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THE EFFECT OF FREE NITROUS ACID PRETREATMENT AND AEROBIC DIGESTION ON THE DYNAMICS OF ANTIBIOTIC RESISTANCE GENES IN RESIDUAL SLUDGE

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Abstract: Free Nitrous Acid (FNA) Pretreatment Enhances Organic Matter Hydrolysis in the Digestion Stage, but Its Impact on the Dynamics of Antibiotic Resistance Genes (ARGs) During Aerobic Digestion of Residual Sludge Remains Unexplored.This study employed an orthogonal experimental design to assess the dynamics of ARGs in the aerobic digestion of residual sludge under varying FNA pretreatment sludge ratios, temperatures, and pH levels. The aim was to identify the optimal conditions for ARG reduction and elucidate the underlying mechanisms. Results indicated that the optimal conditions for ARG reduction were a residual sludge to FNA-pretreated sludge ratio of 2:1, a temperature of 45°C, and pH = 10, yielding reductions of 84.13% and 76.03%, respectively. Range analysis revealed that the key environmental factors influencing ARG reduction were pH > temperature > sludge ratio. Under high-temperature and alkaline conditions, concentrations of ammonia nitrogen (NH_3), soluble extracellular polymeric substances (S-EPS), and soluble chemical oxygen demand (SCOD) increased, suggesting that these conditions facilitate cell lysis, thereby promoting ARG reduction. Microbial community analysis showed that the relative abundance of potential ARG-hosting bacteria (e.g., Saccharimonadales, Caldilineaceae, SC-I-84, Ellin6067, unclassified_Blastocatellaceae, Nitrospira, Gemmatimonadaceae, and OLB12) decreased in parallel with ARG reduction, indicating that the high-temperature, alkaline aerobic digestion environment may mitigate ARG dissemination by inhibiting the proliferation of potential host bacteria.
Keywords: Free Nitrous Acid (FNA) pretreatment; Aerobic digestion; High temperature; Strong alkaline conditions; Antibiotic Resistance Genes (ARGs); Microbial community structure

1 INTRODUCTION

Sewage treatment plants (WWTPs) are considered hotspots for the spread of antibiotic resistance genes (ARGs), which can proliferate within the microbial community through the replication of antibiotic-resistant bacteria and horizontal gene transfer mediated by mobile genetic elements (MGEs)[1],This results in the dissemination of multidrug-resistant pathogens into the environment, posing significant threats to public health and ecological safety. The distribution of ARGs in WWTP effluents and sludge varies. Munir et al. investigated the distribution of ARGs in urban WWTPs and found that the abundance of ARGs in sludge was three orders of magnitude higher than in effluent[2]. Therefore, WWTP sludge serves as a major reservoir and source of ARG contamination. Understanding the dynamics of ARGs during sludge treatment is critical for controlling their spread into the natural environment. As a key component of WWTPs, sludge digestion is commonly used to stabilize biosolids. Aerobic digestion, a widely applied sludge treatment technique, enhances the degradation of volatile solids (VS), improves nitrogen and pathogen removal efficiency, and thereby stabilizes the sludge, making it particularly suitable for small-scale sewage treatment systems. However, research on ARG changes during aerobic digestion remains limited and warrants further investigation.

The rate-limiting step in the digestion process is sludge hydrolysis, primarily due to the extracellular polymeric substances (EPS) and cell walls that form stable microbial aggregates, limiting the hydrolysis rate of macromolecular organics. Numerous studies have shown that pretreatment technologies can disrupt cell walls or lyse cells, thereby releasing intracellular organic matter. Research on pretreatment combined with digestion processes is relatively mature in terms of cell disruption and digestion enhancement. Current studies suggest that cell structure degradation is a key factor in ARG removal. Free Nitrous Acid (FNA) pretreatment, characterized by its environmental friendliness, easy accessibility, and strong oxidative properties, has demonstrated excellent performance in promoting cell lysis and improving digestion efficiency, thereby reducing the operational cost of the digestion process. For example, Wang et al. found that FNA treatment at 2.13 mg/L increased the soluble chemical oxygen demand (SCOD) of the sludge sixfold compared to untreated sludge, and after 24 hours of FNA (2.0 mg/L HNO_2-N) pretreatment, followed by 14 days of aerobic digestion, sludge degradation increased from 32% to 50% [3]. Previous studies have shown that alkali, ultrasound, hydrothermal, and ultrasonic pretreatments can enhance anaerobic digestion efficiency while promoting ARG reduction. However, limited research has been conducted on the effect of FNA pretreatment on the reduction of abundant ARGs in aerobic digestion of residual sludge.

