

Heavy Metal Passivation and Microbial Community Succession during Anoxic-Aerobic Digestion of Municipal Sludge

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Abstract: Although anoxic-aerobic digestion has garnered widespread attention as an emerging sludge treatment technology, the mechanisms governing microbial community succession and heavy metal passivation remain incompletely elucidated. In this study, a sequencing batch reactor (SBR) was operated under alternating anoxic-aerobic conditions (dissolved oxygen = 4 mg/L; sludge retention time = 30 days) to systematically investigate these underlying mechanisms. The alternating environment effectively promoted the migration of heavy metals from the liquid to the solid phase, thereby achieving successful passivation. The liquid-phase zinc (Zn) concentration peaked at 5.76 mg/L before subsequently decreasing, whereas the solid-phase Zn concentration remained stable, reaching a maximum of 57.43 mg/g. Highly toxic cadmium (Cd) was not detected at any point during the operation. Furthermore, the system demonstrated significant nutrient recovery potential, as liquid-phase phosphate accumulated to 2902.81 µg/L. High-throughput sequencing revealed that the alternating redox environment fundamentally reshaped the microbial community structure. Although overall microbial diversity decreased, the functional phyla Chloroflexi (34.08%) and Proteobacteria (19.50%) maintained their dominance. Additionally, the abundance of pathogenic genera, including *Acinetobacter*, was markedly reduced. These specific microbial successions were intrinsically driven by the anoxic-aerobic digestion process and exhibited a strong correlation with heavy metal passivation. Ultimately, these findings offer a sustainable and effective approach for the safe disposal and resource utilization of municipal sludge.

Keywords: Municipal Sludge; Anoxic-Aerobic Digestion; Heavy Metal Passivation; Nitrogen and Phosphorus; Microflora

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1. Introduction

As economic development accelerates, rapid urbanization has driven the continuous expansion of sewage treatment facilities and processing capacities. Consequently, the production of residual sludge from wastewater treatment processes has risen consistently. Current estimates indicate that, assuming an 80% moisture content, the annual production of residual sludge ranges between 35 and 45 million tons. This figure is projected to escalate to 90 million tons by 2025, underscoring the severe challenges associated with sludge treatment and disposal. ^[1,2]

Presently, a substantial disparity exists between sludge generation and available treatment capabilities. Statistics reveal

that less than 20% of sludge undergoes safe, standardized treatment, leaving vast amounts of energy-rich residual sludge underutilized. With evolving environmental philosophies, the governance paradigm is shifting from a water-centric approach to one that places equal emphasis on both sludge and water management. Developing efficient treatment technologies that achieve volume reduction, resource recovery, detoxification, and stabilization has therefore become an urgent priority.^[3,4]

Aerobic composting is currently a traditional technology for the resource utilization of municipal sludge, converting organic components into humus through microbial action while retaining nutrients such as nitrogen and phosphorus^[5,6]. However, this technology has inherent limitations, including a large footprint, the generation of odorous gases, high operating costs, and complex management requirements^[7]. In contrast, the anoxic-aerobic digestion process, as an emerging treatment strategy, demonstrates significant advantages: its intermittent oxygen supply mode effectively reduces aeration energy consumption; the alternating redox environment inhibits ammonia volatilization and odor generation while favoring phosphorus retention; and the digested products can be utilized as soil conditioners for land application, offering broad agricultural application prospects^[8].

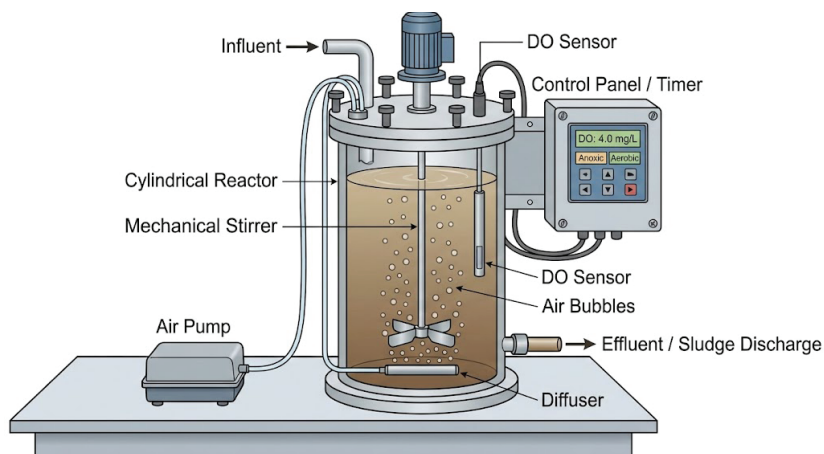
Although the effectiveness of anoxic-aerobic digestion in sludge reduction and stabilization has been preliminarily validated, existing studies have largely focused on it as a volume-reduction method. There is a distinct lack of systematic quantitative assessment regarding the correlations between microbial community shifts and the migration behaviors of heavy metals during this process^[9-12]. In particular, optimizing sludge stabilization through microenvironmental regulation and facilitating heavy metal passivation, rather than treating it merely as a fermentation byproduct, remains a gap in current research. To address this, this study established a sequencing batch reactor (SBR) simulating alternating anoxic-aerobic conditions ($\text{DO} = 4 \text{ mg}\cdot\text{L}^{-1}$, $\text{SRT} = 30 \text{ days}$) to investigate the roles of functional microbial groups in heavy metal passivation and nutrient transformation. By integrating inductively coupled plasma mass spectrometry (ICP-MS), UV spectrophotometry, and 16S rRNA high-throughput sequencing technologies, this study systematically analyzed the migration and distribution of heavy metals, nitrogen and phosphorus metabolic pathways, and the functional succession of microbial communities in both liquid and solid phases^[13,14]. Unlike traditional qualitative descriptions, this research aims to establish a quantitative link between “process parameters, microbial communities, and heavy metal distribution” through multi-dimensional data analysis.

