ACCSCIENCE PUBLISHING

Asian Journal of Water, Environment and Pollution

ORIGINAL RESEARCH ARTICLE

Methanol as an ecological regulator enhancing anaerobic degradation of nitrogenous heterocyclic compounds: Insights into community structure and metabolic function

Yimin Fan[1], Zhuxuhui Huang[2], and Jingxin Shi[2]*

[1]Department of Municipal Engineering, School of Civil Engineering and Architecture, Taizhou University, Taizhou, Zhejiang, China

[2]Jiangsu Key Laboratory of Atmospheric Environment Monitoring and Pollution Control, Jiangsu Collaborative Innovation Center of Atmospheric Environment and Equipment Technology, School of Environmental Science and Engineering, Nanjing University of Information Science and Technology, Nanjing, Jiangsu, China

Abstract

Coal gasification wastewater contains a large amount of refractory nitrogenous heterocyclic compounds (NHCs), which are toxic and difficult to biodegrade. These substances are poorly removed by traditional biological treatment processes, such as anaerobic treatment. Therefore, the anaerobic degradation of NHCs, such as quinoline and indole, presents a significant challenge. This study investigated the efficacy and mechanism of methanol as a co-metabolic substrate for enhancing this process. Two anaerobic reactors—R1 as a control and R2 containing methanol—were operated to treat synthetic wastewater containing quinoline and indole. The results demonstrated that methanol supplementation significantly improved the degradation efficiency of both compounds. Mechanistic studies revealed that methanol fundamentally modified the system's ecology—it altered the physicochemical properties of anaerobic granular sludge by increasing the protein-to-polysaccharide ratio in extracellular polymeric substances, thereby enhancing microbial cohesion. Furthermore, methanol induced a targeted microbial community succession, notably enriching key specialist degraders such as *Levilinea* and *Longilinea* while suppressing generalist competitors. Functional gene prediction analysis indicated a comprehensive activation of metabolic pathways, energy production, and cellular processes. For the archaeal community, methanol increased diversity and facilitated a shift toward a multi-pathway methanogenic pattern, enriching methylotrophic methanogens (*Methanomassiliicoccus*) alongside acetoclastic pathways. This study highlights that methanol acts as a comprehensive ecological modulator, enhancing NHCs degradation by optimizing the sludge matrix, restructuring the microbial community for efficient metabolic division of labor, and activating overall metabolic potential, thereby providing a robust strategy for the treatment of industrial wastewater containing refractory organics.

Keywords: Anaerobic degradation; Co-metabolism; Quinoline; Indole; Methanol; Microbial community

***Corresponding author:**
Jingxin Shi
(003494@nuist.edu.cn)

Citation: Fan Y, Huang Z, Shi J. Methanol as an ecological regulator enhancing anaerobic degradation of nitrogenous heterocyclic compounds: Insights into community structure and metabolic function. *Asian J Water Environ Pollut.* 2026;23(3):026100062. doi: 10.36922/AJWEP026100062

Received: March 6, 2026

Revised: March 19, 2026

Accepted: March 19, 2026

Published online: May 4, 2026

1. Introduction

The coal gasification industry is considered an efficient and clean coal utilization method, and it has developed widely in China.[1] However, coal gasification wastewater (CGW)—consisting of phenolic compounds, nitrogenous heterocyclic compounds (NHCs), polycyclic aromatic hydrocarbons, and some other refractory organic compounds—poses a great threat to human health and the ecological environment.[2,3] NHCs occupy a proportion of 60% in the refractory organic compounds in CGW.[4] Owing to their mutagenic, carcinogenic, and toxic properties, the existence of NHCs has significant negative impacts on human health and environmental quality.[5]

Biological co-metabolism refers to the phenomenon whereby organic matter that cannot be metabolized by microorganisms is degraded in the presence of an additional carbon and energy source.[6-8] As a special type of microbial metabolism, co-metabolism technology has distinct advantages over traditional biodegradation technology in terms of degradation efficiency.[9] In recent years, co-metabolism technology has developed rapidly.[10] In the presence of a co-metabolic substance, anaerobic bacteria can decompose and detoxify the refractory and toxic organic compounds. Under anaerobic conditions with a co-metabolic substance, the typical NHCs (quinoline and indole) undergo different levels of transformation or degradation. Methanol is one of the most common co-metabolic substances. Kumabe *et al.*[11] reported that methanol can be obtained easily from the coal gasification industry, which makes the co-digestion of CGW with methanol a feasible option. Perez *et al.*[12] reported that the presence of methanol could increase biodegradation and decrease the toxicity of phenolic compounds.

Compared to existing studies that primarily focus on the macroscopic enhancement of degradation efficiency by methanol, the innovation of the present study lies in its systematic elucidation, from the perspective of ecological regulation, of the multi-scale enhancement mechanisms of methanol as a co-metabolic substrate on microbial community structure, functional metabolic networks, and systemic ecological functions. The study not only confirms the specific enrichment effect of methanol on key degrading bacteria (such as *Levilinea* and *Longilinea*), but more importantly, reveals that it enhances the physicochemical properties of sludge by optimizing the composition of extracellular polymeric substances (EPS), constructs a degradation pathway with a clear functional division of labor by reshaping the community structure (inhibiting opportunists and stabilizing syntrophic populations), and simultaneously activates the overall metabolic functions ranging from basic metabolism to cell growth and proliferation.

Furthermore, this study highlights, in an anaerobic system treating NHCs, the diversified regulation of methanogenic pathways by methanol, namely the enrichment of methylotrophic archaea, resulting in a synergistic methanogenesis mode where acetoclastic and methylotrophic pathways coexist. This series of findings transcends the traditional perception of methanol as a mere carbon source, establishes its role as a regulatory factor in systemic ecological engineering, and provides a new theoretical basis and in-depth mechanistic insights for the precise application of co-metabolism technology in the treatment of refractory wastewater.

In this study, the main objectives are: (i) to verify the enhancing effect of methanol on the removal efficiency of quinoline and indole; (ii) to elucidate the mechanism by which methanol regulates the physical and chemical properties of anaerobic granular sludge, particularly focusing on the changes in EPS and their impact on microbial cohesion; and (iii) to unravel the response of the microbial community structure—including bacteria and archaea—to methanol addition, thereby linking community dynamics to functional enhancement.

2. Materials and methods

2.1. Preparation of wastewater and acclimation of sludge

Experiments in this study were carried out using synthetic wastewater. The composition of the wastewater was the same as that reported by Shi *et al.*[4] (0.025 g/L monopotassium phosphate [Aladdin, China], 0.1 g/L potassium chloride [Aladdin, China], and 0.1 g/L ammonium chloride [Aladdin, China]). The initial influent chemical oxygen demand values for R1 and R2 reactors were about 400 mg/L.

The anaerobic granular seed sludge was collected from Yima, China. The acclimation of sludge was conducted in a 2.0 L anaerobic bottle. The initial mixed liquor suspended solids (MLSS) was about 20 g/L. The 2.0 L anaerobic bottle was cultivated in a constant temperature incubator. Operating parameters of the constant temperature incubator were 135 rpm, 30 ℃, and a dark environment. The wastewater in the 2.0 L anaerobic bottle was replaced with fresh synthetic wastewater every 48 h. The specific methods of acclimation were divided into three phases:

(i) Phase 1 (1–20 days): 25 mg/L quinoline and 25 mg/L indole were added as the carbon source.
(ii) Phase 2 (21–40 days): 50 mg/L quinoline and 50 mg/L indole were added.
(iii) Phase 3 (41–60 days): 75 mg/L quinoline and 75 mg/L

indole were added.

2.2. Reactor experiments

Reactor experiments were conducted in two 250 mL anaerobic bottles with acclimated anaerobic granular sludge. In each reactor, the MLSS was about 10 g/L. The reactors were cultivated in a constant-temperature incubator. The operating parameters were 135 rpm, 30 °C, and a dark environment. The wastewater in each reactor was replaced with fresh synthetic wastewater (quinoline 100 mg/L and indole 100 mg/L as carbon source) every 48 h. R1 and R2 were operated with 10 g/L anaerobic granular sludge. In addition, R2 was supplemented with methanol. The specific operating conditions of the reactor experiments are shown in Table 1.

