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HIGHLIGHTS

G R A P H I C A L A B S T R A C T

- AnMBR removed NH⁺₄-N, and SO₄2⁻-S simultaneously via Sulfammox and SDAD processes.
- \bullet SO_42 $^-$ enabled TN removal via Sulfammox, while S^2 $^-$ contributed through SDAD.
- Desulfovibrio and Sulfurospirillum microbes led the sulfammox and SDAD processes.
- AnMBR showed stable solution of achieving 45% SO₄²⁻ removal in treating rubber WW.

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Keywords: Ammonium oxidation Anaerobic nitrogen processing Sulfur-dependent Metabolism Integrated COD



ABSTRACT

Global rubber industry, growing 4–6 % annually with 13.76 million Mt of rubber produced in 2019, significantly impacts the economy. This study explores coupling sulfate-dependent ammonium oxidation (Sulfammox) and sulfide-driven autotrophic denitrification (SDAD) within an anaerobic membrane bioreactor (AnMBR) to treat high-strength natural rubber wastewater. Over 225 days, the AnMBR system achieved maximal chemical oxygen demand (COD), total nitrogen (TN), ammonium nitrogen (NH \ddagger -N), and sulfate sulfur (SO \ddagger -S) removal

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Nitrogen Sulfate reduction efficiencies of 58 %, 31 %, 13 %, and 45 %, respectively. TN is predominantly removed through Sulfammox (accounting for 49 % of NH_4^+ -N removal), SDAD, and conventional denitrification pathways. Sulfate removal is achieved via Sulfammox (responsible for 43 % of SO_4^{2-} -S removal), and Dissimilatory sulfate-reducing (DSR) processes (contributing 57 % of SO_4^{2-} -S removal). Microbial analysis identified *Desulfovibrio* and *Sulfurospirillum* as key microbes, while metagenomic analysis highlighted crucial sulfur and nitrogen cycling pathways. The findings support Sulfammox and SDAD as promising eco-friendly strategies for treating ammonia- and sulfate-rich industrial wastewater.

1. Introduction

The global rubber industry, which has been growing at 4–6 % annually, produced 13.76 million metric tons in 2019 and plays a crucial role in economies like Sri Lanka, where it is the third-largest export item (Ngteni et al., 2020; Export Development Board, Sri Lanka, 2023). However, this industry generates highly polluted wastewater, particularly from the centrifuged latex sector, with COD concentrations ranging from 1,650 to 36,400 mg/L, NH₃-N levels between 285 and 5,000 mg/L, and SO₄^{2–}-S levels from 500 to 3,330 mg/L (Pendashteh et al., 2017). Traditional treatment methods like anaerobic filter beds and facultative ponds face limitations due to high ammonia and sulfate levels, reducing COD removal efficiency(Wijerathna et al., 2023). These conventional systems struggle with regulatory compliance, especially when production volumes increase (Ho et al., 2023). Innovative treatment approaches are essential to meet stricter environmental standards for controlling COD, NH⁺₄-N, and SO₄^{2–}-S.

While simultaneous removal of NH₄⁺-N and SO₄²⁻-S shows promise for reducing environmental and financial burdens in wastewater treatment, real-world application faces significant optimization challenges (Dominika et al., 2021). The Sulfammox process, discovered by Fdz-Polanco et al., 2001, achieves 55 % TKN removal and up to 80 % sulfur removal under anaerobic conditions by coupling ammonium oxidation and sulfate reduction. Sulfammox utilizes sulfate as a terminal electron acceptor in the anaerobic oxidation of NH₄⁺-N, producing nitrogen gas (N_2) , sulfide (S^{2-}) , and elemental sulfur (S^{0}) (Liu et al., 2024). Research by Qin et al., 2021; L. Wu et al., 2020 confirmed that Sulfammox connects the nitrogen and sulfur cycles, traditionally treated as separate processes, and can reduce the dependency on conventional nitrogen removal pathways. For instance, Li et al., (2021) demonstrated removal efficiencies of 95 % NH₄⁺-N and 53 % SO₄²⁻-S in synthetic systems using circulating-flow anaerobic bioreactors. However, these studies were performed using synthetic wastewater, leaving a gap in understanding the application of this process in real wastewater treatment systems where the composition is more complex and variable.

Another key approach for simultaneous NH_4^+ -N, and SO_4^{2-} -S removal is the Sulfide-driven autotrophic denitrification (SDAD) process, where autotrophic denitrifying bacteria use sulfide as an electron donor to convert nitrate to nitrogen gas (Zhang et al., 2022a). Studies such as Zhang, Cui, et al., (2019) has highlighted that nitrate and nitrite can significantly enhance ammonium oxidation, thereby facilitating the SDAD process in synthetic wastewater systems. SDAD tending dominated the denitrification process due to its thermodynamic advantage (Gibbs free energy of $-630.5 \text{ kJ/mol NO}_3$) compared to conventional denitrification.In practical applications, nitrogen removal efficiency is critically dependent on the N/S ratio and the availability of sulfide (Wang et al., 2017). For instance, Zhang et al. (2023a,b) employed an innovative process that integrated partial nitrification, nitritedependent Anammox (N-Anammox), and sulfate-dependent Anammox (S-Anammox) to treat mature landfill leachate with high NH₄⁺-N concentrations, achieving 96 % nitrogen removal. Isotope analysis confirmed the roles of N-Anammox and S-Anammox in nitrogen and sulfur cycles, reducing energy consumption by 44 %. However, the metabolic relationship between Sulfammox and SDAD in systems with organic matter in the anaerobic treatment system remains unclear.

