



Research article



Magnetic coconut shell biochar driven leachate activated sludge system treatment efficiency, antibiotic resistance gene response, and microbial community succession

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ABSTRACT

Explored the effects of magnetic coconut shell biochar (MCSB) with different concentrations (5 and 8 g/L) on the anaerobic/aerobic (A/O) biodegradation performance, antibiotic resistance genes (ARGs), and bacterial community of landfill leachate. The optimal concentration of MCSB (5 g/L) promoted the degradation of total organic carbon (80.01 % and 76.36 % removal rates in A/O systems), total nitrogen (65.27 % and 50.26 % removal rates in A/O systems), and tetracycline antibiotics (TCs, including tetracycline, oxytetracycline, and chlortetracycline, with removal rates of 81–88 %), while 8 g/L MCSB had a more significant degradation effect on total phosphorus. 5 g/L MCSB effectively stimulated the secretion of extracellular polymers in sludge (with concentrations reaching 449.46 and 434.23 mg/g VSS in A/O systems), while 8 g/L MCSB increased the deposition of heavy metals in sludge. It was worth noting that the 8 g/L MCSB (anaerobic reactor) reduced the relative abundance of ARGs in the leachate by 28.22 % (compared to the 5 g/L anaerobic reactor), reflected in the decrease in the abundance of *tetA*, *tetB*, *tetM*, and *tetQ*, but increased the reproductive risk of ARGs in anaerobic sludge microorganisms. TCs induction and environmental factor pressure regulated the proliferation of dominant bacterial species in microbial communities, which might alter the composition of ARGs in bacteria through a co selection mechanism. MCSB provided growth space for functional microorganisms and became a vital factor in shaping the spread of ARGs and host microbial transmission by stimulating microbial metabolism, increasing their activity, and altering the abundance of functional microorganisms. From a practical application perspective, the continuous flow A-O combination process was the preferred solution. This study proposes promising magnetic biochar material, which provides technical reference for promoting A/O biodegradation of TCs containing leachate and controlling the environmental pressure of ARGs.

1. Introduction

Tetracycline antibiotics (TCs) are the most widely used globally, mainly including tetracycline (TC), oxytetracycline (OTC), and chlorotetracycline (CTC). The three countries with high TCs consumption are Russia (13,579 tons per year), China (6,950 tons per year), and the United States (3,230 tons per year), with China accounting for 18 % of global consumption (Amangelsin et al., 2023). It was known that CTC and dissolved organic matter could affect the variability of microbial

community function (Antos et al., 2024). OTC also stimulated soil microbial activity through changes in the abundance of Gram-positive bacteria and fungi (Fang et al., 2025). The proposed results indicated that the sustained accumulation of TCs in water and soil environments led to changes in the function and structure of microorganisms (Antos et al., 2024). However, over 70 % of TCs are excreted from the body in their unmetabolized form or as active/inactive metabolites (Kweon et al., 2025), with a final portion entering municipal solid waste (MSW) landfills. During the landfill process, these pollutants will be transferred

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to the leachate, making it complex and difficult to degrade wastewater. Song et al. (2016) found three antibiotics-sulfamethoxazole (SMX), TC, and OTC in fifty-one waste samples from eight locations in a large landfill. Among them, OTC had the highest average concentration ($100.9 \pm 141.8 \mu\text{g}/\text{kg}$), followed by TC ($63.8 \pm 37.7 \mu\text{g}/\text{kg}$) and SMX ($47.9 \pm 8.1 \mu\text{g}/\text{kg}$). Besides, among the 324 antibiotic resistance genes (ARGs) detected, TC resistance genes (*tetA*, *tetB*, *tetO*, *tetM*, *tetQ*, *tetT*, and *tetW*) accounted for 5.7%–34.4% (Jia et al., 2024). The occurrence of antibiotic resistance (AR) in leachate is due to both the presence of antibiotics and environmental factors that have a significant impact on the number of mobile genetic elements (MGEs) and ARGs (Rui et al., 2025). Hence, it is crucial to fully quantify the treatment of leachate and analyze the origin and migration of ARGs.

Due to its ease of use and low cost, biological treatment has been widely used to remove organic matter, nutrients, and various micro pollutants from leachate (Kurniawan et al., 2025). Aerobic process is suitable for the treatment of young leachate, but its efficiency is low when applied to high organic load rates, which can easily generate excess sludge (Ilmasari et al., 2022). In contrast, anaerobic processes have the advantage of resource recovery through bioenergy production. However, the limiting factors are also significant, such as the low specific growth rate and sedimentation rate of anaerobic bacteria, incomplete stability of organic matter, and the presence of excessive ammonia nitrogen ($\text{NH}_3\text{-N}$) and hydrogen sulfide, which affect the effluent quality of anaerobic systems (Talalaj et al., 2019). Therefore, a single treatment method cannot meet practical requirements. A-O combined systems achieve better removal efficiency because they can simultaneously remove chemical oxygen demand (COD) and ammonium (Pashaki et al., 2021). It is worth noting that the performance of A-O processes is influenced by key parameters such as hydraulic retention time (HRT), C/N ratio, pH, temperature and salinity. Usually, this system needs longer HRT and sludge retention time, which requires a larger working surface area (Mishra et al., 2023). Therefore, it is urgent to find entry points to improve the efficiency of biological treatment.

As is well known, biochar (BC) has demonstrated its superiority and ability in removing various organic and inorganic pollutants dissolved in aqueous media through its microporous structure, thermal stability, and expanded surface area (Xie et al., 2025). Meanwhile, BC has excellent biocompatibility, and its potential to support bacterial growth and activity is crucial for biodegradation. Additionally, compared to high production costs and the involvement of expensive carbonaceous materials, the application of BC is more economically beneficial. Therefore, researchers have applied BC to the biological treatment of leachate, which is an integration of various physical chemical and biological methods. Pan et al. (2020) found that bamboo BC improved the removal of organic and nitrogen pollutants from leachate in semi aerobic aged garbage biofilters. BC, with its large specific surface area and functional groups, absorbed and removed large organic molecules through hydrogen bonding, hydrophobic interactions, and other mechanisms. Furthermore, it enhanced the internal porosity and dissolved oxygen content of biofilters, providing favorable carrier and living environment for the growth and reproduction of microorganisms, especially for the large-scale growth of nitrifying bacteria. Furthermore, under OTC stress, BC helps alleviate long-term stress in aerobic denitrification systems, by enhancing bacterial metabolic activity, strengthening sludge structure and substrate transport, and improving community stability and diversity (Mao et al., 2023). Pham et al. (2021) used husk charcoal as an additive to enhance bacterial culture and improved anaerobic fermentation of landfill leachate for hydrogen production, ultimately achieving a hydrogen production of 1620 mL per day. However, for high concentration leachate, the treatment effect of A-O process combined with BC is more viable. Faggiano et al. (2023) developed an A-O moving bed biofilm reactor and BC integrated process, which achieved maximum removal rates of COD, $\text{NH}_3\text{-N}$, and total phosphorus (TP) of 98.5%, 91.2%, and 99.9%, improving the effluent quality. In this process, the removal of CTC relied on hydrolysis, and its chemical stability was easily

affected by environmental conditions such as substrate, pH value, temperature, and metals (Wen et al., 2018).

Considering that BC particles are difficult to separate and recover after adsorption. For this purpose, transition metals (Fe, Co, Ni, etc.) or their oxides are doped into BC to form magnetic biochar (MBC) (Xu et al., 2023a). The doping of iron has significant advantages, for example, Chen et al. (2022) found that modified iron containing BC increased the content of oxygen-containing functional groups. Additionally, Fe, as an essential trace element for microbial growth, facilitates enzyme synthesis (Zhang et al., 2021a). MBC, acts as catalyst and adsorbent, enhancing electron transfer and Fe (II) release efficiency, which stimulates microbial activity and enhances pollutant removal (Zhang et al., 2023). This indicates that using MBC to enhance the biological treatment of leachate is a promising approach. Ma et al. (2023) used MBC combined with anaerobic digestion (AD) and found that the system enhanced pollutant removal by regulating bacterial diversity and activity. However, more research is needed to understand the symbiotic relationship between MBC and bacterial communities, and to optimize the MBC production process to meet the biodegradation applications of specific pollutants, to effectively remediate polluted environments.

Previous studies had shown that the application of MBC in leachate treatment involves the adsorption of organic pollutants and heavy metals (HMs), AD or aerobic biofilters, etc. (Zhang et al., 2024). However, there is limited research on the addition of MBC for leachate treatment in the A/O process, especially regarding its impact on microbial functional bacterial structure and ARG migration. Furthermore, the presence of TCs and oxygen availability are key factors affecting the spread of ARGs (Su et al., 2019). Hence, this study selected leachate containing TCs as the research object, constructed an A/O process, added magnetic coconut shell biochar (MCSB) of different concentrations. The aim was to: (1) evaluate the treatment efficiency and sludge performance of TCs inhibited leachate A/O system with different concentrations of MCSB; (2) explore the response behavior of ARGs after MCSB addition and their relationship with abiotic environmental factors; (3) analyze the microbial community structure, function, and their association with corresponding ARGs; (4) speculate on the reaction mechanism of A/O process for treating leachate driven by MCSB; (5) Examine the overall operational efficiency and differences in microbial and genetic information of the continuous flow A-O combination process under the optimal MCSB dosage.

2. Materials and methods

2.1. Chemicals

Activated sludge was taken from Yuhang WWTP in Hangzhou, Zhejiang Province. The chemicals, such as TC, OTC, CTC, were of analytical purity, and the solutions used in precision instruments were of chromatographic purity. The composition of leachate was shown in Table S1.

