matter before being utilized by denitrifying bacteria, so as to cause a lower denitrification rate [28,29]. Additionally, at the end of the AcoD, the NO$_3^-$ was not removed completely, and its content was slightly higher than the initial stage of the reaction, indicating the incomplete denitrification. The reason for the low denitrification rate is that the solid organic matters in FW were converted by hydrolase before being utilized by denitrifying bacteria [29].

As displayed in Figure 4d, the concentration of NO$_2^-$ dropped rapidly from the beginning of AcoD and then increased slightly and gradually decreased to almost 0 mg/L. The differences between reactors of NO$_2^-$ might be attributed to the unbalanced electron allocations between nitrate reductase (NaR) and nitrite reductase (NiR) during denitrification. As the ratios of FW in the substrate increased, the activity of NaR was enhanced, leading to the accumulation of NO$_2^-$ in the reaction system [30,31]. Combined with the changes of NO$_3^-$, it showed that nitrification and denitrification were not synchronized in

![Figure 4](image_url)

**Figure 4.** Dynamic of nitrogen chemical forms: (a) variations of NH$_3$ during AcoD process; (b) variations of NH$_4^+$ during AcoD process; (c) variations of NO$_3^-$ during AcoD process; (d) variations of NO$_2^-$ during AcoD process.
the process of AcoD. In the first step of anaerobic ammonium oxidation (anammox) process, low concentration of oxygen (O$_2$) by FW provided the conditions for partial nitritation. NH$_4^+$ was partially oxidized to NO$_2^-$ by aerobic ammonia-oxidizing bacteria (AOB), which was further oxidized to N$_2$ later [32].

3.6. Microbial Community Analysis

3.6.1. Microbial Community Diversity and Richness

The richness and diversity of the microbial communities in substrates from each reactor were analyzed on the basis of Miseq sequencing platforms. The Shannon’s, Chao1 and ACE diversity indices and Good’s coverage of the α diversity indices of bacteria and archaea were shown in Tables 2 and 3. All the Good’s coverage in this study were higher than 0.99, indicating that the OTU readings were representative. In R-1 and R-7, the Shannon and Ace indices were higher than others. This suggested that the AcoD played an important role in the selection and enrichment of some specific bacterial. The OTUs, Chao and Ace of archaea community of R-6 were the highest, which was consistent with the CMY conclusion (Section 3.1). Additionally, the α diversity of bacteria was higher than that of archaea, which indicated that the diversity and richness of bacterial community was greater than that of archaea. Therefore, bacterial communities played an important role in the transformation of N and S compounds.

Table 2. OTU and diversity index of bacterial communities under different co-digestion conditions.

| Sample  | OTUs | Shannon | Chao     | Ace     | Coverage |
|---------|------|---------|----------|---------|----------|
| SS      | 904  | 5.05    | 1004.96  | 993.88  | 0.99     |
| Inoculum| 889  | 3.75    | 1142.05  | 1137.53 | 0.99     |
| R-1     | 991  | 4.22    | 1223.69  | 1213.37 | 0.99     |
| R-2     | 878  | 3.81    | 1170.19  | 1156.09 | 0.99     |
| R-3     | 880  | 3.83    | 1120.64  | 1146.37 | 0.99     |
| R-4     | 861  | 3.78    | 1203.01  | 1176.86 | 0.99     |
| R-5     | 810  | 3.78    | 1034.32  | 1053.54 | 0.99     |
| R-6     | 871  | 3.77    | 1170.07  | 1219.51 | 0.99     |
| R-7     | 946  | 4.19    | 1183.03  | 1151.82 | 0.99     |

Table 3. OTU and diversity index of archaea communities under different co-digestion conditions.