2. Materials and Methods

2.1 Materials and Experimental Setup

The sludge samples used in this study were obtained from the secondary sedimentation tank of a wastewater treatment plant (A/A/O process) at Xi'an Polytechnic University (Xi'an, China). The sludge had a sludge retention time (SRT) of 30 days. Key chemical reagents, including hydrochloric acid, nitric acid, and hydrofluoric acid, were of analytical grade and purchased from Xilong Scientific Co., Ltd. (Chengdu, China). The experiment was conducted in a sequencing batch reactor (SBR) with an effective working volume of 1 L (Figure 1). The reactor operated under alternating anoxic-aerobic conditions with a hydraulic retention time (HRT) ratio of 1:1. The operational cycle was set to 6 h, with the temperature maintained at 25°C. During the aerobic phase, dissolved oxygen (DO) was maintained at 4 mg·L⁻¹ using an aeration pump. The total SRT was controlled at 30 days.

Figure 1. Schematic diagram of the sequencing batch reactor (SBR) system used for anoxic-aerobic sludge digestion



2.2 Chemical Analysis and Quantification Methods

Sludge samples were collected every three days and separated into liquid and solid phases via centrifugation (4000 rpm, 10 min) for subsequent analysis. For heavy metal quantification, heavy metals (Cr, Cu, Zn, Cd, Pb) in the liquid phase were digested using an adjustable electric heating plate (Model ML-2.2-4, Kewei Yongxing, Beijing, China) following the standard method GB 7475-87. Solid phase samples were digested using the aqua regia-perchloric acid method according to standard HJ 491-2019. The concentrations of heavy metals in the digests were quantified using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7900X, Agilent Technologies, Santa Clara, CA, USA).

Additionally, nitrogen and phosphorus species were analyzed according to standard protocols. Specifically, ammonia nitrogen (NH_4^+-N) was quantified using Nessler's reagent spectrophotometry. Nitrite nitrogen (NO_2--N) was measured using the N-(1-naphthyl)-ethylenediamine dihydrochloride spectrophotometric method. Nitrate nitrogen (NO_3--N) was analyzed via ultraviolet spectrophotometry, and phosphate ($\text{PO}_4^{3--}\text{P}$) was determined using the molybdenum-antimony anti-spectrophotometric method. For solid samples, Total Nitrogen (TN) was measured using alkaline potassium persulfate digestion followed by UV spectrophotometry. Total Phosphorus (TP) was quantified using the potassium persulfate digestion-molybdenum antimony anti-spectrophotometric method. All absorbance measurements were performed using a UV-Vis spectrophotometer (T6-1650E, Puxi General Instrument Co., Ltd., Beijing, China).

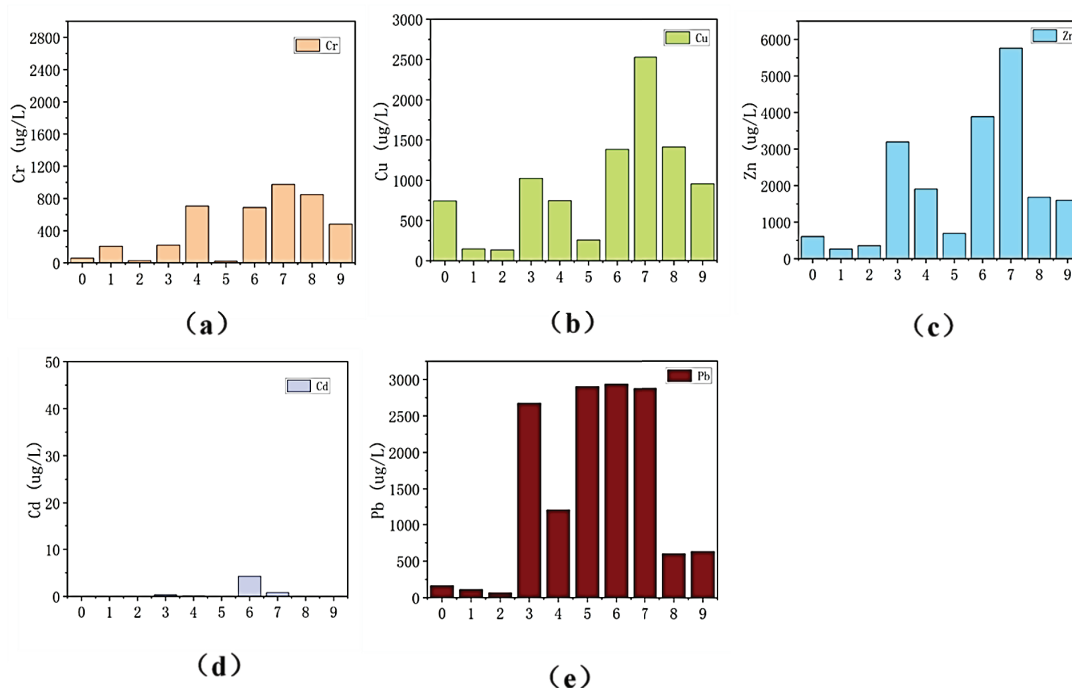
2.3 Microbial Community Analysis

Microbial species and their abundance were identified using 16S rRNA high-throughput sequencing. Sludge samples were collected before the reaction cycle (A0) and after the cycle (A9). DNA was extracted, and the V3–V4 region of the 16S rRNA gene was amplified. For bioinformatics analysis, raw sequencing data were processed to obtain valid sequences. Operational Taxonomic Units (OTUs) were clustered with a 97% similarity cutoff using UPARSE software (version 7.1), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier against the Silva 16S rRNA database (v138) using a confidence threshold of 0.7. Alpha diversity indices (Chao, ACE, Shannon, Simpson, and Coverage) were calculated using MOTHUR to evaluate species richness and diversity.