2.3. Analytical methods

The mixed liquor volatile suspended solids (MLVSS) and MLSS were analyzed based on standard methods.[13] Concentrations of quinoline and indole were analyzed using a liquid chromatograph (Agilent LC-1200, USA). The specific analytical procedures were based on the study by Zhu *et al.*,[14] whereas the extraction protocol of EPS was based on the study by Zhen *et al.*[15] In addition, proteins (PN) were analyzed with the Lowry procedure,[16] and polysaccharides (PS) were measured with the phenol–sulfuric acid method. The granulometric distribution of granular sludge was measured via a laser particle size analyzer (Nicomp 380, Aofa Meijia Technology Co., Ltd., Shanghai, China). At the end of each phase, sludge samples from both reactors were collected to measure the bacterial and archaeal community structure.

3. Results and discussion

3.1. Degradation performance of reactors

Figure 1 shows the degradation efficiency of quinoline and indole in two reactors. The anaerobic sludge was acclimated for 60 days. Thus, in the initial stage of phase 1, R1 and R2 showed stable degradation ratios. Without the addition of methanol, R1 showed a stable degradation ratio from phase 1 to phase 3. In R1, the degradation ratios of quinoline in the three phases were 89.55%, 90.21%, and 90.22%, respectively. The indole degradation ratios in the three phases were 88.74%, 90.68%, and 91.22%, respectively. With the addition of methanol, R2 showed a higher degradation ratio than R1. From phase 1 to phase 3 in R2, the degradation ratios of quinoline were 95.69%, 96.98%, and 97.12%, respectively, while the degradation ratios of indole were 95.29%, 96.82%, and 97.98%, respectively. The findings indicate that when methanol increased from 25 mg/L to 175 mg/L, the degradation efficiency of the typical NHCs showed an increasing trend.

The functions of co-metabolic substances mainly include: on the one hand, providing a carbon source and energy for microorganisms, promoting their reproduction, growth, and metabolic activity; on the other hand, microorganisms can produce non-specific enzymes that degrade both growth substrates and non-growth substrates.[17,18] Most of the co-metabolic substances belong to small-molecule organic matter, which has a simple structure and is easily utilized by microorganisms. While they promote microbial growth, they do not necessarily promote the microbial degradation of certain refractory compounds.[19,20] The findings indicate that methanol is a suitable co-metabolic substrate. In addition, Bacosa and Inoue[21] reported that the dosage of co-metabolic substrates is a key factor in controlling co-metabolic biodegradation efficiency; higher dosages are not conducive to the effective degradation of pollutants. The findings of the present study indicate that 25–175 mg/L of methanol enhanced the anaerobic degradation of quinoline and indole, which did not exceed the dosage that would produce competitive

Table 1. Operating conditions of the anaerobic bottle experiment in different phases

Phase	Day	Quinoline + indole (mg/L)	Methanol (mg/L)		Operating conditions
			R1	R2	
Phase 1	1–40	100 + 100	0	25	Hydraulic retention time: 48 h; temperature: 30 °C; rotate speed: 135 rpm; dark environment
Phase 2	41–80		0	75	
Phase 3	81–120		0	175	

inhibition due to high concentrations of co-metabolic substrates.[22]

3.2. Effect of methanol on anaerobic granular sludge

Table 2 presents the properties of sludge from both reactors after 120 days of operation. The mean surface area particle sizes (MAPZ) of sludge from R1 and R2 were 68.266 μm and 65.579 μm, respectively, whereas the mean volume particle sizes (MVPZ) were 180.236 μm and 183.602 μm, respectively. The results indicate that the addition of methanol decreased the MAPZ while increasing the MVPZ. In addition, the specific surface areas of sludge were 0.0915 m^2/g and 0.0888 m^2/g, respectively, which indicates that methanol decreased the specific surface area of anaerobic granular sludge.

Several studies have reported that the total content of PS and PN accounted for more than 80% of the total content of EPS, and these two substances could more directly reflect the content of EPS.[23] Figure 2 shows the PN, PS, and PN/PS values of sludge in the two reactors. For R1 and R2, PN values were 36.92 and 39.166 mg/g MLVSS, respectively, which indicates that methanol could increase the concentration of protein in the EPS of anaerobic granular sludge. Sheng *et al.*[24] reported that a high concentration of PN in EPS benefits microbial flocculation. The PS values of sludge in the two reactors were 5.81 and 4.44 mg/g MLVSS, respectively, indicating that the addition of methanol decreased the concentration of PS in EPS. Ying *et al.*[25] indicated that higher PS content in EPS could induce stronger hydrophilic characteristics of microorganisms, which further lead to sludge foaming and agglomeration.

Several researchers have reported that the positively charged amino groups in PN can effectively offset the negative charge carried by carboxylic acids and uronic acids in PS, which can increase the hydrophobicity and reduce the surface charge of sludge.[26] For this reason, the ratio of PN to PS in EPS can be used as an indicator of microbial cohesion.[27,28] The findings of the present study indicate that the PN/PS values of sludge in R1 and R2 were 6.35 and 8.82, respectively, indicating that methanol could increase the ratio of PN/PS and improve the microbial cohesion of anaerobic granular sludge.

The addition of methanol as an exogenous co-metabolic substrate fundamentally alters the metabolic activity of the anaerobic microbial community and the biochemical composition of EPS. By providing microorganisms with a readily utilizable carbon and energy source, methanol not only promotes microbial growth and reproduction but, more critically, induces the synthesis of more non-specific enzymes and significantly modifies EPS composition. Specifically, it increases PN synthesis (from 36.92 to 39.166 mg/g MLVSS) while reducing PS production (from 5.81 to 4.44 mg/g MLVSS), resulting in a significant rise in the PN/PS ratio from 6.35 to 8.82.

This core biochemical change carries major physicochemical implications: the abundant protein in EPS, containing positively charged amino groups, effectively neutralizes the negatively charged carboxyl groups and uronic acids in PS. This reduces the electrostatic repulsion at the sludge surface, enhances hydrophobicity, and substantially improves inter-microbial cohesion. This enhanced cohesion leads to a tighter and more compact internal structure of the sludge granules, causing small particles to aggregate and large particles to become more homogeneous and stable. Ultimately, this microstructural remodeling directly translates into changes in macroscopic physical properties. The granule surfaces become smoother and more compact, leading to a slight decrease in the MAPZ (from 68.266 to 65.579 μm). Conversely, the overall volume and density of the granules increase, causing an increase in the MVPZ (from 180.236 to 183.602 μm). The total exposed surface area per unit mass of sludge (specific surface area) decreases (from 0.0915 to 0.0888 m^2/g). Therefore, these alterations in sludge properties are the result of a cascade of events initiated by methanol, driving shifts in microbial metabolism, biochemical modifications of EPS, changes in surface physicochemical characteristics,

Table 2. Characteristics of sludge in the two reactors

Reactor	Mean surface area particle size (μm)	Mean volume particle size (μm)	Specific surface area (m^2/g)
R1	68.266	180.236	0.0915
R2	65.579	183.602	0.0888

