Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

C8-HSL Promotes Activated Sludge Recovery under Combined Environmental Stress: EPS Restructuring, Microbial Community Succession, and Nitrogen and Phosphorus Removal

Lang Xie
Northeast Normal University
Yuan Qi
Northeast Electric Power Design Institute Co.
Jinming Gu
Northeast Normal University
Dandan Zhou
Northeast Normal University
Hongliang Huo
huohl@nenu.edu.cn
Northeast Normal University
Kejian Tian
Ministry of Education, Engineering Research Center of Low-Carbon Treatment and Green Development of Polluted Water in Northeast China

Research Article

Keywords: Quorum sensing, C8-HSL, activated sludge, environmental stress, bioaugmentation

Posted Date: November 23rd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11709214/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

C8-HSL Promotes Activated Sludge Recovery under Combined Environmental Stress: EPS Restructuring, Microbial Community Succession, and Nitrogen and Phosphorus Removal

Lang Xie[a], Yuan Qi[b], Jinming Gu[a], Dandan Zhou[a,c], Hongliang Huo[a*], Kejian Tian[c*]

a School of Environment, Northeast Normal University, Changchun 130117, China
b Northeast Electric Power Design Institute Co. , Ltd. , China Power Engineering Consulting Group, Changchun 130021, China
c Ministry of Education, Engineering Research Center of Low-Carbon Treatment and Green Development of Polluted Water in Northeast China, Changchun 130117, China

Email address of the corresponding author, Hongliang Huo: huohl@nenu.edu.cn; Kejian Tian: tiankj617@nenu.edu.cn

Abstract

Combined environmental stress can severely disrupt microbial activity, extracellular polymeric substance (EPS) stability, and nitrogen and phosphorus removal in activated sludge systems. Promoting functional recovery while maintaining sludge structural stability remains a challenge in biological wastewater treatment. This study investigated the effects of exogenous C8-HSL on sludge recovery under combined stress involving low temperature, low dissolved oxygen, a low carbon-to-nitrogen ratio, and alkaline conditions. The results showed that the addition of 2 μmol/L C8-HSL increased the removal efficiencies of NH_4^+-N, total nitrogen (TN), and total phosphorus (TP) by 19%, 20%, and 14%, respectively. EPS content increased by 94.6%, accompanied by the recovery of tightly bound EPS (TB-EPS) and improved sludge floc stability. Microbial community and functional gene analyses showed that the relative abundances of *Zoogloea*, *Nitrosomonas*, and *Thauera* increased by 151%, 634%, and 118%, respectively. The relative abundances of the genes *hao*, *ppx*, and *pgaC*, which are associated with nitrogen and phosphorus removal and EPS synthesis, increased by 471%, 2.1%, and 173%, respectively. Overall, C8-HSL addition was associated with shifts in microbial community, EPS restructuring, and improved activated sludge

performance under combined environmental stress. These findings provide a potential microbial regulation strategy for maintaining stable wastewater treatment under complex environmental stress.

Keywords

Quorum sensing; C8-HSL; activated sludge; environmental stress; bioaugmentation

1. Introduction

The activated sludge process is one of the most widely used biological wastewater treatment technologies, owing to its well-established operation, high treatment efficiency, and broad applicability. However, the performance of activated sludge systems depends heavily on microbial metabolic activity and community stability, making them susceptible to changes in environmental conditions. Different types of environmental stress can compromise treatment performance by affecting the activity of key functional microorganisms, sludge settling properties, and pollutant transformation processes. For example, at temperatures below 20°C, the contribution of ammonia-oxidizing bacteria (AOB) to nitrogen removal decreases from 88% to 8%[1], indicating a substantial decline in the activity of key functional microbial and nitrogen removal capacity. When dissolved oxygen (DO) in the aerobic zone is maintained at 0.09–0.45 mg/L, the diluted sludge volume index increases from 92.3 mL/g in the inoculated sludge to an average of 210.1 mL/g. The deterioration in sludge settleability is accompanied by a decline in AOB activity and a 21% decrease in ammonium nitrogen removal efficiency[2]. At a low carbon-to-nitrogen ratio (C/N) of 5, TN removal efficiency decreases by 38.8%. This decrease is mainly attributable to insufficient available organic carbon and electron donors, which limit electron transfer during denitrification, thereby reducing nitrate reduction and overall nitrogen removal capacity[3]. When the pH increases from 7.5 to 9.0, the maximum nitrite nitrogen (NO_2^--N) reduction rate decreases from 80 to 42 mg N/(g VSS·h), representing a decline of approximately 47.5%[4]. This indicates that alkaline stress can substantially impair denitrification by inhibiting key denitrifying enzyme activities and disrupting proton gradients and energy metabolism. Therefore, environmental factors such as temperature, dissolved oxygen, carbon availability, and acid–base conditions can affect the structure and biological treatment performance of activated sludge.

However, environmental conditions in full-scale wastewater treatment systems are considerably more complex, with multiple factors often changing simultaneously and jointly affecting system performance. For example, long-term operation of wastewater treatment systems is often accompanied by multiple disturbances, including variations in influent loading, seasonal climatic changes in temperature, and fluctuations in DO. In addition, some wastewaters are characterized by the coexistence of high pH and high nitrogen loading. For instance, nitrate nitrogen concentrations in industrial wastewater at pH 9 can reach 400–1000 mg/L[5-7]. Although single-stressor experiments can clarify the independent effects of individual factors, they cannot fully reproduce the actual stresses encountered by microorganisms under real-world operating conditions. Under combined environmental stress, multiple factors may simultaneously affect microbial metabolism, sludge structural stability, and pollutant transformation processes. Consequently, microbial responses and changes in treatment performance observed under individual stress conditions cannot be directly extrapolated to complex operating environments. Therefore, elucidating the functional responses of activated sludge and the underlying mechanisms under the combined effects of multiple environmental factors is essential for understanding variations in system performance under complex conditions.

Previous studies have shown that supplemental sodium acetate can help maintain nitrogen removal performance to some extent under seasonal cooling and low C/N conditions. When influent NH_4^+-N increased from 26.59 to 39.78 mg/L, effluent NO_3^--N remained at approximately 10 mg/L[5]. Deterioration in sludge settleability caused by low DO can be addressed by restoring aeration to improve oxygen supply, with low-dose NaClO applied when necessary to suppress filamentous bacteria[7]. For wastewater with high pH, acid neutralization is commonly used to adjust the influent pH to an appropriate range[8]. However, these external control measures have inherent limitations. Low temperatures can reduce the efficiency of supplemental carbon source utilization, and active biomass growth may stagnate even when carbon sources and nitrate are sufficiently available, resulting in diminishing returns from further carbon addition. Supplemental carbon sources may also intensify competition for oxygen from heterotrophic bacteria, while increasing operating costs and the carbon burden. In addition, relatively high doses of NaClO may inhibit the metabolism activity of both autotrophic and heterotrophic microorganisms. Although acid neutralization can alleviate high-pH stress, it further increases treatment costs and process complexity.

More importantly, control measures targeting individual operating parameters do not necessarily improve multiple biological processes simultaneously when these processes are constrained by combined environmental stress. Therefore, systematic investigation of activated sludge functional responses under combined environmental stress, together with further elucidation of the underlying microbial regulatory mechanisms, is needed to provide a basis for understanding and improving biological treatment performance under complex operating conditions.

Quorum sensing (QS) is a cell-to-cell communication mechanism through which microorganisms regulate collective behaviors by producing and sensing signaling molecules. Previous studies have shown that QS mediated by N-acyl-homoserine lactone (AHL) can influence microbial collective behavior, EPS secretion, and pollutant removal performance in activated sludge, suggesting that exogenous signaling molecules may provide a means of improving biological treatment performance by modulating microbial communication. Under low-temperature conditions, exogenous AHLs have been used to improve the biological treatment performance of activated sludge[9]. Under high-salinity stress, the addition of 3-oxo-C6-HSL shortened the time required to achieve complete nitrification at 5% salinity from 18 d to 5 d, while TN removal efficiency remained at 79.46% at 8% salinity[10]. Under combined stress involving a low C/N and low DO, the addition of mixed AHLs increased ammonium nitrogen removal efficiency by 10% and TN removal efficiency by 13–25%[11]. Under a severe alkaline shock at pH 10.5, exogenous C4-HSL and C6-HSL significantly enhanced the metabolic activity of activated sludge, reducing the inhibition of specific ammonia oxidation activity by 19–33%[12]. Beyond these stress conditions, AHL-mediated QS has also been investigated under heavy metal and toxic organic compound stress. For example, in activated sludge treatment of wastewater containing cadmium and lead, exogenous AHLs have been reported to promote heavy metal removal and improve nitrogen, phosphorus, and organic matter removal performance, while also affecting EPS secretion, antioxidant responses, and microbial community structure[13]. Collectively, these studies indicate that AHL-mediated regulation has the potential to improve the performance of biological treatment systems under stress, as demonstrated across different types of environmental stress and biological responses.

Although exogenous AHLs can improve activated sludge treatment performance under various environmental stresses, existing evidence is derived primarily from studies of individual stress conditions. Whether these effects and their underlying

mechanisms extend to combined environmental stress remains insufficiently investigated. Accordingly, this study employed C8-HSL as an exogenous signaling molecule to investigate its effects on the recovery of activated sludge treatment performance, EPS restructuring, microbial community succession, and nitrogen metabolism and EPS synthesis under defined combined environmental stress conditions. The potential mechanisms underlying C8-HSL-associated system recovery were also examined. This study aimed to determine whether C8-HSL can promote the structural and functional recovery of activated sludge impaired by combined environmental stress and to further elucidate the microbiological mechanisms underlying this bioaugmentation process. The findings are expected to improve understanding of activated sludge recovery under complex operating conditions and inform the development of microbial regulation strategies for enhancing treatment performance.

2. Materials and Methods

2.1 AHL Standards and Preparation

The AHL standards used in this study were purchased from Sigma-Aldrich (Shanghai, China), including C4-HSL (Cat. No. 09945), 3-oxo-C6-HSL (Cat. No. K3255), C6-HSL (Cat. No. 09926), C8-HSL (Cat. No. 10940), 3-oxo-C8-HSL (Cat. No. 01764), C10-HSL (Cat. No. 17248), C12-HSL (Cat. No. 17247), and 3-oxo-C12-HSL (Cat. No. 09139). Stock solutions were prepared in methanol (HPLC grade, Cat. No. 34860–1 L-R; Sigma-Aldrich) and stored at −20°C until use.

