Mixotrophic S-Anammox Enrichment for Synergistic Nitrogen and Sulfur Removal: Performance, Microbial Community Succession, and Key Functional Taxa

Fangyuan Ren, Li Cui*, Siqi Gang
School of Environmental and Chemical Engineering, Shenyang University of Technology, Shenyang, China
**Corresponding Author*

Abstract: The S-Anammox process couples sulfur (S) and nitrogen (N) cycling in anaerobic environments, yet its metabolic behavior under mixotrophic conditions remains largely unexplored. This study examines the operational performance and microbial community succession in a laboratory-scale UASB bioreactor during a 180-day period of mixotrophic S-Anammox enrichment under intermittent organic carbon (COD) addition. Our results demonstrate that intermittent COD supply significantly enhances sulfate reduction efficiency up to 46.5% (average 32.8%) while preserving robust ammonium removal. 16S rRNA amplicon sequencing revealed a distinct microbial community restructuring under mixotrophic conditions: the anammox genus Candidatus_Brocadia was substantially enriched, while nitrite-oxidizing Nitrospira nearly disappeared, effectively suppressing nitrate accumulation. Concurrently, denitrifying Denitratisoma and sulfate-reducing Thermodesulfobacteriota were stably maintained, establishing a functional consortium for coordinated N-S removal. These findings provide new insights into the mixotrophic S-Anammox process, demonstrating that intermittent COD addition fosters a synergistic autotrophic-heterotrophic system successfully. This strategy effectively overcomes the limitations of purely inorganic or organic conditions, offering a promising technical pathway for treating industrial wastewater containing both ammonium, sulfate and COD in the wastewater.

Keywords: S-Anammox; Mixotrophic Condition; Microbial Community Succession; Nitrogen Removal; Sulfate Reduction

1. Introduction

Sulfur (S) and nitrogen (N) are key elements in the biogeochemical cycle and have received increasing attention in the context of the growing industrialization and the rising demand for sustainable development across the country. Industrial activities such as the combustion of fossil fuels and drainage from mining areas can lead to the continuous accumulation of pollutants such as ammonium nitrogen (NH_4^+-N) and sulfate (SO_4^{2-}) in wastewater [1]. The excessive discharge of NH_4^+ and SO_4^{2-} in wastewater not only poses a serious threat to the stability of aquatic ecosystems but also may endanger human health through food chain transmission and drinking water pollution.

Fdz-Polanco et al. first at 2001 discovered the simultaneous removal of NH_4^+ and SO_4^{2-} in a digestion bioreactor treating diluted distillery wastewater and named this process as SRAO. The process has been recognized as a promising approach for converting NH_4^+ and SO_4^{2-} into nitrogen gas (N_2) and elemental sulfur (S) [2]. There is also growing evidence in natural ecosystems that the nitrogen and sulfur cycles have complex and close interactions [3], providing the possibility for the simultaneous N and S removal. Different studies have used different abbreviations for this reaction, such as SRAO, S-Anammox, and Sulfammox. In this paper, S-Anammox is uniformly used to represent this process.

S-Anammox can occur under both inorganic and organic conditions. Under inorganic conditions, it is more conducive to nitrogen removal, with a high NH_4^+ removal rate and strong activity, but the removal of sulfate is limited, and even the phenomenon of sulfate reduction stagnation may occur. Under organic conditions, it is more conducive to sulfur removal, with a high SO_4^{2-} removal rate, but the NH_4^+-N removal rate is not efficient. Mixotrophic conditions, through the not only heterotrophic bacteria but also autotrophic denitrifying bacteria coexistence, enhance the denitrification capacity, can utilize more carbon and sulfur sources, and reduce the dependence on external carbon sources, thereby achieving a more efficient and sustainable nitrogen removal process [4]. During the addition period, organic carbon stimulates the activity of SRB and heterotrophic denitrifying bacteria, promoting the generation of sulfides and the reduction of nitrate; during the non-addition period, organic carbon is consumed, the activity of heterotrophic bacteria decreases, and AnAOB regains dominance, efficiently completing the oxidation of NH_4^+ and the conversion of nitrite.

However, up to now, there is still a lack of understanding of the impact of mixotrophic conditions on the S-Anammox metabolism. Existing studies have mostly focused on pure inorganic or pure organic conditions, and there is a lack of systematic research on the metabolic interaction between autotrophic bacteria (AnAOB, SOB) and heterotrophic bacteria (SRB, denitrifying bacteria) in the mixotrophic environment formed by intermittent addition of organic matter. Whether mixotrophic conditions can enhance the sulfate reduction efficiency while maintaining the advantages of autotrophic denitrification, and the microbiological and molecular biological mechanisms remain to be further explored.