This study aims to optimize the FNA pretreatment-aerobic digestion process by regulating environmental factors (such as sludge ratio, temperature, and pH) through orthogonal experimental design. The study will investigate the impact of

these environmental factors on the fate of ARGs by examining changes in ammonia nitrogen (NH_3), SCOD, and EPS. It will systematically analyze the mechanisms of ARGs reduction before and after FNA-aerobic digestion treatment, along with changes in microbial community structure, further elucidating the potential hosts of ARGs and their interactions under different environmental conditions. The findings will provide a theoretical basis for environmental pollution management and ARGs control.

2 MATERIALS AND METHODS

2.1 Source of Activated Sludge

The residual sludge was obtained from the secondary sedimentation tank of a sewage treatment plant in Chengdu, Sichuan Province. The sludge was passed through a 0.5 mm sieve to remove large particles, then left to settle, and the supernatant was discarded to obtain the concentrated sludge

2.2 Pretreatment Conditions

Free Nitrous Acid (FNA) exhibits a strong bactericidal effect, and as the FNA concentration increases, the yield of soluble chemical oxygen demand (SCOD) also increases. Among the influencing factors, pH and nitrite nitrogen (NO_2^--N) concentration are key determinants of FNA concentration. However, neither pH nor NO_2^--N alone has a significant effect on cell lysis, indicating that molecular disruption is primarily caused by FNA rather than by H^+ or NO_2^--N acting independently. Higher FNA concentrations (lower pH and higher NO_2^--N) result in more pronounced cell lysis and better ARG removal from residual sludge [3]. Based on both effectiveness and cost considerations, the FNA pretreatment conditions for residual sludge aerobic digestion in this study were selected as pH 5.3 and NO_2-N 753 mg/L (see Supplementary Table S1).

2.3 Setup and Operation of the Aerobic Digestion System

Aerobic digestion was performed under different conditions using the FNA pretreatment combination. The influence of the pretreatment sludge ratio (residual sludge/treated sludge), temperature, and pH on the abundance of ARGs in the aerobic digestion system was examined. An orthogonal experimental design was used to investigate the changes in ARG concentrations under the influence of these three environmental factors, as shown in Table 1. Sampling was conducted at 0, 4, 8, 12, 16, and 20 days to analyze SCOD, NH_3, and EPS. Samples were also collected at 0, 8, 16, and 24 days to assess ARGs, in order to determine the optimal parameters for ARG reduction during aerobic digestion.

Table 1 Orthogonal Experimental Design of Anaerobic Digestion

Number	Factor 1 (sludge ratio)	Factor 2 (temperature)	factor 3(pH)
E1	1（1:1）	1（25 ℃）	1（6）
E2	1	2（35 ℃）	3（10）
E3	1	3（45 ℃）	2（8）
E4	2（2:1）	1	2
E5	2	2	1
E6	2	3	3
E7	3（3:1）	1	3
E8	3	2	2
E9	3	3	1

2.4 Detection Methods for NH_3, SCOD, and EPS

NH_4^+-N and SCOD were determined using national standard methods. pH was measured using a PH SJ-3F Leici pH meter. EPS in this study was divided into two categories: S-EPS and TB-EPS. The mixed sludge-water suspension was centrifuged at 4000 rpm for 15 minutes. The supernatant was filtered through a 0.45 μm membrane to obtain S-EPS. The remaining sludge sample was rehydrated to the original volume, mixed, and incubated in a 85°C water bath for 10 minutes. It was then subjected to high-speed centrifugation at 12000 rpm for 15 minutes. The supernatant was filtered to obtain TB-EPS. The EPS components mainly include proteins (PN) and polysaccharides (PS), which were measured by the BCA method and the phenol-sulfuric acid method, respectively.