2.2. The preparation of MCSB

(1) Coconut shell pretreatment. Used a multifunctional crusher to crush coconut shells and passed them through a 60-mesh sieve. (2) Activation. Mixed coconut shell powder with one mol/L HCl at a solid-liquid ratio of 1:10, dispersed by ultrasound for 30 min, and then let it stand for another 30 min. Subsequently, washed and dried at 70 °C for 12 h (3) Carbonization. Placed the activated precipitate in a sealed crucible and heated it up in a muffle furnace at a rate of 5 °C/min to 500 °C for 2 h. After that, cooled it down to room temperature to obtain coconut shell biochar (CSB). (4) Load. Mixed 2.705 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 1.39 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ to 100 mL of ultrapure water, then added 5 g CSB and dropped five mol/L NaOH to pH 10–11, sonicated for 2 h, and let it stand overnight. Washed with ultrapure water and anhydrous ethanol

until neutral, then dried in a 70 °C oven for 12 h and ground it through a 100-mesh sieve to obtain MCSB. The characterization of CSB and MCSB was shown in Fig. S1. From the scanning electron microscopy (SEM) image, it could be seen that the pore structure of CSB was significant, and the surface became smooth and flat. Many Fe₃O₄ particles attached to the surface and pores, providing numerous active sites. Furthermore, the specific surface area of MCSB was 92.865 m²/g. The energy dispersive spectroscopy (EDS) surface scan results revealed that the Fe element content on the surface of MCSB was 45.6 %, confirming the successful attachment of Fe₃O₄ particles to CSB.

2.3. Reactor setup and operation

Independent A/O process (Fig. S2): Cylindrical reactors with an effective volume of 3 L were set up, divided into A and O. The top of the anaerobic reactor was sealed, and the sludge and wastewater were thoroughly mixed through a stirring device. The aerobic reactor was kept open, and the dissolved oxygen was controlled at 2–4 mg/L using an aeration device. During the domestication stage of activated sludge leachate (37 d), 100 % domestic sewage was initially used for cultivation, and the proportion of leachate gradually increased from 10 % to 40 % until the system reached a stable COD removal rate (70 %). During the TCs cultivation stage (24 d), TC, OTC, and CTC were added, and their concentrations gradually increased (1, 3, 5, and 10 mg/L), until the final TCs concentration reached 10 mg/L. The concentration of TCs was determined based on leachate survey data and relevant pre-experiments (Liu et al., 2023; Xie et al., 2025). The inlet water quality of this leachate was: COD, 720–800 mg/L; total organic carbon (TOC), 140–160 mg/L; NH₃-N, 240–270 mg/L; TP, 6.1–7.0 mg/L; total nitrogen (TN), 320–370 mg/L. During the target experimental phase (24 d), A0/O0 was set as the control group (without BC) for the first 12 d, while A5/O5 were treated with 5 g/L MCSB. Added A8/O8 and MCSB dosage of 8 g/L in the next 12 d. The dosage of MCSB was determined based on previous experimental conclusions and literature research (Zhuang et al., 2020). Additionally, the experimental group reactors were sampled on the last day of operation, while the control group reactor was always present. The reactor operated continuously in a constant temperature chamber with an HRT of 3 d and a displacement inflow of 20 % of the total volume. Sampling at different periods were recorded as A0 (0 d)/O0 (0 d), A0 (12 d)/O0 (12 d), and A0 (24 d)/O0 (24 d). To explore the role of MCSB in biological treatment, the degradation efficiency of leachate, sludge performance, microbial community structure, and succession of ARGs were regularly measured.

Continuous flow A-O process (Fig. S2): Composed of a water storage tank (10 L), A/O reaction vessels (6 L each), and a sedimentation tank (10 L) in sequence. Used a peristaltic pump to automatically inject the leachate into the system at a speed of 2 r/min and a flow rate of 8 L/d (controlled by a flow meter). The anaerobic tank was sealed and equipped with an electric stirrer with a speed of 170 r/min. The anaerobic tank and aerobic tank were connected by pipelines. The effluent from the aerobic tank entered the sedimentation tank, while the rest flowed back to the anaerobic tank. The sludge from the sedimentation tank was returned to the anaerobic tank through pipelines at a flow rate of 2.7 r/min, with a reciprocating circulation and an internal to external reflux ratio of 1:1. 8L of leachate was added to the water storage tank at once, with an HRT of 2 d, and the reactor was pre operated for one week. Subsequently, 5 g/L MCSB was added to the A-O continuous flow reactors and operated continuously at a constant temperature for 12 d. During this period, regular samples were taken to measure the degradation efficiency of the leachate, and the performance of the sludge, microorganisms, and ARGs changes were analyzed at the end of the reactor operation.

2.4. MCSB characterization, conventional indicators, and sludge performance

The quality parameters of leachate effluent, such as total organic carbon TOC, TP, TN, NH₃-N, nitrate nitrogen (NO₃-N) and nitrite nitrogen (NO₂-N) were measured using a visible ultraviolet spectrophotometer (TU1901, China). SEM was used to evaluate the surface morphology of MCSB and sludge. The elemental composition of MCSB was analyzed by EDS scanning, and its specific surface area was determined by BET method. The concentration of TCs was determined by high-performance liquid chromatography (HPLC, Shimadzu, Japan) using aqueous phase (oxalic acid) and organic phase (pure acetonitrile) as mobile phases, referring to previous study (Xie et al., 2025). Before measuring the content of HMs, pre-treatment with aqua regia was carried out, followed by heating, acid removal, filtration, and volume determination. Then, analysis was performed using inductively coupled plasma spectroscopy (ICP-MS Elite, Analytical Jena, Germany). The extracellular polymeric substances (EPS) were extracted using thermal fractionation method, and the concentrations of proteins (PN) and polysaccharides (PS) were determined using Lowry-Folin method and phenol sulfuric acid method, respectively. Using a laser particle size analyzer (LAP-W2000H, EAST, China) to analyze sludge particle size (wet dispersion).

2.5. Analysis of ARGs and microbial community structure

Regularly sample and measure ARGs in activated sludge and leachate, including 0.2 g of freeze-dried sludge and 0.22 μm filter membrane (filtering 80 mL of leachate supernatant). The target genes of this study were *tetA*, *tetB*, *tetO*, *tetM*, *tetQ*, *tetT*, and *tetW*, which had been widely reported in landfill leachate (Li et al., 2024). The sample DNA was obtained by DNeasy PowerOil Pro kit, and its concentration and purity were determined using a UV5 Nano-spectrophotometer (Mettler Toledo, Switzerland). The quantitative PCR reaction system, program settings, and sequence information were based on the method proposed by Liu et al. (2022).

Regularly take equal amounts of microbial samples (on day 0, 12, and 24). Use Illumina MiSeq platform for high-throughput sequencing of microbial samples. The sequencing database was set up by Majorbio Limited. Performed later species diversity analysis based on ASV representative sequence information and abundance information. The petal plot displayed the similarity and uniqueness of species in different reactors. Meanwhile, the composition and abundance of bacteria in each reactor were visually analyzed through bar charts and heat maps. Both co-occurrence networks and related heat maps had been applied to present the correlation between microbial community structure and abundance.

2.6. Statistical analysis and visualization

Conventional data statistics were completed in Excel 2018, and OriginPro 2022 was used to visualize the results. Microbial statistical data analysis was conducted by IBM SPSS v22 software, and *p* < 0.05 was considered significant. The correlation heatmap between ARGs and environmental factors was depicted by R (v4.3.2) (*p* < 0.05). The network co-occurrence model of ARGs and antibiotic-resistant bacteria (ARBs) was implemented using Gephi 0.97 (*r* > 0.5, *p* < 0.05). The microbial function was performed by PICRUSt2.

3. Results and discussion

3.1. Effect of MCSB on the performance of leachate biological treatment system

3.1.1. Removal of TOC, nitrogen, and phosphorus

The experimental process tracked the treatment efficiency of adding

different concentrations of MCSB to the reactor. When the concentration of TCs reached 10 mg/L, the TOC removal rates in A0(0 d) and O0(0 d) were 64.65 % and 69.08 %, respectively (Fig. 1a). As time passed, the TOC concentration continued to increase, and microbial growth and metabolism were inhibited, resulting in a significant decrease in organics degradation efficiency. On the contrary, the presence of MCSB alleviated the negative effects of toxic substances on microorganisms. The degradation efficiency of A5 and O5 was better than that of A8 and O8, and the highest TOC removal rates reached 80.01 % and 76.36 %, respectively. This might be since MCSB itself was an organic carbon source, and excessive addition led to excessively high TOC concentration in the effluent. Besides, MCSB might agglomerate due to high concentration, resulting in a decrease in effective specific surface area and weakening the TOC removal performance.

The degradation process of TN and NH₃-N followed a similar pattern, with the removal rates of TN in A5 and O5 reaching their maximum values (65.27 % and 50.26 %), while the degradation rates of NH₃-N in these two reactors were 56.02 % and 47.87 % (Fig. 1b–c). However, the effluent concentrations of TN and NH₃-N gradually increased in A8 and O8, indicating that in the initial stage, the high concentration of TCs and the persecution of leachate inhibited the growth and metabolism of microorganisms, weakening the degradation of organic pollutants and NH₃-N. A suitable concentration of MCSB provided adhesive space for bacterial growth, promoted the development and activity of microbial communities, facilitated carbon and nitrogen removal, and thus enhanced the removal efficiency of the system (Deng et al., 2024). Besides, the release of Fe²⁺ in MCSB could enhance the permeability of the cell membrane, promote the absorption of nutrients by nitrifying bacteria and the degradation of ammonia nitrogen (Song et al., 2023). Meanwhile, anaerobic microorganisms exhibited comparable tolerance to leachate and TCs.

The concentration changes of TP in each reactor were complex and had the largest fluctuation amplitude (Fig. 1d). At first, with the increase of TCs concentration, the TP effluent concentration in each reactor significantly increased and then showed a downward trend. After adding MCSB, the TP removal rates in A8 and O8 reached 62.92 % and 43.59 %,

respectively, with better treatment effects than A5 and O5. The degradation effect of MCSB concentration on TOC, TN, and NH₃-N was opposite to that of TP. The reason for this was that when the TOC removal rate was high, the microorganisms responsible for removing TOC would compete with phosphorus accumulating organisms (PAO) and limit the removal of TP, among which nitrifying bacteria were also potential competitors for PAO (Faggiano et al., 2023). In addition to the role of microorganisms, physical sedimentation and MCSB adsorption also participated in the removal of TP.

The adsorption of TCs on activated sludge was due to the electrostatic interaction between the negatively charged surface of biological sludge and zwitterions. Besides, antibiotics reacted with environmental substances such as heavy metals to form complexes or chelates (Chu et al., 2023). The addition of MCSB gave rise to improvement in the removal rate of TCs (Fig. S3), and the trends in A/O systems were similar, revealing that the addition of MCSB facilitated the adsorption, degradation, or transformation of TC, OTC, and CTC (He et al., 2022). However, the difference in the effect of adding 5 and 8 g/L MCSB on the removal of TCs was not significant, with their removal rates ranging from 81 % to 88 %. Furthermore, their removal also depended on hydrolysis. According to the report (Yang et al., 2019), they could hydrolyze within 24 h at concentrations of 3–10 mg/L.

