GDAŃSK UNIVERSITY
OF TECHNOLOGY

Faculty of Civil and Environmental Engineering

The author of the PhD dissertation: Dominika Derwis
Scientific discipline: Environmental Engineering, Mining and Energy

DOCTORAL DISSERTATION

Title of PhD dissertation: The impact of sulfur transformations on nitrogen removal processes in systems with granular sludge

Title of PhD dissertation (in Polish): Wpływ przemian siarki na procesy usuwania azotu w układach z osadem granulowanym

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Gdańsk, 2024

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The author of the PhD dissertation: Dominika Derwis

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Gdańsk, 5.12.2024r.

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Faculty of Civil and Environmental Engineering
Doctoral dissertation, Dominika Derwis

DESCRIPTION OF DOCTORAL DISSERTATION

The Author of the PhD dissertation: Dominika Derwis

Title of PhD dissertation: The impact of sulfur transformations on nitrogen removal processes in systems with granular sludge

Title of PhD dissertation in Polish: Wpływ przemian siarki na procesy usuwania azotu w układach z osadem granulowanym

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Cosupervision: dr inż. Joanna Majtacz

Date of doctoral defense:

Keywords of PhD dissertation in Polish: Usuwanie azotu, Redukcja siarczanów, Osad granulowany, Dynamika społeczności mikroorganizmów, Denitryfikacja zależna od siarki

Keywords of PhD dissertation in English: Nitrogen removal, Sulfate reduction, Granular sludge, Microbial community dynamics, S-dependent autotrophic denitrification

Summary of PhD dissertation in Polish: Celem rozprawy było zoptymalizowanie integracji cykli azotu (N), siarki (S) i węgla (C) w systemach oczyszczania ścieków z osadem granulowanym, z naciskiem na wpływ suplementacji siarczanami (SO_4^{2-}). Badania koncentrowały się na ocenie, jak wzrastające stężenia SO_4^{2-} wpływają na efektywność procesów takich jak utlenianie amonu (NH_4^+) przy redukcji SO_4^{2-} (SRAO), autotroficzna denitryfikacja zależna od siarki (SDAD) oraz anammox. Zbadano także, jak obecność lub brak COD i NO_2^- wpływa na działanie tych zintegrowanych cykli. W pracy przeprowadzono przegląd literatury oraz trzy eksperymenty. Pierwszy porównywał procesy anammox i SRAO, analizując wpływ wysokich stężeń SO_4^{2-} na szybkość usuwania NH_4^+. Drugi eksperyment badał połączone procesy anammox i miksotroficznej denitryfikacji w zmiennych warunkach COD. Trzeci dotyczył częściowej denitryfikacji i anammox (PD/anammox) w warunkach autotroficznych i miksotroficznych, z badaniem wpływu SO_4^{2-} przy braku NO_2^-. Wszystkie eksperymenty obejmowały analizę mikrobiologiczną i stechiometryczną, co pozwoliło lepiej zrozumieć interakcje między cyklami N, S i C. Wyniki wykazały, że integracja tych cykli może znacząco poprawić efektywność oczyszczania ścieków, zwłaszcza w zmiennych warunkach operacyjnych.

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Doctoral dissertation, Dominika Derwis

Summary of PhD dissertation in English: The aim of the dissertation was to optimize the integration of nitrogen (N), sulfur (S), and carbon (C) cycles in wastewater treatment systems with granular sludge, with an emphasis on the impact of sulfate (SO_4^{2-}) supplementation. The research focused on evaluating how increasing SO_4^{2-} concentrations affect the efficiency of processes such as ammonium (NH_4^+) oxidation with SO_4^{2-} reduction (SRAO), sulfur-dependent autotrophic denitrification (SDAD), and anammox. The study also examined how the presence or absence of COD and NO_2^- affects the functioning of these integrated cycles. The dissertation included a literature review and three experiments. The first compared the anammox and SRAO processes, analyzing the impact of high SO_4^{2-} concentrations on the NH_4^+ removal rate. The second experiment studied combined anammox and mixotrophic denitrification processes under varying COD conditions. The third focused on partial denitrification and anammox (PD/anammox) under autotrophic and mixotrophic conditions, with the effect of SO_4^{2-} under the absence of NO_2^-. All experiments included microbiological and stoichiometric analysis, which allowed for a better understanding of the interactions between N, S, and C cycles. The results showed that integrating these cycles can significantly improve wastewater treatment efficiency, especially under variable operational conditions.

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GDAŃSK UNIVERSITY
OF TECHNOLOGY

Faculty of Civil and Environmental Engineering
Doctoral dissertation, Dominika Derwis

M.Sc. Dominika Derwis

THE IMPACT OF SULFUR TRANSFORMATIONS ON NITROGEN REMOVAL PROCESSES IN SYSTEMS WITH GRANULAR SLUDGE

Wpływ przemian siarki na procesy usuwania azotu w układach z osadem granulowanym

DOCTORAL DISSERTATION

Supervisor:

prof. dr hab. inż. Jacek Mąkinia

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dr. inż. Joanna Majtacz

GDAŃSK 2024

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I would also like to express my heartfelt thanks to my late father, who was always by my side, especially while I was writing my first articles. He would bring me the best coffee while I worked in our family home, offering his quiet presence and unwavering belief in me. Even though he didn’t always understand the specifics of my research, his support and encouragement in times of doubt were priceless. He didn’t live to see the end of this journey, but he stood by me as long as he could.

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Table of contents

Summary

The aim of the doctoral dissertation was to optimize the integration of nitrogen (N), sulfur (S), and carbon (C) cycles in wastewater treatment systems with granular sludge, with particular emphasis on the impact of sulfate (SO_4^{2-}) supplementation. This work focused on studying how increasing SO_4^{2-} concentrations affect the efficiency of N and S removal, especially in the context of processes such as ammonium (NH_4^+) oxidation with SO_4^{2-} reduction (SRAO), sulfur-dependent autotrophic denitrification (SDAD), and anammox. A key goal was also to understand how the presence or absence of compounds like organic carbon (COD) and nitrites (NO_2^-) affects the functioning of these integrated cycles, ultimately aiming to improve the efficiency and sustainability of wastewater treatment processes.

The first stage of the research focused on a detailed literature review concerning the integration of S cycles with N removal processes, with an emphasis on the sustainable development of wastewater treatment technologies. This review highlighted that S -dependent processes, such as SRAO and SDAD, can be efficient alternatives to traditional N removal methods that require external C sources. S compounds serve as electron donors, enabling effective N removal in C -limited conditions while reducing greenhouse gas emissions. The identified processes can also contribute to reducing the operational costs of wastewater treatment plants.

Experiments comparing the anammox and SRAO processes showed that increasing SO_4^{2-} concentration (up to 952 mg S/L) in the SBR reactor led to a 2.1-fold increase in NH_4^+ removal rate and a 15-fold increase in SO_4^{2-} reduction rate compared to the control reactor. The combination of S-driven processes with anammox in single-stage systems introduced a completely new quality to wastewater treatment, with Nitrosomonas and Thauera playing key roles in N and S metabolism, respectively. These results demonstrated that the cooperation of N and S cycles can significantly improve pollutant removal efficiency, opening new possibilities for more sustainable wastewater treatment.

In the next stage, a 200-day experiment with a combined anammox/mixotrophic denitrification process showed that SO_4^{2-} supplementation significantly improved NH_4^+ oxidation efficiency (with a 10% higher NH_4^+ utilization rate (AUR) in SBR2 compared to SBR1). Total nitrogen (TN) removal efficiency ranged from 62% to 99%, with the SO_4^{2-}-enriched reactor consistently outperforming the control. These results clearly show that the simultaneous application of S-driven processes and anammox, combined with alternating autotrophic and heterotrophic conditions, introduces a new perspective for sustainable wastewater treatment.

The final stage of the research analyzed partial denitrification/anammox (PD/anammox) processes under both autotrophic (N-S cycle) and mixotrophic (N-S-C cycle) conditions. The results showed that increasing SO_4^{2-} concentration significantly improved AUR, with NH_4^+ and TN removal reaching a maximum of 57% in SBR1 and almost 100% in SBR2, where organic compounds were also introduced. Even in the absence of NO_2^-, SO_4^{2-}-dependent systems were able to support N removal. These findings demonstrate that the interaction of N, S, and C cycles can significantly enhance wastewater treatment efficiency under varying operational conditions.

The innovative aspect of this work lies in the combination of S-driven processes, such as SRAO and SDAD, with traditional N removal pathways in single-stage granular sludge systems. Unlike previous studies that examined N and S cycles separately, this dissertation comprehensively investigates their interaction under varying SO_4^{2-}, COD, and NO_2^- concentrations. The simultaneous use of autotrophic and heterotrophic operational conditions was also innovative, opening new perspectives for more sustainable wastewater treatment technologies. Additionally, the advanced stoichiometric models introduced in this work offer new opportunities for optimizing reactor configurations. These results are particularly relevant for SO_4^{2-}-rich systems, increasingly common in industrial applications.

The conclusions of the research indicate that the integration of N, S, and C cycles can significantly improve the efficiency of wastewater treatment, especially in conditions of variable organic matter and SO_4^{2-} content. These findings are particularly important for the treatment of low- C wastewater, as S -dependent processes can effectively compensate for the lack of organic electron donors.

Streszczenie

Celem rozprawy doktorskiej było zoptymalizowanie integracji cykli azotu (N), siarki (S) i węgla (C) w systemach oczyszczania ścieków z osadem granulowanym, ze szczególnym uwzględnieniem wpływu suplementacji siarczanami (SO_4^{2-}). Praca ta koncentrowała się na badaniu, jak wzrastające stężenia SO_4^{2-} wpływają na efektywność usuwania N i S, szczególnie w kontekście procesów takich jak utlenianie amonu (NH_4^+) przy redukcji SO_4^{2-} (SRAO), autotroficzna denitryfikacja zależna od siarki (SDAD) oraz anammox. Kluczowym celem było także zrozumienie, jak obecność lub brak związków takich jak węgiel organiczny (COD) i azotyny (NO_2^-) wpływają na funkcjonowanie tych zintegrowanych cykli, co miało prowadzić do zwiększenia wydajności i zrównoważenia procesów oczyszczania ścieków.

Pierwszy etap badań skupił się na szczegółowym przeglądzie literatury dotyczącej integracji cykli siarki z procesami usuwania azotu, z naciskiem na zrównoważony rozwój technologii oczyszczania ścieków. Przegląd ten podkreślił, że procesy zależne od siarki, takie jak SRAO i SDAD, mogą być wydajnymi alternatywami dla tradycyjnych metod usuwania azotu, które wymagają zewnętrznych źródeł węgla. Związki siarki pełnią funkcję donorów elektronów, co pozwala na skuteczne usuwanie azotu w warunkach ubogich w węgiel, jednocześnie zmniejszając emisję gazów cieplarnianych. Zidentyfikowane procesy mogą również przyczynić się do redukcji kosztów operacyjnych oczyszczalni ścieków.

Eksperymenty porównujące procesy anammox oraz SRAO wykazały, że wzrost stężenia SO_4^{2-} (do 952 mg S/L) w reaktorze SBR prowadził do 2,1-krotnie wyższej szybkości usuwania NH_4^+ oraz 15-krotnie wyższej szybkości redukcji SO_4^{2-} w porównaniu do reaktora kontrolnego. Połączenie procesów napędzanych S z anammox w systemach jednoetapowych wprowadziło zupełnie nową jakość do procesów oczyszczania ścieków, przy czym *Nitrosomonas* i *Thauera* odgrywały kluczową rolę w metabolizmie odpowiednio N i S. Wyniki te dowiodły, że współpraca cykli N i S może znacząco poprawić efektywność usuwania zanieczyszczeń, otwierając nowe możliwości w zakresie bardziej zrównoważonego oczyszczania ścieków.

W kolejnym etapie badania, które trwały 200 dni, z połączonym procesem anammox/miksotroficznej denitryfikacji wykazały, że dodatek SO_4^{2-} znacząco poprawia wydajność utleniania NH_4^+ (o 10% wyższą szybkość wykorzystania NH_4^+ (AUR) w SBR2, w porównaniu do SBR1). Całkowita efektywność usuwania N (TN) wynosiła od 62% do 99%, a reaktor wzbogacony o SO_4^{2-} osiągał stałą przewagę w wydajności. Te wyniki jasno pokazują, że równoczesne stosowanie procesów napędzanych S oraz anammox, w połączeniu z naprzemiennymi warunkami autotroficznymi i heterotroficznymi, wprowadza nową perspektywę do zrównoważonego oczyszczania ścieków.

W ostatnim etapie badań analizowano procesy częściowej denitryfikacji/anammox (PD/anammox) zarówno w warunkach autotroficznych (cykl N-S), jak i miksotroficznych (cykl N-S-C). Wyniki wykazały, że wzrost stężenia SO_4^{2-} znacząco poprawia AUR, a usuwanie NH_4^+ i TN osiągnęło maksymalnie 57% w SBR1 oraz prawie 100% w SBR2, gdzie wprowadzono także związki organiczne. Nawet w warunkach braku NO_2^- systemy zależne od SO_4^{2-} były w stanie wspierać usuwanie N. Wyniki te pokazują, że interakcja cykli N, S i C może znacznie zwiększyć wydajność oczyszczania w zmiennych warunkach operacyjnych.

Nowatorski charakter tej pracy polega na połączeniu procesów zależnych od S, takich jak SRAO i SDAD, z tradycyjnymi ścieżkami usuwania N w jednoetapowych systemach z osadem granulowanym. W przeciwieństwie do wcześniejszych badań, które analizowały cykle N i S oddzielnie, rozprawa ta kompleksowo bada ich współdziałanie w zmiennych warunkach stężeń SO_4^{2-}, COD i NO_2^-. Innowacyjne było także równoczesne stosowanie autotroficznych i heterotroficznych warunków operacyjnych, co otwiera nową perspektywę w kontekście bardziej zrównoważonych technologii oczyszczania ścieków. Ponadto, wprowadzone w pracy zaawansowane modele stechiometryczne oferują nowe możliwości optymalizacji konfiguracji reaktorów. Wyniki te mają szczególne znaczenie dla systemów bogatych w SO_4^{2-}, coraz częściej spotykanych w zastosowaniach przemysłowych.

Wnioski z badań wskazują, że integracja cykli N, S i C może znacząco poprawić efektywność oczyszczania ścieków, szczególnie w warunkach zmiennego składu związków organicznych i SO_4^{2-}. Wyniki te są szczególnie ważne dla oczyszczania ścieków o niskiej zawartości C, ponieważ procesy zależne od S mogą efektywnie kompensować brak organicznych donorów elektronów.

List of papers

The following papers form the backbone of this dissertation and are referred to the text by Roman numerals I-IV:

Paper I: **Grubba, D.,** Yin, Z., Majtacz, J., Al-Hazmi, H. E., & Mąkinia, J. (2022). Incorporation of the sulfur cycle in sustainable nitrogen removal systems - A review. *Journal of Cleaner Production*, 133495. https://doi.org/10.1016/j.jclepro.2022.133495

Paper II: **Derwis, D.,** Majtacz, J., Kowal, P., Al-Hazmi, H. E., Zhai, J., Ciesielski, S., Piechota, G., & Mąkinia, J. (2023). Integration of the sulfate reduction and anammox processes for enhancing sustainable nitrogen removal in granular sludge reactors. *Bioresource Technology*, *383*, 129264. https://doi.org/10.1016/j.biortech.2023.129264

Paper III: **Derwis, D.,** Al-Hazmi, H. E., Majtacz, J., Kowal, P., Ciesielski, S., & Mąkinia, J. (2024). The role of the combined nitrogen-sulfur-carbon cycles for efficient performance of anammox-based systems. *Science of The Total Environment*, *917*, 170477. https://doi.org/10.1016/j.scitotenv.2024.170477

Paper IV: **Derwis, D.,** Al-Hazmi, H. E., Majtacz, J., Ciesielski, S., & Mąkinia, J. (2024). Enhancing nitrogen removal in the partial denitrification/anammox processes for SO4− - Rich wastewater treatment: Insights into autotrophic and mixotrophic strategies. *Journal of Environmental Management*, *358*, 120908. https://doi.org/10.1016/j.jenvman.2024.120908

The points value and basic journal metric based on Impact Factor for each journal are listed in Table below.

Table 1. Journal points based on the Polish Ministry of Science and Higher Education and Impact Factors.

Paper	**Journal**	**Point value**	**Impact Factor (5 years)**	**Impact Factor (2024)**
I	Journal of Cleaner Production	140	10.2	9.7
II	Bioresource Technology	140	9.4	11.889
III	Science of the Total Environment	200	8.6	8.2
IV	Journal of Environmental Management	200	7.9	8.0
Total		**680**	**36.1**	**37.789**

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Authors contribution to the papers

- **Paper I:**

Dominika Derwis: Conceptualization, Investigation, Formal analysis, Writing – original draft, Preparation, Visualization, Funding acquisition, Project administration.

Zhixuan Yin: Conceptualization, Investigation, Writing – original draft, Preparation, Visualization.

Joanna Majtacz: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Preparation, Visualization.

Hussein Ezzi Al-Hazmi: Conceptualization, Methodology, Investigation, Writing – original draft, Preparation, Visualization.

Jacek Mąkinia: Validation, Writing – review & editing, Supervision.

- **Paper II:**

Dominika Derwis: Conceptualization, Investigation, Formal analysis, Writing – original draft, Preparation, Visualization, Funding acquisition, Project administration.

Joanna Majtacz: Conceptualization, Methodology, Investigation, Formal analysis, Preparation, Visualization.

Przemysław Kowal: Conceptualization, Methodology, Investigation, Writing – original draft, Preparation, Visualization.

Hussein E. AL-Hazmi: Methodology, Investigation, Writing – original draft, Preparation, Visualization.

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Other publications from my PhD studies:

- **Grubba, D.** (2019). Comparative analysis of earthships and conventional buildings in terms of investment and operating costs. Gdańsk. University of Technology, Faculty of Civil and Environmental Engineering, ISBN 978-83-60261-62-0.
- **Grubba, D.**, & Mąkinia, J. (2019). Odzysk bioplastiku i celulozy z osadów ściekowych. Wodociągi i Kanalizacja, 6 (184).
- **Grubba, D.**, Al-Hazmi, H., Majtacz, J., & Mąkinia, J. (2019). Charakterystyka procesu deamonifikacji oraz rozwój technologii usuwania azotu w oparciu o ten proces. In Czerwionka, K., Gajewska, M., Matej-Łukowicz, K., & Wojciechowska, E. (Eds.), Innowacje w inżynierii środowiska (pp. 198-224). Gdańsk: Wydawnictwo Polskiej Akademii Nauk.
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- Al-Hazmi, H. E., Maktabifard, M., **Grubba, D.,** Majtacz, J., Hassan, G. K., Lu, X., Piechota, G., Mannina, G., Bott, C. B., & Mąkinia, J. (2023b). An Advanced Synergy of Partial Denitrification-Anammox for Optimizing Nitrogen Removal from Wastewater: A Review. *Bioresource Technology*, 129168. https://doi.org/10.1016/j.biortech.2023.129168

Conference participation during my PhD study:

- Poster presentation, National Conference "Innovations in Environmental Engineering", June 24-25, 2019, Gdańsk, Poland
- Oral presentation in English: "The International Scientific Conference 10th Forum of Ecological Engineering", September 9-11, 2019, Kazimierz Dolny, Poland
- Oral presentation in English: The 3rd International Scientific-Technical Conference "Water Supply and Wastewater Disposal: Designing, Construction, Operation, and Monitoring", October 23-25, 2019, Lviv, Ukraine
- Oral presentation in English: 2nd IWA Polish Young Water Professionals Conference, "Emerging Technologies in Water and Wastewater Sector", February 12-14, 2020, Warsaw, Poland
- Oral presentation in English: 5th IWA Specialized International Conference "Ecotechnologies for Wastewater Treatment (EcoSTP) 2021", June 21-25, 2021, Milan, Italy
- Oral presentation in English: IWA Wastewater Water and Resource Recovery Conference, April 10-13, 2022, Poznań, Poland
- Oral presentation in English: WEF/IWA Innovations in Process Engineering Conference 2023, June 6-9, 2023, Portland, Oregon, USA

Nomenclature and abbreviations

Item	Description
AnAOB	anaerobic ammonium oxidizing bacteria
AOB	ammonium oxidizing bacteria
AUR	ammonium utilization rate
AUR_AOB	AUR by AOB
BR	batch tests
C	carbon
COD	chemical oxygen demand, organic carbon
CUR	COD utilization rate
DO	dissolved oxygen
GHG	greenhouse gas
HCl	hydrochloric acid
HRT	hydraulic retention time
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
N	nitrogen
N_2	nitrogen gas
N_2O	nitrous oxide
NH_4^+	ammonium
NiUR	nitrite utilization rate
NO_2^-	nitrite
NO_3^-	nitrate
NOB	nitrite-oxidizing bacteria
NPR/NUR	nitrate production/utilization rate
OTU	operational taxonomic units
PCoA	principal coordinate analysis
PD	partial denitrification
PE	population equivalents
S	sulfur
S^0	elementar sulfur

S^{2-}/H_2S	sulfide
SBR	sequencing batch reactor
SCC	Spearman’s correlation coefficient
SDAD	S-dependent autotrophic denitrification
SO_3^{2-}	sulfite
SO_4^{2-}	sulfate
SOB	sulfur-oxidizing bacteria
SPR/SUR	sulfate production/utilization rate
SR	sulfate reduction
SRAO	sulfate reduction ammonium oxidation
SRB	sulfate reducing bacteria
TN	total nitrogen
WWTP	municipal wastewater treatment plan

1. INTRODUCTION

1.1. Importance of nitrogen, sulfur and carbon cycles in wastewater treatment and challenges in integrating this cycles

The growing demand for sustainable and efficient wastewater treatment systems is driven by increasing environmental regulations and the necessity to reduce the impacts of nitrogen (N) and sulfur (S) compounds on aquatic ecosystems. Excess N in wastewater, primarily in the form of ionized ammonium (NH_4^+) and nitrate (NO_3^-), can lead to eutrophication and degradation of water quality, while S compounds such as sulfate (SO_4^{2-}) and sulfide (S^{2-}) can cause corrosion and odor issues in treatment plants. Therefore, developing advanced methods for the effective removal of N and S from wastewater has become a key priority in modern wastewater treatment plants (WWTPs).

Conventional nitrification-denitrification is currently the most widely used method for N removal in municipal WWTPs. This process, however, requires significant energy input for aeration and often depends on external carbon (C) sources, making it both costly and energy-intensive (Zhou et al., 2020). Furthermore, the potential for nitrous oxide (N_2O) emissions during denitrification raises concerns about the environmental sustainability of this method. In recent years, anaerobic ammonium oxidation (anammox) has emerged as a promising alternative, offering a more energy-efficient and C-neutral approach to N removal. The anammox process converts NH_4^+ directly into nitrogen gas (N_2) using nitrite (NO_2^-) as an electron acceptor, bypassing the need for external C sources and reducing energy consumption (Zekker et al., 2023). However, maintaining a stable supply of NO_2^- is critical for the anammox process, and competition from nitrite-oxidizing bacteria (NOB) for NO_2^- can hinder its effectiveness (Wu et al., 2022).

In response to these challenges, alternative pathways for N removal have been explored, including partial denitrification (PD), which involves the reduction of NO_3^- to NO_2^-, providing the required electron acceptor for anammox. The combination of PD and anammox has been shown to reduce energy demand and lower greenhouse gas emissions compared to conventional methods (Al-Hazmi et al., 2023). These advancements have opened new avenues for integrating various biochemical cycles, such as N and S cycles, to enhance the overall efficiency and sustainability of wastewater treatment systems.

S compounds, present in wastewater in various forms (e.g., SO_4^{2-}, S^{2-}, elementar sulfur (S^0)), have been less explored in the context of biological N removal. However, S can play a crucial role in driving autotrophic denitrification processes, where S compounds serve as electron donors for the reduction of N compounds. S -dependent autotrophic denitrification (SDAD), for example, utilizes reduced S species such as S^{2-} or S^0 to reduce NO_3^- to N_2, providing an alternative to C-dependent heterotrophic denitrification (Zou et al., 2016). Similarly, sulfate reduction ammonium oxidation (SRAO), also known as sulfammox, can use SO_4^{2-} as an electron acceptor to oxidize NH_4^+ in the absence of NO_2^- (Fdz Polanco et al., 2001).

The integration of N and S cycles has the potential to create synergistic effects, improving the overall removal efficiencies of both N and S compounds. This approach reduces the dependency on external C sources, lowers operational costs, and enhances sustainability by exploiting naturally occurring interactions between N and S transformations. Studies have demonstrated that coupling S-based processes with anammox can significantly improve N removal, especially in wastewater streams rich in SO_4^{2-} (Liu et al., 2017, Yuan et al., 2020). These integrated systems take advantage of the complementary roles of N and S cycles, where S transformations produce intermediates that can serve as substrates in N removal processes, leading to higher overall system performance.

In recent years, there has been growing recognition that C plays a pivotal role in optimizing the integration of N and S cycles in wastewater treatment. The combination of N, S, and C cycles offers a more holistic approach to nutrient removal, allowing for greater flexibility in handling wastewater streams with varying compositions. For example, heterotrophic bacteria, which utilize organic C as an electron donor, can coexist with S -oxidizing bacteria (SOB) that use S compounds to drive denitrification, creating a mixotrophic environment. This integration of C allows for the simultaneous removal of N, S, and C compounds, providing enhanced treatment efficiencies under varying operational conditions (Wei et al., 2017).

Moreover, integrating the N-S-C cycles can provide additional pathways for electron transfers, reducing the reliance on external chemical additives while lowering operational costs. In systems where chemical oxygen demand (COD) is variable, such as in industrial or landfill leachate wastewater, utilizing C sources from the wastewater itself becomes advantageous. Studies have shown that mixotrophic systems, which leverage both autotrophic and heterotrophic processes, can offer improved performance, especially when dealing with fluctuating SO_4^{2-} and C levels (Kosugi et al., 2019). In these systems, the versatility of microbial groups allows for enhanced N removal and S oxidation under varying conditions.

Mixotrophic systems, in which both heterotrophic and autotrophic bacteria coexist, offer another promising avenue for enhancing the capacity of N and S removal. In such systems, heterotrophic denitrifiers utilize organic C, while autotrophic denitrifiers use S compounds as electron donors, leading to a more balanced denitrification process (Yánez et al., 2021). The coexistence of these microbial pathways allows for the simultaneous removal of N and S compounds, optimizing the use of available resources and reducing the need for external C supplementation. Additionally, anaerobic NH_4^+-oxidizing bacteria (AnAOB), such as *Candidatus Kuenenia* and *Candidatus* Brocadia, thrive in mixotrophic environments, contributing further to N removal through the anammox process (Feng et al., 2019).

Despite the benefits of N-S-C integration, challenges remain in balancing these cycles, particularly in optimizing the microbial interactions that govern these processes. SOB, heterotrophic denitrifiers, and anammox bacteria all compete for key electron acceptors such as NO_2^- and NO_3^-, which can affect the overall efficiency of the system. Maintaining a stable balance between the autotrophic and heterotrophic processes, especially in reactors where both C and S are present, is critical for achieving consistent performance (Yánez et al., 2021). Additionally, the accumulation of intermediate compounds such as sulfide (H_2S) or sulfite (SO_3^{2-}) can inhibit microbial activity, requiring careful control of S and C inputs to avoid disruptions in the treatment process.

In conclusion, the integration of N, S, and C cycles in wastewater treatment systems holds significant potential for enhancing the sustainability and efficiency of nutrient removal. By leveraging the synergies between these biochemical cycles, it is possible to optimize the treatment process for wastewater streams with varying compositions of NH_4^+, SO_4^{2-} and organic C. However, future research must focus on fine-tuning the operational conditions that balance these cycles, particularly in terms of microbial community management and reactor stability. Addressing these challenges will be key to fully realizing the potential of N-S-C integrated systems and advancing the development of more sustainable wastewater treatment technologies.

1.2. Originality and innovation of the research

This research introduces an innovative approach to integrating N, S, and C cycles in wastewater treatment by examining the underexplored interactions between S-based processes, such as SRAO and SDAD, and N removal pathways in granular sludge systems. Unlike previous studies that have typically investigated N and S cycles independently, this work uniquely focuses on their combined effects, particularly under varying conditions of SO_4^{2-}, COD and NO_2^-. By addressing these unexplored interactions, the research addresses gaps in understanding the interactions between these cycles.

A key innovation of this work lies in the simultaneous application of S-driven processes alongside anammox in single-stage systems, coupled with the alternation between autotrophic and heterotrophic conditions. Furthermore, this research proposes a pioneering strategy to optimize reactor configurations for simultaneous N and S removal with minimal energy consumption and reduced greenhouse gas emissions.

The work includes the development of advanced stoichiometric models and process control strategies aimed at maximizing real-world applicability. These tools are designed to enhance operational efficiency, particularly for SO_4^{2-}-rich industrial wastewaters, which are becoming increasingly common. The insights gained from this study are expected to advance sustainable wastewater treatment solutions for managing complex wastewater streams.

2. OBJECTIVE AND SCOPE

2.1. General objective and hypothesis of the study

The main objective of this dissertation was to optimize the integration of N, S, and C cycles in granular sludge systems under different operational conditions, with a specific focus on assessing the impact of SO_4^{2-} supplementation. The research aimed to understand how increasing SO_4^{2-} concentrations influenced N and S removal efficiencies, particularly in relation to processes like SRAO, SDAD and anammox. The study also explored microbial dynamics, process efficiencies and stoichiometric relationships, seeking to determine how environmental factors, such as the presence or absence of COD and NO_2^-, affected the performance of these interrelated cycles, ultimately enhancing N removal efficiency.

The central hypothesis was that integrating N, S and C cycles, particularly through S-driven processes such as SRAO and SDAD, would significantly improve N and S removal efficiencies in wastewater treatment systems.

To investigate this, the research explored how S-based processes could be optimized within N removal systems, focusing on the interactions between S compounds (SO_4^{2-}, S^{2-}) and N compounds (NH_4^+, NO_2^-, NO_3^-). Additionally, the study examined how COD influenced the balance between autotrophic and heterotrophic processes, particularly in mixotrophic systems, where both COD and S played crucial roles.

The dissertation also analyzed microbial population dynamics, with special attention to organisms, such as SOB, SO_4^{2-} reducing bacteria (SRB), and anammox bacteria, and their contributions to overall process performance. Detailed stoichiometric analyses were conducted to quantify the removal rates of N and S under different conditions, specifically assessing the impact of SO_4^{2-} supplementation and varying COD levels on process efficiency.

Finally, the research investigated how operational factors like the availability of NO_2^- and fluctuations in COD and SO_4^{2-} levels affected the interactions between N and S cycles. This analysis provided insights into optimizing these processes for improved wastewater treatment efficiency.

2.2. Specific objectives

- Paper I:

This publication reviews the current state of N, S and C cycles in wastewater treatment systems. It consolidates knowledge about SDAD and discusses novel pathways like SRAO (sulfammox). The review explores challenges and opportunities in integrating these cycles, with a focus on improving N removal in SO_4^{2-}-rich wastewaters through processes such as anammox, SRAO and SDAD.

- Paper II:

This study investigates SDAD in COD-free granular sludge systems, evaluating how S compounds act as electron donors in the absence of COD. It assesses the role of SRAO and other S-based processes in N removal. Microbial dynamics are analyzed to understand how COD-free conditions affect microbial populations and N removal efficiency. Stoichiometric calculations quantify N and S process rates and their interactions in the absence of COD.

- Paper III:

This publication examines the effects of COD addition on N and S removal in mixotrophic systems. It evaluates how the balance between S-driven autotrophic processes (such as SRAO and SDAD) and heterotrophic denitrification impacts microbial community structure and process performance. By incorporating COD, the study explores how competition between S oxidizers and heterotrophic denitrifiers affects N removal rates. Stoichiometric analyses are performed to assess the overall process efficiency under mixotrophic conditions, particularly focusing on the influence of COD on S-driven processes.

- Paper IV:

This study focuses on the role of NO_2^- in S-driven N removal processes. The research explores how NO_2^- supplementation affects microbial interactions in granular sludge systems, specifically looking at the interplay between SRAO, anammox and SDAD. Microbiological analysis identifies key microorganisms involved in N and S cycling under different NO_2^-

conditions. Stoichiometric calculations measure N and S removal rates and examine how these processes are influenced by NO_2^-.

3. DISSERTATION METHODOLOGY (Papers II-IV)

3.1. Laboratory setup

The laboratory setup used in the experiments described in papers II, III and IV consists of sequencing batch reactors (SBRs) (Figure 1) designed to investigate the interaction between N and S cycles under varying operational conditions.

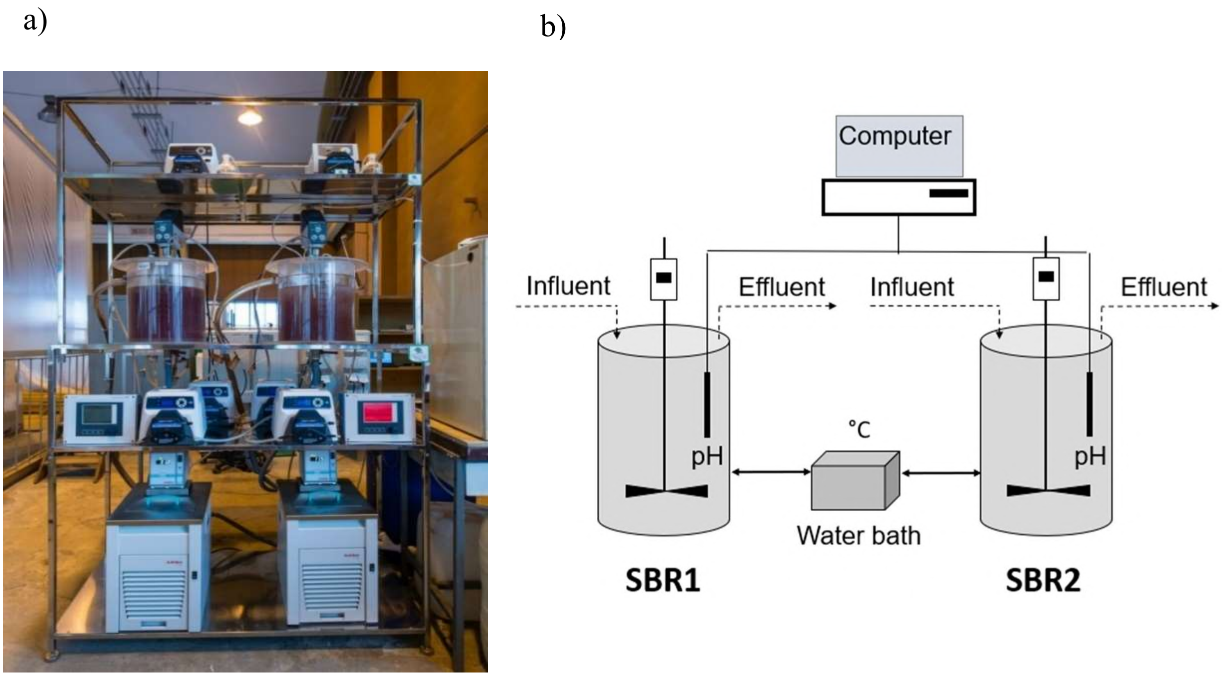


Figure 1. Reactors used in research in papers II-IV: a) photo b) scheme

Two SBRs were used in all experiments, each constructed from transparent plexiglass and equipped with a working volume of either 10 L. The reactors were placed inside water jackets to maintain a controlled temperature throughout the experiment. Temperature regulation was achieved using a Julabo F32 (Germany) water bath, which offered precise control with an accuracy of ±0.1°C. This allowed the process temperature to be consistently maintained at the desired level across all experimental phases.

Each reactor was outfitted with a variable-speed mechanical stirrer (Heidolph RZR 2041 or RZR 2041, Germany) to ensure uniform mixing within the system. Additionally, the pH and dissolved oxygen (DO) levels were continuously monitored using Endress + Hauser (Switzerland) probes (pH sensor EH CPS 471D-7211 and DO sensor COS22D-10P3/O) connected to Hach Lange HQ40D multimeters. These devices provided real-time feedback, ensuring precise control of environmental conditions.

Both reactors were controlled using automated systems that regulated the influent and effluent flow rates, mixing and temperature. The control systems ensured the maintenance of consistent conditions, such as pH and DO, throughout the experiments. Regular calibration of sensors was carried out to guarantee data accuracy.