3. Results

3.1 Heavy Metal Migration and Distribution

Figure 2. Heavy metal content in liquid sludge (a) Cr, (b) Cu, (c) Zn, (d) Cd, (e) Pb



Significant dynamic fluctuations were observed in liquid-phase heavy metal concentrations (Table 1, Figure 2)^[15,16]. Initially, concentrations remained low, but a sharp release was observed mid-cycle. Specifically, Zn concentrations surged to a

peak of 5760.44 $\mu\text{g}\cdot\text{L}^{-1}$ on the 6th sampling day, followed by a substantial decline in the final stages. This surge in liquid-phase Zn coincided with a temporary decrease in solid-phase Zn (Figure 3c), confirming the solubilization of metal-organic complexes. In contrast, Cd levels remained negligible throughout the process. In the solid phase (Table 2, Figure 3), heavy metal concentrations remained relatively stable compared to the liquid phase. Zn was the predominant element, peaking at 57.43 $\text{mg}\cdot\text{g}^{-1}$. Notably, the concentrations of Cr, Cu, and Zn in the solid phase showed a slight downward trend after the 6th sampling but stabilized towards the end of the cycle [17,18].

Figure 3. Heavy metal content in solid sludge (a) Cr, (b) Cu, (c) Zn, (d) Cd, (e) Pb

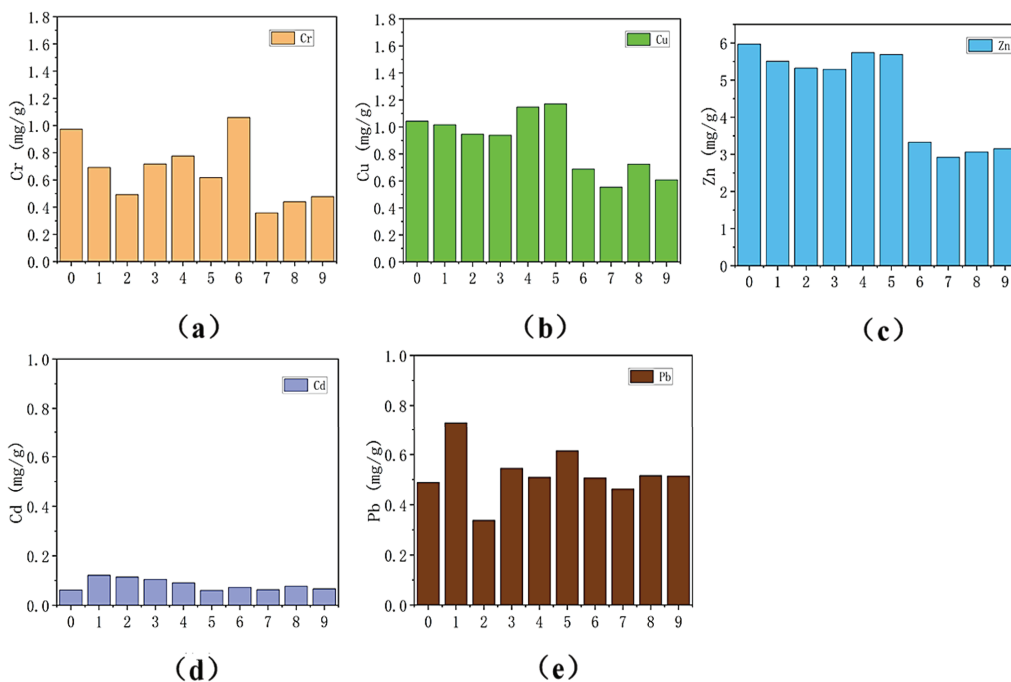


Table 1. Heavy metal content ($\text{mg}\cdot\text{L}^{-1}$) in liquid sludge

Concentration	Cr	Cu	Zn	Cd	Pb
0	57.75	742.4	606.09	0	164.72
1	206.59	147.11	265.94	0	111.27
2	27.64	134.24	356.21	0	66.79
3	219.07	1022.93	3195.56	0.34	2675.67
4	706.11	746.06	1905.42	0.09	1211.17
5	20.87	256.26	691.45	0	2902.81
6	687.7	1382.8	3886.32	4.28	2936.11
7	973.59	2526.4	5760.44	0.81	2875.87
8	847.72	1411.27	1680.97	0	605.83
9	479.92	954.69	1600.88	0	638.32

Table 2. Heavy metal content ($\text{mg}\cdot\text{L}^{-1}$) in solid sludge

Concentration	Cr	Cu	Zn	Cd	Pb
0	3.891	4.173	23.888	0.247	0.165
1	4.152	6.098	33.039	0.727	0.111
2	4.919	9.456	53.249	1.141	0.067
3	7.169	9.393	52.896	1.048	2.676

Concentration	Cr	Cu	Zn	Cd	Pb
4	7.764	11.481	57.426	0.904	1.211
5	6.170	11.716	56.908	0.602	2.903
6	10.589	6.878	33.298	0.726	2.936
7	3.579	5.538	29.215	0.632	2.876
8	4.390	7.237	30.638	0.770	0.606
9	4.766	6.078	31.505	0.664	0.638

3.2 Nitrogen and Phosphorus Transformation

The variations of nitrogen and phosphorus in the liquid phase are illustrated in Figure 4. Phosphate concentrations were remarkably higher than those of nitrogen species, peaking at 2902.81 $\mu\text{g}\cdot\text{L}^{-1}$ on the 18th day (6th sampling) before declining [19,20]. Ammonia nitrogen and nitrate nitrogen also showed accumulation trends, whereas nitrite nitrogen remained at low levels. In the solid phase, Total Nitrogen (TN) (Figure 5a) initially decreased and then stabilized, reaching its lowest point on the 9th day. Total Phosphorus (TP) (Figure 5b) exhibited a trend similar to the liquid phase, increasing initially to a peak in the early stages (2nd sampling) and then gradually decreasing [21,22].