3.1.2. Sludge performance

The SEM images of sludge before and after the addition of MCSB showed a sharp contrast (Fig. 2). Under the stress of TCs and leachate, A0(0 d) sludge contained a large number of rod-shaped bacteria with loose structure, and over time, its surface became denser. In O0(0d) sludge, there were rod-shaped and filamentous microorganisms, but in O0(24 d), the sludge structure presented loose, the surface was smooth, and the microbial amount was reduced. Table S2 depicted the changes in sludge particle size. The average sludge particle size (D50) in A0(24 d) and O0(24 d) decreased by 11.73 % and 17.43 % respectively over time, supplying that the addition of TCs led to the decomposition of activated sludge into smaller particles with a more uniform particle size distribution. When sludge was combined with MCSB, the surface of the sludge

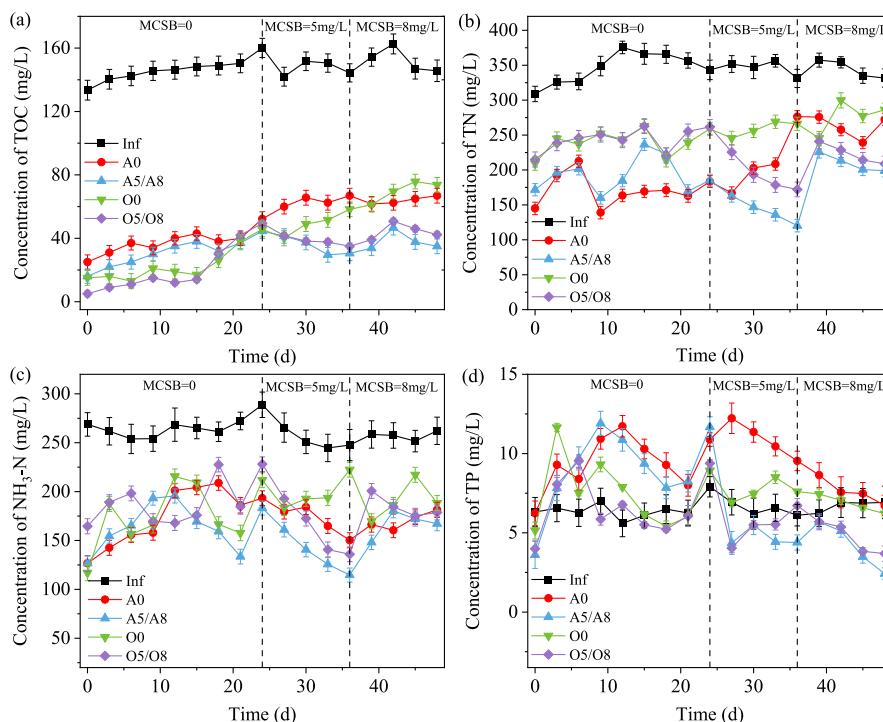


Fig. 1. Effects of MCSB on TOC (a), TN (b), NH₃-N (c), and TP (d) in leachate biological treatment systems. Inf: Influent leachate. A0/O0: Control group. Days 0–24: TCs domestication. Days 24–36: Added 5 g/LMCSB, corresponding to A5/O5. Days 36–48: Added 8 g/LMCSB, corresponding to A8/O8.

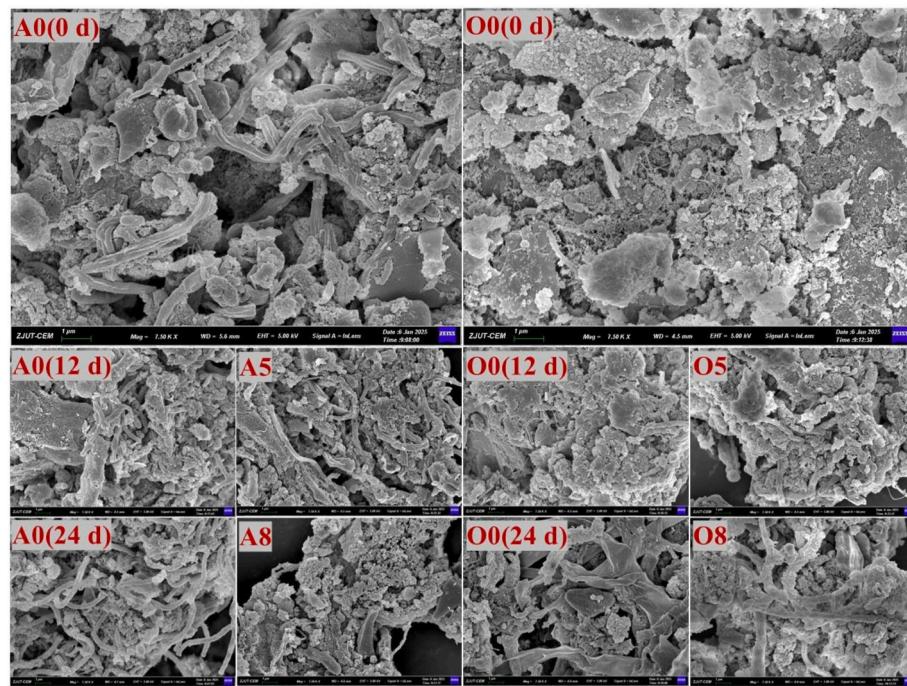


Fig. 2. Morphological changes of anaerobic and aerobic sludge before and after the addition of different concentrations of MCSB ($\times 7500$). According to the operating time of the reactor, the control group was set as A0 (0 d)/O0 (0 d), A0 (12 d)/O0 (12 d), and A0 (24 d)/O0 (24 d). A5/O5: 5 g/L MCSB. A8/O8: 8 g/L MCSB.

in the reactor tends to be rough and has many small protrusions. The microorganisms in the sludge formed a complex biological structure, making it more stable and the surface characteristics change significantly. At the same time, the sludge particle size in the system increased, and the D50 values in A8 and O8 were higher than those in A5 and O5, especially in anaerobic reactors. Research had shown that the adsorption of a portion of TCs by carbon material alleviated the toxicity to microorganisms, facilitated sludge flocculation, improved settling performance, and effectively removed pollutants (Nabi et al., 2023). Zhuang et al. (2020) showed that adding Fe_3O_4 loaded BC was beneficial for sludge granulation and promoted anaerobic processes. In summary, the water quality conditions of leachate had a significant impact on sludge morphology and microbial growth, but the addition of MCSB could improve microbial activity.

3.1.3. EPS and HMs

EPS, as an indicator of the performance of biological treatment systems, is related to pollutant removal. Its main modes of action include adsorption and retention, protection of microorganisms, and improvement of sludge floc structure. EPS has a unique spatial structure and complex composition, including soluble EPS (S-EPS), loosely bound EPS (LB-EPS), and tightly bound EPS (TB-EPS) (Wang et al., 2023). Under the

influence of TCs, the PN content in A0 and O0 first rose and then decreased with time (Fig. 3a), while the PS content showed an upward trend (Fig. 3b). When the dosage of MCSB was 5 g/L, the PN concentration in A5 and O5 reached 229.68 and 154.73 mg/g VSS, higher than the control group at the same stage. Similarly, the PS concentration also exhibited the same pattern (219.78 and 279.50 mg/g VSS in A5 and O5), indicating that the presence of MCSB was more conducive to their secretion of EPS, thereby providing a thicker physical protective barrier for bacteria to avoid the invasion of toxic substances (Feng et al., 2021). The significant increase in TB-EPS/LB-EPS in A5 and O5 promoted denser sludge particles (Jachimowicz et al., 2022). The momentous change in PS was in S-EPS, which might block the diffusion of some organic pollutants into cells, thereby enhancing microbial resistance to the environment. In contrast, when the dosage of MCSB reached 8 g/L, the PN concentration in A8 and O8 decreased to 98.67 and 123.45 mg/g VSS, respectively. The decrease in TB-EPS content revealed the negative impact of excessive MCSB on microorganisms. More seriously, the PS concentration in A5 and O5 was even lower than that of the control group during the same period, manifested as a decrease in S-EPS. The reason for this might be that excessive MCSB formed aggregates in the system and lost its catalytic and adsorption effects. The concentration of PS in the A/O system was

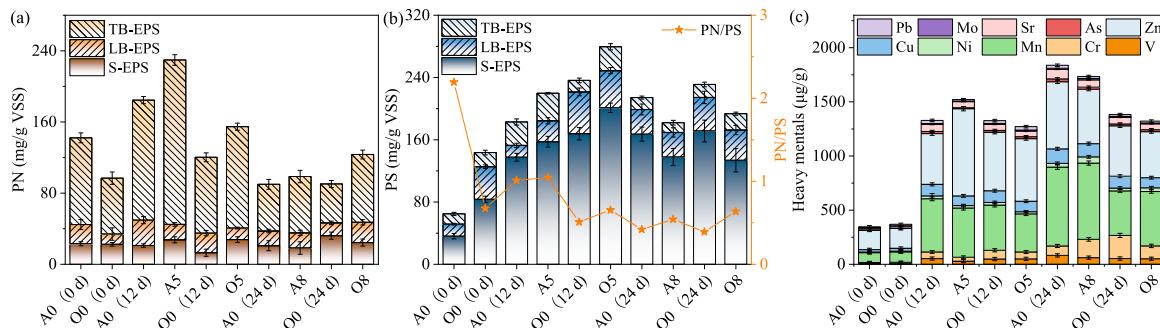


Fig. 3. Variations in PN, PS, and HMs concentrations in activated sludge.

always higher than that of PN, and the synthesis of PS affected cell adhesion ability and facilitated the formation of microbial aggregates, which also proved that PN was more sensitive to environmental changes. According to the fluctuation of PN/PS (Fig. 3b), the ratio continued to decline in A0 and O0, while the experimental group with added MCSB was higher than the control group reactor, testifying that higher PN/PS improved the structural stability of sludge in the leachate system and made it easier to settle and separate (Xue et al., 2024). Furthermore, iron ions in MCSB bound with EPS to form metal EPS chelates, which reduced the toxic effects of metal ions on microorganisms over time (Gao et al., 2024a). In summary, the involvement of MCSB might lead to changes in EPS content through four mechanisms: microbial co-entropy, electron transfer, biofilm formation, and substrate distribution (Nabi et al., 2024), which expedited the growth and activity of specific microorganisms, thereby affecting the composition and quantity of EPS.