Sulfammox and SDAD have demonstrated promise in nutrient

removal, yet significant knowledge gaps remain in understanding their integration in real-world scenarios, especially in high-nitrogen and sulfur-content wastewater. Sulfammox is a novel process that has not been widely studied alongside SDAD, and their integration has not been previously applied to natural rubber industry wastewater, which poses unique challenges due to its high nitrogen and sulfur content. Most studies have used synthetic wastewater, leaving gaps in understanding their performance with actual industrial wastewater over long-term operation. This study addresses these gaps by using an AnMBR system to couple Sulfammox and SDAD for efficient, energy-saving treatment of rubber industry wastewater. MBRs, combining membrane filtration with biological treatment, have become increasingly popular for municipal and industrial wastewater treatment due to their ability to achieve high pollutant removal efficiency, superior effluent quality, reduced sludge production, and a smaller operational footprint compared to conventional systems (Zhang et al., 2021). This study adopted AnMBR to treat pre-treated wastewater from the natural rubber industry to assess wastewater treatment performance with sulfammox and SDAD, as well as microbial communities were studied to explain the mechanism. The objectives of this research were to (1) examine the coupling of N and S cycles in treating NH₄⁺-N- and SO₄²⁻-S-rich wastewater from the natural rubber industry, (2) assess the conversion efficacy of nitrogen and sulfur compounds,(3) evaluate the capacity of these integrated processes for the efficient treatment of NH₄⁺-N- and SO₄²⁻-S-rich wastewater while achieving energy efficiency and environmental sustainability, and (4) investigate the microbial community structure and dynamics at the genus level using metagenomic sequencing to elucidate the key microorganisms involved in the nitrogen and sulfur cycles that support the Sulfammox and SDAD processes.

2. Material & methods

2.1. Raw wastewater and pre-treated wastewater of natural rubber industry

Wastewater samples were collected from a centrifuged latex manufacturing factory in Mawanella, Sri Lanka (7°15'22.2"N; 80°26'27.9"E), where natural latex was used for production. Natural rubber was centrifuged for 7,000 to 10,000 rpm to produce concentrated latex, which was ammoniated for preservation. Then less concentrated latex portion, which is known skim latex was coagulated using sulfuric acid to form skim rubber. This skim rubber was dried and after being milled into sheets. After the coagulum was removed, the remaining water was ejected as wastewater (referred to as skim serum water). Skim serum, acidified effluent, wastewater generated by cleaning of centrifuged machines, and wastewater from milling of rubber sheets constitute the factory's raw wastewater. Concentrations of major pollutants in the raw natural rubber industrial wastewater are elevated, such as COD at 34875 \pm 2652 mg/l, NH_3^-N at 2583 \pm 810 mg/l, and SO_4^2--S at 3130 \pm 1032 mg/l (Table 1). Raw rubber wastewater was pre-treated using a magnetic seed coagulation (MSC) before being introduced into an AnMBR. The feeding-water characteristics of the AnMBR after MSC pretreatment are listed in Table 1. Details of the pre-treatment study is available with our previous publication (Wimalaweera et al., 2024a).

Table 1

Characteristics of raw & pre-treated natural rubber wastewater from centrifuged Latex factory (n = 20) (Average \pm S.D.).

Parameter	Raw Wastewater	Pre-treated Wastewater
COD, mg/l	$34,\!875 \pm 2,\!652$	$23,\!647 \pm 3,\!826$
TOC, mg/l	$12,034 \pm 1,617$	$\textbf{7,438} \pm \textbf{1,068}$
рН	5.0 ± 0.8	$6.8 {\pm} .0.3$
EC, mS/cm	$\textbf{28.4} \pm \textbf{8.6}$	24.6 ± 8.7
TSS, mg/l	$5,695 \pm 2,595$	$1,275\pm45$
NO ₃ -N, mg/l	$1,461 \pm 245$	113 ± 37
NO ₂ -N, mg/l	50.5±24.7	35.6 ± 20.2
NH ₃ -N, mg/l	$\textbf{2,583} \pm \textbf{810}$	$1,545\pm97$
TN, mg/l	$4,137 \pm 1,103$	$3{,}529\pm312$
$SO_4^{2-}-S, mg/l$	$3,130 \pm 1,032$	425 ± 181
$S^{2-}, mg/l$	102.3 ± 18.7	8.3±3.7
PO ₄ ³⁻ -P, mg/1	995 ± 333	156 ± 35

2.2. Experimental setup

The AnMBR was a cylindrical tank with an effective volume of 15 L (diameter = 230 mm, height = 450 mm). The system was operated with a hydraulic retention time (HRT) of 72 h and a flow rate of 5 L/day. The external tubular membrane was made of ceramic (yttria-stabilized zirconia) with a nominal pore size of 0.1 μ m, and a total area of 0.11 m² (Hefei Shijie Membrane Engineering Co. Ltd., Hefei, China) was coupled with the bioreactor. The operating temperature ranged from 27 to 30 °C, and the pH of the influent was maintained between 6.7 and 7.3. A peristaltic pump (SHENCHEN, LanN1, YZ1515x Pump, China) was used to feed the pre-treated wastewater into the bioreactor. The ceramic membrane pressure (TMP). Another peristatic pump (ALLEDOSIEREN, V series, China) was used to feed the membrane. The bioreactor contained sensors and a gas outlet, which were used to track the temperature, pH, and EC of the system.

Ceramic employed a time-based backwashing strategy aimed at maximizing pump lifespan, minimizing costs, and optimizing energy consumption. Backwashing was carried out daily using 1 L of permeate for 60 s. The membrane was backwashed at a pressure of 150 kPa and a flux rate of 150 L/m^2 /h. When the TMP reached 85 kPa, ex-situ chemical cleaning was performed. This cleaning process involved sequential rinsing with water, followed by treatment with a 500 ppm NaOCl solution and a 500 ppm citric acid solution, each accompanied by a 4-hour water soak (Nilusha et al., 2020; Wimalaweera et al., 2024b).

