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Effects of ozone on activated sludge: performance of anaerobic digestion and structure of the microbial community

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ABSTRACT

The treatment and disposal of activated sludge are currently challenging tasks in the world. As a common biological engineering technology, biological fermentation exists with disadvantages such as low efficiency and complex process. Ozone pretreatments are commonly applied to improve this problem due to their high efficiency and low cost. In this study, the significant function of ozone in anaerobic fermentation gas production was verified with excess sludge. Compared with other untreated sludge, ozone pretreatment can effectively degrade activated sludge. After ozone treatment and mixing with primary sludge, the methane production of excess sludge increased by 49.30 and 50.78%, and the methanogenic activity increased by 69.99 and 73.83%, respectively. The results indicated that the mixing of primary sludge with excess sludge possessed synergistic effects, which contributed to the anaerobic fermentation of excess sludge. The results of microbial community structure exhibited that methanogenic processes mainly involve hydrogenogens, acidogens and methanogens. The relative abundance of both bacteria and microorganisms changed significantly in the early stage of hydraulic retention time, which coincided exactly with the gas production stage. This study provided a feasible pretreatment strategy to improve sludge biodegradability and revealed the role of microorganisms during anaerobic digestion.

Key words: activated sludge, anaerobic digestion, methane, microbial community, ozone, sludge reduction

HIGHLIGHTS

- Ozone exhibited significant decomposition effects on sludge.
- The methanogenic activities and methane production of the excess sludge were significantly increased after it was oxidized by ozone and then mixed with the primary sludge.
- The relative abundance of both bacteria and microorganisms changed significantly in the early stage of hydraulic retention time, which coincided with the gas production stage.

1. INTRODUCTION

With the growing world's population, daily water consumption is also increasing, which will exacerbate the pressure on sewage treatment plants. In the sewage treatment process, microorganisms use biodegradable substances in sewage as an energy source to support physiological metabolism. Some organic matter is also involved in the proliferation of microorganisms, in which inactivated or less active microorganisms are periodically discharged from the primary, secondary or sedimentation tanks, producing sludge. As the world's largest developing country, China has produced a large amount of sewage and sludge with industrialization and urbanization (Sun *et al.* 2022a), which exceeds the load capacity of sewage treatment plants. Sludge contains a highly complex molecular mixture of organic materials with some toxic compounds (Karn & Kumar 2019), which can cause secondary damage to the environment if not treated properly. It is a great challenge for sewage treatment plants to deal with these serious environmental problems and the rising cost of sludge treatment (Xu *et al.* 2014). It may cost 50–60% of the total operating cost of a sewage treatment plant to dispose of excess sludge (ES) (Sun *et al.* 2022b). Traditional landfill, incineration and other treatments can no longer meet economic and environmental needs (Jin *et al.* 2016a, 2016b; Zhu *et al.* 2017). For example, landfill and incineration have several disadvantages: landfill may pollute

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groundwater sources, and incineration will produce harmful gases affecting the air quality (Cameron *et al.* 1997; Leila *et al.* 2017).

As an effective, sustainable and versatile sludge treatment method with the advantages of mass reduction, pathogen removal and methane gas production, anaerobic digestion (AD) is an effective way to achieve sludge reduction, soundness and resource utilization (Pei *et al.* 2015). The organic matter is decomposed in sludge through anaerobic fermentation bacteria in an anaerobic environment, eventually producing carbon dioxide, methane and water. AD involves four reaction steps including hydrolysis, acidification, hydrogen production, and acetic acid production and methanation (Zhen *et al.* 2017; Wang *et al.* 2018), among which the hydrolysis stage is regarded as the rate-limiting step in AD of sludge. Most of the organic matter present in the waste activated sludge (WAS) is isolated in the microbial cell membrane. As a semirigid structure, microbial cell walls contain sugar chains cross-linked with peptide chains to protect the cells from osmotic cleavage. Only the release of these organic substances from the broken cell walls can be digested by anaerobic bacteria. Therefore, it is necessary to pretreat the sludge in order to improve the AD performance of the sludge.

Pretreatment technologies of WAS mainly include mechanical (Balasundaram *et al.* 2022), chemical (Barrios *et al.* 2017), biological (Jiang *et al.* 2022), pulsed electrical field (Kuscu *et al.* 2022), ultrasound (Jiang *et al.* 2022) and heat (Chen *et al.* 2020; Yan *et al.* 2022) treatment methods. As a strong oxidant, ozone is commonly used in many phases of wastewater treatment and water purification processes. Ozone pretreatment produces the highest degree of sludge decomposition without introducing substances that may cause secondary contamination. Ozone possesses the advantages such as reducing ES production, destroying sludge flocculation cells, dissolving organic matter in the cells, shortening the fermentation phase and improving AD performance. A large number of highly sensitive microbial life forms exist in the activated sludge system, which is easily affected by the external reduction 'energy' and undergoes qualitative changes (cell lysis). The microorganisms in the sludge system are inactivated and dissolved by the strong oxidation ability of ozone. Low-dosage ozonation is proved to result in reduction in accumulated biomass and an improvement in microbial aggregate properties like sludge dewaterability (Meng *et al.* 2016). Ozone disintegrates zoogloeal structure, rendering microorganisms more accessible to cell envelop rupture by peroxidative attack (Yuan *et al.* 2019a). Three ways of sludge ozonation processes are commonly used, including floc decomposition, dissolution and subsequent oxidation of the released organic matter to carbon dioxide (mineralization). Following ozonation, the sludge's flocs were first broken down into fine, dispersed particles. Subsequently, due to sludge dissolution, soluble organic matter was released into the liquid phase (Chu *et al.* 2009). More volatile fatty acids (VFAs) are produced in ozonated samples, which are easily exploited by methanogens, which became the dominant bacteria, resulting in an increase in the percentage of methane in biogas (He *et al.* 2021).