The fluctuation of HMs concentration in sludge was the result of comprehensive regulation by multiple factors, including MCSB performance, sludge characteristics, water quality conditions, and environmental factors. At first, the concentration of HMs in A0(0 d) and O0(0 d) was not high (346.79 and 369.446 µg/g), but over time, the concentration of HMs in both reactors significantly increased, with the highest concentrations reaching 1838.26 and 1383.17 µg/g (Fig. 3c). Meanwhile, the concentration of HMs in sludge in anaerobic systems was higher than that in aerobic systems. On the one hand, the surface structure and functional groups of MCSB in sludge contributed to the formation of insoluble inclusion complexes with metal ions through chelation and complexation processes (Jayawardane and Dayanthi, 2024). On the other hand, microorganisms produced more EPS under the induction of TCs and MCSB to form aggregation effects. The three-dimensional structure of EPS molecules gave it strong binding ability to HMs. The anionic functional groups on PN and PS could calculate with metal ions through ion exchange, chelation, and electrostatic interactions (Du et al., 2019). Under the influence of MCSB, only the concentration of HMs in A5 increased. Therefore, further increasing the dosage of MCSB did not demonstrate a corresponding increase in the adsorption efficiency of HMs in the sludge. This phenomenon was attributed to the complexity of the leachate, where HMs interwovened with various other pollutants and interfered with the adsorption process (Priya et al., 2022). Another contributing factor might be the increase in quality, resulting in overlapping and accumulation of adsorption sites on the adsorbent surface, reducing effective surface area (Abyaneh et al., 2024). The metals with the most significant changes throughout the entire reactor operation cycle were Mn, Zn, and Cr. The concentration of Zn gradually increased in A0 and showed a trend of first increasing and then decreasing in O0, with maximum concentrations of 619.60 µg/g (A0 (24 d)) and 539.92 µg/g (O0 (12 d)), respectively. 5 g/L MCSB enhanced the adsorption of Zn by sludge, while 8 g/L MCSB had the opposite effect. The content of Cr in the aerobic system went up significantly, and different concentrations of MCSB hindered the accumulation of Cr in the sludge. Mn increased from 90.85 µg/g (A0 (0 d)) to 491.41 µg/g (A0 (12 d)), reaching a maximum value of 728.18 µg/g (A0 (24 d)), while the highest concentration of Mn in the aerobic reactor was only 417.36 µg/g (O0 (12 d)). However, the addition of MCSB reduced the adsorption of Mn by activated sludge, but the Mn concentration in A8 and O8 was still 1.55 and 1.43 times higher than that in A5 and O5. This proved that the competitive adsorption of MCSB in the solution reduced the enrichment of Mn in sludge, but the concentration of MCSB still had a remarkable impact on the adsorption effect of individual metals. However, the adsorption properties of MCSB were easily affected by environmental factors such as pH and temperature.

3.2. Effect of MCSB on the propagation of tet genes

3.2.1. Distribution of tet genes

Fig. 4a described the relative abundance of seven target ARGs in the leachate. Originally, the relative abundance of initial ARGs in A0(0 d) and O0(0 d) was 52.01 and 46.57 log₁₀ copies/L, respectively, with slight change thereafter. The abundance of ARGs in A5 and O5 was not significantly different from the control group. It was worth noting that when the concentration of MCSB reached 8 g/L, the relative abundance of ARGs in A8 decreased by 28.22 % compared to A5, mainly reflected in *tetA* (from 9.45 to 6.91 log₁₀copies/L), *tetB* (from 9.17 to 7.05 log₁₀copies/L), *tetM* (from 7.06 to 5.04 log₁₀copies/L), and *tetQ* (from 9.72 to 6.65 log₁₀copies/L). This indicated that 8 g/L MCSB significantly weakened the diffusion of ARGs in the leachate and inhibited the development of AR in host bacteria. This indicated that 8 g/L MCSB significantly weakened the diffusion of ARGs in anaerobic systems and inhibited the development of antibiotic resistance in host bacteria. This might be since ARGs (such as plasmid DNA) free in the filtrate supernatants bound to biochar through electrostatic interactions. However, more importantly, high-dose iron ions could disrupt the DNA structure of ARGs and impair the activity of ARGs host bacteria (Wang et al., 2018). The total abundance of ARGs in activated sludge was always lower than that in leachate (Fig. 4b). The relative abundance of ARGs in A0(0 d) and O0(0 d) was 43.03 and 40.09 log₁₀copies/g, respectively. The relative abundance of ARGs in A0 showed an increasing and then decreasing trend over time, reaching a maximum of 52.39 log₁₀ copies/g. The relative abundance of ARGs in A5 and O5 increased compared to the control group during the same period, demonstrating that the dominant bacterial population selectively survived or their ARGs were improved (Tong et al., 2019). Low concentration of MCSB (5 g/L) promoted the proliferation of aerobic microorganisms and accelerated the spread of ARGs, while high concentration of MCSB enhanced the diffusion of ARGs in anaerobic microorganisms. In addition, the total relative abundance of ARGs in A5 and A8 was consistently higher than that in O5 and O8, indicating that anaerobic condition was more conducive to the proliferation of ARGs, in line with the reported conclusion (Fan et al., 2022). Overall, whether it was leachate or activated sludge, *tetA* was the most abundant, followed by *tetB* and *tetW*, which were antibiotic target protective proteins and tetracycline resistance genes.

3.2.2. Impact of abiotic environmental factors on the transmission of tet genes

Spearman correlation analysis based on bilateral testing was used to study the key environmental factors affecting microbial succession in leachate and activated sludge, as well as their potential correlation with ARGs transmission. With the increase of MCSB concentration, *tetM*, *tetO*, and *tetW* in the leachate were always positively correlated with these water quality parameters (Fig. 4c). The AR of bacteria was closely related to the nutrients in wastewater. If there was a lack of energy, the stability of plasmids would be reduced, giving rise to a decrease in AR (Sun et al., 2022). However, the genes *tetA* and *tetB* were negatively correlated with TOC, NH₃-N, and TN. In addition, TP was significantly positively correlated with seven *tet* genes. Under the regulation of 10 mg/L TCs, most *tet* genes were positively correlated with TC, OTC, and CTC. In summary, the selection pressure of antibiotics was considered the main cause of antibiotic gene and microbial deposition, while the presence of MCSB was an additional driving force to alter the growth and distribution of ARB and genes.

The distribution of ARGs in activated sludge was influenced by the combined pressure of MCSB, TCs, HMs and EPS (Fig. 4d). The *tetA* of the efflux pump mechanism encoded the resistance of activated sludge to TCs, controlling the process of toxins being squeezed from the interior of cells to the external environment, and this gene might be horizontally transferred (Liu et al., 2023). The ribosomal protective protein genes (*tetO*, *tetM*, and *tetQ*) were positively correlated with some metals (Zn,

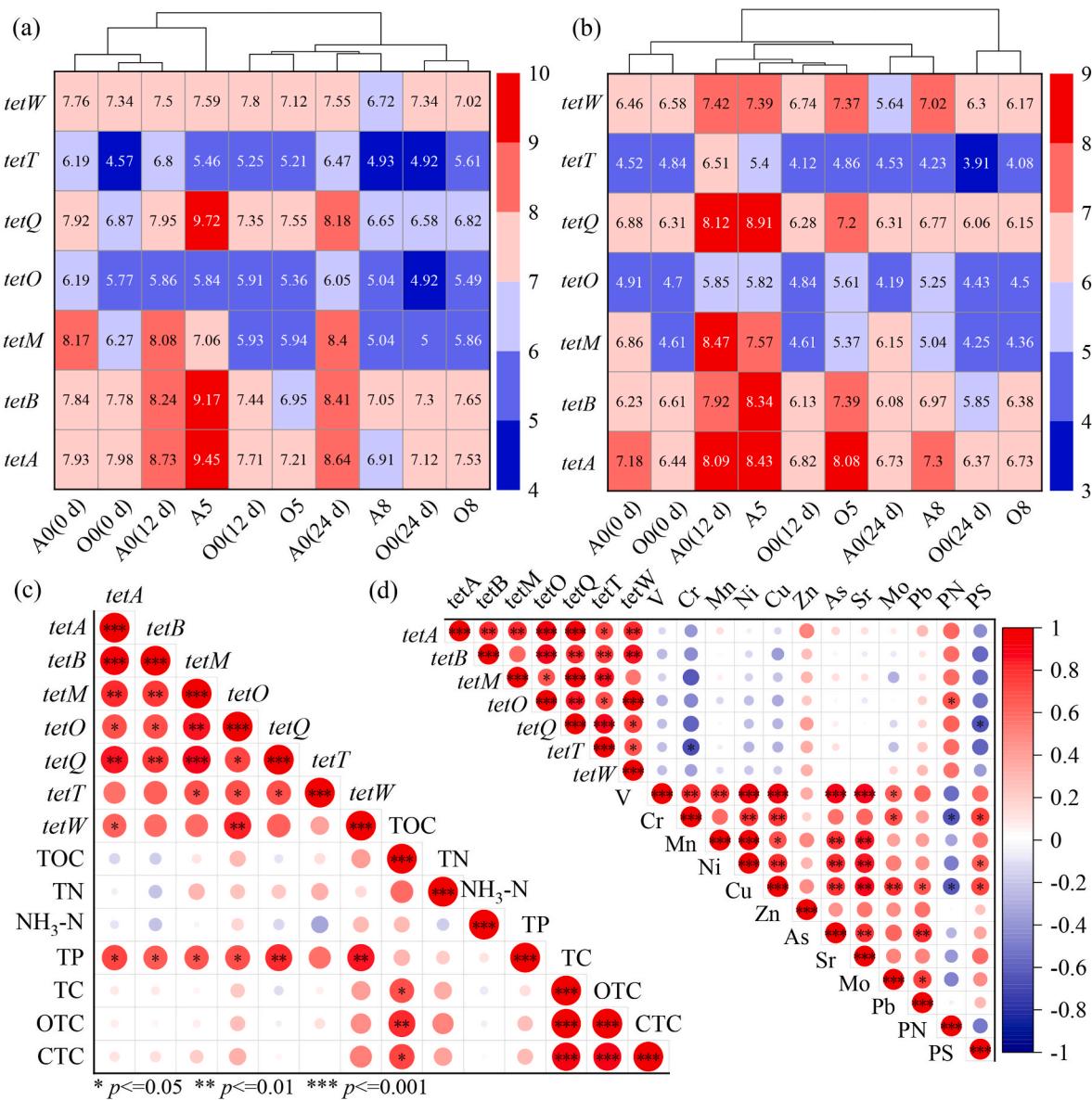


Fig. 4. The relative abundance (log₁₀ copies/g or L) of *tet* gene in leachate (a) and activated sludge (b). Correlation analysis between the distribution of resistance genes and their respective environmental factors (c) and (d). According to the operating time of the reactor, the control group was set as A0 (0 d)/O0 (0 d), A0 (12 d)/O0 (12 d), and A0 (24 d)/O0 (24 d). A5/O5: 5 g/L MCSB. A8/O8: 8 g/L MCSB.