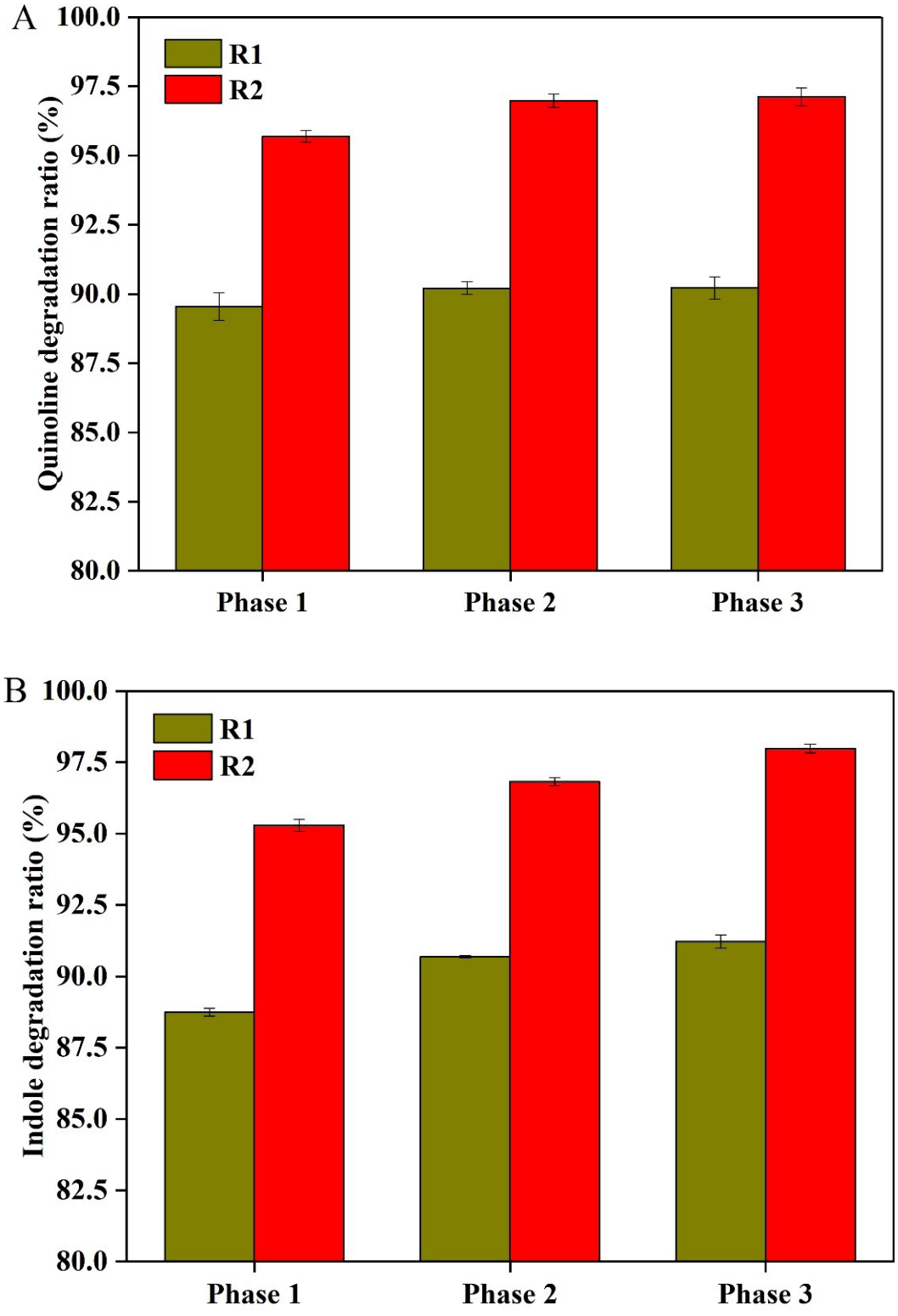


Figure 1. Degradation ratio of (A) quinoline and (B) indole in the two reactors across three phases

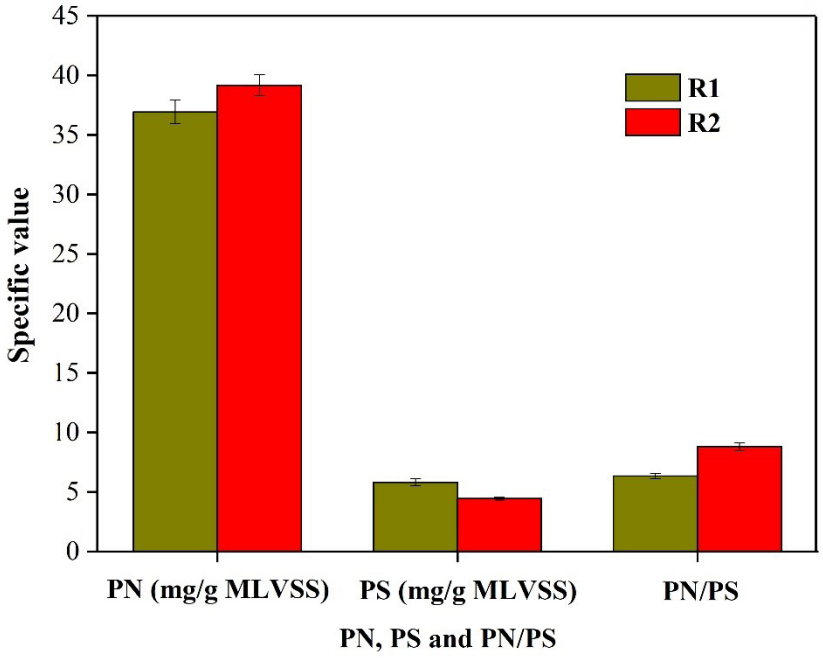


Figure 2. PN, PS, and PN/PS values of anaerobic granular sludge in two reactors
Abbreviations: MLVSS: Mixed liquor volatile suspended solids; PN: Protein; PS: Polysaccharide.

and culminating in the remodeling of macroscopic structure.

3.3. Bacterial community structure

Based on Table 3, the operational taxonomic units (OTUs) of R2 were higher than those of R1, indicating that methanol increased the sequence richness of the bacterial community. In addition, R2 had a higher Chao1 index and abundance-based coverage estimator (ACE) index, indicating that methanol enriched the bacterial community. Furthermore, R2 had a higher Shannon index and a lower Simpson value, indicating that R2 had a more diverse bacterial community structure with methanol.

Figure 3 shows the bacterial community structure of the two reactors at different levels. Figure 3A shows that the main phyla include Proteobacteria, Chloroflexi, Bacteroidetes, Aminicenantes, Euryarchaeota, and Actinobacteria. The percentage of Proteobacteria in R1 and R2 was 32.66% and 20.08%, respectively, indicating that the addition of methanol was not conducive to the enrichment of the phylum Proteobacteria. The percentage of Chloroflexi in R1 and R2 was 17.2% and 22.83%, respectively, indicating that methanol promoted the enrichment of the phylum Chloroflexi.

The addition of methanol as a co-metabolic substrate appears to induce niche reconstruction within the microbial community, leading to enhanced degradation efficiency of quinoline and indole. The core mechanism lies in the fact that methanol alters the substrate landscape, thereby triggering community succession: it suppresses Proteobacteria (decreasing from 32.66% to 20.08%), which initially dominated in R1 as "generalist opportunists," as the resource competition initiated by methanol diminishes their advantage. Concurrently, it selectively enriches bacterial phyla with specialized functions in degrading complex organic compounds, particularly Chloroflexi (increasing from 17.2% to 22.83%)—key "collaborative degraders"—and its class Anaerolineae.

This shift in community structure from "quantity-oriented" to "quality-oriented" optimizes the functional division of labor: one group of microorganisms specializes in utilizing readily degradable methanol to provide an energy foundation, while the enriched specialist populations more efficiently degrade quinoline and indole. Such an assembly line-like metabolic collaboration not only improves contaminant removal rates but, more importantly, the change in EPS composition (increased PN, decreased PS, higher PN/PS ratio) driven by the microbial community shift may underlie improvements in sludge physical properties, such as enhanced cohesion and altered particle size distribution.