Most previous studies have focused on the ozone pretreatment of ES or mixtures of ES and primary sludge (PS) (Chacana *et al.* 2017; Chiavola *et al.* 2019). However, research studies have shown that ozone has no effect on the oxidation of the PS (Chacana *et al.* 2017). ES consists mainly of microorganisms, extracellular polymeric substances and inorganic matter, which were considered to be relatively low biodegradable substrates. PS usually consists of natural fibers, fats and other solids, which was more readily biodegradable than ES. As the main object of the experiment, the ES has a high solid content. PS refers to the sludge that settles at the bottom of the primary sedimentation tank after simple physical and chemical treatments of the influent of the sewage plant. Most of the suspended and dissolved organic matter of the activated sludge remains unhydrolyzed and digested by microorganisms at this stage. The digested sludge (DS) is used as inoculation sludge. Although the advantages of ozone in increasing methane production have been demonstrated in several studies, the relative applicability of ozone pretreatment has not been fully evaluated for different types of sludge. Accordingly, the main objectives of this study were to evaluate the effect of ozone pretreatment on the ES and the mixed system of different types of sludge and to assess the production and fermentation effect of methane by the change of microbial biochemical methane potential (BMP) in the later stage. It also provided reference for evaluating the relative applicability of ozone pretreatment to different types of sludge. Meanwhile, the advantages of anaerobic fermentation gas production and sludge reduction and its microbial community were analyzed.

2. MATERIALS AND METHODS

2.1. Activated sludge

WAS was taken from the secondary sedimentation tank of the Qingdao wastewater treatment plant in Shandong, China, which uses the Anaerobic–Anoxic–Oxic–Modified Sequencing Batch Reactor (A^2/O-MSBR) process. The collected samples were screened through a sieve to separate large debris from the activated sludge. The sludge samples were precipitated for 24 h and stored in a 4 °C refrigerator before the experiment. Part of the process flow chart is shown in Supplementary Figure 1. ES used in the ozone oxidation experiment was obtained from the secondary sedimentation tank. The PS was the sludge discharged from the primary sedimentation tank. The mixed sludge (MS) was obtained from the mixing tank and the DS taken from the biological fermentation tank. After retrieving the raw sludge, the initial values of total suspended solids (TSS), volatile suspended solids (VSS), total chemical oxygen demand (tCOD), soluble chemical oxygen demand (sCOD) and pH were determined within 1 day. The properties of activated sludge are shown in Table 1. All samples were measured in triplicate.

2.2. Ozone pretreatment of WAS

The ozone experimental device is shown in Supplementary Figure 2 and consists of an ozone generator (CF-G-3-10G, Qingdao, China), an ozone analyzer (UV-2300C, China), an ozone reaction device, an ozone exhaust analyzer (UV-2100, Zibo, China), and a KI absorbent. Ozonation was performed with an ozone gas generator (CF-G-3-10G, Qingdao, China). Pure oxygen was used as the gas source and converted to ozone with a high-voltage converter. The sludge (sample volume of 1 L) is transferred to a 2 L contact chamber where it was maintained at a predetermined time for the ozone flow. The ozone was fully mixed with the sludge by magnetic stirring at a speed of 600 rpm to improve ozone utilization. The ozone concentration was controlled at 160 g/m^3 by changing the current of the ozone generator to 0.5 A. At a flow rate of 0.3 L/min, 1 L of activated sludge was pumped into the ozone gas generator, with the oxidation time set to 90 min (0.16g·O_3/g VSS) and 150 min (0.27g·O_3/g VSS). The residual ozone from the outlet gas is pyrolyzed with an exhaust gas treatment unit and then absorbed with KI liquid.

2.3. AD batch experiment

BMP tests were carried out to assess the methane production of sludge under different pretreatment conditions. Eight pretreatment methods were applied to WAS (Table 2), where W.1 was the fermentation process of the raw wastewater treatment plant. Pretreated sludge samples of 200 mL mixed with 200 mL DS were placed into the 600 mL fermentation flasks, and then each of the flasks was sparged with nitrogen gas for 5 min to create an anaerobic condition. To provide better contact between the pretreated sludge lysis substrate and the anaerobe, the fermentation flasks were placed on a magnetic stirring apparatus and stirred at 500 rpm at 35 ± 1 °C. Each experimental condition was carried out in triplicate.

2.4. Quality analysis

During the sludge pretreatment period, the tCOD, sCOD, VSS, TSS and pH were analyzed according to standard methods (Carranzo 2012). The pH value was measured with a pHS-25 pH meter (Shanghai, China).