| Sample  | OTUs | Shannon | Chao     | Ace     | Coverage |
|---------|------|---------|----------|---------|----------|
| SS      | 81   | 2.07    | 82.5     | 82.31   | 0.99     |
| Inoculum| 85   | 2.12    | 107.67   | 100.11  | 0.99     |
| R-1     | 63   | 2.30    | 74       | 80.71   | 0.99     |
| R-2     | 77   | 2.20    | 89.0     | 94.09   | 0.99     |
| R-3     | 82   | 2.26    | 88.5     | 93.61   | 0.99     |
| R-4     | 70   | 2.15    | 75.14    | 76.29   | 0.99     |
| R-5     | 75   | 2.26    | 105      | 92.76   | 0.99     |
| R-6     | 90   | 2.13    | 96       | 97.19   | 0.99     |
| R-7     | 73   | 2.30    | 80.5     | 79.65   | 0.99     |

3.6.2. Taxonomic Composition of the Microbial Community

The classification of bacterial sequences from substrates were summarized at the phylum and genus level (Figure 5a,b). The dominant phylum of bacteria were Firmicutes (4.49–59.02%), Bacteroidetes (11.58–22.17%), Proteobacteria (4.74–37.87%), Actinobacteria (1.84–5.37%), Candidatus_Saccharibacteria (0.93–4.73%), Synergistetes (0.03–4.79%) and Planctomycetes (0.88–2.55%). It is reported that Firmicutes, Proteobacteria and Bacteroidetes play important roles in SO$_4^{2-}$ and NO$_3^-$ co-reduction system [33]. The highest abundance of Firmicutes was 59.01% in R-6, which was close to that of inoculum. This phenomenon showed that the distribution of microorganisms in the inoculum has a great influence on the results of AcoD. The abundance of Proteobacteria in R-6 was much less than that of
inoculum and SS, which was observed in desulfurization and denitrification process with increasing influent NO$_3^-$ concentrations [34]. Additionally, Proteobacteria, Planctomycetes, Chloroflexi, Bacteroidetes and Acidobacteria were often found to play key roles in anammox reactors [35].

Figure 5. Microbial community structure in AcoD systems: (a) bacteria at the phylum level; (b) bacteria at the genus level; (c) archaea at the genus level.
At the genus level, the relative abundance of dominant bacteria over 1% reduced from 13 genera to 5 genera compared with SS, which indicated the difference of substrate composition leading to the change of relative abundance of different functional microorganisms after the AcoD process. It has been reported that Clostridium sensu stricto and Lutispora belonging to Firmicutes play an important role in converting various organic compounds to VFAs during anaerobic digestion [36]. Methylothermus was confirmed to carry out heterotrophic denitrification by the utilization of methanol [37]. Sporanaerobacter contains known fermentative species coupled with the reduction of SO$_4^{2-}$, suggesting its potential ability of extracellular electron transfer [38]. Petrimonas is capable of converting complex substrates to acetate in the presence of the elemental sulfur as an electron acceptor and is considered to be an electron-donating bacterium in DIET [39]. Caldimina is observed to play significant roles in phosphorus removal and denitrification along with Acinetobacter [40].

The methanogenic capacity of the AcoD process is primarily related to the archaea communities’ structure. As showed in Figure 5c, the predominant archaea at the genus level (with relative abundance over 1%) were Methanobacterium (14.28–53.43%), Methanosarcina (2.88–33.31%), Methanobrevibacter (9.07–25.68%), Methanomassiliicoccus (2.81–19.16%), Methanospirillum (1.65–26.97%) and Methanoculleus (0.57–13.49%). Methanotrichus species are capable of accepting electrons via direct interspecies electron transfer (DIET) for the reduction of carbon dioxide to methane. As another predominated genus, Methanosarcina is known to generate methane by both acetoclastic and the hydrogenotrophic methanogenesis pathways. Methanoculleus could not only ensure efficient CH$_4$ production in a stable stage but also be tolerant to high ammonia in anaerobic digestion with organic wastewater [41].