3.2. Biomass inoculum

In all studies (Papers II, III and IV), the biomass inoculum was sourced from a full-scale sidestream granular deammonification system at a WWTP in Słupsk, Poland. This WWTP treats wastewater for approximately 200,000 PE (population equivalents) and has a working volume of 700 m^3. The granular sludge from this system provided an excellent starting point for all experiments, containing high microbial activity suited for N and S removal processes.

3.3. Operational conditions

The operational conditions in all three series (Papers II, III and IV) were designed to explore the interaction between N-S-C cycles under various scenarios. The studies involved consistent core parameters, such as temperature, pH, DO levels and synthetic influents, while varying the influent compositions and conditions for N, S and C across the experiments. The temperature in all reactors was maintained at 30 ± 1°C to ensure optimal conditions for N removal processes, including anammox and SDAD. The pH was automatically controlled within the range of 7.5–7.8, an ideal range for anammox and S-driven processes. It was adjusted using 6 M hydrochloric acid (HCl) based on pH probe readings. All reactors were operated under low oxygen conditions, with DO levels consistently kept below 0.2 mg O_2/L, promoting anaerobic conditions crucial for SRAO and anammox processes. Hydraulic Retention Time (HRT) was adjusted dynamically based on process rates, ranging between 0.5 and 3 days. The HRT was shortened as the process rates increased.

3.3.1. 1st series (II paper)

This series focused on SDAD and SRAO under COD-free conditions. The experiment lasted for 160 days. In the first reactor with anammox (SBR1_AMX), NH_4^+ and NO_2^- were dosed in typical proportions for the conventional anammox process (1.3). Although no external SO_4^{2-} was added to this reactor, the initial SO_4^{2-} concentration fluctuated between 45.2 and 58.6 mg S/L, likely due to the release of SO_4^{2-} from biomass and rapid, unmeasurable reactions of S compounds. In the second reactor (SBR2_SRAO), which also operated with anammox, the influent concentrations of SO_4^{2-} were gradually increased from 0.0 to 950.0 mg S/L. Additionally, batch tests (BR_SR) were conducted on days 4, 29, 57, 84, 119, and 153, without the addition of NO_2^-, using high initial SO_4^{2-} concentrations ranging from 46.1 to 856.0 mg S/L. Samples for these tests were taken from SBR2_SRAO. In both SBR1_AMX and SBR2_SRAO reactors, the influent concentrations of NH_4^+ and NO_2^- were gradually increased in both reactors (Figure 1 in Paper II).

3.3.2. 2nd series (III paper)

This series introduced COD to explore mixotrophic conditions where both SDAD and heterotrophic denitrification occurred. The focus was on how COD influenced N and S removal processes. This series lasted for 200 days. The dynamic changes in influent concentrations of NH_4^+, NO_2^-, NO_3^-, COD, and SO_4^{2-} are depicted in Figure 1a-e in Paper III. The influent NH_4^+ concentrations ranged between 70 and 200 mg N/L, while NO_2^- concentrations varied from 90 to 260 mg N/L in both reactors. To optimize the anammox process, a consistent NO_2^-/NH_4^+ ratio of approximately 1.3 was maintained throughout. During the phases when COD was added, the influent concentrations of NH_4^+ and NO_2^- were increased to support both the anammox reaction and mixotrophic denitrification. In contrast, during periods without COD addition, the NH_4^+ and NO_2^- concentrations were lowered to focus on the anammox process. SO_4^{2-} was not introduced into SBR1, whereas the influent SO_4^{2-} concentrations in SBR2 ranged between 150 and 400 mg S/L. Throughout the study, alternating phases of COD addition (I, III, V, VII) and no COD addition (II, IV, VI) were implemented in both reactors. In general, the influent COD concentrations were held at 250 mg COD/L, with the exception of phase V, where the concentrations were progressively increased, reaching a maximum of 500 mg COD/L.

3.3.3. 3rd series (IV paper)

This series explored the effect of NO_2^- supplementation on S-based N removal processes, with a focus on SDAD, anammox, and SRAO. The study lasted 180 days. The dynamic behavior of influent and initial concentrations during the reaction phase in both bioreactors, including NH_4^+, NO_3^-, COD, and SO_4^{2-}, is depicted in Supplementary Material in Figure S2 and Figure S3 in Paper IV. NH_4^+, NO_3^-, and SO_4^{2-} were introduced into both reactors, while COD was specifically added to SBR2 to enable a comparison between N-S cycle conditions in SBR1 and N-S-C conditions in SBR2. Influent NH_4^+ concentrations ranged between 10-50 mg N/L for SBR1 and 20-40 mg N/L for SBR2. Concurrently, NO_3^- concentrations varied from 0-20 mg N/L in SBR1 and 10-40 mg N/L in SBR2. These variations in NH_4^+ and NO_3^- concentrations were due to the accumulation of these compounds during the process, leading to periodic adjustments in dosing. The influent SO_4^{2-} concentration was maintained the same in both reactors, ranging from 150 to 800 mg S/L. The experiment was divided into two stages: the first stage was conducted before the influent SO_4^{2-} concentration was elevated, while the second stage occurred after the concentration increase. This transition took place around day 100. In the first stage, the maximum influent SO_4^{2-} concentration was 300 mg S/L, whereas in the second stage, it increased to 800 mg S/L. The introduction of SO_4^{2-} was meant to simulate a SO_4^{2-}-rich wastewater scenario, anticipating its reduction to reduced S forms (S^{2-}, S^0, and $S_2O_3^{2-}$) through SRAO in the N-S process, and additionally by SRB in the N-S-C process. In SBR2, the influent COD concentration varied between 100-200 mg COD/L, with an initially higher concentration but ultimately stabilizing at 100 mg COD/L for most of the experiment duration.

3.4. Determination of specific process rates based on stoichiometric calculations

The determination of specific process utilization/production rates was based on the maximum slopes of key variables, including AUR, NO_2^- utilization rate (NiUR), NO_3^- production/utilization rate (NPR/NUR), COD utilization rate (CUR), and SO_4^{2-}

production/utilization rate (SPR/SUR). Additionally, the efficiency of N, SO_4^{2-} and COD utilization/production was determined by comparing the differences between influent and effluent concentrations.

Stoichiometric analysis was performed using the SOLVER tool in MS Excel, assuming the reaction stoichiometries of processes such as anammox, SRAO, SDAD, heterotrophic SO_4^{2-} reduction and heterotrophic denitrification, depending on the study. The stoichiometric calculations were based on reactions described in the literature (Fdz-Polanco et al., 2001, Huang et al., 2019, Sun et al., 2018). This set of reaction equations enabled the breakdown of total rates and the attribution of specific rates to individual processes, guided by the assumptions specific to each study, which are detailed in their respective methodologies.

Analyses were conducted on the days when microbiological sampling occurred to assess the correlation between process rates and microbial composition. This strategic timing allowed for a thorough examination of how microbial communities responded to changing conditions throughout the study. The results were visualized through Sankey diagrams generated using the GPS-X simulation platform (ver. 7.0, Hydromantis, Canada), providing a clear depiction of the flow and distribution of N and S throughout the various processes.

3.5. Analytical methods

In all three studies (Papers II-IV) analytical methods were used to monitor reactor performance and nutrient removal.

Biomass was assessed by determining the mixed liquor volatile suspended solids (MLVSS) as a fraction of mixed liquor suspended solids (MLSS), following Standard Methods (APHA, 2005). Samples were filtered using disposable MFV-3 glass microfiber filters (Sartorius, Germany).

Concentrations of NH_4^+, NO_2^-, NO_3^-, SO_4^{2-}, S^{2-} and COD were measured using certified cuvette tests and a DR 3900 spectrophotometer (Hach Lange GmbH). Filtered mixed liquor samples were analyzed immediately for accuracy. All measurements used certified cuvette tests with verified accuracy and were repeated as necessary to ensure reliability.

3.6. Microbiological analyses

3.6.1. Sample Collection

Granular activated sludge samples were collected from the SBRs at various operational days, depending on the specific study:

Paper II: Samples were taken on days 1 (inoculum), 100 and 160.

Paper III: Samples were collected on days 49, 75, 126, 154 and 187.

Paper IV: Samples were taken on days 1 (inoculum), 94, 114 and 164.

3.6.2. DNA Extraction and Quality Control

For all studies, genomic DNA was extracted from the collected sludge samples using the FastDNA Spin kit for soil (MP Biomedicals, USA). Approximately 200 mg of semi-dry biomass was bead-beaten in an Uniequip device for 5 minutes. The DNA extraction was performed in duplicates, and the quality of the extracted DNA was verified using agarose gel electrophoresis. DNA concentrations were measured with a Quant-iT BR DNA Assay (Thermo Fisher Scientific, USA).

3.6.3. 16S rRNA Gene Sequencing

The microbial communities were characterized by sequencing the V3-V4 hypervariable regions of the 16S rRNA gene. This was performed using high-throughput Illumina sequencing with pair-end technology on the MiSeq platform. The primers used were 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′). Sequencing was performed with the MiSeq Reagent Kit v3, ensuring a read length of 300 base pairs for all samples.

3.6.4. Data Processing and OTU Identification

Raw reads were processed with the QIIME2 pipeline. Sequences were merged using the fast-join algorithm, and unmerged sequences were excluded from further analysis. Low-quality sequences (quality score < 20) were filtered out using Cutadapt, and chimeric sequences were removed using USEARCH. Operational Taxonomic Units (OTUs) were clustered with a 97% identity threshold, ensuring coverage of the 16S rRNA V3-V4 region.

For further taxonomic classification, the sequences were compared against the Silva (138 release) and EzBioCloud databases. OTU numbers and rarefaction curves were computed, and alpha-diversity indices (Chao1, Shannon) were calculated for each sample.

3.6.5. Alpha and Beta Diversity Analyses

Alpha diversity indices were calculated to estimate microbial richness and evenness across the samples. Beta diversity was assessed using generalized UniFrac distances, combining weighted and unweighted UniFrac metrics to evaluate phylogenetic distances between samples. Principal Coordinate Analysis (PCoA) was used to visualize microbial community structure differences between reactor conditions and sampling days. PERMANOVA tests were applied to assess the significance of genetic distance differences.

3.6.6. Statistical and Network Analyses

Significant correlations between microbial taxa abundance and operational parameters were identified using the Wilcoxon rank-sum test. Network analysis was conducted to explore the interactions between different microbial taxa. Spearman's correlation coefficients (SCC) greater than 0.70 ($P < 0.01$) were used to define relationships between bacterial taxa and reactor conditions. The Gephi platform was used to visualize the network, where nodes represented taxa, and edges indicated statistically significant relationships. Nodes with high betweenness centrality were considered key taxa influencing the microbial network.

4. Critical review on incorporation of the sulfur cycle in sustainable nitrogen removal systems (Paper I)

Paper I offers an extensive review of the integration of S cycles into N removal systems, with a focus on enhancing the sustainability of wastewater treatment processes (Figure 2). The paper highlights S-driven processes, such as SRAO and SDAD, as promising alternatives to conventional N removal pathways, which typically rely on C-based denitrification.

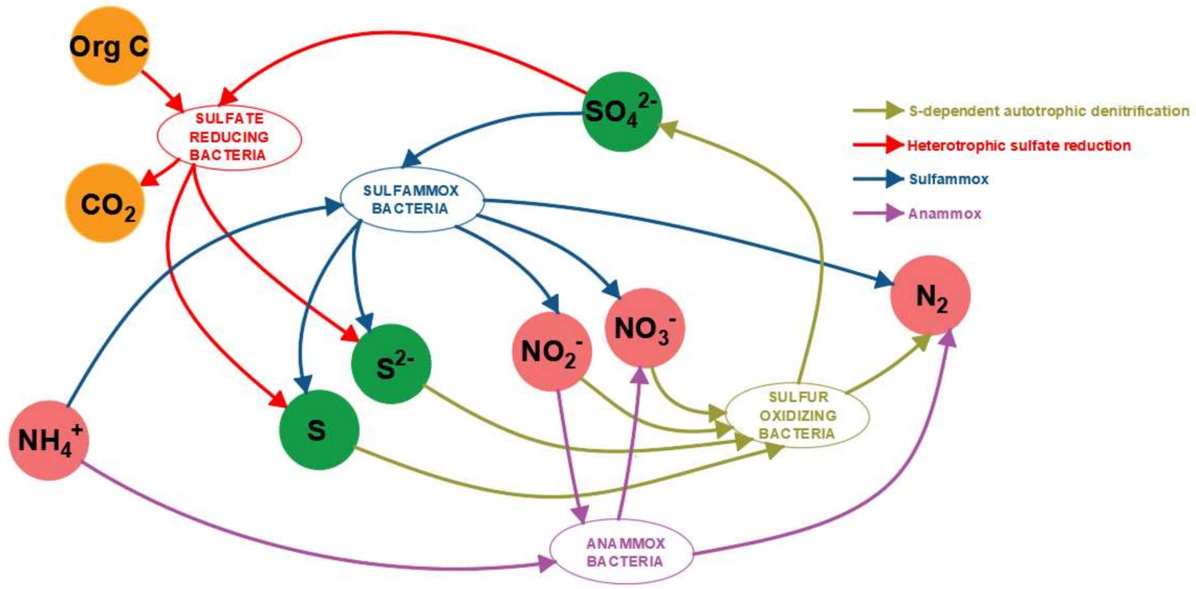


Figure 2. Interactions between S-dependent autotrophic denitrification, heterotrophic SO_4^{2-} reduction, anammox and sulfammox process (Figure 1 from Paper I)

The review emphasizes the ability of S compounds, such as SO_4^{2-} and S^0, to serve as electron donors in N removal processes. These S-based pathways allow for effective N removal without the need for significant external C sources, making them particularly relevant for wastewater systems low in organic matter. Paper I showcases the efficiency of SRAO in facilitating NH_4^+ oxidation through S reduction and SDAD in reducing NO_3^- and NO_2^-, both of which align with goals of reducing operational costs and energy usage in wastewater treatment. These processes contribute to minimizing the C footprint of treatment facilities by reducing the need for external C sources and decreasing greenhouse gas (GHG) emissions, such as N_2O, often associated with traditional methods.

A key component of the review is the exploration of microbial communities, particularly SOB, which play a crucial role in enabling S-based N removal. The paper explains how these bacteria interact with other microbial groups, such as anammox bacteria and heterotrophic denitrifiers, to drive N and S cycling. This microbial synergy is particularly important in systems that combine both autotrophic and mixotrophic conditions, allowing for greater flexibility and enhanced nutrient removal performance.

Paper I also addresses several challenges associated with integrating S cycles into N removal processes. One of the primary concerns is the formation of intermediate S compounds, such as H_2S, which can inhibit microbial activity and reduce the efficiency of N removal processes. Additionally, the competition between different microbial groups for electron donors, whether S compounds or organic C, can impact the overall stability and performance of the system. The review stresses the importance of controlling operational parameters, such as S dosing and oxygen levels, to prevent the accumulation of toxic intermediates and maintain efficient microbial activity.

In its conclusion, paper I highlights key areas for future research, calling for the optimization of S-based processes such as SRAO and SDAD, particularly under varying conditions of COD and N. The review advocates for the development of hybrid systems that combine S-driven processes with traditional N removal techniques, such as anammox and heterotrophic denitrification, to address a wider range of wastewater compositions. Additionally, the paper emphasizes the need for further research into microbial dynamics, particularly through advanced techniques like metagenomics, to gain a deeper understanding of the functional roles of SOB and other microbial populations involved in N and S removal.

Overall, paper I establishes a strong foundation for the further integration of S cycles into N removal systems. It underscores the potential of these S-driven processes to enhance the sustainability, efficiency, and cost-effectiveness of wastewater treatment, while identifying the challenges that must be addressed to optimize these systems for practical applications.

5. RESULTS AND DISCUSSION (Papers II-IV)

5.1. Impact of sulfate on process performance in different operational conditions

5.1.1. Nutrients removal under autotrophic conditions (without COD) (Paper II)

The results showed a clear link between SO_4^{2-} supplementation (Figure 3) and improved N removal efficiency (Figure 2 in Paper II). In SBR1_AMX, which followed the traditional anammox process, AUR increased by 27.8 mg N/g VSS/h over 160 days, while NiUR increased by 13.5 mg N/g VSS/h. The SUR in this reactor was relatively low, only increasing by 1.4 mg S/g VSS/h. This suggests that the primary N removal mechanism in SBR1_AMX was driven by the anammox process, with limited involvement of S-related processes.

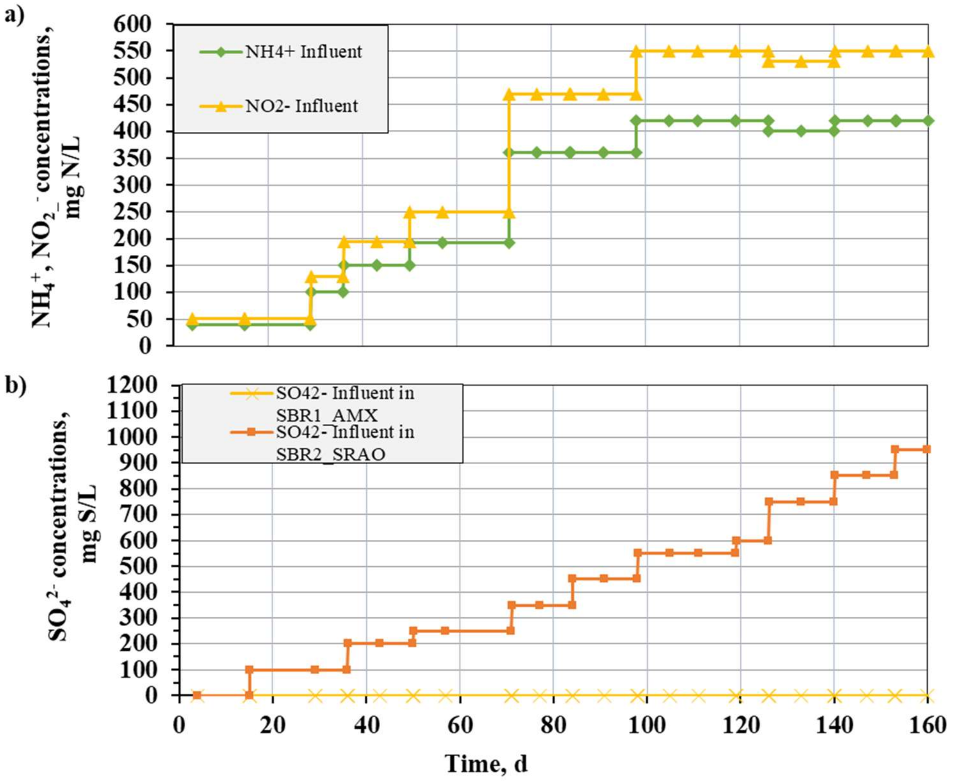


Figure 3. The influent concentrations of a) NH_4^+, NO_2^- b) SO_4^{2-} (Figure 1 from Paper II)

In contrast, in SBR2_SRAO, where SO_4^{2-} played a more significant role, the N removal efficiency was substantially higher. The AUR increased by 51 mg N/g VSS/h, while the SUR by 19.8 mg S/g VSS/h. The NiUR initially increased by 11.9 mg N/g VSS/h on day 100 but decreased by 6.5 mg N/g VSS/h until the end of the study. This two-fold increase in AUR and a 15-fold improvement in SUR in SBR2_SRAO demonstrate the effectiveness of SO_4^{2-} as an electron acceptor in boosting N removal through the SRAO process. The NH_4^+ removal efficiency was high in both reactors, exceeding 90% in SBR1_AMX and 96% in SBR2_SRAO after day 36.

In the batch tests - BR_SR, where NO_2^- was not added, N removal performance was notably lower compared to reactors with both NO_2^- and SO_4^{2-}. The AUR in BR_SR only increased by 8.1 mg N/g VSS/h over 153 days, which was three times lower than in SBR1_AMX and significantly lower than in SBR2_SRAO. The lack of NO_2^-, which is a crucial electron acceptor in the anammox process, limited the efficiency of NH_4^+ removal in BR_SR, peaking at 64% on day 84 and decreasing to 17% by the end of the study.

While the NH_4^+ removal efficiency in BR_SR was low, the SO_4^{2-} removal efficiency was the highest among all reactors, reaching an average of 45% compared to 12% in SBR2_SRAO. SUR in BR_SR increased by 22 mg S/g VSS/h, the fastest increase observed in the study. This suggests that in the absence of NO_2^-, SO_4^{2-} became the dominant electron acceptor, promoting S reduction but at the cost of lower N removal efficiency.

5.1.2. Nutrients removal under mixotrophic conditions (with COD) (Paper III)

The study found that the addition of COD significantly enhanced both AUR and NiUR (Figure 2 in Paper III). The most notable results occurred during phase VII, where the maximum AUR reached 22.8 mg N/(g VSS·h) in SBR1 and 25.8 mg N/(g VSS·h) in SBR2. The higher AUR in SBR2 suggests that SO_4^{2-} supplementation improved NH_4^+ removal in combination with COD (Figure 4). Similarly, the NiUR peaked at 24 mg N/(g VSS·h) in SBR1 and a much higher 34.6 mg N/(g VSS·h) in SBR2, indicating that the presence of both COD and SO_4^{2-} enhanced NO_2^- removal, especially in SBR2. The N removal efficiency across both reactors varied from 62% to 99%, with SBR2 consistently showing higher efficiency by approximately 4% compared to SBR1.

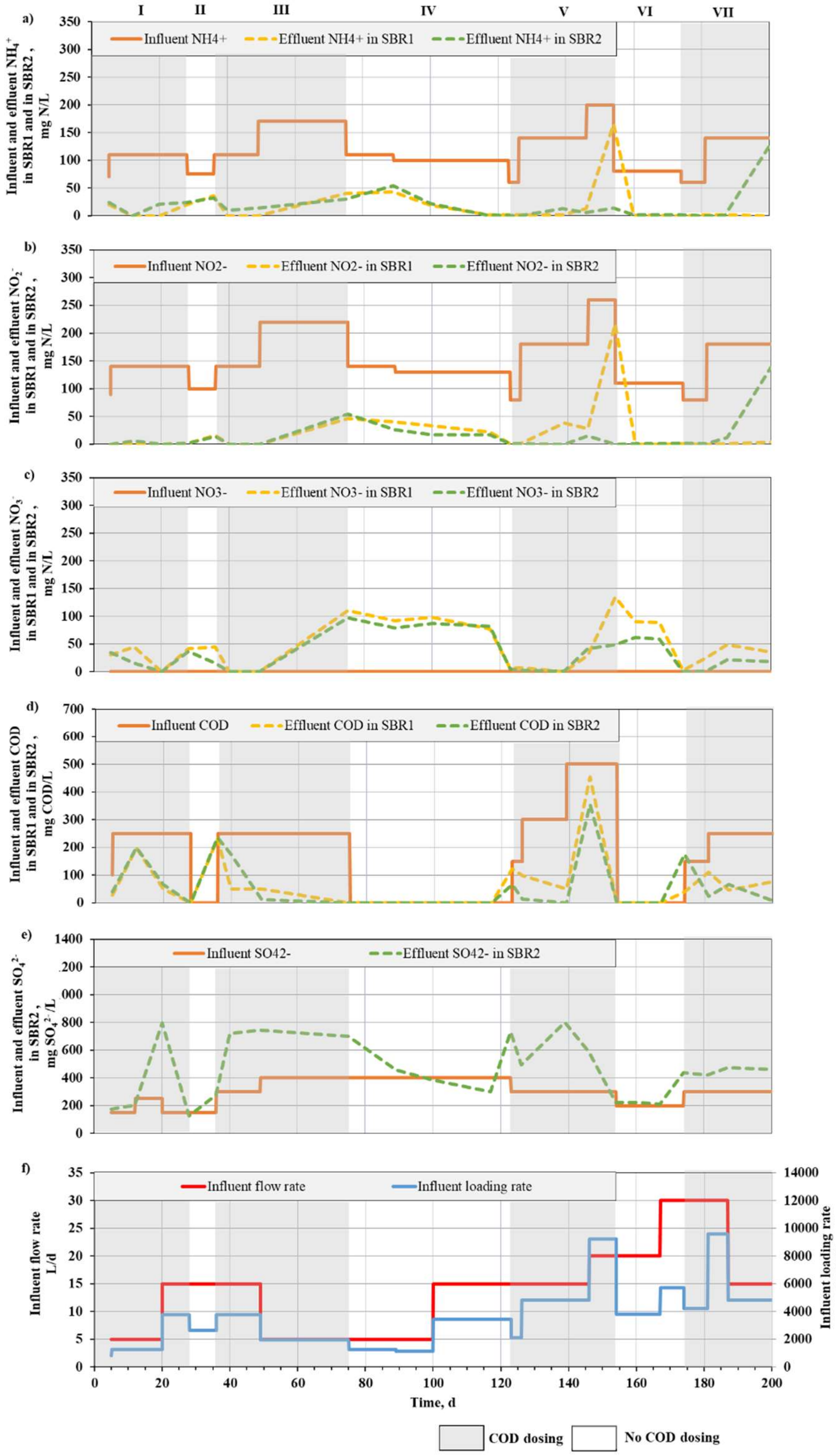


Figure 4. Influent and effluent concentrations of a) NH_4^+, b) NO_2^-, c) NO_3^-, d) COD, e) SO_4^{2-}; f) Influent flow rate and influent loading rate (Figure 1 from Paper III)

In phase III, SBR2 achieved the highest CUR of 46 mg COD/(g VSS·h), compared to 36.3 mg COD/(g VSS·h) in SBR1. These elevated CUR values suggest that SBR2 utilized C more efficiently when SO_4^{2-} was present.

During phases where COD was added, the SPR increased significantly, while the SUR dominated during COD-free phases. The highest SPR in SBR2 was observed in phase III, reaching 45.5 mg S/(g VSS·h), but this rate declined to 20.9 mg S/(g VSS·h) in phase VII. This decline in SPR suggests that the SPR was most efficient during early COD phases but diminished as COD levels remained elevated. The SUR were consistently lower, with the highest SUR in SBR2 recorded at 7 mg S/(g VSS·h) in phase IV.

5.1.3. Nutrients removal under autotrophic and mixotrophic conditions without NO_2^- addition (Paper IV)

In SBR1, AUR remained between 0.5 and 2 mg N/(g VSS·h) until day 118, whereas in SBR2, the range was slightly higher, between 1.1 and 2 mg N/(g VSS·h) until day 103 (Figure 1 in Paper IV). After these initial phases, a notable increase in AUR was observed in both reactors, with SBR1 reaching a maximum of 6.1 mg N/(g VSS·h) on day 180 and SBR2 peaking at 4.8 mg N/(g VSS·h) on day 148. This increase in AUR coincided with the rise in SO_4^{2-} addition after day 100, suggesting that SO_4^{2-} supplementation enhanced NH_4^+ oxidation in both reactors (Figure 5).

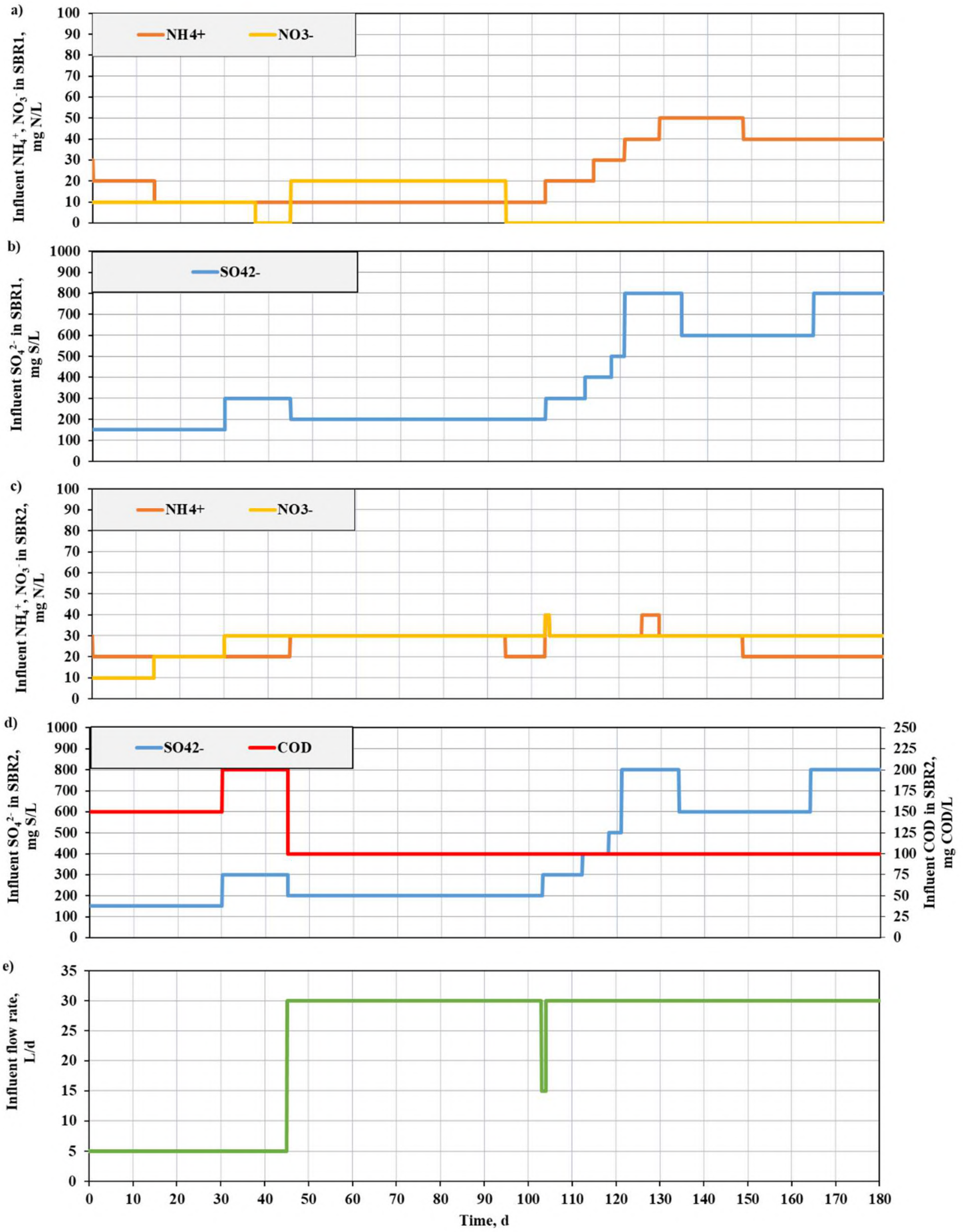


Figure 5. Influent NH_4^+, NO_3^-, SO_4^{2-}, COD concentrations in SBR1 (a-b) and SBR2 (c-d); Influent flow rate in SBR1 and SBR2 (e) (Figure S2 from Paper IV)

SO_4^{2-} dynamics also showed marked differences between the two reactors (Figure 1 in Paper IV). In SBR1, the SUR increased gradually from 1.6 mg S/(g VSS·h) to 12.7 mg S/(g VSS·h) by day 118, reaching a peak of 38.6 mg S/(g VSS·h) on day 129. On the other hand, SBR2 initially exhibited SO_4^{2-} production, with SPR in the range of 6.8 to 10 mg S/(g VSS·h) until day 103. After this point, SO_4^{2-} utilization began in SBR2, and SUR peaked at 44.9 mg S/(g VSS·h) by day 125. The presence of COD in SBR2 significantly enhanced NH_4^+ removal efficiency, which reached 100% by day 100, compared to a maximum of 64% in SBR1 (Figure 3 in Paper IV). Similarly, total nitrogen (TN) removal efficiency was higher in SBR2, nearly reaching 100%, while SBR1 only achieved a maximum of 57%. The addition of COD enhanced the performance of N and S removal processes in SBR2 by integrating C into the N-S cycle.

5.2. Microbial community dynamics and interactions

Depending on the operational conditions, such as the availability of SO_4^{2-} and COD, significant differences in microbial composition and diversity were observed. By comparing the results from Papers II, III, and IV, notable trends in microbial community structure are evident, with different microbial populations adapting to the specific conditions in each reactor.

In Paper II, both reactors, SBR1 and SBR2, operated without COD, with varying SO_4^{2-} concentrations. Following a 100-day adaptation period, the bacterial communities in both reactors developed compositions that, while similar to each other, were distinctly different from the original inoculum (Figure 6). Over the next 60 days, the adaptation process accelerated, resulting in the formation of highly diverse bacterial populations, each specialized for the dominant processes occurring in their respective reactors. This shift in community structure highlighted the influence of SO_4^{2-} in SBR2_SRAO, where its presence played a significant role in shaping the microbial dynamics specific to S -driven processes.

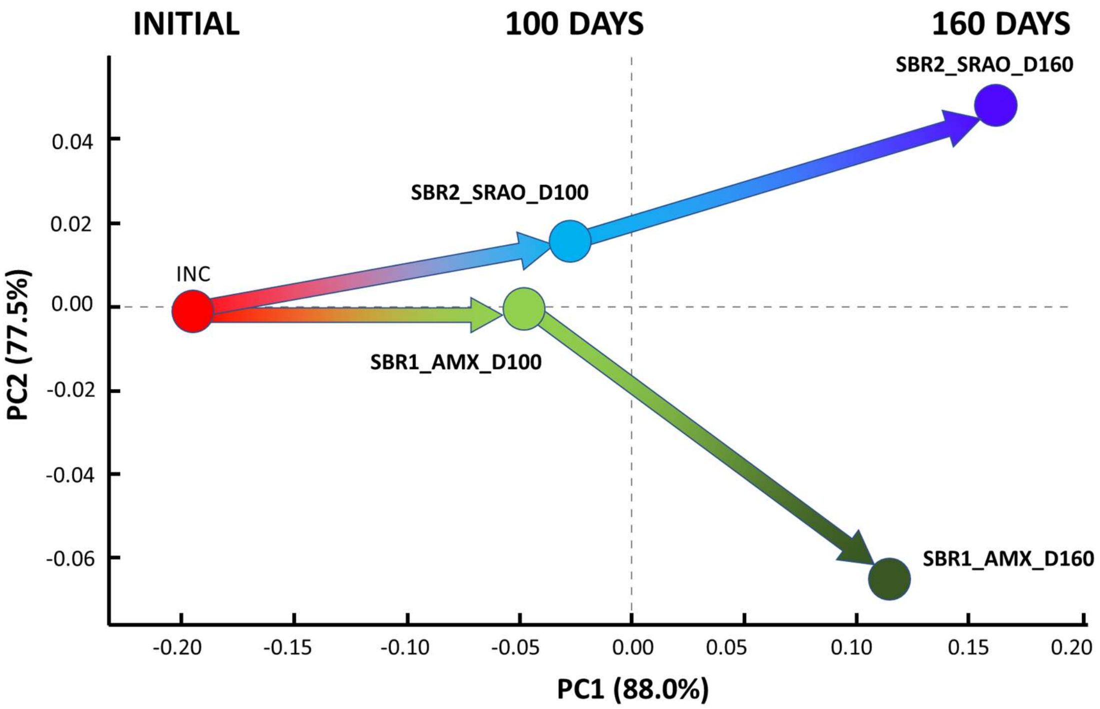


Figure 6. Differentiation of the bacterial community structure between SBR1_AMX and SBR2_SRAO after 160 days of the operation, analyzed by the PCA of the 16 S rRNA sequencing data at the genus level (Figure 5 from Paper II)

Throughout the study, *Proteobacteria* and *Chloroflexi* were the dominant phyla in both reactors, each making up over 20% of the microbial community. By day 160, notable shifts occurred (Table 1 in Paper II). In SBR2_SRAO, *Proteobacteria* increased to 50%, while *Chloroflexi* remained stable at around 24%. *Nitrosomonas*, an ammonia-oxidizing bacterium (AOB), was one of the key genera within *Proteobacteria*. By the end of the study, *Nitrosomonas* constituted 14.7% of the microbial population in both reactors, indicating its dominant role in N cycling under autotrophic conditions. *Thauera* was a key player in the S metabolism due to its high abundance in SBR2_SRAO (>15.0% of the total bacterial community).