The solubilization of the WAS was calculated according to Equation (1) (Tulun & Bilgin 2019):

$$\%\,\text{Solubilization} = \frac{\text{soluble COD after pretreatment}}{\text{Total COD}} \times 100. \tag{1}$$

COD was measured after the samples were centrifuged at 6,000 rpm/min for 10 min and filtered through 0.45 μm microfiltration membranes. Methane contents were analyzed using gas chromatography (Shimadzu GC-2014). High purity argon (99.999%, 0.6 MPa) was used as the carrier gas at a flow rate of 40 mL/min. The gas volume was measured by the drainage method.

Table 1 | Properties of the activated sludge

Species/parameter	sCOD (mg·L^{-1})	tCOD (mg·L^{-1})	sCOD/tCOD	pH	VSS (g·L^{-1})	TSS (g·L^{-1})	VSS/TSS
MS	433 ± 1	24,270 ± 570	0.018	6.83	13.95 ± 0.23	23.68 ± 0.52	0.59
DS	332 ± 2	11,500 ± 20	0.029	7.47	10.26 ± 0.09	20.74 ± 0.41	0.49
PS	1,091 ± 15	12,650 ± 30	0.086	6.21	16.84 ± 0.6	25.47 ± 0.68	0.66
ES	358 ± 8	34,430 ± 230	0.010	7.35	26.44 ± 0.29	83.50 ± 0.48	0.32

Table 2 | Experimental process and batches

Batch	Sludge	DS (mL)	Ozone oxidation (min)	Mechanically stirred (h)	PS (mL)	Substrate/inoculum
W.1	MS	200	–	–	–	1:1
W.2	ES	200	–	6	–	1:1
W.3	PS	200	–	6	–	1:1
W.4	ES	200	–	6	200	2:1
W.5	ES	200	90	6	–	1:1
W.6	ES	200	90	6	200	2:1
W.7	ES	200	150	6	–	1:1
W.8	ES	200	150	6	200	2:1

2.5. Principal component analysis

The principal component analysis (PCA) was performed using the SPSS software (IBM SPSS Statistic 21.0, IBM Corp., Armonk, NY, USA) for better understanding the interrelations between all measured quality indexes (including tCOD, sCOD, VSS, TSS and pH for all samples) during AD after ozone pretreatment. For this purpose, all measurements of the AD series were analyzed separately as summary data after standardization treatment for each index in order to obtain general information on the differences of the interrelations between the quality indicators of AD.

2.6. DNA extraction and high throughput sequencing

Samples were washed with the phosphoric acid buffer for three times. Microbial DNA was directly extracted from samples with the E.Z.N.A.® Soil DNA Kit (D5625-01, USA) and the 16S rRNA gene of V3–V4 according to the manufacturer's instructions (Xu *et al.* 2016). The polymerase chain reaction and analysis of high-quality sequences were performed according to the description of Wu *et al.* (2018).

3. RESULTS AND DISCUSSION

3.1. Effect of ozone treatment on sludge solubilization

The effect of ozone pretreatment on sludge solubility was investigated by measuring the changes in sCOD and tCOD of sludge samples. Supplementary Table 1 shows the raw sCOD and the sCOD and tCOD after ozone pretreatment for each batch of sludge, and the data are plotted in Figure 1. As the process of the original sewage plant, W.1 has a solubility of only 1.78%. The solubility after 6 h of mechanical stirring was 1.67% (W.2). Evidently, the sludge was not decomposed by simple mechanical agitation, thereby disrupting the cell wall; thus, the change in solubility was not significant (Zhou *et al.* 2018). Mechanical stirring mainly contributed to the role of stirring evenly for better material exchange and bacterial contact. The solubility of W.5 is 22.78%, which is 13.64 times that of W.2; while the solubility of W.7 is 27.89%, which is 16.70 times that of W.2. Consequently, sludge solubility was used to show the effect of ozone oxidation, and sludge solubility increased with increasing ozone dose. However, the increase in solubility was not significant due to mineralization. The potential pathways for sludge ozonation in this experiment were hypothesized by the analysis of the data of W.5 and W.7. The cell walls and cell membranes of microorganisms were damaged or broken down by ozone, which resulted in reactions with proteins and DNA. In addition, ozone could damage the flocs in the sludge. As the oxidation time increased, most of the ozone interacted with the dissolved organic matter, resulting in the production of carbon dioxide. The solubility of W.5 and W.7 decreased to 13.96% (W.6) and 13.98% (W.8) after 1:1 mixing with PS, respectively, which was caused by the difference in the properties of PS and ES. Microorganisms are the main component and production source of sCOD in ES, while sCOD in PS is mainly derived from particulate organic matter, which is easier to hydrolyze than microbial cells. The results showed that ozone had a significant decomposition effect on sludge. However, excessive ozone doses caused mineralization reactions, which increased the decomposition effect insignificantly.