2.2 Reactor Operation

Activated sludge collected from the aeration tank of a wastewater treatment plant in Changchun, China, was used as the inoculum to investigate microbial responses under combined environmental stress. Before being introduced into the experimental reactors, the sludge was acclimated under laboratory conditions for 2 months to improve its adaptation to the synthetic wastewater. In the unstressed sequencing batch reactor (SBR), the mixed liquor suspended solids (MLSS) concentration was maintained at approximately 3200 mg/L, and the effective working volume was 2000 mL. Each operating cycle lasted 10 h and consisted of a 30-min influent feeding phase, a 300-min aeration phase, a 120-min anoxic phase, a 120-min settling phase, and a 30-min effluent discharge phase. Synthetic wastewater was prepared to simulate municipal

wastewater (Table S1), with chemical oxygen demand (COD), NH_4^+-N, and TP concentrations maintained at approximately 296.75, 34.37, and 6 mg/L, respectively (Table S2).

Two sets of experiments were conducted: single-environmental-stressor experiments and combined environmental stress experiments. The single-stressor experiments were designed to investigate changes in AHLs under different stress conditions, whereas the combined-stress experiments were conducted to evaluate the effects of C8-HSL on activated sludge treatment performance, sludge characteristics, and microbial responses. Four individual stress conditions were examined: low C/N, low DO, low temperature, and alkaline conditions. In the low-C/N reactor (A1), the influent composition of the synthetic wastewater was adjusted to maintain a C/N ratio of approximately 4. In the low-DO reactor (A2), a motorized air pump (YM1139-220V, Guangdong, China) and a dissolved oxygen meter (JPB-607A, Shanghai, China) were used to maintain the DO concentration at 0–0.5 mg/L. In the low-temperature reactor (A3), the temperature was maintained at 10℃ using a low-temperature thermostatic bath (LC-DC-0506, Shanghai, China). In the alkaline reactor (A4), an appropriate amount of $NaHCO_3$ was added to the synthetic wastewater to adjust the pH to 8.5–8.8. The combined environmental stress reactor (R) was simultaneously subjected to low temperature (10℃), a low influent C/N ratio (approximately 4), alkaline conditions (pH 8.5–8.8), and low DO (DO < 0.5 mg/L).

2.3 Extraction and Quantification of Endogenous AHLs

AHLs were extracted according to a previously reported method with appropriate modifications[14]. After the SBR had operated stably for 2 h, 200 mL of activated sludge mixed liquor was collected and centrifuged at 4000 rpm for 10 min at 4℃. The supernatant was collected and filtered through a 0.45 μm glass fiber filter. The filtrate was concentrated using an Oasis HLB solid-phase extraction (SPE) cartridge (6 cm^3/200 mg, Waters, USA; Cat. No. WAT106202). The cartridge was conditioned sequentially with 3 mL of methanol and 3 mL of ultrapure water. After sample loading, the cartridge was washed with 3 mL of ultrapure water and dried under nitrogen at 4–5 psi for 30 min. The AHLs were then eluted with 6 mL of methanol. The eluate was concentrated to approximately 0.5 mL under a gentle stream of nitrogen and then evaporated to dryness. The residue was reconstituted in 2 mL of methanol. The resulting solution was stored at −20℃ until analysis.

All AHLs were quantified using ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS; 1260 Infinity II–6470, Agilent, Germany). Chromatographic separation was performed using a ZORBAX Eclipse Plus C18 column (2.1 mm × 50 mm, 1.8 μm, Agilent, Germany; Cat. No. 959757–902). The column temperature was maintained at 40℃, the injection volume was 3 μL, and the flow rate was 0.3 mL/min. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient elution program was as follows: the proportion of mobile phase B was increased from 15% to 70% over 5 min, then increased to 95% over 4 min and maintained at 95% for 4 min, decreased to 15% within 0.1 min, and held at 15% for 4 min for column re-equilibration. The total run time was 17 min. Mass spectrometric detection was performed in positive electrospray ionization (ESI+) mode using multiple reaction monitoring (MRM). The precursor/product ion transitions and associated mass spectrometric parameters for each target AHL are provided in Table S3. Each treatment was analyzed using three biological replicates.

2.4 C8-HSL Bioaugmentation Experiments

The experimental SBR was operated in three phases: a stabilization phase from day 0 to day 5 (R-stable phase), a combined environmental stress phase from day 5 to day 15 involving low temperature, a low carbon-to-nitrogen ratio (C/N), low dissolved oxygen (DO), and alkaline conditions (R-stress phase), and a C8-HSL bioaugmentation phase from day 15 to day 35 (R-C8-HSL phase). Although AHL-mediated QS is concentration-dependent, previous studies have shown that an AHL concentration of 2 μmol/L can accelerate activated sludge performance recovery under environmental stress[15] and increase nitrogen removal rates by enhancing specific oxidation activity[16]. Therefore, C8-HSL was added to the reactor to achieve a final concentration of 2 μmol/L in the reactor. To account for the potential effects of methanol introduced during C8-HSL addition, a methanol control group (R-methanol) was established by adding methanol at a concentration of 0.002% (v/v). Three parallel replicates were established for each group.

2.5 EPS Extraction and Analysis

A 30 mL aliquot of activated sludge mixed liquor was transferred to a 50 mL centrifuge tube and thoroughly mixed, followed by centrifugation at 4000 rpm for 10 min. The supernatant was collected and filtered through a 0.45 μm membrane filter. The filtrate was used to determine soluble EPS (S-EPS). After removal of the supernatant,

the sludge pellet was resuspended in 0.05% NaCl solution preheated to 70°C, and the volume was adjusted to 30 mL. After thorough mixing, the suspension was sonicated at 20 kHz and 50–100 W for 10 min, and then centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was collected and filtered through a 0.45 μm membrane filter to obtain the filtrate for loosely bound EPS (LB-EPS) analysis. After removal of the supernatant, 0.05% NaCl solution was added to restore the original volume. The suspension was then incubated in a 60°C water bath for 30 min and centrifuged at 8000 rpm for 15 min at 4°C. The resulting supernatant was collected for TB-EPS analysis[17, 18].

Protein (PN) and polysaccharide (PS) were analyzed as the two major components of EPS. Polysaccharide content was determined using the phenol–sulfuric acid method[19], while protein content was measured using the Lowry–Folin method[20]. EPS characteristics were further characterized using three-dimensional excitation–emission matrix (3D-EEM) fluorescence spectroscopy using a spectrofluorometer (Hitachi F-2700, Japan). The measurement parameters were as follows: emission (Em) wavelengths ranged from 250 to 550 nm at 2 nm intervals, excitation (Ex) wavelengths ranged from 220 to 550 nm at 5 nm intervals, and the scanning speed was 12,000 nm/min. Scattering effects were removed[21]. After fluorescence data acquisition, regional integration analysis was performed. The zeta potential of the sludge was measured using a zeta potential analyzer (Zetasizer Nano, USA).

2.6 Microbial Community and QS Gene Analysis

Activated sludge samples were collected on day 5 of the stabilization phase, on the final day of the environmental stress phase (day 15), and on the final day of the C8-HSL bioaugmentation phase (day 35). Three parallel sludge samples were collected from each treatment group. The samples were centrifuged at 8000 rpm and 4°C, and the supernatants were discarded. The resulting sludge pellets were collected for metagenomic sequencing. Total genomic DNA was extracted from the sludge samples, and paired-end sequencing libraries were prepared for Illumina sequencing. After quality control, high-quality clean reads were obtained from the raw sequencing data and subsequently assembled into contigs. Open reading frames were predicted from the assembled sequences, and redundant genes were removed to construct a nonredundant gene catalog. Genes were annotated for taxonomy and function using public databases, including NR, KEGG, and eggNOG. Based on the annotated gene profiles, microbial

community composition, functional genes, and metabolic pathways were further analyzed. The raw sequencing data have been deposited in the NCBI Sequence Read Archive under the BioProject accession number PRJNA1542428.

2.7 Other Analytical Methods

COD was determined using the potassium dichromate method. TN was measured using the alkaline potassium persulfate digestion–UV spectrophotometric method, ammonium nitrogen (NH_4^+-N) was determined using the Nessler reagent spectrophotometric method, and TP was measured using the ammonium molybdate spectrophotometric method[22]. All measurements were performed in triplicate.

2.8 Data Analysis

Bar and line charts were generated using Origin 2025. All experiments were conducted in triplicate, and the results are presented as means ± standard deviations. Statistical analyses were performed using SPSS Statistics 26.0. Spearman's rank correlation analysis was used to assess correlations among microbial taxa and between microbial taxa and environmental variables. Correlations were considered strong and statistically significant when $|r| > 0.7$ and $p < 0.05$. Microbial association networks were visualized using Gephi version 0.9.5.

3. Results and Discussion

3.1. Dynamic Changes in AHLs under Different Environmental Stresses

Under unstressed conditions, 10 types of AHLs were detected in the activated sludge, with their concentrations fluctuating within certain ranges and showing no apparent trend of sustained accumulation or decline. Among these, the short-chain AHLs (C4-HSL, C6-HSL, and C8-HSL) were the predominant signal molecules detected in the system, with concentration ranges of 184.84–496.48 ng/g VSS, 99.36–329.18 ng/g VSS, and 5.21–157.06 ng/g VSS, respectively. In contrast, long-chain AHLs (C10-HSL and C12-HSL) and oxo-AHLs were generally present at lower concentrations. The concentration ranges of C10-HSL and C12-HSL were 0–10.30 ng/g VSS and 1.07–2.41 ng/g VSS, respectively. The concentration ranges of the oxo-AHLs (C4-OXO-HSL, C6-OXO-HSL, C8-OXO-HSL, C10-OXO-HSL, and C12-OXO-HSL) were 25.50–54.76, 16.66–46.30, 2.30–72.56, 0.23–7.26, and 1.23–7.89 ng/g VSS,

respectively (Fig. 1a). Previous studies have shown that the levels of C4-HSL, C6-HSL, and C8-HSL in activated sludge systems are positively correlated with biofilm formation. In addition to promoting biofilm formation in sludge aggregates, C8-HSL can also increase the nitrification rate[23]. Therefore, further comparisons of changes in AHL profiles under different environmental stresses should be compared to identify candidate signal molecules for further investigation.

