Pol. J. Environ. Stud. Vol. XX, No. X (XXXX), 1-12
DOI: *10.15244/pjoes/211965* ONLINE PUBLICATION DATE: 2025-12-10

Original Research

Influence of AHLs on the Characteristics and Development of Biofilms in a PHBV-Supported SPD System

Shusong Zhang[1, 2, 3#], Huanhuan Ding[1#], Mingfan Si[1], Anqi Yang[1], Peng Xu[2, 3°*]

[1]College of Ocean and Bioengineering, Yancheng Teachers University, Yancheng 224007, China
[2]Jiangsu Key Laboratory for Bioresources of Saline Soil, Yancheng Teachers University, Yancheng 224007, China
[3]Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, YanchenTeachers University, Yancheng 224007, China

Received: 7 July 2025
Accepted: 12 October 2025

Abstract

The application of solid-phase denitrification (SPD) as a system for treating wastewater with biofilms underscores the necessity of developing efficient acclimation strategies to cultivate resilient biofilms. This research examined how N-acyl homoserine lactones (AHLs) influence the characteristics and development of biofilms within a PHBV-supported SPD system. By incorporating five distinct AHLs into the SPD setup, it was found that the addition of 3-oxo-C14-HSL AHL significantly enhanced nitrate removal efficiency. Conversely, the introduction of exogenous C8-HSL and C14-HSL AHLs appeared to hinder the removal of pollutants. The quorum-sensing (QS) mechanism, mediated by AHLs, identified 3-oxo-C14-HSL as the pivotal AHL governing the production of tightly bound extracellular proteins (TB-PN) in the SPD apparatus. The introduction of exogenous 3-oxo-C14-HSL AHL proved to be instrumental in promoting the synthesis of amino acids akin to tryptophan, as well as in the formation of humic substances. This finding highlights the significant impact that this specific N-acyl homoserine lactone (AHL) can have on biological processes. Through the use of confocal laser scanning microscopy (CLSM), it was documented that the QS system, which is mediated by 3-oxo-C14-HSL, plays a crucial role in the formation of bioaggregates. This phenomenon is further characterized by an observable increase in the thickness of biofilms during biological denitrification processes. These findings underscore the importance of AHL-mediated QS in regulating the properties and development of biofilms within SPD systems. The insights gained from this study contribute significantly to the understanding of quorum-sensing mechanisms, thereby enhancing the theoretical framework surrounding this field of research. Furthermore, the practical

# equal contribution
*e-mail: 18705185250@163.com
°ORCID iD: 0000-0002-6400-1171

implications of the study provide valuable recommendations for improving biofilm acclimation strategies in SPD applications, ensuring more effective and efficient biological treatment processes.

Keywords: solid-phase denitrification, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), quorum sensing, extracellular polymeric substances, biofilm

Introduction

A form of cellular communication, commonly referred to as quorum sensing (QS), is employed by bacteria to synchronize the expression of particular genes in response to their population density [1]. This phenomenon is a well-studied mechanism of bacterial intercellular communication. By synthesizing and responding to specific signaling molecules that accumulate, bacteria can modulate their physiological activities and traits, including biofilm development, pathogenicity, sporulation, and luminescence [2]. Since QS was discovered in 1979, numerous studies have been conducted on various bacteria, leading to the identification of a small soluble molecule that functions as a signal for intercellular communication [3]. The molecules in question are frequently referred to as QS signal molecules, or more colloquially, "autoinducers". These autoinducers play a critical role in the population-sensing system, which is fundamentally governed by the interactions and concentrations of these signaling molecules. The ability of these molecules to facilitate communication among bacterial populations is essential for coordinating their collective behavior [4]. In bacterial communities, a variety of signaling molecules have been discovered, which can generally be categorized into three primary types: acyl-homoserine lactones (AHLs) synthesized by Gram-negative bacteria, modified oligopeptides or autoinducible peptides (AIP) frequently utilized by Gram-positive bacteria, and autoinducer-2 (AI-2), which facilitates interspecies communication in both Gram-negative and Gram-positive bacterial strains [5, 6].

In recent years, the significance of population sensing has been increasingly acknowledged by researchers within the realm of environmental engineering. Wastewater treatment systems that focus on biological nitrogen removal harbor a variety of dense microbial communities, manifesting as flocculating agents, biofilms, or granular sludge. Advances in molecular biology techniques, along with enhanced analysis and detection methods, have led to the identification of QS signal molecules across diverse wastewater treatment systems [7]. Themeli et al. disclosed the existence of AHLs as signaling molecules involved in sensing within activated sludge environments [8]. The research conducted by Ren et al. focused on how QS affects the development of aerobic granular sludge. Their findings revealed that the signaling molecules involved in sensing influence the growth of cell attachment, which thereby aids in the granulation of sludge [9]. Biofilm formation is one of the many roles of QS that has been thoroughly examined, given its crucial importance. The initial investigation documenting the link between QS and biofilm formation was performed by Ge et al. [10]. Additionally, it was discovered that the activity of bacteria can be significantly enhanced through the roles of the QS system mediated by AHLs in the process of biological nitrogen removal. Consequently, the QS system mediated by AHLs is vital in regulating the biological nitrogen removal processes within wastewater treatment systems.

QS, which operates through signaling molecules (AHLs), has been shown to play a significant role in regulating various aspects of microbial behavior. Specifically, it impacts the adhesion properties of microorganisms, which are crucial for the initial stages of biofilm formation. Additionally, QS influences the overall thickness of biofilms, affecting their stability and resilience. Moreover, this signaling mechanism is integral to the organization and structuring of microbial communities, leading to the development of complex interactions among different microbial species. Through these processes, QS contributes to the functionality and ecology of microbial populations in various environments. This interaction plays a critical role in maintaining the activity and stability of biofilms, which fosters an optimal setting for the secretion of AHLs and improves the efficiency of sewage treatment processes. Feng et al. highlighted the crucial contributions of N-Octanoyl-L-homoserine lactone (C8-HSL), N-Decanoyl-L-homoserine lactone (C10-HSL), and N-dodecanoyl-L-homoserine lactone (C12-HSL) in shaping microbial proliferation, aggregation, the formation of extracellular polymeric substances (EPS), and particle development, which collectively facilitate enhanced anaerobic ammonia oxidation while lowering ammonia nitrogen levels [11]. Throughout the process of biofilm formation in systems utilizing aerobic granular sludge for treating ammonia and low concentrations of organic wastewater, the presence of AHLs, especially long-chain variants like C10-HSL and C12-HSL, has been observed [12]. These discoveries have prompted additional investigations into the effects of QS on various wastewater treatment systems. Furthermore, research indicates that N-Hexanoyl-L-homoserine lactone (C6-HSL) and C12-HSL could play a role in the metabolism of ammonium oxidation by ammonia-oxidizing bacteria [13]. It has been suggested that the QS mediated by AHLs is vital throughout the biofilm formation process in water treatment, ultimately influencing the effectiveness of wastewater treatment [14].

In summary, the QS system facilitated by AHLs enhances microbial adherence, which is crucial for biofilm development.