3.2. Effect of ozone treatment on methane production and sludge reduction

The cumulative methane yield was measured during the entire BMP period. The trends of cumulative methane production from different batches of sludge are shown in the graphs of Figure 2(a) and 2(b). The increase in gas production was not

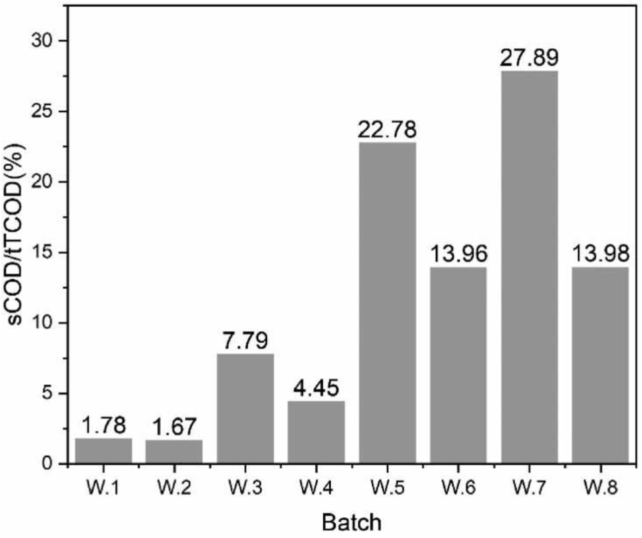

Figure 1 | Influence of pretreatment on sCOD/tCOD ratio (W.1 = MS, W.2 = ES + M, W.3 = PS + M, W.4 = ES + M + PS, W.5 = ES + M + O90, W.6 = ES + M + O90 + PS, W.7 = ES + M + O150 and W.8 = ES + M + O90 + PS).

significant from day 10 (Figure 2(a)). The raw wastewater treatment plant process (W.1) exhibited the lowest production among all batches. Among the first four batches, the gas production of the PS (W.3) was the most abundant. The organic matter in the PS was mostly particulate organic matter, which could be rapidly hydrolyzed by the action of hydrolytic enzymes and therefore easily absorbed and utilized by anaerobic microorganisms. In contrast, cell walls and bacterial micelles were present in the sludge flocs, which were not easily utilized by anaerobic microorganisms, resulting in difficulty in degradation (Chiavola *et al.* 2019). Therefore, it is necessary to take some measures that can effectively destroy the sludge floc structure, break the cell wall and dissolve the cell inclusions in order to improve the AD performance of the remaining sludge. When the AD system of a wastewater treatment plant is inadequate and no other wall-breaking measures are available, it is recommended that all PS be treated first and ES be supplemented to increase the methane production of the system.

Figure 2(b) shows that the methane production of the ES after ozone oxidation increased by 15.94% (W.5) and 18.06% (W.7), respectively, compared to the original ES (W.2). The methane production of the ES oxidized and then mixed with the PS increased by 49.30% (W.6) and 50.78% (W.8), respectively. The results showed that the methane production of the ES was significantly increased after it was oxidized by ozone and then mixed with the PS, which is consistent with the results of previous studies (Sun *et al.* 2022a). The amount of methane produced from the dissolved substrate from the particulate organic matter in the mixture of ES and PS is significantly abundant compared to single-component sludge such as W.6 and W.8, which was attributed to a synergistic effect that is produced when the two sludges get together.

Sludge reduction was another parameter to assess the properties of AD. Most of the organic matter in the sludge is decomposed and even mineralized after AD. VSS was often used to describe the rate of sludge reduction. Figure 2(c) shows the methanogenic activity of the sludge. During the hydraulic retention time (HRT) period, methane production activity was concentrated in the early period (days 0–12), with a significant decrease in production in the late period (days 12–23). The CH_4/VSS of all batches of sludge in the pre-HRT period was 3.40, 1.90, 5.21, 3.81, 1.86, 2.59, 2.36, and 2.57 times higher than that in the post-HRT period, which was also reflected in the reduction of VSS (Figure 2(d)). In terms of cumulative gas production, all batches of sludge remained at a stable low level in the late HRT. Throughout the HRT, the yield of the original wastewater treatment plant process (W.1) was 484.26 mL CH_4/g VSS, the ES (W.2) was 412.55 mL CH_4/g VSS, and the PS (W.3) was 668.02 mL CH_4/g VSS. Compared to W.2, the yield of oxidized residual sludge (W.5 and W.7) increased by 24.86 and 61.65%, respectively. Meanwhile, W.6 and W.8 were 69.99 and 73.83% greater than W.4, respectively. Both compared to the original process and to the unoxidized process, the increase in methanogenic activity of the ES after ozone oxidation exceeded previous studies (Erden & Filibeli 2011). It was evident that the PS exhibited great potential to stimulate the synergistic effect of organic matter in the ES to promote anaerobic fermentation, both for gas production and methanogenic activities.