3.6.3. Relationships of the Bacterial Community with N and S Compounds

RDA was used to determine the correlation between the bacterial community and different dynamic parameters. As shown in Figure 6, the effect of S compounds on bacterial genera was displayed as follows: S$_2^-$ > SO$_4^{2-}$ > H$_2$S > SO$_3^{2-}$, while the effect of N was NO$_2^-$ > NH$_3$ > NH$_4^+$ > NO$_3^-$ . Obviously, the dominant genus Syntrophomonas and Aminobacterium were positively correlated to H$_2$S, NH$_3$, NH$_4^+$ and SO$_3^{2-}$, but negatively correlated to NO$_2^-$ and SO$_4^{2-}$ which positively correlated with Saccharibacteria genera_incertae_sedis and Thermomonas. The Syntrophomonas and Aminobacterium could not only participate in the degradation process of butyric acid and amino acids but also could indirectly intensify the performance of AD [42]. Notably, the SO$_3^{2-}$ had a positively relationship with NO$_2^-$, and electron transfer might have occurred between them under the action of specific microorganisms. It has been confirmed that Pseudomonas has denitrification and desulphurization functions [43]. Thermomonas was always associated with N removal, especially with denitrification [39]. As for Candidatus Cloacamonas, which was positively correlated to NO$_3^-$, it was commonly associated with amino acid degradation [34]. These results suggested that N and S compounds had great influence on the structures of bacterial communities in the AcoD process and could be used as important indicators to effectively regulate the microbial ecosystem during AcoD.

3.6.4. Functional Prediction of Bacterial Community

FAPROTAX (Annotation of Prokaryotic Taxa) was adopted to evaluate the metabolic pathways of N and S cycles in the AcoD. As shown in Figure 7, the abundance of functional groups involved in the cycles of N and S showed an increase with the ratio of SS:FW. In the reactor R-7, the presence of anaerobic regions in FW resulted in notable ureolysis, nitrate respiration, nitrogen respiration and nitrate reduction, thereby contributing to high NH$_4^+$. Bacterial functions for the S cycle were dominantly composed of sulfite and sulfate respiration. These functions made contributions to the notable H$_2$S emission. Compared to the R-1, these bacterial activities were more abundant. Given their more severe anaerobic conditions, they would lead to much higher H$_2$S and NH$_3$ emission inside the composting pile with merely the FW only. These results were also consistent with previous bacterial community analysis.
bic conditions, thereby con-
24
24
2
2
b

(b)\[45\]. This phenomenon was proved by the presence of
2
the more the H
2
HS

(c)\[45\]. Figure 5. Microbial community structure in AcoD systems: ... pathways of the sulfur cycle (blue solid line), nitrogen cycle (yellow solid line) and carbon cycle (black solid line).

Figure 6. Redundancy analysis (RDA) of the relationship between N and S compounds and bacterial genera.

Figure 7. Heatmap of the abundance of the metabolic pathways of the sulfur cycle (blue solid line), nitrogen cycle (yellow solid line) and carbon cycle (black solid line).

3.7. Integrated Dynamic of N and S

Although N2 was used to create anaerobic conditions at the beginning of the test, there was still a small amount of O2 between the pores FW and SS, so that microorganisms with nitrification could oxidize NH4+ to NO2− and NO3−. As another key nitrogen cycle, denitrifying microorganisms utilize NO3− and NO2− instead of O2 as the terminal electron acceptor in the oxidation of organic compound. As shown in Figure 8, the concentrations of NH4+ and NO2− changed greatly, while NO3− remained at a low level. It indicated that the accumulation of NH4+ over time might inhibit nitrification or denitrification. At the same time, sulfide has been found to inhibit nitrification [44]. Most sulfide existed in HS− form at pH 8.0, and in the form of H2S at pH 6.0. Therefore, the lower the pH was, the more the H2S was formed in aqueous solution, which was consistent the results in Figure 2 [45]. This phenomenon was proved by the presence of Sporanaerobacter involved in sulfur reduction in this study. The Advenella involved in sulphide-oxidation coupled to nitrate reduction seems to be the predominant denitrification process in the AcoD of SS with FW. In addition, sulfate reduction also contributed to ammonium accumulation by organic matter mineralization [46]. At the end of AcoD, 2200 mg/L of NH4+ remained, which was much higher than other ions in the study. Hence, it could be inferred that nitrate reduction was indeed prior to NH4+ oxidization. Recent studies demonstrated the
process of sulfate reducing ammonium oxidation (SRAO) could combine NH$_4^+$ and SO$_4^{2-}$ as Equations (2)–(4) [47]. *Sporanaerobacter* was found to have this function. It is interesting to notice that sulfide-dependent autotrophic denitrification (Equation (5)) is one of the reactions involved in the sulfammox process. The autotrophic denitrification reactions use S$^2-$ and SO$_3^{2-}$ as electron donors and NO$_3^-$ and NO$_2^-$ as electron acceptors (Equation (5)). In this study, *Caldivinea*, *Thermomonas* and *Acinetobacter* could participate in the process. Because the FW used in this experiment contains less NO$_3^-$ and NO$_2^-$, the denitrification activity might be limited.