At the class level (Figure 3B), the main classes

Table 3. Summary of sequencing metrics for bacterial communities

Sample	Sequence number[a]	OTU number[b]	Shannon index[c]	ACE index[d]	Chao1 index[e]	Coverage[f]	Simpson[g]
R1	68,410	1,422	4.131	2,578.4	2,221.45	0.99	0.046
R2	66,416	1,544	4.572	2,663.3	2,346.78	0.99	0.030

Notes: [a]Sequence number represents the number of high-quality reads obtained per sample. [b]OTU number reflects the number of operational taxonomic units identified, with higher values indicating greater species richness. [c]Shannon index measures community diversity, with higher values indicating greater diversity. [d]ACE index estimates community richness, with higher values indicating greater richness. [e]Chao1 index estimates community richness based on rare taxa, with higher values indicating greater richness. [f]Coverage fraction of the sample library. [g]Simpson index quantifies community diversity, with lower values indicating greater diversity.
Abbreviations: ACE: Abundance-based coverage estimator; OTU: Operational taxonomic unit.

included Deltaproteobacteria, Anaerolineae, Bacteroidia, Gammaproteobacteria, Methanomicrobia, Actinobacteria, and Betaproteobacteria. The findings of this study showed that Deltaproteobacteria occupied a similar percentage in R1 and R2, with proportions of 13.94% and 13.97%, respectively. In addition, R2 had a higher percentage of Anaerolineae and Bacteroidia, indicating that methanol was beneficial for the enrichment of these two classes. Oba *et al.*[29] reported that the class Anaerolineae has the ability to degrade phenolic compounds and NHCs. Notably, the percentage of Gammaproteobacteria in R1 and R2 was 15.67% and 2.66%, respectively, indicating that the addition of methanol inhibited the enrichment of the class Gammaproteobacteria.

The addition of methanol triggered a targeted functional remodeling of the microbial community at the class level. Its most prominent feature was the strong suppression of Gammaproteobacteria, whose relative abundance sharply decreased from 15.67% to 2.66%. This indicates that methanol introduction did not uniformly stimulate all microorganisms but altered the niche competition landscape, effectively reducing "opportunistic" taxa that preferentially utilize simple substrates, thereby creating space for functionally specialized groups. Methanol may promote the growth of obligate anaerobic bacteria, thereby indirectly inhibiting Gammaproteobacteria with facultative characteristics by altering the redox potential or producing specific metabolic intermediates.

In contrast, Anaerolineae (under the phylum Chloroflexi) and Bacteroidia (under the phylum Bacteroidota), which are responsible for degrading complex organic compounds, were significantly enriched. This suggests that methanol, as a cometabolic substrate, created a microenvironment favorable for these specialized "decomposers," enabling them to more efficiently target and degrade refractory pollutants such as quinoline and indole. Notably, Deltaproteobacteria, the core functional class responsible for syntrophic oxidation processes, remained remarkably stable throughout the process, maintaining an abundance of around 14%. This suggests that methanol optimized different stages of the degradation pathway, while preserving the core "downstream" metabolic processes responsible for converting intermediates like fatty acids into acetate and hydrogen. Thus, this community shift represents an efficient functional reorganization: by suppressing Gammaproteobacteria to reduce nonproductive competition, enriching Anaerolineae and Bacteroidia to enhance targeted pollutant degradation capability, and simultaneously ensuring the unimpeded flow of the syntrophic metabolic network led by Deltaproteobacteria, an efficient cooperative metabolic process—from initial degradation to final methanogenesis—was established.

Figure 3C shows the bacterial community structure at the order level. The main bacterial groups included Anaerolineales, Bacteroidales, Pseudomonadales, Syntrophobacterales, Methanosarcinales, Desulfuromonadales, and Coriobacteriales. The findings demonstrate that the addition of methanol enriched the Anaerolineales and Bacteroidales, while greatly reducing the proportion of Pseudomonadales.

Figure 3D shows the bacterial community structure at the family level. The main bacterial groups included *Anaerolineaceae*, *Prolixibacteraceae*, *Moraxellaceae*, *Syntrophaceae*, *Methanotrichaceae*, *Geobacteraceae*, *Coriobacteriaceae*, and *Desulfomicrobiaceae*. The findings

demonstrate that methanol increased the percentage of *Anaerolineaceae* and *Prolixibacteraceae*, while greatly decreasing the percentage of *Moraxellaceae*.

Analysis at the order and family levels further reveals the targeted effects of methanol co-metabolism. By performing a "targeted surgical" restructuring of the microbial community, methanol refines the system's degradation function. The addition of methanol specifically enriched the order *Anaerolineales* (primarily the family *Anaerolineaceae*) and the family *Prolixibacteraceae* within the order *Bacteroidales*. These two groups act as specialized "assault units" for degrading complex aromatic compounds in anaerobic environments. In particular, the family *Anaerolineaceae* directly includes key genera such as *Levilinea* and *Longilinea*, which are known to degrade quinoline and indole. Their enrichment enhances the system's primary attack capability against the target pollutants.

Concurrently, methanol strongly suppressed the order *Pseudomonadales* (within the class *Gammaproteobacteria*) and its core family *Moraxellaceae*. This indicates that the methanol-shaped environment selectively reduced "redundant" taxa that are less competitive under strict anaerobic conditions and functionally inclined toward opportunistic growth, thereby freeing ecological niches and resources for specialized degraders. Most critically, throughout the restructuring process, the core functional modules responsible for system stability—including the order *Syntrophobacterales* (e.g., family *Syntrophaceae*) and the order *Desulfuromonadales* (e.g., family *Geobacteraceae*), which perform syntrophic oxidation—remained highly stable. This ensured the uninterrupted operation of the "downstream metabolic engine" that converts degradation intermediates into methane.

Figure 3E shows the bacterial community structure at the genus level. The main genera included *Aminicenantes* genera incertae sedis, *Acinetobacter*, *Methanothrix*, *Levilinea*, *Longilinea*, *Smithella*, *Geobacter*, and *Desulfomicrobium*. The findings demonstrate that methanol enriched the *Aminicenantes* genera incertae sedis, *Levilinea*, and *Longilinea*, while sharply decreasing the percentage of *Acinetobacter*. *Aminicenantes* genera incertae sedis have potential roles in anaerobic hydrolysis and fermentation.[30] In addition, *Levilinea* and *Longilinea* have the ability to degrade quinoline, indole, and phenolic organic compounds.

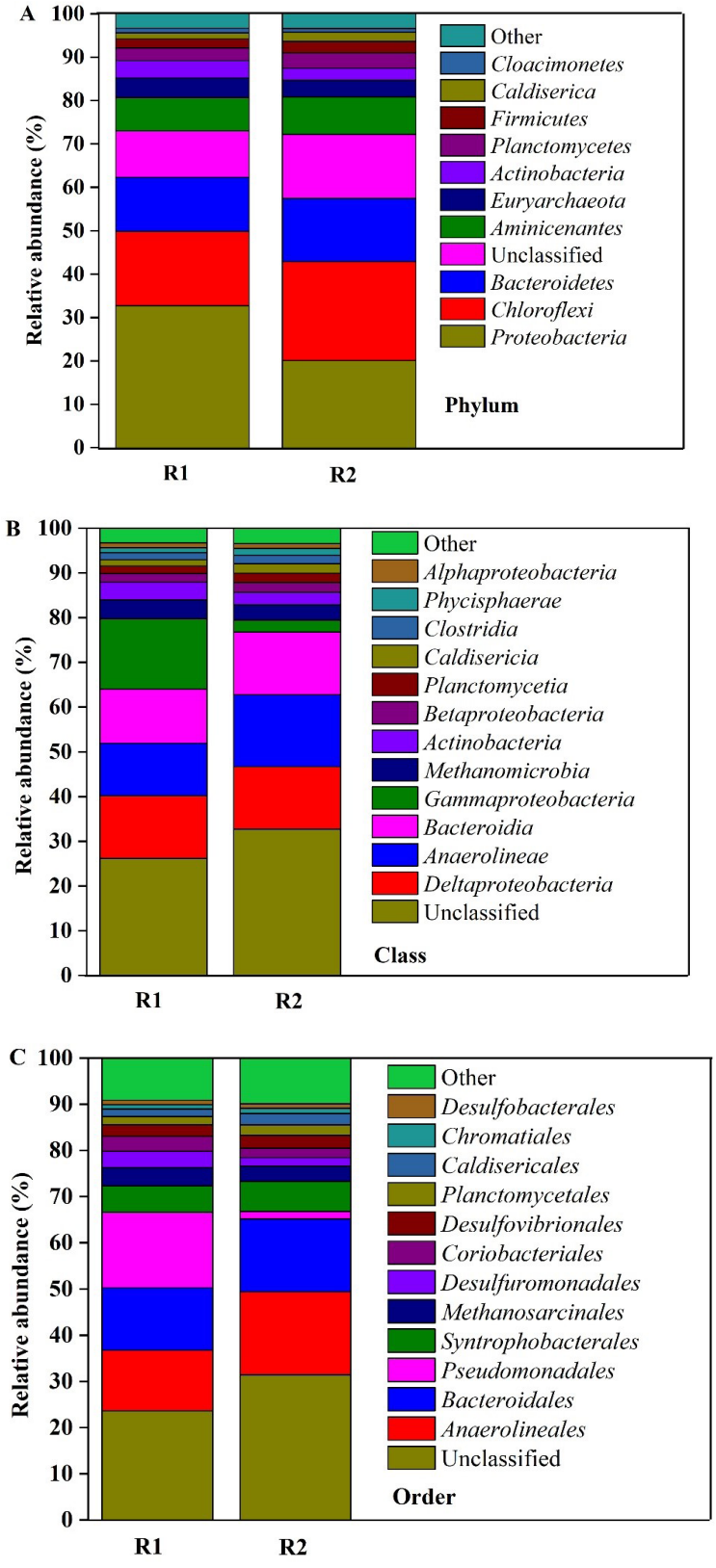
Analysis at the genus level provides the most direct and compelling evidence for the methanol-enhanced co-metabolism mechanism, clearly demonstrating that this process constitutes a highly specific functional regulation of the microbial community. The addition of methanol achieves targeted optimization for the efficient degradation of refractory organic compounds through an integrated mechanism. Firstly, it enhances key genera with explicit degradation functions by directly enriching the key genera *Levilinea* and *Longilinea*, known degraders of quinoline and indole, thereby translating the beneficial changes observed at higher taxonomic levels into functional outcomes. Secondly, it supports auxiliary steps in the degradation chain by co-enriching potentially hydrolytic and fermentative taxa such as *Aminicenantes genera incertae sedis*, which act to convert complex intermediates into more utilizable molecules, thus optimizing the efficiency of the entire metabolic network. Most critically, it reduces the facultative anaerobic opportunist *Acinetobacter*, thereby limiting inefficient competition and creating a more favorable ecological niche for functionally specialized strict anaerobes.