Figure 4. Nitrogen and phosphorus content in liquid sludge (a) nitrite nitrogen, (b) ammonia nitrogen, (c) phosphate, (d) nitrate nitrogen

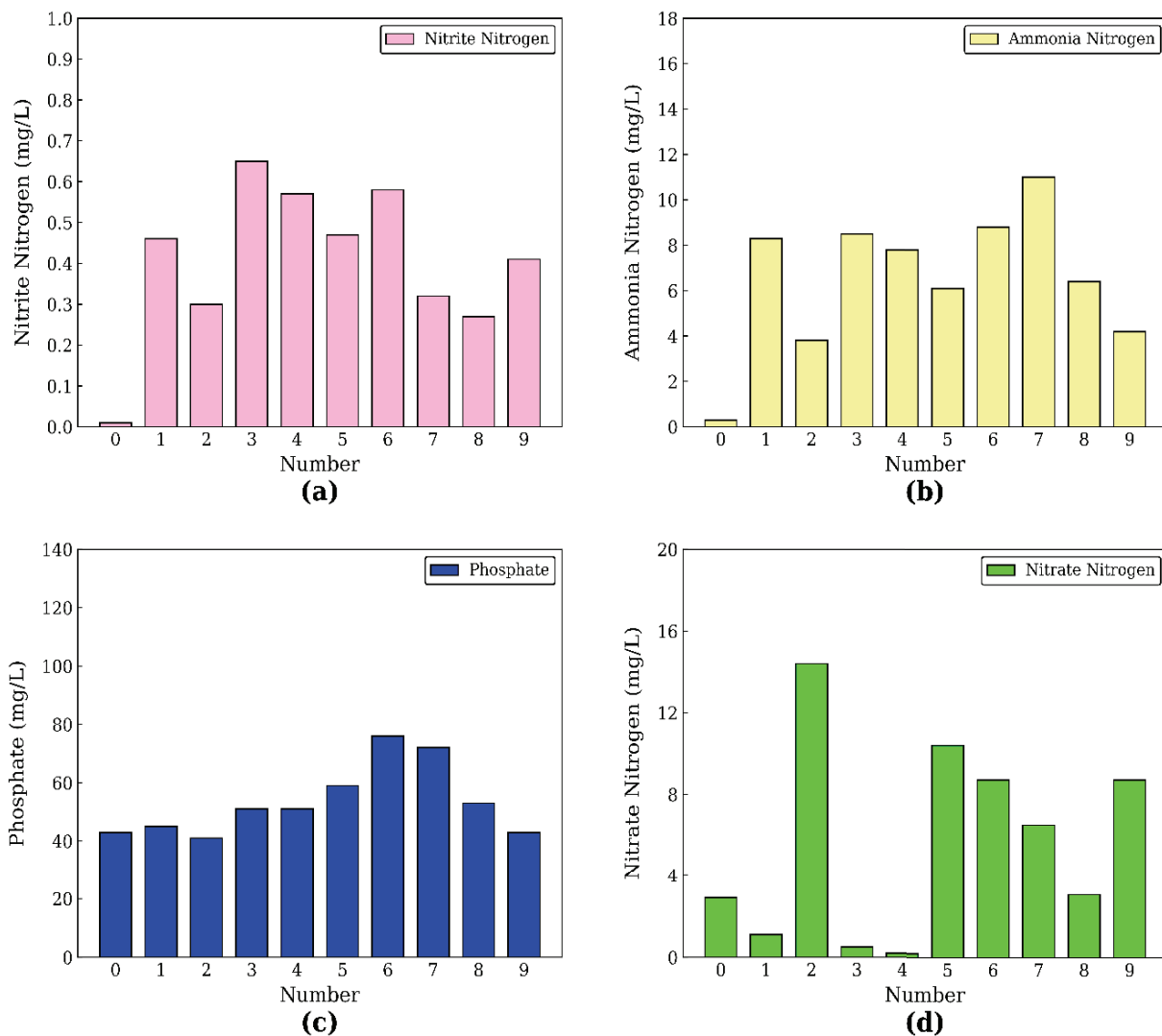
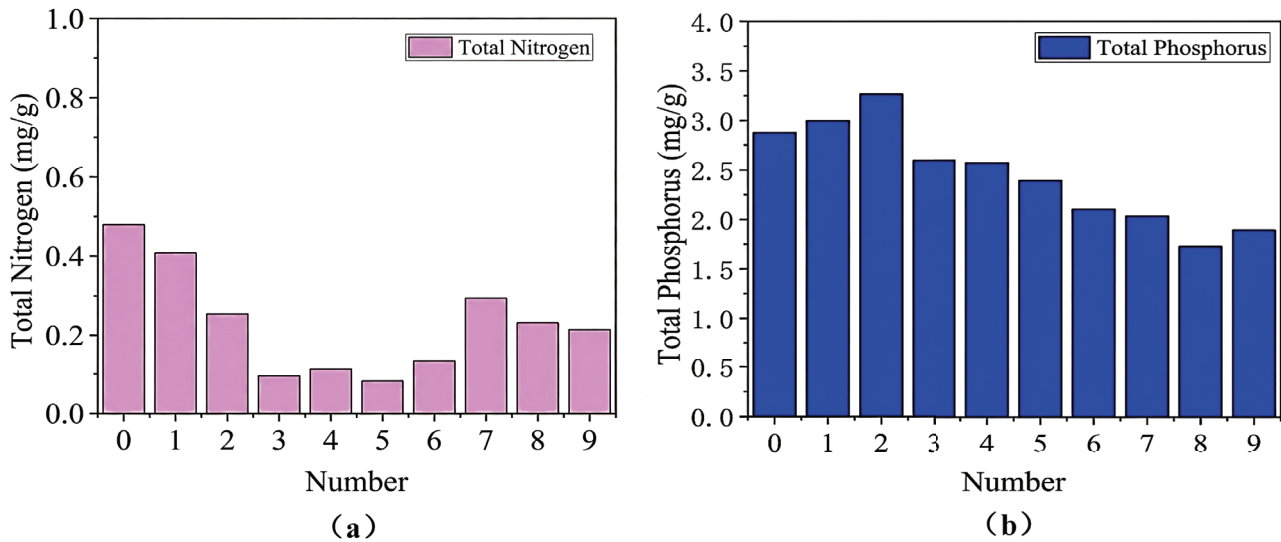


Figure 5. Total nitrogen content in solid sludge and Total phosphorus content in solid sludge



3.3 Microbial Community Diversity, Succession, and Association Analysis

The high-throughput sequencing yielded 49,252 valid sequences per sample. As shown in the Pan and Core OTU analyses (Figure 6), the total species richness increased with sample accumulation, while the number of core OTUs shared between A0 and A9 decreased by approximately one-third, indicating a turnover of microbial populations to adapt to the alternating environment. The adequacy of sequencing depth was confirmed by the rarefaction curves (Figure 7), which reached a clear plateau for both the Sobs and Shannon indices, indicating that the majority of microbial phylotypes were captured. Alpha diversity indices (Figure 8) further quantified the shifts in community complexity. The Goods coverage for all samples exceeded 0.99, reinforcing the reliability of the sequencing data^[23,24]. While the richness indices (Chao and ACE) showed no statistically significant differences between A0 and A9, a notable decline was observed in the diversity indices (Shannon and Simpson). This reduction in evenness implies that the anoxic-aerobic digestion process exerted selective pressure, leading to the dominance of specific functional microbial groups^[25,26].