2.3. Operation of the reactor

Seed sludge was collected from the same centrifuged latex manufacturing factory in Mawanella, Sri Lanka, where wastewater samples also been collected. This factory has a suspended growth anaerobic and aerobic wastewater treatment system. In this experiment, the AnMBR was operated for 225 days, which was divided into four periods based on the influent quality and loading rates. To establish the process and optimize its factors of the sulfammox, dissimilatory sulfate reduction (DSR), and SDAD processes, concentrations of major pollutants in the influent and effluent were monitored and analysed. These periods encompassed a sequential influent loading approach for the reactor. Starting from the day 71, the system was fully loaded with full concertation of real wastewater. Supplementary information presents details of the primary operational parameters of the AnMBR (see supplementary material). The AnMBR system started with low substrate concentrations, which were gradually increased over 70 days. Optimal environmental conditions were maintained, including controlled temperatures (25.1-30.0 °C) and pH levels (7.0-8.5), creating a favorable environment for the growth and activity of slow-growing anaerobic bacteria (Dominika et al., 2021).

2.4. Batch test

To explore microbial process behaviors under controlled anaerobic conditions, we conducted a series of batch tests using five 500 ml vessels, designed to simulate various biochemical reactions reflective of the AnMBR system. Each vessel contained 250 ml of washed sludge from the lab-scale AnMBR system. The vessels were placed in a thermostatic shaker at 30 °C and 150 rpm to ensure homogeneity and optimal metabolic activity, with pH maintained between 7.0 and 7.5. Synthetic solutions of NH₄Cl, KNO₃, glucose, and Na₂SO₄ were used as substrates. Vessel 1 focused on the Sulfammox process, vessel 2 examined interactions between Sulfammox and SDAD processes, vessel 3 integrated Sulfammox with DSR and anaerobic digestion, vessel 4 served as a comprehensive model incorporating all processes, and vessel 5 operated as a control with only sludge and water to provide a baseline for microbial activity. Samples were collected at specific intervals and all batches had been repeated three times. Schematic diagram of the batch test experiment and specific operation parameters of batch tests are available at the supplementary document (see supplementary material).

2.5. Analytical methods

2.5.1. Chemical and physical parameters analysis

A standard method (APHA, 2017) was used to analyse all parameters. pH and conductivity were measured using a HACH HQ 40d multiparameter water quality analyser (Hach, USA). Dissolved components were analysed after passing the supernatant samples through a 0.45-µm membrane filter (GD/XP Syringe Filters, Whatman, UK). The COD, S²⁻, alkalinity, and total phosphorus were determined using spectrophotometry (HACH DR 6000). The Phenate method was used to quantify NH⁴₄ – N. Concentrations of SO²₄⁻, NO³₃ and NO²₂ were measured using an ion chromatography (IC, ICS 1000, Dionex, Sunnyvale, CA, USA). COD and alkalinity were determined using a prefabricated tube reagent (HACH, USA). TOC and TN were measured using a TOC analyser (LCPH/CPN, Shimatzu, Japan). Mixed liquor suspended soilds (MLVSS) of sludge samples were determined by the standard method.

2.5.2. Microbial analysis

Samples were collected from the mixed liquor on Days 34, 62, 90, 119, 140, 181, and 220 to analyse the bacterial and archaeal communities. DNA was extracted using the FAST DNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). The extracted DNA samples were transported to Majorbio Bio-Pharm Technology Co., Ltd in Shanghai, China, for analysis. PCR amplification of the 16S rRNA genes was performed using the 515F/806R primer pair. Paired-end Illumina MiSeq sequencing (Illumina MiSeq, USA) was conducted at the Majorbio sequencing facility. Raw sequences were demultiplexed, quality-filtered using Trimmomatic, and merged with FLASH. The sequences were quality-controlled and denoised using the DADA2 tool to obtain ASV (Amplicon Sequence Variant) representative sequences and abundance data. The sequences were then rarefied to the minimum sample size (50,850). ASV taxonomy was assigned by aligning the sequences against the SILVA 16S rRNA database (version 138). To identify dominant microbial genera, relative abundances were determined by normalizing the number of sequences assigned to each genus. Genera were ranked based on their relative abundance, with the dominant genera defined as those exhibiting the highest proportions across all samples. Dominant genera were tracked at multiple sampling points to evaluate their contribution to nitrogen and sulfur cycling within the AnMBR system. The Qiime2 was used to assess the alpha and beta diversity and structural integrity of microbial communities.

Metagenomic analysis using shotgun sequencing was performed on samples collected on days 34 and 140. These time points were selected to capture different phases of reactor operation and microbial community development. The library construction (350 bp) and paired-end sequencing (150 bp) of this DNA samples were conducted on the HiSeq 4000 platform at Shanghai Majorbio Biopharm Technology Co., Ltd (Shanghai, China). After quality control, involving the removal of reads containing three or more ambiguous nucleotides, with quality scores of less than 20 for more than 36 bases, or splice contaminated reads. The protein database of Ncyc and SCyc database were used for the associated genes blast analysis through the diamond v0.9.22 (blastx, --query-cover 75, --id 90, --evalue 1e - 5) to find genes or enzyme related to nitrogen and sulphur metabolism routes. Furthermore, GraphPad Prism software was used to generate visual representations of metagenomic data, including bar charts and other graphical elements. The microbial communities and metagenomic data generated in this study is available at NGDC (National Genomics Data Center, https://ngdc.cncb.ac.cn/) with the accession number CRA019750.