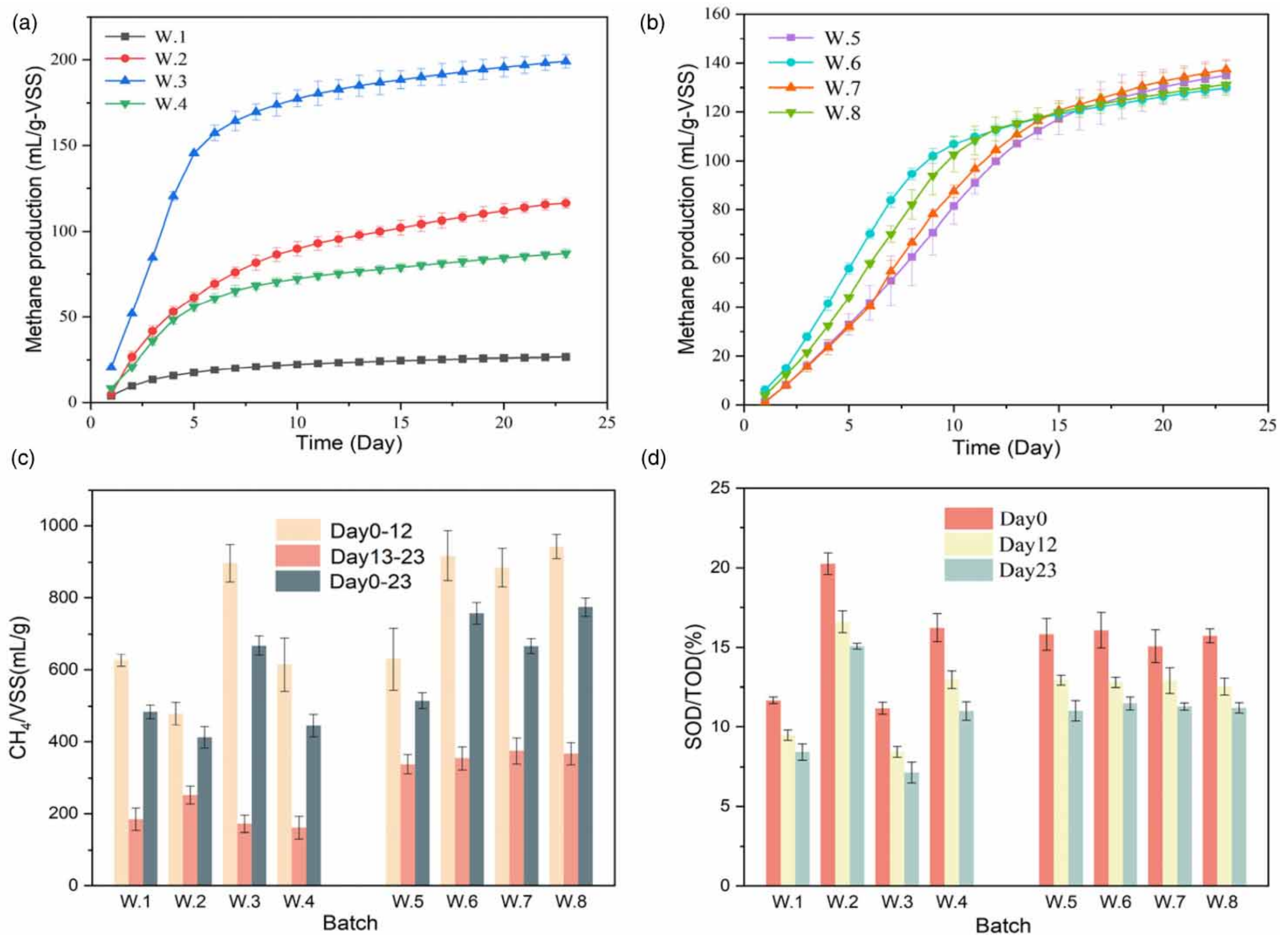

Figure 2 | Cumulative methane production and sludge reduction effect (W.1 = MS, W.2 = ES + M, W.3 = PS + M, W.4 = ES + M + PS, W.5 = ES + M + O90, W.6 = ES + M + O90 + PS, W.7 = ES + M + O150 and W8 = ES + M + O90 + PS).

How to increase the gas production and methanogenic activities is the major concern in terms of environment and economy. The dose of ozone W.8 is 1.5 times higher than that of W.6; in terms of the methane production, W.6 increased by 49.30% and W.8 increased by 50.78%. For methanogenic activities, W.6 and W.8 were 69.99 and 73.83% greater than W.4, respectively. Longer ozone pretreatment time did not give additional advantage and it also increased costs. The past research (Du *et al.* 2021; Sun *et al.* 2022b) showed that the effects of ozonation are strictly dependent upon the applied ozone dosage: increasing the dosage usually provides higher benefits in terms of solid reduction and methane production enhancement. However, mineralization of dissolved organic matter can occur as a result of excessive ozone dosage or treatment time and should be avoided (Braguglia *et al.* 2012). Overall, the experimental group with ozone oxidation time of 90 min showed good results. The additional ozone dosing time did not significantly promote the methane production and the methanogenic activities in the entire system. Subsequently, physical pretreatment and ozone pretreatment can be combined to reduce the energy requirements.