2.5 ARGs High-Throughput Sequencing

DNA extraction was performed using the MoBio Soil Genomic DNA Extraction Kit (DNeasy PowerSoil) to ensure the DNA concentration and purity, with an A260/280 ratio of approximately 1.8. DNA markers and PCR amplification products were loaded into gel wells, and the brightest bands were identified through gel imaging. The brightest bands corresponding to the PCR amplification products were sent for cloning into vectors for the subsequent establishment of

standard curves. Plasmid extraction from bacterial liquid was conducted according to the Plasmid DNA Mini-Prep Kit (SanPrep Column Plasmid Mini-Preps Kit) instructions. The plasmid DNA concentration and purity were determined using a microvolume UV-visible spectrophotometer. The qualified plasmids were used to establish standard curves, and the extracted plasmid DNA was stored at -20°C for future use.

2.6 Microbial Community Structure Analysis

Microbial changes are an important indicator of the aerobic digestion process in sludge treatment. The microbial community structure in the pretreatment phase reflects the survival and death of microbes during the FNA pretreatment process. Changes in microbial community structure can influence ARG abundance. Analyzing the dynamics of microbial community structure during digestion is essential for understanding the fate of ARGs. Correlation analysis between ARGs and microbial community structure was performed to identify potential ARG hosts. Samples were taken on days 0, 8, 16, and 24. The sludge samples from the best FNA pretreatment, anaerobic digestion, and aerobic digestion stages were centrifuged at high speed, and the supernatant was discarded. The remaining sludge samples were frozen at -20°C for preservation. These samples were sent to Shanghai Meiji Biological and Pharmaceutical Technology Co., Ltd., for Illumina MiSeq sequencing analysis. DNA extraction, concentration and purity measurement using a micro-volume UV spectrophotometer, PCR amplification, purification, and quantification using Picogreen dye fluorescence were performed before sequencing. The purified products were then sequenced using the Illumina MiSeq platform.

2.7 Data Analysis

This study used Origin software to analyze the changes in NH_3, SCOD, and EPS during the aerobic digestion process, as well as the abundance of ARGs and MGEs. The relationships between environmental factors, pollutants, ARGs, and MGEs were investigated using Meiji platform and R4.4.1 software. Spearman correlation analysis was performed to assess the correlations between these factors. The changes in microbial communities at the phylum and genus levels during FNA pretreatment and aerobic digestion were also analyzed. Pearson correlation analysis was used to evaluate the relationships between ARGs and MGEs, as well as between bacterial communities, ARGs, and MGEs.

3 MATERIALS AND METHODS

3.1 Changes in Pollutant Indicators, EPS and ARGs

3.1.1 NH_3 and SCOD

As shown in Figure 1(A), aerobic digestion led to the release of NH_3. High-temperature reactors, such as E3, E6, and E9 (45°C), exhibited increases in NH_3 by 14.08, 17.41, and 25.86 times, respectively, compared to day 0, indicating a significant release of NH_3. This suggests that high temperature promotes NH_3 release, and the strong positive correlation between high temperature and NH_3 release is confirmed in Figure 1(F). However, after 12 days of aerobic digestion, the release rate slowed down. High temperature can inhibit nitrification and denitrification, and cell disruption can produce nitrogen in the form of ammonia [4]. Proteins are hydrolyzed into amino acids, which are then converted into NH_3. Therefore, NH_3 release can indirectly reflect microbial cell death. After 12 days, substrate depletion, reduced microbial metabolic activity, weakened endogenous respiration, and the inhibitory effect of high concentrations of NH_3 on microbial activity occurred.