The incorporation of AHLs into nitrifying biofilms has the potential to enhance biomass production and facilitate the swift recovery of damaged biofilms [15]. Additionally, the presence of exogenous AHLs markedly boosts bacterial activity [16]. According to Li et al., an autotrophic nitrifying sludge community may be regulated using select AHL molecules [13]. The incorporation of AHLs into the system promoted cell adhesion, nitrification, and sludge granulation. These findings suggest that the efficiency of a wastewater treatment bioreactor can be optimized by selectively adjusting AHL activity. While earlier studies have shown that AHL-mediated QS may influence bacterial behavior, the focus of most research has primarily been on AHLs related to the nitrification process, seldom addressing their implications for heterotrophic denitrification systems [17]. Previous studies have primarily focused on the role of AHLs in the nitrification processes, while their impact on the heterotrophic denitrification, particularly in SPD systems, remains underexplored. Our study addresses this gap by investigating how exogenous AHLs influence biofilm formation, EPS production, and nitrogen removal efficiency in a PHBV-supported SPD system, which relies on heterotrophic denitrifying bacteria. Consequently, the existing literature lacks comprehensive insights into the effects of QS on the heterotrophic denitrification process. Hence, it is crucial to investigate whether the operation of a heterotrophic denitrification system can remain stable due to the influence of AHLs.

The process known as SPD utilizes insoluble biodegradable polymers as a carbon source for biological nitrogen removal [18]. This SPD framework allows for straightforward regulation and management, effectively avoiding the carbon source overdosing typically observed in conventional methods, thereby ensuring stable operation within water treatment systems [19]. Nevertheless, the sluggish rate of denitrification continues to be a significant limitation of the SPD system, hindering its broader application in wastewater treatment. In light of this, we introduce an innovative approach aimed at enhancing the efficiency of the SPD system. We explore whether the addition of AHLs could expedite biofilm development by modifying the behavior of microbial consortia for improved nitrogen removal effectiveness. To evaluate this hypothesis, we employed biodegradable polymers, particularly poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), serving as both a carbon source and a biofilm support within the denitrification framework. Our research thoroughly examines two primary components: (1) the assessment of the feasibility and performance associated with QS regulation in SPD for nitrogen elimination and (2) the characteristics and formation of biofilms within the SPD system when exogenous AHLs are introduced. The outcome of this investigation may present a viable strategy to enhance reactor efficiency by aligning bacterial reactions to environmental stresses, thereby offering a novel approach to improve pollutant removal and reduce the start-up duration of the reactor.

Materials and Methods

Carbon Source, Inoculum, and Wastewater Characteristics

The PHBV pellets utilized in the study, which served a dual purpose as both the carbon source and the carrier, were obtained from Ningbo Tianan Biomaterials Co., Ltd., located in Zhejiang, China. These pellets are characterized by their cylindrical shape and distinct milky white coloration, with dimensions measuring 2 mm in diameter and 3 mm in height. Importantly, they possess a specific surface area of 0.015 m^2/g, which is a critical factor for their effectiveness in various biochemical processes. For the experimental setup, the inoculum was sourced from the effluent of operational PHBV-SPD reactors that are maintained in our laboratory. This particular inoculum has been shown to be more effective at significantly reducing the enrichment period required for denitrifying bacteria, particularly when contrasted with the traditional activated sludge inoculum often used in similar processes. To simulate a realistic wastewater scenario, an artificial wastewater mixture was prepared using sodium nitrate as the sole nitrogen source. This formulation resulted in an initial nitrate concentration of 50 mg/L, providing a controlled environment for subsequent testing and evaluations. The PHBV pellets were added to the synthetic wastewater as the sole carbon source, which is essential for denitrification. Additionally, for optimal microbial activity and growth, several supplementary components were added to the system. These included 10 mg/L of magnesium ions sourced from magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 10 mg/L of phosphate ions (PO_4^{3-}-P) derived from potassium dihydrogen phosphate (KH_2PO_4), 10 mg/L of calcium ions from calcium chloride ($CaCl_2$), 5 mg/L of ferrous ions (Fe^{2+}) originating from ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), along with 1 mL/L of a trace element solution. The formulation of the trace element solution adhered to the preparation methods outlined by Zhang et al., ensuring that the necessary microelements were available for the denitrifying bacteria to thrive [20].

Shake Flask Experimental Setup

The experimental procedure involved utilizing 1,000 mL conical flasks that were filled with synthetic wastewater, accompanied by 20,000 mg of denitrification sludge taken from a laboratory-operated Sequential Batch Reactor. The primary objective of the experiment was to reduce the nitrate concentration

in the synthetic wastewater to below 3 mg/L. The experiment was deemed complete once this target was achieved, indicating that the denitrification process was successful. In the course of the study, four standard AHL reagents were used, which included C8-HSL, N-tetradecanoyl-L-homoserine lactone (C14-HSL), N-(3-Oxotetradecanoyl)-L-homoserine lactone (3-oxo-C14-HSL), and N-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL). These reagents were sourced from Sigma-Aldrich, a reputable supplier based in the United States. To ensure the integrity of microbial activity during the experiments, stock solutions of these compounds were prepared in methanol at millimolar concentrations, thus minimizing the adverse effects that organic solvents can have on microbial processes. These stock solutions were stored at -20°C and were subsequently diluted in sterile water to reach the required concentration. At the start of the reaction, C8-HSL, C14-HSL, 3-oxo-C14-HSL, and 3-oxo-C12-HSL were introduced into the conical flasks (designated as R1-R4). The final AHL concentration within the reactor was set at 500 mmol/L. R5 contained an equal-volume mixture of the four AHLs (C8-HSL, C14-HSL, 3-oxo-C14-HSL, and 3-oxo-C12-HSL), while R6 served as a control with an equivalent volume of methanol added. The entire experiment was conducted at room temperature (25°C). Weekly samples were collected and filtered through a 0.45-μm membrane prior to analysis, which included measuring the concentrations of nitrate and nitrite. The levels of nitrate and nitrite were assessed using ultraviolet spectrophotometry and N-(1-naphthyl)-ethylene diamine photometry, respectively. The experimental methodology adhered to the previously established protocol [21].

Extraction and Analysis of EPS Components and Content

The extraction of loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) was performed following the protocol outlined by Malamis [22]. Initially, a specific quantity of biofilm was manually removed from the surface of PHBV to create a biofilm suspension. This suspension underwent centrifugation at 5,000 r/min for a duration of 15 min at a temperature of 4ºC, with the resulting supernatant being designated as the LB-EPS fraction. To obtain the TB-EPS fraction, a heating technique was utilized. Biofilm samples from the earlier stage were combined with deionized water and subjected to heating in a water bath at 100ºC for one hour. Upon returning to room temperature, the mixture was centrifuged once more under identical conditions, yielding the supernatant corresponding to the TB-EPS fraction. The quantification of extracellular polysaccharides (PS) and proteins (PN) in the LB-EPS and TB-EPS fractions was performed utilizing the methodologies developed by Malamis et al. and Dubois et al., respectively [22, 23]. To determine the concentration of TB-EPS, we calculated it as the aggregate of the tightly bound proteins (TB-PN) and the tightly bound polysaccharides (TB-PS). Conversely, the content of LB-EPS was derived from the sum of the loosely bound proteins (LB-PN) and the loosely bound polysaccharides (LB-PS).