The study aims to compare the operational performance of S-Anammox under anaerobic ammonium oxidation and mixotrophic conditions. Through continuous operation, the transformation and removal effects of carbon, nitrogen, and sulfur were investigated, under different process conditions, dynamic shifts in the microbial community were analyzed via 16S rRNA amplicon sequencing, revealing key bacterial populations involved in combined nitrogen–sulfur–carbon (N-S-C) metabolism. These findings offer valuable guidance for optimizing sulfur-driven anammox under anaerobic ammonium oxidation and mixotrophic conditions.

2. Materials and Methods

2.1 Experimental Design of Mixotrophic S-Anammox Enrichment in Bioreactor

To explore the characterization of coupled N and S removal pathways, Mixotrophic S-Anammox enrichment was performed in bioreactor. A laboratory-scale UASB bioreactor with an effective volume of 2 L was used for continuous flow experiments. This synthetic influent mainly consisted primarily of NH_4Cl, Na_2SO_4, $NaNO_2$, COD and $NaHCO_3$. Compared with low-molecular-weight volatile fatty acids (VFA), glucose is relatively complex and can be further decomposed to produce VFAs. To minimize the impact of carbon sources on the selection of sulfate-reducing bacteria, we chose glucose as the COD in the bioreactor in this experiment. The reactor was maintained at a temperature of 30 ± 1℃ through a temperature water bath, and wrapped with aluminum foil externally to avoid light influence and reduce heat loss. The system pH was maintained at 7.5 ± 0.2 by adding $NaHCO_3$. The HRT was set to 48 hours and subsequently to 24 hours, depending on the removal effect. The total of inoculated sludge in the reactor was 500 mL, including 200 mL of anammox granular sludge and 300 mL of anaerobic sludge taken from a WWTP in Shenyang. To enhance substrate mixing, the internal liquid in the bioreactor was circulated internally by a peristaltic pump. The preparing influent was stored in a influent tank connected to the UASB, and before each influent, high-purity argon gas (purity 99.99%) was used to purge for 30 minutes. To ensure that DO concentration in the influent was below 0.05 ± 0.03 mg/L. To rapidly enrich the S-Anammox process, the influent of NH_4^+, SO_4^{2-} and COD (Table 1) were set according to a previous study that successfully enriched anammox bacteria and the S-Anammox process.

Table 1. Concentration of Inlet Matrix

Phase	AS	TN	ME	MS
Time (d)	0-40	41-70	71-120	121-180
NH_4^+ (mg N/L)	30-60	60	60	120
NO_2^- (mg N/L)	70-166	166-0	0	0
SO_4^{2-} (mg S/L)	0	0-133	133	266
COD (mg/L)	0	0	50/0	100/0

2.2 Amplicons Sequencing and Data Analysis

To compare microbial communities with Anammox and microbial communities with S-Anammox, DNA was extracted from bioreactor collected from Day 40 (the end of AS) and Day 180 (the end of MS) respectively. Because the functional bacterial communities of both stages have completed sufficient enrichment at these two time points. Total genomic DNA was extracted with DNA concentration and integrity quality control performed using the Qubit 4 Fluorometer and agarose gel electrophoresis. The bacterial 16S rRNA gene was amplified using primers 338F/806R, and microbial diversity was analyzed using the QIIME2 workflow. Sequencing for this study was outsourced to Majorbio Bio-pharm Technology Co., Ltd. Relative bacterial abundance at the genus levels was determined by calculating the ratio of assigned reads to the total number of ASV. ASV can detect rare species with an abundance lower than 0.1%, which is important for understanding the microbial response mechanism under environmental stress. Therefore, the application of the ASV method can provide a more comprehensive analysis of the functions in the sludge system, offering a basis for optimizing the denitrification process in wastewater treatment.

2.3 Chemical Analysis

All water samples were filtered before the test, through 0.45μm pore size polyethersulfone membranes prior to analysis. The NH_4^+, NO_2^-, and nitrate concentrations were determined using a UV spectrophotometer (HACH, DR5000, USA). The concentration of S^{2-} was measured immediately using the methylene blue method. SO_4^{2-} was analyzed using ion chromatography (DIONEX, ICS1000, USA).