Figure 6. OTU analysis of sludge samples. (a) Pan OTU curve representing total species richness; (b) Core OTU curve representing shared species

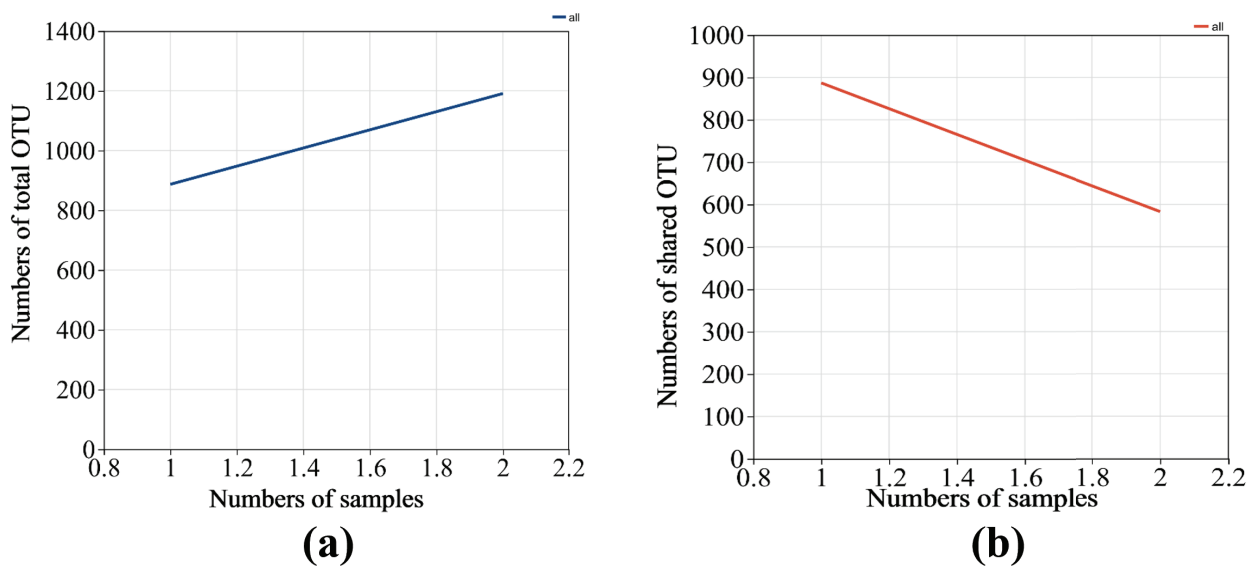


Figure 7. Rarefaction curves verifying sequencing depth suitability. (a) Sobs index; (b) Shannon index