2.6. Calculations

2.6.1. Nitrogen removal performance

The nitrogen removal efficiency (NRE) and nitrite accumulation ratio (NAR) were calculated using Eqs. (1) and (2), respectively. The ammonium utilisation rate (AUR) and nitrate utilisation rate(NUR) are shown in Eqs.(3) and (4), respectively (Derwis et al., 2023).

$$NRE = \frac{(TIN_{inf} - TIN_{eff})}{(TIN_{inf})}$$
(1)

$$NAR = \frac{(\Delta NO_2^- - N)}{(\Delta NO_2^- - N + \Delta NO_3^- - N)}$$
(2)

$$AUR = \frac{(\Delta NH_4^+ - N)}{(HRT)x(VSS)}$$
(3)

$$NUR = \frac{(\Delta NO_3^- - N)}{(HRT)x(VSS)}$$
(4)

2.6.2. Sulphate removal performance

The sulphate removal efficiency (SRE) and sulphate utilization rate (SUR) were calculated using Eqs.(5) and (6), respectively. The reactor performance in terms of sulfate removal was analyzed considering the relationship between sulfate reduction and nitrate removal via the SDAD process. The efficiency of nitrate removal was closely linked to the presence of sulfate-reducing bacteria, which contributed to the overall nitrogen-sulfur cycle in the anaerobic system.

$$SRE = \frac{(SO_4^{2-}eff - SO_4^{2-}inf)}{SO_4^{2-}inf} \times 100\%$$
(5)

$$SUR = \frac{(\Delta SO_4^{2-} - S)}{(HRT)x(VSS)}$$
(6)

The loading rates (kg N/m³·d⁻¹) were calculated according to the Eqs.(7), where C_{inf} is influent concentration and T is hydraulic retention time.

$$Loading Rate = C_{inf}/1000.T$$
⁽⁷⁾

Formulas to quantify the pollutant removal rates during batch tests is shown at <u>supplementary documents</u>. All data were statistically analyzed using IBM SPSS Statistics software to ensure the robustness and validity of the results.

3. Results and Discussion

3.1. Treatment performance of the reactor system

The removal of COD fluctuated notably across the reactor's phases, largely due to the interaction between different metabolic processes. In

Phase 1 (Day 0–40), the COD removal was moderate at 28 % (Fig. 1(a)). During this phase, Sulfammox and SDAD processes, which do not rely on organic matter, also began to initiate nitrogen removal. In the initial phases of the study, methanogenesis was the dominant process for COD reduction, despite having less favorable Gibbs free energy (-369.4 kJ/ mol) compared to conventional denitrification (-567.7 kJ/mol) and DSR (-137.1 kJ/mol) (Tang et al., 2013). This dominance occurred primarily due to the limited availability of NO₃-N in the reactor, which restricted the more energetically favorable denitrification and sulfate reduction processes. As the reactor transitioned to Phase 2, COD removal increased to 40 %, though ammonia removal efficiency decreased (from 22 % to 11 %). This indicates that DSR and conventional denitrification processes, which utilize organic matter, began to dominate, particularly as organic loading increased and sulfate availability reduced. In Phase 3 (Day 71–130), COD removal reached its peak efficiency of 58 %, and by Phase 4 (Day 131-225), COD removal remained stable at 41 % corresponding with enhanced organic matter consumption through DSR and conventional denitrification.

The AnMBR system exhibited notable fluctuations in NH₄⁺-N removal efficiency throughout its operational phases (Fig. 1(c)). In Phase 1, NH₄⁺⁻ N removal started at 22 %, driven by Sulfammox. This relatively high efficiency can be linked to the abundance of sulfate, facilitating the ammonia oxidation process. However, in Phase 2, ammonia removal efficiency decreased to 11 %, a decline attributed to either high loading rates or insufficient sulfate levels to drive Sulfammox effectively. By Phase 4, NH₄⁺-N removal dropped to a low of 6 %, correlating with a shortage of electron acceptors and inhibitory effects on sulfammox bacteria. Notable enhancements in nitrate and nitrite removal efficiencies suggested (Fig. 1(d)) a shift toward the predominance of SDAD, likely fuelled by sulphide from ongoing sulphate reduction. This is supported by Gibbs energy values, where SDAD and sulfate reduction processes are more favorable compared to methanogenesis, with Gibbs energy values of -492 kJ/mol for sulfate reduction, and -428 kJ/mol for methanogenesis (Pokorna and Zabranska, 2015; Moraes et al., 2012). The high alkalinity consumption in second and third stages correlated with high TOC consumption (see supplementary material) due to increased denitrification activity(Inaba et al., 2020).

Sulfate removal efficiency was at elevated level of 52 % in Phase 1 (see supplementary material), primarily due to active Sulfammox processes, as evidenced by the concurrent high NH⁺₄-N removal. Based on stoichiometric calculations and prior research, it is estimated that by the sulfammox process approximately 40 % of the removed sulfate was reduced to sulfide (S²⁻), while the remaining 60 % was converted to elemental sulfur (S°)(Liu et al., 2024a). As the system progressed to Phase 3&4, sulfate removal efficiency stabilized at 45 % (Fig. 1(b)). The transition from Phase 1 to later phases suggested a shift from Sulfammox to SDAD and DSR, evidenced by the reduction in sulfate levels. The declining availability of sulfate limited Sulfammox, while the predominance of SDAD processes likely drove the nitrogen removal. This observation aligns with findings by Qin et al., (2021), who reported that increasing SO²₄-S concentrations led to a decrease in NH⁺₄-N removal efficiency, with negligible changes in SO²₄-S removal efficiency.

The increasing NH⁺₄-N/SO^{2–}₄-S (N/S) ratio from influent to effluent across all phases indicates a growing inefficiency in maintaining the ideal conditions for the Sulfammox and SDAD processes(see supplementary material). For example, in Phase 1, the influent N/S ratio was 3.97, increasing to 4.30 in the effluent. In Phase 2, this imbalance became more pronounced, with the ratio increasing from 5.10 to 7.25, while in Phase 3, the ratio further increased from 4.14 to 11.44. These fluctuations suggest that the Sulfammox process became less effective as operational conditions progressed. The increased effluent N/S ratios indicate that Sulfammox, which ideally requires a balanced N/S ratio, was less effective under the given operational conditions. Moreover, the discrepancy between sulfate reduction and S^{2–} production may have resulted from re-oxidation of sulfide into sulfate, elemental sulfur deposition during the SDAD process, and hydrogen sulfide (H₂S)



Fig. 1. Operational performance during the operation of AnMBR, (a) variation of COD level; (b) Operation performance of sulphur compounds during operational period; (c) Concentration of N compounds (TN, NH₃-N and NOx); (d) Removal efficiencies of N compounds and ratios of Δ SO₄²⁻-S/ Δ COD and Δ TN/ Δ COD. (Operational phases are denoted by vertical dotted lines)(n = 96).

volatilization (DeLeon et al., 2012).