To identify the primary fluorescent substances in EPS, the fluorescence peaks were examined within the three-dimensional fluorescence spectrum. The fluorescence spectrophotometer (F-2700; Hitachi High-Tech Corporation, Japan) was utilized to assess the excitation-emission matrix (EEM) of the EPS, adhering to the procedure outlined by Malamis et al. [22]. The scanning conditions were as follows: a xenon lamp served as the light source, with the excitation wavelength (Ex) varying from 250 to 450 nm in 5 nm increments and the emission wavelength (Em) extending from 300 to 600 nm in increments of 5 nm. A slit width of 5 nm was employed, alongside a scanning speed of 12,000 nm/min.

Analysis of Biofilm Properties

The thickness of the biofilm was evaluated using an inverted microscope (Nikon Ti2000; Japan), with measurements captured through a CCD camera. The adhesins present in the EPS of the biofilms were stained with Thioflavin T, in accordance with the protocol outlined by Srivastava et al. [24]. Digital images for quantification were acquired using confocal laser scanning microscopy (CLSM; Nikon Corporation, Japan). CLSM was also employed to examine cell distribution and assess biofilm thickness, as detailed in the study by Wu et al. [25].

Test for Biofilm Formation Ability

The assessment of biofilm formation was conducted using CLSM, specifically with the UltraVIEW VOX model from PerkinElmer (PE), USA. To enable effective labeling of the biofilms, fluorescein isothiocyanate-concanavalin A (FITC-ConA), sourced from Sigma-Aldrich, USA, was utilized. Prior to the labeling process, the PHBV particles underwent thorough rinsing using a 1× PBS buffer solution. This step was crucial in eliminating any residual surface salts that could interfere with subsequent analyses. Following the rinsing procedure, the PHBV particles were incubated in a solution containing 100 μg/mL of FITC-ConA for 10 min at room temperature. After incubation, the particles were carefully washed three times with the 1× PBS buffer solution to remove any unbound FITC-ConA. Once adequately stained, the PHBV particles were transferred to blood cell culture dishes for further observation. Imaging was conducted using the designated instrument, and the collected images were processed using Imaris software for image reconstruction. Additionally, ImageJ software was used for further processing of the images, allowing for a comprehensive analysis of biofilm formation.

Data Analysis

The mean and standard deviation of the water quality and EPS content were determined from three replicate tests. To assess the relationships among various indices, Pearson's correlation analysis was performed utilizing Origin 8.5 software. A p-value of less than 0.05 was deemed statistically significant.

Results and Discussion

Effects of AHLs on NO_3^--N Removal and System Performance

In this study, the influence of quorum sensing (QS) on the SPD system was examined using various AHLs. Fig. 1 illustrates the performance in nitrate removal and nitrite accumulation. Specifically, the NO_3^--N reduction in the SPD system is depicted in Fig. 1a). During the shake-flask experiment, the introduction of exogenous 3-oxo-C14-HSL (R3) showed the most significant impact on nitrogen removal efficiency, achieving a NO_3^--N removal rate of 95%. This performance markedly surpassed that of the blank control group (R6), with a p-value of less than 0.05. Concurrently, the nitrite concentration remained below 0.1 mg/L, indicating minimal accumulation, as shown in Fig. 1b). In contrast, 3-oxo-C12-HSL (R4), which shares a structural resemblance with 3-oxo-C14-HSL, yielded a nitrate removal rate of 63.7%. By the end of the reaction, only trace amounts of nitrite had accumulated. It is noteworthy that while both AHLs contain a carbonyl substituent at the 3-carbon position, they exhibited differing efficiencies in nitrate removal. The presence of the carbonyl group at this position correlated with nitrogen elimination, yet the denitrification effects varied among the AHLs. The data presented in Fig. 1a) indicate that exogenous C8-HSL significantly inhibited the rate of nitrate removal ($p<0.05$), with a measured nitrate removal rate of 48.4%. The nitrite variation demonstrated that the introduction of C8-HSL, C14-HSL, 3-oxo-C12-HSL, and a mixture within the SPD system resulted in nitrite accumulations of 0.10, 0.20, 0.13, and 0.15, respectively, as shown in Fig. 1b). Each of these values surpassed the nitrite accumulation observed with the exogenous addition of 3-oxo-C14-HSL. Previous research has indicated that exogenous AHLs enhance the microbial breakdown of resistant organic pollutants [26]. Given that the SPD process involves both carbon source degradation and denitrification by microbial populations, it constitutes a sophisticated nitrogen removal mechanism reliant on the collaborative action of various microorganisms [27]. Consequently, the impact of QS on SPD is also considerably more intricate when juxtaposed with traditional denitrification processes. These findings imply that the presence of AHLs facilitates the denitrifying bacteria's more effective utilization of biodegradable carbon substrates, ultimately leading to improved nitrogen removal in the SPD system.

Effects of AHLs on EPS Properties

Evaluation of EPS Production

As illustrated in Fig. 2, the concentrations of TB-EPS and LB-EPS changed with the addition of various AHLs. TB-EPS serves as the principal component of the biofilm's EPS. PS and PN constitute the key elements of extracellular polymers, which play a vital role in enhancing biofilm adhesion, microbial structural development, and the overall formation of biofilms. PS promotes the aggregation of bacterial cells, which boosts their attachment to solid surfaces and aids

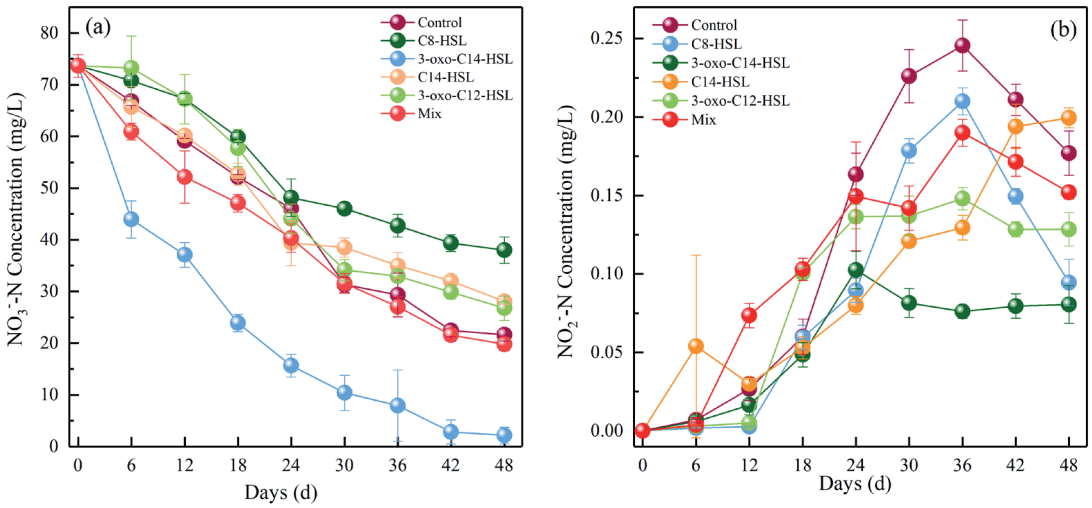

Fig. 1. The effect of different AHLs on nitrate removal rate during the SPD process. a) The concentration of NO_3^-; b) The concentration of NO_2^-. The data are shown as the mean ±SD of 3 replicates.