In Paper III, study revealed significant microbial shifts influenced by COD and SO_4^{2-} availability, with the anammox process, driven by AnAOB, being the primary pathway for NH_4^+ removal. *Candidatus* Brocadia, a dominant anammox bacterium, was more abundant in SBR2 (7.1%) compared to SBR1 (3.6%), correlating with higher AUR and NiUR in SBR2, indicating that SO_4^{2-} enhanced anaerobic NH_4^+ oxidation as an additional electron acceptor. *Proteobacteria* and *Chloroflexi* were the most abundant phyla in both reactors, with *Anaerolineae* contributing to microbial aggregation and N removal, especially in SBR2. Additionally, *Denitratisoma* sp. and PHOS-HE36, linked to denitrification, were present in both reactors, reflecting the balance of autotrophic and heterotrophic processes. The addition of COD in SBR2 stimulated both heterotrophic and autotrophic transformations, enhancing AnAOB activity, which adapted to utilizing organic C sources like acetate. The increased presence of *Candidatus* Brocadia in SBR2 and improved mixotrophic denitrification confirm the successful integration of N-S-C cycles.

In Paper IV, the dominant bacterial phyla in both reactors were *Chloroflexi* and *Proteobacteria*, with relative abundances of 26% and 20% in SBR1, and 24% and 23% in SBR2, respectively. *Bacteroidota* accounted for 17% in SBR1 and 18% in SBR2, while *Planctomycetota* comprised 14% in SBR1 and 15% in SBR2. At the genus level, *Candidatus* Brocadia was more abundant in SBR2 (9.38%) compared to SBR1 (7.73%), reflecting enhanced anammox activity in SBR2. PHOS-HE36, another prominent genus, exhibited similar abundances in both reactors, at 7.95% in SBR1 and 7.89% in SBR2. A notable increase was observed for *Thauera*, which was more prevalent in SBR2 (5.12%) compared to SBR1 (2.35%), particularly after 100 days of operation. This increase is likely linked to improved NH_4^+ utilization and SO_4^{2-} reduction, given *Thauera* role in denitrification. Additionally, *Denitratisoma* was present at 6.08% in SBR1 and 5.07% in SBR2, further contributing to N removal processes, alongside *Candidatus Brocadia*. These results suggest that the presence of SO_4^{2-} in SBR2 not only enhanced the proliferation of key denitrifying bacteria but also played a crucial role in improving overall N and S removal efficiency.

This comparison highlights how SO_4^{2-} and COD availability drive microbial dynamics in N and S removal processes. The balance between these microbial groups is essential for optimizing the efficiency of N and S removal in wastewater treatment systems.

5.3. Stoichiometric calculations of process rates

The results of the stoichiometric analysis are presented in Figures 7 and S1 in Paper II, Figures 5 and S1 in Paper III, and Figures 5, 6, and S4 in Paper IV. They are the basis for summarizing the results in this chapter.

5.3.1. Calculation of AUR under autotrophic and mixotrophic conditions

In Paper II, the stoichiometric analysis demonstrated that, initially, the AUR in both reactors was primarily driven by the anammox process. By day 100, the AUR in SBR2_SRAO increased significantly due to the combined effect of NH_4^+ oxidation by AOB (AUR_AOB) and SDAD. By day 160, the SRAO process also contributed substantially to AUR, which reached 3.1 mg N/(g VSS•h).

In Paper III, the mixotrophic conditions in SBR2, with the introduction of both SO_4^{2-} and COD, significantly enhanced NH_4^+ oxidation. SRAO process, alongside COD-supported denitrification, drove the AUR increase in SBR2, which reached 25.8 mg N/(g VSS•h) by day 187. In contrast, AUR in SBR1 was mainly driven by the anammox process and remained lower (22.8 mg N/(g VSS•h)).

In Paper IV, the stoichiometric analysis showed a significant shift in the processes driving AUR after SO_4^{2-} supplementation. In SBR1, AUR initially involved both anammox and minor contributions from S-driven processes. However, by day 164, SRAO process became the dominant contributor to AUR. Meanwhile, in SBR2, SRAO process gradually replaced anammox as the primary driver of NH_4^+ oxidation, indicating a full transition to S-driven processes.

5.3.2. Stoichiometric analysis of SRAO and anammox pathways

The stoichiometric analysis across Papers II, III, and IV revealed key differences in the roles of SRAO and anammox pathways in N removal processes. In Paper II, SRAO became a significant driver of NH_4^+ oxidation in SBR2 as SO_4^{2-} was added. Anammox activity remained important but was overshadowed by the growing influence of S-driven processes.

In Paper III, SRAO played a central role in the mixotrophic system (SBR2), where the integration of S and C cycles further enhanced N removal. By day 187, SRAO pathway, combined with mixotrophic denitrification, contributed to higher NH_4^+ utilization and efficient N transformations, reducing the relative impact of anammox compared to SRAO.

In Paper IV, SBR1 maintained a balance between SRAO and anammox, with both pathways contributing to N removal. However, in SBR2, the stoichiometric data showed that SRAO completely replaced anammox by day 164, highlighting the dominance of S-related processes in the system. These results demonstrate that while anammox is initially important, SRAO becomes increasingly significant in systems with added SO_4^{2-}, especially in mixotrophic environments.

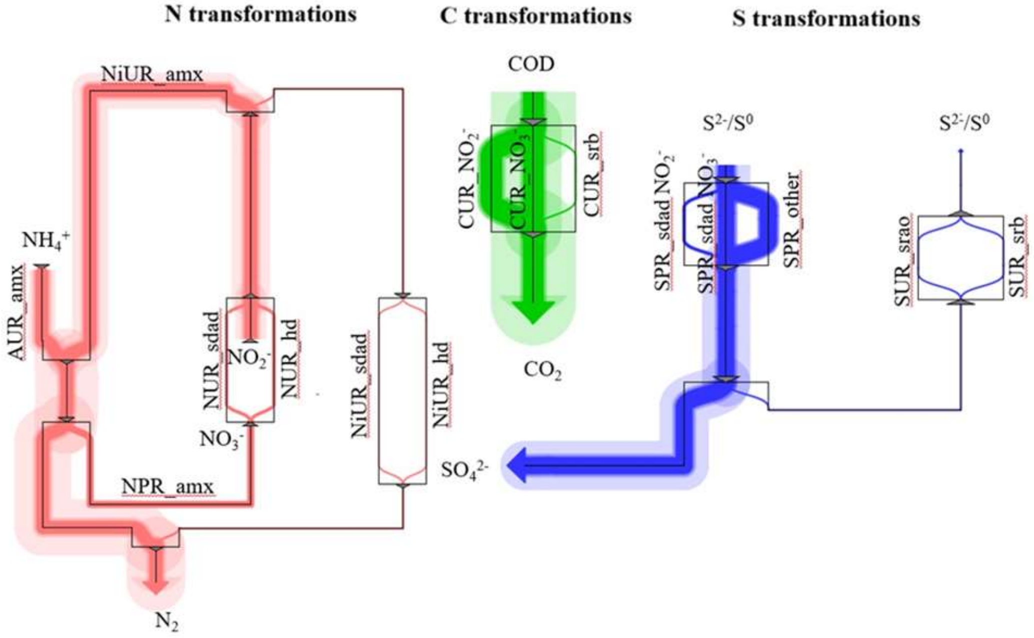
5.3.3. Comparative stoichiometric efficiency of N removal with and without NO_2

The stoichiometric analysis in Papers II, III, and IV compared N removal efficiencies under conditions with and without NO_2^-, revealing notable differences.

In Paper II, NO_2^- was consistently supplied during regular operation in both reactors. The presence of NO_2^- allowed for efficient N removal through both anammox and SDAD. However, in batch tests, NO_2^- was excluded, and the stoichiometric analysis showed a substantial decrease in N removal efficiency when NO_2^- was absent, indicating its critical role in driving both anammox and SDAD processes. SBR2, which had increased SO_4^{2-} concentrations, exhibited higher N removal rates due to S-driven pathways that utilized NO_2^-, further emphasizing its importance.

In Paper III, NO_2^- was also supplied in both reactors, with the stoichiometric analysis highlighting that the presence of NO_2^- facilitated efficient N removal. In SBR2, where SO_4^{2-} and COD were added, NO_2^- played a key role in driving mixotrophic denitrification and anammox processes, leading to superior N removal efficiency compared to SBR1. The high NiUR in SBR2 demonstrated the effectiveness of NO_2^- in enhancing N transformation pathways (Figure 7).

a) SBR1 (187d)



b) SBR2 (187d)

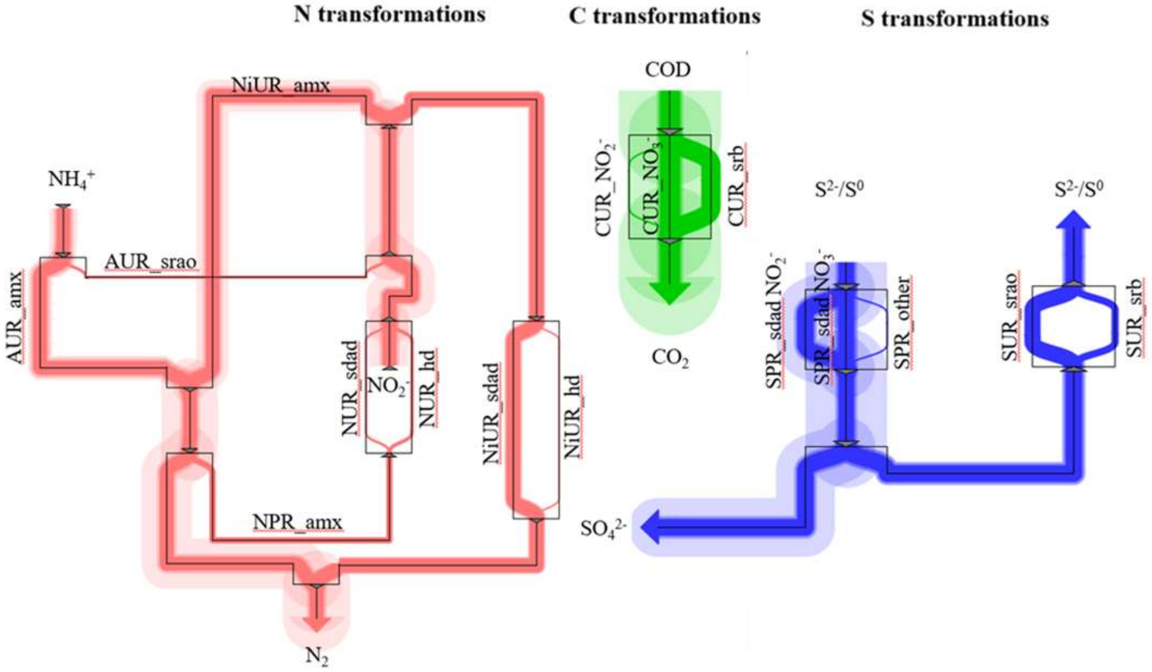


Figure 7. Sankey diagram showing the transformation pathways of N, S and COD based on the stoichiometric analysis on day 187 a) in SBR1, b) in SBR2 (thickness of the arrows is proportional to the rate) (Figure 5 from Paper III)

In Paper IV, NO_2^- was not added to either reactor, and the stoichiometric analysis revealed a marked reduction in N removal efficiency. In the absence of NO_2^-, N transformations relied solely on NO_3^-, leading to a shift in process dominance from anammox to SDAD and SO_4^{2-} reduction. The lack of NO_2^- inhibited anammox, significantly lowering N removal efficiency, particularly in SBR2, which transitioned entirely to S-related pathways by the end of the study.

In summary, the stoichiometric analyses across all three publications show that N removal is more efficient in systems where NO_2^- is present. The absence of NO_2^-, as seen in Paper IV, led to a reduced reliance on anammox and lower N removal rates, highlighting the critical role NO_2^- plays in supporting both anammox and S-driven processes like SDAD.

5.3.4. Optimization of N and S removal through stoichiometric modeling in reactors with and without COD

The stoichiometric modeling in Papers II, III, and IV analyzed the optimization of N and S removal in reactors with and without COD, revealing key differences in process efficiencies.

In Paper II, COD was not introduced in either reactor, and the N and S removal processes were primarily driven by SDAD and anammox. The stoichiometric modeling showed that, even in the absence of COD, both SBRs achieved efficient N removal, particularly in SBR2, where SRAO played a significant role. However, the lack of organic C limited the extent of SDAD, which could have been further enhanced with COD.

In Paper III, COD was added alternately in both SBRs during specific phases, allowing for a direct comparison between the presence and absence of organic C. The stoichiometric analysis showed that the introduction of COD significantly enhanced N and S removal, particularly in SBR2, where mixotrophic denitrification (involving both heterotrophic and autotrophic pathways) was more efficient. The COD on phases increased both AUR and SUR, driving higher overall process efficiency. Conversely, during COD off phases, anammox and S-dependent processes (such as SRAO) became more dominant, maintaining N removal but with slightly reduced S utilization.

In Paper IV, COD was only added in SBR2, which allowed for a comparison between COD-supported processes in SBR2 and autotrophic processes in SBR1. The stoichiometric modeling demonstrated that SBR2, with COD, showed superior N and S removal efficiency compared to SBR1. The presence of COD in SBR2 stimulated mixotrophic denitrification, leading to higher SO_4^{2-} reduction rates and more efficient N removal, while SBR1 relied on SRAO and anammox with lower overall removal rates. By the end of the experiment, the absence of COD in SBR1 resulted in reduced N and S transformations compared to the COD-driven processes in SBR2.

In summary, stoichiometric modeling across the three publications highlighted that the addition of COD significantly enhanced the efficiency of both N and S removal. In Paper III, alternating COD phases optimized the balance between heterotrophic denitrification and autotrophic processes, while in Paper IV, the presence of COD in SBR2 led to much higher removal efficiencies compared to the autotrophic-only processes in SBR1.

5.4. Integration of N-S-C cycles in wastewater treatment

5.4.1. Impact of NO_2^- and SO_4^{2-} on the performance of combined N-S-C cycles

The presence and concentration of NO_2^- and SO_4^{2-} are pivotal in influencing the performance of integrated N-S-C cycles in wastewater treatment. The efficiency of key processes, such as SRAO and SDAD, as well as overall N and S removal, is heavily dependent on the availability of these compounds, as demonstrated across Papers I-IV.

NO_2^- plays a significant role in driving N removal within integrated N-S-C systems. It acts as an electron acceptor in both the denitrification process and the anammox pathway, directly enhancing N removal efficiency. Paper II demonstrated that in systems integrating S cycles, concentrations of NO_2^- between 10-15 mg/L significantly improved N removal efficiency through SDAD, where S compounds acted as electron donors. This pathway was especially effective in low-COD wastewater streams, where the reliance on organic C is minimized. Conversely, NO_2^- concentrations below 5 mg/L led to incomplete N removal, indicating that a deficiency in NO_2^- could reduce the system's performance and limit the effectiveness of S-based N removal (Kosugi et al., 2019).

The role of SO_4^{2-} is equally critical, particularly in S-driven processes like SRAO and SDAD, where it serves as an electron acceptor. The reduction of SO_4^{2-} facilitates NH_4^+ oxidation in SRAO systems, enabling simultaneous N and S removal. The studies from Paper III found that SO_4^{2-} concentrations ranging from 200 to 300 mg/L significantly boosted N removal efficiency, as the S cycle actively contributed to the overall N transformations. Systems with SO_4^{2-} concentrations at this level showed stable performance, with optimal NH_4^+ oxidation and SO_4^{2-} reduction. However, at concentrations exceeding 500 mg/L, SO_4^{2-} reduction became less efficient, and the system exhibited diminished N removal rates due to imbalances in the S cycle (Derwis et al., 2023).

In systems where autotrophic and heterotrophic processes are combined, the interaction between NO_2^- and SO_4^{2-} becomes particularly important. Paper IV highlighted that when NO_2^- concentrations were maintained above 10 mg/L, SDAD was the dominant N removal process. Under these conditions, SO_4^{2-} reduction supported the oxidation of nitrogenous compounds, leading to efficient N and S removal, even in COD-limited environments. In contrast, when NO_2^- levels dropped below 5 mg/L, SRAO processes became more prominent, compensating for the lack of NO_2^- by using SO_4^{2-} to drive N removal. The balance between NO_2^- and SO_4^{2-} concentrations was found to be crucial in maintaining system stability and maximizing overall treatment efficiency (Grubba et al., 2021).

The integration of N-S-C cycles in wastewater treatment benefits from the strategic manipulation of NO_2^- and SO_4^{2-} levels. Optimal concentrations of NO_2^- (10-15 mg/L) and SO_4^{2-} (200-300 mg/L) enhance the contribution of S-based processes like SDAD and SRAO to N and S removal. Additionally, these processes allow the system to remain flexible, effectively handling variations in COD and other influent characteristics. Managing NO_2^- and SO_4^{2-} concentrations is critical not only to maximize removal efficiencies but also to prevent operational issues such as process inhibition due to excessive SO_4^{2-} (Kosugi et al., 2019).

5.4.2. Role of COD in driving mixotrophic denitrification and SRAO processes

The role of COD in driving mixotrophic denitrification and SRAO processes is essential for optimizing N and S removal in wastewater treatment systems. COD influences the balance between autotrophic and heterotrophic processes. Across the studies, it has been observed that the presence of COD can enhance N and S transformations, particularly through its impact on mixotrophic denitrification and SRAO.

In Paper III, COD played a key role in mixotrophic denitrification, where both S compounds and organic C served as electron donors. It was shown that when COD concentrations fluctuated between 200 and 400 mg/L, N removal was significantly improved due to the stimulation of heterotrophic denitrification. During COD-on phases, heterotrophic denitrifiers utilized the organic C available in the system to reduce NO_3^- and NO_2^-, improving the overall N removal efficiency. The optimal COD concentration for maintaining this balance was observed to be between 250 and 350 mg/L, allowing mixotrophic denitrification to work alongside S-based processes like SDAD (Kosugi et al., 2019).

The balance between COD and S-driven processes was further explored in Paper IV, which demonstrated that in low-COD environments (<100 mg/L), S-driven processes like SRAO became dominant, driving both N and S removal. As the COD concentration increased to 200-300 mg/L, the system shifted towards mixotrophic denitrification, utilizing both C and S as electron donors. This dynamic adjustment allowed for efficient N removal without compromising the S reduction processes, as mixotrophic conditions supported both autotrophic and heterotrophic pathways (Grubba et al., 2021, Derwis et al., 2023).

The alternation between COD-on and COD-off phases, as observed in Paper III, was found to have a significant effect on N and S removal processes. During COD-off phases, S-driven autotrophic processes like SRAO maintained N removal, while during COD-on phases, heterotrophic denitrification accelerated N removal. This flexibility allowed the system to adapt to varying levels of COD, maintaining high levels of performance in both N and S removal despite fluctuations in organic load (Kosugi et al., 2019).

5.4.3. Technological benefits and limitations of N-S-C cycle integration in wastewater treatment

The integration of N-S-C cycles in wastewater treatment systems provides numerous technological benefits that enhance the efficiency and sustainability of nutrient removal processes. One of the most notable advantages is the improved TN removal efficiency compared to traditional separation processes and standalone methods, including anammox and SDA. The synergistic interactions among different microbial groups in integrated systems allow for the simultaneous reduction of NH_4^+ and SO_4^{2-}, leading to higher N and S removal rates.

This integration also significantly reduces the dependency on external C sources, as S-driven processes do not require additional C for SO_4^{2-} reduction. As a result, wastewater treatment facilities can achieve effective N removal while minimizing operational costs associated with chemical inputs. Studies have shown that integrating anammox and mixotrophic denitrification leads to lower energy consumption compared to traditional denitrification methods, further enhancing the system's efficiency.

The flexibility of these systems is another advantage, allowing for the use of alternating COD on/off conditions. This operational strategy can increase the activity of AnAOB during COD-off phases while promoting denitrifier activity during COD-on phases. Such adaptability enables the treatment of complex wastewaters, which often contain varying concentrations of N, S and C compounds.

Moreover, the N-S-C cycle integration leads to a reduced need for chemicals, as the products of one microbial process often serve as substrates for another. This closed-loop approach not only lowers operational complexity but also results in decreased sludge production, thereby reducing waste generation and the environmental impact associated with sludge handling.

Additionally, the incorporation of anammox and mixotrophic denitrification can mitigate the production of N_2O, a potent greenhouse gas commonly associated with conventional denitrification processes. By minimizing N_2O emissions, integrated systems align with sustainable development goals aimed at reducing the C footprint of wastewater treatment facilities.

Lastly, the adaptability of N-S-C cycle integration makes it particularly promising for treating complex wastewaters from various sources, such as mining, fermentation processes, landfill leachate, and the pulp and paper industry. By effectively managing N-S-C content, these systems can mitigate the environmental impacts of industries with high pollutant loads, leading to a more sustainable approach to wastewater treatment

However, alongside these advantages, several challenges and limitations must be addressed to optimize the performance of integrated N-S-C cycle systems. One significant challenge is the limited understanding of the mechanisms underlying SRAO and the specific microorganisms involved. The complexity of microbial interactions and the precise roles of different species, such as SRB and AnAOB, require further research to fully leverage their potential.

Competition between AnAOB and SRAO bacteria is another major concern. In mixed microbial communities, the presence of other microorganisms can interfere with AnAOB activities, potentially impacting N removal efficiency. This competition becomes particularly evident in environments where both SRAO and anammox processes are expected to coexist. In non-limited NO_3^- conditions, the concentration of SO_4^{2-} may rise due to SDAD, complicating the management of S cycles within the treatment system.

Moreover, achieving and maintaining optimal operating conditions for both mixotrophic denitrification and anammox can be challenging. Parameters such as pH, temperature, nutrient concentrations, and COD availability must be carefully monitored to ensure efficient and stable operation. The right balance of C/N ratios is particularly vital for AnAOB, yet maintaining this balance can be difficult in certain wastewater streams.

The applicability of these processes is limited by their suitability for specific wastewater characteristics, primarily those with high N and S content but low COD. This restricts their range of application, making them less effective in wastewaters with varying compositions. The potential accumulation of NO_3^- poses another limitation - if the denitrification step is not effectively balanced with anammox, increased levels of NO_3^- can inhibit the activity of AnAOB, thereby reducing overall N removal efficiency.

Temperature dependence is an additional concern, as the efficiency of both anammox and mixotrophic denitrification processes may significantly decrease at low temperatures, limiting their use in colder climates. AnAOB are also sensitive to oxygen, which may restrict their application in systems where oxygen control is challenging. Some studies have indicated that

AnAOB might be susceptible to certain heavy metals and antibiotics, as well as S^{2-}, which could further limit their use in specific types of wastewater.

In conclusion, while the integration of N-S-C cycles in wastewater treatment offers substantial benefits, addressing the associated challenges is essential for optimizing system performance. By understanding and mitigating these limitations, wastewater treatment facilities can effectively leverage the advantages of N-S-C cycle integration to achieve sustainable and efficient nutrient removal.

6. CONCLUSIONS

The research presented in this dissertation provides advancements in understanding the integration of N-S-C cycles in wastewater treatment systems. The findings demonstrate the potential of S-driven processes, such as SDAD and SRAO, as efficient alternatives to traditional N removal pathways. By focusing on the role of S transformations and their interactions with the anammox process under various operational conditions, this work provides a solid foundation for optimizing wastewater treatment processes, especially for SO_4^{2-}-rich streams.

The addition of SO_4^{2-}has a substantial positive effect on N removal efficiency. The experimental results revealed significant increases in both AUR and SUR in systems employing S -driven processes. These findings confirm that S compounds, such as SO_4^{2-}, can effectively serve as electron acceptors in N removal processes, even in the absence of organic C. In COD-limited or COD-free environments, S-driven pathways compensated for the lack of organic substrates, leading to improved N removal.

NO_2^- was identified as a critical electron acceptor in enhancing the overall performance of S-based N removal processes, particularly when integrated with anammox. The absence of NO_2^- reduced N removal efficiency considerably, although S-based processes were still able to partially compensate when SO_4^{2-} and COD were present. This suggests that while S compounds can support N removal to some extent, the integration of NO_2^- significantly enhances the efficiency of these systems.

Introducing COD into S-based N removal systems significantly improved N removal efficiency by facilitating the coexistence of autotrophic and heterotrophic processes. The presence of COD facilitated the activity of heterotrophic denitrifiers, such as *Thauera*, which played a significant role in SDAD. The interaction between autotrophic and heterotrophic processes enhanced the removal of both N and S compounds, leading to higher overall removal rates. This finding underscores the importance of operational flexibility, as systems capable of alternating between autotrophic and heterotrophic processes are better suited to handle wastewater with with fluctuating COD and SO_4^{2-} levels.

Effective management of electron donors, such as S compounds and organic C, is crucial to avoid microbial competition that could diminish efficiency. Excessive COD levels can lead to heterotrophic bacteria outcompeting autotrophic SOB, reducing N removal efficiency. Conversely, in COD-limited environments, autotrophic processes driven by S transformations dominate, improving N removal. Therefore, optimizing the balance between these processes is critical for sustaining high system performance.

The research highlighted the crucial role of microbial community dynamics in driving the efficiency of S-based N removal processes. The synergy between SOB and anammox bacteria was essential for effective N and S removal. SOB thrived in S-enriched environments, significantly contributing to SDAD, while anammox bacteria efficiently converted NH_4^+ and NO_2^- into N_2. Stable environmental conditions are necessary to maintain microbial activity, as shifts in operational parameters, such as SO_4^{2-} or COD concentrations, can influence microbial community structure and system performance.

This research provides a comprehensive framework for understanding and optimizing S-driven N removal processes. The insights gained from this work offer promising opportunities for developing more sustainable and energy-efficient wastewater treatment technologies, capable of handling diverse wastewater compositions while minimizing environmental impact and operational costs.

7. FUTURE RESEARCH

The research presented in this dissertation provides valuable insights into the integration of N-S-C cycles in wastewater treatment systems, emphasizing the potential of S-driven N removal processes. While these findings provide a robust foundation, several areas remain open for further investigation, optimize system performance, and address challenges related to operational stability and scalability.

One of the most critical areas for future research is the optimization of operational conditions in S-based N removal systems. Although this research demonstrated the effectiveness of S-driven processes, such as SDAD and SRAO, the balance between electron donors and acceptors, particularly in systems with fluctuating influent compositions, requires further attention.

Another promising direction for future research lies in the investigation of microbial community dynamics, especially the interactions between SOB and anammox bacteria. While this research has highlighted the importance of these microbial populations in driving N and S removal, a deeper understanding of the functional roles and interspecies relationships within the microbial community is essential.

The scalability of S-driven N removal processes also presents an important challenge for future research. Most of the experimental work presented in this dissertation was conducted at a laboratory scale, and while the results were promising, translating these findings to full-scale wastewater treatment plants will require further investigation. Pilot-scale studies should be conducted to assess the feasibility of implementing S-driven N removal processes in real-world settings, with particular focus on the operational challenges associated with larger systems, such as hydraulic retention times, mixing efficiency, and nutrient loading variability. Moreover, it will be important to evaluate economic viability, including energy consumption, chemical usage, and sludge production, is necessary to support the adoption of these processes on a larger scale.

In addition to the technical aspects of system optimization and scalability, future research should explore the environmental implications of S-driven N removal processes. While this research demonstrated the potential for energy savings and reduced reliance on organic C, further studies are needed to quantify the overall environmental impact of these systems, particularly in terms of GHG emissions. Finally, future studies should investigate the integration of S-driven processes with other emerging technologies in wastewater treatment, such as resource recovery and energy generation.

In conclusion, while the research provides a solid foundation for understanding S-driven N removal processes, many areas remain for future exploration. By focusing on the optimization of operational conditions, microbial dynamics, scalability, environmental impacts, and integration with emerging technologies, future research can help in unlocking the potential of these processes, leading to more efficient, sustainable, and economically viable wastewater treatment systems.

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Paper I

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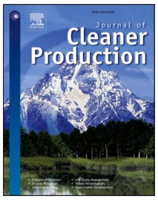

Review

Incorporation of the sulfur cycle in sustainable nitrogen removal systems - A review

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ABSTRACT

In wastewater treatment systems, sulfur (S) removal processes are generally based on heterotrophic sulfate (SO_4^{2-}) reduction by sulfate reducing bacteria and S-dependent autotrophic denitrification by sulfur oxidizing bacteria. A combination of either two cycles (N and S) or three cycles (N, S and C) appears to be a viable approach to sustainable wastewater treatment, resulting in energy savings and reduction of sludge production. This review shows how the S cycle can be coupled with the other cycles in single systems for efficient N and S removal. Operating conditions, advantages, limitations and challenges of such systems are described. S removal processes are generally based on heterotrophic sulfate (SO_4^{2-}) reduction by sulfate reducing bacteria and S-dependent autotrophic denitrification by sulfur oxidizing bacteria. In terms of pH and temperature, the optimum conditions are determined by the narrowest ranges for heterotrophic SO_4^{2-} reduction (pH of 7–7.6, T = 28–30 °C). The combined processes allow for almost complete N removal, while the efficiency of SO_4^{2-} removal can reach up to 75%. Among all the processes linking the N, S and C cycles, SANI (sulfate reduction, autotrophic denitrification and nitrification integrated) has been best recognized. Recently, the growing attention has been paid to the novel sulfammox process, which involves SO_4^{2-} dependent, anaerobic ammonia oxidizing bacteria. Numerous systems have been developed to combine SO_4^{2-} reduction, S-dependent autotrophic denitrification and partial nitritation/anammox processes. The coexistence of several bacterial groups and their competition for the substrates is thus a key issue to be considered. Specific inhibitors for each bacterial group also need to be recognized before full-scale implementations. Moreover, modeling the transformations of S compounds has been incorporated with respect to all the processes responsible for those transformations.

1. Introduction

High concentrations of ammonium (NH_4–N) lead to eutrophication of surface waters and pose a threat to the aquatic life and human health (Qin et al., 2021a,b). NH_4–N can effectively be converted to nitrogen gas by combined nitrification-denitrification, but this method has a few important disadvantages, including a high demand of energy and carbon, and high sludge production. On the other hand, sulfate (SO_4^{2-}) is a type of the secondary pollutant because reduction of sulfide (S^{2-}) under anaerobic conditions is harmful for the aquatic environment (Hao et al., 2014). S compounds have not been widely used as substrates in wastewater treatment processes. Simultaneous removal of these two compounds (N and S) from wastewater, with or without involving the carbon (C) cycle, can be a viable approach to the sustainable wastewater management. In particular, this approach may be an effective alternative in the case of many types of industrial wastewater, which are characterized by high concentrations of pollutants, such as NH_4–N, SO_4^{2-} (>1000 mg/L of both N and S) and chemical oxygen demand (COD) (>60,000 mg COD/L) (Rikmann et al., 2016; Jarvis and Younger, 2000; Chapman, 1992).

A viable sustainable approach to biological wastewater treatment comprises a combination of nitrogen (N), sulfur (S) and carbon (C) removal. Lower operating costs result from the use of some products in one process as the substrates in other processes and the use of shared reactors. Moreover, no carbon is needed for S-dependent autotrophic denitrification, less sludge is generated, and the environmentally neutral

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compounds, such as nitrogen gas (N_2) and elementar sulfur (S^0), are the final products of biochemical reactions (Lin et al., 2018).

Conventional nitrification/denitrification for N removal is now being replaced by more sustainable N-shortcut processes, such as "nitrite shunt" or deammonification. In the case of S compounds, biological removal is based on heterotrophic SO_4^{2-} reduction by sulfate reducing bacteria (SRB) and S-dependent autotrophic denitrification by sulfur oxidizing bacteria (SOB). Recently, the growing attention has been paid to the novel sulfate reducing ammonia oxidizing (sulfammox) process, which involves anaerobic ammonium oxidizing bacteria (AAOB). These bacteria use SO_4^{2-}, instead of nitrite nitrogen (NO_2^--N), as an electron acceptor to oxidize NH_4^+-N under anaerobic conditions.

The growing importance of using the combined N, S and C cycles in biological wastewater treatment processes has been confirmed by the increasing number of review papers on various aspects of S transformations. According to Web of Science database, 15, 12 and 3 review papers have been published specifically on S-dependent autotrophic denitrification, heterotrophic reduction of SO_4^{2-} and sulfammox (–see Fig. S1 in the Supporting Information (SI)). Several papers focused on particular issues, including a detailed description of mechanisms of the individual processes, responsible microorganisms, reactors used, optimal operational conditions or inhibiting factors in S-dependent autotrophic denitrification (Wu et al., 2021; Cui et al., 2019; Lin et al., 2018), heterotrophic sulfate reduction (Sinharoy et al., 2020b) and sulfammox (Liu et al., 2021; Grubba et al., 2021).

However, only a combination of either two cycles – (N and S) or three cycles (N, S and C) would be the rational approach to wastewater treatment in order to save energy and the amount of sludge generated, especially for NH_4^+-N and SO_4^{2-} rich industrial wastewater. Due to the variety of N, S and C removal processes, the research interests have been shifting to the use of single- and multi-stage systems based on the combination of several processes, such as heterotrophic sulfate reduction, S-dependent autotrophic denitrification, nitrification, denitrification, anaerobic ammonia oxidation (anammox) and sulfammox (Wu et al., 2020, Yuan et al., 2020, Sun et al., 2018, Liu et al., 2017, Qian et al., 2015a, b, c, Jiang et al., 2013, Wang et al., 2009b).

Only two review papers (Hao et al., 2014; Show et al., 2013) described simultaneously S-dependent autotrophic denitrification and heterotrophic sulfate reduction. Hao et al. (2014) described a relationship between the N, S, C and P cycles in biological wastewater treatment systems. These authors focused on the acceptors and electrons used in the transformations of S compounds, key microorganisms, developed technologies, factors influencing the process performance, and achieved SO_4^{2-} reduction efficiencies. In the review of Show et al. (2013), existing models of the transformations of S compounds were additionally described (see – Table S1 in SI).

The present review provides updated results of research on S transformations, which have been revised and extended with new understanding and discoveries. A novel aspect is the inclusion of sulfammox in these transformations as no paper has synthesized autotrophic S-dependent denitrification, heterotrophic sulfate reduction and the sulfammox process in one review. In addition, the present study describes how sulfammox can increase the efficiency of N and S removal. Various process configurations and technologies, which are based on the three (N–S–C) cycles, are described and compared in terms of their efficiency. Moreover, modeling the transformations of N, S and C compounds has been incorporated with respect to all processes responsible for those transformations. Such a review provides a deeper insight into the conversions of S in biochemical processes, including sulfammox.

2. Single S-dependent biochemical processes integrating N, S and C conversions

There are three known processes combining sulfur and nitrogen conversions: S-dependent autotrophic denitrification, heterotrophic sulfate reduction and autotrophic sulfammox. The detailed description of those processes, including the metabolic mechanisms, biochemical reactions, influencing environmental factors can be found in the SI (S1–S3).

S-dependent autotrophic denitrification consists of oxidation of S compounds, including S^{2-}, S^0, thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}), coupled with reduction of NO_3^--N and/or NO_2^--N. *T. denitrificans*, *Thiomicrospira denitrificans*, *Thiobacillus versutus*, *Thiosphaera pantotropha* and *P. denitrificans* are the known microorganisms responsible for that process. *P. denitrificans* is the chemotrophic α-proteobacteria which can grow on organic monocarbon compounds (methanol, methylamine) while using reduced forms of S and hydrogen as electron donors in denitrification (Baker et al., 1998). *T. denitrificans* belongs to β-proteobacteria that can use $S_2O_3^{2-}$ and thiocyanates under aerobic conditions, and additionally S^{2-} and S^0 under anaerobic conditions. *Sulfurimonas denitrificans* belongs to the ε-proteobacteria and is capable of oxidizing SO_3^{2-}, $S_2O_3^{2-}$ and S^0, while both NO_3^--N and oxygen are used as electron acceptors. *T. thioparus* is one of the representatives of autotrophic denitrifiers that reduce NO_3^--N to NO_2^--N by oxidation of S^{2-} (Tang et al., 2009). Although autotrophic denitrifying bacteria are chemolithotrophic, there are many denitrifying bacteria capable of adapting to autotrophic, heterotrophic and even mixotrophic growth (*P. versatus*, *P. denitrificans*, *Beggiatoa* sp.) (Pokorna and Zabranska, 2015).

Heterotrophic sulfate reduction is SO_4^{2-} reduction which takes place in two independent different paths. The first is the use of organic electron donors, which are also the carbon source for the SRB. The second is the use of inorganic electron donors, which must be supplemented with a carbon source, such as CO_2 (Sinharoy et al., 2020a). The SRB can be divided into 7 phylogenetic lines, including five for bacteria and two for archaea. Most of the SRB found in sulfate reduction reactors belong to 23 genera within *Deltaproteobacteria* (*Desulfovibrio, Desulfobacteraceae, Desulfobulbaceae, Syntrophobacteraceae, Desulfomicrobium, Desulfohalobium*). Another SRB belong to the gram-positive genera *Clostridia* (*Desulfotomaculum, Desulfosporosinus and Desulfosporomusa*). Three lineages, *Nitrospirae* (*Thermodesulfovibrio*), *Thermodesulfobacteria* (*Thermodesulfobacterium*) and *Thermodesulfobiaceae* (*Thermodesulfobium*), contain only thermophilic SO_4^{2-} reducing agents. Archaeal SRB are *Euryarchaeota* and *Crenarchaeota* (Muyzer and Stams, 2008).