Regarding $\Delta SO_4^{2-}/\Delta COD$ and $\Delta TN/\Delta COD$ ratios(Fig. 1(d)), Phase 1 showed moderate sulfate reduction ($\Delta SO_4^{2-}/\Delta COD \approx 0.2$). As the system progressed to Phase 3, sulfate reduction decreased slightly ($\Delta SO_4^{2-}/\Delta COD \approx 0.15$), while nitrogen removal improved ($\Delta TN/\Delta COD \approx 0.10$). By Phase 3, $\Delta SO_4^{2-}/\Delta COD$ further decreased to 0.01, while $\Delta TN/\Delta COD$ reached 0.15, reflecting enhanced denitrification and reduced sulfate reduction activity. By Phase 4, both ratios stabilized ($\Delta SO_4^{2-}/\Delta COD$ and $\Delta TN/\Delta COD \approx 0.12$), indicating a balanced interaction between sulfur and nitrogen removal processes. The consistently low $\Delta TN/\Delta COD$ ratio across all phases suggests that nitrogen reduction was driven primarily by Sulfammox and SDAD, rather than conventional denitrification, which depends on COD. Meanwhile, the slight elevation in the $\Delta SO_4^{2-}/\Delta$

 Δ COD ratio in later stages reflects a growing role of DSR in sulfur removal as the system adapted to increasing organic loads. This balance supports stable TN removal via SDAD and conventional denitrification observed in later phases.

3.2. Dynamic interplay of Sulfammox and sulfide driven autotrophic denitrification processes in nutrient removal

The presence of sulphate in wastewater plays a crucial role in linking the N and S cycles, with practical and ecological implications, such as presenting an opportunity for enhancing treatment efficiency, promoting resource utilisation, reducing the formation of undesirable compounds, and aligning with sustainable and holistic wastewater management practices (Derwis et al., 2023). In the Phase 1 of the AnMBR system, as indicated by the utilization rates and ratios from the Fig. 2(a), there is a clear dominance of the sulfammox process. This is evidenced by the enhanced ammonium utilization rate and sulfate utilization rate, which are harmonizing with the thermodynamic favorability reflected in the lower Gibbs free energy (Wang et al., 2018).

As the system progresses into the Phase 2 and 3, a discernible shift is observed. The Nitrate Utilization Rate (NUR) and Nitrate Accumulation Rate (NAR) begin to ascend, indicative of an emerging shift toward SDAD, which the Gibbs free energy data corroborates as more energetically favorable than Sulfammox. Denitrifying microorganisms, crucial in removing N from wastewater, can perform NO_3^-/NO_2^- reduction with S^{2-}/S^{0} . As a result, increasing SO_4^{2-} loading during the treatment to a certain level may increase the overall effectiveness of N removal (Wu et al., 2020b). During SDAD, NO₂ was gradually produced as intermediate product. When S^{2-} was oxidised to S_0 , the liquid phase of the system turned pale yellow. The intermediate product S_0 as an electron donor continued to react with NO3-N/NO2-N and was eventually reduced to N_2 and SO_4^{2-} . This variation is explained in Fig. 2(a), in which a higher NUR is observed at higher SUR levels. This further explains the relationship between the N and S cycles. In the phase 3 and 4, depicted by the second graph, the ratios of $\Delta SO_4^{2-}-S/\Delta NH_3-N$ and $\Delta NO_3^{-}-N$ $/\Delta NH_3$ - provide a nuanced view of the ongoing processes. The declining ΔSO_4^{2-} -S/ ΔNO_3^{-} -N ratio, coupled with the NAR (Fig. 2(b)), suggests a well-established and energetically efficient SDAD process (T.-T. Zhang et al., 2023). Overall this system not only illustrates the integral roles of Sulfammox, and SDAD in the removal of NH₃-N, TN, and sulfate but also highlights the complex interplay between these processes over time.

Nutrient removal within the AnMBR is facilitated by a diverse array of processes. Notably, TN is predominantly removed through the sulfammox, SDAD, and conventional denitrification pathways. Meanwhile, sulphate reduction is achieved via sulfammox, SDAD and DSR processes. Additionally, organic matter utilization is managed through anaerobic digestion and methanogenesis with Gibbs free energy values of -492 kJ/mol for sulfate reduction and -428 kJ/mol for methanogenesis, supplemented by conventional denitrification and assimilation by DSR process (Zhang et al., 2022b). Among these, the sulfammox process's contribution to ammonia removal is directly quantifiable, being the sole process targeting ammonia. The core achievement of this continuous operation of lab reactor is the effective simultaneous reduction of COD, ammonium, and sulphate at an organic loading rate of 6.07-10.14 kg COD/m^3 day, with COD removal efficiency observed at range of 28 - 76%. Stable operation of sulfammox, SDAD, and heterotrophic denitrification pathways have collectively contributed up to 4-48 % to TN removal. Among all bioprocesses examined, the Sulfammox pathway is unique in its direct impact on ammonia removal. This process alone has contributed to an increase in the TN removal efficiency, ranging between 30 % to 82 % of the TN removal percentage, highlighting its vital role in enhancing the system's overall performance. Conversely, the interconnectivity of the other processes presents a complex challenge for precise quantification.