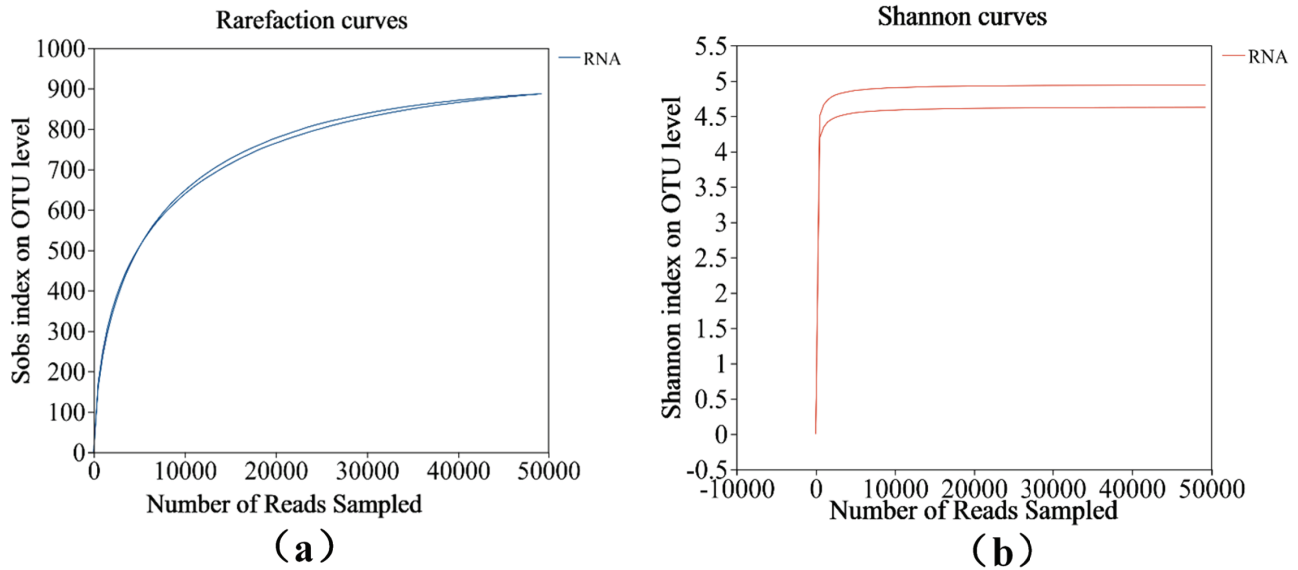
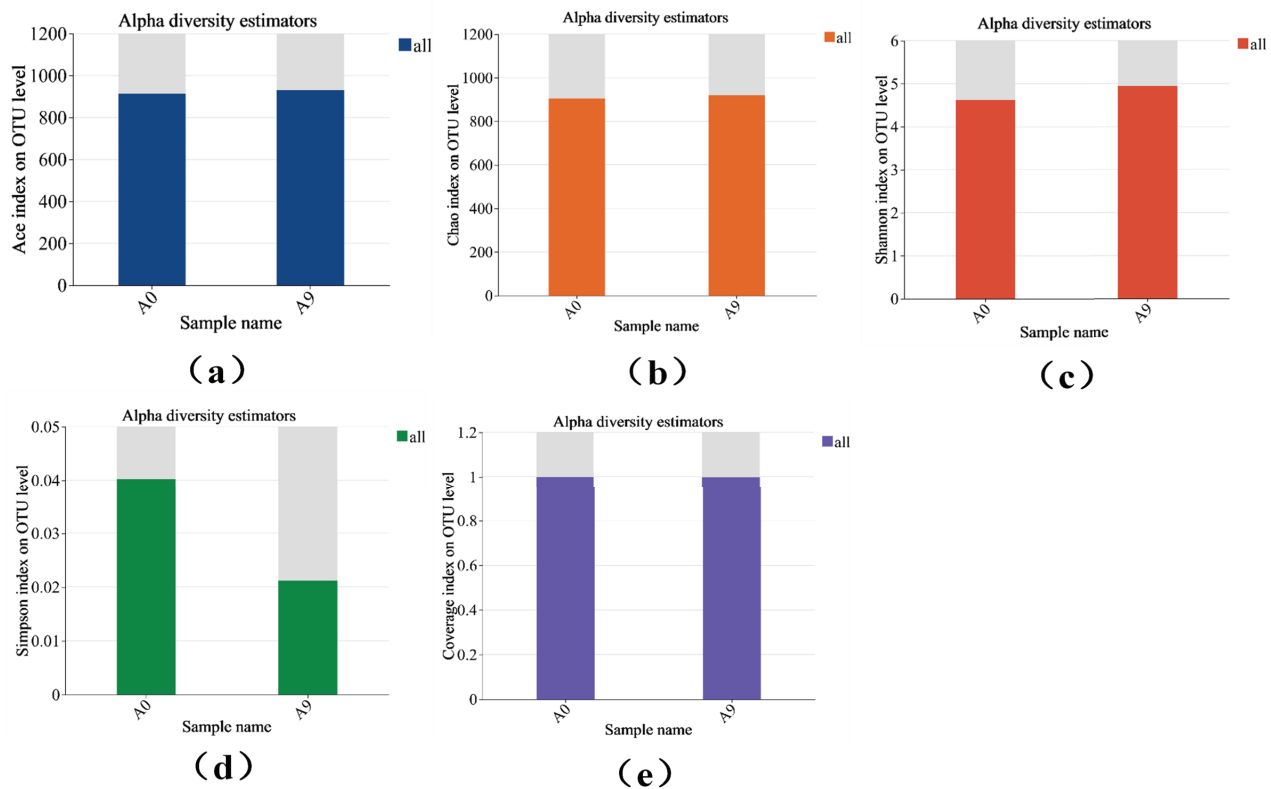


Figure 8. Alpha diversity indices: (a) Ace index, (b) Chao index, (c) Shannon index, (d) Simpson index, (e) Coverage index



The taxonomic composition at the phylum level was visualized using a community bar chart (Figure 9). Chloroflexi and Proteobacteria remained the predominant phyla throughout the process. However, the relative abundance of Chloroflexi decreased from 38% to 34%, and Proteobacteria dropped from 33% to 19% after the 30-day cycle. Conversely, Bacteroidetes and Patescibacteria exhibited an upward trend, with Bacteroidetes increasing from 9.4% to 15.6%. These shifts indicate a significant restructuring of the microbial backbone in response to the alternating redox environment [27,28]. At the genus level, the community succession and the distribution of key functional groups were further elucidated via Heatmap analysis (Figure 10). The hierarchical clustering clearly separated the initial sludge (A0) from the digested sludge (A9), indicating a fundamental shift in the dominant taxa. Specifically, the A0 sample was characterized by a high intensity of Acinetobacter (approx. 15% relative abundance), a genus often associated with opportunistic pathogens. Following the anoxic-aerobic

treatment, the signal for Acinetobacter in the A9 sample was significantly suppressed to negligible levels (<1%). Meanwhile, the heatmap revealed the concurrent enrichment of functional genera such as Dechloromonas and various members of the Saprospiraceae family, which are instrumental in nutrient cycling and organic matter stabilization [29,30].