Concurrently, the stable presence of the methanogenic archaeon *Methanothrix* ensures the unimpeded operation of the acetoclastic methanogenesis pathway at the terminal end of the system. This targeted community restructuring effectively channels the community's metabolic capacity toward the degradation of the target pollutants quinoline and indole, ultimately achieving a significant enhancement in system performance.

3.4. Potential function of bacterial community

Figure 4 shows the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) functional prediction based on the Clusters of Orthologous Groups database from R1 and R2. To analyze the pollutant degradation capacity, three functional gene groups related to metabolism, information storage and processing, and cellular processes and signaling were investigated. The function predictions in the metabolism group mainly included energy production and conversion, amino acid transport and metabolism, nucleotide transport and metabolism, carbohydrate transport and metabolism, coenzyme transport and metabolism, and lipid transport and metabolism.

The findings demonstrate that, with the addition of methanol, R2 exhibited higher levels of energy production and conversion, amino acid transport and metabolism, nucleotide transport and metabolism, carbohydrate transport and metabolism, and coenzyme transport and metabolism. This indicates that methanol, as a co-metabolic substance, could comprehensively improve the metabolic capacity of microorganisms based on the analysis of functional genes. Furthermore, in the functional gene group of information storage and processing, the findings


A
Relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
R1
R2
Other
Cloacimonetes
Caldiserica
Firmicutes
Planctomycetes
Actinobacteria
Euryarchaeota
Aminicenantes
Unclassified
Bacteroidetes
Chloroflexi
Proteobacteria
Phylum
B
Relative abundance (%)
R1
R2
Other
Alphaproteobacteria
Phycisphaerae
Clostridia
Caldisericia
Planctomycetia
Betaproteobacteria
Actinobacteria
Methanomicrobia
Gammaproteobacteria
Bacteroidia
Anaerolineae
Deltaproteobacteria
Unclassified
Class
C
Relative abundance (%)
R1
R2
Other
Desulfobacterales
Chromatiales
Caldisericales
Planctomycetales
Desulfovibrionales
Coriobacteriales
Desulfuromonadales
Methanosarcinales
Syntrophobacterales
Pseudomonadales
Bacteroidales
Anaerolineales
Unclassified
Order

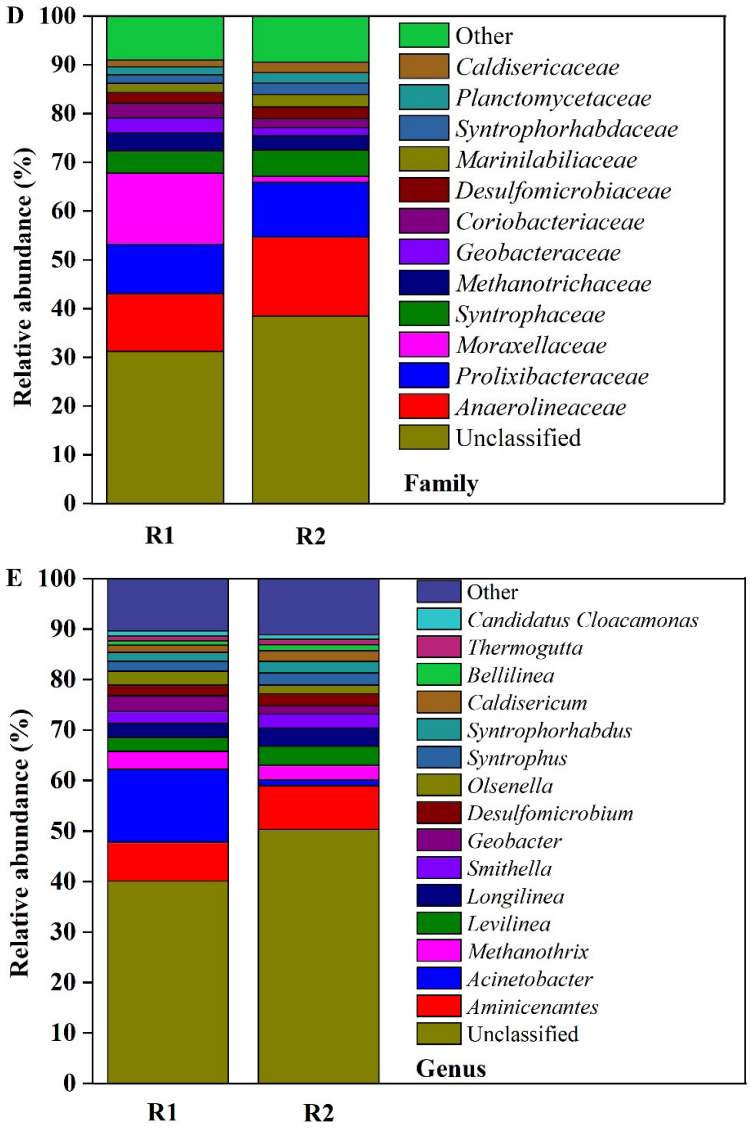


Figure 3. Analysis of bacterial community structure of different reactors at the (A) phylum, (B) class, (C) order, (D) family, and (E) genus levels. "Other" represents all classified taxa with relative abundance <1% in all samples

demonstrate that the addition of methanol improves the functions of translation, ribosomal structure and biogenesis, transcription, replication, recombination, and repair.

In-depth analysis based on PICRUSt functional prediction reveals that methanol, as a co-metabolic substrate, induces substantial and systematic reshaping of the microbial community's functional profile. Its role extends beyond merely providing a simple carbon and energy source, instead triggering a comprehensive activation spanning from basal metabolism to collective behaviors. At the core metabolic level, the microbial community exhibits metabolic reprogramming, with the most significant increase observed in carbohydrate transport and metabolism (absolute increase of 638,143), indicating a substantial enhancement in carbon source utilization capacity. Concurrently, the widespread enhancement of key metabolic pathways—such as energy production, amino acid metabolism, and nucleotide metabolism—provides an abundant substrate and energy foundation for pollutant degradation.