3.3. Batch experiment

3.3.1. Performance evaluation

The primary purpose of the batch tests was to evaluate the distinct biological processes such as Sulfammox, SDAD, DSR, AD, and conventional denitrification under controlled conditions, simulating an actual laboratory reactor environment. The design aimed to ensure that these processes could be observed separately, providing a clear understanding of their individual contributions and interactions within the system. After 12 days of operation, the microbial communities adapted and reached a steady state, indicating successful simulation and validation of these biological processes in the experimental setup (see supplementary



Fig. 2. Trends in AUR, NUR, and SUR at AnMBR Operational Phases(a); Comparative analysis of nutrient conversion ratios and NAR in AnMBR over Time(b) (n = 96).

material).

In Vessel 1, Sulfammox was the primary process, effectively reducing NH₄⁺-N and SO₄²⁻-S, while maintaining stable COD levels and minimal nitrate levels remained low, indicating minimal sulfide production. In the Sulfammox-only vessel, ammonia oxidation occurred with minimal nitrate formation, in agreement with the thermodynamic constraints predicted by the Gibbs energy for the Sulfammox process (-45.35 kJ/mol SO₄²⁻), which efficiently couples ammonium oxidation with sulfate reduction. Vessel 2 combined Sulfammox with SDAD, resulting in a more significant reduction in SO₄²⁻-S and a notable decrease in NO₃-N. This behavior is consistent with the thermodynamic preference for SDAD with sulfide oxidation, which has a lower Gibbs energy (-630.5 kJ/mol NO₃⁻). However, SO₄²⁻-S was less pronounced compared to other

vessels due to the re-oxidation of S²⁻ back to SO₄²⁻-S, as indicated by minimal sulfide levels (lowest at 0.2 mg/L). Vessel 3, integrating Sulfammox with DSR and anaerobic digestion, showed substantial reductions in NH₄⁺⁻N, SO₄²⁻-S, and COD, with an increase in sulfide levels (from 0.1 mg/L to 1.1 mg/L), confirming the activity of SRB supported by the Gibbs free energy for DSR (-137.1 kJ/mol SO₄²⁻). Finally, Vessel 4, which integrated all processes, achieved the highest overall removal efficiencies for NH₄⁺⁻N, SO₄²⁻-S, COD, and NO₃³⁻N, with controlled S²⁻ levels. The synergy between processes contributed to this enhanced performance.

The AUR (Fig. 3) are compared across the same four setups. Sulfammox process been identified as the only pathway to reduce the ammonia in this system. In the Sulfammox setup, the AUR shows peaks



Fig. 3. Utilization rates of substrates in each vessel during the batch test: (a) Sulfammox process, (b) Sulfammox + SDAD, (c) Sulfammox + DSR + AD, and (d) All processes integrated. NO_3^- was spiked on Days 1, 6, 12, 16, and 20 due to depletion of available NO_3^- , which was monitored daily. AUR = ammonia utilization rate; SUR = sulfate utilization rate; NUR = nitrate utilization rate (n = 48).

up to 10 mg $NH_{4}^{+}-N/g$ VSS/day, indicating active ammonium oxidation. The Sulfammox + SDAD setup exhibits similar peaks, reflecting active ammonium oxidation with some fluctuations due to interactions with SDAD. In the Sulfammox + DSR + AD setup, the AUR displays slightly lower peaks compared to Sulfammox alone, suggesting that while Sulfammox is active, other processes like DSR might compete for sulfate. The Sulfammox + DSR + AD + SDAD setup shows moderate peaks in AUR, reflecting a balance between ammonium oxidation and other processes related to N cycle (Statiris et al., 2022), including SDAD and DSR. The SUR for the Sulfammox (Fig. 3) setup is relatively stable with minor fluctuations around zero, indicating consistent sulfate reduction. In the Sulfammox + SDAD setup, the SUR shows periodic negative spikes, indicating moments when sulfate is regenerated from sulfide due to the SDAD process, which converts sulfide back to sulfate. The Sulfammox + DSR + AD setup exhibits higher positive SUR values compared to Sulfammox alone, demonstrating enhanced sulfate reduction due to active sulfate-reducing bacteria. The Sulfammox + DSR + AD + SDAD setup shows significant fluctuations in SUR, with both high positive and negative spikes, reflecting the complex interactions between all processes, including sulfate reduction by sulfammox, DSR and regeneration by SDAD.

In the Sulfammox setup, NUR is close to zero (Fig. 3), indicating minimal nitrate involvement in the Sulfammox process. The Sulfammox + SDAD setup shows distinct periodic spikes and drops (up to 5 mg NO₃⁻-N/g VSS/day), corresponding to nitrate consumption and external NO₃ addition. In the Sulfammox + DSR + AD setup, the NUR remains close to zero, similar to Sulfammox alone, indicating that nitrate is not significantly involved in this setup. The Sulfammox + DSR + AD + SDAD setup shows rapid reduction of NO₃⁻ similarly to Sulfammox + SDAD, reflecting the active nitrate reduction and regeneration processes occurring in this comprehensive setup. In summary, the Sulfammox + DSR + AD setup shows the highest sulfate reduction due to active SRB, while the

SDAD process in Sulfammox + SDAD and Sulfammox + DSR + AD + SDAD setups causes periodic sulfate regeneration. All setups show active ammonium oxidation, with Sulfammox alone and Sulfammox + SDAD exhibiting the highest AUR peaks. The presence of SRB and AD slightly reduces the AUR. Significant nitrate utilization is observed in setups involving SDAD, reflecting active nitrate reduction, while Sulfammox and Sulfammox + DSR + AD setups show minimal nitrate involvement.