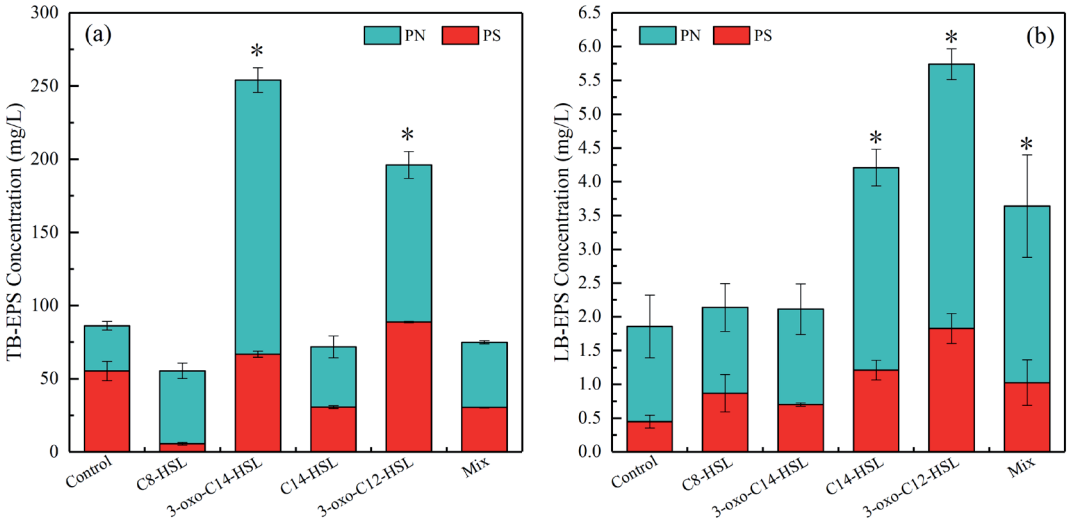

Fig. 2. Exogenous addition of different AHLs, EPS concentrations of biofilm during the whole operation process. a) TB-EPS; b) LB-EPS. *The value indicates a significant difference compared with the blank control. Every experiment was repeated 3 times for the statistical test.

in the establishment of initial microcolonies within the biofilm [28]. Analysis of EPS Production: Prior research has established that PS functions as the structural framework for biofilms, supporting the arrangement of microbial cells within. Additionally, PN networks, primarily comprising cell surface appendages, significantly contribute to the stability and formation of biofilms. These appendages play a crucial role in promoting bacterial migration and their adherence to solid substrates, thereby facilitating biofilm development [29]. Moreover, PN can modify the charge and hydrophobic properties of cell surfaces, which enhances cell adhesion capabilities [30].

The impact of AHLs on EPS characteristics is illustrated in Fig. 2. As shown in Fig. 2a), the external introduction of 3-oxo-C14-HSL led to the highest level of TB-EPS production, reaching 254.2 mg/L. Notably, the concentration of the TB-PN made up over 73.7% of this total, measured at 187.4 mg/L, while the TB-PS concentration was comparatively lower at just 66.8 mg/L. This indicates that 3-oxo-C14-HSL significantly influences TB-PN formation. In biofilm samples with the supplementary addition of 3-oxo-C12-HSL, TB-PN levels were recorded at 107.4 mg/L. These results imply that TB-PN is the predominant component of TB-EPS, and the external application of 3-oxo-C14-HSL notably enhances EPS production, specifically TB-EPS ($p<0.05$). TB-EPS is recognized as the primary variant of EPS, aligning with earlier research findings. Li et al. [13] noted that adding AHLs containing a carbonyl group at the third carbon position facilitated the development of EPS in attached autotrophic nitrifying sludge. The findings in Fig. 2a) additionally indicate a trend toward increased microbial EPS secretion when AHLs are present, with 3-oxo-C14-HSL exhibiting a significantly more favorable effect on TB-EPS compared to other AHLs. Moreover, TB-EPS is rich in hydroxyl and some carboxyl groups, providing numerous sites for cation binding. The relatively relaxed secondary protein structure within TB-EPS further encourages its aggregation, which is vital for the clumping of sludge [31]. The findings from this experiment indicate that incorporating 3-oxo-C14-HSL AHLs improves both biofilm aggregation and stability. When considered alongside the data presented in Fig. 1a), the external supplementation of 3-oxo-C14-HSL exhibited the most significant nitrate removal rate. It can be inferred that the external introduction of 3-oxo-C14-HSL supports the secretion of TB-EPS, which enhances biofilm development and subsequently boosts nitrate removal effectiveness.

The results from our investigation are consistent with previous findings, indicating that the concentration of TB-EPS exceeded that of LB-EPS across all biofilm samples. The introduction of 3-oxo-C12-HSL from an external source led to an increase in LB-EPS by nearly three times compared to the control group. However, the levels of LB-EPS remained considerably low, with a concentration of only 5.75 mg/L (Fig. 2b)). Earlier studies by Feng et al. have pointed out that an overproduction of LB-EPS has detrimental effects on cell attachment and the stability of aggregation [32]. This phenomenon occurs because LB-EPS generally carries a negative charge, which results in electrostatic repulsion, thereby diminishing the capacity for cell attachment. Consequently, a greater quantity of LB-EPS correlates with reduced adhesion of microbial cells. This observation provides an explanation for the diminished nitrate removal rate associated with exogenous 3-oxo-C12-HSL in comparison to the control group.

Examination of 3D-EEM Spectroscopy

The 3D-EEM technique was utilized to assess the EPS contents across various biofilms. Research conducted by Shi et al. categorized the fluorescence regions into five segments, marked from I to V, which corresponded to fulvic acid-like, humic acid-like substances, microbial by-products, and aromatic proteins, respectively [33]. Although the types and locations of EPS peaks were consistent among the different sample groups, the fluorescence intensities exhibited variations. When exposed to different AHLs, two prominent peaks were identified for the EPS (refer to Fig. 3). The initial peak (A), which had excitation/emission wavelengths of 275-300 nm/300-375 nm, indicated the presence of microbial by-products, including proteinaceous substances such as tyrosine-like, tryptophan-like, and other protein-like compounds [28]. The second peak (B), characterized by excitation/emission wavelengths of 325-400 nm/425-475 nm, signaled the existence of humic acid-like materials [34]. The A-peak exhibited a higher fluorescence intensity compared to the B-peak, which was predominantly made up of proteins. This observation aligns with the greater proportion of proteins found within the measured TB-EPS. Predominantly represented by tryptophan-like proteins, the microbial by-product-like proteins reflected in the A-peak indicate that AHLs can influence the chemical composition of EPS by promoting the production of these proteins. Tryptophan-like proteins, being hydrophobic in nature, can enhance the development of more compact structures within the biofilm and collaborate with the aromatic amino acid structures found in EPS to strengthen the biofilm's structural integrity [35]. Among