The synchronous increase in genetic information-

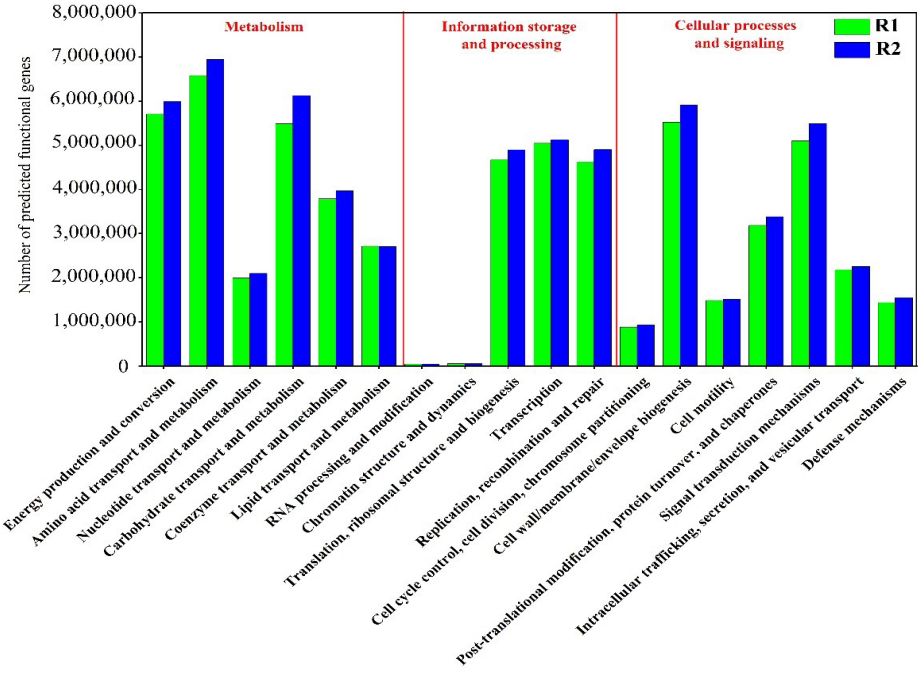


Figure 4. Functional prediction using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States based on the Clusters of Orthologous Groups database

related functions (e.g., translation, replication, repair, and transcription), coupled with a substantial increase in cell wall/membrane biogenesis (increase of 390,456), collectively suggests a state of rapid growth and proliferation of microbial populations. More notably, the sharp rise in signal transduction mechanisms (increase of 388,198) suggests that methanol addition may enhance intercellular communication processes, such as quorum sensing, thereby promoting synergistic degradation within the community.

The relative stability of a few functions, such as lipid metabolism, further reflects the selective optimization of resource allocation by microorganisms, focusing capabilities on addressing current environmental conditions. Thus, this study suggests at the functional gene level that the co-metabolic effect of methanol transforms the microbial community from a basal state into a more efficient biodegradation system characterized by enhanced metabolism, active growth, and coordinated interactions, providing a theoretical basis for the effective application of co-metabolism technology in bioremediation.

3.5. Archaeal community structure

Table 4 shows that R2 had higher OTU, ACE index, and Chao1 index, indicating that methanol enriched the archaeal community structure. In addition, R2 had a higher Shannon index and a lower Simpson value, indicating that R2 had a more diverse archaeal community structure. In general, the richer and more diverse archaeal community structure in R2 provides a basis for better methane production.

Figure 5 shows the archaeal community structure from the two reactors at the genus level. In the two reactors, the most dominant genus was *Methanothrix*, which accounted for 67.12% and 60.64%, respectively. Jetten *et al.*[31] reported that *Methanothrix* was an extremely strict anaerobic archaeon and degrades acetic acid into methane. The percentages of *Methanomassiliicoccus* in the two reactors were 7.26% and 14.67%, respectively, indicating that the methanol in R2 facilitated the enrichment of *Methanomassiliicoccus*. Furthermore, *Methanomassiliicoccus* is generally considered to be mainly methanogenic archaea (obligate methylotrophic) that use hydrogen to reduce methanol or methylamine.[32] The percentages of *Methanolinea* in the two reactors were 12.39% and 10.31%, respectively. Imachi *et al.*[33] reported that *Methanolinea* utilized formate and H_2 for methane production. In general, in anaerobic degradation of NHCs wastewater, acetic acid-type metabolism was the dominant methane-producing mode, with a small amount of formic

Table 4. Summary of sequencing metrics for archaeal communities

Sample	Sequence number[a]	OTU number[b]	Shannon index[c]	ACE index[d]	Chao1 index[e]	Coverage[f]	Simpson[g]
R1	61,498	248	2.030	735.21	450.22	0.99	0.323
R2	72,802	299	2.046	860.06	586.34	0.99	0.283

Notes: [a]Sequence number represents the number of high-quality reads obtained per sample. [b]OTU number reflects the number of operational taxonomic units identified, with higher values indicating greater species richness. [c]Shannon index measures community diversity, with higher values indicating greater diversity. [d]ACE index estimates community richness, with higher values indicating greater richness. [e]Chao1 index estimates community richness based on rare taxa, with higher values indicating greater richness. [f]Coverage fraction of the sample library. [g]Simpson index quantifies community diversity, with lower values indicating greater diversity.
Abbreviations: ACE: Abundance-based coverage estimator; OTU: Operational taxonomic unit.

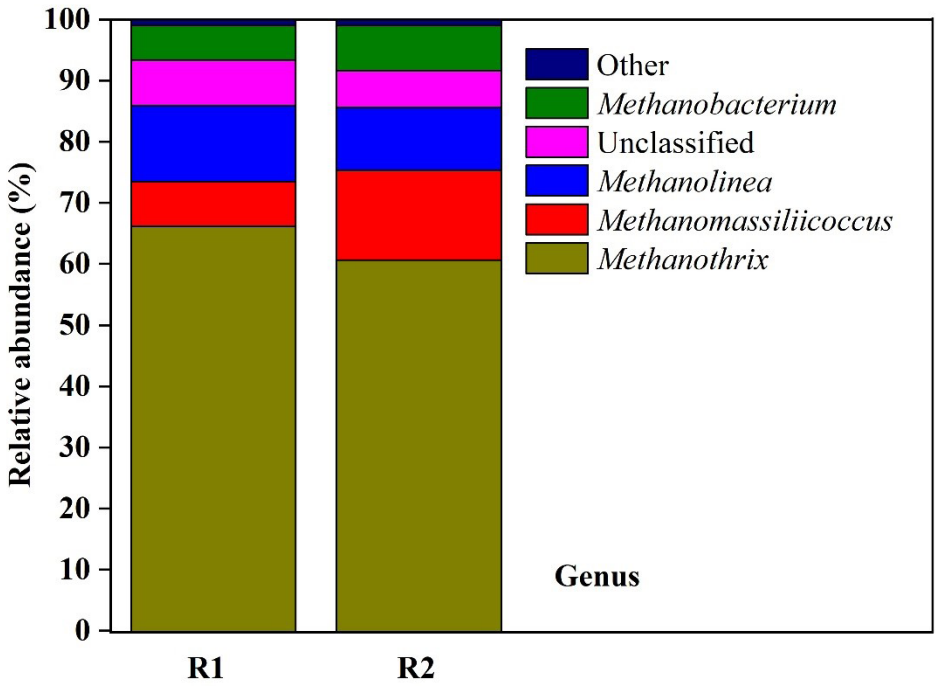


Figure 5. Archaeal community structure at the genus level

acid-type metabolism.