3.3. Community diversity analysis of different sludge batches

The differences in bacterial community compositions between the different sludge batches were illustrated by PCA (Figure 3(a)). PC1 and PC2 accounted for 15.34 and 8.22% of the total variance in the dataset, respectively. It was evident that two distinct regions could be identified within the PCA profile. Region A showed the original sludge batches at the beginning of the experiment, and Region B included sludge batches from the middle and late stages of the experiment. It can be seen that the sludge community of the initial batches was dispersed, which was attributed to the different types of sludge in each batch. Differences were found between the microbial communities of the batches not oxidized by ozone and the

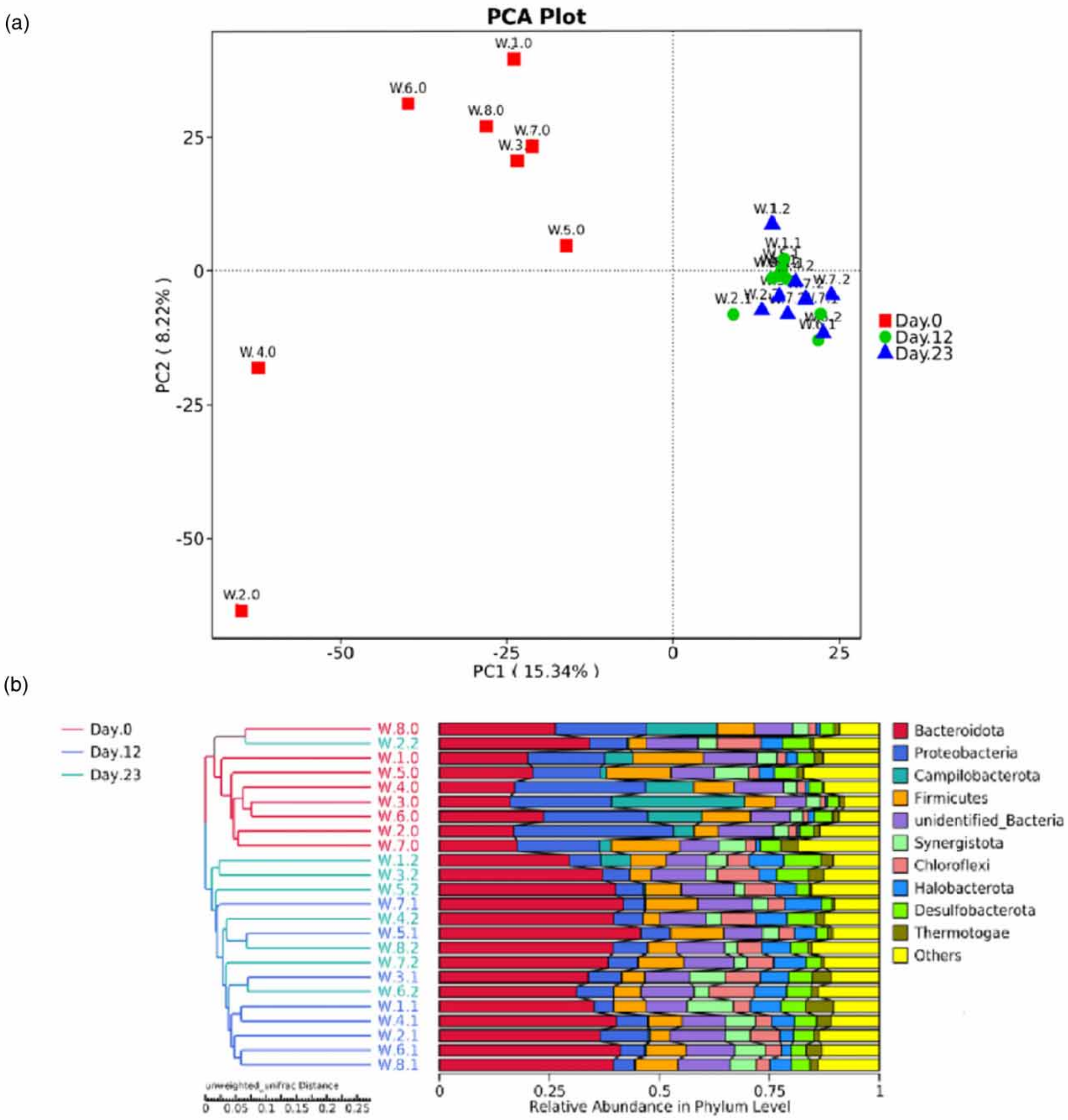

Figure 3 | The shift of microbial community diversity in activated sludge in each group according to the 16S rRNA data. (a) PCA. (b) Clustering tree based on OUT (W.1 = MS, W.2 = ES + M, W.3 = PS + M, W.4 = ES + M + PS, W.5 = ES + M + O90, W.6 = ES + M + O90 + PS, W.7 = ES + M + O150 and W.8 = ES + M + O90 + PS).

ozone-oxidized batches. In addition, the distribution of the sludge batches after ozone oxidation was also different, suggesting that ozone was responsible for the differences in the microbial communities. The microorganisms in the sludge were degraded by ozone, resulting in changes in the microbial community (Hashimoto *et al.* 2021). The dissolution of organic matter in the sludge caused changes in the microbial community structure, which fundamentally determined the performance of the anaerobic fermentation process.