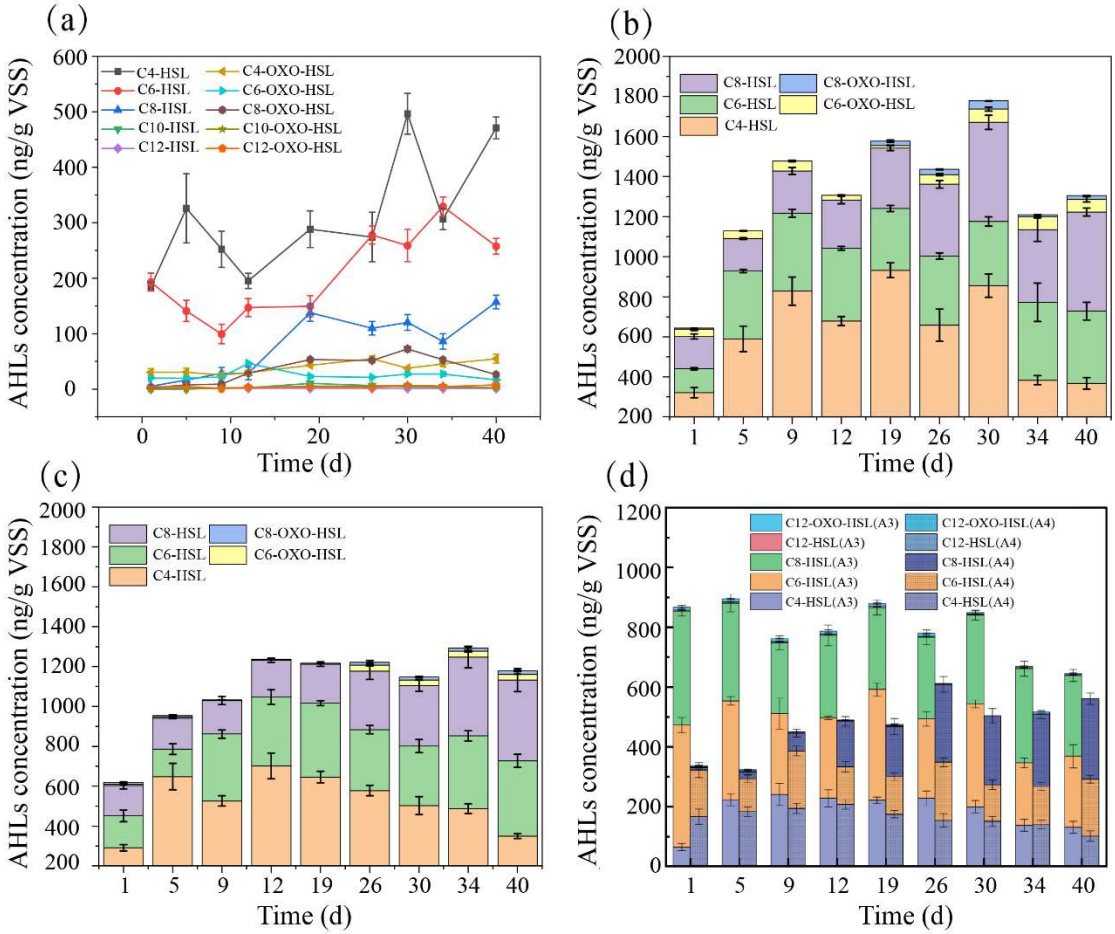


Fig. 1. Dynamic changes in AHLs in activated sludge systems under different operating conditions. (a) unstressed conditions, (b) low C/N stress, (c) low DO stress, (d) low-temperature and alkaline stress.

Under different environmental stress conditions, the AHL profiles of activated sludge were markedly remodeled, but the response patterns differed depending on the stressor, indicating that sludge microbial communities may adjust the composition of QS signal molecules in response to different types of environmental pressure[24]. Under low C/N conditions, the sludge primarily exhibited increases in multiple AHLs. Specifically, C4-HSL, C6-HSL, C8-HSL, and C6-OXO-HSL increased by 36%, 58%, 304%, and 81%, respectively, reaching 423.8 ng/g VSS, 325.83 ng/g VSS, 309.6 ng/g

VSS, and 44.9 ng/g VSS, respectively. In contrast, C8-OXO-HSL decreased by 37.4% to 13.5 ng/g VSS (Fig. 1b). Under low DO conditions, the sludge exhibited increases primarily in non-oxo AHLs concentrations, with C4-HSL, C6-HSL, and C8-HSL reaching 325.4 ng/g VSS, 299.7 ng/g VSS, and 250.3 ng/g VSS, respectively, representing increases of 4.9%, 45.5%, and 226% relative to unstressed sludge (Fig. 1c). Meanwhile, the levels of some oxo-AHLs (C6-OXO-HSL and C8-OXO-HSL) decreased by 37.4% and 73.9%, respectively, reaching 15.5 ng/g VSS and 8.8 ng/g VSS, respectively (Fig. 1c). Under low-temperature conditions, the response shifted from short-chain AHLs toward medium- and long-chain AHLs. C8-HSL, C12-HSL, and C12-OXO-HSL increased to 294.2 ng/g VSS, 3.6 ng/g VSS, and 8.2 ng/g VSS, respectively, representing increases of 283.7%, 137.4%, and 89.1% relative to unstressed sludge. In contrast, C4-HSL decreased by 40% to 185.7 ng/g VSS (Fig. 1d). Under alkaline conditions, C8-HSL and C12-HSL levels increased by 104% and 23%, respectively, reaching 156.7 ng/g VSS and 1.91 ng/g VSS, respectively. Conversely, C4-HSL and C6-HSL levels decreased by 47% and 27%, respectively, reaching 163.1 ng/g VSS and 149.3 ng/g VSS, respectively (Fig. 1d). Overall, AHL profiles did not exhibit a consistent overall increase or decrease under different stress conditions but instead showed stressor-dependent changes. Among the AHLs, C4-HSL and C6-HSL exhibited relatively pronounced responses under low C/N and low DO conditions, whereas C12-HSL increased under low-temperature and alkaline conditions. In contrast, C8-HSL showed an increasing trend under all four individual environmental stress conditions, indicating a relatively consistent response pattern.

Comparison of the changes in signal molecules under different environmental conditions revealed that C4-HSL and C6-HSL primarily responded to low C/N and low DO conditions, whereas C12-HSL primarily responded to low-temperature and alkaline conditions. Although C4-HSL and C6-HSL exhibited pronounced responses to environmental stress, their short acyl side chains may be associated with lower environmental stability, making them susceptible to rapid degradation in response to changes in pH and temperature and thereby potentially limiting their persistence and long-term roles in QS regulation[25, 26]. In addition, C12-HSL exhibited relatively poor stability during stress exposure and was present at low absolute concentrations under some conditions. In contrast, C8-HSL showed a consistent increasing trend under various environmental stresses, including low C/N, low DO, low temperature, and alkaline conditions, with its levels increasing by 304%, 226%, 283.7%, and 104%,

respectively, relative to those in unstressed sludge. Furthermore, C8-HSL is a medium-chain AHL that can diffuse across membranes. Unlike long-chain AHLs, it does not require specific transport proteins, and it may exhibit different diffusion and partitioning behaviors in the sludge matrix. Previous studies have shown that C8-HSL can restore ammonia oxidation activity under low-temperature conditions, promote the expression of functional genes, and reduce oxidative damage[27]. In addition, C8-HSL can promote EPS synthesis and enhance sludge structural stability under quinoline stress[28]. These findings provide a basis for further investigating the relationships between C8-HSL and changes in activated sludge function and structure. Given the consistently increasing trend of C8-HSL under each of the four individual environmental stress conditions examined in this study, C8-HSL was selected for subsequent experiments under combined environmental stress to further evaluate the effects of exogenous C8-HSL supplementation on activated sludge treatment performance, sludge structure, and microbial functional responses.

3.2. C8-HSL Promotes the Recovery of Activated Sludge Performance under Combined Environmental Stress

Combined environmental stress caused a rapid decrease in the MLSS concentration in the reactor from 3,411 to 2,410 mg/L (Fig. 2a), indicating a reduction in sludge concentration and a substantial decrease in sludge biomass. Previous studies have shown that environmental stressors, such as low temperature and low dissolved oxygen, can alter the microbial community structure of activated sludge by inhibiting microbial activity, thereby restricting microbial growth and reducing sludge biomass[29]. Meanwhile, the sludge volume index 30 min (SVI_{30}) decreased to 44.6 during the stress period (Fig. 2a), indicating a marked change in sludge settling characteristics under environmental stress. Further analysis revealed that environmental stress significantly impaired pollutant removal performance in the activated sludge system. Compared with the stable operating period, the TN removal efficiency decreased by 27%, with the average effluent TN concentration reaching 19.9 mg/L (Fig. 2b). The NH_4^+-N removal efficiency decreased by 71%, with the average effluent NH_4^+-N concentration remaining at 22.92 mg/L (Fig. 2c), while the TP removal efficiency decreased by 49%, with the average effluent TP concentration remaining at 3 mg/L (Fig. 2d). These results indicate that combined environmental stress substantially impaired NH_4^+-N and TP removal by activated sludge. This impairment may be associated with the suppression

of metabolic activity in AOB and polyphosphate-accumulating organisms (PAOs), potentially restricting NH_4^+-N oxidation and subsequent nitrogen transformation processes[29, 30] and reduces TP removal efficiency[31].

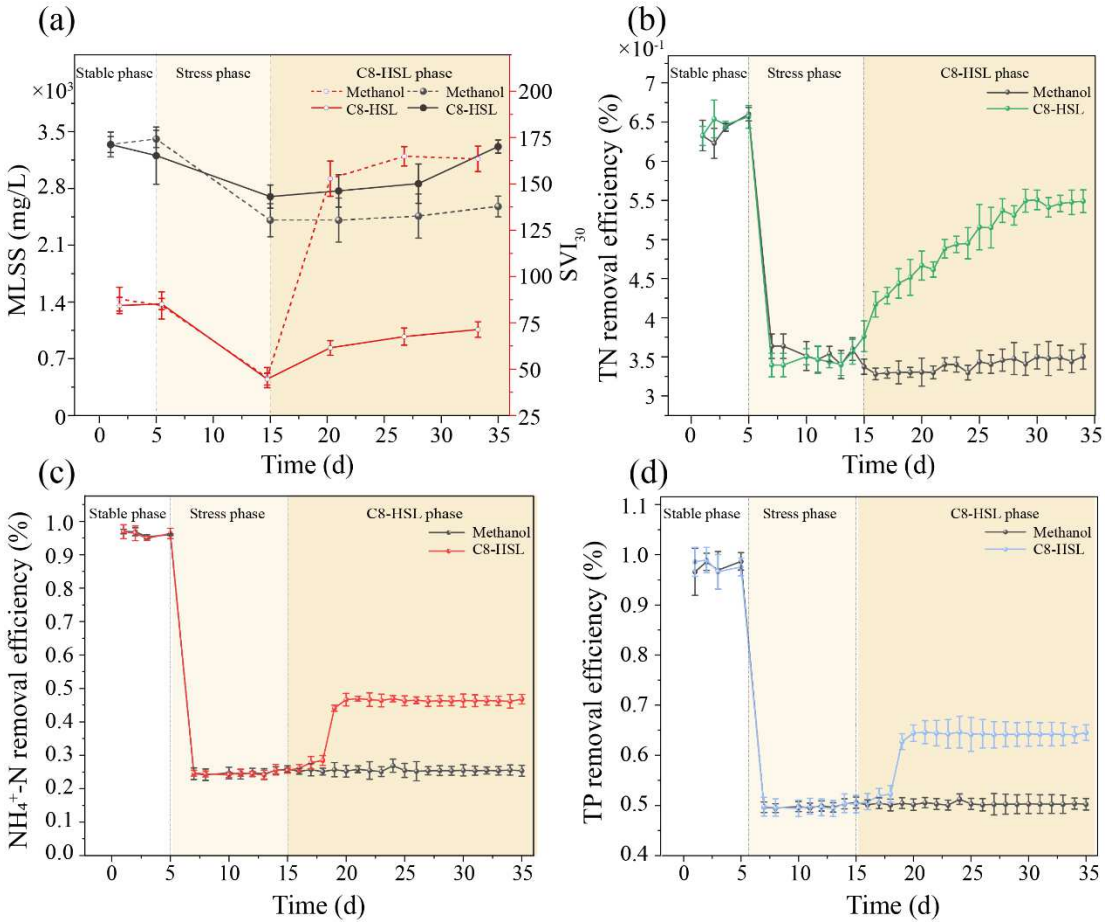


Fig. 2. Characterization of activated sludge and its wastewater treatment performance. (a) MLSS concentration and SVI_{30} of activated sludge, (b) TN concentration, (c) NH_4^+-N concentration and (d) TP concentration.