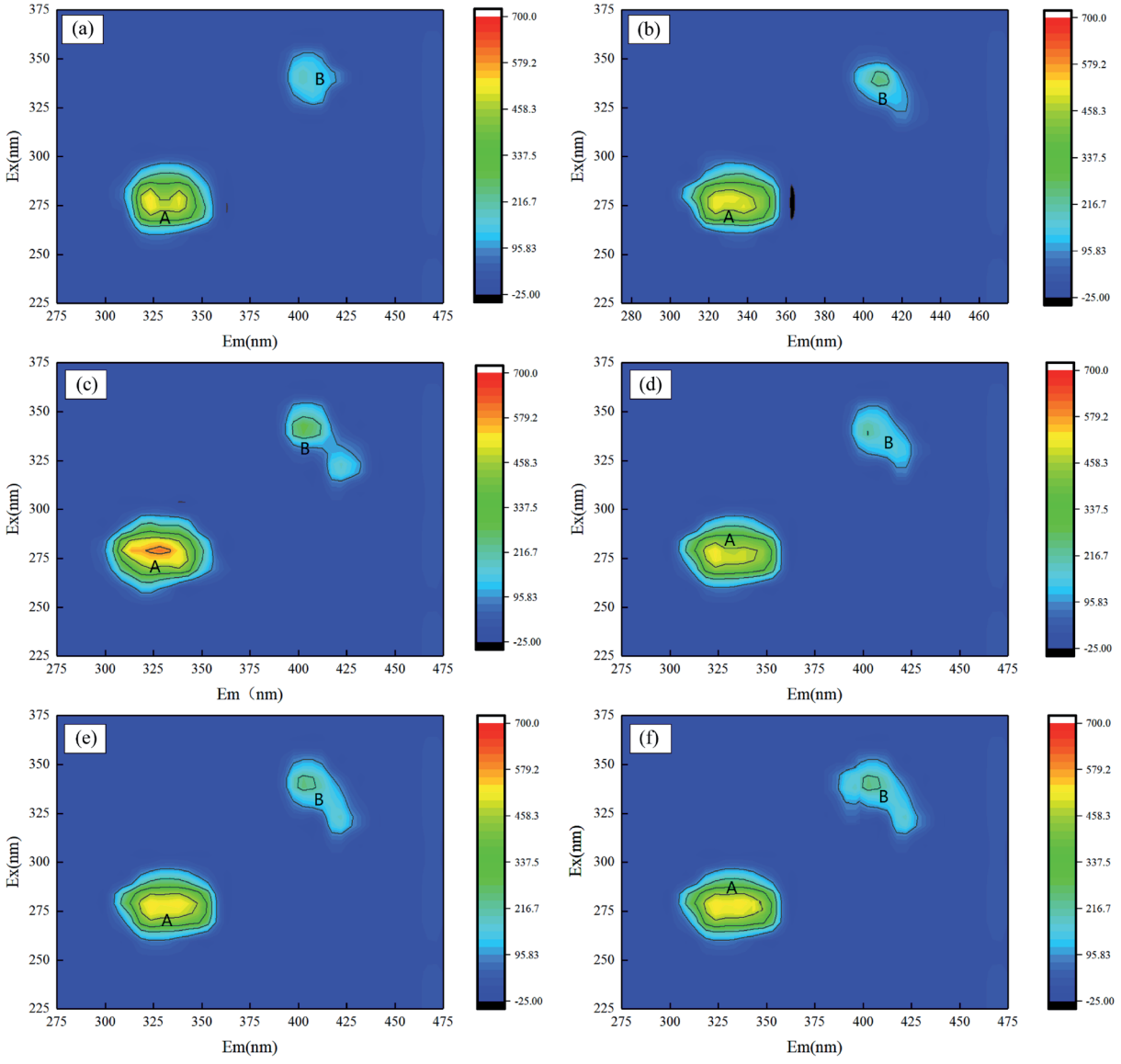

Fig. 3. 3D-EEM spectra of TB-EPS under the condition of adding different AHLs. a) Control; b) C8-HSL; c) 3-oxo-C14-HSL; d) C14-HSL; e) 3-oxo-C12-HSL; f) Mix.

all experimental groups, the addition of the 3-oxo-C14-HSL sample yielded the highest A-peak fluorescence intensity. This leads to the conclusion that 3-oxo-C14-HSL may stimulate the secretion of tryptophan-like proteins in TB-EPS, enabling the detection of pronounced fluorescence peaks for these proteins in R3, with peak intensities significantly surpassing those of the control group. Interestingly, similar results were observed concerning the fluorescence intensity of the B-peak. This implies that the external addition of 3-oxo-C14-HSL can promote the secretion of tryptophan-like proteins as well as enhance the formation of humic acids. Humic acids are crucial for adhesion and serve as electron donors or acceptors, and they also play a minor role in the flocculation and biosorption of EPS, with their effects being highly dependent on their characteristics and concentration [32]. As a result, the introduction of 3-oxo-C14-HSL AHLs resulted in a rise in EPS content, aligning with the observations presented in Fig. 2. The existence of proteins along with humic acids facilitates effective electron transfer, which in turn promotes significant electrochemical activity in EPS biofilms rich in protein-like and humic acid-like components [36]. Previous studies have indicated that the introduction of exogenous AHLs enhances the conductive properties found in the EPS of electroactive biofilms [37]. This is a crucial factor contributing to the superior denitrification efficacy of the externally supplied 3-oxo-C14-HSL within denitrification systems.

Physico-Chemical Characterization of Biofilms

Evaluation of Biofilm Thickness and Adhesive Strength

The characteristics of biofilms in the presence of AHLs within the SPD system were assessed, focusing on biofilm thickness as well as the adhesive strength of EPS. The reversible adhesion stage of biofilm development is characterized by the collaborative interactions among various microorganisms within the community, instead of individual cell activity [38]. Typically, biofilm thickness serves as a physical measure for the quantitative analysis of the early stages of biofilm development [39]. As illustrated in Fig. 4a), the observed biofilm thicknesses were 15.75 μm (R1, C8-HSL), 12.15 μm (R2, C14-HSL), 47.75 μm (R3, 3-oxo-C14-HSL), 10.75 μm (R4, 3-oxo-C12-HSL), and 22.50 μm (R5, Mix). It is evident that AHLs exhibit divergent effects on biofilm thickness. Specifically, the incorporation of 3-oxo-C14-HSL led to a notable increase in biofilm thickness ($p<0.05$). The effects on microbial QS vary among different AHLs. Moreover, the SPD process represents a multifaceted water treatment approach that involves the breakdown of PHBV particles into a soluble carbon source and the subsequent denitrification utilizing this source. In summary, the influence of QS in environmental contexts is intricate, and variations in AHL structures can lead to different impacts on the QS dynamics of the system.

The adhesion force is a critical property of cell-membrane surfaces, serving a key function in facilitating effective attachment and biofilm development [31]. To evaluate the impact of AHLs on adhesion, we measured biofilm adhesion forces under different treatments. As illustrated in Fig. 4b), exogenous C14-HSL did not significantly alter adhesion force compared to the control group ($p>0.05$). Conversely, the introduction of C8-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL, and the Mix yielded a notable increase in biofilm adhesion when contrasted with the control group ($p<0.05$). Among the various treatments, the adhesion force of biofilms was significantly greater in R3 (3-oxo-C14-HSL) ($P<0.05$) than in the other reactors. Microorganisms, which are the main actors in the adhesion process, significantly