Based on an in-depth analysis of the archaeal community structure and function, the following conclusions can be drawn: the addition of methanol, as an effective eco-engineering strategy, significantly enhanced the species richness (higher OTU numbers, ACE, and Chao1 indices) and diversity (higher Shannon index and lower Simpson value) of the archaeal community, establishing a more stable functional redundancy foundation for the system. In terms of population structure, it maintained the dominance of *Methanothrix*—the predominant archaeon utilizing acetate as a substrate—confirming the central role of acetoclastic methanogenesis. On the other hand, it specifically enriched the methylotrophic archaeon *Methanomassiliicoccus* (increasing from 7.26% to 14.67%), which can directly utilize methanol, thereby introducing an additional methanogenic pathway. Meanwhile, the stable presence of the hydrogenotrophic

archaeon *Methanolinea* preserved an important cross-feeding channel. This metabolic shift from a "single-core driven" mode to a "synergistic multi-pathway model" integrating acetoclastic, methylotrophic, and hydrogenotrophic methanogenesis, coupled with the previously observed enhancement of overall metabolic functions in the bacterial community, collectively transformed the anaerobic system into a more functionally diverse, tightly coordinated, and resilient ecosystem. This restructuring provides a solid microbiological foundation for sustained efficient methane production and the removal of refractory pollutants.

3.6. Enhancement mechanism of methanol

The enhancement mechanism of methanol on the anaerobic degradation of nitrogen heterocyclic compounds (quinoline and indole) is a multilevel and systematic ecoengineering process. Its role extends far beyond merely providing a carbon source; instead, it regulates the physicochemical properties of sludge, restructures the microbial community, and activates cellular metabolic functions, ultimately establishing a highly efficient and synergistic degradation pipeline.

At the physicochemical level, the addition of methanol acts as an environmental signal that significantly alters the biochemical composition of EPS in anaerobic granular sludge. Specifically, the PN concentration increased from 36.92 mg/g MLVSS to 39.166 mg/g MLVSS, while the PS concentration decreased from 5.81 mg/g MLVSS to 4.44 mg/g MLVSS, resulting in a notable rise in the PN/PS ratio from 6.35 to 8.82. This core biochemical change enhanced the neutralization of negatively charged carboxyl groups in PS by positively charged amino groups in PN, thereby reducing the surface charge of the sludge, increasing hydrophobicity, and substantially improving microbial cohesion. These modifications led to a more compact (with a decrease in the MAPZ from 68.266 μm to 65.579 μm) and stable structure of the sludge granules, creating an optimized physical carrier for microbial aggregation and functional expression.

At the microbial community structure level, methanol intervention triggered a precise community succession and functional remodeling. For the bacterial community, methanol demonstrated a highly selective enrichment capacity: it significantly suppressed *Gammaproteobacteria* (decreasing from 15.67% to 2.66%), which had a relatively high abundance in R1 but functionally tended toward opportunistic growth, along with its subordinate taxa such as the order *Pseudomonadales* and the family *Moraxellaceae*, particularly causing a sharp reduction in the genus *Acinetobacter*. Concurrently, it specifically enriched microbial groups with specialized functions in degrading complex organic matter, including the phylum *Chloroflexi* (increasing from 17.2% to 22.83%), its class *Anaerolineae*, and the family *Anaerolineaceae*, ultimately leading to the enrichment of key functional genera capable of directly degrading quinoline and indole—*Levilinea* and *Longilinea*. Meanwhile, bacteria with potential for anaerobic hydrolysis and fermentation, such as *Aminicenantes genera incertae sedis*, were co-enriched. Crucially, the core functional groups responsible for syntrophic oxidation—converting intermediates like fatty acids into acetate and hydrogen, including the class *Deltaproteobacteria* (stable at approximately 14%), the order *Syntrophobacterales*, and the family *Syntrophaceae*—remained highly stable.

For the archaeal community, methanol similarly enhanced its richness and diversity (higher OTU numbers, ACE, Chao1, and Shannon indices). While maintaining the core status of the dominant archaeon *Methanothrix* (performing acetoclastic methanogenesis, proportion changing from 67.12% to 60.64%), it specifically enriched the methylotrophic archaeon *Methanomassiliicoccus*, capable of directly utilizing methanol (its proportion doubling from 7.26% to 14.67%), and preserved the functional pathway of the hydrogenotrophic archaeon *Methanolinea* (proportion changing from 12.39% to 10.31%).

At the molecular functional level, PICRUSt-based functional prediction analysis confirmed that methanol triggers a comprehensive metabolic activation of the microbial community. This is reflected in the widespread enhancement of functional genes directly associated with pollutant degradation, such as carbohydrate, amino acid, and nucleotide transport and metabolism, as well as energy production and conversion, providing ample substrates and energy for the degradation process. Simultaneously, the synchronous upregulation of genetic information processing functions—including translation, transcription, replication, and repair—along with cellular structure synthesis functions, such as cell wall/membrane biogenesis, indicates that the microbial population is in a state of rapid growth and proliferation. Furthermore, the significant enhancement of signal transduction mechanisms suggests that methanol may promote intercellular communication behaviors, such as quorum sensing, thereby improving the synergistic interactions within the community.

Ultimately, the aforementioned multi-level effects collectively constitute the enhancement mechanism of methanol: by optimizing the EPS composition, methanol improves the micro-physical environment; through precise community restructuring, it suppresses "redundant" microbial populations while enriching key degraders of quinoline and indole, such as *Levilinea* and *Longilinea*, as

well as auxiliary hydrolytic bacteria, all while maintaining the stability of syntrophic bacterial groups and the diversity of archaeal pathways. On this basis, the overall metabolic potential of the system is fully activated. Through these integrated actions, the anaerobic system is transformed from a basal state into an efficient "metabolic assembly line": functional bacteria specialize in the primary degradation of target pollutants, a stable syntrophic network efficiently converts intermediate products, and a diversified archaeal community, comprising acetoclastic, methylotrophic, and other pathways, ensures the smooth and efficient terminal methanogenesis process, thereby systematically achieving enhanced removal of typical NHCs.

4. Conclusion

Based on comprehensive experimental results, this study conclusively demonstrates that methanol is a highly effective co-metabolic substrate. It significantly enhances the anaerobic degradation of refractory NHCs, specifically for quinoline and indole. The enhancement mechanism operates systematically across multiple levels. Primarily, methanol addition altered the EPS composition of anaerobic granular sludge, increasing the PN/PS ratio and thereby improving microbial cohesion and sludge physical structure, thus creating a favorable microenvironment. Furthermore, methanol induced a precise microbial community succession, notably enriching key specialist degraders like *Levilinea* and *Longilinea* while suppressing generalist competitors, and ensuring the stability of syntrophic bacterial consortia. Concurrently, it increased the diversity of the archaeal community and diversified methanogenic pathways by enriching methylotrophic methanogens such as *Methanomassiliicoccus* alongside the acetoclastic pathway. At the molecular level, functional gene prediction confirmed a comprehensive activation of microbial metabolic capabilities, energy production, and cellular processes. In essence, methanol orchestrates the formation of a highly efficient metabolic assembly line, where specialized bacteria perform targeted degradation, syntrophic communities facilitate intermediate conversion, and diverse archaea ensure efficient methanogenesis. This work provides valuable theoretical and practical insights into the application of co-metabolism technology for treating industrial wastewater containing refractory organic compounds.

Acknowledgments

None.

Funding

This study was supported by the Taizhou Science and Technology Project (Grant No. 24sfa01).

Conflict of interest

The authors declare they have no competing interests.

Author contributions

Conceptualization: Jingxin Shi
Formal analysis: Yimin Fan, Zhuxuhui Huang
Investigation: Yimin Fan, Zhuxuhui Huang
Methodology: Yimin Fan
Writing–original draft: Yimin Fan, Zhuxuhui Huang
Writing–review & editing: All authors

Availability of data

Not applicable.