Following C8-HSL supplementation, both activated sludge biomass and wastewater treatment performance showed varying degrees of recovery. During the C8-HSL bioaugmentation phase, the MLSS concentration gradually recovered and eventually stabilized at 3,349 mg/L, approaching the level observed during the stable operating period. Meanwhile, the SVI_{30} remained within the range of 66–72 mL/g (Fig. 2a). These results indicate that C8-HSL supplementation was accompanied by the recovery of sludge biomass and improvements in settling characteristics, suggesting that exogenous signal molecules may help maintain sludge condition under combined environmental stress. Further analysis revealed that C8-HSL supplementation

effectively promoted the recovery of wastewater treatment performance in the activated sludge system. Compared with the stress period, the removal efficiencies of TN, NH_4^+-N, and TP during the C8-HSL bioaugmentation phase increased by 20%, 19%, and 14%, respectively. The TN and TP removal efficiencies reached 54% and 64%, respectively, indicating improvements in total nitrogen and total phosphorus removal. In contrast, although NH_4^+-N removal improved, its removal efficiency did not recover to the pre-stress level, and the effluent NH_4^+-N concentration was 46% lower than the influent concentration (Fig. 2b–d). This finding indicates that the beneficial effect of C8-HSL on ammonia removal was limited, suggesting that ammonia transformation may be one of the treatment processes that are more difficult to restore under combined environmental stress. A previous study reported that C8-HSL supplementation at 10°C alleviated the inhibition of QS caused by low temperature and restored 59.3% of ammonia oxidation activity, although activity remained below the original level[27]. This finding is consistent with the partial improvement in ammonia removal observed in the present study, suggesting that the effectiveness of exogenous C8-HSL supplementation may depend on the specific stress conditions and microbial functional status. In addition, high pH alters the proportion of free ammonia, and elevated free ammonia concentrations inhibit nitrite-oxidizing bacteria (NOB) and AOB[32]. Therefore, the combined effects of low temperature and alkaline conditions may further constrain ammonia transformation. Regarding phosphorus removal, low dissolved oxygen has been reported to substantially inhibit the activity and energy metabolism of PAOs[33]. This may help explain the changes in TP removal performance under combined environmental stress. However, the specific effects of C8-HSL on phosphorus removal require further examination in light of the EPS, microbial community, and functional gene results of the present study.

In contrast, in the control reactor supplemented with methanol alone, the MLSS concentration did not increase, and the SVI increased to 163 mL/g. Meanwhile, no substantial improvement in pollutant removal performance was observed (Fig. 2). These results indicate that the observed improvements in sludge condition and treatment performance were not attributable solely to methanol addition and were associated with C8-HSL supplementation. Overall, under the combined environmental stress conditions investigated in this study, C8-HSL promoted the recovery of activated sludge biomass and the removal of certain pollutants. The improvement in TN removal was particularly evident, whereas NH_4^+-N removal remained substantially constrained.

Therefore, C8-HSL has potential as an approach for enhancing activated sludge performance, although its underlying mechanisms and effects on different functional processes require further elucidation through subsequent microbiological analyses.

3.3. C8-HSL Promotes EPS Restructuring and Maintains Sludge Floc Stability

The zeta potential of activated sludge reflects the electrostatic interactions between sludge particles and plays an important role in floc aggregation and settling[34]. Under combined environmental stress, the absolute value of the sludge zeta potential increased by 27.7%, reaching 23.1 mV. This change may have been an important contributor to the deterioration in sludge settleability during the stress phase. During the C8-HSL bioaugmentation phase, the absolute value of the zeta potential decreased by 34.8% to 16.4 mV, largely returning to its level during the stabilization phase. In contrast, the methanol-only control group showed no clear trend toward zeta potential recovery (Fig. 3a). These changes indicate that C8-HSL addition was associated with a recovery of the sludge surface charge toward its stabilization-phase level, potentially reducing electrostatic repulsion between particles and promoting microbial aggregation and floc structural recovery[35].

Biofilms can substantially enhance bacterial resistance to environmental stress, and EPS play a key role in microbial aggregate formation and biofilm development[36]. During the combined-stress phase, the total EPS content and TB-EPS content of activated sludge decreased by 43.5% and 54.5%, respectively, reaching 66.1 and 40 mg/g VSS (Fig. 3b,c). The greater decrease in TB-EPS than in total EPS indicates that TB-EPS was particularly responsive to the combined environmental stress conditions investigated in this study. Previous studies have shown that environmental stressors, including low temperature and alkaline conditions, can affect EPS synthesis and composition, thereby altering cell-to-cell adhesion and sludge floc structure and further impairing nitrogen and phosphorus removal[15, 37, 38]. Therefore, the decline in EPS content, particularly the TB-EPS content, observed in this study may be associated with changes in sludge structural stability during the stress phase and may partly explain the marked deterioration in effluent quality under combined environmental stress.

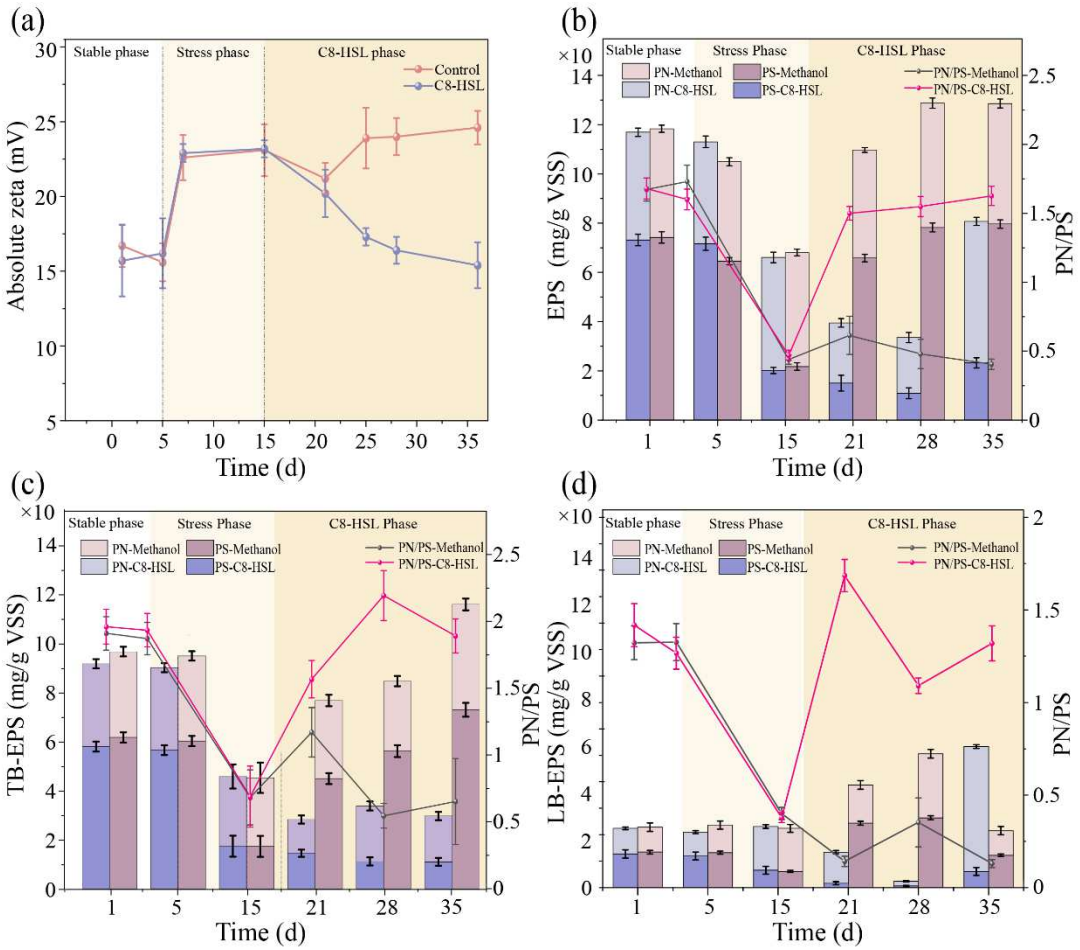


Fig. 3. Measurements of (a) zeta potential, (b) EPS content, (c) TB-EPS content, and (d) LB-EPS content in activated sludge following C8-HSL supplementation.

During the C8-HSL bioaugmentation phase, both total EPS and TB-EPS contents increased, reaching 128.6 and 107.1 mg/g VSS, respectively, on day 35, corresponding to increases of 94.6% and 167.8%. In contrast, neither EPS nor TB-EPS content increased in the methanol-only control group (Fig. 3b,c). This comparison indicates that C8-HSL addition was associated with EPS recovery, with a more pronounced change in TB-EPS, suggesting a stronger promoting effect on the recovery of tightly bound EPS. Previous studies have reported a significant positive correlation between AHLs and EPS during the early stages of biofilm formation, while mature biofilms can provide resistance to adverse environmental conditions[39]. Under low-temperature stress at 15°C, C8-HSL increased EPS content from 92.5 to 234.1 mg/g VSS[40]. The increase in EPS content observed in the present study was comparatively smaller, potentially reflecting the multiple effects of combined environmental stress on microbial physiology and EPS formation. However, the experimental conditions differed between the two studies, and the effects of C8-HSL cannot be directly

compared solely on the basis of changes in EPS content.

In addition, the PN/PS ratio has been reported to be positively correlated with biofilm stability [41]. A higher PN/PS ratio has also been associated with a more positive cell-surface charge and greater cell-surface hydrophobicity[42]. During the C8-HSL bioaugmentation phase, the PN/PS ratios of total EPS, TB-EPS, and LB-EPS increased significantly by 244%, 177%, and 254%, respectively, reaching 1.62, 1.69, and 1.32. No comparable increases were observed in R-methanol (Fig. 3b–d). The increased PN/PS ratios indicate a higher proportion of protein relative to polysaccharides in EPS, with this shift observed in total EPS, TB-EPS, and LB-EPS. Because proteins generally exhibit strong adhesive properties that can enhance microbial interactions and sludge floc stability[43], C8-HSL may have promoted the recovery of protein-enriched EPS fractions, thereby helping maintain sludge floc structural stability.