In a novel sulfammox process, NH_4^+-N is oxidized to N_2, whereas SO_4^{2-} plays the role of an electron acceptor which is reduced to S^0 under anaerobic conditions. *Brocadia Anammoxoglobus Sulfate* (Liu et al., 2008) is a functional microorganism responsible for simultaneous removal of NH_4^+-N and SO_4^{2-} and ended the conversion of NH_4^+-N and SO_4^{2-} by producing NO_2^--N as an intermediate. The second isolated species, *Bacillus Benzoevorans*, is responsible for carrying out the entire sulfammox reaction (Cai et al., 2010). *Verrucomicrobia* has also been reported to be involved in the sulfammox process (Rikmann et al., 2016). Some *Proteobacteria*, which may potentially perform sulfammox, include the following species: *Sulfurimonas, Desulfuromonadales, Desulfovibrio, Desulfuromonas, Desulfobulbus, norank Rhodobacteraceae* and *Thiobacillus* (Rios-Del Toro et al., 2018; Wang et al., 2017).

The key issues and challenges of S-dependent autotrophic denitrification, heterotrophic sulfate reduction and sulfammox are presented in Table 1. Fig. 1 below shows the interactions between S-dependent autotrophic denitrification, heterotrophic sulfate reduction and sulfammox process.

3. Operational conditions and performances of single S-dependent processes

Each of the discussed processes (S-dependent autotrophic denitrification, heterotrophic sulfate reduction and sulfammox) can be carried out independently, as evidenced by numerous studies (Tables 2 and 3). However, the challenge is to combine these processes, in either single- or multi-stage systems, in order to make biological wastewater treatment systems more efficient.

Table 1
Key issues and challenges of S-dependent autotrophic denitrification, heterotrophic sulfate reduction and sulfammox.

Topic	Process		
	S-dependent autotrophic denitrification	Heterotrophic sulfate reduction	Sulfammox
Key issues	consists of oxidation of S compounds, including S^{2-}, S^0, thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}), coupled with reduction of NO_3^--N and/or NO_2^--N	SO_4^{2-} reduction, which involves the use of organic electron donors or inorganic electron donors, which must be supplemented with a carbon source	NH_4^+-N is oxidized to N_2, whereas SO_4^{2-} plays the role of an electron acceptor and is reduced to S^0 under anaerobic conditions
Challenges and opportunities	a) a good alternative to heterotrophic denitrification due to the lack of carbon dosing; b) reduction of toxic S2-; c) the possibility of treating wastewater poor in organic content; d) residual SO42- in wastewater; e) a long incubation time is needed before a fully adapted culture is obtained; f) precise control strategy (from S2- to S0) and novel S0 recovery technology at the source; g) acclimation and adjustment of microorganisms: the concentration of S2- should be controlled; maintaining the denitrification efficiency of autotrophic denitrification systems at low temperatures; alkalinity and pH control is necessary to prevent the formation of NO2–N; influence of the N/S ratio on the reactions and bioproducts, the optimal N/S ratio = 0.5–0.9 for S oxidation and NO3–N reduction (see Eqs. 1-8 in the SI); h) when the dissolved oxygen (DO) concentration is > 1.6 mg O2/L, denitrification is completely inhibited	a) SO_4^{2-} reduction, especially in SO_4^{2-} rich industrial wastewater; b) use of wastewater rich in organic compounds; c) high concentrations of SO_4^{2-} inhibit SRB activity; d) elevated levels of heavy metals may reduce or terminate SRB activity	a) anaerobic oxidation of NH_4–N without carbon addition; b) SO_4^{2-} reduction in wastewater; c) knowledge of microorganisms, mechanisms and their metabolic pathway is still limited; d) temperature, DO and pH would influence its practical applications; e) inhibition of sulfammox activity due to S^{2-} accumulation; f) with a high concentration of NO_3–N, SO_4^{2-} concentration may increase due to autotrophic denitrification

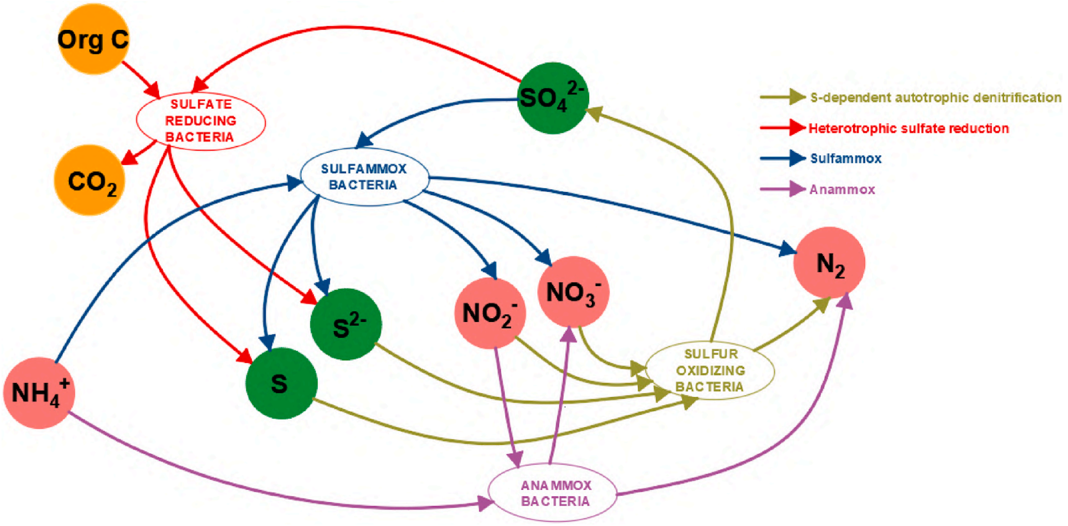

Fig. 1. Interactions between S-dependent autotrophic denitrification, heterotrophic sulfate reduction, anammox and sulfammox process.

3.1. S-dependent autotrophic denitrification

In S-dependent autotrophic denitrification, the most frequently used electron donors are S^0 and S^{2-} (Table 2). The experiments were mainly carried out in packed bed reactors, but several other types of reactors were also used. The reported rates of denitrification varied in a wide range - from 0.03 to 8.13 kg $N/m^3/d$, depending mainly on the temperature and influent NO_3^--N concentrations. The effects of pH in the investigated range (6.0–9.0) and S concentrations were less significant. For a detailed description of previous research related to S-dependent autotrophic denitrification, see the SI (S1). This process allowed for the efficient (>90%) removal of N and S^{2-} (Yang et al., 2016; Jing et al., 2010) with the NO_3^--N concentration in the range of 20–1230 mg N/L (Zhu et al., 2019; Zou et al., 2016; Kim et al., 2004).

During S-dependent autotrophic denitrification, SO_4^{2-} can be produced from different electron donors. Frequently, the S balance in the process is not 1/1 for the removed electron donor to SO_4^{2-} produced (Zou et al., 2016). In Table 2, the initial donor concentrations and the amount of SO_4^{2-} produced are similar. The observed imbalances result from the production of other S intermediates. The most common electron acceptor is NO_3^--N, but several studies comparing NO_3^--N and NO_2^--N have been reported (Sun and Nemati, 2012; Moraes et al., 2012; Jing et al., 2010).

Different aspects of S-dependent autotrophic denitrification have been addressed in several reviews (Wu et al., 2021; Cui et al., 2019; Lin et al., 2018; Sabba et al., 2016). Wu et al. (2021) summarized all types of biofilm denitrification in terms of the reactor configuration, microbial transformations, factors influencing the process, and especially focused on N_2O emissions. The coexistence of S-dependent denitrification with anammox was also reported and S-driven denitrifiers were identified,

Table 2
Process conditions and observed NO_3^- utilization rates during S-dependent autotrophic denitrification in different types of reactors.

Reactor type	Electron donor	Temperature	pH	S-compound	Initial NO_3^-N concentration	SO_4^{2-} production	Denitrification rate	References
		(°C)	(–)	(mg S/L)	(mg N/L)	(mg S/L)	(kg N/m3/d)	
Fluidized-bed reactor	$S_2O_3^{2-}$	20–30	7	184–2260	100–1230	150–320	1.24–3.25	Zou et al.. (2016)
Fluidized-bed reactor	S^0	28–30	7.2–9	na	25–75	100–600	0.07–0.2	Sahinkaya and Dursun (2015)
Fluidized-bed reactor	$S^0/S_2O_3^{2-}$	20	6.8–8.2	na	20–700	na	2.53–3.37	Kim et al.. (2004)
Packed-bed reactor	S^0	28–30	6–8	na	50–75	200–600	0.07–0.1	Sahinkaya and Kilic (2014a)
Packed-bed reactor	S^0	10–26	6–8	na	30–60	191–483	0.03–0.24	Sahinkaya et al.. (2014b)
Packed-bed reactor	S^0	15.2–29	6.7–8.4	592.42–5924.17	20–25	640	0.2	Kimura et al., 2002
Packed-bed reactor	S^0	20–25	8.3–8.7	na	60–251	na	0.27–0.87	Koenig and Liu (2002)
Packed-bed reactor	S^0	20–25	na	na	60–400	na	0.48–0.77	Koenig and Liu (2001)
Up-flow continuous reactor	S^{2-}	29–31	7	160–1000	30.4–169.6	na	0.15–0.61	Jing et al. (2010)
Up-flow anaerobic sludge blanket	S^{2-}	30	7.5	0.62[a]	0.33[a]	na	0.09–0.31	Yang et al. (2016)
Up-flow column reactor	S^0	30	7.3	na	20	6.15–7.92[b]	0.22	Zhu et al.. (2019)
Vertical fixed-bed reactors	S^{2-}	30	7–7.5	49.3	20	20	na	Moraes et al. (2012)

[a] $kg/m^3/d$.
[b] g/g $N_{removal}$.

including *Thiobacillus denitrificans* and *Thiobacillus thioparus*.

Cui et al. (2019) described S-dependent autotrophic denitrification in terms of the functional enzymes, electron donors, types of reactors, and operational factors. They also emphasized a significant advantage regarding S-dependent autotrophic denitrification compared to heterotrophic denitrification with respect to N_2O emissions. It was shown that autotrophic denitrification mediated by S compounds (S^0, S^{2-}) emitted significantly less N_2O than heterotrophic denitrification with methanol, ethanol or acetate.

Sabba et al. (2016) focused mainly on SO_3^{2-} and its occurrence in the environment, chemistry, microbiology, and the role in denitrification. It was emphasized that SO_3^{2-} is an intermediate in the S oxidation pathway and should be chosen as the most economical electron donor. Lin et al. (2018) focused primarily on S oxidation, including biological gas desulphurization, phototrophic S^{2-} oxidation, S-dependent autotrophic denitrification, biological sulfur oxidation associated phosphorous removal, dye treatment. They also indicated viable applications of the products, such as Li batteries, production of S concrete by mixing S^0 with aggregates, biologically produced S fertilizer, oxidation of S^{2-} in microbiological fuel cells, and reclamation of metals from sewage sludge.

3.2. Heterotrophic sulfate reduction

Table 3 presents the diversity of research carried out so far on heterotrophic SO_4^{2-} reduction in terms of the electron donor, type of reactor and operating conditions. Most studies have been carried out in the gas lift reactor and fluidized-bed reactor. Both organic and inorganic donors were used, including carbon monoxide, methane, methanol, ethanol, hydrogen, crab shell, compost and many others. The use of different donors resulted in a different SO_4^{2-} reduction efficiency. A detailed description of the research can be found in SI (S2). The use of different electron donors and SO_4^{2-} content resulted in a wide range of SO_4^{2-} removal efficiencies (51–98%) and rates (0–3400 mg SO_4^{2-}/L/d). Nielsen et al. (2019) used methanol and ethylene glycol which resulted in reduction of SO_4^{2-} by 71.2% and 36.9%, respectively. The decrease of SO_4^{2-} concentration was limited to 13.8 and 5.3%, respectively, with the use of peat and straw. Low temperatures (below 10 °C) significantly affected the SO_4^{2-} removal rates. For example, Virpiranta et al. (2019) carried out studies at various temperatures (22 °C, 16 °C, 6 °C) and found gradually decreasing SO_4^{2-} removal rates, i.e. 169, 98 and 13–42 mg SO_4^{2-}/L/d, respectively.

Sulfate reduction is less popular compared to S-dependent autotrophic denitrification, but that process has also been addressed in several reviews (Kumar et al., 2021; Costa et al., 2020; Sinharoy et al., 2020b; Serrano et al., 2019; Van den Brand et al., 2015). Kumar et al. (2021) and Costa et al. (2020) focused on the use of SO_4^{2-} reduction for treatment of metal-rich wastewater and recovery of these metals, showing a high degree of SO_4^{2-} reduction (>90%) along with the efficient (>99%) recovery of metals (Fe, Zn, Cd, Cu).

Similarly, Sinharoy et al. (2020b) described treatment of acid mine drainage (AMD) with biological reduction of SO_4^{2-}. Heavy metals present in AMD can be removed by S^{2-} precipitation. The review discussed various gaseous substrates, such as H_2, CO, CH_4, as electron donors that could be used in this process. It was emphasized that only the microorganisms capable of using gaseous substrates are appropriate for the AMD treatment systems.

Serrano et al. (2019) focused on the optimum conditions for SRB. They presented the recommended conditions for biomass, electron donor and acceptor and an experimental setup of three SRB tests: (1) to assess the activity of SRB culture, (2) to determine the reduction potential of an electron donor, and (3) to determine the possibility of using various sources of SO_4^{2-} as an electron acceptor. They collected methodologies and results from many publications and recommended setup and monitoring conditions to increase the comparability and reproducibility of the SRB tests. Sodium sulfate and lactate were used as an electron acceptor and electron donor, respectively.

Van den Brand et al. (2015) analyzed important parameters, such as pH, organic substrates, COD/SO_4^{2-} ratio, substrate composition, SO_4^{2-}, salt, temperature and DO. They found that the presence of SRB reduced pathogens, heavy metals and sludge produced. Sulfate reduction, autotrophic denitrification and nitrification integrated (SANI) was identified as a process combining the advantages of SRB and S-dependent autotrophic denitrification. However, they indicated that in order to ensure the benefits of using SRB, a sufficient SO_4^{2-} concentration in the influent wastewater would be required to maintain the COD/SO_4^{2-} ratio below 0.67.

Table 3
Reactor types, operational (environmental) conditions, influent S concentrations and efficiency of heterotrophic SO_4^{2-} reduction and sulfammox.

Reactor type	Electron donor	Temperature	pH	SO_4^{2-} concentration	SO_4^{2-}removal efficiency or rate	References
		(°C)		(mg/L)		
HETEROTROPHIC SULFATE REDUCTION						
Gas lift reactor	Carbon monoxide	30	7	250–1000	62.5–97.5%	Sinharoy et al.. (2020a)
Moving bed biofilm reactor	Carbon monoxide	30	7	250–1000	67.1–95.2%	Sinharoy et al.. (2019)
Batch	Succinic acid, yeast extract	22 16 6	–	1700	169 mg SO_4^{2-}/L/d 98 mg SO_4^{2-}/L/d 13–42 mg SO_4^{2-}/L/d	Virpiranta et al.. (2019)
Batch	Methanol	5	7	–	26.7 mg SO_4^{2-}/L/d	Nielsen et al. (2019)
Packed bed reactor	Ethylene glycol	30	7	250–1000	4.1 mg SO_4^{2-}/L/d	Kumar et al.. (2018)
Inverse fluidized bed reactor	Scourer Cork	30	7	700	34 mg SO_4^{2-}/gVSS/d 6.1 mg SO_4^{2-}/gVSS/d	Reyes-Alvarado et al.. (2018)
Packed bed reactor	Molasses	4–8	6.5–7.1	287–548.2	0–22 mg SO_4^{2-}/L/d	Nielsen et al. (2018)
Batch	Crab shell Potato Filter paper	30	7	721–738 764–766 752–823	6–9 mg SO_4^{2-}/gVSS/d 22–34 mg SO_4^{2-}/gVSS/d 50–65 mg SO_4^{2-}/gVSS/d	Reyes-Alvarado et al.. (2017)
Fluidized-bed reactor	Glycerol	23	5.5–8.5	2000–3000	167 mg SO_4^{2-}/gVSS/d	Bertolino et al.. (2014)
Stirred tank reactor	Hydrogen + carbon dioxide	30	6.95–7.05	–	3400 mg SO_4^{2-}/L/d	Sáez-Navarrete et al., 2012
Fluidized-bed reactor	Ethanol Ethanol + lactate	35	7.5	–	211 mg SO_4^{2-}/gVSS/d 2016 mg SO_4^{2-}/gVSS/d	Nevatalo et al.. (2010)
Gas lift reactor	Hydrogen	30–35	7–7.5	5000–30000	7080 kg SO_4^{2-}/d	Van Houten et al.. (2009)
Anaerobic filter	Ethanol, spent manure Methanol, spent manure	6	2.5–4.3	900	961–1345 mg SO_4^{2-}/L/d 1057–1441 mg SO_4^{2-}/L/d	Tsukamoto et al. (2004)
SULFAMMOX						
Upflow anaerobic sludge bed reactor	Ammonium nitrogen	35	7.9–8.3	80	8.18 mg S/L/d	Qin et al. (2021a)
Circulating flow completely anaerobic reactor	Ammonium nitrogen	30	8.1–8.6	88 223 154	2–27% 2–27% 18–64%	Zhang et al. (2020)
Self-designed circulating flow reactor	Ammonium nitrogen	35	8.1–8.3	183 216 116 100	approx. 40% approx. 0% approx. 30% approx. 45%	Zhang et al. (2019a)
Self-designed circulating flow reactor	Ammonium nitrogen	30	8.1–8.6	90 170 360	approx. 30% approx. 30% approx. 5%	Zhang et al. (2019b)
Sequencing batch reactor	Ammonium nitrogen	–	–	261	19%	Prachakittikul et al. (2016)
Batch	Ammonium nitrogen	30	8.5	163	40%	Cai et al. (2010)
Upflow anaerobic sludge blanket reactor	Ammonium nitrogen	35	7.5–8.5	240	30%	Yang et al. (2009)
Non-woven rotating biological contactor	Ammonium nitrogen	35	8–8.2	–	–	Liu et al. (2008)

3.3. Sulfammox

Sulfammox is a new process that has been addressed in the literature, especially review papers, only very recently. Sulfammox has mainly been carried out in an upflow anaerobic sludge bed reactor and circulating flow reactor (Table 3). The obtained SO_4^{2-} removal efficiencies are normally much lower compared to heterotrophic sulfate reduction. However, sulfammox is an important process linking the N and S cycles, therefore the effect of sulfammox on the overall reduction of SO_4^{2-} and NH_4^+-N should not be neglected. In the studied systems, the typical influent concentrations of SO_4^{2-} ranged from 80 to 360 mg/L (Qin et al., 2021a,b; Zhang et al., 2019b) and the highest obtained SO_4^{2-} removal efficiency was 45% (Zhang et al., 2019a). A detailed description of the research can be found in the SI (S3).

Liu et al. (2021) summarized the current understanding of sulfammox, including the mechanisms, responsible microorganisms and factors influencing the process. It was emphasized that the understanding of sulfammox has improved significantly in recent years, but more attention should be paid to recognizing the microbial community and its metabolic pathways. In addition, a variety of sulfammox end products were described that could be substrates for various N and S (anammox, S-dependent autotrophic denitrification) processes and coexist together in wastewater treatment systems. However, a challenge for the process is to ensure optimal environmental factors, such as temperature, pH, DO, for its practical applications. It was also emphasized tha t residual organic carbon could have a significant positive effect on sulfammox, but this requires further research. A significant limitation of sulfammox is that the process was mostly investigated under laboratory scale. Practical applications should focus on implementations at low temperatures in full-size reactors.

In order to increase the efficiency of S removal in the sulfammox process, it is important to maintain the optimal pH of 8.5 and temperature of 30 °C (Cai et al., 2010). The N/S ratio is also an important factor affecting that efficiency. When increasing the influent NH_4^+-N concentration from 166 to 666 mg N/L to 1000–2000 mg N/L, then the SO_4^{2-} removal efficiency increased from 64% to 71%. However, after increasing the influent NH_4^+-N concentration further to >3000 mg/L, the SO_4^{2-} reduction efficiency decreased to 28% (Wang et al., 2017). Also, reducing the concentration of SO_4^{2-} from 223 to 154 mg/L had a positive effect on the removal of SO_4^{2-} in the sulfammox process (Zhang et al., 2020). The N/S ratio also influenced the SO_4^{2-} removal efficiency, as the SO_4^{2-} removal efficiency at N/S = 2:1 and 4:1 was 38.8% and 30.5%, respectively (Zhang et al., 2019a).

3.4. Optimal conditions for S-dependent autotrophic denitrification, heterotrophic sulfate reduction and the sulfammox process

Fig. 2 shows a summary of the reported pH and temperature ranges

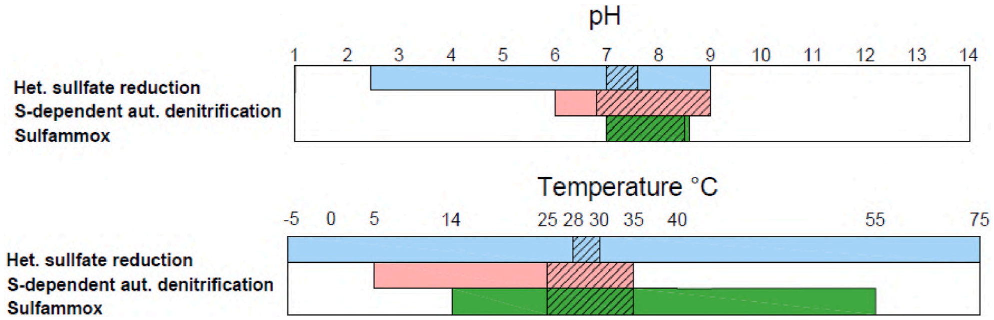

Fig. 2. Ranges of pH and temperatures and their optimal values ("[▨]" – optimum conditions) reported in literature for the S-dependent processes.

and their optimal values for the three S-dependent processes. The overall optimum conditions are explicitly determined by the narrowest ranges for heterotrophic sulfate reduction, which are 7–7.6 and 28–30 °C for pH and temperature, respectively. The processes of S-dependent autotrophic denitrification, heterotrophic sulfate reduction and sulfammox can occur simultaneously with deammonification or its component processes, i.e. partial nitritation and anammox.

For comparison, for partial nitritation, the optimal ranges were 25–35 °C for temperature (Zhu et al., 2008; Kanders et al., 2014) and 7–8.6 for pH, with the optimal value of 8 (Jaroszynski et al., 2011). On the contrary, too low temperatures (10–15 °C) cause the excessive activity of NOB (Kouba et al., 2017), which can grow faster than AOB under such conditions (Hellinga et al., 1998). The optimal pH range for NOB is 6–7.5, with the maximum at 7 (Yin et al., 2016). For the anammox process, the optimal temperature and pH is respectively 35–40 °C (Dosta et al., 2008) and 6.7–8.3 (Jetten et al., 2001). The recommended ranges for efficient deammonification are as follows: T = 20–35 °C (Kanders et al., 2014) and pH of 7.5–8 (Oshiki et al., 2011).

When coupling sulfammox with S-dependent autotrophic denitrification and heterotrophic SO_4^{2-} reduction to increase the efficiency of S removal, it is important to keep the optimal temperature of 28–30 °C and pH of 7–7.6. The N/S ratio should be adjusted based on the stoichiometry of all the processes involved, so that products of one process can be the substrates for another process. Deviations from the optimal ratio can cause either production of unwanted residues or bacterial competition for the substrates. SRB can compete with sulfammox bacteria for SO_4^{2-}. Moreover, heterotrophic SO_4^{2-} reduction and sulfammox contribute to formation of S^{2-} and/or S^0, which is the substrate for S-dependent autotrophic denitrification. Too intensive production of S^{2-} may lead to the persistence of this toxic compound in the effluent. The presence of carbon in heterotrophic SO_4^{2-} reduction may also contribute to the development of heterotrophic bacteria responsible for heterotrophic denitrification. Then NO_3^--N and/or NO_2^--N may become limited due to their use in both autotrophic and heterotrophic denitrification. In such a case, it is recommended to use full or partial nitrification to produce NO_3^--N and/or NO_2^--N. The competition and interactions of microorganisms participating in the aforementioned processes are shown in Fig. 1.

4. Wastewater treatment systems integrating the N–S–C cycles

4.1. Systems integrating the sulfur cycle with nitrification-denitrification - sulfate reduction, autotrophic denitrification and nitrification integrated (SANI) and its modifications

Biological SO_4^{2-} reduction along with biological oxidation of S in the form of SO_3^{2-}, S^0 or $S_2O_3^{2-}$ are two main pathways responsible for S conversions in wastewater treatment systems (Cardoso et al., 2006). An integrated process for SO_4^{2-} reduction, autotrophic denitrification and nitrification (SANI) was aimed to primarily remove organic compounds and N (Wang et al., 2009b). This process was originally developed for saline wastewater in Hong Kong and demonstrated there in full-scale (Wu et al., 2016; Wang et al., 2009b).

With that innovative approach, the conventional wastewater treatment, incorporating C and N cycles, can be extended with the S cycle, as shown in Fig. 3. In the first anaerobic zone, COD is removed by SRB, which results in SO_4^{2-} reduction to S^{2-}. In the second anoxic zone, autotrophic reduction of NO_3^--N occurs with dissolved S^{2-} formed in the first zone. In the third aerobic zone, NH_4^+-N is oxidized to NO_3^--N, which is then recirculated to the second anoxic zone (Wang et al., 2009b). The SANI process and its modifications combine the advantages of energy saving, reduced sludge production and smaller footprint. Wang et al. (2009b) noted that the total cost reduction for SANI would be >50% for a WWTP with an influent flow rate of 10,000 m^3/d.

The SANI process can be used for treatment of SO_4^{2-}-poor wastewater provided that low-cost and S-rich sources are available. For example, wet flue gas desulfurisation (FGD) systems used in boilers, coal-fired furnaces and power plants, can be reduced to alkaline flue gas sorption for production of liquid waste containing SO_4^{2-} and SO_3^{2-} (Srivastava and Jozewicz, 2001). Such a waste stream can be co-treated in the main wastewater stream in wet FGD-SANI after removing suspended solids and heavy metals (Qian et al., 2013).

The Mixed Denitrification (MD) - SANI process has also been proposed (Qian et al., 2015a,b,c). MD-SANI generates $S_2O_3^{2-}$, S^{2-}, and some volatile fatty acids (VFA), which are subsequently converted in both heterotrophic denitrification (VFA) and autotrophic denitrification (S^{2-} and $S_2O_3^{2-}$) (Qian et al., 2015a). It should be noted that the latter process is induced faster by $S_2O_3^{2-}$ than S^{2-} (Cardoso et al., 2006). Fig. 3b–d shows the SANI, FGD-SANI and MD-SANI processes depending on the available substrates.

4.2. Systems integrating the S cycle with anammox-based nitrogen removal processes

In recent years, the growing attention has been paid to N removal using the anammox process. The anammox process completely eliminates the need for organic C source, reduces the amount of sludge produced by 80% and related energy costs for aeration by 60% compared to conventional nitrification/denitrification. The anammox process also has economic advantages in the context of co-treatment of wastewater containing S compounds, especially S^{2-} (Kosugi et al., 2019).

The anammox-based systems for combined N and S removal comprise (1) Sulfate Reduction, Denitrification/Anammox and Partial Nitrification (SRDAPN), (2) Partial Nitrification/Anammox and S-dependent autotrophic Denitrification (PNASD), (3) Anammox and S-dependent autotrophic Denitrification (ASD), and (4) S-dependent autotrophic Partial Denitrification/Anammox (SPDA).

The SRDAPN process is similar to the SANI process, but enhanced with anammox (Fig. 4a). As a consequence, instead of full nitrification, only PN is needed to produce NO_2^--N (Kosugi et al., 2019).

The PNASD process uses PN/A to remove NH_4^+-N under aerobic (PN) – anoxic (anammox) conditions. With S-dependent autotrophic

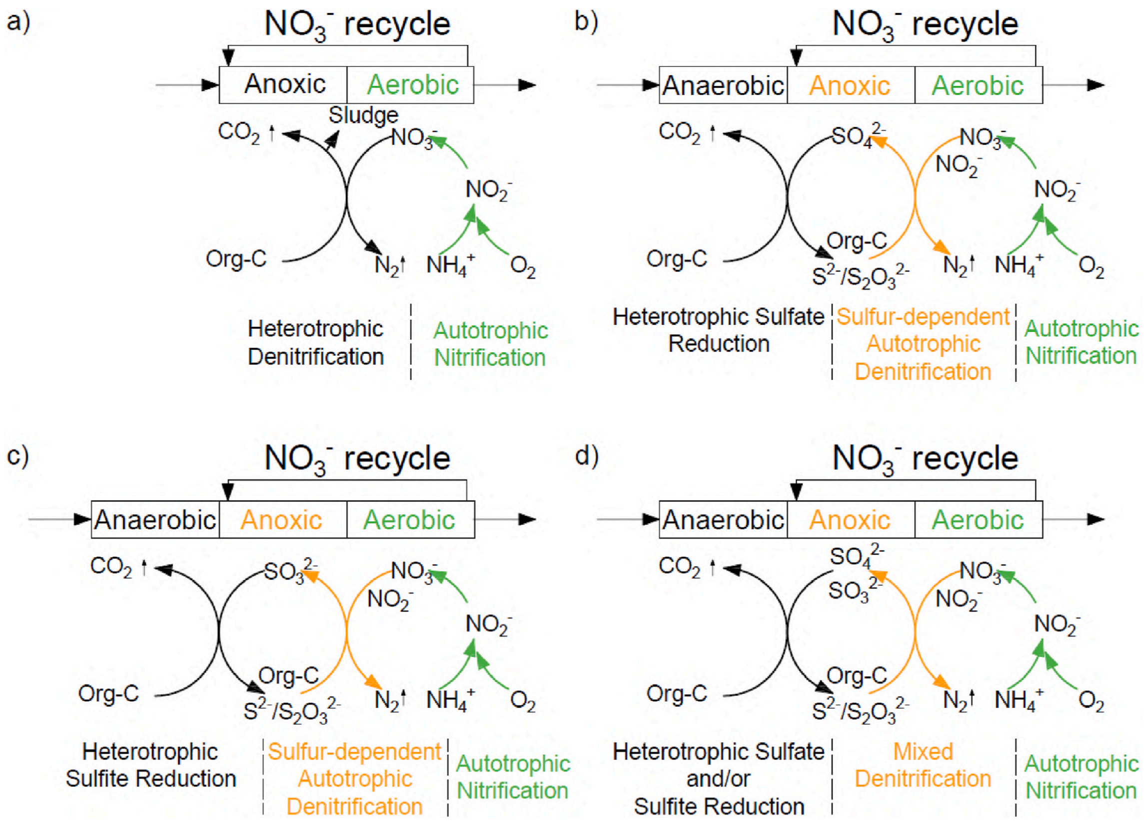

Fig. 3. Biological wastewater treatment systems using a) conventional heterotrophic denitrification with autotrophic nitrification b) SANI c) FGD-SANI d) MD-SANI.

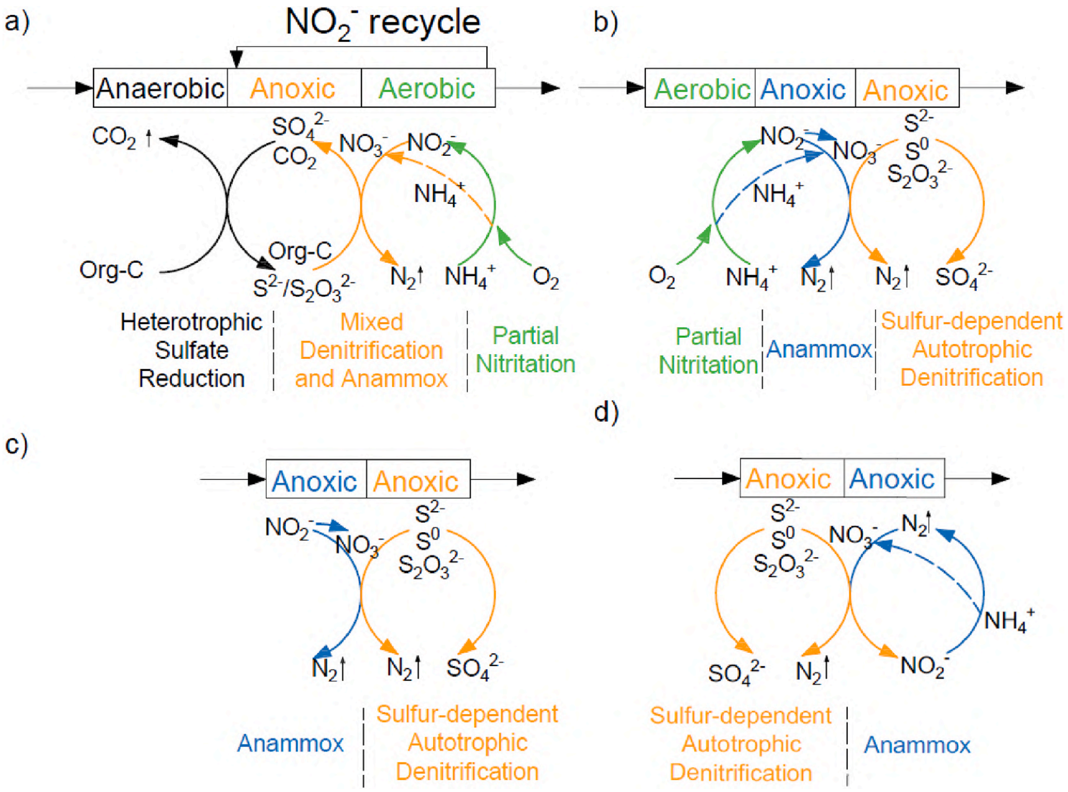

Fig. 4. Wastewater treatment systems using the anammox process a) SRDAPN b) PNASD c) ASD d) SPDA.

denitrification, the produced NO_3^--N can further be reduced to N_2, as shown in Fig. 4b. The PNASD process has been implemented as both two-stage (Dasgupta et al., 2017) and one-stage system (Yuan et al., 2020).

The PNASD system can also be limited to an ASD system that ignores the share of PN, as shown in Fig. 4c. Then the NO_2^--N acceptor for anammox is not obtained from the conversion of NH_4^+-N, but supplied from external sources. Accordingly, the costs of energy used to produce NO_2^--N by AOB in PN are neglected, but the costs of process substrates increase. The residual NO_3^--N from anammox can be removed along with S compounds (S^{2-}, S^0, $S_2O_3^{2-}$) by S-dependent autotrophic denitrification. The ASD process has been implemented in both one-stage (Guo et al., 2016) and two-stage (Sun et al., 2018) systems.

If NO_2^--N can be obtained by partial autotrophic denitrification of NO_3^--N with oxidation of S compounds (S^{2-}, S^0, $S_2O_3^{2-}$), then it can be used as a substrate in the anammox process. Liu et al. (2017) and Wu et al. (2019) used a UASB reactor to perform S-dependent denitrification with S^{2-} (Liu et al., 2017) and $S_2O_3^{2-}$ (Wu et al., 2019) for NH_4^+-N removal from wastewater, as shown in Fig. 4d.

4.3. Systems including the sulfammox process

Both sulfammox and anammox incorporate “anaerobic” oxidation of NH_4^+-N. The coexistence of both processes was found in marine sediments (Rios-Del Toro et al., 2018) and anaerobic sludge (Rikmann et al., 2016). In conventional sulfammox, SO_4^{2-} is an electron acceptor, which is reduced to S^0 or S^{2-}, while NH_4^+-N is oxidized to N_2, NO_2^--N and/or NO_3^--N. Sulfammox may occur on its own, as shown in Fig. 5a. Alternatively, the formed NO_2^--N may be used as an electron acceptor for anammox in the combined Sulfammox/Anammox (SA) system (Fig. 5b).