3.3.2. Mass balance analysis

The mass balance diagram derived from the batch test data illustrates (Fig. 4(b)) the flow and transformation of key compounds through various microbial processes. The stoichiometric ratios used to calculate these transformations ensure accurate representation of the system's efficiency. The sulfate pathway begins with 2.3 mg/L.d of SO_4^2 -S entering the system (Fig. 4(a)). Through the Sulfammox process, sulfate is partially reduced to sulfide (0.5 mg/L·day) via the sulfammox reaction. SRB further reduce sulfate to sulfide (1.1 mg/L·day). This results in a combined sulfide production of 1.6 mg/L·day. Sulfammox process account for 52 % of SO_4^2 -S removal and DSR process then utilizes the produced sulfide, converting it back to sulfate and contributing to dinitrogen production. The sulfate regeneration (0.7 mg/L·day of SO_4^2 -S) through SDAD shows efficient recycling, with part of it directed towards dinitrogen production (0.6 mg/L·day).

The ammonia pathway starts with 5.5 mg/L·day of NH⁺₄-N as input. The Sulfammox process primarily converts ammonia to dinitrogen (5.2 mg/L·day). Additionally, conventional denitrification converts 0.1 mg/L.day part of NO₃-N to dinitrogen. The mass balance diagram, supported by stoichiometric calculations, highlights the efficient cycling of sulfate, ammonia, and nitrate through the integrated processes of Sulfammox, SRB, SDAD, and conventional denitrification(Du et al., 2022; Han et al., 2024).



Fig. 4. Sankey diagram of mass balance analysis of the batch test (a), schematic diagram summarizing the mechanisms of nitrogen (N), carbon (C), and sulfur (S) transformations (b).

3.4. Microbial community analysis

3.4.1. Microbial diversity and community structure

The microbial communities on day 34 of Phase 1, day 62 of Phase 2, and day 90, 119 of Phase 3 and day140, 181, and 220 of Phase 4 were analysed using Illumina high-throughput sequencing technology. Over time, the abundance-based coverage estimator (ACE) values generally increased, which suggested that, as more samples are collected, new ASV are being discovered, indicating higher diversity. In this study, ASVs were used to capture microbial diversity with single-nucleotide precision, allowing for more accurate differentiation of closely related microbial sequences. Similar to that of ACE, the Chao1 value also tended to increase over time, which indicated that more unique ASVs were being discovered in each subsequent sample, and the richness of the microbial community was higher than initially observed. However, the values were generally within a similar range, indicating that the evenness of their distributions may vary over time. The Simpson index gradually decreased over time, which suggests that the microbial community was becoming less dominated by specific ASVs and was more evenly distributed. Overall, the trends in the ACE, Chao1, Shannon, and Simpson indices indicated (see supplementary material) that the dynamic and diverse microbial communities continued to evolve as the study period progressed (Nilusha et al., 2021; Zuo et al., 2020). A principal coordinates analysis (PCoA) at the genus level explained 73.4 % of the variation in microbial community composition. Two clusters emerged: Group A, representing increasing wastewater loading, and Group B, representing full-strength wastewater treatment. The separation along PC1 reflected major changes in microbial structure due to loading rates, while PC2 suggested subtler shifts influenced by environmental factors like nutrient and sulfate levels. The statistically significant R2 value of 0.3305 (P = 0.029) highlights the reactor's operational conditions as a key driver of microbial community adaptation and resilience.

3.4.2. Alterations to the primary Functioning bacterial genera

The relative taxonomic abundance of microbial communities in the AnMBR system at the phylum level is described in supplementary material (Fig. 5(a)). Numerous phylum, including those belonging to Bacteroidota, Firmicutes, Proteobacteria, and Actinobacteriota, were found, indicating a flexible microbial community capable of breaking down complex organic waste. Bacteroidota, which often play a role in the degradation of complex carbohydrates, ranged from 14.39 % on Day 34 to a peak of 20.63 % by Day 140, slightly declining to 16.62 % on Day 220. Proteobacteria, including SRB, fluctuated from 19.20 % on Day 34 to 5.71 % on Day 62, before rising to 25.54 % by Day 220. The presence of Desulfobacterota (which remained relatively stable with values between 3.88 % and 9.51 %) and other SRB indicates the potential for sulfammox, which involves simultaneous sulphate reduction and ammonium oxidation. The presence of diverse bacterial phylum from phyla, including Proteobacteria, Desulfobacterota, indicates a complex microbial community with potential involvement in sulphur cycling and SDAD. Genera within the phyla Firmicutes and Proteobacteria include several SRB that can reduce sulphate in anaerobic environments. Thermoplasmatota exhibited an initial abundance of 5.15 % on Day 34, peaking at 43.42 % by Day 90 before decreasing to 11.24 % by Day 220. This phylum includes thermophilic SRB that thrive in high-temperature environments (Frolov et al., 2021). Genera within Campilobacterota have the capacity to oxidise ammonia. This feature is especially important for wastewater treatment with elevated ammonia levels. Campilobacterota, known for ammonia oxidation, also fluctuated, starting at 15.66 % on Day 34, dipping to 3.66 % by Day 62, and then increasing to 19.29 % on Day 140, eventually reaching 7.65 % by Day 220. Although genera from Campilobacterota is not directly linked to the decomposition of organic matter, its existence obliquely aid in the degradation of organic compounds in wastewater by means of interactions with other microbial processes (Hassan et al., 2021).

To better understand the microbial community at the genus level, key genera were selected and analysed using heatmaps (Fig. 5(b)). The Candidatus_Methanogranum, Candidatus_Methanoplasma, and Candidatus_Methanosaeta genera are frequently associated with methanogenesis and may contribute to methane synthesis when organic matter decomposes and methanogenesis occurs under favourable conditions. Over time, these genera became less noticeable, which explained the COD variation in the reactor. Desulfovibrio and Sulfurospirillum frequently engage in sulphur cycling activities, such as sulfammox and sulphate reduction (Dominika et al., 2021). A constant concentration of these genera was observed over time, indicating the stability of sulphate removal efficiency during the study period. The Arcobacter and Sporanaerobacte genera are frequently associated with anaerobic digestion. They aid in organic material degradation and the breakdown of complicated substrates into simpler substances. Their abundance increased with time, peaked, and then decreased at the end of the period, following the same patterns as the TOC removal efficiency. The Acinetobacter, Comamonas, and Petrimonas genera are involved in fermentation by breaking down organic matter into various by products. Acinetobacter and Comamonas increased in abundance over time (Pokorna and Zabranska, 2015; Liu et al., 2023).