Sr, Pb) and negatively correlated with others (V, Cr, Ni, Cu, Mo). These HMs could trigger common and cross selection among various ARB, thereby promoting or inhibiting the spread of ARGs (Wang et al., 2022). However, there were some metals that had little effect on the transfer of some ARGs, such as As and Sr. Surprisingly, the proliferation and transfer of ARGs were related to the formation of EPS (Li et al., 2021), and all seven *tet* genes were positively correlated with PN but negatively correlated with PS secreted by microorganisms. The *tetM* was related to the synthesis of cytoplasmic proteins, which protected ribosomes from the effects of TCs (Wang et al., 2020). EPS facilitated the accumulation of biofilm on MCSB, making for the diffusion and proliferation of ARG. As the biofilm fell off, ARG proliferated in the liquid phase. Wang et al. (2021) demonstrated that EPS related ARGs are closely clustered with intracellular genes, while extracellular ARGs may also be transformed by horizontal gene transfer (HGT) through EPS related ARGs. In summary, these correlations highlighted the potential role of environmental factors in the migration and transmission of ARGs under the influence of MCSB.

3.3. Microbial functional metabolism and gene regulation driven by MCSB

3.3.1. Microbial community structure

Table S3 listed the alpha diversity index of sludge microorganisms in the reactor. Among them, the Ace, Chao, Shannon, and Sobs indices in A0 decreased over time, while in O0, these indicators showed a trend of first increasing and then decreasing. As the concentration of MCSB went up, the richness and diversity of microbial communities in A5 and A8 significantly increased, especially when MCSB reached 8 g/L. However, the richness and diversity of microbial communities in aerobic reactors presented opposite trends at different MCSB dosages. Among them, the Ace, Chao, and Shannon values in O8 reached 1144.881, 1139.509, and 5.129 (the highest values among all samples), indicating that MCSB alleviated the toxicity of TCs and leachate to microorganisms, further improving species abundance, diversity, and uniformity. Meanwhile, high concentration of MCSB (8 g/L) had significantly positive impact on aerobic biological systems. The results of sample microbial typing

analysis based on ASV showed that MCSB promoted differences in the dominant microbial community structure in A/O reactors (Fig. 5a). According to the petal diagram, the total number of species in all microbial samples was 167 (Fig. 5b). When there was no MCSB, the number of unique species in anaerobic systems decreased, while the number of species in aerobic systems increased. As the concentration of MCSB rose, compared to A5, A8, and O5, O8, their unique species numbers varied significantly, and microbial reproduction was vigorous at high concentrations of MCSB.

Among all the samples, Pseudomonas, Bacteroidota, Bacillota, Chloroflexita, Actinomycota, Acidobacteriota, Patescibacteria, and Planctomycota were the main bacterial phyla in the MCSB driven

leachate activated sludge system (Fig. 5c). The abundance of Bacillota in A0 increased significantly over time, dominating in A0(12 d) (40.92 %) and A0(24 d) (49.82 %). Therefore, it was speculated that Bacillota could tolerate environmental toxicity under anaerobic conditions, exhibited strong vitality, and promoted the degradation of organic matter. In addition, the decrease in the relative abundance of Bacteroidota and Chloroflexita in A0(24 d) might be related to the decrease in particle structure stability and nutrient removal ability. The difference in oxygen concentration created species differences between A0 and O0, and microorganisms with significantly increased abundance in O0(24 d) in the later stage include Pseudomonas, Chloroflexita, and Actinomycota. With the addition of MCSB, Pseudomonas remained the most

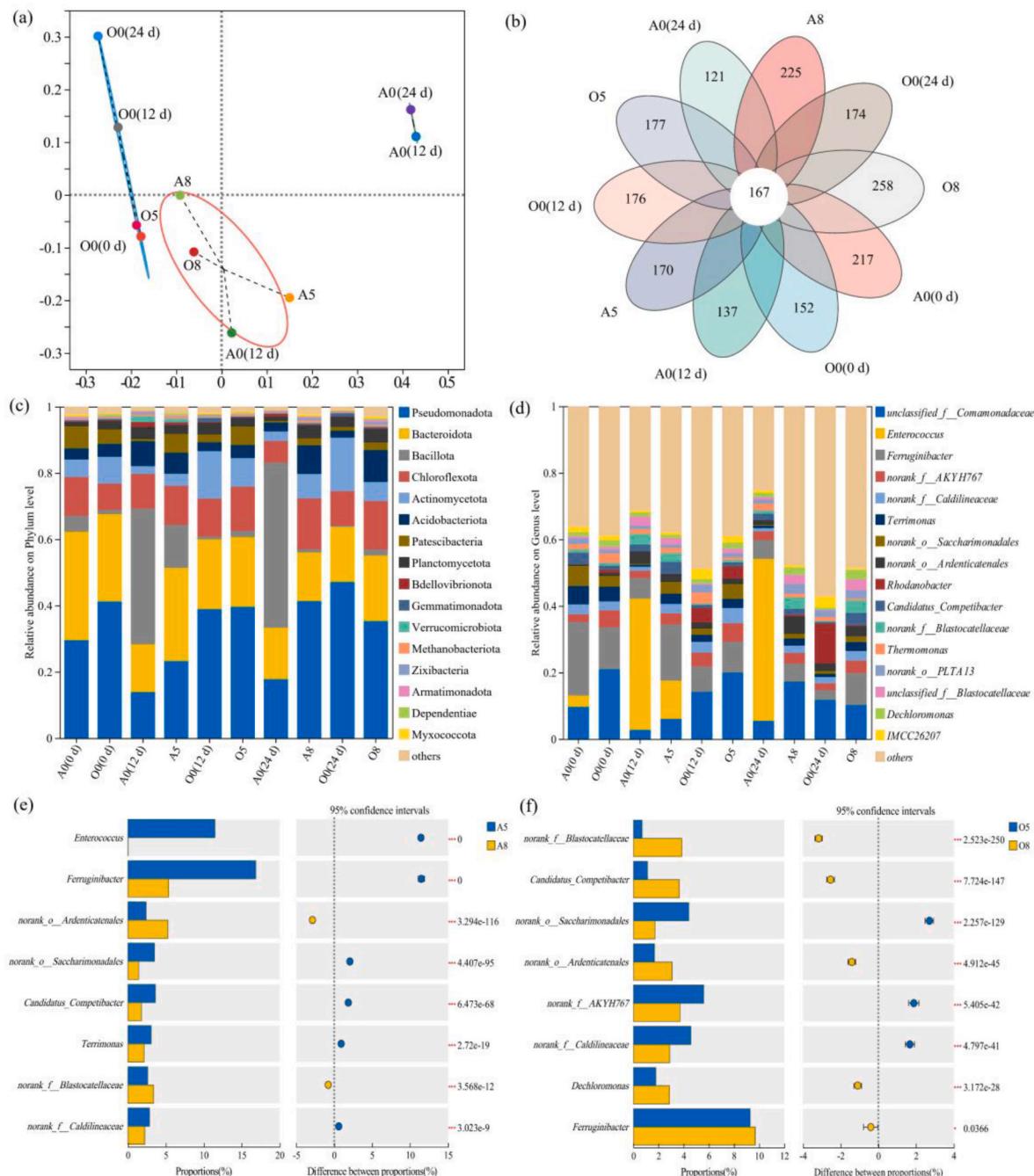


Fig. 5. (a) Dilution curve of microbial samples. (b) Venn petal diagram based on ASV. The relative abundance of microbial communities at the phylum (c) and genus (d) levels. Analysis of species differences caused by different concentrations of MCSB: Wilcoxon rank sum test bar chart at the genus level (95 % confidence interval). (e) A5 and A8; (f) O5 and O8. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. According to the operating time of the reactor, the control group was set as A0 (0 d)/O0 (0 d), A0 (12 d)/O0 (12 d), and A0 (24 d)/O0 (24 d). A5/O5: 5 g/L MCSB. A8/O8: 8 g/L MCSB.

common and abundant phylum, accounting for over 35 % of its abundance in aerobic systems, with increases of 67.39 % and 132 % in A5 and A8 (compared to the A0(12 d) and A0(24 d)). So, Pseudomonas could grow in different environments with various carbon sources, and most denitrifying bacteria belonged to these bacterial species (Si et al., 2018), some of which had biochemical activity and helped tolerate HMs conditions (Martínez-Martínez et al., 2023). Surprisingly, the relative abundance of Bacteroidota in A0 and O0 gradually decreased over time, while the addition of MCSB enhanced the proliferation of this species in A5 (28.08 %) and O8 (19.78 %). According to reports, Bacteroidetes could produce various bacterial hydrolytic enzymes and lipase for the degradation of organic compounds. In addition, according to the research of Xiong et al. (2023), Bacteroidota had been shown to exist under some harsh conditions, owned the ability to resist toxic substances, and could secrete more EPS to enhance sludge hydrophobicity, thereby accelerating the sludge granulation process. Chlorflexi had similar abundance in all samples and played an important role in degrading carbohydrates and cellular substances, often appearing in autotrophic denitrification reactors (Deng et al., 2020). The relative abundance of Actinomycota in A5 and A8 was higher than that in the control group, which could degrade cellulose and in connection with organic matter conversion (He et al., 2022). After adding MCSB, the abundance of Pseudomonas and Patescibacteria increased, supplying their potential to resist various toxicities of leachate. In addition, non-dominant bacteria *Gemmimonadetes* had a nitrite reducing effect (Gao et al., 2024b), which was conducive to the removal of nitrogen-containing organic matter. Broadly speaking, the effects of dissolved oxygen concentration, MCSB, and TCs might be the reasons for the above differences, leading to varying degrees of changes in the structure of microbial communities.