Figure 9. Community bar chart

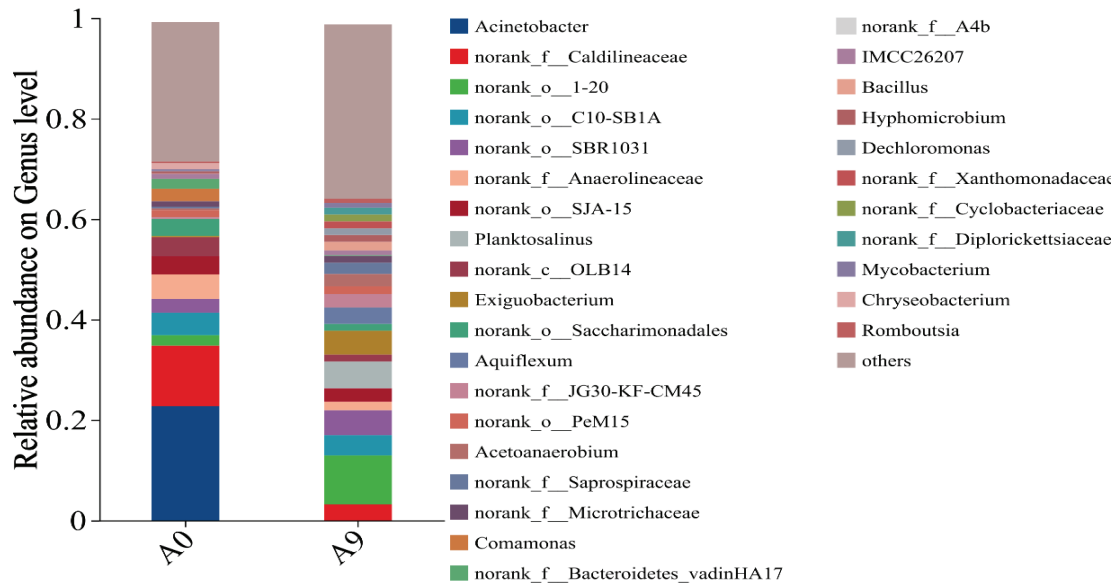
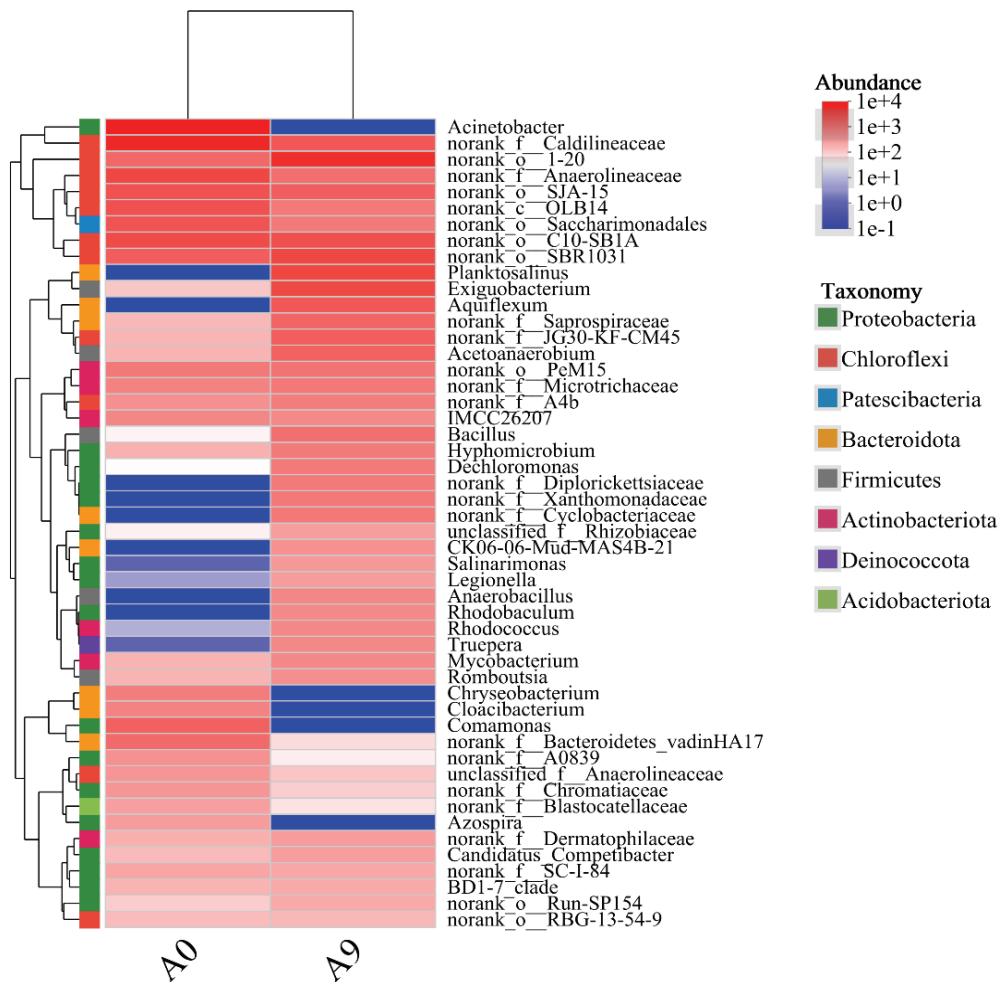
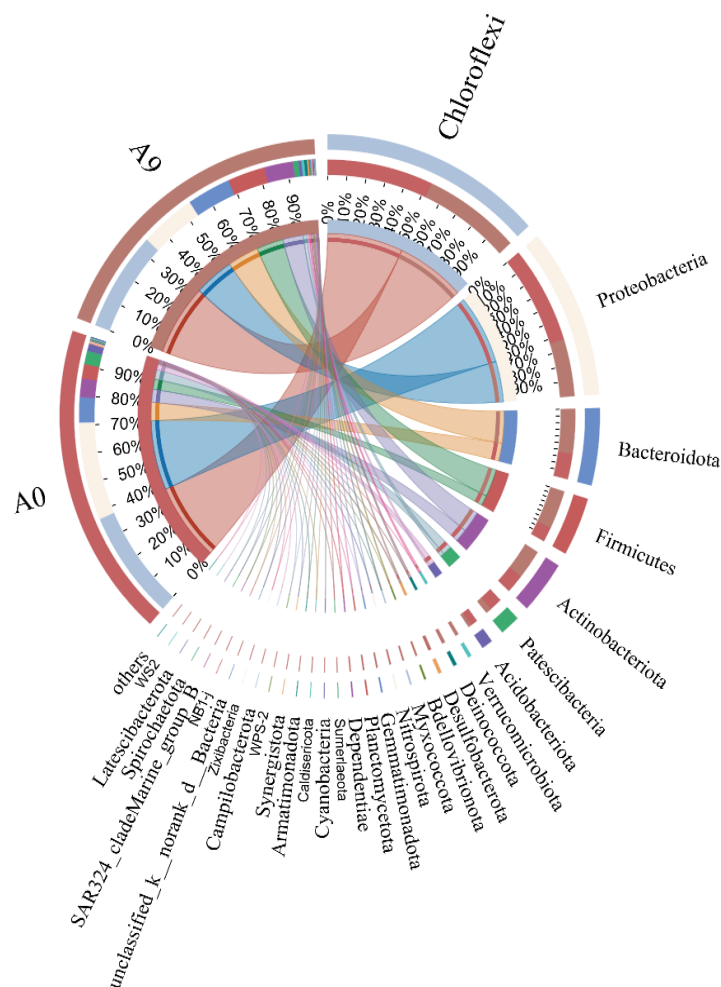


Figure 10. Genus-level heatmap showing the taxonomic succession and relative abundance of microbial communities before (A0) and after (A9) anoxic-aerobic digestion