As NO_2^--N and NO_3^--N are generated in sulfammox, the process can be combined with autotrophic S-dependent denitrification in an Sulfammox - S-dependent autotrophic Denitrification (SSD) system, as shown in Fig. 5c (Liu et al., 2021; Grubba et al., 2021). The formed S^0 and S^{2-} in sulfammox can be oxidized again to SO_4^{2-}, while NO_X-N are reduced to N_2. The SSD system can be expanded with anammox in SASD (Sulfammox – Anammox - S-dependent autotrophic denitrification), as shown in Fig. 5d. In this case, NO_2^--N can be reduced by both AAOB and autotrophic denitrifiers (Liu et al., 2021; Grubba et al., 2021).

5. Operational conditions and performances of the systems integrating the N–S–C cycles

The biochemical processes associated with the C, N and S conversions and the microorganisms responsible for those conversions can be found in the SI (Fig. S2).

5.1. SANI, FGD-SANI, MD-SANI

The S cycle, which is part of the SANI process, ensures a more efficient use of electrons (Wu et al., 2020) and eliminates the production of toxic S^{2-} (Qian et al., 2015c). In addition, it reduces sludge production by 90% compared to the conventional biological N removal processes. This is possible due to very low yield coefficients of the microorganisms responsible for SO_4^{2-} reduction, autotrophic denitrification and nitrification, i.e., 0.02 kg VSS/kg COD, 0.01 kg VSS/kg NO_3^--N and 0.07 kg VSS/kg NH_4^+-N, respectively (Lu et al., 2011; Wang et al., 2009b). In addition, there are other significant reductions, including energy consumption by 35% (Lu et al., 2011), greenhouse gas emission (GHG) by 36% (Lu et al., 2011), and the space required for the process of wastewater treatment and sludge handling by 30%–40% (Liu et al., 2016).

As shown in Table 4, SANI shows a relatively high level of performance compared to the conventional systems. The efficiencies of SO_4^{2-}, total nitrogen (TN) and COD removal vary in the ranges of 72–98%, 55–74% and 82–97%, respectively (Hao et al., 2015; Lu et al., 2009). The SANI modifications (FGD-SANI and MD-SANI), which use

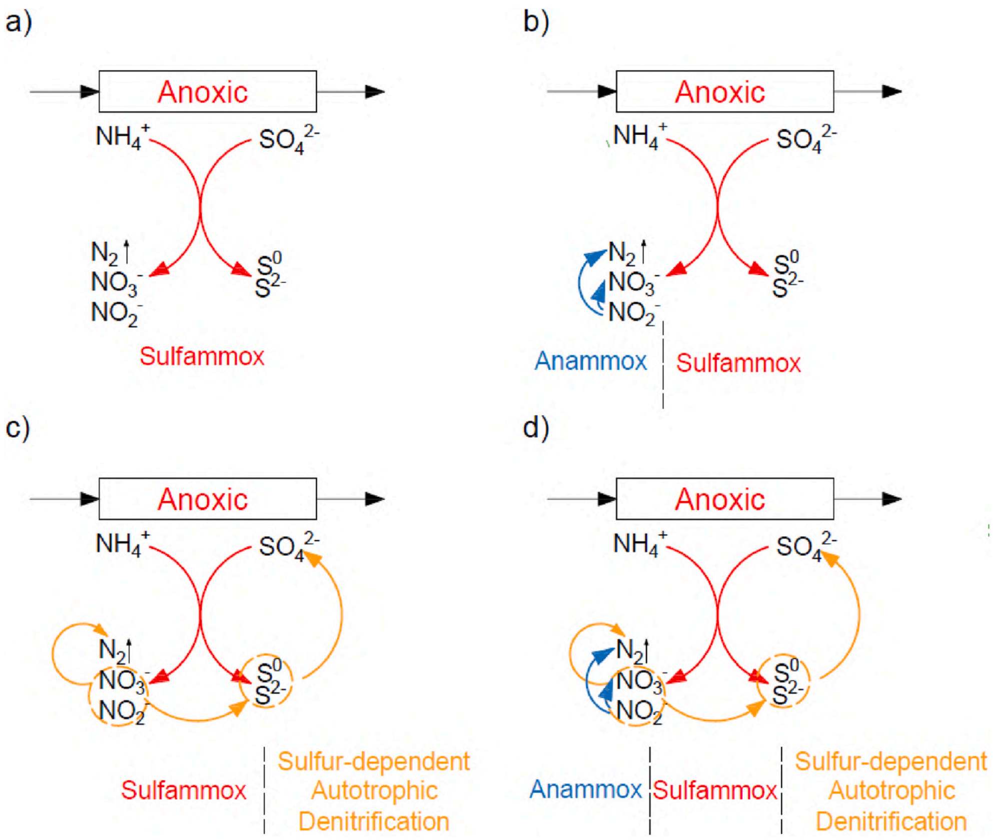

Fig. 5. Wastewater treatment systems incorporating the sulfammox process a) Sulfammox b) SA c) SSD d) SASD.

Table 4
Technologies for integrated S, N, COD removal and the observed removal efficiencies for S, N and COD.

Process	Reactor type	S removal efficiency	N removal efficiency (N form)	COD removal efficiency	References
Sulfate reduction, Autotrophic denitrification and Nitrification Integrated (SANI)	Up-flow anaerobic sludge bed, an anoxic filter, an aerobic filter	16–68 mg S^{2-}/L	74% (TN)	95%	Wang et al.. (2009b)
SANI	Up-flow sludge bed reactor, an anoxic reactor and an aerobic reactor	98% S^{2-}	55% (TN)	87%	Lu et al.. (2012)
SANI	Up-flow anaerobic sludge bed, an anoxic filterand an aerobic filter	97% S^{2-}	74% (TN)	97%	Lu et al. (2009)
SANI	Sulfate-reducing up-flow sludge bed	75% SO_4^{2-}	–	90%	Hao et al. (2013)
SANI	Sulfate-reducing up-flow sludge bed	72% SO_4^{2-}	–	82%	Hao et al. (2015)
Flue gas desulphurization - Sulfate reduction, Autotrophic denitrification and Nitrification Integrated (FGD-SANI)	Sulfite-reducing upflow anaerobic sludge bed	~54% S^{2-}	~98% (TN)	94%	Jiang et al.. (2013)
Mixed Denitrification - Sulfate reduction, Autotrophic denitrification and Nitrification Integrated (MD-SANI)	Sulfate/sulfite reducing upflow sludge bed and anoxic up-flow sludge bed	–	100% (NO_3^--N)	80%	Qian et al.. (2015a)
MD-SANI	Sulfur-reducing upflow sludge bed and the anoxic upflow sludge bed	~100% SO_3^{2-}	100% (TN)	81%	Qian et al.. (2015b)
Sulfate reduction, denitrification/anammox and partial nitrification (SRDAPN)	Laboratory scale up-flow anaerobic-anoxic biological filter reactor	400–500 mg S^{2-}/d	79% (TN)	500–2300 mg/d	Kosugi et al. (2019)
Partial Nitrification/Anammox and S-dependent autotrophic Denitrification (PNASD)	PN/A reactor and an elemental sulfur-supported packed bed autotrophic denitrification	–	97% (TN)	–	Dasgupta et al. (2017)
PNASD	Single reactor under mainstream conditions	~100% S^{2-}	84% (TN)	–	Yuan et al.. (2020)
Anammox and S-dependent autotrophic Denitrification (ASD)	Expanded granular sludge bed	90–100% $S_2O_3^{2-}$	98% (TN)	–	Sun et al.. (2018)
ASD	Up-flow anaerobic sludge blanket reactor	99.6% S^{2-}, 330 mg S^{2-}/L	88% (TN), 252 mg NH_4^+-N/L	–	Guo et al.. (2016)
S-dependent autotrophic Partial Denitrification and Anammox (SPDA)	Up-flow anaerobic sludge blanket reactor	~100% $S_2O_3^{2-}$	>90% (TN)	–	Wu et al. (2019)
SPDA	Up-flow anaerobic sludge blanket reactor	70% S^0	90% (NO_2^--N)	–	Liu et al.. (2017)
Sulfammox/Anammox (SA) with COD	Moving Bed Biofilm Reactor	10% SO_4^{2-}	30% (NH_4^+-N)	–	Rikmann et al.. (2016)

wastewater streams from wet flue gas desulphurization, reveal even a greater performance potential (Qian et al., 2015a, b, Jiang et al., 2013). The biological reduction of SO_3^{2-} in FGD-SANI and MD-SANI provides more energy for bacterial growth, which is associated with a higher sludge efficiency compared to the biological reduction of SO_4^{2-} (Jiang et al., 2013). Moreover, SO_3^{2-} is an intermediate in SO_4^{2-} reduction, which may result in faster reduction by SRB.

Jiang et al. (2013) found that the removal rates of specific organics in the SO_3^{2-} and SO_4^{2-} reducing reactors were similar. At the extremely low temperatures (<10 °C), incomplete reduction of SO_3^{2-} in an anaerobic reactor (Fig. 3c) resulted in accumulation of $S_2O_3^{2-}$ and reduction in the removal rate of organics. However, the anoxic and aerobic reactors (Fig. 3c) still provided a high removal efficiency of organics (>94%), while NH_4^+-N and NO_3^--N were almost completely removed.

The MD reaction can lead to a much higher reduction of NO_3^--N and NO_2^--N compared to the S^{2-} based SANI process (Qian et al., 2015a). Qian et al. (2015b) reported that the denitrification rate increased sevenfold in MD-SANI compared to SANI. Furthermore, in comparison with SANI, FGD-SANI shows higher TN and COD removal efficiencies (98% and 94%). The complete removal of SO_3^{2-} and TN was achieved in MD-SANI, while the COD removal efficiency in that process was 81% (Table 4).

5.2. SRDAPN and PNASD - challenges resulting from the combination of aerobic and anaerobic conditions

The presence of S^{2-} in the influent wastewater imposes a significant risk of inhibition of the AAOB responsible for anammox. Threshold levels of S^{2-} inhibiting AAOB were found in the range of <1–64 mg S/l (Jin et al., 2013; Carvajal-Arroyo et al., 2013; Dapena-Mora et al., 2007). The study by Wisniewski et al. (2019) determined the half maximal inhibitory concentration (IC_{50}) under two different S^{2-} conditions. The IC_{50} was 4.25 mg H_2S–S/L at a constant S^{2-} concentration of 11 mg TS-S/L and pH in the range 7–7.9 vs. 4.67 mg H_2S–S/L at a varying concentration of S^{2-} ranging from 1 to 15 mg TS-S/L and a constant pH of 7. The decrease in AAOB activity was due to the pH-dependent non-ionized form of H_2S. In addition, heterotrophic bacteria may coexistence with AAOB but also outcompete AAOB at high influent C/N ratios (Chamchoi et al., 2008).

The PNASD process has been implemented in both one- and two-stage systems. The two stage-systems are easier to maintain and allow to avoid the negative impact of S^{2-} on AAOB and the competition between AOB and SOB for DO (Sahinkaya and Kilic, 2014).

Zhang et al. (2020) used S^0 for denitrification and observed only a small effect, when DO was kept at the level of 0.4–0.8 mg/L. When the DO concentration increased to 1.2 mg O_2/L, the concentrations of NO_3^--N and SO_4^{2-} also increased. This indicates excessive oxidation of S^{2-} or its reduced compounds in aerobic systems. Under non-limited DO conditions, autotrophic SOB can readily utilize oxygen, which leads to accumulation of SO_4^{2-}. On the other hand, too low DO concentrations in the PNASD process can reduce the NO_2^--N production rate in PN.

5.3. ASD, SPDA and sulfammox systems - coexistence of AAOB and denitrifiers

Under anaerobic conditions, the combination of anammox process and S-dependent autotrophic denitrification can work with high removal efficiencies of TN (88–96%) and S (90–100%) (Table 4). AAOB and T. *denitrificans* can assist in the combined N and S removal without inhibition by S^{2-} (Guo et al., 2016). In that study, most of S^{2-} was oxidized to S^0 at the influent ratios of NH_4^+-N/S^{2-} and NO_2^--N/S^{2-} at 1.74 and 2.2–2.27, respectively. Two S forms can accumulate depending on the S/N ratio in the reactor, i.e., SO_4^{2-} (at S/N ratio <1) or S^0 (at S/N ratio >1) (Cardoso et al., 2006).

When NO_2^--N is fed to the anammox process, S-dependent autotrophic denitrification may occur. When both NO_2^--N (anammox substrate) and NO_3^--N (anammox product) are simultaneously present in the influent, the latter form is the preferred electron acceptor for denitrification (Guo et al., 2016). However, a small portion of NO_2^--N can also be used by T. *denitrificans* and increase the overall efficiency of N and S removal.

Instead of complete denitrification, partial reduction to NO_2^--N can be achieved. This approach is advantageous for the Partial Denitrification/Anammox (PD/A) systems by continuously producing NO_2^--N for anammox (Wu et al., 2019). In addition, the consumption of electron donors can be reduced in comparison with the conventional biological nitrogen removal processes. The reported TN removal efficiencies exceeded 90% in SPDA (Table 4).

The novel sulfammox process has been applied in SO_4^{2-} and NH_4^+-N-rich wastewater treatment systems. One of the intermediates in the sulfammox reaction is NO_2^--N, which can be used by either AAOB or S-dependent autotrophic denitrification along with the residual NO_3^--N from anammox. Wu et al. (2020) combined sulfammox and anammox and obtained high removal efficiencies of NH_4^+-N (98.5%) and SO_4^{2-} (53%). Furthermore, the sulfammox and anammox processes can also be combined with S-dependent autotrophic denitrification (Rios-Del Toro et al., 2018).

6. Modeling N, S and C conversions in wastewater treatment systems

Modeling has been proven to be an effective tool to understand complex, interrelated N, S and C transformations (Show et al., 2013). In principle, two modeling approaches are possible, including empirical models, such as artificial neural networks (ANNs), and mechanistic models based on the Activated Sludge Model (ASM) family.

6.1. *Artificial neural networks (ANNs)*

The ANN model does not require a detailed process description, and it can be established by simple input and output parameters. Therefore, the ANN has been known for a long time as a tool in setting control mechanisms and performance models of biological wastewater treatment processes (Choi and Park, 2001). Wang et al. (2009a) developed an

Table 5
Overview of the reported mechanistic models linking C, S and N transformations.

No.	Reactor type	Substrate	Influent concentrations			Model structure				References
			Organic (mg COD/L)	S^{2-} (mg S^{2-}-S/L)	NO^{3-}-N (mg –N/L)	No. of processes	No. of components	No. of parameters	S and N involved processes	
1	Bench-scale EGSB reactor	Synthetic wastewater	200–800	200–800	75–275	7	10	18	Hydrolysis: Particulate N → Organic N Ammonification: Organic N → NH_4^+-N Heterotrophic: NO_3^--N → N_2 Autotrophic: NO_3^--N → N_2	Wang et al. (2010)
2	Bench-scale EGSB reactor	Synthetic wastewater	275–2300 mg C/L	156–1490	100–800	6	8	31	Autotrophic: S^{2-}→S_0 → SO_4^{2-} Autotrophic: NO_3^--N → NO_2^--N →N_2 Heterotrophic: NO_3^--N → NO_2^--N → N_2	Xu et al. (2014)
3	Bench-scale SBR	Synthetic wastewater	–	194 145	321 202	4	5	9	Autotrophic: S^{2-} → S_0 → SO_4^{2-} Autotrophic: NO_3^--N → NO_2^--N → N_2	Xu et al. (2016)
4	Bench-scale EGSB reactor	Synthetic wastewater	2700	1000 mg SO_4^{2-}-S/L	200–700	14	15	38	Autotrophic: S^{2-} → S_0 Autotrophic: NO_3^--N → NO_2^--N Heterotrophic: NO_3^--N → NO_2^--N → N_2 Heterotrophic: SO_4^{2-} → S^{2-}	Xu et al. (2017)
5	MBfR	Anaerobic digestion liquor	50–100	30	50–1000	18	17	60	Autotrophic: NH_4^+-N → NO_2^--N → NO_3^--N Autotrophic: NH_4^+-N, NO_2^--N → N_2, NO_3^-N Heterotrophic: NO_3^--N → N_2 Autotrophic: S^{2-} → S^0 → SO_4^{2-} Autotrophic:CH_4 → CO_2	Chen et al. (2016)
6	Coastal upwelling system	Sea water	–	0.1 mmol S/m^3	0.1 mmol N/m^3	9	14	46	Autotrophic: NH_4^+-N → NO_2^--N → NO_3^--N Heterotrophic: NO_3^--N → NO_2^--N → N_2 Heterotrophic: SO_4^{2-} → S^{2-} Autotrophic: S^{2-} → SO_4^{2-}	Azhar et al. (2014)

SBR: sequencing batch reactor, EGSB: expanded granular sludge bed, MBfR: membrane biofilm reactor.

ANN model to monitor a denitrifying S^{2-} removal (DSR) process. The proposed model revealed that the comparative influences of four input factors on DSR performance were as follows: hydraulic retention time (HRT) > S^{2-} concentration > C/S ratio > N/S ratio. Even though the ANN model is capable of predicting an intricate function between input and output parameters, it cannot help in understanding mechanisms of the complex biochemical processes.

6.2. Mechanistic models

The International Water Association Activated Sludge Models (ASMs) No. 1, 2, 2d and 3 (Henze et al., 2000) describe conversions of organic C and N compounds (ASM1 and ASM3), and additionally P compounds (ASM2 and ASM2d). However, to simplify the model structure, all the ASMs only considered NO_3^--N reduction as a one-step heterotrophic process using readily biodegradable organic compounds as electron donors. Moreover, one-step NH_4^+-N oxidation to NO_3^--N was the only autotrophic N transformation.

S-dependent autotrophic denitrification and the synergistic and competitive relationships among microorganisms were subsequently integrated with the ASMs. On one hand, developing realistic models is essential for practical applications in simultaneous N, C and S removal systems. On the other hand, due to the complex interactions between autotrophic and heterotrophic denitrifiers, developing an exhaustive model and appropriate control strategy becomes challenging. The existing models (Table 5) have been used in bench-scale reactors to predict the process involving intricate metabolic pathways with synthetic substrates. However, further work is still necessary to confirm the models in practical applications with real wastewater.

A detailed description of the mechanistic models can be found in SI (S4).

7. Implications of combining the N, S and C cycles in wastewater treatment systems

7.1. Processes application opportunities

S^0 and S^{2-} are considered good alternatives to organic matter in the denitrification process due to the absence of organic residues in the treated wastewater. It is thus strongly recommended to use S-dependent autotrophic denitrification instead of heterotrophic denitrification, especially for wastewater with a low organic content. Attention should also be paid to the water-insoluble S^0, which can physically be removed from wastewater and reused for production of sulfuric acid, pesticides, fertilizers, in construction (Lin et al., 2018). It is economic, effective and readily available source of electrons. On the other hand, $S_2O_3^{2-}$ is readily bioavailable and may mediate a higher rate of denitrification compared to S^0 and H_2S. S^{2-} is often used in municipal and industrial areas requiring desulphurization. Depending on the local conditions, S-dependent autotrophic denitrification can occur with a wide spectrum of S compounds. Moreover, it can get them from the initial SO_4^{2-} reduction stage in the integrated systems combining N–S–C cycles.

Biological SRB-based methods are a sustainable way of treating AMD compared to physico-chemical methods (Sinharoy et al., 2020b). SRB are capable of using toxic metals in their metabolism, thus reducing environmental and human health problems. SRB can grow in a wide range of environmental conditions, which provides many opportunities for the development of technologies based on their metabolism, with SO_4^{2-} reduction being recognized as a key step in all S- dependent processes (Hao et al., 2014).

Among the various gaseous substrates for SO_4^{2-} reduction, H_2 is most energetic for SRB. The resources that can be recovered from this process are metal sulfides and S^0, which has also been identified by Kumar and Pakshirajan (2020) as a potential substrate for S-dependent autotrophic denitrification.

The combination of the N, S and C cycles could lead to the development of economically feasible and sustainable wastewater treatment systems that produce less sludge and reduce carbon footprint compared to the existing systems. The SANI process has already been used in several full-scale wastewater treatment installations in Hong Kong due to the practice of flushing toilets with seawater (Jiang et al., 2013). The process can also be applied to freshwater wastewater, even in cold inland areas that do not contain enough SO_4^{2-} or SO_3^{2-} rich wet flue gas desulphurization (Qian et al., 2015a, b, Jiang et al., 2013). It can also be adapted to treat industrial wastewater by adding SO_4^{2-}, seawater or some SO_4^{2-}-rich wastewater. Lu et al. (2009, 2012) suggested that the SANI process could be a good solution in densely populated cities to treat saline wastewater as an economic source in terms of water scarcity and wastewater treatment in water-poor coastal areas.

Other technologies that include anammox and SANI processes have discovered the advantages of AAOB coexisting with SRB, SOB, and AOB. In addition, compared to the SANI process, the combination of SO_4^{2-} reduction, denitrification/anammox and partial nitrification will further reduce aeration energy consumption due to the lack of full nitrification required for NO_3–N production. The presence of anammox in the SRDAPN process resulted in an increased NO_2^--N removal efficiency by over 30% (Kosugi et al., 2019).

For wastewater with a low organic content, PNASD can be considered a viable option. The two-step PNASD system was more efficient for N and S removal, and easier to maintain than the one-step system (where bacteria competed for DO) (Dasgupta et al., 2017). Moreover, it has also been proven that the process can be applied in a single reactor under mainstream conditions (Yuan et al., 2020).

Instead of combining the heterotrophic SO_4^{2-} reduction with anammox, sulfammox can replace or accompany both processes by using a SO_4^{2-} dependent AAOB. Recent studies have proposed the use of sulfammox based on the combined reduction of NH_4^+-N and SO_4^{2-}. If SO_4^{2-} was reduced to S^{2-} or S^0 with organic compounds, this process would be replaced with sulfammox, while eliminating the addition of external carbon. Another suggested solution is to combine the sulfammox process with heterotrophic SO_4^{2-} reduction in order to increase the reduction rate of SO_4^{2-}. Moreover, if sulfammox is used upstream of an S-dependent autotrophic denitrification reactor, it contributes to oxidation of NH_4^+-N to N_2 (which increases the overall efficiency of NH_4^+-N removal) or NO_2^--N and NO_3^--N (which can be used in S- dependent autotrophic denitrification). By combining sulfammox and anammox, the efficiency of NH_4^+-N removal and SO_4^{2-} reduction to S^0 can be simultaneously increased (Liu et al., 2021; Grubba et al., 2021).

7.2. Advantages and disadvantages of two cycles or three cycles in wastewater treatment

The advantages and disadvantages of the systems based on the N–S–C cycles and their coupling are summarized below.

Advantages:

1. Approximately 35% reduction in energy consumption and up to 90% reduction in sludge production compared to full nitrification-denitrification.
2. Reduction or even no external carbon dosing for S-dependent autotrophic denitrification.
3. For the combined processes, almost complete N and S^{2-} removal and up to 75% efficiency of SO_4^{2-} removal.
4. Products of one process used as the substrates for another process.
5. When replacing heterotrophic denitrification with S-dependent autotrophic denitrification, carbon consumption is reduced by 100%. If heterotrophic SO_4^{2-} reduction is replaced by sulfammox, carbon consumption is also reduced by 100%.
6. Removal of a few harmful compounds (NH_4^+, NO_2^-,SO_4^{2-}, S^{2-}) in one system.
7. Approximately 30–40% reduction of volumes required for wastewater and sludge treatment processes.

8. Reduction of GHG emissions by 36% compared to conventional nitrification-denitrification.

Disadvantages:

1. Limited use in cold regions due to the high optimal temperature range (28–30 °C).
2. Complex interactions and competition for substrates between the functional microorganisms.
3. Greater complexity of the systems potentially resulting in higher investment costs.
4. The operating conditions must be compatible with all the N–S–C processes.
5. Some substrates/products involved in one process may be inhibitors for other processes, e.g. S^{2-}.

7.3. Processes application limitations

One of the most important limitations of technologies combining N, S and C cycle processes is the narrow optimal range of temperature (28–30 °C) and pH (7–7.6). Thus, cold weather in inland areas also restricts the use of coupled systems.

An important factor that should be considered when implementing technologies containing the S-dependent autotrophic denitrification process is the inhibition of this process caused by S^{2-} (Cardoso et al., 2006) as well as NO_2^--N, NO_3^--N and free nitric acid (FNA) (Cui et al., 2019). Even though S^0 is an inexpensive and non-toxic electron donor, but it provides a low denitrification rate due to its low solubility. The use of smaller S granules with a larger surface area improves the reaction efficiency, however it can cause low porosity and clogging and fouling of the reactors due to small S grain size or cracking (Wu et al., 2021). Moreover, as S^0 and H_2S reveal a much lower rate of NO_3^--N reduction, mainly the use of $S_2O_3^{2-}$ is recommended in the process. However, its natural content of wastewater is rather limited due to its instability (Cui et al., 2019).

In the case of heterotrophic SO_4^{2-} reduction, the presence of DO, NO_3^--N and NO_2^--N inhibits reduction of SO_4^{2-} and enhances oxidation of S^{2-} to S^0 or SO_4^{2-} (Mohanakrishnan et al., 2009). Moreover, the activity of SRB is inhibited by heavy metals, including Pb and Cd (Sinharoy and Pakshirajan, 2019). The toxicity of heavy metals depends mainly on the type of metal, responsible microorganisms, presence of other pollutants, and process conditions (Mal et al., 2016). Therefore, the systems based on heterotrophic SO_4^{2-} reduction cannot be used for wastewater rich in heavy metals. Moreover, a significant limitation is the limited number of microorganisms that are able to carry out SO_4^{2-} reduction with the use of gaseous substrates. Moreover, the low gas-liquid mass transfer also makes it difficult to scale-up the process.

A significant limitation in the implementation of integrated systems connecting N–S–C cycles is also the insufficient knowledge about the mechanism of sulfammox and responsible microorganisms. Until now, there has been no genomic evidence to support the ability of AAOB to use SO_4^{2-} as an electron acceptor. The growth rate of potential functional bacteria is also low, which limits their unambiguous identification (Liu et al., 2021). Moreover, the organic matter present in the wastewater stimulates the survival of heterotrophic bacteria, including denitrifiers. This leads to a competition between these bacteria and the sulfammox bacteria, thus destroying the sulfammox process.

7.4. Processes application challenges

Using specific N, S and C removal processes independently of each other is much easier to maintain than the processes combining these cycles. To link those processes in the combined technologies as presented in this review, it is important to recognize the effects of S^{2-} on N removal processes, such as autotrophic/heterotrophic denitrification and anammox, as well as the competition between AOB and SOB for DO. S^{2-} and organic matter, which are fed to an anaerobic compartment, can inhibit AAOB in anammox-coupled systems (Kosugi et al., 2019). Chen et al. (2018) showed that DO can react with S^{2-} while reducing the NO_3^--N removal rate. In addition, S^{2-} was reduced to S^0 and then converted to SO_4^{2-g} due to the presence of DO. These findings highlight the challenges faced by single-stage integrated systems.

In order to avoid the inhibition of SO_4^{2-} reduction by heavy metals, it is recommended to use an upstream reactor in order to remove metals from AMD using S^{2-}. In order to use SO_4^{2-} reduction coupling systems, it is also necessary to consider selection of the appropriate type of reactor, use of resistant microorganisms, and presence of other pollutants. Designing novel reactor configurations with high gas-liquid mass transfer can also help in applying the process in full scale. Moreover, instead of obtaining pure gases, a cost-effective solution would be production of gaseous substrates by thermochemical or biochemical methods from various compounds (e.g. waste) (Sinharoy et al., 2020b).

In the case of sulfammox, more research is needed to identify potential applications and integration with other systems. The key enzymes involved in the metabolism of NH_4^+-N and SO_4^{2-} should also be investigated. For this purpose, it is important to develop appropriate reactor configurations and create operational conditions that can enrich functional bacteria and allow for simultaneous removal of NH_4^+-N and SO_4^{2-}. Under non-limited NO_3^--N conditions, the SO_4^{2-} concentration may increase due to S-dependent autotrophic denitrification. The role of organic matter also requires further investigation with regard to the existence of the sulfammox process.

The combination of anammox, S-dependent autotrophic denitrification and sulfammox processes is challenging due to the different requirements of the microorganisms responsible for each process. The S-dependent autotrophic denitrification process may result in the production of SO_4^{2-} from S^{2-} or S^0, which negatively affects sulfammox, where SO_4^{2-} must be reduced to S^0 (Liu et al., 2021). More focused research on the coexistence of sulfammox with other bacteria and the development of a mechanistic model are needed to better understand and predict N and S dynamics. Moreover, the S/N ratio also plays an important role in determining the S-dependent autotrophic denitrification end products, requiring a closer look at the N and S dynamics. On the other hand, in order to avoid fouling and clogging of the reactors due to the presence of S^0, it is important to search for the appropriate sulfur grain size.

Wang et al. (2009b) identified three main challenges for the SANI process. First of all, it is the low efficiency of both SO_4^{2-} reduction during heterotrophic and S-dependent autotrophic denitrification reduction. Secondly, high concentrations of SO_4^{2-} are required, which may increase residual S^{2-} in the treated wastewater. Thirdly, transfer of NO_3^--N from the nitrification reactor to the S-dependent autotrophic denitrification reactor can also be difficult.

8. Conclusions

In terms of sustainability, the combination of N–S–C cycles processes has a few important benefits, including energy savings and lower sludge production. The combined processes allow for almost complete N and S^{2-} removal, while the efficiency of SO_4^{2-} removal can reach up to 75%.

Among all the processes linking the N–S–C cycles, SANI has been best recognized, but is rather not applicable in the case of wastewater with low organic content. Instead, it is worth of considering the sulfammox process that can reduce SO_4^{2-} and increase NH_4^+-N removal rate under anoxic conditions without the addition of external carbon.

Practical applications of the reviewed systems still face many challenges, especially in the single-stage configurations. In particular, the coexistence of several bacterial groups (AOB, AAOB, sulfammox bacteria, SOB, SRB) and their competition for the substrates is a key issue to be considered. Moreover, practical applications of the coupled S and N/C cycles require realistic models. However, due to the complex interactions between autotrophic and heterotrophic denitrifiers, development of a mechanistic model and appropriate control strategy becomes challenging.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2022.133495.

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Paper II

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Integration of the sulfate reduction and anammox processes for enhancing sustainable nitrogen removal in granular sludge reactors

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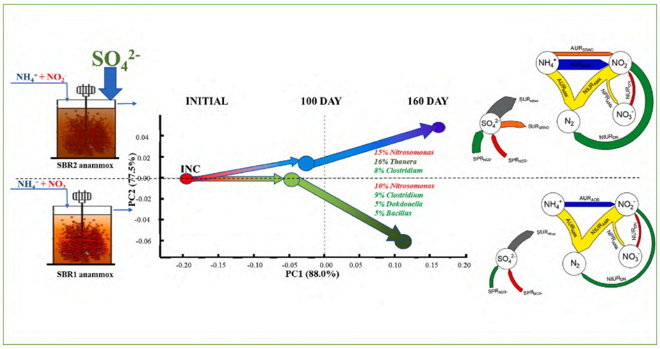
HIGHLIGHTS

- The effect of SO_4^{2-} on anammox was studied in two sequencing batch reactors.
- The increased SO_4^{2-} concentrations improved utilization rates of NH_4^+ and NO_2^-.
- The highest SO_4^{2-} utilization rates were obtained in the absence of NO_2^-.
- The predominant role in the N-S metabolism was related to *Nitrosomonas* and *Thauera.*
- N and SO_4^{2-} rich wastewater can be treated in a sustainable way in one-stage system.

GRAPHICAL ABSTRACT



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ABSTRACT

The Anammox and Sulfate Reduction Ammonium Oxidation processes were compared in two granular sequencing batch reactors operated for 160 days under anammox conditions. It was hypothesized that increasing the concentration of SO_4^{2-} may positively influence the rate of N removal under anaerobic conditions and it was tested whether SO_4^{2-} reduction and anammox occur independently or are related to each other. The cooperation of N-S cycles by increasing the concentration of influent SO_4^{2-} to 952 mg S/L in the second reactor, a higher ammonium utilization rate and sulfate utilization rate was achieved compared to the first reactor, i.e., 2.1-fold and 15-fold, respectively. *Nitrosomonas* played the dominant role in the N metabolism, while *Thauera* – in the S metabolism. This study highlights the benefits of linking the N-S cycles as an effective approach for the treatment of NH_4^+ and SO_4^{2-} – rich wastewater, including lower substrate removal cost and reduced energy consumption.

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1. Introduction

Currently, the principal N removal methods comprise both traditional nitrification -denitrification and a more energy- and C-efficient alternative, anaerobic ammonium oxidation (anammox). Wastewater treatment from N compounds at low cost with high efficiency is the main interest of recent years. In contrast, biological removal of S has received less attention until now. A combination of N, S and C cycles may be a promising way to develop innovative technologies for biological wastewater treatment, where products from one process can be used as substrates in other processes (Al-Hazmi et al., 2023a, 2022; Sun et al., 2018). For example, Liu et al. (2008, 2017) demonstrated the effectiveness of an integrated partial autotrophic denitrification-anammox process, while Yuan et al. (2020) explored partial nitrification/anammox and S-dependent autotrophic denitrification. Kosugi et al. (2019) also investigated the potential of combining sulfate reduction, denitrification/anammox, and partial nitrification.

It is important to note that S is present in wastewater in various forms, which can impact N removal differently. These forms include SO_3^{2-}, $S_2O_3^{2-}$, S_8^0, S^{2-}, and SO_4^{2-} (Al-Hazmi et al., 2023b). The latter two forms (S^{2-} and SO_4^{2-}) are the dominant S forms in wastewater treatment systems (Fisher et al., 2017). Researchers have identified three different S conversions, as reported in various studies. These include heterotrophic reduction of SO_4^{2-} (Virpiranta et al., 2019), S-dependent autotrophic denitrification (Zou et al., 2016), and sulfate reducing ammonium oxidation (SRAO or sulfammox) (Fdz-Polanco et al., 2001). The process of S-dependent autotrophic denitrification involves the oxidation of S compounds such as S^{2-}, S_8^0, $S_2O_3^{2-}$, and SO_3^{2-}, while simultaneously reducing NO_3^- and/or NO_2^-. On the other hand, heterotrophic reduction of SO_4^{2-} occurs using organic matter (COD) and reduces SO_4^{2-} to S^{2-}/S_8^0 (Grubba et al., 2022). Sulfammox is a variation of anammox using SO_4^{2-} as an electron acceptor instead of NO_2^-. In this process, SO_4^{2-} is transformed into S^{2-}/S_8^0 (Fdz-Polanco et al., 2001).

In the anammox process, the addition of NO_2^- can trigger S-dependent autotrophic denitrification by sulfur-oxidizing bacteria (SOB). However, in the presence of both NO_2^- and NO_3^-, NO_3^- becomes the preferred electron acceptor for denitrification. Guo et al. (2016) have reported that a small fraction of NO_2^- can also enhance the overall efficiency of N and S removal through specific SOB, such as *Thiobacillus denitrificans*. Similarly, in the sulfammox process, NO_2^- can be utilized by anaerobic ammonia oxidizing (anammox) bacteria (AnAOB) or SOB, along with residual NO_3^- from the anammox process. Furthermore, under anaerobic conditions and in the presence of COD, sulfate reducing bacteria (SRB) can convert SO_4^{2-} to S^{2-}/S_8^0.

There have been several studies on the SRAO process under different conditions, either with COD addition (Qin et al., 2021, Rikmann et al., 2016; Rikmann et al., 2014, Zhao et al., 2006) or without COD addition (Wu et al., 2020, Zhang et al., 2020, Zhang et al., 2019a; Zhang et al., 2019b). However, to date, there has been a lack of research on the use of granular sludge in sequencing batch reactors (SBRs), leading to a significant gap in knowledge. While one study has examined the effect of increasing NO_2^- and NO_3^- concentrations on the SRAO process (Zhang et al., 2019a), it did not include a gradual increase in SO_4^{2-} concentrations, and no comparison was made to the anammox process. In addition, there have been no studies comparing the SRAO efficiency without and with NO_2^- addition under autotrophic conditions (without COD addition). Previous studies have reported NH_4^+ and SO_4^{2-} removal efficiency, but none have calculated indicators, such as ammonium utilization rate (AUR), sulfate utilization rate (SUR), or nitrite utilization rate (NiUR) and nitrate production rate (NPR), which represents another knowledge gap.