\[
3\text{SO}_4^{2-} + 4\text{NH}_4^+ = 4\text{NO}_2^- + 3\text{S}^2- + 4\text{H}_2\text{O} + 8\text{H}^+ \quad (2)
\]

\[
2\text{NO}_2^- + 2\text{NH}_4^+ = 2\text{N}_2 + 4\text{H}_2\text{O} \quad (3)
\]

\[
5\text{S}^2- + 8\text{NO}_3^- + 8\text{H}^+ = 5\text{SO}_4^{2-} + 4\text{N}_2 + 4\text{H}_2\text{O} \quad (4)
\]

\[
3\text{S}^2- + 2\text{NO}_2^- + 8\text{H}^+ = 3\text{S}^0 + \text{N}_2 + 4\text{H}_2\text{O} \quad (5)
\]

**Figure 8.** Integrated variation of N and S species during AcoD at ratio 2:8: (a) nitrogen and sulfur in gas phase; (b) nitrogen and sulfur inorganic compounds in supernatant.
4. Conclusions

In this study, the AcoD of SS with FW were performed to investigate the temporal change of N-compounds and S-compounds under different VS ratios. The Gompertz model demonstrated that 2:8 of SS and FW was the optimum condition for methane production, whose cumulative methane yield was 15.12 L. When methane was no longer produced, the concentrations of NO$_3^-$, NO$_2^-$, NH$_4^+$, SO$_4^{2-}$, SO$_3^{2-}$ and S$^2-$ were 0.00, 0.00, 2118.35, 0.00, 0.00 and 0.60 mg/L at the optimum ratio, respectively. In addition, **Syntrophomonas** and **Aminobacterium** were positively correlated to H$_2$S, NH$_3$, NH$_4^+$ and SO$_3^{2-}$, while **Saccharibacteria_genera_incertae_sedis** and **Thermomonas** were positively correlated to SO$_4^{2-}$ and NO$_2^-$ . According to the Functional prediction, the ureolysis, nitrate respiration, nitrogen respiration and nitrate reduction contributed to high NH$_4^+$ , while sulfate respiration and sulfate respiration contributed to the S cycle. The results of this study revealed the change mechanism of typical N-compounds and S-compounds in the AcoD, which could provide a basis for improving the efficiency for engineering application.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14094982/s1, Table S1: The definitions of the $\alpha$ diversity indices.

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Abbreviations

| Abbreviation | Definition                                      |
|--------------|-------------------------------------------------|
| AcoD         | Anaerobic co-digestion                          |
| FW           | Food waste                                      |
| SS           | Sewage sludge                                   |
| N            | Nitrogen                                        |
| S            | Sulfur                                          |
| SCOD         | Soluble chemical oxygen demand                  |
| VFAs         | Volatile fatty acids                            |
| CMY          | Cumulative methane yield                        |
| OTUs         | Operational taxonomic units                     |
| RDA          | Redundancy analysis                             |
| FAPROTAX     | Functional Annotation of Prokaryotic Taxa       |

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