At the genus level (Fig. 5d), it could be seen that the abundance of most microorganisms in A0 and O0 gradually decreased over time. Except for *Enterococcus*, which dominated in A0(12 d) and A0(24 d) (39.48 % and 48.71 %), this species was suitable for survival under anaerobic conditions. On the contrary, after adding MCSB, the relative abundance of *Ferruginibacter*, *norank_f AKYH767*, *norank_f Caldilineaceae*, *Terrimonas*, and *norank_o Saccharimonadales* in the reactors of the four experimental groups were higher than that of the control group, with *Ferruginibacter* showing the most significant proliferation. *Ferruginibacter* were more adapted to anaerobic environments, due to their metabolic pathways under anaerobic conditions. However, driven by MCSB, they improved their tolerance to the environment and were able to reproduce under aerobic conditions (9.28 % and 9.69 % in O5 and O8). However, excessive concentration of MCSB led to the extinction of some microorganisms in aerobic or anaerobic reactors, such as *Ferruginibacter*, *unclassified_f Comamonadaceae*. This might be related to the Fe loaded in MCSB. Zhang et al. (2021b) confirmed that an appropriate iron concentration (<10 mg/L) boosted microbial metabolism, but excessive concentration could lead to inhibition. In addition, *Rhodanobacter* was enriched in O5 and had highly differentiated copper containing nitrite reductase (*nirK*) gene sequence, thereby promoting the removal of TP (Xu et al., 2023b).

Detecting species differences and significance levels based on sample community abundance data. Longitudinal comparison (Fig. 5e–f) showed that under anaerobic conditions, the abundance of *Enterococcus* and *Ferruginibacter* in A5 was 11.44 % and 11.48 % higher than in A8, respectively, when different concentrations of MCSB were added. According to reports, the enriched *Ferruginibacter* was sensitive to changes in oxygen concentration and other external environments (Cui et al., 2021). On the contrary, the abundance of *norank_o_Ardauthenticatenales* in A8 was 2.19 times that in A5, and 8 g/L MCSB induced its growth and enrichment. On the other hand, under aerobic conditions, 8 g/L MCSB provided more microbial growth carriers, promoting the enrichment of *norank_f_Blastocatellaceae*, *Candidats Competibacter*, *norank_o_Ardau thenticatenales*, and *Dechloromonas*. Horizontal comparison (Fig. S4a–b) revealed significant differences in microorganisms between A5 and O5

at 5 g/L MCSB. *Enterococcus* was only grown in A5, while *Rhodanobacter* was only enriched in O5. At high concentrations of MCSB, the significantly different microorganisms were *Ferruginibacter*, whose relative abundance in O8 was twice that of A8. Different concentrations of MCSB and dissolved oxygen significantly altered the composition of dominant bacterial species in the leachate system in multiple dimensions.

3.3.2. Analysis of microbial community functional potential

The prediction of microbial community function in PICRUSt2 was shown in Fig. 6a, with metabolism, genetic information processing, environmental information processing, cellular processes, human diseases, and biological systems being the main functional features. Among them, metabolism and genetic information processing were the main functions of bacteria in various reactors, which was consistent with recent studies, indicating that sludge domesticated with TCs leachate had unique metabolic functions and superior conditions for facilitating ARG transmission (Mamo et al., 2024). The porous structure of MCSB enhanced microbial cell viability and metabolic activity and promoted intercellular communication through quorum sensing (QS) (Yan et al., 2023). However, only these two main functions in O8 were weakened. The second level KEGG pathway showed thirty metabolic pathways (Fig. S5a). Some highly abundant metabolic pathways, such as carbohydrate metabolism, amino acid metabolism, energy metabolism, as well as the metabolism of cofactors and vitamins, were worth paying attention to. This proved that MCSB maintained the basic metabolic activities of microorganisms and alleviated the growth pressure caused by unfavorable environmental factors.

The abundance prediction of the top 30 metabolic functions based on KEGG (KO level) was shown in Fig. 6b. KO enrichment was significant in the anaerobic system, mainly belonging to the functions of bacterial motility proteins (KO3406), ATP-binding cassette transporters (ABC transporters) (KO1995, KO1996, KO1997, KO1998, KO1999), and bacterial cytoskeleton proteins (KO3496), and transporters (KO7114). Among them, some KO were closely related to the resistance and spread of ARGs, such as ABC transporters responsible for regulating the function of the ABC transport system. Their enrichment might lead to multidrug resistance (A5, O5, and A8), but their decrease in O8 was related to the obstruction of ARGs diffusion (Li et al., 2022). For KO1897, it was an important signal transduction factor involved in bacterial environmental information processing. However, it was a dominant inhibitor of guanylate diphosphate and had negative effects on biofilm formation on MCSB, such as flocculation and separation of biofilms (Li et al., 2023). Hence, these core functions promoted the production of QS signaling molecules in microorganisms, regulated the formation and structural stability of microbial biofilms on MCSB, created favorable environment for gene exchange, and thus promoted the transfer of ARGs. Based on the PCA sorting method, Procrustes analysis demonstrated a significant correlation between metabolites and microorganisms ($M^2 = 0.097, p = 0.001$), and their changes were highly consistent (Fig. S5b).

3.3.3. Association of tet genes and succession of sludge microbial community

The existence of ARGs endowed hosts with specific functions, thereby regulating the growth and expansion of species and their environmental relevance. As depicted in Fig. 6c, *tetM* was significantly positively correlated with *Enterococcus* ($p < 0.01$), which promoted the enrichment of *tetM* in A0. There was a positive correlation ($p < 0.05$) between *tetB* and *unclassified_f Slastocatellaceae* and *norank_o_RBG-13-54-9*. However, *tetM*, *tetQ*, and *tetT* showed significant negative correlations ($p < 0.05$) with various microorganisms, such as *unclassified_f Rhodanobacteraceae*, *Longivirga*, and *norank_o_KIST-JJY010*. In addition, *tetM* was significantly negatively correlated with *Rhodanobacter* and *Nakamuraella* ($p < 0.01$). MCSB provided a carrier for microbial proliferation. The symbiotic mode between ARGs and dominant bacteria confirmed the shaping of microbial community structure driven by MCSB and TCs

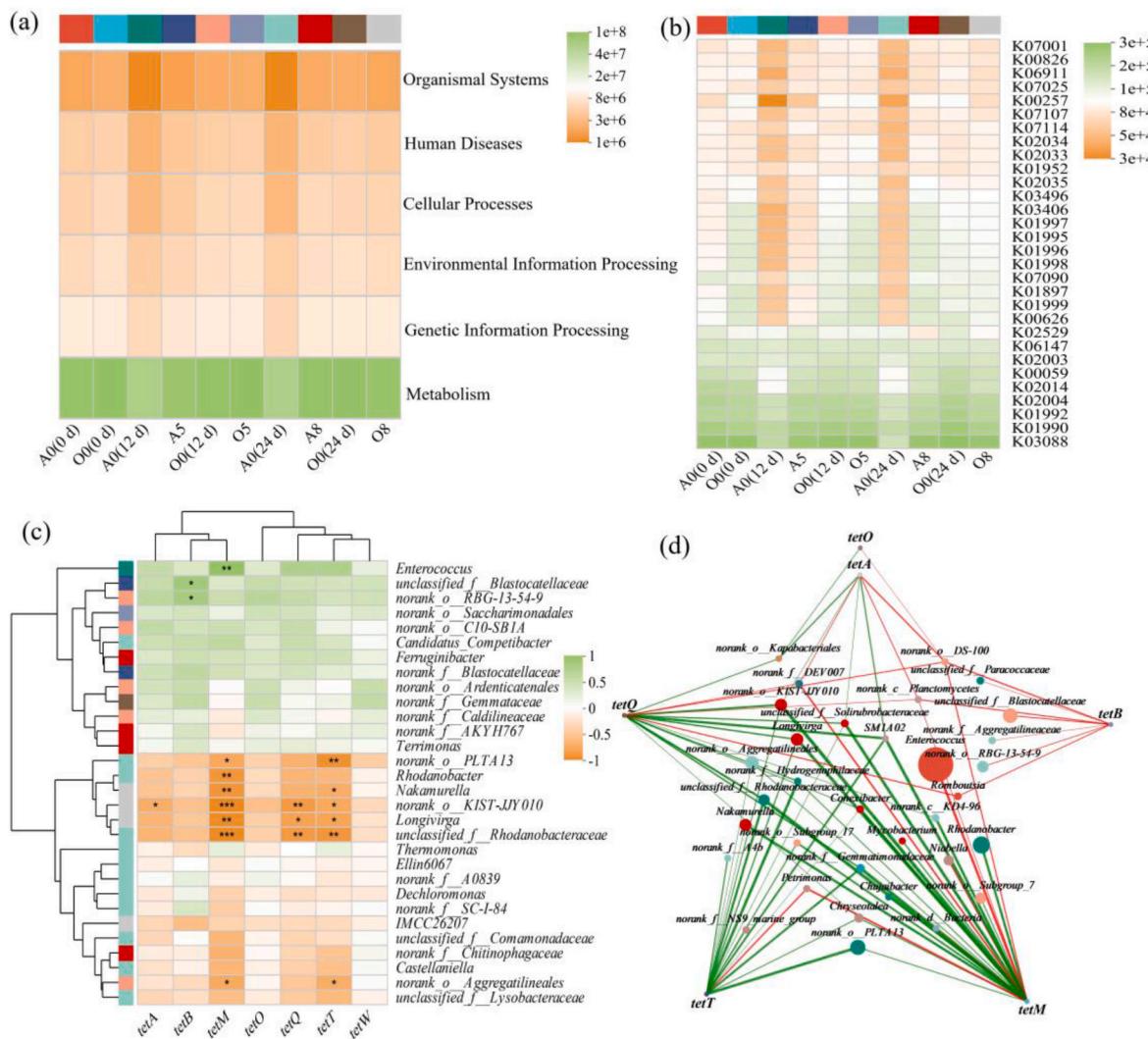


Fig. 6. (a) PICRUSt2 prediction of microbial community function (based on KEGG pathway level 1). (b) The abundance of the top 30 metabolic functions based on the KEGG database (KO level). (c) Correlation heatmap showing the correlation between the top 30 taxonomic units at the genus level and antibiotic resistance genes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (d) The regulatory mode of tet genes and the corresponding collinear network of potential microbial hosts ($r > 0.5$, $p < 0.05$). The size of the node represents the abundance of the taxon in the microbial community. Red lines represent a positive correlation, and green lines represent a negative correlation; the thickness of the line indicates the size of the correlation coefficient, the thicker the line, the higher the correlation. The more lines, the closer the connection between nodes. According to the operating time of the reactor, the control group was set as A0 (0 d)/O0 (0 d), A0 (12 d)/O0 (12 d), and A0 (24 d)/O0 (24 d). A5/O5: 5 g/L MCSB. A8/O8: 8 g/L MCSB.