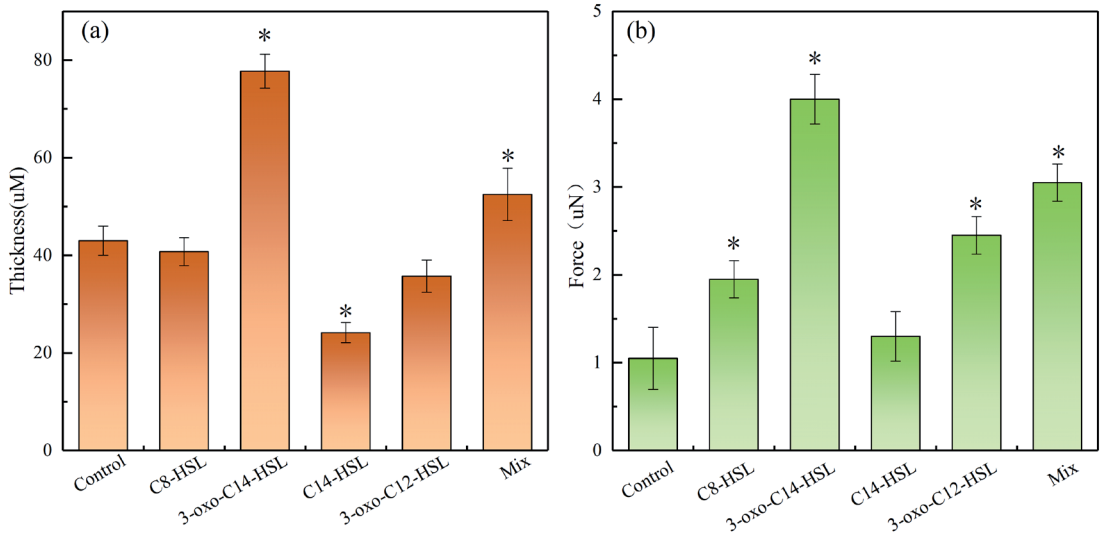

Fig. 4. Biofilm properties, including a) biofilm thickness, b) biofilm adhesion force during biofilm development. *The value indicates a significant difference compared with the blank control. Every experiment was repeated 3 times for the statistical test.

impact this process through their surface free energy and charge characteristics. Most microorganisms exhibit a negative charge, leading to a relatively stable adhesion effect; however, variations in the microorganisms' surface free energy can affect microbial adhesion outcomes [40]. Differences in cell surface components, such as lipopolysaccharides, EPS, extracellular DNA, and proteins, play a role in influencing the surface free energy of these microorganisms [41]. Consequently, the external addition of 3-oxo-C14-HSL stimulated the microorganisms to produce more EPS, thereby enhancing adhesion.

Examination of Biofilm Morphology

Biofilm serves as a mechanism for survival, wherein microorganisms adhere to tissues in the body or to various surface carriers as they grow, thereby enabling them to adjust to environmental changes. This capability facilitates enhanced adaptability to different ecological conditions. In this research, the influence of QS molecules on biofilm development in SPD systems was evaluated through CLSM, as illustrated in Fig. 5.

The results in Fig. 5c) showcase the most intense green fluorescence and the broadest area, signifying

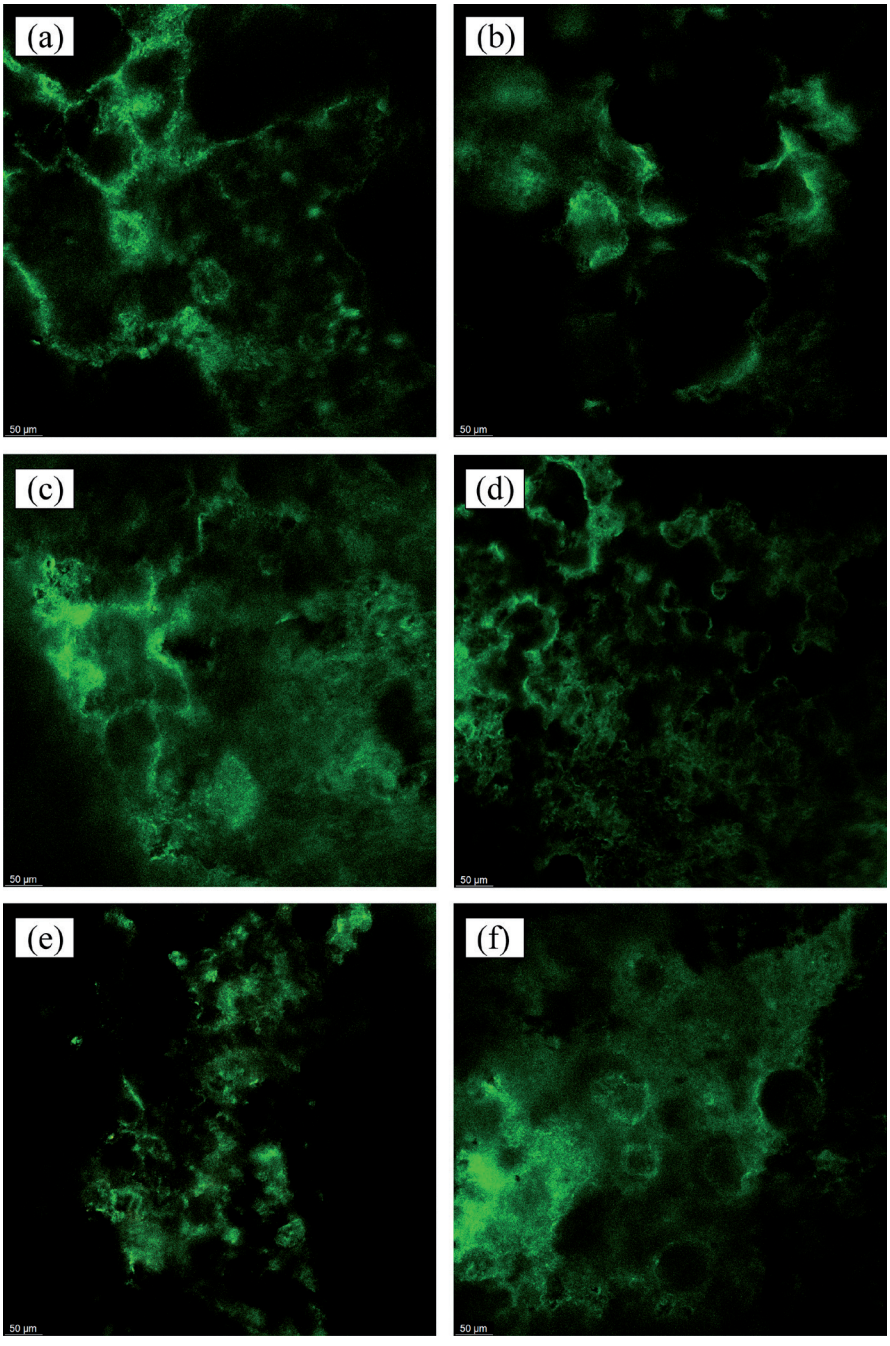

Fig. 5. Effect of AHLs on the biofilm formation process of SPD systems. a) Control; b) C8-HSL; c) 3-oxo-C14-HSL; d) C14-HSL; e) 3-oxo-C12-HSL; f) Mix.

the formation of a robust biofilm characterized by a considerable polysaccharide matrix. These findings imply that 3-oxo-C14-HSL enhances microbial density and stimulates the release of extracellular polysaccharides. Serving as the biofilm's "skeleton", polysaccharides contribute to the cohesiveness among microorganisms [42]. Figs 5b) and 5e) present minimal green fluorescence, suggesting that the addition of C8-HSL and 3-oxo-C12-HSL from external sources diminishes biofilm activity and disrupts the structure compared to the control group. The data depicted in Fig. 2 indicated that the external addition of 3-oxo-C12-HSL raised the levels of negatively charged LB-EPS, which in turn led to electrostatic repulsion and a decrease in cell adhesion capacity, thereby reducing the overall microbial attachment. This observation aligns with the limited presence of the biofilm matrix evidenced in Fig. 5e) within this section.