Furthermore, in reactors E4, E5, E7, and E8, NH_3 showed a decreasing trend over time, suggesting that low FNA-treated sludge content and non-alkaline, low-temperature conditions are unfavorable for microbial cell lysis. Conversely, microbial cell disruption aids in the release of organic matter from the sludge into the waste liquid, resulting in an increase in SCOD, as shown in Figure 1(B). Among these, reactors E2 and E6 (pH = 10) exhibited higher SCOD release than the others, indicating that alkalinity facilitates SCOD release. A significant positive correlation between pH and SCOD ($r = 0.59$, $P < 0.01$) is shown in Figure 1(F). However, E7 showed lower SCOD release, which was inhibited by temperature, suggesting that high temperature promotes SCOD growth, a relationship also confirmed by the mental test (Figure 1(F)). Moreover, reactor E6, under high temperature and high alkalinity, displayed the highest SCOD release, consistent with previous research, which concluded that high temperature and strong alkalinity promote SCOD increase during the later stages of aerobic digestion [5].

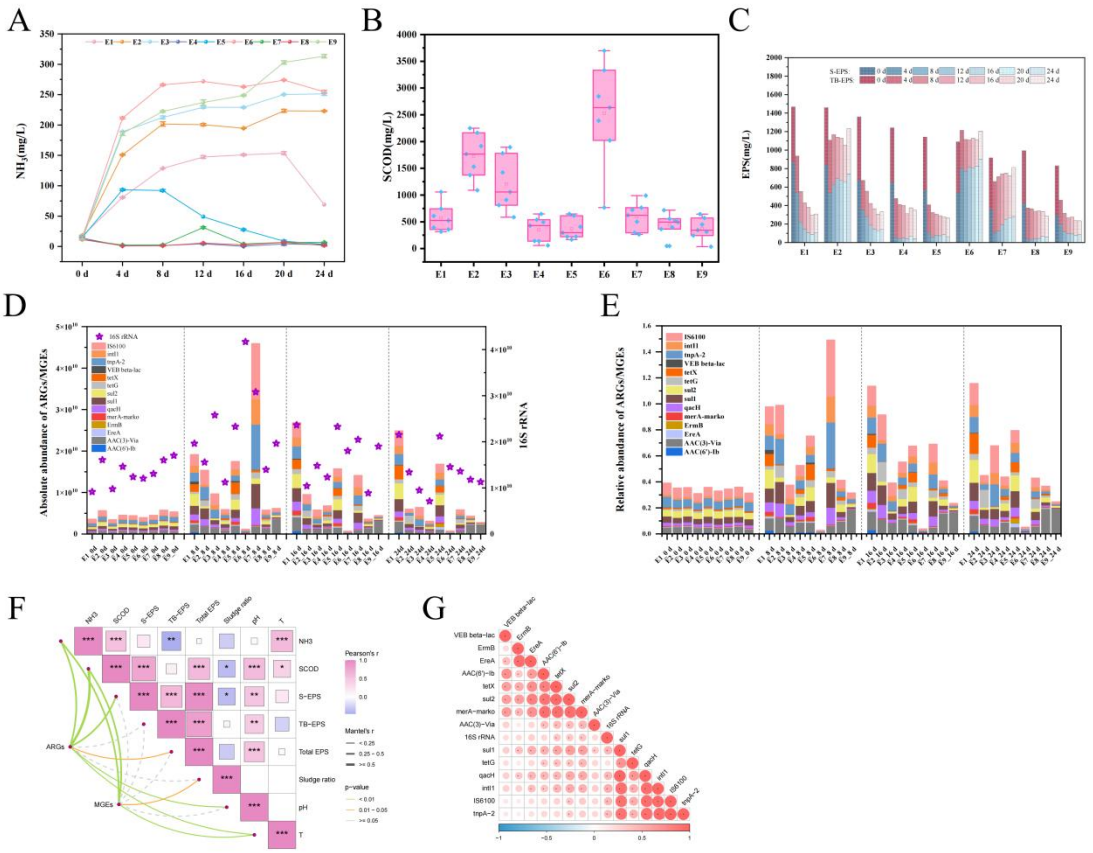

Figure 1 Variations of NH_3(A), SCOD (B), EPS (C), Absolute Abundance of ARGs/MGEs (D), and Relative Abundance of ARGs/MGEs (E) during Aerobic Digestion in Reactors E1–E9. Correlation between ARGs/MGEs and Environmental Factors, NH_3, SCOD, and EPS(F) . Correlation between ARGs and MGEs(G)