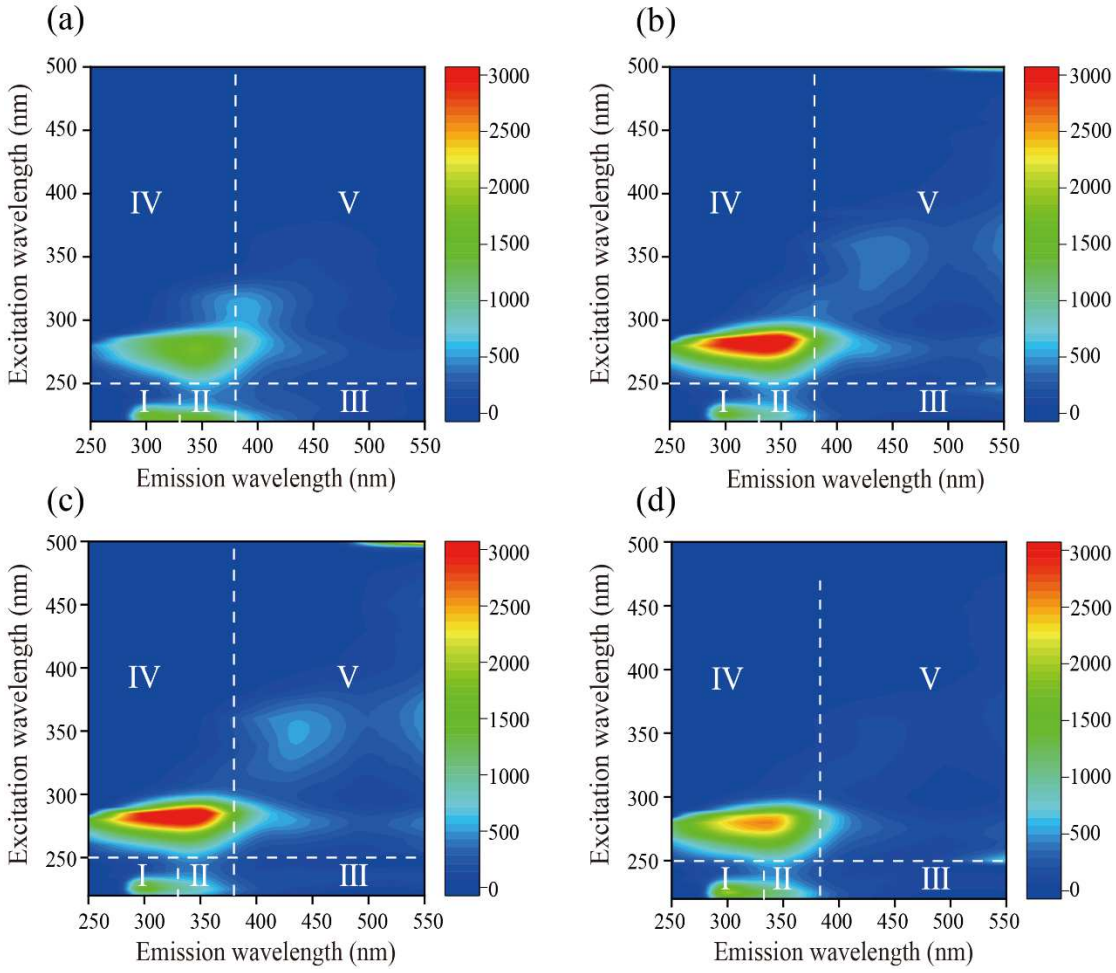


Fig. 4. 3D-EEM fluorescence spectra of the major components of TB-EPS in activated sludge under different conditions. (a) stable phase, (b) combined environmental stress phase, (c) R-methanol, and (d) R-C8-HSL.

Aromatic proteins in TB-EPS are essential for maintaining EPS structural integrity

and sludge settleability[44, 45]. To further characterize changes in EPS composition, 3D-EEM fluorescence spectroscopy was used to characterize the relative distribution of different fluorescence regions in TB-EPS. In the 3D-EEM spectra used to characterize the major components of activated sludge EPS, Regions I (Ex/Em = 200–250/280–300 nm) and II (Ex/Em = 200–250/330–380 nm) were assigned to aromatic proteins. During the combined-stress phase, the relative proportions of aromatic proteins in Regions I and II of TB-EPS were 16% and 21%, respectively, representing decreases of 4% and 5%, respectively, compared with the end of the stabilization phase (Fig. 4a,b and Figure S1). These findings indicate that environmental stress reduced the relative proportions of aromatic proteins in TB-EPS, potentially compromising activated sludge structural integrity. During the C8-HSL bioaugmentation phase, the relative proportions of aromatic proteins in Regions I and II of TB-EPS increased by 8% and 11%, respectively, compared with the combined-stress phase (Fig. 4d and Figure S1). Aromatic proteins not only contribute to microbial aggregation and sludge floc formation but also exhibit relatively high bioavailability and can serve as carbon sources for denitrification, providing substrates for nitrogen transformation[44, 45]. Together with the changes in TB-EPS content and the PN/PS ratio, these results further indicate that C8-HSL addition was associated with the recovery of protein-related components in TB-EPS.

Region IV (Ex/Em = 270–290/320–350 nm) was assigned to soluble microbial by-products in the TB-EPS fluorescence spectrum. During the combined-stress phase, the fluorescence signal assigned to soluble microbial by-products accounted for 42% of the total TB-EPS fluorescence signal, representing an increase of 4% compared with the end of the stabilization phase. During the C8-HSL bioaugmentation phase, this proportion decreased by 10% relative to the combined-stress phase (Fig. 4 and Figure S1). These results indicate that C8-HSL addition was also accompanied by a decrease in the relative contribution of fluorescence associated with soluble microbial by-products in TB-EPS. Previous studies have reported that a decrease in soluble microbial by-products may be associated with enhanced organic matter uptake by activated sludge microorganisms[46]. Overall, C8-HSL addition was associated with EPS restructuring and improvements in indicators associated with sludge floc stability under combined environmental stress, with TB-EPS recovery and relative protein enrichment emerging as important features. These findings provide structural-level evidence that may help explain the recovery of sludge biomass and treatment performance described above. However, whether C8-HSL directly regulates EPS synthesis through specific quorum-

sensing pathways remains to be verified through further analysis of the microbial community and functional genes.

3.4. C8-HSL Modulates Functional Microbial Communities and Gene Profiles

During the C8-HSL bioaugmentation phase, the relative abundances of the putative AHL-responsive bacterial strains *Alexandriicola marinus*, *Histidinibacterium aquaticum*, and *Frigidibacter* sp. RF13 increased by 285%, 626%, and 312%, respectively, compared with the combined-stress phase. In contrast, their relative abundances in the methanol-only control group decreased by 34%, 12%, and 32%, respectively (Fig. 5a). These results indicate that C8-HSL addition was accompanied by changes in the relative abundances of certain microbial taxa in activated sludge, suggesting that exogenous signaling molecules may influence microbial community composition under combined environmental stress. Previous studies have reported that exogenous AHLs can be recognized by LuxI/LuxR-type QS systems in microbial communities, inducing changes in the expression of QS-related genes and thereby regulating functional processes such as EPS synthesis, biofilm formation, nutrient utilization, and environmental adaptation[47]. Accordingly, this study further examined changes in functional microbial communities and their associated genes, focusing on microorganisms and functional genes related to nitrogen removal, phosphorus removal, and protein and polysaccharide synthesis. This analysis aimed to elucidate the potential microbiological mechanisms through which C8-HSL promotes the recovery of activated sludge performance under combined environmental stress.

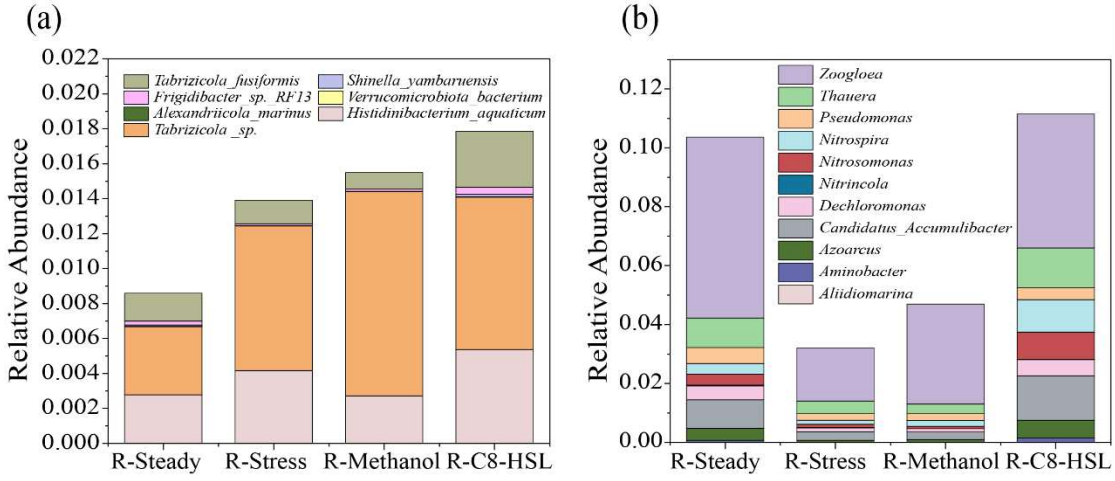


Fig. 5. Effects of C8-HSL on the relative abundances of functional microbial taxa and genes. (a) relative abundances of AHL-responsive taxa, (b) relative abundances of taxa

involved in nitrogen and phosphorus removal and biofilm synthesis.