3. Results and Discussion

3.1 Stage-dependent S and N Removal across the S-Anammox Enrichment

The S-Anammox enrichment process in the reactor under mixotrophic conditions is divided into four stages. The transformation of N and S in the influent and effluent was continuously monitored during each stage, as shown in Figure 1. In the anammox stable phase (AS), the initial concentration of SO_4^{2-} was set at 30 mg N/L and that of NO_2^- at 70 mg N/L. After day 9, the removal rates of NH_4^+ and NO_2^- gradually increased and stabilized. To maintain the stable operation of the reactor, the concentration of NH_4^+ was increased to 60 mg N/L and that of NO_2^- to 166 mg N/L on day 21. At the end of this stage, the average molar NO_2^-/NH_4^+ was approximately 1.24, close to the theoretical stoichiometric ratio of anammox. The color of the sludge in the reactor changed from black to brick red, indicating that the anammox process might have been successfully established and stably operated. The second stage of this experiment was the transition phase (TN), where the influent NH_4^+ concentration remained unchanged, and NO_2^- was replaced by SO_4^{2-} in the influent. The concentration changes of NH_4^+, SO_4^{2-}, NO_2^-, and NO_3^- in the influent and effluent are shown in Figure 1. During this stage, the average removal load of NH_4^+ remained at a stable level of 57.29 mg N/(L·d), while the removal load of SO_4^{2-} gradually increased with the increase in influent concentration, reaching an average of 16.01 mg S/(L·d). In the third stage, the mixotrophic S-Anammox enrichment phase (ME), the concentration of NH_4^+ was fixed at 60 mg N/L and that of SO_4^{2-} at 133 mg S/L. COD was intermittently added (with an intermittent period of 5 days) to maintain a mixotrophic environment in the reactor. In the ME stage, the removal efficiency of SO_4^{2-} showed obvious periodic fluctuations, closely related to the intermittent addition of COD. During the period without COD addition, the removal rate of SO_4^{2-} remained between 22% and 33%; while during the period with COD addition, the removal rate significantly increased to 30%-39%. This indicates that heterotrophic sulfate-reducing bacteria rapidly activated their metabolic activity under the condition of available organic matter, enhancing the conversion efficiency of sulfate. In the fourth stage, the mixotrophic S-Anammox stable phase (MS), the concentration of NH_4^+ was set to 120 mg N/L and that of SO_4^{2-} to 266 mg S/L. During the period with COD addition, the removal rate was stably maintained at 37% - 46%. The highest removal rate observed in this stage was 46.5% on day 168. Notably, as the operation time increased, the SO_4^{2-} removal rate during the period without COD addition gradually increased from 22% - 33% in the third stage to 35% - 44% in the fourth stage. This result indicates that the sulfate-reducing bacterial community was effectively enriched and acclimated under intermittent COD stimulation and could still maintain a high sulfate reduction activity without COD addition, consistent with previous studies [5]. This indicates that the reactor successfully constructed a stable mixotrophic S-Anammox system through intermittent COD addition, achieving stable simultaneous removal of S and N.

The transformation of NO_2^- and NO_3^- under mixotrophic conditions is shown in Figure 2. The NO_2^- concentration increased from 40%

initially to 93%-100% in the later stage, and the effluent NO_2^- concentration was 0 on multiple occasions after day 53, demonstrating excellent nitrite conversion capacity. In the anammox system, the intermittent supply of organic carbon sources not only supported the activity of denitrifying bacteria but also enhanced the competitiveness of S-Anammox bacteria for nitrite, thereby inhibiting the accumulation of nitrate [6]. In addition, intermittent COD addition helps maintain the diversity, promotes the synergistic action of autotrophic and heterotrophic denitrification processes, and achieves efficient denitrification. Therefore, the co-nitrifying condition, that is, the intermittent COD addition method, optimizes the dynamic balance of nitrite and nitrate in the S-Anammox system, and enhances the denitrification performance and stability. In the ME stage, the actual concentration of COD added was on average 59.52 mg/L, and the concentration of the effluent was on average 4.24 mg/L. In the MS stage, the COD removal effect remained excellent, indicating that the system was operating stably.

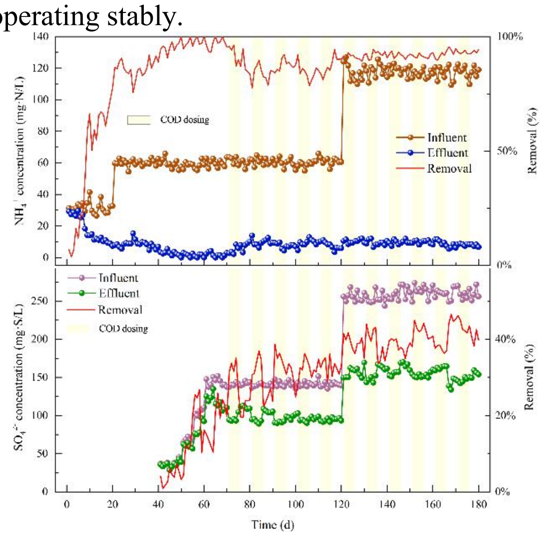

Figure 1. The nitrogen and Sulfur Removal Effects of S-Anammox: The Concentrations of NH_4^+ and SO_4^{2-} in Reactor under Mixotrophic Conditions