3.1.2 Variation of EPS

Figure 1(C) shows the changes in Total EPS during the aerobic digestion process, including the concentrations of S-EPS and TB-EPS. In the initial stage, as the proportion of FNA-pretreated sludge decreased (E1/2/3 sludge ratio = 1; E4/5/6 sludge ratio = 2; E7/8/9 sludge ratio = 3), the S-EPS concentration gradually decreased. This phenomenon suggests that FNA pretreatment helps promote cell rupture, thereby releasing more EPS, which results in an increase in S-EPS concentration. Compared to other reactors, E2/6/7 (pH 10) exhibited consistently higher Total EPS, but analysis of Total EPS at 24 days revealed the following trend: E6 (45°C) > E2 (35°C) > E7 (25°C). Under pH 10 conditions, as the temperature increased, the S-EPS/TB-EPS ratio also increased. Figure 1(F) shows a significant positive correlation between pH and S-EPS, indicating that an alkaline environment (pH 10) facilitates microbial cell rupture and metabolism during aerobic digestion, and high temperature enhances this effect[6]. Except for E6, all other reactors exhibited a decrease in TB-EPS and Total EPS at 24 days. This is because under aerobic conditions, ammonium is generated from the degradation of bacterial cells and their EPS, and with the help of ammonia-oxidizing bacteria (AOB), it is converted into nitrite, which is eventually oxidized to nitrate by nitrite-oxidizing bacteria (NOB)[7], The rate of Total EPS decrease was faster after the first 12 days, which may be related to substrate sufficiency and strong microbial activity in the earlier stages.

3.1.3 Variation of ARGs

Figures 1(D and E) show the trends in the absolute abundance (AA) and relative abundance (RA) of ARGs and MRGs, respectively. During the aerobic digestion process, except for reactor E6, all other reactors exhibited a trend of enrichment followed by removal of ARGs and MRGs. This suggests that the intracellular and extracellular substances released during the pretreatment phase were utilized in the early stage, leading to microbial proliferation. The initial bacterial regrowth is the main reason for the increase in ARGs during the maturation phase [8]. As digestion progressed, in high-temperature environments (E3, E6, and E9 at 45°C), the AA of Total ARGs decreased in all cases. For example, at the end of digestion, the RA of Ermb, tetX, and VEB beta-lactam were almost undetectable, indicating that high-temperature environments not only reduce the AA of ARGs but also reduce the RA of target genes [2]. Notably, E6 exhibited the most significant reduction, with ARGs and MGEs decreasing by 84.13% and 76.03%, respectively. Additionally, when comparing SCOD values, E6 showed significantly higher SCOD than other reactors, suggesting that cell lysis inhibited the transfer of resistance genes and that high alkalinity and high temperature conditions promoted the reduction of ARGs.

Furthermore, a Mantel test correlation analysis was performed on the relationships between resistance genes, environmental factors, and pollutant indicators. The results revealed that both ARGs and MGEs were significantly positively correlated with S-EPS and Total EPS. As reported by Sheng et al. [9], EPS can significantly affect the surface

interactions of microbial cells, such as surface charge, mass transfer, and hydrophilicity/hydrophobicity. These properties influence the interaction between microbial cells and extracellular ARGs, as well as the adsorption of extracellular ARGs by microbial cells, thereby altering the transformation capacity of extracellular ARGs [10] .
SCOD showed a significant positive correlation with S-EPS, indicating that cell lysis not only increased SCOD but also contributed to an increase in S-EPS, which further promoted Total EPS growth. pH exhibited a significant negative correlation with SCOD and EPS reduction, which can be attributed to the continuous cell lysis in an alkaline environment, leading to the sustained increase in EPS and SCOD. According to the range analysis (Table 2), the factors affecting the removal efficiency of resistance genes were ranked as follows: pH > Temperature > Sludge ratio. In the orthogonal experiment, only E6 (sludge ratio = 2; pH = 10; 45°C) demonstrated the highest ARGs reduction rate, due to the strong alkaline and high-temperature environment. Since ARGs are closely related to microbial community composition, further investigation of the microbial community is necessary.