References

1. Shi J, Han Y, Xu C, Han H. Biological coupling process for treatment of toxic and refractory compounds in coal gasification wastewater. *Rev Environ Sci Biotechnol*. 2018;17(4):765-790.

   doi: 10.1007/s11157-018-9481-2

2. Zhu X, Ni J, Lai P. Advanced treatment of biologically pretreated coking wastewater by electrochemical oxidation using boron-doped diamond electrodes. *Water Res*. 2009;43(17):4347-4355.

   doi: 10.1016/j.watres.2009.06.030

3. Zhang Z, Han Y, Xu C, *et al*. Microbial nitrate removal in biologically enhanced treated coal gasification wastewater of low COD to nitrate ratio by coupling biological denitrification with iron and carbon micro-electrolysis. *Bioresour Technol*. 2018;262:65-73.

   doi: 10.1016/j.biortech.2018.04.059

4. Shi J, Xu C, Han Y, Han H. Enhanced anaerobic biodegradation efficiency and mechanism of quinoline, pyridine, and indole in coal gasification wastewater. *Chem Eng J*. 2019;361:1019-1029.

   doi: 10.1016/j.cej.2018.12.162

5. Shi J, Xu C, Han Y, Han H. Anaerobic bioaugmentation hydrolysis of selected nitrogen heterocyclic compound in coal gasification wastewater. *Bioresour Technol*. 2019;278:223-230.

   doi: 10.1016/j.biortech.2018.12.113

6. Yang Y, Huang F, Kang S. Mechanism of Penetration Rate Improvement in Hot Dry Rock Under the Coupling of Impact Load and Confining Pressure Release. *Reserv Sci*. 2026;2(1):52-64.

   doi: 10.62762/RS.2025.804345

7. Wu J, Ansari U. From CO2 Sequestration to Hydrogen Storage: Further Utilization of Depleted Gas Reservoirs. *Reserv Sci*. 2025;1(1):19-35.

doi: 10.62762/RS.2025.860510

8. Hu Y, Yang Y. A Comparative Study on Drag Reduction Methods for Continental Shale Drilling in the Fuxing Block, Southeastern Sichuan Basin. *Reserv Sci*. 2026;2(2):81-96.

   doi: 10.62762/RS.2025.790790

9. Fan C, Wang SC. Co-metabolic enhancement of organic removal from waste water in the presence of high levels of alkyl paraben constituents of cosmetic and personal care products. *Chemosphere*. 2017;179:306-315.

   doi: 10.1016/j.chemosphere.2017.03.120

10. Xiao M, Yin X, Gai H, *et al*. Effect of hydroxypropyl-β-cyclodextrin on the cometabolism of phenol and phenanthrene by a novel Chryseobacterium sp. *Bioresour Technol*. 2019;273:56-62.

    doi: 10.1016/j.biortech.2018.10.087

11. Kumabe K, Fujimoto S, Yanagida T, Ogata M, Fukuda T. Environmental and economic analysis of methanol production process via biomass gasification. *Fuel*. 2008;87(7):1422-1427.

    doi: 10.1016/j.fuel.2007.06.008

12. Perez M, Rodriguez-Cano R, Romero L, Sales D. Anaerobic thermophilic digestion of cutting oil wastewater: Effect of co-substrate. *Biochem Eng J*. 2006;29(3):250-257.

    doi: 10.1016/j.bej.2006.01.011

13. APHA. *Standard Methods for the Examination of Water and Wastewater*. 20th ed. Washington, DC, USA: American Public Health Association; 1998.

14. Zhu H, Han Y, Ma W, Han H, Ma W. Removal of selected nitrogenous heterocyclic compounds in biologically pretreated coal gasification wastewater (BPCGW) using the catalytic ozonation process combined with the two-stage membrane bioreactor (MBR). *Bioresour Technol*. 2017;245:786-793.

    doi: 10.1016/j.biortech.2017.09.029

15. Zhen GY, Lu XQ, Li YY, Zhao YC. Innovative combination of electrolysis and Fe(II)-activated persulfate oxidation for improving the dewaterability of waste activated sludge. *Bioresour Technol*. 2013;136:654-663.

    doi: 10.1016/j.biortech.2013.03.007

16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265-275.

    doi: 10.1016/S0021-9258(19)52451-6

17. Boyd TJ, Montgomery MT, Steele JK, *et al*. Dissolved oxygen saturation controls PAH biodegradation in freshwater estuary sediments. *Microb Ecol*. 2005;49(2):226-235.

    doi: 10.1007/s00248-004-0279-0

18. Rothermich MM, Hayes LA, Lovley DR. Anaerobic, sulfate-dependent degradation of polycyclic aromatic hydrocarbons in petroleum-contaminated harbor sediment. *Environ Sci Technol*. 2002;36(22):4811-4817.

    doi: 10.1021/es0200241

19. Torres LG, Rojas N, Bautista G, Iturbe R. Effect of temperature, and surfactant's HLB and dose over the TPH-diesel biodegradation process in aged soils. *Process Biochem*. 2005;40(10):3296-3302.

    doi: 10.1016/j.procbio.2005.03.032

20. Teng Y, Luo Y, Ping L, Zou D, Li Z, Christie P. Effects of soil amendment with different carbon sources and other factors on the bioremediation of an aged PAH-contaminated soil. *Biodegradation*. 2010;21(2):167-178.

    doi: 10.1007/s10532-009-9291-x

21. Bacosa HP, Inoue C. Polycyclic aromatic hydrocarbons (PAHs) biodegradation potential and diversity of microbial consortia enriched from tsunami sediments in Miyagi, Japan. *J Hazard Mater*. 2015;283:689-697.

    doi: 10.1016/j.jhazmat.2014.09.068

22. Xu P, Ma W, Han H, Hou B, Jia S. Biodegradation and interaction of quinoline and glucose in dual substrates system. *Bull Environ Contam Toxicol*. 2015;94(3):365-369.

    doi: 10.1007/s00128-014-1388-1

23. Li WW, Yu HQ. Insight into the roles of microbial extracellular polymer substances in metal biosorption. *Bioresour Technol*. 2014;160:15-23.

    doi: 10.1016/j.biortech.2013.11.074

24. Sheng GP, Yu HQ, Li XY. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. *Biotechnol Adv*. 2010;28(6):882-894.

    doi: 10.1016/j.biotechadv.2010.08.001

25. Ying W, Yang F, Bick A, Oron G, Herzberg M. Extracellular polymeric substances (EPS) in a hybrid growth membrane bioreactor (HG-MBR): Viscoelastic and adherence characteristics. *Environ Sci Technol*. 2010;44(22):8636-8643.

    doi: 10.1021/es102309y

26. Tay JH, Liu QS, Liu Y. The role of cellular polysaccharides in the formation and stability of aerobic granules. *Lett Appl Microbiol*. 2001;33(3):222-226.

    doi: 10.1046/j.1472-765x.2001.00986.x

27. Zhu L, Qi H, Lv M, Kong Y, Yu Y, Xu X. Component analysis of extracellular polymeric substances (EPS) during aerobic sludge granulation using FTIR and 3D-EEM technologies. *Bioresour Technol*. 2012;124:455-459.

    doi: 10.1016/j.biortech.2012.08.059

28. Wang ZW, Liu Y, Tay JH. The role of SBR mixed liquor volume exchange ratio in aerobic granulation. *Chemosphere*.

2006;62(5):767-771.

doi: 10.1016/j.chemosphere.2005.04.081

29. Oba Y, Futagami T, Amachi S. Enrichment of a microbial consortium capable of reductive deiodination of 2,4,6-triiodophenol. *J Biosci Bioeng*. 2014;117(3):310-317.

doi: 10.1016/j.jbiosc.2013.08.011

30. Wang W, Wu B, Pan S, Yang K, Hu Z, Yuan S. Performance robustness of the UASB reactors treating saline phenolic wastewater and analysis of microbial community structure. *J Hazard Mater*. 2017;331:21-27.

doi: 10.1016/j.jhazmat.2017.02.025

31. Jetten M. Methanogenesis from acetate: a comparison of the acetate metabolism in Methanothrix soehngenii and Methanosarcina spp. *FEMS Microbiol Lett*. 1992;88(3-4):181-198.

doi: 10.1111/j.1574-6968.1992.tb04987.x

32. Yin C, Shen Y, Yu Y, Yuan H, Lou Z, Zhu N. In-situ biogas upgrading by a stepwise addition of ash additives: Methanogen Adaption and CO2 sequestration. *Bioresour Technol*. 2019;282:1-8.

doi: 10.1016/j.biortech.2019.02.110

33. Imachi H, Sakai S, Sekiguchi Y, *et al*. Methanolinea tarda gen. nov., sp. nov., a methane-producing archaeon isolated from a methanogenic digester sludge. *Int J Syst Evol Microbiol*. 2008;58(1):294-301.

doi: 10.1099/ijs.0.65394-0