Perspectives for Future Research

The formation of biofilms represents a multifaceted process governed by QS. There has been considerable focus on how to regulate biofilm formation within wastewater treatment systems. A comprehensive understanding of the key factors that significantly affect biofilm development is crucial for enhancing and fine-tuning control strategies. This article highlights the critical impact of AHL-mediated QS on biofilm formation in suspended-growth systems, particularly regarding bacterial activity, EPS production, and the aggregation of microorganisms. Nonetheless, a number of unresolved challenges remain. The QS mechanism in SPD processes is still inadequately understood, highlighting the need for systematic investigations into the QS networks of relevant microorganisms through the application of emerging biotechnological tools. Techniques in genetic engineering, including metabolic engineering, omics-based methodologies, genome editing, and bioinformatics, offer promising avenues to enhance research on biofilms in the context of wastewater treatment. Furthermore, additional investigation is essential to unravel the metabolism, distribution, and eventual fate of QS signaling molecules produced during wastewater processing in SPD systems, alongside shifts in the microbial community at a molecular scale. From an economic viewpoint, it is important to consider the cost implications and sustained effects of adding exogenous AHLs. Therefore, identifying bacteria that produce AHLs and incorporating them into the biological nitrogen removal process could lead to more cost-effective, energy-efficient, and environmentally sustainable wastewater management practices.

Conclusions

This research thoroughly explored the impact of AHL-mediated QS and the characteristics of biofilms in the SPD system. It was observed that the external introduction of 3-oxo-C14-HSL AHLs within the PHBV-supported SPD system resulted in an increased efficiency of nitrogen removal. Furthermore, it was found that the SPD system yielded greater amounts of TB-PN and TB-PS upon the addition of 3-oxo-C14-HSL, in contrast to other signaling agents and controls. This indicates that 3-oxo-C14-HSL AHLs enhance the release of EPS in biofilms, significantly influencing the development and composition of biofilm EPS. The presence of AHLs also stimulates the generation of tryptophan-like proteins and humic substances. In summary, these results enhance our comprehension of how QS regulates biofilm formation within the SPD system.

Acknowledgments

We extend our gratitude to Editage (www.editage.cn) for their assistance in English language editing during the preparation of this manuscript. This research was funded by the National Natural Science Foundation of China (32201427); the Basic Science (Natural Science) Research Project of Jiangsu Higher Education Institution (22KJD61005); the Basic Research Programme of Yancheng City (YCBK2023012); the Key Laboratory Open Projects at Yancheng Teachers University (JKLBS2019009, JKLBZ2020004); and the Transversal Research Project of Jiangsu (2022320907000240).

Conflict of Interest

The authors affirm that they do not possess any recognized financial conflicts or personal connections that might have seemed to affect the research presented in this document.

Reference

1. SHORT J.D., NERENBERG R. Monitoring bacterial twitter: does quorum sensing determine the behavior of water and wastewater treatment biofilms? Environmental Science & Technology. **46** (4), 1995, **2012**.
2. LI Y.F., LI S.J., KUN Y., GUO R.X., ZHU X., SHI Y.A., HUANG A.X. Antibiofilm mechanism of a novel milk-derived antimicrobial peptide against Staphylococcus aureus by down regulating agr quorum sensing system. Journal of Applied Microbiology. **133** (4), 2198, **2022**.
3. NEALSON K.H., HASTINGS J.W. Bacterial Bioluminescence: Its Control and Ecological Significance. Microbiological Reviews. **43** (4), 496, **1979**.
4. LI D., HUANG T., WU B.R., WANG C.X., JIANG X.P., HUANG S., DAI X.H., CHAI X.L. Facilitating anaerobic digestion of kitchen waste through diatomite-mediated spatial ecological niches construction. Waste Management. **201**, 114792, **2025**.
5. PATEL R.J., NERURKAR A.S. Thauera sp. for efficient nitrate removal in continuous denitrifying moving bed