To further elucidate the internal relationships within the microbial community, hierarchical clustering and association visualizations were performed. The heatmap analysis (Figure 10) provided a high-resolution view of the distribution patterns of the top 35 microbial genera. A clear bifurcation in the clustering tree was observed between A0 and A9, indicating that the anoxic-aerobic treatment significantly reshaped the community hierarchy at the genus level. Notably, the heatmap confirmed the drastic depletion of acinetobacter and the concurrent enrichment of functional groups such as dechloromonas and various members of the sapsprospiraceae family in the A9 sample, suggesting a coordinated response to the digestion process. The circos diagram (Figure 11) visualized the distribution of major phyla across the experimental samples and the degree of contribution from each taxon. The diagram illustrated that chloroflexi and proteobacteria maintained the dominant representation (visualized as broad ribbons) toward both A0 and A9, reinforcing their roles as the core functional backbone. Consistent with the taxonomic shifts, the ribbon thickness for Bacteroidetes widened significantly toward the A9 sample, visually confirming its proliferation. This holistic visualization integrates the sample-species relationships, demonstrating that while the core phyla remained stable, the fine-tuning of microbial proportions at the genus level was the key driver for successful sludge stabilization and sanitization.

Figure 11. Circos diagram illustrating the distribution and relative abundance of major microbial taxa across sludge samples A0 and A9. The ribbons (flows) represent the correspondence and contribution of each phylum to the respective samples, with the width of the bands indicating relative abundance



4. Discussion

4.1 Mechanisms of Heavy Metal Passivation and Potential Role of Organic Transformation

The observed “release-passivation” pattern of heavy metals in this study (Section 3.1) underscores the dynamic phase transfer driven by the digestion process. The initial surge in liquid-phase metals is attributed to the biodegradation of extracellular polymeric substances (EPS) and the hydrolysis of the organic matrix, which releases bound metal ions. However, the

subsequent decline and passivation in the solid phase are strongly linked to the stabilization of organic matter. The migration of Zn and Cu to the solid phase (Figure 3) implies the formation of stable complexes. Although the chemical structure of organic matter (e.g., humic substances) was not directly characterized in this study, the biological data offers insight into the underlying mechanism. The dominant phylum Chloroflexi, which maintained high abundance (34.08%), has been widely reported as a key player in the hydrolysis of organic matter and the synthesis of humic acid precursors^[31,32]. The enzymatic polymerization mediated by such functional microbes can generate carboxyl and phenolic groups, which act as powerful chelators for divalent cations^[33-35]. Therefore, it is inferred that the microbial succession promoted the transformation of organic substrates into stable complexes, likely humic-like substances, which immobilized the heavy metals [36]. This suggests that biologically mediated organic transformation is a crucial pathway for detoxification in this system^[37,38].

4.2 Nutrient Recovery Potential

The synchronous variations of nitrogen and phosphorus (Section 3.2) reveal migration mechanisms modulated by microbial metabolic activities under alternating redox conditions. The significant accumulation of soluble phosphate in the liquid phase (peaking at $\sim 2.9 \text{ mg}\cdot\text{L}^{-1}$) creates a highly concentrated stream ideal for resource recovery. Advanced technologies can leverage these enriched supernatants to reclaim phosphorus in the form of struvite or vivianite, thereby realizing the high-value utilization of sewage sludge^[39-41]. Furthermore, the stabilization of nitrogen in the solid residues ensures that the dewatered sludge retains nutrient value for potential land application as a soil conditioner.

4.3 Microbial Functional Succession and Pathogen Removal

The reshaping of the microbial community (Section 3.3) provides the biological basis for the observed chemical changes^[42]. The alternating anoxic-aerobic environment imposes a severe “metabolic selection pressure,” acting as a biological filter. This is evidenced by the drastic reduction of the opportunistic pathogen *Acinetobacter* (Section 3.3), likely due to its inability to adapt to the periodic anoxic stressors and competition from adapted taxa. While some *Acinetobacter* species are known Polyphosphate Accumulating Organisms (PAOs), the *Acinetobacter* detected here were predominantly associated with pathogenic groups, and their removal outweighs the potential loss in biological phosphorus removal capacity. Furthermore, the loss of biological P-removal is likely compensated by chemical precipitation and organic chelation mechanisms contributing to P retention in the solid phase. The succession favors versatile functional phyla like Chloroflexi and Proteobacteria^[8]. Chloroflexi, serving as the skeletal structure of sludge flocs, plays a pivotal role in hydrolyzing complex organic substrates. Meanwhile, specific genera within Proteobacteria (e.g., *Dechloromonas*) facilitate nutrient removal and denitrification^[43]. Moreover, the accumulated stable organic substances can act as electron shuttles to facilitate interspecies electron transfer, enhancing the overall stability of the digestion system. From a life cycle perspective, this biological selection not only improves sludge safety by degrading antibiotic resistance genes but also supports carbon footprint reduction through soil carbon sequestration when the treated sludge is applied to land^[44].

5. Conclusions

This study establishes a quantitative relationship between process parameters, microbial community succession, and heavy metal fate within an anoxic-aerobic SBR system. The results demonstrate that the proposed process effectively drives the migration of typical heavy metals, particularly Zn, from the bioavailable liquid phase to the stable solid phase, thereby achieving efficient heavy metal passivation and mitigating the environmental risks associated with sludge. Simultaneously, the substantial accumulation of liquid-phase phosphate offers a promising pathway for high-value nutrient recovery. The alternating redox environment exerts significant selective stress, which reduces overall microbial diversity but successfully suppresses opportunistic pathogenic genera such as *Acinetobacter*, thereby enhancing the biosafety of the digested sludge. These performance improvements are primarily attributed to the enrichment of functional phyla, notably Chloroflexi and Proteobacteria, which facilitate the humification of organic matter and further promote the chelation and immobilization of heavy metals. Overall, anoxic-aerobic digestion represents a highly reliable technical strategy for the integrated detoxification, stabilization, and resource recovery of municipal sludge. Future research should prioritize parameter optimization for pilot-scale operations and conduct long-term assessments regarding the agricultural and environmental risks of applying treated sludge to land.

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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