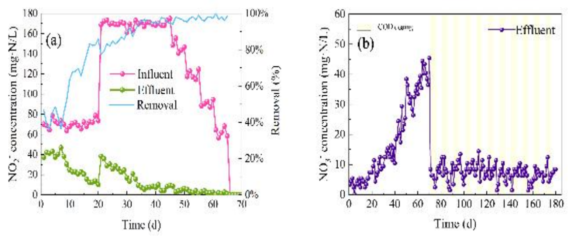

Figure 2. Concentrations of NO_2^- (a) and NO_3^- (b) in Influent and Effluent in Reactor under Mixotrophic Conditions

3.2 Microbial Community Succession and Diversity

To elucidate the microbial mechanisms driving the S-Anammox performance in reactor, 16S rRNA was used to characterize the dynamic shifts in microbial communities. The changes are shown in Figure 3. The main genera shared by the two stages include Candidatus_Brocadia, Nitrospira, Denitratisoma, Gemmatimonadaceae, Aggregatilineaceae, Alphaproteobacteria, and Thermodesulfobacteriota. In the AS stage, the community structure was characterized by the coexistence of multiple genera. The core anammox functional genus Candidatus_Brocadia occupied a relatively high proportion and was one of the dominant genera in this stage, which was highly consistent with the performance of the reactor achieving over 90% nitrogen removal efficiency in the AS stage. Meanwhile, Liu et al. suggested that Candidatus_Brocadia could utilize SO_4^{2-} and NO_3^- as electron acceptors to remove NH_4^+ [7]. The denitrifying genus Denitratisoma also showed a high abundance in the AS stage, indicating that the denitrification process might cooperate with the anammox process to jointly achieve nitrogen removal. The anammox process generates a small amount of nitrate, and denitrifying bacteria such as Denitratisoma can utilize organic carbon sources (possibly from cell lysis) to reduce it to nitrogen gas, thereby further enhancing the total nitrogen removal efficiency. The nitrite-oxidizing genus Nitrospira presented a moderate abundance in the AS stage, which was consistent with the detection of a certain concentration of nitrate in the effluent of this stage, suggesting that part of the nitrite was oxidized to nitrate by this genus. The AS stage also enriched a large number of Chloroflexota-related genera, including Caldilineaceae, Aggregatilineaceae, Ardenticatenales, etc. Bovio-Winkler et al. pointed out that these genera usually act as framework bacteria in anammox systems, and their filamentous structure helps form and maintain sludge granules, providing a carrier for the attachment and growth of anammox bacteria [8].

In the MS stage, Candidatus_Brocadia increased significantly, indicating that the co-culture conditions not only did not pose a threat to the enrichment of anammox bacteria but also had a strong promoting effect. Nitrospira almost completely disappeared. As a representative of nitrite-oxidizing bacteria (NOB) [9], the disappearance of Nitrospira cut off the key pathway for the conversion of nitrite to nitrate, thereby reducing the net production of nitrate and improving the total N removal. This change is an important manifestation of the optimization of the microbial community structure under co-culture conditions. Sulfate-reducing bacteria of the Thermodesulfobacteriota remained at a certain level during the MS stage, and their stable presence provided a microbiological basis for the significant improvement of sulfate reduction efficiency in this stage [10,11]. The genus Denitratisoma of denitrifying bacteria remained at a relatively high level during the MS stage, indicating that the facultative conditions greatly promoted the enrichment of denitrifying bacteria, which might be related to the addition of COD providing a usable organic carbon source - denitrifying bacteria can utilize COD for heterotrophic denitrification, reducing nitrate or nitrite in the system to nitrogen gas. The enrichment of Denitratisoma and the disappearance of Nitrospira formed a functional complementarity: the disappearance of Nitrospira reduced the generation of nitrate, while the enrichment of Denitratisoma enhanced the removal of nitrate. The combined effect of both improved the removal efficiency of total N.