3.4.1. Enrichment of Nitrogen-Removal-Related Microorganisms and Genes

To further investigate the potential microbiological mechanisms underlying C8-HSL-mediated recovery of nitrogen-removal performance in activated sludge, changes in nitrogen-removal-related microorganisms and their associated functional genes during the bioaugmentation phase were analyzed at both the microbial community and functional gene levels. Previous studies have shown that the phylum Pseudomonadota contains diverse bacteria capable of producing AHLs and participating in QS regulation. Their QS activities may influence microbial interactions and activated sludge treatment performance[28, 48]. In addition, C8-HSL has been reported to increase the relative abundance of Pseudomonadota, thereby improving pollutant removal efficiency[18]. During the C8-HSL bioaugmentation phase, the relative abundance of Pseudomonadota in activated sludge increased by 36% compared with the combined-stress phase, reaching 0.73 (Figure S2). Among the nitrifying microorganisms belonging to this phylum, the relative abundances of the ammonia-oxidizing genus *Nitrosomonas* and the nitrite-oxidizing genus *Nitrospira* increased by 7.34-fold and 7.25-fold, respectively, compared with the combined-stress phase, reaching 0.009 and 0.011 (Fig. 5b). Previous studies have reported that C8-HSL can promote the enrichment of nitrifying bacteria carrying *luxR*, including *Nitrosomonas* and *Nitrospira*, thereby enhancing ammonia oxidation and nitrite oxidation[49]. Thus, the increased relative abundances of *Nitrosomonas* and *Nitrospira* observed in this study suggest that C8-HSL addition may have facilitated the recovery of nitrifying microorganisms under combined environmental stress. Meanwhile, during the C8-HSL bioaugmentation phase, the relative abundances of *amoA*, *amoB*, *amoC*, and *hao* increased by 2.49-fold, 2.19-fold, 1.48-fold, and 5.71-fold, respectively, compared with the combined-stress phase, reaching 0.000016, 0.00002, 0.000026, and 0.00005 (Fig. 6a). Previous studies have reported that *amo* and *hao* genes are involved in nitrification and may be regulated by AHL-mediated QS[50]. The increased relative abundances of these genes suggest an bioaugmentation of the genetic potential for ammonia oxidation in the sludge during the bioaugmentation phase. Together with the observed changes in the *Nitrosomonas* and *Nitrospira* populations, these findings suggest that shifts in nitrifying microbial communities and associated functional genes may have contributed to improved

nitrogen transformation during C8-HSL bioaugmentation.

In addition to nitrifying microorganisms, the relative abundances of several denitrification-related genera also increased during the C8-HSL bioaugmentation phase. Compared with the combined-stress phase, the relative abundances of *Thauera*, *Aliidiomarina*, and *Pseudomonas* increased by 2.18-fold, 0.40-fold, and 0.74-fold, respectively, reaching 0.013, 0.00005, and 0.004 (Fig. 5b). Previous studies have reported that C8-HSL addition is associated with changes in denitrification-related microorganisms and improved TN removal performance, with TN removal efficiency increasing by 36%[18]. In the present study, the increased relative abundances of multiple denitrification-related genera indicate changes in the composition of the denitrification-related community during the C8-HSL bioaugmentation phase, which may be associated with improved TN removal performance. In parallel, the relative abundances of the denitrification-related functional genes *nirK* and *nirS* increased by 3.7% and 23.7%, respectively, compared with the combined-stress phase, reaching 0.00029 and 0.00025 (Fig. 6a). A previous study reported that C8-HSL increased TN removal efficiency by 24.51% through the upregulation of key denitrification-related genes (*nirK*, *nirS*, and *napA*)[51]. Therefore, changes in denitrification-related microbial communities and functional genes provide community-level and genetic evidence that may help explain the improvement in TN removal performance during the bioaugmentation phase. C8-HSL may enhance TN removal by promoting the enrichment of denitrifying microorganisms and key denitrification-related genes.

Notably, the relative abundance of *Nitrincola* decreased by 14% compared with the stable phase, reaching 0.00012 (Fig. 5b). Given that *Nitrincola* is capable of heterotrophic nitrification and aerobic denitrification[52], this change suggests that different nitrogen-removal-related genera responded differently to combined environmental stress and C8-HSL addition. Considering that ammonium nitrogen removal performance had not fully recovered during the bioaugmentation phase, the decline in the relative abundance of *Nitrincola* may represent a community shift warranting further investigation. Further analysis revealed significant co-occurrence patterns between AHL-responsive taxa and nitrogen-removal-related microorganisms ($r = 0.75–1.00$, $p < 0.05$; Table S4). In particular, the denitrification-related genera *Achromobacter* and *Azoarcus*, along with other potential nitrogen-cycling microorganisms, exhibited strong positive correlations with AHL-responsive taxa (Fig. 6b, Table S4). These findings indicate that changes in the relative abundances of AHL-

responsive taxa were consistent with those of several groups of nitrogen-removal-related microorganisms, suggesting potential ecological associations between them. Together with the microbial community and functional gene analyses, these results suggest that quorum-sensing-related processes may be involved in the recovery of nitrogen removal functions under combined environmental stress.

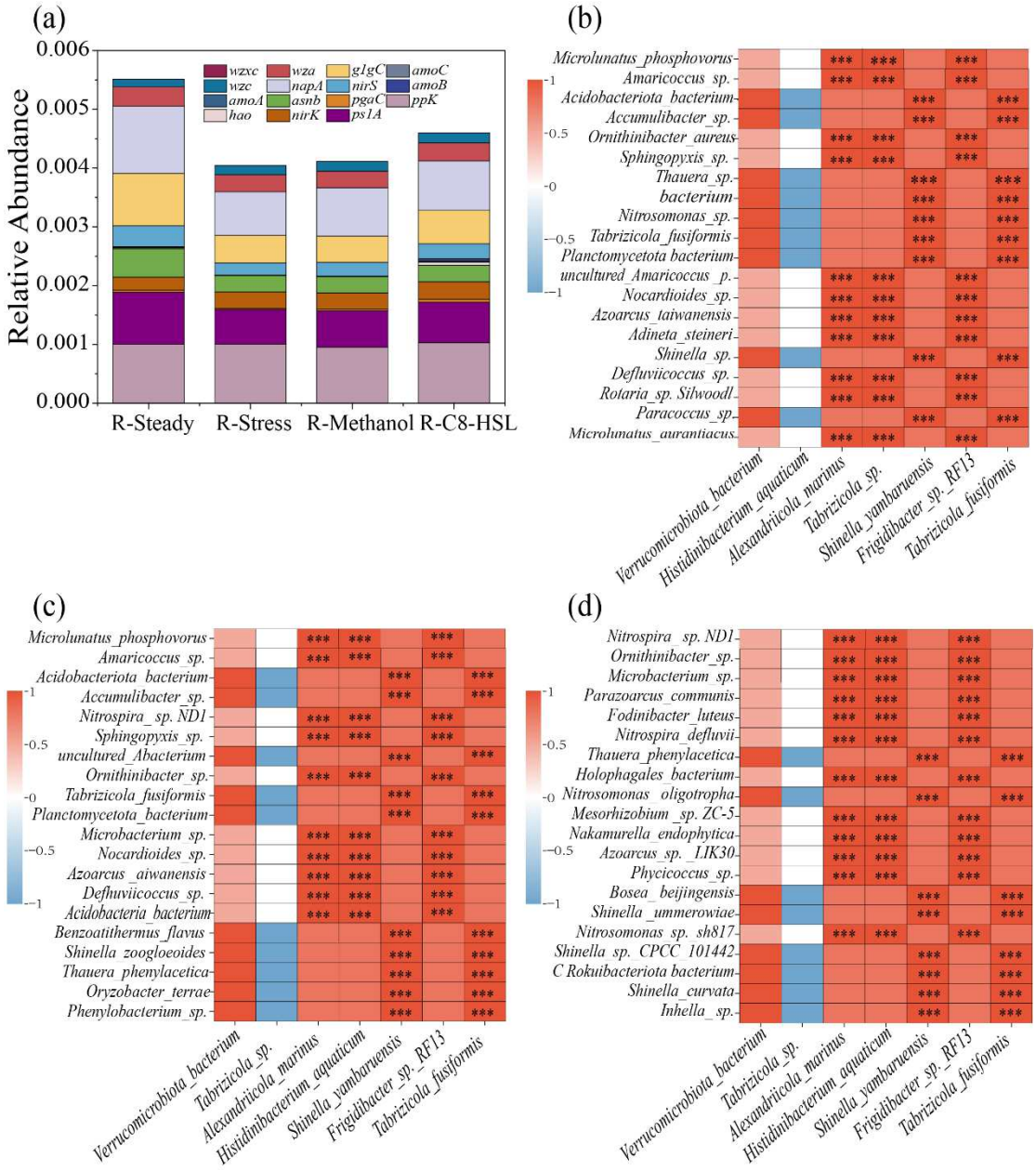


Fig. 6. Changes in the relative abundances of genes involved in nitrogen and phosphorus removal and biofilm synthesis (a), and heatmaps of interspecies correlations between AHL-responsive taxa and the 20 most abundant taxa with potential roles in nitrogen removal (b), phosphorus removal (c), and EPS synthesis (d). Asterisks indicate statistically significant Spearman correlations * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.4.2. Enrichment of Phosphorus-Removal-Related Microorganisms and Genes

During the C8-HSL bioaugmentation phase, the relative abundances of the putative phosphorus-removal-related microorganisms (PAOs) *Aminobacter*, *Candidatus Accumulibacter*, and *Dechloromonas* increased by 2270%, 427%, and 323%, respectively, compared with the combined-stress phase, reaching 0.0015, 0.015, and 0.0054 (Fig. 5b). Previous studies have shown that C8-HSL can increase the relative abundance of *Aminobacter*, thereby improving TP removal efficiency by 39%[18]. In the present study, the relative abundances of all three phosphorus-removal-related microbial taxa increased, suggesting that C8-HSL bioaugmentation was associated with changes in the microbial community involved in phosphorus removal. These changes may have contributed to the improvement in TP removal performance during the bioaugmentation phase. Meanwhile, the relative abundance of the *ppx* gene increased by 2.1% compared with the combined-stress phase, reaching 0.00102 (Fig. 6a). Previous studies have reported that C8-HSL can affect the abundance of genes involved in polyphosphate metabolism, such as *ppx* and *ppk*, and promote phosphorus accumulation in microorganisms[53]. Together with the changes in the abundances of phosphorus-removal-related microorganisms, these results suggest that C8-HSL bioaugmentation may have been accompanied by changes in the genetic potential associated with phosphorus removal. In addition, a decrease in the C/N ratio can intensify competition for ecological niches between PAOs and glycogen-accumulating organisms, thereby affecting enhanced biological phosphorus removal[31]. Low temperatures can also reduce the metabolic activity of key nitrogen- and phosphorus-removal microorganisms in activated sludge, leading to decreased phosphorus release and uptake by PAOs such as *Candidatus Accumulibacter*[31]. These findings provide potential explanations for the limitations on phosphorus removal under combined environmental stress. Although the relative abundances of phosphorus-removal-related microorganisms increased during the C8-HSL bioaugmentation phase, TP removal performance did not recover to the level observed during stable operation. This suggests that changes in the abundance of functional microbial populations may have been insufficient to fully offset the effects of multiple stressors on phosphorus removal.

Furthermore, AHL-responsive taxa were also significantly positively correlated with phosphorus-removal-related microorganisms ($r = 0.70–1.00$, $p < 0.05$; Table S5).

Notably, strong associations were observed with phosphorus-removal-related microorganisms such as *Candidatus Accumulibacter*, *Defluviicoccus*, and *Amaricoccus* (Fig. 6c and Table S5). These findings indicate that changes in AHL-responsive taxa were associated with concurrent changes in some phosphorus-removal-related microorganisms, potentially reflecting ecological relationships among microbial populations under combined environmental stress.