The research novelty lies in the investigation of whether increasing the concentration of SO_4^{2-} in the reactor may positively influence the efficiency of NH_4^+ removal and whether the SRAO and anammox processes take place independently of anammox or are related to each other. The aim of this study was to compare the performance of anammox to SRAO with and without NO_2^- addition in an SBR with granular sludge. In addition, the bacterial community structure was characterized in response to the different experimental conditions and the key bacterial groups responsible for the mutual N and S metabolism were identified. According to the findings of this study, connecting the N and S cycles could prove to be a sustainable and efficient strategy for the remediation of wastewater containing high levels of NH_4^+ and SO_4^{2-}. A sustainable strategy meeting both energy neutrality and carbon footprint minimization targets" (Maktabifard et al., 2020).

2. Materials and methods

2.1. Laboratory configuration

The laboratory arrangement comprised of a computer and a control box connected to two SBRs, each having a working volume of 10 L. The SBRs were constructed using transparent plexiglass and positioned within an outer tube of the same material with a larger diameter, thus creating a water jacket. The Julabo F32 (Germany) water bath was utilized to regulate the temperature within the water jacket, with a precise temperature control range of −35.0 to +200.0 °C and an accuracy of ±0.1 °C. The bath was connected to the water jacket of the two reactors. The reactors could be emptied through manual ball valves. Each reactor had also a variable-speed RZR 2041 mechanical stirrer. Two Hach Lange HQ40D (Switzerland) multimeters were placed in the cabinet, and the Endress + Hauser (Switzerland) probes for measuring pH and dissolved oxygen (DO), EH CPS 471D-7211 and COS22D-10P3/O, respectively, were placed in the main reactor.

2.2. Inoculum biomass and operational conditions

The biomass for the reactors was obtained from a sidestream deammonification system in Słupsk WWTP (Poland). The study lasted for 160 days, during which both reactors were maintained at a constant temperature of 30.0 °C (±1.0 °C). The DO concentration in the SBRs did not exceed 0.2 mg O_2/L. The pH was kept between 7.5 and 7.8 by the automatic addition of 6 M hydrochloric acid (HCl). The synthetic substrates utilized in this investigation were identical to those employed in our earlier study (Al-Hazmi et al., 2021), which facilitated the ideal environment for anammox. The key components, including NH_4^+, NO_2^-, and SO_4^{2-}, were supplied as NH_4Cl, $NaNO_2$, and $MgSO_4$, respectively. The experiments were conducted with biomass concentrations ranging from 2000 to 4000 mg MLVSS/L.

In both reactors (SBR1_AMX and SBR2_SRAO), NO_2^- and NH_4^+ were dosed in the ratio of 1.3 which is typical for the anammox process. No external SO_4^{2-} was dosed to this reactor, however, the initial SO_4^{2-} concentration still varied in the range 45.2–58.6 mg S/L – probably as a result of the release of SO_4^{2-} from biomass and fast, unmeasurable reactions of S compounds. The SBR2_SRAO was operated with anammox, while gradually increasing the influent concentrations of SO_4^{2-} from 0.0 to 950.0 mg S/L. It should be noted that the maximum value is similar to the concentrations found in some types of industrial wastewater, such as effluent from yeast production (Rikmann et al., 2016) and landfill leachate (Wu et al., 2020). Additional batch tests (BR_SR) were carried out on a regular basis (approximately once per month), without the addition of NO_2^- and with high initial SO_4^{2-} concentrations in the range 46.1–856.0 mg S/L. Samples for these batch tests were taken from SBR2_SRAO, so the initial NH_4^+ concentrations were the same as for SBR2_SRAO. The aim of those tests was to check the effect of the absence of NO_2^- on the oxidation of NH_4^+ and the reduction of SO_4^{2-}. The influent concentrations of NH_4^+ and NO_2^- were gradually increased in the SBR1_AMX and SBR2_SRAO reactors (Fig. 1) in response to the increase in the bacterial activity and AUR during the test in both reactors.

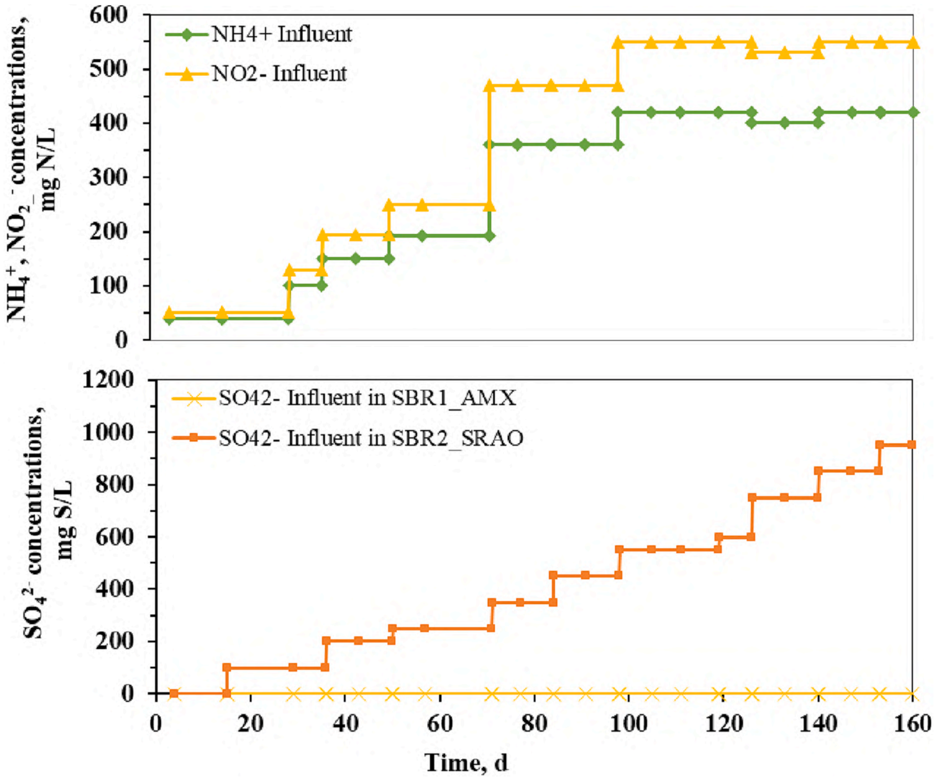

Fig. 1. The influent concentrations of a) NH_4^+, NO_2^- b) SO_4^{2-}.

2.3. *Microbiological and chemical analytical methods*

To analyze the microbial composition, granular activated sludge samples were collected from SBR1_AMX and SBR2_SRAO on day 1 (inoculum), 100, and 160. Genomic DNA was extracted, sequenced using Illumina NGS, and classified based on taxonomical levels. Statistical analysis and network analysis were performed to examine differences in taxonomy and function, as well as the relationship between Operational Taxonomic Units (OTUs) and influent concentrations. The Gephi platform was used for network analysis, considering significant correlations and centrality measures.

Biomass concentrations were determined by calculating the volatile suspended solids (MLVSS) fraction of the total mixed liquor suspended solids (MLSS), following Standard Methods (APHA, 2005). Mixed liquor samples were filtered immediately using disposable glass microfiber filters MFV-3 (47 mm diameter) from Sartorius (Germany). The filtered samples were analysed for concentrations of NO_3^-, NO_2^-, NH_4^+, SO_4^{2-}, and S^{2-} using cuvette tests from Hach Lange GmbH (Dusseldorf, Germany) and a DR 3900 spectrophotometer.

2.4. *Calculations of the process rates and efficiencies and optimization of the results*

The maximum slopes of NH_4^+ (AUR), NO_2^- (NiUR), NO_3^- (NPR), and SO_4^{2-} (SUR) were used to determine the specific process utilization rates. The efficiencies of NH_4^+ and SO_4^{2-} removal in the reactors were calculated based on the influent and effluent concentrations. A stoichiometric analysis was carried out using the SOLVER utility in MS Excel from the following reactions:

1) Anammox: $NH_4^+ + 1.32NO_2^- \rightarrow 1.02\ N_2 + 0.26NO_3^- + 2H_2O$
2) SRAO: $3SO_4^{2-} + 4NH_4^+ \rightarrow 3S_8^0 + 2NO_2^- + N_2 + 4H_2O$
3) S-dependent autotrophic denitrification with NO_3^-: $5S_8^0 + 6NO_3^- + 2H_2O \rightarrow 5SO_4^{2-} + 3\ N_2 + 4H^+$
4) S-dependent autotrophic denitrification with NO_2^-: $3S_8^0 + 6NO_2^- \rightarrow 3SO_4^{2-} + 3\ N_2$

As a result, the overall rates were decomposed and attributed to the specific processes based on the reaction equations and the following assumptions:

1) Anammox, anaerobic NH_4^+ oxidation, SRAO and S-dependent autotrophic denitrification, and other SO_4^{2-} reduction were considered,
2) SRAO did not occur in SBR1_AMX,
3) The SRAO-related AUR (AUR_{SRAO}) in SBR2_SRAO was established based on the AUR in BR_SR,
4) The same anammox-related AUR (AUR_{AMX}) was both in SBR1_AMX and SBR2_SRAO,
5) The other SO_4^{2-} reduction process (SUR_{other}) was not related to the N cycle.

The calculations were performed on the microbiological sampling days (initial day, 100 and 160) and the results were presented in the form of Sankey graphs.

3. Results and discussion

3.1. *The role of SO_4^{2-} in linking the N and S cycles*

In SBR1_AMX, the AUR and NiUR increased by 27.8 mg N/g VSS/h and 13.5 mg N/g VSS/h in 160 days, respectively, while the SUR increased by only 1.4 mg S/g VSS/h, (Fig. 2). In contrast, SBR2_SRAO exhibited a 2-fold increase in AUR (51 mg N/g VSS/h) and a 15-fold increase in SUR (19.8 mg S/g VSS/h). Both reactors experienced a rapid AUR increase followed by stabilization. The NH_4^+ removal efficiency was high in both SBR1_AMX (>90%) and SBR2_SRAO (>96%). SBR2_SRAO had a significantly higher AUR (59.5 mg N/g VSS/h), likely attributed to the activity of S-dependent AnAOB and *Nitrosomonas* in response to the increased influent SO_4^{2-} concentrations. In BR_SR, the

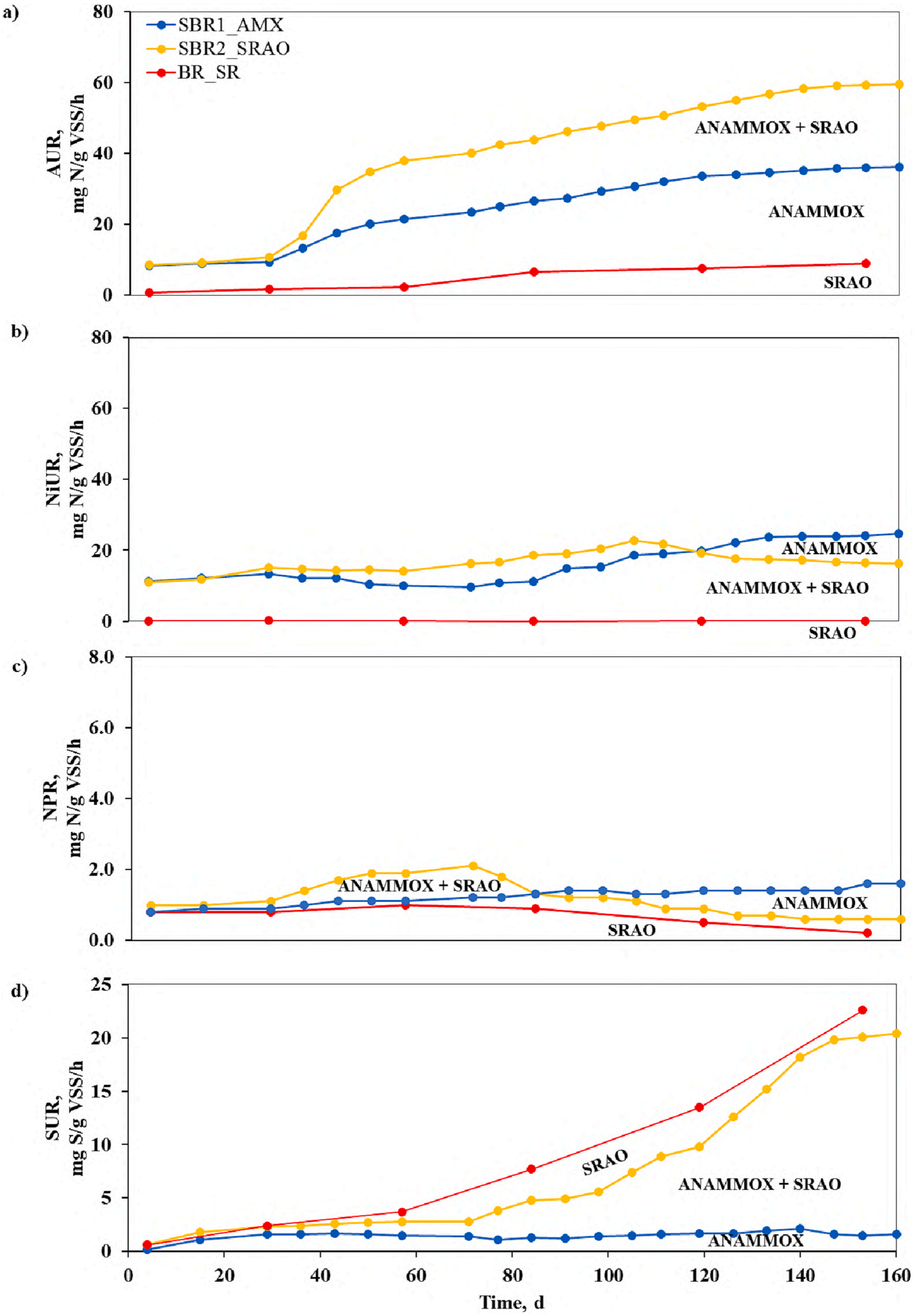

Fig. 2. Indicators and responsible processes in SBR1_AMX, SBR2_SRAO and BR_SR a) AUR b) NiUR c) NPR d) SUR.

AUR increased gradually by 8.1 mg N/g VSS/h in 153 days, while the SUR increased rapidly by 22 mg S/g VSS/h. The SUR trends in BR_SR and SBR2_SRAO were significantly different. The rate in BR_SR continued to increase and reached much higher levels compared to SBR2_SRAO, which showed stabilization at the end of the study.

Fluctuations in the NH_4^+ removal efficiency were observed in BR_SR, dropping to 17% on day 153. Significantly higher efficiencies were observed in SBR1_AMX and SBR2_SRAO. On the other hand, BR_SR exhibited a maximum SO_4^{2-} removal efficiency approximately 4 times higher than SBR2_SRAO. The NPRs were small in both reactors compared to the AURs and NiURs. SBR1_AMX showed a continuous increase in NPR, doubling in 160 days, while SBR2_SRAO initially increased and then decreased. BR_SR showed a similar trend to SBR2_SRAO, with S^{2-} concentrations detected below 1 mg S/L.

The impact of elevated SO_4^{2-} concentrations on anaerobic NH_4^+ oxidation is a subject of conflicting findings in the literature. In the study of Zhang et al. (2019b), increasing SO_4^{2-} concentration from 90 to 170 mg S/L improved NH_4^+ removal from 40 to 90% and SO_4^{2-} removal remained unchanged, but further increase to 360 mg S/L decreased both NH_4^+ removal to 20% and SO_4^{2-} to 5%. Rikmann et al. (2014) showed that increasing influent SO_4^{2-} from 71 to 193 mg S/L improved both NH_4^+ and SO_4^{2-} removal from 18 to 30% and 10 to 20%, respectively. Qin et al. (2021) found that increasing SO_4^{2-} from 32 to 80 mg S/L decreased NH_4^+ removal efficiency from 71% to 29%, while SO_4^{2-} removal was negligible.

The presence of SO_4^{2-} in wastewater plays a critical role in linking the N and S cycles, with practical and ecological implications. SRB utilize SO_4^{2-} as electron acceptors, facilitating their reduction to S^{2-}/S_8^0. This not only drives the S cycle but also influences N transformations. Denitrifying microorganisms, crucial in removing N from wastewater, can perform NO_3^-/NO_2^- reduction with S^{2-}/S_8^0 even under DO-limited conditions. This has practical implications for wastewater treatment systems that may encounter DO limitations or fluctuations. Including SO_4^{2-} in the treatment process can improve the overall N removal efficiency. Furthermore, SO_4^{2-} serves as an electron acceptor in the SRAO process, where NH_4^+ is oxidized to N_2. In addition, the presence of S^{2-} resulting from SO_4^{2-} reduction can affect the activity and efficiency of AnAOB, thus affecting the overall N removal in wastewater treatment systems.

Further research is necessary to explore the complex interactions between S, N, and microbial communities and their implications for efficient wastewater treatment and environmental impact. Understanding these interactions is essential for optimizing treatment strategies, improving process stability, and minimizing the release of N and S compounds into the environment.

3.2. *Effect of NO_2^- on the SRAO process*

Chen et al. (2018) determined the theoretical NO_2^-/NH_4^+ ratio optimal for the anammox process is 1.32. In the present study, despite that ratio was maintained in the influent, the ratio of NO_2^-/NH_4^+ consumption was decreasing in both SBR1_AMX and SBR2_SRAO. A similar observation was made by Yang et al. (2009) in a study on SRAO in an anaerobic bioreactor under inorganic feeding conditions, where the ratio of NO_2^-/NH_4^+ consumption was 1.15 and simultaneous NH_4^+ and SO_4^{2-} removal was detected. In BR_SR, the tests were carried out without adding NO_2^- (electron acceptor) and consequently the NH_4^+ removal efficiency was limited, on day 84 of the study, it only peaked at 64% and then decreased to 17% by the end of the study. The poor process efficiency in BR_SR was also reflected in the low AUR, which was an average of 4.6 mg N/g VSS/h, with a maximum value of 8.9 mg N/g VSS/h. This is almost six times less compared to SBR1_AMX and nine times less compared to SBR2_SRAO. Therefore, SBR2_SRAO, which had an increased concentration of SO_4^{2-} with the presence of NO_2^-, appears to be the best solution for the oxidation of NH_4^+ in anaerobic conditions, as shown in Fig. 3.

In terms of SO_4^{2-} removal efficiency, BR_SR exihibited the highest values, averaging 45%, while SBR2_SRAO only reached an average of 12%. The average and maximum SURs were comparable in both reactors, with BR_SR averaging 8.4 mg S/g VSS/h and a maximum of 22.6 mg S/g VSS/h, and SBR2_SRAO averaging 8.1 mg S/g VSS/h with a maximum of 20.4 mg S/g VSS/h. These results suggest that the highest SO_4^{2-} removal efficiency can be achieved in the absence of NO_2^- and with increased SO_4^{2-} concentration, as observed in BR_SR. When analyzing NO_2^- removal, both SBR1_AMX and SBR2_SRAO exhibited similar average NiUR levels, i.e., 16.3 mg N/g VSS/h vs. 16.7 mg N/g VSS/h, respectively. The NPRs were consistently low and similar across all the reactors, with average values of 1.2 mg N/g VSS/h, 1.2 mg N/g VSS/h, and 0.7 mg N/g VSS/h in SBR1_AMX, SBR2_SRAO, and BR_SR, respectively.

Other studies have explored the SRAO process either with or without the addition of NO_2^-. Liu et al. (2021) reported that AnAOB would prefer NO_2^- over SO_4^{2-} as an electron acceptor, leading to a decrease in SO_4^{2-} reduction. Zhang et al. (2019a) found that increasing NO_2^- from 0 to 34 mg N/L improved NH_4^+ removal to 60%, but completely halted SO_4^{2-} removal. Wu et al. (2020) found that a high NO_2^- concentration (230 g N/L) resulted in 4 times greater NH_4^+ removal by SRAO compared to anammox. Qin et al. (2021) found that reducing NO_2^- from 70 to 0 mg N/L and increasing SO_4^{2-} from 32 to 80 mg S/L reduced NH_4^+ removal by over 50%. Wang et al. (2017) observed a negative effect of accumulated NO_2^- on SRAO, resulting in decreasing NH_4^+, SO_4^{2-} and COD removal efficiencies from 70, 80, 70% to 10, 28 and 20%, respectively. Rikmann et al. (2012, 2014) compared NO_2^- and SO_4^{2-} as electron acceptors and found higher TN removal efficiencies for NO_2^-, (>75%) than SO_4^{2-} (<25%).

The impact of NO_2^- on the SRAO process is primarily related to the competition for electron acceptors and the metabolic preferences of different microbial communities. Both NO_2^- and SO_4^{2-} can serve as electron acceptors in the SRAO process. AnAOB utilize NO_2^- during anaerobic (anoxic) NH_4^+ oxidation, while SRB or S-dependent AnAOB can reduce SO_4^{2-} to S^{2-}. The presence of both NO_2^- and SO_4^{2-} can lead to a competition among microbial communities for these electron acceptors. Different microbial communities have varying preferences for electron acceptors. The presence of NO_2^- can influence the microbial community balance, potentially impacting the SO_4^{2-} reduction efficiency. In contrast, the absence of NO_2^- in the SRAO process can prevent the inhibition of SO_4^{2-} reduction but may provide limiting conditions for AnAOB.

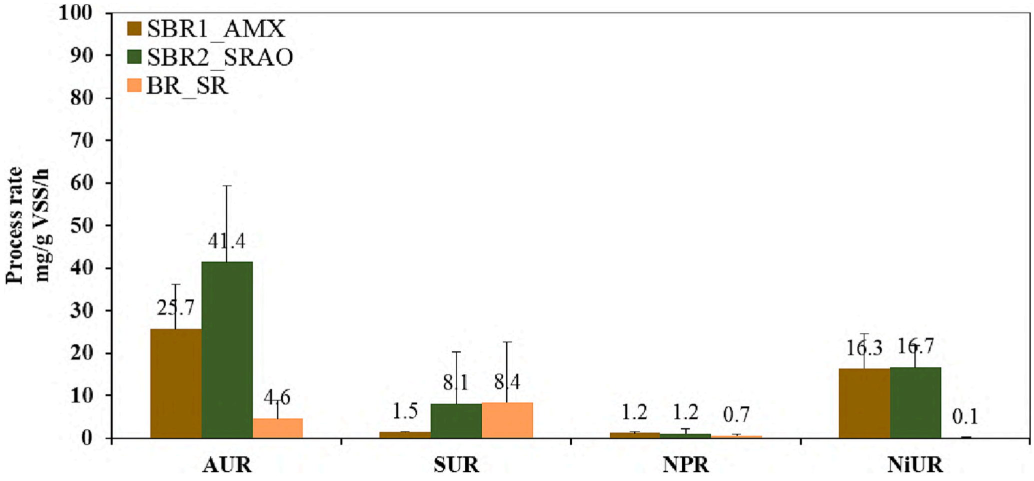

Fig. 3. Comparison of the average and maximum values of the process efficiencies in SBR1_AMX, SBR2_SRAO and BR_SR.

3.3. Comparison of the performance of SBR2_SRAO and BR_SR compared to SBR1_AMX

A comparative analysis of AUR and SUR was conducted over the study period to evaluate the difference in their rates between SBR2_SRAO, BR_SR, and SBR1_AMX (Fig. 4). The results showed that the SRAO process in SBR2_SRAO had a positive impact on the NH_4^+ removal rate, as indicated by the continuously increasing the deviation ΔAUR from 0.1 to 23.3 mg N/g VSS/h between the AUR in SBR2_SRAO and SBR1_AMX. On the other hand, the BR_SR showed a negative impact on the NH_4^+ removal rate, as indicated by the decreasing ΔAUR from −7.6 to −27.2 mg N/g VSS/h, with a clear decrease observed between 29th and 57th day of the study.

In terms of SO_4^{2-} removal, both SBR2_SRAO and BR_SR showed higher efficiencies compared to the conventional anammox process in SBR1_AMX, as indicated by the increasing the deviation ΔSUR from 0.4 to 18.8 mg S/gVSS•h for SBR2_SRAO and 21.1 mg S/gVSS•h for BR_SR. Furthermore, BR_SR showed a slightly higher ΔSUR compared to SBR1_AMX than SBR2_SRAO, with a noticeable increase after 70 days of the study.

The results of this study have several implications and offer important insights into the performance of different wastewater treatment processes. The positive impact of the SRAO process in SBR2_SRAO on NH_4^+ removal suggests its potential for more efficient NH_4^+ oxidation compared to the conventional anammox process in SBR1_AMX. Incorporating SO_4^{2-} reduction enhances NH_4^+ removal, as evidenced by the consistently higher AURs in SBR2_SRAO. On the other hand, the absence of NO_2^- in the SO_4^{2-} reduction process of negatively affects AUR, as observed in BR_SR, highlighting the need for addressing the limitations and challenges associated with the process conditions in that reactor.

Regarding SO_4^{2-} removal, both SBR2_SRAO and BR_SR showed higher efficiencies compared to SBR1_AMX, indicating the effectiveness of incorporating SO_4^{2-} reduction in wastewater treatment. These findings contribute valuable insights to optimize wastewater treatment processes, emphasizing the importance of NO_2^- in SO_4^{2-} as electron acceptors in reduction processes. Further research should focus on understanding the underlying mechanisms and optimizing the operation of these processes to maximize their efficiency and improve nutrient removal while minimizing the environmental impact of effluent wastewater.

3.4. Key microorganisms involved in the S and N metabolism under anoxic conditions

Microbial community structure adaptation to the operational process conditions has been analyzed in SBR1_AMX and SBR2_SRAO. The Principal Component Analysis (PCA) of the 16S rRNA sequencing data, at the genus level, have revealed directed rearrangements within bacterial consortia over the experimental period (Fig. 5). After 100 days of adaptation, the bacterial consortia in the both reactors reflected a similar composition, but noticeably different from the inoculum sample. During the following 60 days of the operation, the adaptation process was intensified and lead to the establishment of highly diverse bacterial communities specified for the predominant processes in the particular reactor.

In the course of the study, *Proteobacteria* and *Chloroflexi* were the most abundant phyla in both reactors, each accounting for over 20% of the community. However, after 160 days of the operation, significant changes in the relative abundances were observed, what is shown in Table 1. *Proteobacteria* increased their share up to 50% in SBR2_SRAO, *Chloroflexi* remained stable at around 24%, and *Bacteroidetes* decreased from 30% to 20% in SBR1_AMX, and 6% in SBR2_SRAO. Several other phyla showed alterations, such as an increased proportion of *Verrucomicrobiota* and *Firmicutes*, which elevated their representation from 0.4%

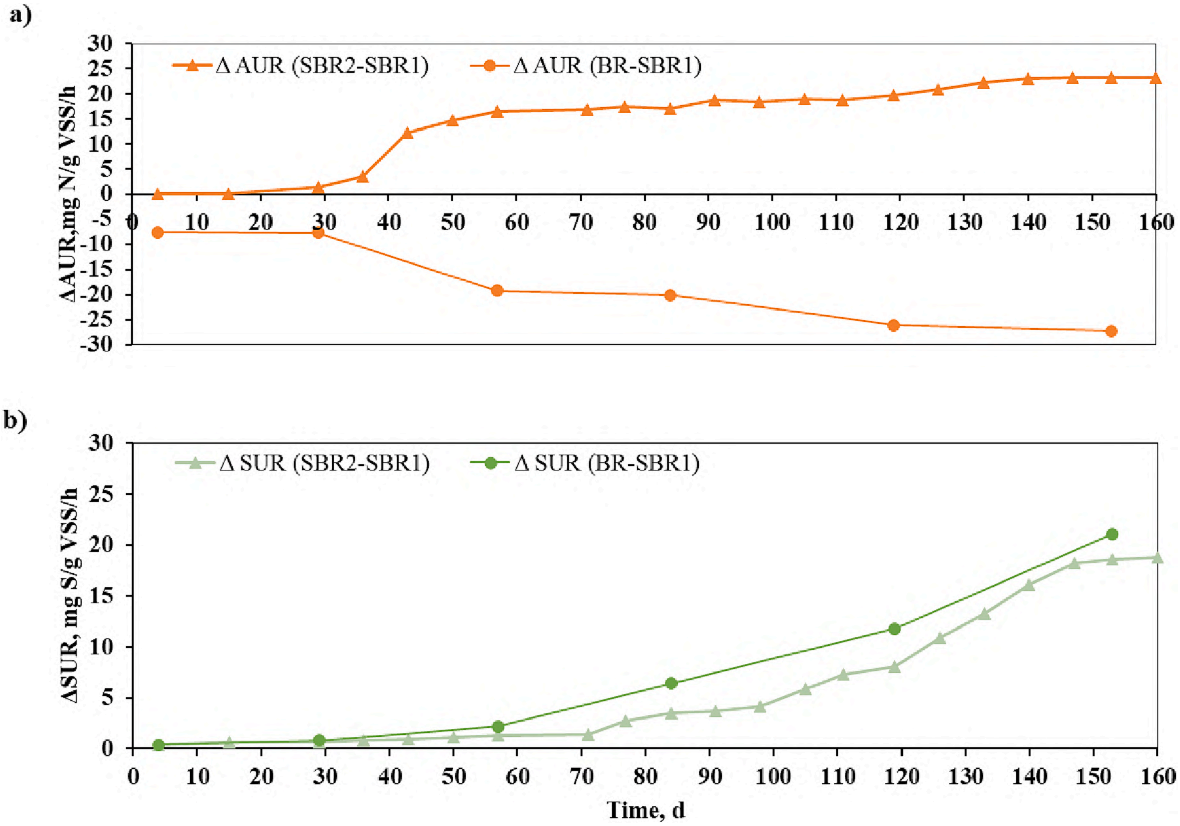

Fig. 4. Differences in the observed AUR and SUR in the three reactors a) ΔAUR between SBR2_SRAO and SBR1_AMX; b) ΔAUR between BR_SR and SBR1_AMX c) ΔSUR between SBR2_SRAO and SBR1_AMX; d) ΔSUR between BR_SR and SBR1_AMX.

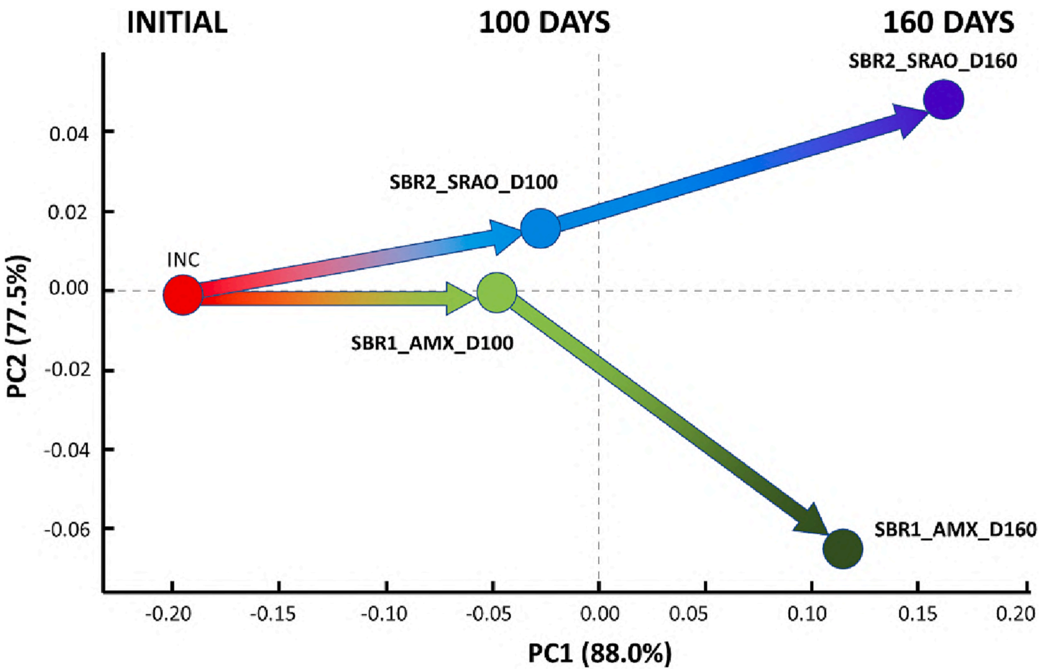

Fig. 5. Differentiation of the bacterial community structure between SBR1_AMX and SBR2_SRAO after 160 days of the operation, analyzed by the PCA of the 16 S rRNA sequencing data at the genus level.

Table 1
Structure of the bacterial communities at the phylum level in the initial day (INC) and samples collected after 100 and 160 days of the operation. The colors correspond to the percentage of specific phyla in the total bacterial community according to the heat map.

Phylum/Reactor/Exp. day	INC	SBR1_AMX		SBR2_SRAO	
		100 d	160 d	100 d	160 d
Unit	%				
Bacteroidota	30.5	18.6	19.7	4.6	5.8
Proteobacteria	26.4	36.3	34.4	39.9	50.6
Chloroflexi	18.8	22.1	23.1	29.5	24.6
Acidobacteriota	7.9	3.9	5.1	3.6	1.3
Planctomycetota	4.5	1.1	1.8	2.1	2.1
Patescibacteria	2.9	0.3	0.5	2.7	0.9
Actinobacteriota	2.9	2.0	2.6	3.0	3.2
Armatimonadota	2.0	3.0	0.8	2.0	1.1
Other	1.4	1.4	1.6	3.7	1.9
Myxococcota	1.0	1.7	1.3	0.9	0.1
Verrucomicrobiota	1.0	1.7	2.0	2.0	2.1
Firmicutes	0.4	7.3	6.1	3.8	3.4
Gemmatimonadota	0.2	0.2	0.3	1.3	1.6
Nitrospirae	0.1	0.5	0.6	0.8	1.0

to 6% (SBR1_AMX) and 3.4% (SBR2_SRAO) at the study's culmination.

Zhang et al. (2019a) observed similar proportions of major phyla in their study, which investigated the effects of NO_2^- and NO_3^- on the integration of NH_4^+ oxidation and SO_4^{2-} reduction. In their study, *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes* were the most abundant phyla, comprising 32.9, 15.1, and 13.9%, respectively, in the reactor with NO_2^- addition. A Zhang et al. (2021) also reported comparable abundances of major phyla in the SRAO process, with *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes* representing 26.4, 21.4, and 15.8%, respectively.

After 100 days, the bacterial community in both reactors was subjected to significant changes at the genus level (see supplementary material). *Nitrosomonas* increased from 0.4% to 14.7% in SBR1_AMX and 18.6% in SBR2_SRAO, while *Candidatus* Brocadia decreased from 2.7% to 0.1% in both reactors. Additionally, the genera *Chloroflexi* (from the order RBG-13–54-9) and midas_g_179 showed a decline in their contributions.

In the final stage of the study, *Candidatus* Brocadia (0.9%), *Nitrosomonas* (9.5%), and *Chloroflexi* (8.7%) were the major phyla in SBR1_AMX, while *Thauera* (15.8%), *Nitrosomonas* (15.3%) dominated in SBR2_SRAO. Notable genera with higher abundances in SBR2_SRAO compared to SBR1_AMX included *Chloroflexi*. *Acinetobacter* (1.1%), *Nitrospira* (1.0%), and *Terrimicrobium* (1.1%).

The relatively high abundances of *Nitrosomonas* (15.3%) and *Thauera* (15.8 %) contributed to the relatively high abundance of the *Proteobacteria* phylum (50%), which differs from the results reported by other authors. The abundance of *Nitrosomonas* at 15% is much higher than that reported by Zhang et al. (2021), who found *Nitrosomonas* to constitute approximately 2% of the total community. In contrast, the elevated abundances of *Thauera* (9.26–13.63%) were previously observed in a denitrifying S conversion process supported by enhanced biological phosphorus removal (Zhang et al., 2017).

Network analysis of the SBR1_AMX reactor identified three modules consisting of OTUs and influent concentrations (see supplementary material). One module included 9 nodes representing OTUs, with *Nitrosomonas, Candidatus Brocadia, Denitratisoma, Thioprofundus, Limnobacter*, and *Terrimicrobium* exhibiting numerous linkages but a negative correlation with the influent concentrations. *Nitrosomonas* had the highest betweenness centrality and showed a positive correlation with all the influent concentrations. Another module comprised 7 nodes representing both OTUs and influent concentrations, with *Thauera* and *Nitrospira* showing a positive correlation and high betweenness centrality. *Dokdonella* and midas_g_9708 bridged the two modules with high betweenness centrality.

Similarly, the network analysis of the SBR2_SRAO reactor revealed two modules (see supplementary material). The first module consisted of 10 nodes representing OTUs, with 5 nodes exhibiting a negative correlation with influent concentrations. The second module included 8 nodes representing both OTUs and influent concentrations, with *Nitrosomonas* exibiting the highest number of linkages. Most OTUs showed a positive correlation with the influent concentrations. Notably, *Blastocatellaceae* and SO_4^{2-} concentration showed the highest betweenness centrality values in the network analysis.