3.2 Microbial Communities

3.2.1 Differences in microbial communities before and after FNA pretreatment and aerobic digestion

The microbial communities of FNA pretreatment and the FNA-based aerobic digestion process exhibited significant differences. The FNA pretreatment stage led to a notable reduction in microbial diversity in the sludge, as evidenced by the Shannon and Simpson indices (Figure 2). During the aerobic digestion phase, both microbial diversity (Shannon and Simpson indices) and richness (Chao 1 and Ace indices) decreased. Additionally, in our study, the diversity index (Shannon index) significantly decreased from 4.78 to 4.05 at the end of the digestion process. The goods coverage index indicated that the major bacterial community types present in each sample were thoroughly explored in this study[2].

Table 2 Range Analysis of ARGs Absolute Abundance Reduction by Aerobic Digestion for 24 days

Number	Sludge ratio	temperature	pH	ARGs cut amount (%)
E1	1:1	25℃	6	-787.20
E2	1:1	35℃	10	-40.88
E3	1:1	45℃	8	0.47
E4	2:1	25℃	8	24.04
E5	2:1	35℃	6	-351.32
E6	2:1	45℃	10	84.13
E7	3:1	25℃	10	-44.34
E8	3:1	35℃	8	-6.22
E9	3:1	45℃	6	15.99
K1	-827.61	-807.52	-1122.53	
K2	-243.15	-398.42	-1.09	
K3	-34.58	100.59	18.8	
R	264.34	302.70	380.28	
The best level of factors	3	45℃	8	
Primary and secondary order of factors	PH> Temperature> Sludge ratio			

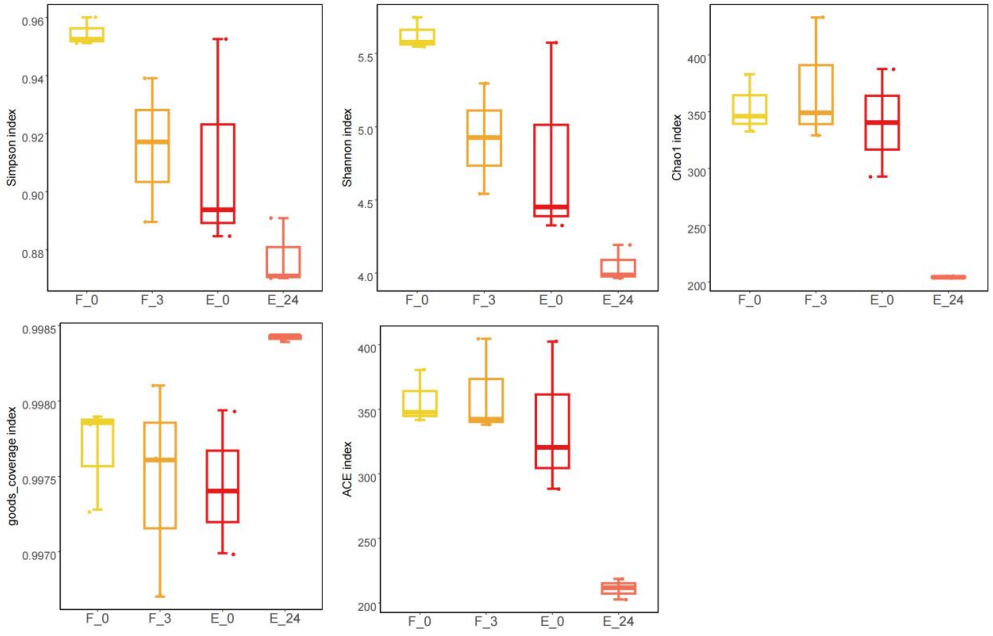

Figure 2 Community Structure Diversity Analysis Before and After FNA-Aerobic Digestion