biofilm reactor. Bioprocess and Biosystems Engineering. **47** (3), 429, **2024**.
6. FLEMMING H.C., WUERTZ S. Bacteria and archaea on Earth and their abundance in biofilms. Nature Reviews Microbiology. **17** (4), 247, **2019**.
7. WATERS C.M., BASSLER B.L. Quorum sensing: cell-to-cell communication in bacteria. Annual Review of Cell and Developmental Biology. **21**, 319, **2005**.
8. THEMELI E., KOUMAKI E., KAPERONIS P., MICHALAKI S., MAMAIS D., NOUTSOPOULOS C., MALAMIS S. Development of an integrated fixed-film activated sludge (IFAS) reactor treating landfill leachate for the biological nitrogen removal through nitritation-denitritation. Journal of Environmental Management. **365**, 121709, **2024**.
9. REN T.T., YU H.Q., LI X.Y. The quorum-sensing effect of aerobic granules on bacterial adhesion, biofilm formation, and sludge granulation. Applied Microbiology and Biotechnology. **88**, 789, **2010**.
10. GE S.M., YANG H.T., LI Y.R., CHEN X.H., YANG R.X., DONG X.J. Mitigation of biofouling in membrane bioreactors by quorum quenching bacteria during the treatment of metal containing wastewater. Environmental Science and Pollution Research. **31**, 32126, **2024**.
11. FENG Z.L., WU Y.H., SHI Y.H., XIAO L.W., WU G. Successful startup of one-stage partial nitritation and anammox system through cascade oxygen supply and potential ecological network analysis. Science of The Total Environment. **696**, 134065, **2019**.
12. CHEN H., LI A., CUI C.W., MA F., CUI D., ZHAO H.P., WANG Q.L., NI B.J., YANG J.X. AHL-mediated quorum sensing regulates the variations of microbial community and sludge properties of aerobic granular sludge under low organic loading. Environment International. **130**, 104946, **2019**.
13. LI A.J., HOU B.L., LI M.X. Cell adhesion, ammonia removal and granulation of autotrophic nitrifying sludge facilitated by N-acylhomoserine lactones. Bioresource Technology. **196**, 550, **2015**.
14. CHEN Y.C., DING K., ZHANG Y.M., ZHENG J.J., JIANG M.J., WANG D.Q., ZHANG X.H., HUANG X.W., ZHOU L.J., LI H.X. Enhancing biofilm formation in the hydrogen-based membrane biofilm reactor through bacterial Acyl-homoserine lactones. Frontiers of Environmental Science &Engineering. **18** (11), 142, **2024**.
15. BATCHELOR S.E., COOPER M., CHHABRA S.R., GLOVER L.A., STEWART G.S., WILLIAMS P., PROSSER J.I. Cell density-regulated recovery of starved biofilm populations of ammonia-oxidizing bacteria. Applied And Environmental Microbiology. **63** (6), 2281, **1997**.
16. TANG X., LIU S.T., ZHANG Z.T., ZHUANG G.Q. The release and impacts of AHLs within anammox cultures for bacterial communication were identified. Chemical Engineering Journal. **273**, 184, **2015**.
17. ZHANG G.S., ZHANG F., DING G., LI J., GUO X.P., ZHU J.X., ZHOU L.Q., CAI S.C., LIU X.L., LUO Y.M., ZHANG G.F., SHI W.Y., DONG X.Z. Acyl homoserine lactone-based quorum sensing in a methanogenic archaeon. ISME Journal. **6**, 1336, **2012**.
18. HIRAISHI A., KHAN S.T. Application of polyhydroxyalkanoates for denitrification in water and wastewater treatment. Applied Microbiology and Biotechnology. **61**, 103, **2003**.
19. ASLAN S., TURKMAN A. Nitrate and pesticides removal from contaminated water using biodenitrification reactor. Process Biochemistry. **41**, 882, **2006**.
20. ZHANG S.S., SUN X.B., FAN Y.T., QIU T.L., GAO M., WANG X.M. Heterotrophic nitrification and aerobic denitrification by Diaphorobacter polyhydroxybutyrativorans SL-205 using poly(3-hydroxybutyrate-co-3-hydroxyvalerate) as the sole carbon source. Bioresource Technology. **241**, 500, **2017**.
21. ZHANG S.S., SUN X.B., WANG X.M., QIU T.L., GAO M., SUN Y.M., CHENG S.T., ZHANG Q.Q. Bioaugmentation with Diaphorobacter polyhydroxybutyrativorans to enhance nitrate removal in a poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-supported denitrification reactor. Bioresource Technology. **263**, 499, **2018**.
22. MALAMIS S., ANDREADAKIS A. Fractionation of Proteins and Carbohydrates of Extracellular Polymeric Substances in a Membrane Bioreactor System. Bioresource Technology. **100** (13), 3350, **2009**.
23. DUBOIS M., GILLES K.A., HAMILTON J.K., REBERS P.A., SMITH F. Colorimetric method for determination of sugars and related substances. Analytical Chemistry. **28** (3), 350, **1956**.
24. SRIVASTAVA A., RUSHIKESH S.A., GUPTA B., CHANDRA S.T., GUPTA A.K. Schwarzite-based 3D-printed carriers for enhanced performance of sequencing batch biofilm reactor (SBBR) for wastewater treatment. Journal of Environmental Chemical Engineering. **12** (1), 111794, **2024**.
25. WU X.Y., CHEN Z.X., LV Z.P., ZHANG L.N., XIN F.X., LI Y., LIU G.N., DONG W.L., WEI P., JIA H.H. Enhanced chloramphenicol-degrading biofilm formation in microbial fuel cells through a novel synchronous acclimation strategy. Journal of Cleaner Production. **317**, 128376, **2021**.
26. LV L.Y., FANG C.D., LI W.G., ZHANG G.M., WANG P.F., REN Z.J. Exogenous N-acyl-homoserine lactones promote the degradation of refractory organics in oligotrophic anaerobic granular sludge. Science of The Total Environment. **761**, 143289, **2021**.
27. LIU S.J., LI Y., LU L.L., HUANG G.R., CHEN F.M. Efficient nitrogen removal from municipal wastewater using an integrated fixed-film activated sludge process in a novel air-lifting loop reactor: A pilot-scale demonstration. Journal of Environmental Management. **360**, 121108, **2024**.
28. ZHANG B., LENS P.N., SHI W.X., ZHANG R.J., ZHANG Z.Q., GUO Y., BAO X., CUI F.Y. The attachment potential and N-acyl-homoserine lactone-based quorum sensing in aerobic granular sludge and algal-bacterial granular sludge. Applied Microbiology and Biotechnology. **102** (2), 5343, **2018**.
29. ZHANG J.C., HE J., ZHAI C.H., MA L.Z., GU L.C., ZHAO K. Effects of PslG on the surface movement of Pseudomonas aeruginosa. Applied and Environmental Microbiology. **84** (13), 219, **2018**.
30. ZHANG G.Y., NIE Q., LIU B.R., GUO H.F., XU Y.H. Effect of N-hexanoyl-L-homoserine lactone mediated quorum sensing on degradation of phthalic acid wastewater in microbial fuel cells. Journal of Environmental Chemical Engineering. **13** (5), 117587, **2025**.
31. XIONG F.Z., ZHAO X.X., WEN D.H., LI Q.L. Effects of N-acyl-homoserine lactones-based quorum sensing on biofilm formation, sludge characteristics, and bacterial community during the start-up of bioaugmented reactors. Science of The Total Environment. **735**, 139449, **2020**.
32. FENG Q., LUO L.W., CHEN X.D., ZHANG K.J., FANG F., XUE Z.X., LI C., CAO J.S., LUO J.Y. Enhancing

the biofilm development of Pseudomonas aeruginosa through the stimulation of exogenous N-Acyl-L-homoserine lactones: Effects on bacterial motility, adhesive properties, and metabolic function. Bioresource Technology. **341**, 125727, **2021**.
33. SHI Y.H., HUANG J.H., ZENG G.M., GU Y.L., CHEN Y.N., HU Y., TANG B., ZHOU J.X., YANG Y., SHI L.X. Exploiting extracellular polymeric substances (EPS) controlling strategies for performance enhancement of biological wastewater treatments: an overview. Chemosphere. **180**, 396, **2017**.
34. PALUCH E., SOROCZYŃSKA J.R., JĘDRUSIK I., MAZURKIEWICZ E., JERMAKOW K. Prevention of biofilm formation by quorum quenching. Applied Microbiology and Biotechnology. **104** (5), 1871, **2020**.
35. HE C.S., DING R.R., CHEN J.Q., LI W.Q., LI Q., MU Y. Interactions between nanoscale zero valent iron and extracellular polymeric substances of anaerobic sludge. Water Research. **178**, 115817, **2020**.
36. DONG J.J., ZHANG Z.M., YU Z.D., DAI X., XU X.Y., ALVAREZ P.J., ZHU L. Evolution and functional analysis of extracellular polymeric substances during the granulation of aerobic sludge used to treat p-chloroaniline wastewater. Chemical Engineering Journal. **330**, 596, **2017**.
37. KUMAR S.S., BASU S., BISHOI N.R. Effect of cathode environment on bioelectricity generation using a novel consortium in anode side of a microbial fuel cell. Biochemical Engineering Journal. **121**, 17, **2017**.
38. DU C.Y., LIU W.L. Defending against environmental threats: Unveiling household adaptation strategies and population heterogeneity. Environment International. **190**, 108858, **2024**.
39. LIU Y., XU H.L., YANG S.F., TAY J.H. Mechanisms and models for anaerobic granulation in upflow anaerobic sludge blanket reactor. Water Research. **37** (3), 661, **2003**.
40. BRUCHMANN J., KIRCHEN S., SCHWARTZ T. Sub-inhibitory concentrations of antibiotics and wastewater influencing biofilm formation and gene expression of multiresistant Pseudomonas aeruginosa wastewater isolates. Environmental Science and Pollution Research. **20** (6), 3539, **2013**.
41. ZHANG X.R., JIANG Z.Y., LI M.Y., ZHANG X.X., WANG G., CHOU A.H., CHEN L., YAN H., ZUO Y.Y. Rapid spectrophotometric method for determining surface free energy of microalgal cells. Analytical Chemistry. **86** (17), 8751, **2014**.
42. LI Y., FAN C.L., LIU L.S., ZHAI X.Y., ZANG B., LI Y.Y., CHEN R. Biochar-induced quorum sensing enhances methane production by strengthening direct interspecies electron transfer. Bioresource Technology. **44**, 132845, **2025**.