The noticeable discrepancy in the microbial structure between both reactors (SBR1_AMX and SBR2_SRAO) began only after >100 days of the operation with the increasing influent SO_4^{2-} concentration above 500 mg S/L. The microbiological revealed that *Nitrosomonas* and *Thauera* played the dominant role in the N and S metabolism, respectively.

As a typical ammonia oxidizing bacterium (AOB), *Nitrosomonas* is known to tolerate high SO_4^{2-} levels. Wu et al. (2020) found *Nitrosomonas* to be dominant (up to 33%) in a landfill leachate treatment system with SO_4^{2-} levels ranging from 1870 to 1920 mg S/L. *Nitrosomonas* has a versatile metabolism and can reduce NO_2^- using hydrogen or organic compounds as electron donors. This is due to the presence of genes encoding nitrite reductase (NirK) and iron-containing membrane-bound cyt c-dependent nitrite reductase (cNOR; NorBC) in its genome (Sedlacek et al., 2020). In earlier studies, Schmidt and Bock (1998) and Juliette et al., (1993) suggested that *Nitrosomonas* could also perform anaerobic ammonium oxidation with NO_2^- using nitrogen dioxide or nitrogen tetroxide from denitrification and contribute to the S metabolism through thioether oxidation to sulfoxides. Bagchi et al. (2012) confirmed the possibility of NH_4^+ anaerobic oxidation by *Nitrosomonas*. In the present study, despite the lack of aeration, representatives of *Nitrosomonas* were the most abundant component of the bacterial communities and was considered as the key AnAOB reflecting a tolerance to high levels of SO_4^{2-}.

Thauera was a key player in the S metabolism due to its high abundance in SBR2_SRAO (>15.0% of the total bacterial community). *Thauera* is a highly versatile genus known for its ability to remove different contaminants during wastewater treatment. It is a typical denitrifying heterotroph but Huang et al. (2015) identified *Thauera* as the population responsible for SO_4^{2-} reduction as the electron donor. *Thauera* is highly resistant to high S^{2-} concentrations in wastewater, and even exposure to S^{2-} can enrich its population. It has been reported that *Thauera* can either oxidize S^{2-}/S_8^0 to SO_4^{2-} (Guo et al., 2019) or use S^{2-} as an alternative electron donor to reduce NO_3^- and NO_2^- to N_2 (Liang et al., 2020).

3.5. Stoichiometric analysis of the processes involved in the N and S cycles

Based on the results of the stoichiometric analysis, Sankey diagrams for 0, 100d, 160d were prepared (see Fig. 6 and supplementary material). The optimization results, revealing the specific process rates, were very accurate. The optimization criterion was to obtain the most similar AUR, NiUR, NPR and SUR indices in the measurement and calculation results. The absolute sum of errors remained below 0.036 in all the three cases when using one set of the stoichiometric coefficients.

The Sankey diagrams from the beginning of the research show the similarity of the N and S cycles in both reactors – the main influence on the AUR and NiUR was the anammox process, SUR and sulfate production rate (SPR) were minimal and insignificant in N transformations. On day 100, an increased NH_4^+ oxidation by AOB (AUR_{AOB}) and S-dependent denitrification with NO_2^- as well as a 2-fold higher SUR_{other} were found in SBR2_SRAO.

On day 160, the stoichiometric analysis revealed that the addition of SO_4^{2-} in SBR2_SRAO increased the total AUR due to the increased contribution of AUR_{AOB} and the additional effect of AUR_{SRAO}. The share of *Nitrosomonas* was 9.5% and 15.3%, respectively, in SBR1_AMX and in SBR2_SRAO. This difference can explain the increased AUR_{AOB} in the latter bioreactor. The nitrate utilization rate (NUR_{DN}) was similar in both reactors, but NO_2^- reduction significantly higher in SBR2_SRAO. *Thauera* may be responsible for this change as its share was only 1.2% in SBR1_AMX compared to 15.8% in SBR2_SRAO.

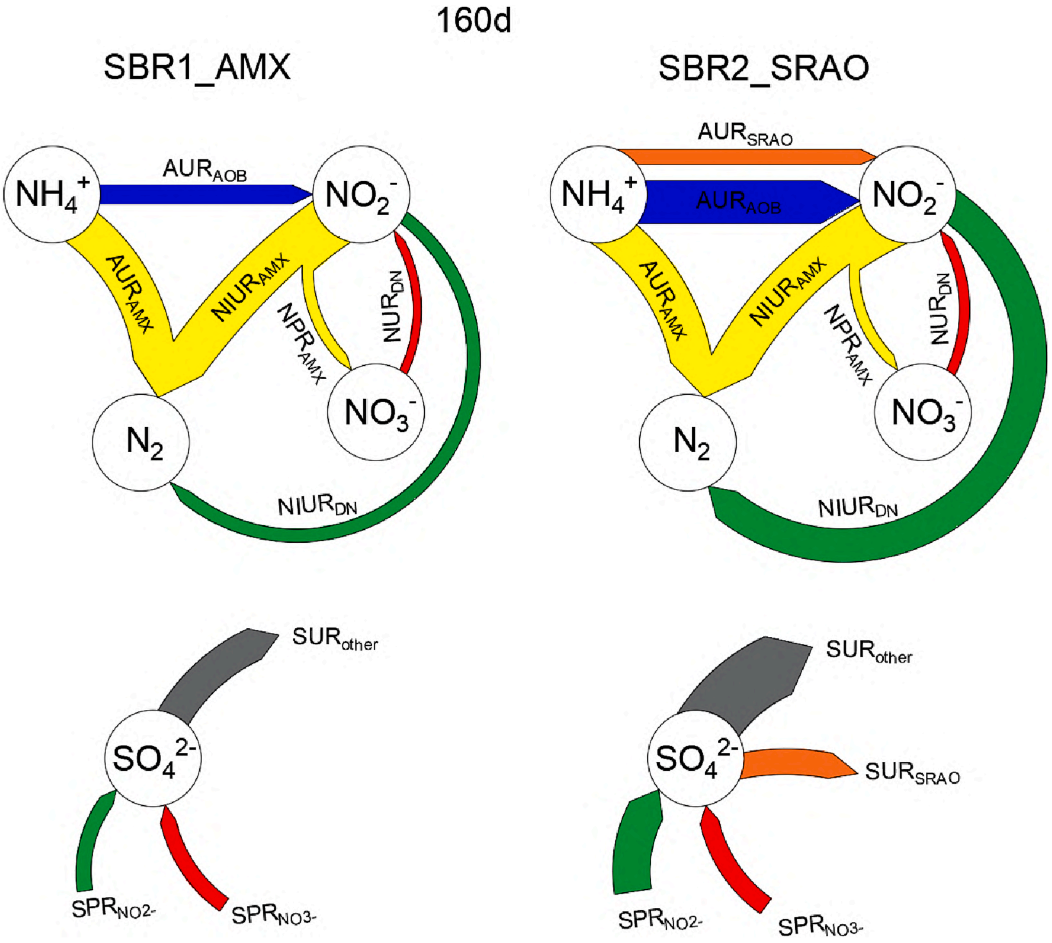

Fig. 6. Sankey diagrams for N and S transitions on day 160 of the study in SBR1_AMX and SBR2_SRAO.

The production of SO_4^{2-} also increased in SBR2_SRAO with the contribution of NO_2^- to S-dependent denitrification but on the other hand SUR_{other} and SUR_{SRAO} also increased. The addition of SO_4^{2-} affected the N transformations, but more noticeably changed the S cycle. The increase in SPR was apparently due to *Thauera*, as is the increase in denitrification. The increased SUR could also be caused by *Chloroflexi*. According to Zhu et al. (2022), the proliferation of *Chloroflexi* (31.8%) and *Proteobacteria* (31.8%) phyla suggests a connection between the SRAO process and thiosulfate-driven denitrification and anammox. The introduction of SO_4^{2-} may trigger processes that are separate from the N cycle (SUR_{other}), but it also leads to an elevation in S-dependent autotrophic denitrification (SPR_{NO2-}) linked to the N cycle.

3.6. Perspectives on the application of SRAO in existing wastewater treatment systems

The SR process effectively removes SO_4^{2-}, as shown in BR_SR. For a higher removal efficiency of both SO_4^{2-} and NH_4^+, an integrated SRAO process could be considered (Liu et al., 2021, Grubba et al., 2021). Table 2 shows that SRAO (better for NH_4^+) and SR (better for SO_4^{2-}) can replace anammox, based on the need to remove NH_4^+ and SO_4^{2-} from wastewater.

The main process for SO_4^{2-} removal was the independent reduction of SO_4^{2-} by sulfate-reducing bacteria (SRB) using organic compounds. By integrating the N-S cycles, the addition of an external carbon source can be avoided. Autotrophic SRAO can provide substrates for sulfur-oxidizing bacteria (SOB) by reducing SO_4^{2-}. In addition, S-dependent autotrophic denitrification is becoming a preferred alternative due to the absence of organic matter in effluents. This is particularly advantageous for wastewater with a low organic matter content.

Several systems have been developed based on N-S-C cycles, which combine processes for removing SO_4^{2-} and N, including Partial Nitrification/Anammox and S-dependent autotrophic denitrification (PNASD) (Yuan et al., 2020; Prachakittikul et al., 2016), Sulfate Reduction, Denitrification/Anammox, and Partial Nitrification (SRDAPN) (Kosugi et al., 2019), S-dependent autotrophic partial denitrification/Anammox (SPDA) (Wu et al., 2019), Biodegradation and Electron transfer process with S Integrated metabolism (BESI) (Wei et al., 2018), Anammox and S-dependent autotrophic denitrification (ASD) (Guo et al., 2016; Liu et al., 2015), and S-dependent Autotrophic denitrification and Nitrification Integrated (SANI) (Lau et al., 2006). Compared to conventional wastewater treatment methods, the integrated systems provide notable benefits, including the simultaneous elimination of N, S, and C compounds, a 90% decrease in sludge production, a 36% decrease in greenhouse gas (GHG) emissions and a 35% reduction in energy consumption (Lu et al., 2011), which confirms their sustainable development.

Table 2
Opportunities, limitations and challenges of the integrated SRAO and anammox process and the standalone SRAO process.

Topic	Process	
	Integrated SRAO	Standalone SRAO
Opportunities	1) simultaneous reduction of NH_4^+ and SO_4^{2-} 2) increased NH_4^+ oxidation compared to anmmox 3) increased the NH_4^+ removal rate compared to anammox 4) no carbon needed to reduce SO_4^{2-} 5) possible combination with partial nitritation	1) simultaneous reduction of NH_4^+ and SO_4^{2-} 2) increased SO_4^{2-} reduction compared to integrated SRAO 3) increased SO_4^{2-} removal rate compared to SRAO 4) no carbon nor NO_2^- needed to reduce SO_4^{2-}
Challenges and limitations	1) Limited understanding of the mechanism of SRAO and the microorganisms involved 2) Competition between AnAOB and SRAO bacteria 3) In non-limited NO_3^- conditions, SO_4^{2-} concentration may rise due to S-dependent autotrophic denitrification 4) AnAOB has a greater affinity for NO_2^- than SO_4^{2-} as an electron acceptor 5) Reduced SO_4^{2-} reduction when compared to a standalone SR process 6) Analytical methods for determining S compounds	1) Limited understanding of the mechanism of SR and the microorganisms involved 2) Potential functional SR bacteria have a slow growth rate 3) In non-limited NO_3^- conditions, SO_4^{2-} concentration may increase due to S-dependent autotrophic denitrification 4) Inadequate NH_4^+ removal efficiency without an additional electron acceptor such as NO_2^- 5) Analytical methods for determining S compounds

While the above technologies typically rely on multi-stage systems where each process operates independently, our research has explored the potential for accomplishing simultaneous NH_4^+ oxidation and SO_4^{2-} reduction in a single-stage system, which still poses various challenges. One critical area of investigation is the coexistence of AOB, AnAOB, SOB, and their competition for the same substrates. Moreover, to achieve optimal outcomes, it is essential to identify the microorganisms involved in each process and establish conducive conditions for their effective coexistence.

To overcome the limitations presented in Table 2, future research should focus on investigating the specific enzyme pathways and microbial communities responsible for SRAO. Advanced molecular techniques, such as metagenomics and metatranscriptomics, can be employed to unravel the complexity of this process. Additionally, the coexistence and competition dynamics between AnAOB and SRAO bacteria is crucial. Identifying the factors that drive competitive interactions and developing strategies to promote the coexistence of SO_4^{2-} reduction and NH_4^+ oxidation, such as optimizing reactor conditions or utilizing specialized microbial consortia, warrant exploration.

Optimal operating conditions and reactor configurations should be explored to effectively control and minimize SO_4^{2-} accumulation during the SRAO process, ensuring effective simultaneous removal of N and S compounds. Strategies to increase SO_4^{2-} reduction efficiencies, such as optimizing the ratio of NO_2^- to SO_4^{2-} or identifying microbial strains with a higher affinity to SO_4^{2-} as an electron acceptor, should be investigated. Additionally, the development of advanced analytical methods for real-time monitoring and analysis of S compounds in the SRAO process would be beneficial.

Addressing these limitations and furthering research in these areas will result in a deeper understanding of the SRAO process, enabling the optimization of their performance and facilitating their practical applications in wastewater treatment systems.

4. Conclusions

The higher SO_4^{2-} concentrations improved the AUR and SUR compared to the system without SO_4^{2-} supplementation, indicating linkages between the biological N-S removal pathways. On the other hand, the highest SUR values were obtained after 160 days of the operation in the absence of NO_2^- in BR_SR, while the AUR was significantly reduced. This means that S removal reflects a more independent nature, with NO_2^- being the key metabolite providing simultaneous S removal and anaerobic NH_4^+ oxidation. *Nitrosomonas* played the dominant role in the N metabolism, while *Thauera* (responsible for S-dependent autotrophic denitrification), in the S metabolism.

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.

Data availability

Data will be made available on request.

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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.2023.129264.

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Paper III

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The role of the combined nitrogen-sulfur-carbon cycles for efficient performance of anammox-based systems

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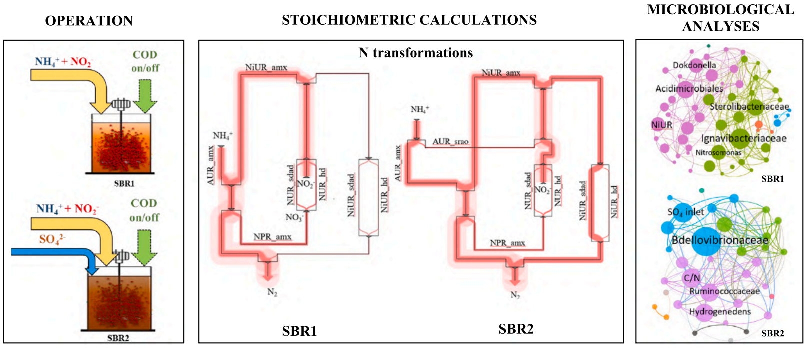
HIGHLIGHTS

- The effect of SO_4^{2-} and COD on anaerobic ammonia oxidation was studied in two SBRs.
- The SO_4^{2-} addition improved utilization rates of NH_4^+ and NO_2^-.
- AnAOB showed organotrophic traits, but alternating COD on/off was needed.
- *Candidatus* Brocadia and *Chloroflexi* played key roles in N-S-C metabolism.
- Wastewater rich in N-S-C can be sustainably treated in a single-stage system.

GRAPHICAL ABSTRACT



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ABSTRACT

The combined anammox/mixotrophic denitrification process was conducted in two granular sequencing batch reactors (SBRs) during a 200-day operation. Both reactors were fed with synthetic medium, but SBR2 was enriched with additional sulfate (SO_4^{2-}) which influenced sulfate reduction ammonium oxidation (SRAO) and heterotrophic reduction of SO_4^{2-} by sulfate reducing bacteria. It was hypothesized that the addition of SO_4^{2-} could positively impact the removal rates of N-S-C compounds. A low C/N ratio (0.4–1.6) was maintained to prevent inhibition of anaerobic ammonium oxidizing bacteria (AnAOB), and alternating chemical oxygen demand (COD) on/off conditions were used to regenerate AnAOB during COD-off phases and heterotrophic denitrifiers during COD-on phases. Stoichiometric analysis showed that introducing SO_4^{2-} in SBR2 enhanced the ammonium utilization rate, which was approximately 10 % higher compared to SBR1 in the final stage of the experiment (25.8 vs. 22.8 mg N/(g VSS·h)). The total nitrogen removal efficiencies ranged from 62 % to 99 % in both reactors, with SBR2 consistently exhibiting approximately 4 % higher efficiency than SBR1. In SBR2, the maximum overall SO_4^{2-} utilization efficiency reached 27 % under COD-off conditions, while overall COD utilization was almost complete under COD-on conditions. A strong correlation ($R^2 = 0.98$) was observed between SO_4^{2-} production and COD utilization. The key players responsible for N and S transformations in response to SO_4^{2-} addition were

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Candidatus Brocadia and *Chloroflexi* - *Anaerolineae*. This study highlights the potential to enhance the overall efficiency of N-S-C removal by implementing an integrated anammox/mixotrophic denitrification process. The combination of cycles emerges as a sustainable approach for treating wastewater rich in N-S-C compounds.

Abbreviations

%	a unit of the abundance of bacteria
°C	a unit of temperature
AnAOB	anaerobic ammonium oxidizing bacteria
AnUSB	anoxic upflow sludge bed
AUR	ammonium utilization rate
AUR_amx	ammonium utilization rate from anammox process
AUR_srao	ammonium utilization rate from sulfate reduction ammonium oxidation process
C	carbon
CH_3COONa	sodium acetate
COD	chemical oxygen demand, organic carbon
CUR	COD utilization rate
CUR_NO$_2^-$	COD utilization rate from NO_2^- in the heterotrophic denitrification
CUR_NO$_3^-$	COD utilization rate from NO_3^- in the heterotrophic denitrification
CUR_srb	COD utilization rate from sulfate reducing bacteria
DO	dissolved oxygen
GHG	greenhouse gas
HCl	hydrochloric acid
MBBR	moving bed biofilm reactor
mg/(g VSS·h)	a unit of utilization rate
mg/L	a unit of dissolved oxygen and concentrations
$MgSO_4$	magnesium sulfate
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
N	nitrogen
N_2	nitrogen gas
$NaNO_2$	sodium nitrite
NH_4^+	ammonium
NH_4Cl	ammonium chloride
NiUR	nitrite utilization rate
NiUR_amx	nitrite utilization rate from anammox process
NiUR_hd	nitrite utilization rate from heterotrophic denitrification
NiUR_sdad	nitrite utilization rate from sulfur dependent autotrophic denitrification
NO_2^-	nitrite
NO_3^-	nitrate
NPR/NUR	nitrate production/utilization rate
NPR_amx	nitrate production rate from anammox process
NUR_hd	nitrate utilization rate from heterotrophic denitrification
NUR_sdad	nitrate utilization rate from sulfur dependent autotrophic denitrification
PCoA	principal coordinate analysis
PD/A	partial denitrification/anammox
PN/A	partial nitritation/anammox
S	sulfur
SANI	Sulfate reduction-Autotrophic denitrification-Nitrification Integrated
SBR	sequencing batch reactor
SCC	Spearman's correlation coefficient
SDAD	S-dependent autotrophic denitrification
SO_4^{2-}	sulfate
SPR/SUR	sulfate production/utilization rate
SPR_sdad NO$_2^-$	sulfate production rate from NO_2^- in the sulfur dependent autotrophic denitrification
SPR_sdad NO$_3^-$	sulfate production rate from NO_3^- in the sulfur dependent autotrophic denitrification
SPR_other/SUR_other	sulfate production/utilization not directly associated with the N cycle
SRAO	sulfate reduction ammonium oxidation
SRB	sulfate reducing bacteria
SRDAPN	Sulfate Reduction, Denitrification/Anammox and Partial Nitrification
SRDN	Sulfate Reduction, Sulfur Denitrification and Nitrification
SRUSB	SO_4^{2-}/SO_3^{2-} reducing upflow sludge bed
SUR_srao	sulfate utilization rate from sulfate reduction ammonium oxidation process
SUR_srb	sulfate utilization rate from sulfate reducing bacteria
TN	total nitrogen
WWTP	municipal wastewater treatment plan

1. Introduction

Approaching carbon (C) neutrality, postulated in the updated European Union urban wastewater treatment directive, become a critical operational challenge for municipal wastewater treatment plants (WWTPs). To meet this challenge, conventional nitrogen (N) removal processes via nitrification-denitrification can be shifted to less energy intensive alternatives based on anaerobic ammonia oxidation (anammox), such as partial nitritation/anammox (PN/A) and partial denitrification/anammox (PD/A) (Du et al., 2023; Zhao et al., 2022). In addition, the N removal processes can be integrated with sulfur (S) conversions to provide alternative pathways for S-driven transformations, such as S-dependent autotrophic denitrification (SDAD) (Zou et al., 2016) and sulfate (SO_4^{2-}) reducing ammonium oxidation (SRAO) (L.-Y. Liu et al., 2021). This integration enhances both overall N removal efficiency and sustainability by reducing the need for external C (Zou et al., 2016; L.-Y. Liu et al., 2021).

To further enhance the sustainability of wastewater treatment, the N-S removal cycles can be combined with organic C (COD) removal in single systems (Kosugi et al., 2019; Wei et al., 2017; Qian et al., 2015). A few innovative processes have been developed based on this approach, including the Sulfate Reduction-Autotrophic Denitrification-Integrated Nitrification (SANI) (Wu et al., 2016), Sulfate Reduction, Sulfur Denitrification and Nitrification (SRDN) (Yamamoto-Ikemoto and Komori, 2003), and Sulfate Reduction, Denitrification/Anammox and Partial Nitrification (SRDAPN) (Kosugi et al., 2019). The latter process (SRDAPN), utilizes PN/A instead of full nitrification (as SANI and SRDN), which results in reduced operational costs compared to other technologies. Furthermore, a unique feature of the SRDAPN process is mixotrophic denitrification with COD and S compounds (Kosugi et al., 2019).

In mixotrophic systems, heterotrophic and autotrophic denitrifying bacteria coexist, leading to an enhanced denitrification capacity (Yánez et al., 2021). A concept of the mixotrophic system has emerged as a solution to mitigate the requirements for COD, minimize SO_4^{2-} accumulation, and prevent NO_2^- accumulation (Kong et al., 2016). Mixotrophy allows for the use of a wider range of C and S sources, facilitating more efficient conversion of nitrate (NO_3^-) to N_2. Moreover, it reduces the dependence on external C sources, as autotrophic denitrifiers can use S compounds as an energy source. This can result in cost savings and a more sustainable denitrification process (Yánez et al., 2021). Anaerobic ammonium oxidizing bacteria (AnAOB), such as *Candidatus Kuenenia* and *Candidatus* Brocadia, can thrive under mixotrophic conditions as these microorganisms possess relevant genes for metabolizing acetate and propionate (Feng et al., 2019). Indeed, Zhang et al. (2021) achieved efficient N removal (85 %) under mixotrophic conditions with a substantial contribution (nearly 50 %) of the *Planctomycetes* phylum.

To date, however, no study has combined mixotrophic denitrification with anammox in a single reactor to treat wastewater rich in NH_4^+, SO_4^{2-} and COD. In our previous study (Derwis et al., 2023), we investigated the coexistence of N-S cycle processes without the addition of COD in granular sludge sequencing batch reactors (SBRs). The introduction of higher concentrations of SO_4^{2-} led to improvements in both NH_4^+ and SO_4^{2-} utilization rates (AUR and SUR) compared to a system without SO_4^{2-} supplementation, indicating favorable interactions between biological N-S removal pathways. By integrating the N-S-C cycles in a single reactor, the complex interactions between N, S and C compounds can be exploited to optimize the removal efficiency of NH_4^+, SO_4^{2-} and COD simultaneously.

This study aimed to evaluate and compare the performance of the combined anammox/mixotrophic denitrification process in two parallel granular sludge SBRs with and without SO_4^{2-} addition. It was hypothesized that supplementary SO_4^{2-} in one SBR could positively impact the removal rates of N-S-C compounds by facilitating the activation of SRAO and heterotrophic SO_4^{2-} reduction. Moreover, variable COD on/off periods were implemented to foster suitable conditions for the growth of both AnAOB (COD off-dosing) and heterotrophic denitrifiers (COD on-dosing). The contributions of specific processes were determined based on stoichiometric reactions and the observed dynamics of N compounds (NH_4^+, NO_2^-, NO_3^-), SO_4^{2-}, and COD. Moreover, changes in the bacterial community structure were observed in response to different process conditions and the key bacterial groups, responsible for the combined N-S-C metabolism, were identified. The findings from this comprehensive analysis can provide valuable insights and guidance for optimizing the combined anammox/mixotrophic denitrification process. The potential benefits of such optimization include improved treatment efficiency, lower operating costs and enhanced resource recovery in WWTPs treating S-rich streams. Wastewater streams are often complex and can contain a mixture of N, S and C compounds, as seen in landfill leachates or industrial wastewater from mining operations or paper production (Ma et al., 2022).

2. Materials and methods

2.1. Inoculum biomass and laboratory setup

The inoculum biomass used for the experiments was obtained from a sidestream granular deammonification system at a large municipal WWTP (200,000 PE) situated in the city of Słupsk (Poland). 30 L of inoculum biomass was sourced from a full-scale sidestream granular deammonification system, with a working volume of 700 m^3, located at a large WWTP (200,000 PE) in the city of Słupsk, Poland. The MLSS concentration in the seed sludge was 4500 mg/L. The laboratory setup consisted of two parallel, automated SBRs, each possessing a working volume of 15 L. Detailed information on the design and operational methodology of the setup can be found in the work of Al-Hazmi et al. (2020).

2.2. Operational conditions

The reactors were operated for 200 days with the process temperature consistently maintained at 30 ± 1 °C. The pH level was maintained within the range of 7.5–7.8, which had been identified as optimal for anammox in our previous studies (Al-Hazmi et al., 2020). The pH was controlled automatically by adding 6 M hydrochloric acid (HCl) solution based on the readings of a pH probe (Endress + Hauser EH CPS 471D-7211, Basel, Switzerland). The reactors were not aerated, and the measured dissolved oxygen (DO) concentration did not exceed 0.2 mg/L. The SBRs were fed with a synthetic medium with microelements, as in the previous study of Al-Hazmi et al. (2020). Crucial components, i.e. NO_2^-, NH_4^+, COD and SO_4^{2-}, were supplied in the form of sodium nitrite ($NaNO_2$), ammonium chloride (NH_4Cl), sodium acetate (CH_3COONa), magnesium sulfate ($MgSO_4$), respectively. Standardized measurement procedures were adopted, including regular calibration of probes and maintaining consistent operating conditions.

In the course of the study, no excess sludge was removed from the reactors and the mixed liquor suspended solids (MLSS) concentrations were continuously increasing from 2000 to 4000 mg/L. The volatile fraction (MLVSS) concentrations ranged from 1300 to 2700 mg/L, which constituted 65–68 % of MLSS. The identical hydraulic retention time (HRT) was kept in both reactors and ranged from 0.5 to 3 d, proportionally to the influent flow rate (Fig. 1f). The shorter HRTs were adopted in response to the increasing process rates in the course of the study.

The dynamic variations in the influent concentrations of NH_4^+, NO_2^-, NO_3^-, COD and SO_4^{2-} are illustrated in Fig. 1a–e. The influent NH_4^+ and NO_2^- concentrations varied in the range of 70–200 mg N/L and 90–260 mg N/L, respectively (Fig. 1a,b). To optimize anammox reaction, a consistent NO_2^-/NH_4^+ ratio of approximately 1.3 was maintained. During phases with COD addition, the influent concentrations of NH_4^+ and NO_2^- were elevated. This augmentation was necessary not only for anammox, but also to support mixotrophic denitrification. Conversely, in periods without COD addition, the concentrations of NH_4^+ and NO_2^- were reduced in the anammox process. Ongoing adjustments of NH_4^+ and NO_2^- concentrations were based on the attained AUR during the study to maintain optimal activity of AnAOB. SO_4^{2-} was not added to SBR1, while the influent SO_4^{2-} concentration introduced to SBR2 varied within the range of 150–400 mg S/L (Fig. 1e). During the study period, alternating phases of COD dosing (I, III, V, VII) and no COD dosing (II, IV, VI) were implemented in both reactors. In general, the influent COD concentrations were maintained at 250 mg COD/L. Only during phase V, the concentrations were gradually increasing to a maximum of 500 mg COD/L (Fig. 1d).

2.3. Determination of specific process rates based on stoichiometric calculations

Due to the complexity of concurring processes, the contributions of specific process utilization/production rates cannot be determined from isolated activity tests. Therefore, these contributions were assessed based on stoichiometric reactions and the maximum slopes of several variables, including NH_4^+ utilization rate (AUR), NO_2^- utilization rate (NiUR), NO_3^- production/utilization rate (NPR/NUR), COD utilization rate (CUR), and SO_4^{2-} production/utilization rate (SPR/SUR). All calculated process rates underwent rigorous statistical analysis and were subjected to N-S-COD mass balances (Table S1). In addition, the efficiencies of N, SO_4^{2-} and COD removal were calculated based on the influent and effluent concentrations, with the negative values indicating SO_4^{2-} production in the periods with COD dosing.

The stoichiometric analysis, coupling the forementioned rates, was carried out with the MS Excel SOLVER tool assuming the following reactions:

1) Anammox:

$$NH_4^{+} + 1.32NO_2^{-} \rightarrow 1.02N_2 + 0.26NO_3^{-} + 2H_2O$$

2) SRAO:

$$3SO_4^{2-} + 4NH_4^{+} \rightarrow 3S^0 + 2NO_2^{-} + N_2 + 4H_2O$$

3) SDAD with NO_3^-:

$$5S^0 + 6NO_3^{-} + 2H_2O \rightarrow 5SO_4^{2-} + 3\,N_2 + 4H^{+}$$

4) SDAD with NO_2^-:

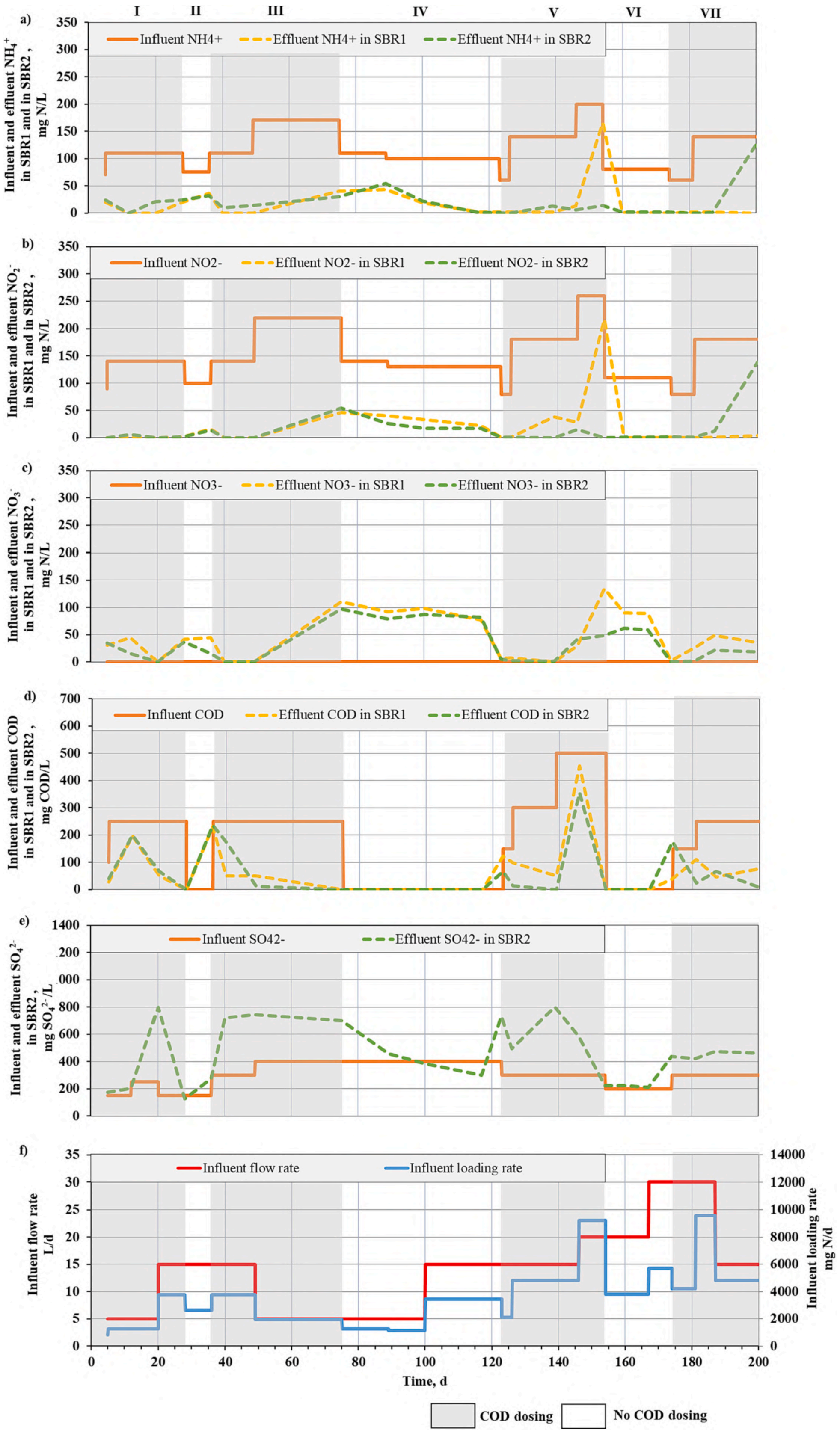

Fig. 1. Influent and effluent concentrations of a) NH$_4^+$, b) NO$_2^-$, c) NO$_3^-$, d) COD, e) SO$_4^{2-}$; f) Influent flow rate and influent loading rate.

$$3S^0 + 6NO_2^- \rightarrow 3SO_4^{2-} + 3\ N_2$$

5) Heterotrophic SO_4^{2-} reduction:

$$CH_3COO\blacksquare + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$$

6) Heterotrophic denitrification with NO_3^-:

$$5CH_3COO^- + 8NO_3^- + 3H^+ \rightarrow 10HCO_3^- + 4\ N_2 + 4H_2O$$

7) Heterotrophic denitrification with NO_2^-:

$$5CH_3COO^- + 4NO_2^- + 6H^+ \rightarrow 2\ N_2 + 5CH_3COO^- + 3H_2O$$

The separate catabolic and anabolic reactions have been added to the Supplementary Material.

With this set of reaction equations, allowed for the overall rates were decomposed and assigned to the individual processes under the following assumptions:

1. Anammox, mixotrophic denitrification, SRAO, heterotrophic SO_4^{2-} reduction, and SO_4^{2-} production (other than SDAD) were considered in the phases with COD addition,
2. Anammox, SDAD, SRAO, and SO_4^{2-} reduction (other than SRAO) were considered in the phases without COD addition,
3. SRAO was confirmed by dedicated activity tests in the presence of SO_4^{2-} (Grubba et al., 2022); in the present study, the process was neglected in SBR1 (without SO_4^{2-} addition),
4. AUR associated with anammox (AUR_{AMX}) was the same in both reactors,
5. The processes of other SO_4^{2-} production (SPR_other) were not directly associated with the N cycle and might include oxidation of reduced S species using trace amounts of leaked oxygen (SPR_other were estimated based on the mass balance of S transformations during the COD-on periods),
6. The processes of other SO_4^{2-} utilization (SUR_other) were not directly associated with the N cycle and might include SO_4^{2-} reduction using bacterially produced organic matter (SUR_other were estimated based on the mass balance of S transformations during the COD-off periods).

Calculations were made on the days of microbiological sampling (49 d, 75 d, 126 d, 154 d, and 187 d) to compare trends in the process rates and shifts in the microbial composition. The results were illustrated using Sankey diagrams developed in the simulation platform GPS-X ver. 7.0 (Hydromantis, Canada).

2.4. Microbiological analyses

Biomass samples for microbiological examination were collected from the inoculum, and then from both reactors on 49, 75, 126, 154 and 187 d. DNA was extracted with FastDNA Spin kit for soil (MP Biomedicals, USA).