Before the operation, as shown in Figure 3(A), the relative abundance of the top five dominant phyla were 82.43% and 88%, respectively. At the end of the operation, these values increased to 89.88% and 97.95%, respectively. A comparison reveals that FNA pretreatment and aerobic digestion promoted the accumulation of Actinobacteria and Firmicutes. Previous studies have also reported higher relative abundances of Actinobacteria and Firmicutes during aerobic digestion[11]. During the aerobic digestion process, the relative abundance (RA) of Proteobacteria, Bacteroidetes, and Verrucomicrobia decreased. However, Actinobacteria, as one of the most abundant phyla in the sludge, maintained a stable abundance range of 23.97%–25.09% at both the end of FNA pretreatment and aerobic digestion, indicating its relative stability throughout the pretreatment and digestion phases. After FNA pretreatment, the relative abundance of Chloroflexi (anaerobic bacteria) increased by 10%, possibly because FNA provided nitrate and nitrite as electron acceptors for facultative anaerobes, promoting their metabolic activity. After aerobic digestion, the relative abundance of most dominant phyla decreased, but the growth of Firmicutes (particularly the Bacillales class) was the most significant, reaching 41.69%. This phylum predominantly grows under thermophilic conditions and is associated with antibiotic resistance.[12]

Based on this, a more detailed investigation of the microbial composition at the genus level during aerobic digestion was conducted, followed by Linear Discriminant Analysis (LDA), as shown in Figure 3(C). The aerobic digestion process identified 15 differential microbes, among which 12 genera were included in the dominant microbial communities. The genera Nakamurella and Bacillus exhibited the greatest increases. Nakamurella (increased by 2.24 times) is associated with antibiotic resistance, while Bacillus, a spore-forming bacterium, showed the most significant growth, increasing by 14.19 times, indicating its potential to thrive in extreme environments. At the same time, the relative abundance of Saccharimonadales, SC-I-84, Ellin6067, unclassified_Blastocatellaceae, and Nitrospira decreased. Saccharimonadales is involved in carbon source degradation, SC-I-84 is associated with organic matter degradation, and Nitrospira and Ellin6067 are known for their excellent denitrification capabilities[13]. The unclassified_Blastocatellaceae genus can degrade complex protein compounds, while TM7a, as a symbiont, affects the relative abundance of its host by inhibiting host growth dynamics or directly killing the host. As aerobic digestion progressed, the establishment of a characteristic microbial community in the aerobic digestion tank was observed.

3.2.2 Correlation between ARGs and community structure

Increasing evidence suggests that ARGs and MRGs profiles are significantly correlated with bacterial communities, particularly in the context of wastewater sludge treatment[14-15]. Therefore, Pearson correlation analysis was conducted to assess the relationship between ARGs/MGEs and dominant bacterial genera. The results revealed that 10 out of the 11 ARGs in this study were significantly positively correlated with 10 dominant genera, and 8 out of the 3 MGEs were significantly positively correlated with 8 dominant genera ($p < 0.05$, $r > 0.6$), Figure 3 (D).The common host genera for both MGE and ARGs (8 genera) included *Saccharimonadales, Caldilineaceae, SC-I-84, Ellin6067, unclassified_Blastocatellaceae, Nitrospira, Gemmatimonadaceae,* and *OLB12*. These genera, as hosts for resistance genes, all showed a decrease in relative abundance, as shown in Figure 3(B). The abundance of MGEs was closely related to the abundance of transferred ARGs (Figure 1(G)), suggesting that controlling the abundance of common host bacteria and MGEs could be an effective strategy for controlling the spread of ARGs[16].