3.4.3. C8-HSL Enriches Protein- and Polysaccharide-Producing Taxa and Functional Genes

To further investigate the potential mechanisms by which C8-HSL promotes microbial aggregation and sludge structural stability, EPS-producing genera and related functional genes were analyzed. During the C8-HSL bioaugmentation phase, the relative abundances of *Zoogloea* and *Azoarcus* increased by 151% and 830%, respectively, compared with those during the combined-stress phase, reaching 0.045 and 0.006, respectively (Fig. 5b). Previous studies have shown that *Accumulibacter*, *Nitrosospira*, *Nitrosomonas*, *Dechloromonas*, *Thauera*, and *Zoogloea* can contribute to extracellular matrix formation through the synthesis of extracellular polysaccharides and proteins, thereby enhancing cell adhesion, biofilm formation, and sludge aggregation[54]. In addition, C8-HSL can enhance the ability of microbial communities to survive under unfavorable environmental conditions by promoting the relative abundance of *Zoogloea*[55]. In the present study, the relative abundance of *Zoogloea* increased significantly, suggesting that C8-HSL may enhance the resistance of activated sludge to combined environmental stress by regulating microbial community succession.

C8-HSL has been reported to participate in the adhesion and maturation stages of biofilm formation by regulating the expression of target genes[56]. Compared with the combined environmental stress phase, the relative abundances of *pslA*, *pgaC*, and *glgC* increased by 17.6%, 173%, and 20.4%, respectively, during the C8-HSL bioaugmentation phase, reaching 0.0006, 0.00005, and 0.0005, respectively (Fig. 6a). The *pslA* gene is an important component of the *Psl* exopolysaccharide biosynthesis gene cluster. Its expression is regulated by AHL-mediated QS, and it is involved in biofilm formation and cell aggregation[57]. The *pgaC* gene encodes a key glycosyltransferase involved in the synthesis of poly-β-1,6-N-acetyl-D-glucosamine, promoting the formation of the extracellular polysaccharide matrix and enhancing

699 microbial adhesion. The *glgC* gene encodes ADP-glucose pyrophosphorylase, a key
700 enzyme in glycogen biosynthesis. Its increased abundance may enhance microbial
701 carbon storage capacity and provide energy to sustain cellular metabolism under stress
702 conditions[58]. These genes are involved in exopolysaccharide biosynthesis and
703 intracellular carbon storage, respectively, and their increased relative abundances
704 suggest changes in the genetic potential associated with these functions during the C8-
705 HSL bioaugmentation phase. Together with the changes in EPS content and the PN/PS
706 ratio described above, these results suggest that C8-HSL bioaugmentation may be
707 associated with remodeling of the extracellular matrix of activated sludge. Furthermore,
708 previous studies have shown that the addition of C12-OXO-HSL under low-
709 temperature stress can regulate the expression of these genes[9]. Considering the
710 increased relative abundances of these genes during the C8-HSL bioaugmentation
711 phase and the increased PS content in EPS, we hypothesize that C8-HSL enhances the
712 microbial potential for exopolysaccharide biosynthesis.
713 Meanwhile, during the C8-HSL bioaugmentation phase, the relative abundances
714 of *wza*, *wzc*, and *asnB* increased by 3.8%, 10%, and 14%, respectively, reaching 0.0003,
715 0.00017, and 0.0008, respectively (Fig. 6a). The *wza* gene is associated with EPS
716 transport and assembly, whereas *wzc* encodes a protein tyrosine kinase involved in
717 regulating EPS chain length and can therefore affect the structural stability of
718 extracellular polysaccharides. The *asnB* gene encodes an enzyme involved in
719 asparagine biosynthesis, potentially providing the material basis for EPS protein
720 components by supporting amino acid biosynthesis and protein metabolism[54].
721 Changes in these genes may therefore reflect shifts in the functional potential associated
722 with extracellular polysaccharide processing and transport, as well as cellular
723 metabolism. Previous studies have shown that C8-HSL can regulate the *floc* gene
724 cluster through QS, thereby promoting biofilm maturation and microbial aggregation[59].
725 Taken together with the changes in the EPS-related genes and the EPS parameters
726 described above, these findings suggest that C8-HSL bioaugmentation may be
727 associated with changes in extracellular matrix composition and improved sludge floc
728 stability. C8-HSL may promote the remodeling of the EPS protective layer, thereby
729 enhancing sludge floc stability and microbial tolerance to environmental stress.
730 To further investigate the mechanisms underlying the enhanced stress tolerance of
731 activated sludge microorganisms following C8-HSL supplementation, we
732 systematically analyzed the potential synergistic relationships between AHL-

responsive taxa and EPS-producing microorganisms. Spearman correlation analysis revealed significant positive correlations between AHL-responsive taxa and EPS-producing taxa, including *Parazoarcus communis* and *Salipiger* sp. (Spearman's r = 0.78–1.00, $p < 0.05$, Fig. 6d and Table S6). Among these taxa, AHL-responsive taxa such as *Frigidibacter* sp. and *Tabrizicola* sp. exhibited strong associations with other taxa. These findings suggest potential ecological associations between AHL-responsive taxa and EPS-producing microorganisms, which may be related to changes in microbial aggregation and sludge structural stability.

4. Conclusions

This study demonstrated that exogenous C8-HSL effectively promoted the recovery of activated sludge systems under combined environmental stress. The addition of 2 μmol/L C8-HSL increased the removal efficiencies of NH_4^+-N, TN, and TP by approximately 19%, 20%, and 14%, respectively. The total EPS content reached approximately 129 mg/g VSS, accompanied by improved sludge floc stability. Analyses of microbial community structure and functional genes further revealed changes in the abundances of EPS-associated microorganisms, nitrogen- and phosphorus-removing microorganisms, and related functional genes during C8-HSL bioaugmentation. Specifically, the relative abundances of *Zoogloea*, *Nitrosomonas*, and *Thauera* increased by 151%, 734%, and 218%, respectively, while the relative abundances of functional genes such as *hao* and *pgaC* increased by 571% and 173%, respectively. Together with the changes in EPS characteristics and the significant positive correlations among different functional microbial taxa, these findings suggest that C8-HSL-mediated signaling may contribute to sludge matrix remodeling, shifts in functional microbial communities, and recovery of treatment performance. The results suggest that C8-HSL-mediated QS may contribute to the multilevel responses and recovery of activated sludge under combined environmental stress, providing experimental evidence for the use of signaling molecules to enhance the stress tolerance of biological wastewater treatment systems.

CRediT authorship contribution statement

Lang Xie: Writing – original draft, Data curation, Conceptualization. Yuan Qi: Methodology, Investigation. Jinming Gu: Formal analysis, Data curation. Dandan Zhou:

Funding acquisition. Hongliang Huo: Writing – review & editing. Kejian Tian: Writing – review & editing.

Acknowledgments

We thanks to the teachers in The Analysis and Testing Center (https://hjxy.nenu.edu.cn/kxyj/cszx/zxjs.htm) for their help. We acknowledge the financial support provided by National Natural Science Foundation of China (No. 52230003).

Data availability

Data will be made available on request.

References

[1] ZHOU W, ZHANG Q, WANG B, et al. Temperature-based strategy for enhanced nitrogen removal in mainstream via selectively strengthening anammox or denitrification [J]. npj Clean Water, 2025, 8(1): 23.

[2] ZHENG Z, WANG J, LI J, et al. Effects of Increasing Dissolved Oxygen on Sludge Bulking and Nitrogen Removal Performance under High Sludge Loading Conditions [J]. Water, Air, & Soil Pollution, 2025, 236(12): 792.

[3] WANG Y, TIAN L, ZHENG J, et al. Enhancing nitrogen removal in low C/N wastewater with recycled sludge-derived biochar: A sustainable solution [J]. Water Research, 2024, 267: 122551.

[4] SHI W, QIU S, HE Z, et al. Nitrifiers and denitrifiers under unconventional acidic/alkaline conditions: Nitrogen transformation, community shift, adaptive mechanisms [J]. Chemical Engineering Journal, 2025, 519: 165622.

[5] CHEN N, WANG X, CHEN S, et al. Managing low C/N municipal wastewater in full-scale IFAS: Interactive effects of acetate dosing and seasonal cooling [J]. Process Safety and Environmental Protection, 2026, 213: 108958.

[6] YAN L, MIAO J, LI M, et al. Removal of nitrate by aerobic denitrification granular sludge under

strongly alkaline and low carbon to nitrogen ratio conditions: Performance and mechanism [J]. Bioresource Technology, 2026, 449: 134362.

[7] LI Z-H, MA Z-B, YU H-Q. Respiration adaptation of activated sludge under dissolved oxygen and hypochlorite stressed conditions [J]. Bioresource Technology, 2018, 248: 171–8.

[8] YAN L, MIAO J, MA Y, et al. Removal of nitrate nitrogen by aerobic denitrification granular sludge under strongly alkaline conditions: Performance and mechanism [J]. Chemical Engineering Journal, 2024, 500: 157153.

[9] GU J, XIE L, ZHOU D, et al. Multivariate analysis study on mechanism of 3-oxo-C12-HSL enhancing low temperature stress resistance of activated sludge [J]. Bioresource Technology, 2027, 464: 135811.

[10] JIANG Y, WANG Y, YUE Z, et al. Overcoming salinity inhibition in biological wastewater treatment: AHLs synchronize aniline mineralization and nitrogen removal [J]. J Hazard Mater, 2026, 503: 141171.

[11] LIU T, XU J, TIAN R, et al. Enhanced simultaneous nitrification and denitrification via adding N-acyl-homoserine lactones (AHLs) in integrated floating fixed-film activated sludge process [J]. Biochemical Engineering Journal, 2021, 166: 107884.

[12] ZHANG J, PENG Y-Z, ZHANG L-H, et al. Improving the resistance of Anammox granules to extreme pH shock: The effects of denitrification sludge EPS enhanced by a fluctuating C/N ratio cultivation on granules [J]. Science of The Total Environment, 2021, 763: 144610.

[13] ZENG T, WANG L, REN X, et al.

The effect of quorum sensing on cadmium- and lead-containing wastewater treatment using activated sludge: Removal efficiency, enzyme activity, and microbial community [J]. Environmental Research, 2024, 252: 118835.

[14] ORTORI C A, DUBERN J-F, CHHABRA S R, et al. Simultaneous quantitative profiling of N-acyl-l-homoserine lactone and 2-alkyl-4(1H)-quinolone families of quorum-sensing signaling molecules using LC-MS/MS [J]. Analytical and Bioanalytical Chemistry, 2011, 399(2): 839–50.

[15] LV L, FENG C, LI W, et al. Accelerated performance recovery of anaerobic granular sludge after temperature shock: Rapid construction of protective barriers (EPS) to optimize microbial community composition base on quorum sensing [J]. Journal of Cleaner Production,

2023, 392: 136243.