(Fig. 6d). According to the phylogenetic tree of the system (Fig. S6a), most microorganisms belonging to the phyla Proteobacteria, Bacteroidetes, and Actinobacteria had been identified as potential hosts of ARGs (He et al., 2022). *Petrimonas* might be a potential host for *tetM* and *tetT*. The potential pathogens and ARBs of *tetA* and *tetB* included *norank_o_Planctomycetes* and *norank_o_SS-100*. In addition, the microorganisms associated with *tetO* were the least, resulting in lower abundance in each reactor. The main gene associated with TC targeted protection was *tetT*, but its transferability was limited (Zheng et al., 2023), which resulted in its relative abundance being at its lowest level. Because most of the genera related to ARGs came from the phyla Proteobacteria, Bacteroidetes, and Actinobacteria, they were the main carriers and spreaders of ARGs. The distribution of ARGs was closely related to the characteristics of ARBs and the localization of genes (plasmids or chromosomal DNA) and changed in bacterial communities to drive the release of ARGs (Gao et al., 2025). ARB transferred its ARGs to other bacterial cells or through vertical gene transfer via plasmids, transposons, integrons, and phage mediated HGT (Hazra et al., 2022). Sometimes, the uptake of bacterial genes occurred through

environmental transformation processes. In A/O systems, bacteria adapted to TCs grow well and gradually dominated, while bacteria without AR weakened or disappeared with prolonged operation time (Liu et al., 2023). In summary, exploring potential hosts for pollution control of ARGs in the environment is of great significance, and taking effective measures to inhibit the adhesion and transformation of ARGs can help reduce environmental risks. The RDA analysis implied that only *tetM* had the most significant impact on species distribution ($p < 0.05$) (Fig. S6b), with a determination coefficient r^2 of 0.72. Except for *tetW*, *tetM* was positively correlated with other tet genes.

3.4. Potential regulatory effect of MCSB on A/O leachate system

The adsorption of MCSB and the degradation of microorganisms contributed to improving the removal performance of activated sludge leachate systems and regulating ARGs and microbial community structure (Fig. 7). The adsorption of BC could remove some organic pollutants and TCs from leachate, and biodegradation was the main way to truly remove pollutants. MCSB provided growth space for functional

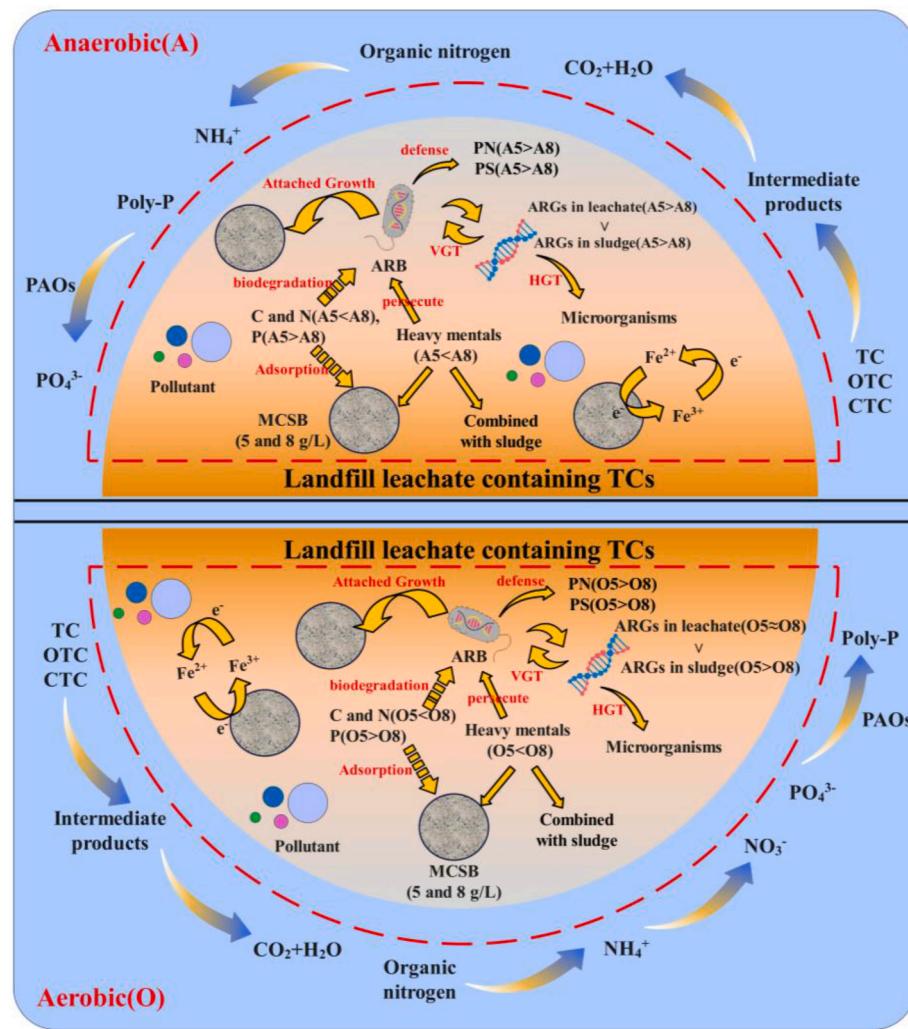


Fig. 7. The potential mechanism of MBC acting on *tet* genes and microbial development in leachate biological treatment systems.

microorganisms and improved the efficiency of biodegradation by stimulating microbial metabolism and altering the abundance of functional microorganisms (Wu et al., 2022). At the same time, the loaded Fe had excellent biocompatibility and great conductivity, which could promote the electron transfer process within the system and directly participated in the growth and synthesis metabolism of microorganisms (Zhang et al., 2021a,b), thereby promoting the mineralization of pollutants. In addition, changes in TCs and MCSB concentrations regulated the proliferation of dominant bacterial species in the microbial community, thereby affecting the migration of ARGs in leachate and activated sludge. This work provides a better understanding of the resistance of MBC mediated ARGs and microbial communities to stress in leachate wastewater containing TCs and offers a feasible approach for controlling ARGs.

3.5. Operation effect, microbial and ARGs changes of continuous flow A-O reactor under optimal MCSB dosage

Research on continuous flow technology is a hot topic and trend, as it can reduce energy consumption and provide high-quality treatment and wastewater. Therefore, selecting an appropriate MCSB concentration (5 g/L) and examining its applicability in guiding the full-scale integrated continuous flow A-O process for TCs containing leachate, systematically comparing similarities and differences, and providing effective guidance for achieving high-performance and complex leachate treatment. As shown in Fig. S7, the maximum removal rates of TOC in A-O were 90.42

% and 91.73 %, respectively (on day 12), which increased by 36.95 % compared to A5 and O5. For TP, the average degradation efficiency of the continuous flow A-O system reached 67.89 %, which was higher than that of the independent A/O system (approximately 36.55 %). Moreover, the removal rate of TN reached 70.08 %, 7.37 % higher than that of A5.

Regarding sludge performance, the total EPS production in the continuous flow A-O reactor was like that in A5 and O5 (Fig. 8a). The components with the highest content in PN and PS were TB-EPS and S-EPS. In addition, the content of S-EPS, LB-EPS, and TB-EPS in the PN of the continuous flow A system was higher than that of O, but this conclusion was exactly the opposite in terms of PS concentration, which was consistent with those in the independent A/O system. Similarly, system A had a higher PN/PS value (0.84), further indicating the structural stability of anaerobic sludge. It was worth noting that compared with A5 and O5, the sludge in the continuous flow A/O reactor was enriched with higher concentrations of HMs (Fig. 8b), with the highest concentration in Set (1923 µg/g), followed by A (1676 µg/g) and O (1363 µg/g). The dominant HMs among them were Zn, Mn, Cu, and Sr, but the content of Mn (374.78 µg/g) decreased by 21.33 % compared to A5.

As shown in Fig. 8c, the abundance of ARGs in the leachate of the continuous flow system was always higher than that in the sludge. The total abundance of ARGs in the A, O, and Set reactors in the leachate was almost the same, about 53.57 log₁₀ copies/L, which was 18.48 % higher than O5 and slightly lower than A5. Furthermore, the abundance of the

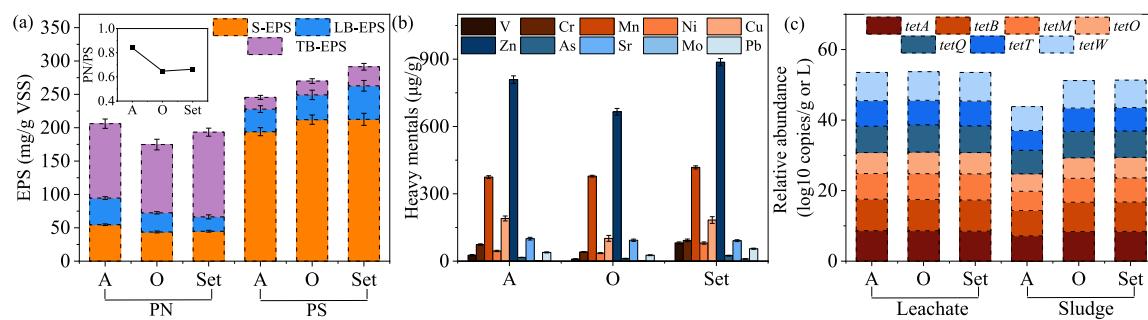


Fig. 8. EPS content (a), HMs concentration (b), and distribution of ARGs (c) in continuous flow A/O reactor.

seven *tet* genes did not vary significantly in each reactor. The abundance of ARGs in continuous flow anaerobic reactor sludge ($43.82 \text{ log}_{10} \text{ copies/L}$) was lower than that in aerobic sludge ($51.21 \text{ log}_{10} \text{ copies/L}$), which was completely opposite to the trend of A5 and O5. The genes with decreased abundance included *tetA*, *tetB*, *tetO*, *tetM*, *tetQ*, *tetT*, and *tetW*.