The clustering results were integrated with the relative abundance of each sample at the phylum level (Figure 3(b)). *Bacteroidetes*, *Proteobacteria* and *Campylobacter* were the three most dominant phyla. *Bacteroidetes* were known as a group of fermentation bacteria in the acidogenic stage of digestion process (Ma *et al.* 2019). Studies have shown that the *Proteobacteria* consumes the production of short-chain fatty acids during AD, and the decrease in abundance facilitates the accumulation of fatty acids (Nelson *et al.* 2011; She *et al.* 2020). *Campylobacter* belongs to aerobic bacteria, which can cross-weave between bacterial micelles in sludge or adhere to the surface of flocs, and a minority of species can also be free-floating between sludge flocs. *Campylobacter* possessed better ability to oxidize and decompose organic matter and played a certain role in purification. *Campylobacter* decreased most significantly among the three species, which is mainly attributed to the depletion of degradable organic matter and the decrease of bacterial abundance.

Significant changes in the microbial community structure and the abundance of some bacteria occurred during the initial phase of the experiment, which could be attributed to the accumulation of various substrates after sludge domestication that stimulated microbial activity. In contrast, there was almost no difference in the microbial community at the later stages of the experiment, which was due to the depletion of substrates available for microbial growth. Evidently, the whole biological community exhibited an extremely strong temporal effect. In addition, the microbial community of the ES was more abundant before it was oxidized by ozone and the differences appeared after oxidation because most of the microorganisms had been oxidized and decomposed. There was no significant change between different ozone doses.

3.4. Analysis of specific microbial community structure analysis

Microbial communities were detected to further investigate the reaction mechanism involved in sludge reduction and methane production. During the entire AD phase, the methane production process mainly involves hydrogenogens, acidogens and methanogens (Yuan *et al.* 2019a) (Figure 4(a)). The main hydrogenogens were counted, including *Clostridium*, *Bacteroides*, *Megasphaera*, *Eubacterium*, *Acetivibrio* and *Ruminococcus*. In the first four batches of sludge, the amount of hydrogenogens in the MS (W.4) was more than twice that of the ES (W.2). The largest amount of hydrogen remained in the PS (W.3). The number of hydrogenogens increased rapidly by nearly 10 times after the ES was oxidized by ozone, which is consistent with previous studies (Wu *et al.* 2013). The oxidation of sludge by ozone disrupted the rate-limiting hydrolysis process, leading to a rapid increase in substrates favoring AD, which stimulated a rapid increase of hydrogenogens. Throughout the HRT phase, the content of hydrogenogens in all batches showed a gradual decrease and was mainly concentrated in the initial phase, which showed the same phenomenon as the previous gas production. As an intermediate product of AD, H_2 is rapidly converted to other products. A portion of H_2 is used for the synthesis of acetic acid, and another portion is used for the reduction of CO_2 to produce methane (Zhao *et al.* 2017; Leng *et al.* 2018). During the pre-HRT period, the

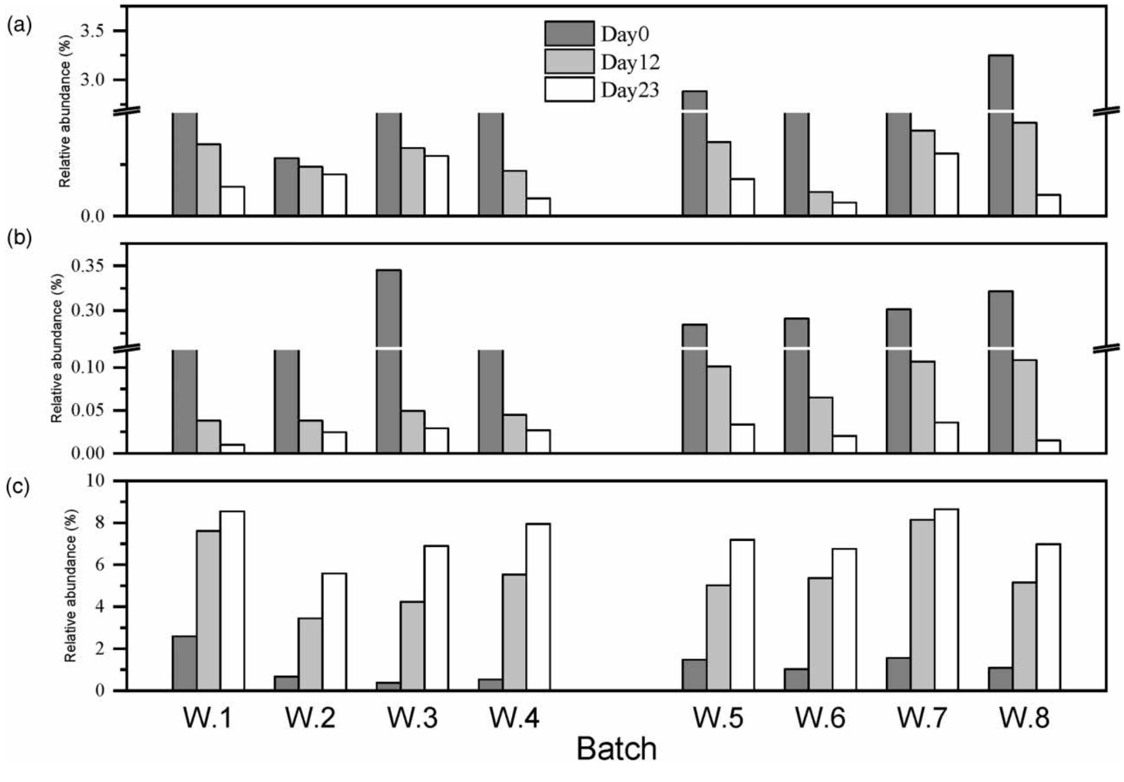

Figure 4 | Impact of ozone on microbial community during sludge AD. (a) *Hydrogenogens bacteria*, (b) *Acidogens* and (c) *Methanogenesis* (W.1 = MS, W.2 = ES + M, W.3 = PS + M, W.4 = ES + M + PS, W.5 = ES + M + O90, W.6 = ES + M + O90 + PS, W.7 = ES + M + O150 and W.8 = ES + M + O90 + PS).