[16] TANG X, GUO Y, WU S, et al. Metabolomics Uncovers the Regulatory Pathway of Acyl-homoserine Lactones Based Quorum Sensing in Anammox Consortia [J]. Environmental Science & Technology, 2018, 52(4): 2206–16.

[17] WU J-Y, HUA Z-L, GU L. Iron–Nitrogen Amendment Reduced Perfluoroalkyl Acids' Phyto-Uptake in the Wheat–Soil Ecosystem: Contributions of Dissolved Organic Matters in Soil Solution and Root Extracellular Polymeric Substances [J]. Environmental Science & Technology, 2023, 57(42): 16065–74.

[18] QIN S, DENG L, LIN Y, et al. Quorum sensing signaling molecules enhance the treatment performance of the HN-AD bacteria-Chlorella symbiotic system in MABR [J]. Journal of Environmental Management, 2025, 392:

126783.

[19] DUBOIS M, GILLES K A, HAMILTON J K, et al. Colorimetric Method for Determination of Sugars and Related Substances [J]. Analytical Chemistry, 1956, 28(3): 350–6.

[20] HARTREE E F. Determination of protein: A modification of the lowry method that gives a linear photometric response [J]. Analytical Biochemistry, 1972, 48(2): 422–7.

[21] REN J, CHENG W, WAN T, et al. Characteristics of the extracellular polymeric substance composition in an up-flow biological aerated filter reactor: The impacts of different aeration rates and filter medium heights [J]. Bioresource Technology, 2019, 289: 121664.

[22] WANG B, LI X, CHEN D, et al. Development of an electronic nose to characterize water quality parameters and

odor concentration of wastewater emitted from different phases in a wastewater treatment plant [J]. Water Research, 2023, 235: 119878.

[23] FENG Z, SUN Y, LI T, et al. Operational pattern affects nitritation, microbial community and quorum sensing in nitrifying wastewater treatment systems [J]. Science of The Total Environment, 2019, 677: 456–65.

[24] LV L, WEI Z, LI W, et al. Regulation of extracellular polymers based on quorum sensing in wastewater biological treatment from mechanisms to applications: A critical review [J]. Water Research, 2024, 250: 121057.

[25] YATES E A, PHILIPP B, BUCKLEY C, et al. N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of

935 Yersinia pseudotuberculosis and
936 Pseudomonas aeruginosa [J]. Infect Immun,
937 2002, 70(10): 5635–46.
938 [26] HMELO L R. Quorum Sensing
939 in Marine Microbial Environments [J].
940 Annual Review of Marine Science, 2017,
941 9(Volume 9, 2017): 257–81.
942 [27] CUI B, ZHANG S, LI C, et al.
943 Collapse of quorum sensing networks
944 underlies low-temperature failure of
945 ammonia oxidation [J]. Bioresource
946 Technology, 2026, 439: 133392.
947 [28] LIU S, LI J, SONG J, et al. AHL-
948 Mediated Quorum Sensing Drives Aerobic
949 Granular Sludge Formation and Stability
950 under Quinoline Stress [J]. ACS ES&T
951 Water, 2026, 6(8): 5009–20.
952 [29] WANG Y-C, LV Y-H, WANG S,
953 et al. The role of bacterial communication
954 in activated sludge at low temperatures [J].
955 Cell Reports Physical Science, 2024, 5(9):

102169.

[30] THAKUR I S, MEDHI K. Nitrification and denitrification processes for mitigation of nitrous oxide from waste water treatment plants for biovalorization: Challenges and opportunities [J]. Bioresource Technology, 2019, 282: 502–13.

[31] LI Z, ZHOU P, CUI Q, et al. Nitrogen and phosphorus removal performance and response mechanisms of the integrated fixed-film activated sludge process under low-temperature conditions on plateaus [J]. Bioresource Technology, 2026, 459: 135263.

[32] LI Y, CHEN B, ZHANG X, et al. Elemental sulfur autotrophic partial denitrification (S0-PDN) with high pH and free ammonia control strategy for low-carbon wastewater: From performance to microbial mechanism [J]. Chemical

977 Engineering Journal, 2023, 474: 145419.
978 [33] BI Z, WU J, HUANG Y, et al.
979 Influence of dissolved oxygen on
980 phosphorus removal by polyphosphate-
981 accumulating organisms biofilm:
982 Performance and metabolic response [J].
983 Biochemical Engineering Journal, 2023,
984 199: 109048.
985 [34] LI Z, LU P, ZHANG D, et al.
986 Population balance modeling of activated
987 sludge flocculation: Investigating the
988 influence of Extracellular Polymeric
989 Substances (EPS) content and zeta
990 potential on flocculation dynamics [J]. Sep
991 Purif Technol, 2016, 162: 91–100.
992 [35] SU B, QU Z, SONG Y, et al.
993 Investigation of measurement methods and
994 characterization of zeta potential for
995 aerobic granular sludge [J]. Journal of
996 Environmental Chemical Engineering,
997 2014, 2(2): 1142–7.

998 [36] FULAZ S, VITALE S, QUINN L,
999 et al. Nanoparticle–Biofilm Interactions:
1000 The Role of the EPS Matrix [J]. Trends in
1001 Microbiology, 2019, 27(11): 915–26.
1002 [37] BEN HAMED H, MAINARDIS
1003 M, MORETTI A, et al. Extracellular
1004 polymeric substances (EPS) in sewage
1005 sludge management: A call for
1006 methodological standardization [J]. Journal
1007 of Environmental Management, 2025, 376:
1008 124407.
1009 [38] SHAO L, WANG X, XU H, et al.
1010 Enhanced anaerobic digestion and sludge
1011 dewaterability by alkaline pretreatment and
1012 its mechanism [J]. Journal of
1013 Environmental Sciences, 2012, 24(10):
1014 1731–8.
1015 [39] BAO Y, YANG B, YANG R, et al.
1016 Regulation of microbial activity based on
1017 quorum sensing: Implications for
1018 biological wastewater treatment [J].

International Biodeterioration & Biodegradation, 2025, 199: 106029.

[40] LIU L, XU S, WANG F, et al. Effect of exogenous N-acyl-homoserine lactones on the anammox process at 15 ℃: Nitrogen removal performance, gene expression and metagenomics analysis [J]. Bioresource Technology, 2021, 341: 125760.

[41] SUN H, JU X, WANG H, et al. Ammonia nitrogen affects bacterial virulence and conditional pathogenic bacterial growth by regulating biofilm microbial metabolism and EPS secretion in laboratory scale distribution systems [J]. Science of The Total Environment, 2025, 958: 178150.

[42] ZHANG Y, FANG X, YE J, et al. Effect of free ammonia control strategy on partial nitrification-denitrification in MBR reactor under low C/N conditions:

Performance, SMP and EPS, and microbial community [J]. Journal of Environmental Chemical Engineering, 2025, 13(3): 116273.

[43] XU J, SHENG G-P, MA Y, et al. Roles of extracellular polymeric substances (EPS) in the migration and removal of sulfamethazine in activated sludge system [J]. Water Research, 2013, 47(14): 5298–306.

[44] ZHANG H, QUAN H, ZHOU S, et al. Enhanced performance and electron transfer of sulfur-mediated biological process under polyethylene terephthalate microplastics exposure [J]. Water Research, 2022, 223: 119038.

[45] YANG L, REN Y-X, CHEN N, et al. Organic loading rate shock impact on extracellular polymeric substances and physicochemical characteristics of nitrifying sludge treating high-strength

ammonia wastewater under unsteady-state conditions [J]. RSC Advances, 2018, 8(73): 41681–91.

[46] GAO C, YANG F, TIAN Z, et al. Pathways of inhibition of filamentous sludge bulking by slowly biodegradable organic compounds [J]. Journal of Environmental Sciences, 2025, 150: 104–15.

[47] PAPENFORT K, BASSLER B L. Quorum sensing signal–response systems in Gram-negative bacteria [J]. Nature Reviews Microbiology, 2016, 14(9): 576–88.

[48] CHUGANI S, GREENBERG E P. An evolving perspective on the Pseudomonas aeruginosa orphan quorum sensing regulator QscR [J]. Front Cell Infect Microbiol, 2014, 4: 152.

[49] TAN C H, YEO Y P, HAFIZ M, et al. Functional metagenomic analysis of

quorum sensing signaling in a nitrifying community [J]. npj Biofilms and Microbiomes, 2021, 7(1): 79.

[50] NI Q, CHEN Y, LU L, et al. C4-HSL-mediated quorum sensing regulates nitrogen removal in activated sludge process at Low temperatures [J]. Environmental Research, 2024, 244: 117928.

[51] ZHANG Q, NIE B, YANG C, et al. Boosting domestic wastewater treatment with quorum signal-augmented heterotrophic nitrification-aerobic denitrification bacterial-algal aerobic granular sludge [J]. Journal of Environmental Sciences, 2026, 168: 381–91.

[52] PAN Z, ZHOU J, LIN Z, et al. Effects of COD/TN ratio on nitrogen removal efficiency, microbial community for high saline wastewater treatment based

on heterotrophic nitrification-aerobic denitrification process [J]. Bioresource Technology, 2020, 301: 122726.

[53] LI L, LI J, YU X, et al. N-octanoyl-DL-homoserine lactone-mediated quorum sensing enhances microbial degradation of petroleum hydrocarbons in saline-alkali soils [J]. Biochemical Engineering Journal, 2025, 220: 109768.

[54] AN W, GUO F, SONG Y, et al. Comparative genomics analyses on EPS biosynthesis genes required for floc formation of Zoogloea resiniphila and other activated sludge bacteria [J]. Water Research, 2016, 102: 494–504.

[55] WANG X, JIANG C, WANG D, et al. Quorum sensing responses of activated sludge to free nitrous acid: Zoogloea deformation, AHL redistribution, and microbiota acclimatization [J]. Water Research, 2023, 238: 119993.

[56] CHEN L, LI Y, ZHANG P, et al. Quorum sensing in anaerobic wastewater treatment: Potential microbiological mechanisms and enhancement strategies [J]. Chemical Engineering Journal, 2024, 499: 156150.

[57] HENDIANI S, PORNOUR M, KASHEF N. Sub-lethal antimicrobial photodynamic inactivation: an in vitro study on quorum sensing-controlled gene expression of Pseudomonas aeruginosa biofilm formation [J]. Lasers in Medical Science, 2019, 34(6): 1159–65.

[58] WANG M, LIU Q, KANG X, et al. Glycogen Metabolism Impairment via Single Gene Mutation in the glgBXCAP Operon Alters the Survival Rate of Escherichia coli Under Various Environmental Stresses [J]. Frontiers in Microbiology, 2020, 11: 588099.

[59] WANG X, HUANG Y, XU J, et

al. Exogenous floc-granule replacement regulates particle-size distribution and signaling-associated ecological responses in aerobic granular sludge [J]. Bioresource Technology, 2026, 457: 135039.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.docx
- SupplementaryTables.xlsx