According to the Venn diagram (Fig. 9a), the total number of species in the three microbial samples was 458, and their unique species were represented as A (298) > Set (297) > O (260), significantly higher than A5 and O5. At the phylum level (Fig. 9b), the dominant microbial species did not change, but their abundance underwent significant changes. For example, the relative abundance of *Pseudomonas* in continuous flow A (46.42 %) was 1.99 times that in A5, thereby enhancing the removal of carbon and nitrogen at a deeper level. Notably, the abundance of *Actinomycota* increased by 188 %, accounting for 10.48 %. Nevertheless, compared with A5, the relative abundance of *Bacteroidota*, *Chloroflexita*, and *Bacillota* slightly decreased. Consistently, these microorganisms also exhibited similar trends of change in the continuous flow of aerobic reactors and O5. At the genus level (Fig. 9c), the abundance of *unclassified_f_Comamonadaceae* and *Nakamurella* in continuous flow anaerobic system (17.86 % and 6.20 %, respectively) was 2.93 and 17.22 times higher than in A5. *Rhodobacter*, which did not appear in A5, proliferated and grew in continuous flow anaerobic system, promoting the cycling and transformation of phosphorus. According to the analysis of sample differences between anaerobic system and A5 (Fig. 9d), *Ferruginibacter* and *Enterococcus* were enriched in A5, but decreased by 242 % and 190 % in continuous flow aerobic system ($p < 0.001$). Microorganisms with doubled relative abundance in the continuous flow aerobic system include *Nakamurella* (6.75 %), *Nitrosomonas* (3.88 %), and *norank_f_Chitinophagaceae* (3.53 %) (Fig. 9e). However, the relative abundance of *Ferruginibacter*, *norank_f_AKYH767*, *Rhodanobacter*, and *norank_o_Saccharimonales* all decreased to varying degrees ($p < 0.001$). Furthermore, the average abundance of *Enterococcus*, which did not exist in O5, reached 3.97 % in continuous flow A, O, and Set.

From Fig. 8, it could be seen that the screening of dominant microbial communities made for a change in the correlation between some ARGs and microorganisms. *Longivirga*, *Thermomonas*, *Nakamurella*, and *Nitrosomonas* were significantly positively correlated with *tetO* and *tetW* ($p < 0.001$), but some were negatively correlated with them, such as *unclassified_o_Sphingobacteriales*, *Agriterricibacter*, and so on. There were some common microbial hosts for *teta*, *tetB*, *tetM*, *tetQ*, and *tetT*, such as *unclassified_f_f_Chitinophagaceae*, *Terrimonas*, *norank_f_AKYH767*, and *Feruginibacter* (positively correlated, $p < 0.001$), but these five *tet* genes were negatively correlated with *unclassified_f_f_paraccaceae*, *Candidatus_Contibacter*, and *norank_f_Hydrogenophilaceae* ($p < 0.001$).

Overall, the MCSB driven continuous flow A-O process demonstrated better performance in controlling TOC, TN, and TP concentrations. The EPS production of the continuous flow A-O reactors was similar to that of A5 and O5, while the sludge in the continuous flow A-O reactors was rich in higher concentrations of HMs, with the main heavy metal species

remaining unchanged. Moreover, due to the continuous operation of the reactor, the total abundance of ARGs in the A, O, and Set reactors in the leachate was almost the same, all lower than A5, while the abundance of ARGs in the sludge was significantly lower than A5 and O5. The microbial richness in the A, O, and Set reactors was significantly higher than A5 and O5, with no change in the dominant microbial species, but their abundance had undergone significant changes. The A-O process, through the synergy of anaerobic degradation and aerobic mineralization, significantly outperformed independent process in ensuring effluent, ARGs control, and operational economy, making it the preferred solution for treating antibiotic containing leachate. Therefore, considering economic and long-term benefits (Text S1), MCSB is a promising material that is expected to promote further development of A-O combination processes.

4. Conclusion

Explored the effects of different concentrations of MCSB on the A/O biological system of leachate. The optimal concentration of MCSB (5 g/L) improved the effluent quality of leachate and stimulated high-level secretion of EPS, while 8 g/L MCSB increased the deposition of HMs in sludge. The high concentration of MCSB (anaerobic reactor) reduced the relative abundance of ARGs in the leachate by 28.22 % (compared to the 5 g/L anaerobic reactor) but increased the reproductive risk of ARGs in anaerobic sludge microorganisms. TCs induction and environmental factor pressure regulated the proliferation of dominant bacterial species in microbial communities, which might alter the composition of ARGs in bacteria through a co selection mechanism. MCSB provided growth space for functional microorganisms and became a vital factor in shaping the spread of ARGs and ARBs by stimulating microbial metabolism, increasing microbial activity, and altering the abundance of functional microorganisms. From a practical application perspective, the continuous flow A-O combination process is the preferred solution that combines treatment efficiency and economy, but it still needs to be validated and optimized in actual leachate. The MBC prepared in this study provides a technical reference for promoting A/O biodegradation of TCs containing leachate and controlling the environmental pressure of ARGs.

CRediT authorship contribution statement

Qiaona Xie: Writing – original draft, Conceptualization. **Kangbo Zhao:** Resources, Investigation. **Yiting Lian:** Visualization, Formal analysis. **Siyi Li:** Methodology, Data curation. **Hongyuan Liu:** Writing – review & editing, Funding acquisition. **Yuyang Long:** Supervision, Project administration. **Chengran Fang:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial

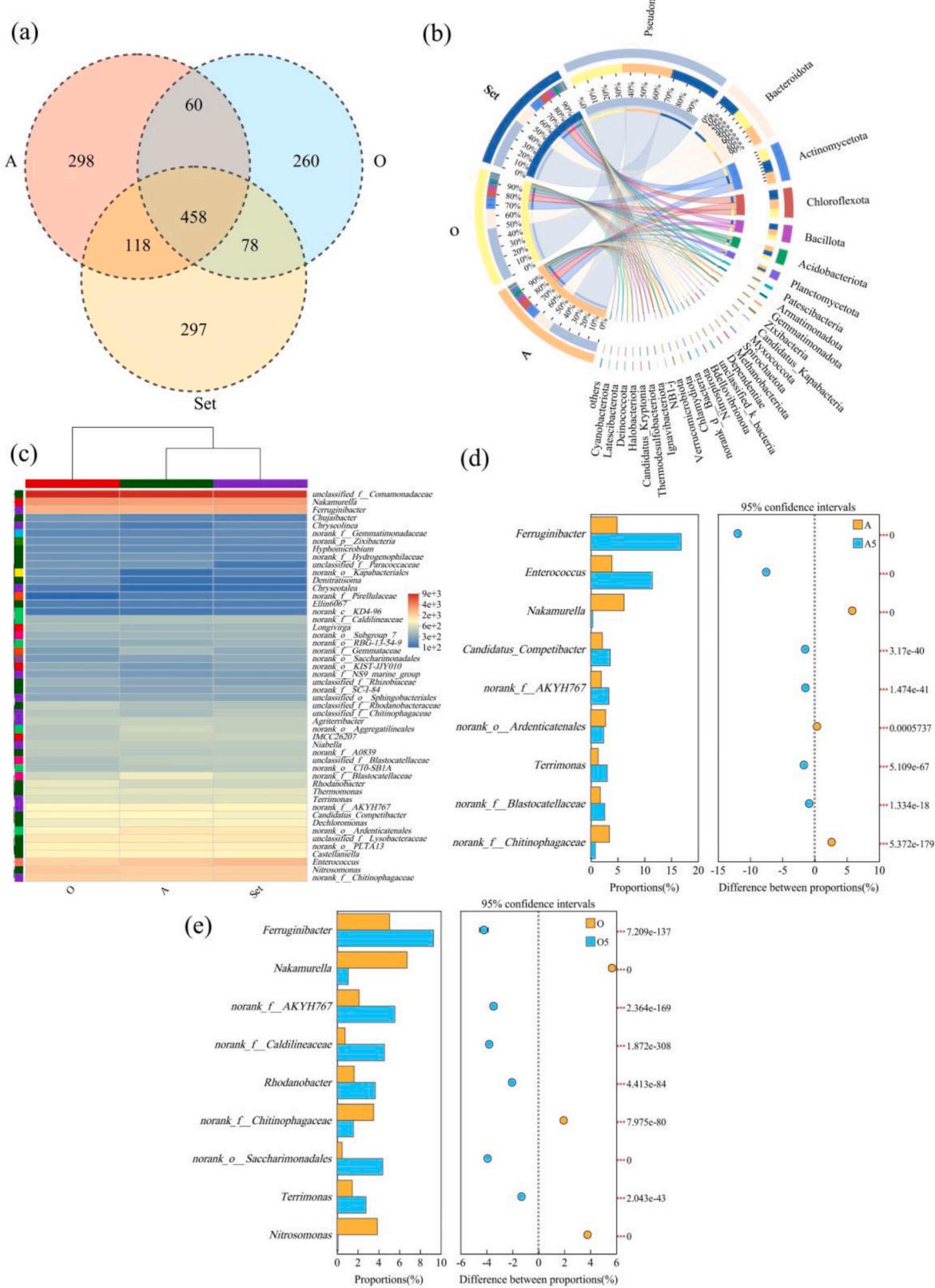


Fig. 9. Venn diagram of species distribution in a continuous flow A/O reactor. The relative abundance of microbial communities at the phylum (b) and genus (c) levels. Wilcoxon rank sum test bar chart at the genus level (95 % confidence interval): (d) A and A5, (e) O and O5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2025.126662>.

Data availability

The data that has been used is confidential.

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