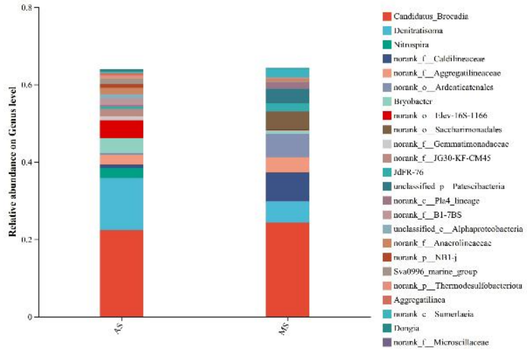

Figure 3. 16S rRNA Sequencing Reveals the Microbial Community at the Gene Level

6. Conclusion

This study successfully established a stable mixotrophic S-Anammox system through intermittent COD addition in a UASB reactor. The process effectively overcame the limitations of purely inorganic and purely organic conditions, achieving synergistic nitrogen and sulfur removal. The main findings are summarized as follows:

(1) Intermittent COD supply significantly enhanced sulfate reduction efficiency (peak 46.5%, average 32.8%) while preserving robust ammonium oxidation and near-complete nitrite conversion.

(2) 16S rRNA amplicon sequencing revealed a distinct microbial community restructuring: the anammox genus Candidatus_Brocadia was substantially enriched, while nitrite-oxidizing Nitrospira nearly disappeared, effectively suppressing nitrate accumulation.

(3) Denitrifying Denitratisoma and sulfate-reducing Thermodesulfobacteriota were stably maintained, forming a functional consortium for coordinated nitrogen-sulfur removal.

These findings provide a theoretical and technical foundation for applying the S-Anammox process to treat industrial wastewater containing both ammonium and sulfate, highlighting the potential of mixotrophic strategies in anaerobic biotechnology.

Acknowledgments

The authors would like to thank the Science and Technology Joint Program of Liaoning Province (Key R&D Program Project) 2025JH2/101800383.

References

[1] CHEN X, ZHENG L G, ZHU M Z, et al. Quantitative identification of nitrate and sulfate sources of a multiple land-use area impacted by mine drainage. Journal of Environmental Management, 2023, 325.

[2] FDZ-POLANCO F, FDZ-POLANCO M, FERNANDEZ N, et al. New process for simultaneous removal of nitrogen and sulphur under anaerobic conditions. Water Research, 2001, 35(4): 1111-1114.

[3] RIOS-DEL TORO E E, VALENZUELA E I, LóPEZ-LOZANO N E, et al. Anaerobic ammonium oxidation linked to sulfate and ferric iron reduction fuels nitrogen loss in marine sediments. Biodegradation, 2018, 29(5): 429-442.

[4] YANEZ D, GUERRERO L, BORJA R, et al. Sulfur-based mixotrophic denitrification with the stoichiometric S^0/N ratio and methanol supplementation: effect of the C/N ratio on the process. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering, 2021, 56(13): 1420-1427.

[5] SHU W H, DU B, WU G X. Strategies for enriching targeted sulfate-reducing bacteria

and revealing their microbial interactions in anaerobic digestion ecosystems. Water Research, 2025, 270.
[6] LI J L, PENG Y Z, YANG S H, et al. Successful Application of Anammox Using the Hybrid Autotrophic-Heterotrophic Denitrification Process for Low-Strength Wastewater Treatment. Environmental Science & Technology, 2022, 56(19): 13964-13974.
[7] LIU L-Y, WANG X, DANG C-C, et al. Anaerobic ammonium oxidation coupled with sulfate reduction links nitrogen with sulfur cycle. Bioresource Technology, 2024, 403.
[8] BOVIO-WINKLER P, GUERRERO L D, ERIJMAN L, et al. Genome-centric metagenomic insights into the role of Chloroflexi in anammox, activated sludge and methanogenic reactors. Bmc Microbiology, 2023, 23(1), 45.
[9] CHAI Z M, TIAN Z C, ZHENG M S, et al. The functional dominance and metabolic diversity of comammox Nitrospira in recirculating aquaculture systems. Water Research, 2025, 273.
[10] MALTSEVA A I, ELCHENINOV A G, KLYUKINA A A, et al. Thermodesulfovibrio autotrophicus sp. nov., the first autotrophic representative of the widespread sulfate-reducing genus Thermodesulfovibrio, and Thermodesulfovibrio obliviosus sp. nov. that has lost this ability. Systematic and Applied Microbiology, 2024, 47(6), 126561.
[11] OSHIKI M, TAKAHASHI K, KAWASAKI S, et al. Microbial Community Structure of Mesophilic and Low-temperature Partial Nitrification-anammox Reactors: Distribution and Functional Roles of the Core Microbiome. Microbes and Environments, 2025, 40(2), Me25001.