hydrogenogens declined sharply. The main reason for this phenomenon was that the hydrogenogens were used to generate other products resulting in an insufficient substrate to produce H_2 rapidly again, in addition to a decrease in the enzymatic activity of hydrogenogens. The results for hydrogenogens were consistent with the previous results for the bacterial communities, with significant changes occurring in the pre-HRT period.

The main acidogenic bacteria were counted, including *Syntrophobacter*, *Clostridium*, *Acetobacterium*, *Bacillus* and *Microbacterium* (Figure 4(b)) (Budhavaram & Fan 2009; Lu *et al.* 2020). The relative abundance of acidogens was significantly less than that of hydrogenogens. The amount of acidogenic bacteria showed a decreasing trend and was mainly concentrated in the initial period of HRT. The amount of acidogens in the MS was superior to that of the ES. The relative abundance of acidogens doubled after the ES was oxidized by ozonation. This indicated that a large amount of monosaccharides, amino acids and short-chain fatty acids were hydrolyzed after sludge ozonation, which are necessary substrates for acetic acid production (Antonopoulou *et al.* 2015). Consequently, the amount of acidogens decreased significantly in the early stage of HRT with the gradual consumption of substrates. In the post-HRT period, the decreasing trend of acid-producing bacteria was relatively smooth due to the lack of substrate, which was consistent with the phenomenon of hydrogenating bacteria. Compared with the oxidized ES, the organic matter in the PS (W.3) was easier to be utilized by various bacteria, which might explain its higher gas production potential.

During the whole anaerobic fermentation stage, methanogenesis was the final process that determined the effectiveness of gas production. As seen in Figure 4(c), the amount of methanogenic bacteria in the MS of the original process was several times higher than the other three sludges. After the ES was oxidized by ozone, the amount of methanogenic bacteria increased by more than three times. It was indicated that the ozone oxidation was extremely beneficial for the main types of bacteria in the methane production process. The production of methane at all stages increased significantly with the increase of these bacteria and the release of the substrate. In addition, the trend of methanogenic bacteria in the eight batches of sludge was increasing throughout the HRT period in contrast to hydrogenogens and acidogenic. The acid-producing bacteria and methanogenic bacteria showed opposite changes in abundance due to their competitive relationship (Yuan *et al.* 2019b). From the relative abundance of the above three bacteria, the PS (W.3) was more abundant in hydrogen- and acid-producing bacteria, while there was no significant difference in methanogenic bacteria. The changes in specific bacterial groups were consistent with the overall changes in microbial community structure, showing significant phases. Consequently, the increase in the abundance of dominant bacteria contributed only a fraction of the potential to promote methanogenesis. More importantly, PS could provide a more abundant fermentation substrate.

4. CONCLUSIONS

In this work, the fate of ozone pretreatment in activated sludge and AD was evaluated. Different oxidant contents exhibited different effects on the degradation of surplus sludge. Ozone oxidation for 60 min increased ES solubility by 21.11%, and ozone oxidation for 90 min increased ES solubility by 26.11%. However, the increase in sludge solubility with increasing ozone oxidation time was not significant due to mineralization. The ozone-oxidized ES mixed with PS showed a superior fermentation effect; after ozone treatment and mixing with PS, the methane production of ES increased by 49.30 and 50.78%, and the methanogenic activity increased by 69.99 and 73.83%, respectively, indicating that the combination of different types of sludge could produce a synergistic effect. This also provided further insights for subsequent anaerobic fermentation and practical applications. Biogas production through AD can be converted to be used as biofuel to generate heat and electricity, which addresses the challenging issue of clean renewable energy generation. Biogas generation using AD of sludge is an excellent solution for an integrated approach to global challenges, growing energy demand and WAS treatment. The changes of the whole microbial community, typical bacteria and anaerobic fermentation gas production process coincide with each other. The analysis of bacterial and microbial communities clarified the methanogenic processes throughout the HRT phase, which is mainly concentrated in the former half. For sludge, the external properties of gas production and sludge reduction correspond to the changes in the structure of its internal biological community. The technique should be scaled up in future work to better assess environmental and energy effects.

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AUTHOR CONTRIBUTIONS

P.G. wrote the original draft of the article and did the formal analysis. X.M. reviewed and edited the manuscript. X.W. contributed to the investigation and methodology. Z.C. contributed to the data curation. Y.L. contributed to the investigation. X.L. contributed to the supervision. D.Z. contributed to the project administration and funding acquisition.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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