3.5. Improved mechanism analysis at the gene level

3.5.1. Key genes for nitrogen removal

By examining the functional genes linked to nitrogen metabolic pathways, a thorough understanding of nitrogen removal at mechanisms was gained (Fig. 6(a)). The abundance of NirK, which is linked to nitrite reduction, increases with time, indicating its involvement in the SDAD process. Increases in the nitric oxide reduction-related abundance of NorBC suggest possible involvement in the denitrification pathway. Nitrite reduction-related enzyme genes NirB [EC:1, NirD [EC:1.7.1.15], NirA [EC:1.7.7.1] and nitrate-reducing enzymes, NarG [EC:1.7.5.1 1.7.99.-], NarH [EC:1.7.5.1 1.7.99.-], NarI [EC:1.7.5.1 1.7.99.-], exhibit varied abundances, indicating possible participation in conventional denitrification as well. NosZ [EC:1.7.2.4] is engaged in the reduction of nitrous oxide, and its abundance rises with time, indicating the prominent SDAD process at the latter stages (Huang et al., 2023). Research on the particular enzymes genes involved in the sulfammox pathway's is still continuing. The pmoA-amoA complex [EC:1.14.18.3 1.14.99.39] is notable among the mentioned enzymes genes because this complex is essential for catalysing the sulfammox pathway's critical intermediate, hydroxylamine, from ammonia. The study's observed reduction in its abundance over time lead to reduction of sulfammox activity at the latter part of the experiment (Zhang et al., 2020).

3.5.2. Key genes for the sulphur removal

By using metagenomic analysis, the microbial metabolism in the sulphate-reducing system was also investigated (Fig. 6 (b)). Among the noteworthy enzyme genes exhibiting temporal patterns, *Cys*H [EC:1.8.4.8 1.8.4.10], which shows a notable increase in abundance during the first 90 days, indicating that it may be involved in the early stages of sulphur metabolism, indicating its involvement in the early stages of sulfur metabolism, possibly in processes involving Sulfammox (Kushkevych et al., 2020). Sulphide quinone oxidoreductase (sqr) [EC:1.8.1.2] exhibits significant increases in abundance, particularly on Days 34 and 220, indicating that it actively participates in sulphide-driven autotrophic denitrification processes. *Apr*AB (EC:1.8.99.2), is an enzyme that involved in sulphur oxidation and its expression increases with time, and it may play essential step in SDAD process(Zhang et al., 2020, Liu et al., 2024b).

In the AnMBR system, key enzymes such as NirK and NorB indicate potential involvement in sulfide-driven autotrophic denitrification and conventional denitrification pathways, respectively. The dynamic variations in the pmoA-amoA complex emphasize its reactivity to environmental factors and its catalytic importance in the Sulfammox pathway.





Fig. 5. The microbial community barplot analysis at phylum(a), Heatmap of the abundance at genus level(b).



Fig. 6. Relative abundances of genes encoding key enzymes (a) N metabolism cycle analysis based on metagenomics sequencing, (b) S metabolism cycle analysis based on metagenomics sequencing. Sample 1 was collected on Day 34 (phase1), representing early reactor operation, and Sample 2 was collected on Day 140 (Phase 4) during the mature, steady-state phase. The bar charts show the abundance of specific genes related to nitrogen and sulfur pathways, providing insight into microbial activity over time.

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Enzymes like CysH, sqr, and AprA in sulfur metabolism show temporal patterns, suggesting their.

roles in sulfate reduction and sulfide-driven autotrophic denitrification. The future development of this research focuses on scaling up the Sulfammox and SDAD processes to a pilot-scale application in the rubber industry, aiming for broader industrial applicability. This step is crucial for validating the efficiency of these processes in treating high-strength wastewater with elevated $\rm NH_4^+-N$ and $\rm SO_4^2-S$ concentrations. Upscaling will facilitate the treatment of larger wastewater volumes and contribute to sustainable practices, such as biogas generation, in alignment with Sustainable Development Goals. Additionally, integrating the Sulfammox process with advanced treatment technologies offers significant potential for its application in various industries beyond rubber manufacturing, enhancing its overall versatility and environmental benefits.

4. Conclusions

Research illustrates the efficient treatment of rubber industry wastewater using Sulfammox and SDAD processes within an AnMBR, achieving significant reductions in COD, ammonia, and sulfate. Sulfammox particularly decreased TN between 30 % to 82 %, while sulfate reduction consistently ranged between 33 % and 57 %. *Desulfovibrio* and *Sulfurosprillum* were identified as key microorganisms supporting these processes. Sulfate removal was driven by Sulfammox (43 %) and the DSR process (57 %), while batch tests confirmed the AnMBR's ability to reduce ammonium, sulfate, and COD efficiently. Findings highlight the role of specific enzymes genes, with Sulfammox process being particularly effective for wastewater high in ammonia and sulfate.

CRediT authorship contribution statement

Ishanka Wimalaweera: Conceptualization, Methodology, Investigation, Formal Analysis, Writing – original draft, Writing – review & editing. Fumin Zuo: Writing – review & editing, Validation, Software. Qihe Tang: Software, Formal analysis, Visualization, Writing – review & editing. Qianwen Sui: Writing – review & editing. Shameen Jinadasa: Writing – review & editing, Supervision, Resources. Sujithra Weragoda: Writing – review & editing, Resources. Tharindu Ritigala: Resources, Methodology. Rohan Weerasooriya: Writing – review & editing. Yawei Wang: Supervision, Project administration. Hui Zhong: Project administration. Madhubhashini Makehelwala: Resources, Project administration. Yuansong Wei: Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131785.

Data availability

Data will be made available on request.

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