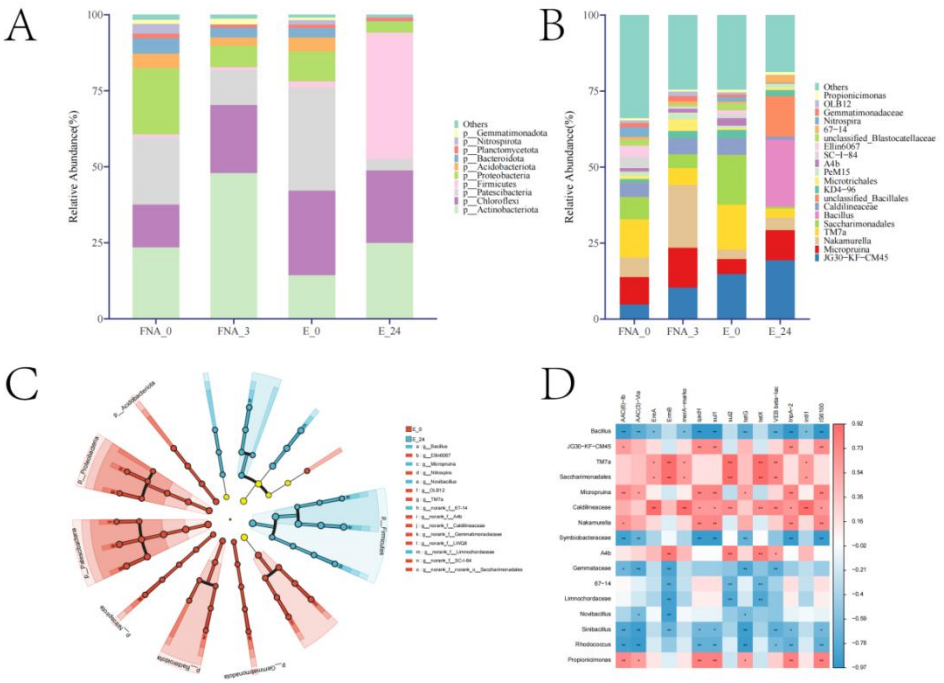

Figure 3 Microbial Community Abundance at the Phylum Level (A)the Genus Level by top 20 (B).LDA Differential Analysis of Microbial Community Composition(C). Heatmap of the Correlation Between ARGs/MGEs and Microbial Community Structure(D)

4 CONCLUSION

This study systematically explored the dynamics of ARGs during the sludge treatment process with FNA pre-treatment combined with aerobic digestion through orthogonal experimental design. The experimental results showed that under the optimal conditions (a sludge ratio of 2:1 for residual sludge to FNA pre-treated sludge, temperature of 45℃, and pH of 10), the removal efficiencies of ARGs and MGEs were significant, with the removal rate of ARGs reaching 84.13% and the removal rate of MGEs at 76.03%. Additionally, the release levels of NH_3, SCOD, and EPS under these conditions were the highest, further demonstrating that the combination of FNA pre-treatment and aerobic digestion significantly enhanced sludge digestion performance. Range analysis revealed that the influence of pH, temperature, and sludge ratio on ARG removal followed the order: pH > temperature > sludge ratio, indicating that high temperature and alkaline conditions facilitate cell disruption, which aids in the removal of ARGs. Further microbial community structure analysis indicated that the reduction of ARGs was closely correlated with the simultaneous reduction of MGEs and potential host bacteria, suggesting that high temperature and alkaline aerobic digestion environments not only remove resistance genes but may also inhibit their transmission. The findings of this study provide new insights into the application of FNA pre-treatment combined with aerobic digestion in sludge treatment, demonstrating that this approach effectively removes resistance genes from sludge and regulates microbial community structure. By properly controlling environmental factors (such as pH, temperature, and sludge ratio), the ARG removal process can be significantly optimized. Future research could further investigate the impact of different types of sludge, FNA concentrations, and directed microbial community regulation on ARG removal, providing deeper theoretical support and technical guidance for ARG control and sludge digestion efficiency enhancement in wastewater treatment.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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Appendix

Table S- 1 PH and NO2-N Concentration Gradient Experiments and the Corresponding Reduction Rates of Total ARGs

Gradient experiments	project	T (℃)	pH	NO_2^--N（mg/L)	Total ARGs reduction rate（%）
pH gradient	CK1	25	7.3	0	-20.70
	A1	25	5.3	700	53.67
	A2	25	5.5	700	-107.81
	A3	25	5.8	700	-157.56
	A4	25	6.1	700	41.96
	A5	25	6.4	700	48.60
NO2-N concentration gradient	CK2	25	5.3	0	-6.37
	B2	25	5.3	69	-1.42
	B3	25	5.3	144	-17.56
	B4	25	5.3	294	15.24
	B5	25	5.3	445	15.23
	B6	25	5.3	589	52.48
	B7	25	5.3	753	62.93