The taxonomic composition of the analyzed samples was determined by sequencing the V3-V4 hypervariable regions of the 16S rRNA gene. High-throughput Illumina sequencing was performed with 341F (5′-CCT ACG GGN GGC WGC AG-3′) and 785R (5′-GAC TAC HVG GGT ATC TAA TCC-3′) primers (Klindworth et al., 2012) and Q5 Hot Start High-Fidelity 2× Master Mix following the manufacturer's manual. The sequencing reaction was performed on a Miseq sequencer with a MiSeq Reagent Kit v3 (Illumina, USA), applying pair-end technology with a read length of 300 base pairs. The raw sequencing data were analyzed using the QIIMEII package. Pairs of sequences were merged using the fast-join algorithm. Unmerged sequences were excluded from further analysis. Low quality sequences (under 20) were filtered by the Cutadapt algorithm. Chimeric sequences were detected and excluded from analyses using USEARCH. 16S rRNA OTUs that were picked from the sequences were clustered at 97 % identity and trimmed to span only the 16S rRNA V3-V4 region flanked by the sequencing primers. For further analysis of samples, trimmed reads were uploaded to EzBioCloud. Using the EzBioCloud MTP pipeline, the OTU numbers and the rarefaction curve were computed, and the alpha-diversity indices (Chao1, Shannon) were calculated to estimate the richness of each group. Statistical significance was tested in this case Wilcoxon rank-sum test. Phylogenetic distance between groups was calculated using generalized UniFrac distances that unify weighted UniFrac and unweighted UniFrac distances. The PERMANOVA, a distance-based non-parametric test procedure, was applied to assess the significance of genetic distance difference by calculation of pseudo-F ratios, and finally to obtain the P-values. Beta diversity was presented by principal coordinate analysis (PCoA).

The links between operating parameters and bacterial taxa abundance were explored by network analysis based on significant correlations ($P < 0.01$) and Spearman's correlation coefficient (SCC) values higher than 0.70. The network analysis was on the Gephi platform, where nodes were connected by edges if a statistically significant relationship existed between them. The framework of the network was defined through modularity analysis, based on the edges linking the nodes. The size of a node in these networks reflected its betweenness centrality, which measures how often a specific node appears on paths connecting two other nodes. A high betweenness centrality signifies a central position of the node within the network, whereas a low value suggests it occupies a more peripheral spot.

2.5. Analytical methods

Mixed liquor samples were immediately filtered through disposable glass microfiber filters MFV-3 (47 mm diameter) from Sartorius (Germany). Concentrations of NO_3^-, NO_2^-, NH_4^+, COD and SO_4^{2-} were determined using the DR 3900 spectrophotometer using the dedicated cuvette tests from Hach Lange GmbH (Dusseldorf, Germany). Cuvette measurements were closely monitored for any deviations, and if necessary, measurements were repeated to ensure accuracy. Furthermore, certified cuvette tests were used. These certificates include standard deviation, coefficient of variation, and the relationship between nominal and actual values. The standard deviation for each measured and calculated value can be found in the Supplementary Material (Table S2). The MLSS and MLVSS concentrations were determined in accordance with the Standard Methods (Greenberg et al., 2005).

3. Results

3.1. Exploring process indicators and compound relationships in N removal pathways

Fig. 2 shows the trends of AUR and NiUR in SBR1 and SBR2 over the 200-day operational period. Overall, elevated AURs and NiURs occurred in SBR2 with SO_4^{2-} addition, particularly during phases I, III, V, and VII, when COD was concurrently dosed. In contrast, the AURs and NiURs were lower during phases II, IV, and VI, with the absence of COD addition.

The maximum attained AURs were 22.8 mg N/(g VSS·h) in SBR1 and 25.8 mg N/(g VSS·h) in SBR2 during phase VII. Similarly, the NiURs reached their peak at 24 mg N/(g VSS·h) in SBR 1 and notably higher at 34.6 mg N/(g VSS·h) in SBR2 during the same phase. Moreover, the AUR and NiUR showed a positive trend during the phases with COD addition, subsequently declining upon cessation of COD dosing. Although the last phase (VII) showed the highest AUR and NiUR, but the rates experienced a subsequent breakdown attributed to the prolonged presence of COD.

The total nitrogen removal efficiencies ranged from 62 % to 99 % in both reactors, with SBR2 consistently exhibiting approximately 4 % higher efficiency than SBR1. In addition, SBR2 demonstrated higher

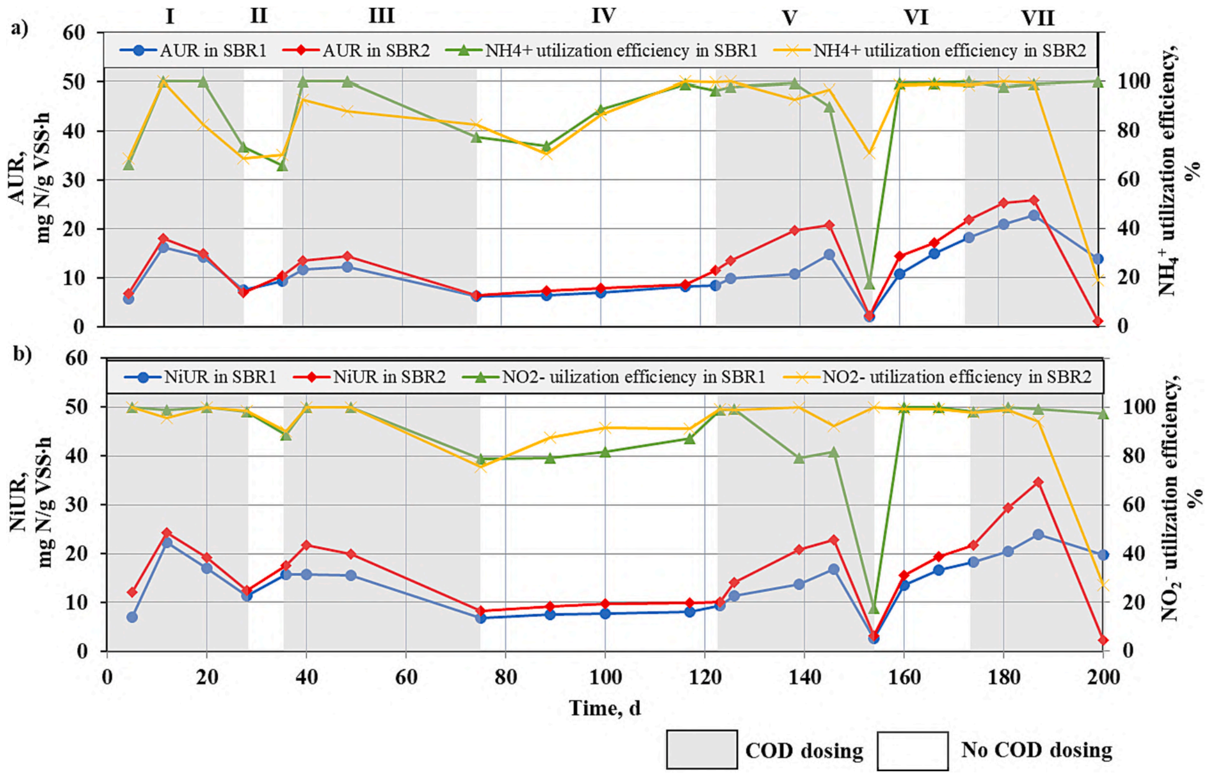

Fig. 2. Variations in the N transformation rates in both reactors: a) AUR, b) NiUR.

NH_4^+ and NO_2^- utilization efficiencies. The NH_4^+ utilization efficiencies in SBR1 under COD-on and COD-off conditions ranged from 66 to 100 % and 69–99 %, respectively. The corresponding efficiencies in SBR2 were 70–100 % and 69–100 %. For NO_2^- utilization efficiency, SBR1 achieved 79–100 % under COD-on conditions and 74–99 % under COD-off conditions. SBR2 consistently outperformed SBR1 with 90–100 % efficiencies under COD-on conditions and 75–100 % efficiencies under COD-off conditions.

When comparing the CURs between SBR1 and SBR2, significantly higher values (minimum 20 %) were consistently observed in SBR2

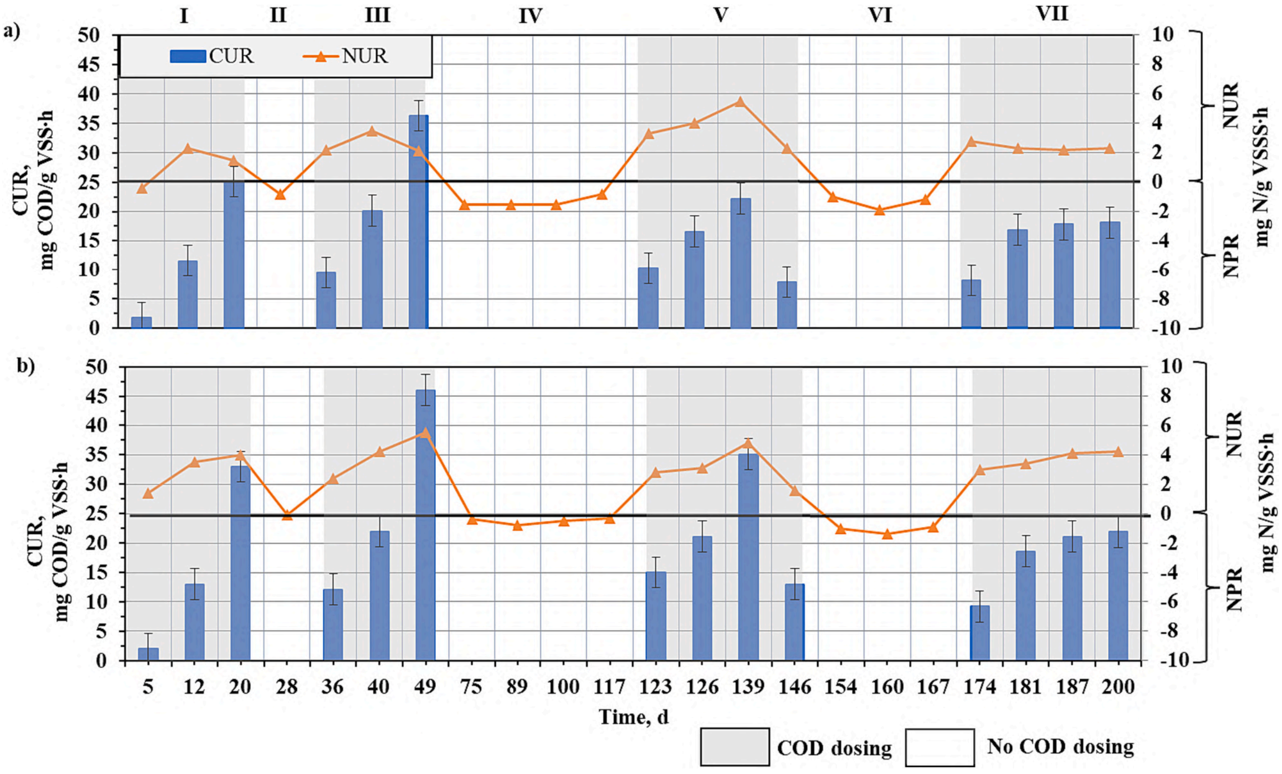

Fig. 3. Variations in the CURs and NPR/NUR ratios: a) in SBR1, b) SBR2 (note: NPR is a negative NUR).

during the phases with COD addition. The highest CURs occurred in both reactors in phase III, i.e., 46 mg COD/(g VSS·h) in SBR2 vs. 36.3 mg COD/(g VSS·h) in SBR1. In phase V, the influent COD concentrations were gradually increasing compared to phases I and III to investigate, which resulted in lower CURs. The purpose of increasing the COD concentration was to check the effect of gradually increasing the COD concentration from 0 to 500 mg COD/L on the rates of the processes. This was most evident on day 146 when the maximum influent COD concentration was 500 mg COD/L and the CUR decreased from 22.2 mg COD/(g VSS·h) to 7.9 mg COD/(g VSS·h) in SBR1, and from 35.1 mg COD/(g VSS·h) to 13 mg COD/(g VSS·h) in SBR2.

NO_3^- production was observed in the phases without COD addition, while the observed NO_3^- reduction occurred in phases with COD addition (Fig. 3). The NPRs were low in both reactors, i.e., 0.4–1.9 mg N/(g VSS·h) in SBR1 and 0.1–1.4 mg N/(g VSS·h) in SBR2. However, the NURs were 1.5–5.5 mg N/(g VSS·h) in SBR1 and 1.4–5.4 mg N/(g VSS·h) in SBR2.

SO_4^{2-} production occurred during periods when COD was added, while SO_4^{2-} reduction took place during periods without COD addition (Fig. 4). The responsible processes for these changes are described in assumptions 1–2 in Section 2.3. The highest SPR in SBR2 increased from 28 mg S/(g VSS·h) during phase I to 45.5 mg S/(g VSS·h) during phase III, and then the rate was decreasing to 33.5 mg S/(g VSS·h) during phase V and 20.9 mg S/(g VSS·h) during phase VII. The SURs in SBR2 were much lower and ranged from 2.9 mg S/(g VSS·h) on day 154 (phase VI) to 7 mg S/(g VSS·h) on day 75 (phase IV). In terms of the efficiency, the maximum SO_4^{2-} production efficiency in SBR2 was 44 % during phase I, 60 % during phase III, 129 % during phase V, and 39 % during

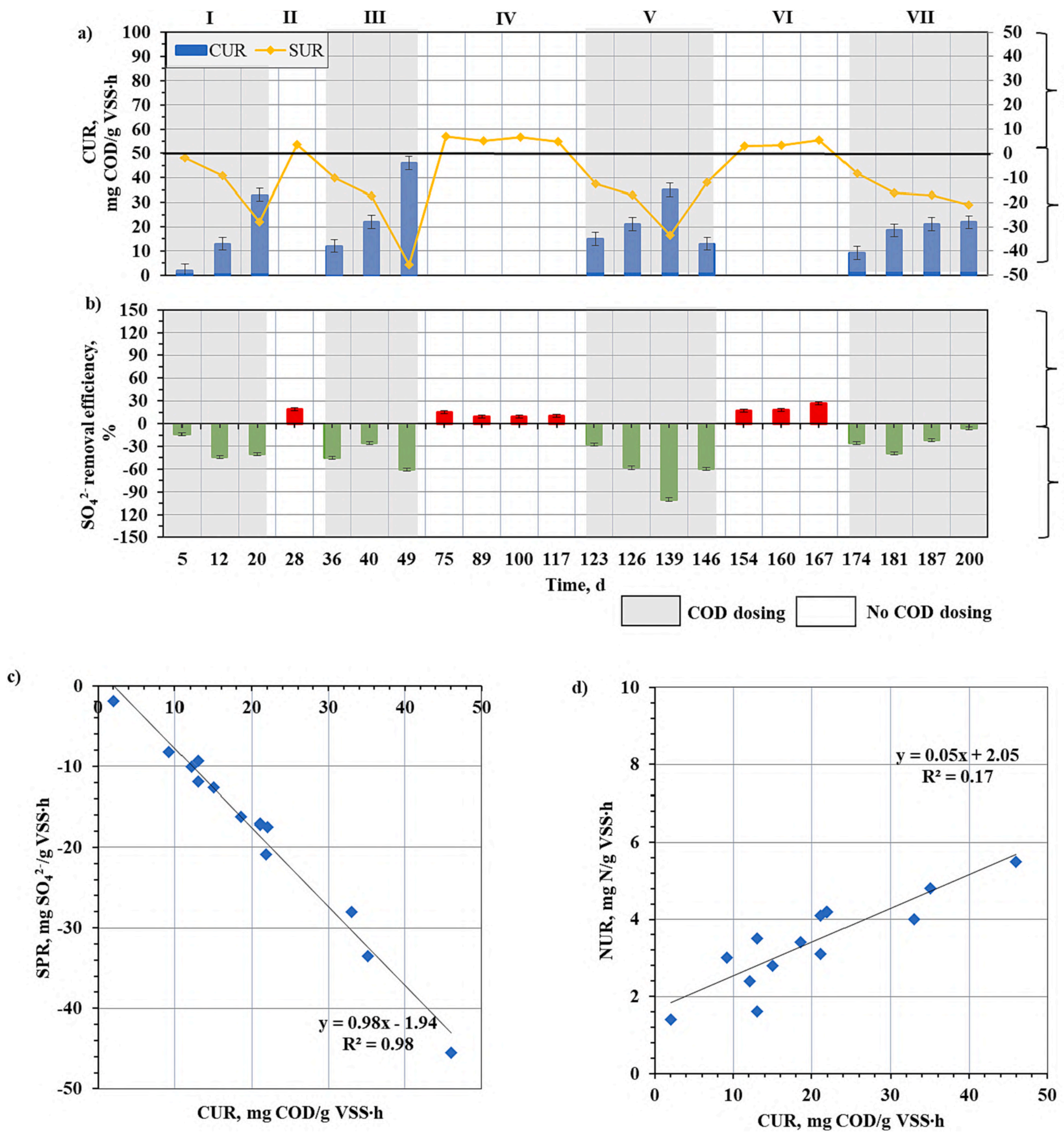

Fig. 4. C and S transformations in SBR2 with COD addition: a) variations in the CUR and SPR/SUR, b) variations in the SO_4^{2-} production/removal, c) relationship of CUR and SPR, d) relationship of CUR and NUR (note: SPR is a negative SUR).

phase VII. On the other hand, the highest efficiency of SO_4^{2-} reduction in the absence of COD, reaching 27 %, was observed in phase VI.

The relationship between the CUR and SPR was linear with the slope close to 1.0 and exhibited excellent correlation ($R^2 = 0.98$) (Fig. 4c). In contrast, the correlation between CUR and NUR was much poorer with $R^2 = 0.17$ (Fig. 4d).

3.2. Calculations of the contributions of specific processes based on stoichiometric reactions and observed process rates

Fig. 5 shows a graphical representation of N, S, and COD transformations (Sankey diagrams), which were estimated in both reactors based on the stoichiometric analysis on day 187. Similar diagrams for the remaining days (49, 75, 126 and 154) are shown in the Supplementary Material (Fig. S1). The optimization process yielded highly accurate results with a single set of stoichiometric coefficients derived from Eqs. (1)–(7). The measured and calculated values of rates and ratios, including AUR, NiUR, NPR/NUR, SPR/SUR and CUR, were matched very accurately, as total absolute errors did not exceed 0.13 mg/(g VSS·h) in the all analyzed scenarios. However, noticeable differences between SBR1 and SBR2 emerged due to the SO_4^{2-} addition and alternating COD on/off phases.

In the absence of COD, SBR1 primarily exhibited anammox, while SBR2 displayed a dual mechanism involving anammox along and a minor contribution from SDAD in reducing NO_2^- and NO_3^- on days 75 and 154. The addition of COD increased the total AUR in both reactors on days 49, 126 and 187. In all these phases, the NURs and NiURs remained elevated in comparison with the COD off phases. The SBR2 exhibited the NiUR from mixotrophic denitrification that was twice as high as that in SBR1, accompanied by sustained high levels of the NUR. Furthermore, the involvement of SRAO contributed to the increased AUR in SBR2 on days 49, 126 and 187.

In SBR1, on days 75 and 154, transformations involving S compounds were modest, primarily linked to N cycle-independent reductions of SO_4^{2-}. On the other hand, in SBR2, the share of SDAD increased for both the denitration and denitrition phases on days 75 and 154. Furthermore, during the COD on phases, both reactors exhibited an increased share of SDAD, which influenced the production of SO_4^{2-} through the utilization of NO_2^- and NO_3^- on days 49, 126, and 187. This production reached >30 times higher levels in SBR2 on day 187 due to SO_4^{2-} addition. Moreover, the presence of COD influenced SO_4^{2-} production independent of the N cycle in both reactors. In SBR2, the addition of SO_4^{2-} led to reduction of SO_4^{2-} through the activity of SRB and the SRAO reaction. In SBR2, the latter reaction contributed to SO_4^{2-} removal more than twice as effectively as SRB.

With regard to COD transformations, the CUR in SBR1 was driven by the heterotrophic denitrification reaction, resulting in a progressive decline in the proportion of NO_2^- and a corresponding rise in the proportion of NO_3^- on days 49, 126 and 187. The CUR attributed to NO_3^- was comparable in both reactors, while SBR2 demonstrated a higher CUR attributed to NO_2^-. Additionally, in SBR2, a noticeable increase was observed in the share of CUR related to SRB activity.

Overall, the addition of SO_4^{2-} in SBR2 led, in the final stage, to the integration of SRAO processes and heterotrophic reduction of SO_4^{2-} involving SRB in the N-S-C cycle (Fig. 5). Concurrently, the reduction of SO_4^{2-} to reduced S forms triggered SDAD and SO_4^{2-} production in SBR2. Furthermore, on day 187, the second phase of heterotrophic denitrification (NO_2^- - N_2) was inhibited in the favor of SDAD, but the first phase (NO_3^- - NO_2^-) was mainly caused by heterotrophic changes. At the same time, the CUR resulted from the utilization of NO_3^- by heterotrophic bacteria and the activity of SRB.

From the observations, it is evident that introducing SO_4^{2-} into SBR2 led to a significant increase in SDAD conversions, in contrast to SBR1. This addition also boosted NH_4^+ oxidation and SO_4^{2-}reduction, attributed to the SRAO process. Furthermore, in the presence of COD, there was a noticeable increase in anammox activity. Alongside SRB, the denitrification processes, particularly heterotrophic denitrification, were more pronounced than in phases without supplementary COD.

3.3. Shifts in the microbial composition in the SBRs

Microbial investigations revealed noticeable shifts in the bacterial community structure in response to the modifications in the feeding patterns and availability of COD and SO_4^{2-}. The experimental period could be divided into two main stages (Fig. 6a). In the initial stage of approximately one moth, the biomass from both reactors showed a relatively close genetic distance (yellow cluster). The subsequent stage was characterized by prolongated COD feeding, followed by the suppression of COD addition, as well continuous SO_4^{2-} supply to SBR 2. These specific conditions initialized direct speciation of the bacterial communities between the reactors, which was reflected by the separated clusters (green and red) indicated by the PCoA. The addition of COD appeared to promote the bacterial biodiversity, but when combined with the supply of SO_4^{2-}, a decrease in biodiversity (Chao1 index) was observed in SBR2 (Fig. 6b).

At the phylum level, representatives of *Proteobacteria* and *Chloroflexi* were the dominant components of the bacterial community, with the average abundances of up to 30 % in both reactors. Relatively numerous were also *Bacteroidota* (almost 20 %) and *Planctomycetota* (6–9 %) (Fig. S2).

At the genus level, members of the *Anaerolineae* family were most abundant (Fig. S3), including the RBG-13-54-9 genus in SBR2 (10.99 %) and the C10-SB1A genus in SBR1 (8.55 %). Other genera exhibiting high abundances belonged to the *Ignavibacteriales* (PHOS-HE36) and *Burkholderiales* (*Denitratisoma* sp.) orders. Both bacteria were identified as potential denitrifiers and showed higher prevalence in SBR1 compared to SBR2. Specifically, the abundances of PHOS-HE36 and *Denitratisoma* sp. were 7.23 % vs. 5.35 % and 6.08 % vs. 5.07 %, respectively.

The abundance of *Candidatus* Brocadia was high in both reactors. In SBR1, these bacteria accounted for 3.81 %, while in SBR2, they were almost two times more abundant (approx. 7.53 %). *Candidatus* Brocadia is widely occurring anammox bacteria oxidizing ammonium under anoxic conditions. The abundance of the main ammonia oxidizing bacteria - *Nitrosomonas* was significantly lower in both reactors. These bacteria were more common in SBR1 (0.48 %) than in SBR2 (0.11 %). Nitrite oxidizers were exclusively represented by *Nitrospira* with the negligible abundance in both reactors (0.26 % and 0.35 % in SBR1 and SBR2, respectively).

To show the interactions between bacterial taxa and operational parameters, co-occurrence network analysis was performed separately for SBR1 and SBR2 (Fig. 7). The color and size of each node refers to its modularity class parameter and betweenness centrality, respectively. Strong (Spearman's correlation coefficient > 0.70) and significant ($P < 0.05$) correlations were identified. The SBR1 network had more nodes (56) and edges (232) compared to SBR2 (35 and 146, respectively). The average path length, representing the number of edges in the shortest path between pairs of nodes, was longer in the SBR1 network than in the SBR2 network (3.03 vs. 2.38). There were five modules in SBR1 and nine modules in SBR2. The main nodes in SBR1, with the values of betweenness centrality above 150, were bacteria from the following taxa: *Ignavibacteriaceae* (239), *Acidimicrobiales* (196), *Sterolibacteriaceae* (181), *Nitrosomonas* (163), *Dokdonella* (156), and additionally NiUR (151). These edges are considered the main hubs responsible for network functioning control. Bacteria from *Ignavibacteriaceae* family are the core components of module 3, which is directly linked with *Nitrosomonas, Nitrospira* and *Sterolibacteriaceae*. The abundance of *Ignavibacteriaceae* is relatively high (mean value of 7.2 %) and positively correlated with *Nitrosomonas, Nitrospira* and *Sterolibacteriaceae*. The second important module is represented by *Acidimicrobiales* order, which is linked with *Dokdonella*. The NiUR is positively correlated with *Limisphaera*, and negatively correlated with *Ignavibacteriaceae* and *Sterolibacteriaceae*.

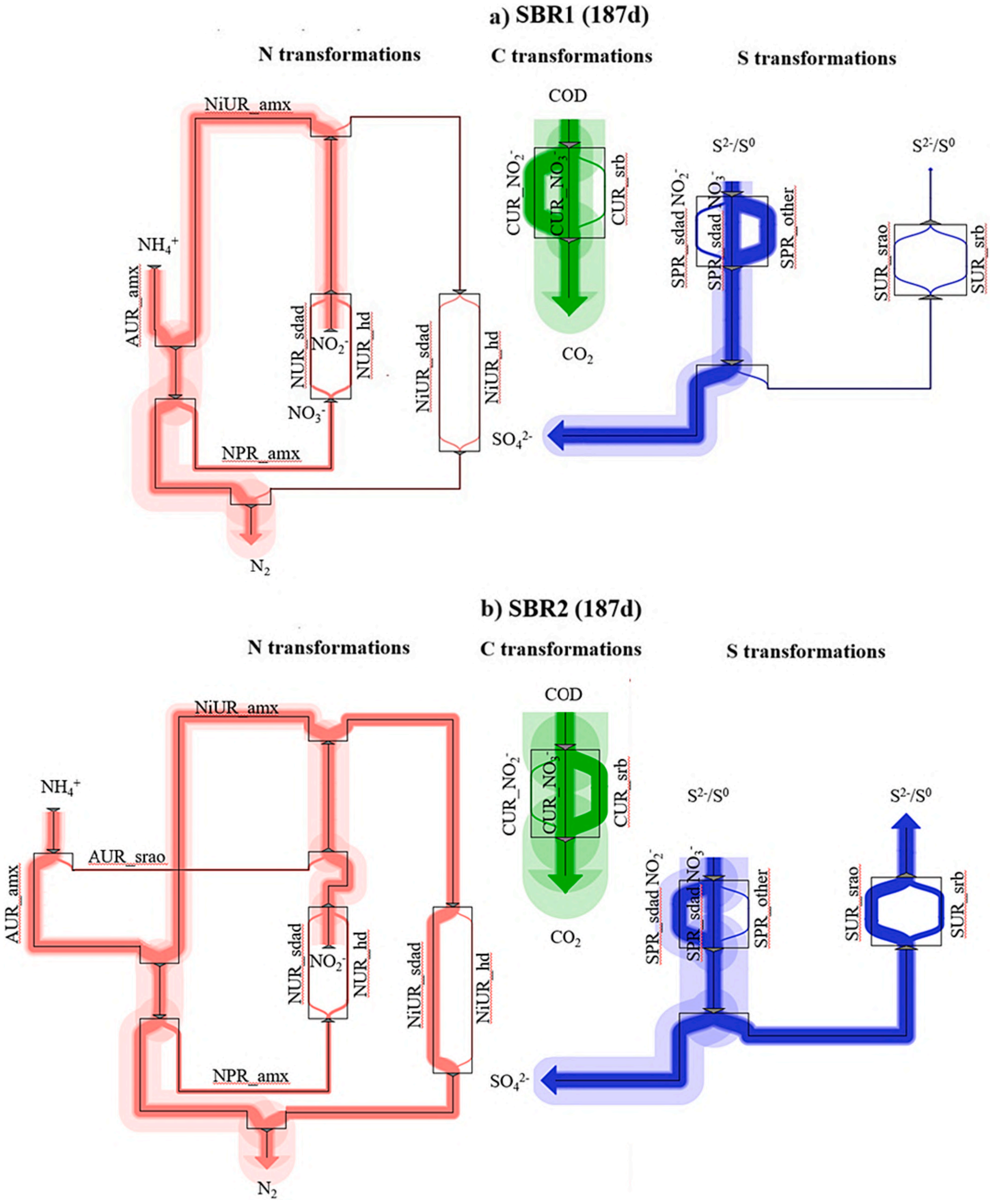

Fig. 5. Sankey diagram showing the transformation pathways of N, S and COD based on the stoichiometric analysis on day 187 a) in SBR1, b) in SBR2 (thickness of the arrows is proportional to the rate).

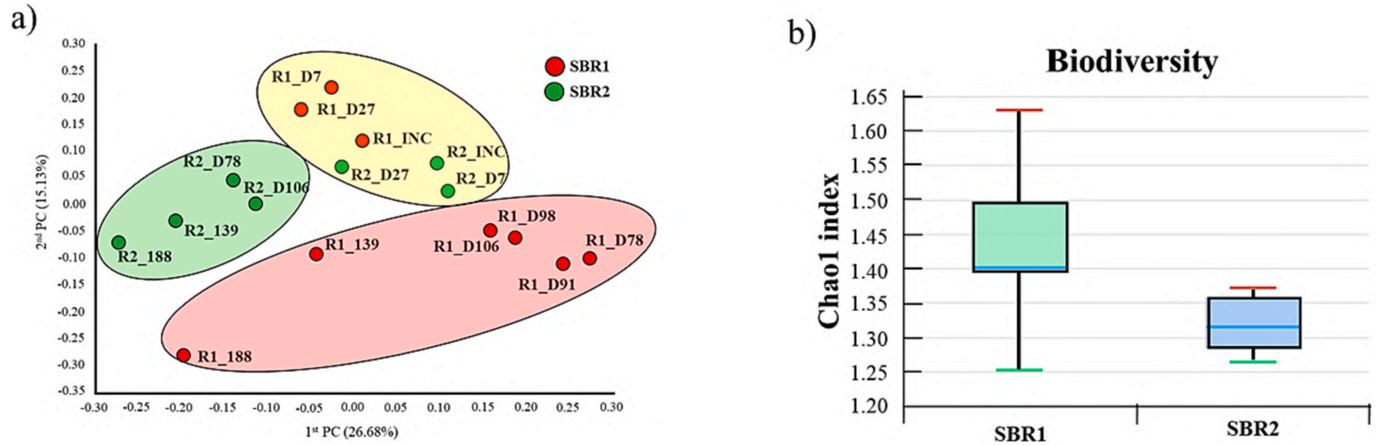

Fig. 6. a) Differentiation of the bacterial community structure between SBR1 and SBR2, analyzed by the PCoA of 16S rRNA sequencing data at the genus level, b) Chao 1 biodiversity index for SBR1 and SBR2.

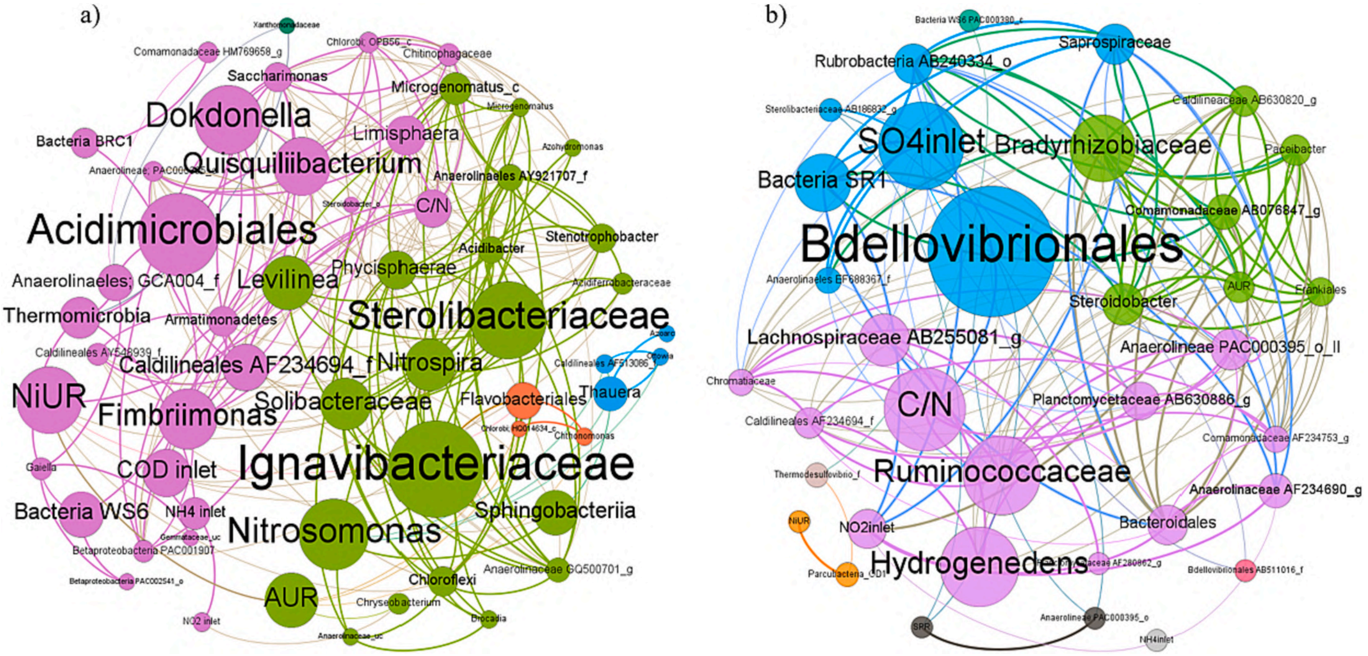

Fig. 7. Co-occurrence network analysis of bacteria and operational parameters; network modularity is represented by differently colored nodes; node size represents betweenness centrality: a) in SBR1, b) in SBR2.

The main nodes in SBR2, with the values of betweenness centrality above 50, were bacteria *Bdellovibrionales* (105) and the influent SO_4^{2-} concentration (64) from module 2, and bacteria *Hydrogenedens* (56) with *Ruminococcaceae* (55) from module 1. The abundance of *Bdellovibrionales* was low (mean value of 0.35 %), but it was strongly and positively correlated with the C/N ratio, and negatively correlated with the influent SO_4^{2-} concentration and SPR. *Ruminococcaceae* showed the high mean abundance (5.0 %) and a negative correlation with the influent SO_4^{2-} concentration. Other bacterial genus negatively correlated with the influent SO_4^{2-} was from *Planctomycetaceae* family.

4. Discussion

4.1. Effect of SO_4^{2-} addition on the microbial community performing integrated C-N-S removal

The findings of the present study suggest that in both reactors, NH_4^+ removal was primarily driven by the anammox process, evident from a significant contribution of AnAOB in both reactors. However, the relative abundance of *Candidatus* Brocadia was approximately twofold higher in SBR2 (7.1 %) compared to SBR1 (3.6 %). Throughout the study period, SBR2 consistently demonstrated elevated AURs and NiURs compared to SBR1, attributed to the presence of SO_4^{2-}. This improved performance of SBR2 supports the hypothesis that SO_4^{2-} had a beneficial effect as an additional electron acceptor, thereby fostering the proliferation of bacteria responsible for anaerobic NH_4^+ oxidation. In the study by Kosugi et al. (2019) in SRDAPN to remove organic matter and N, the coexistence of anammox and denitrification was predominant in the presence of a NO_2^--rich medium and S compounds. This was confirmed by the high content of AnAOB, heterotrophic and autotrophic denitrifying bacteria (mixotrophic denitrification) in the anoxic column to which organic substances and S^{2-} were introduced. *Candidatus* Brocadia was identified within the NO_2^--fed reactor, indicating the coexistence of AnAOB with denitrification bacteria, even under conditions of elevated S^{2-}levels. Moreover, Jenni et al. (2014) and Wang et al. (2021) validated the advantageous role of specific AnAOB strains, highlighting the influence of S^{2-}-mediated DNRA and specific gene correlations (nrfA and dsrA) in driving anammox activity.

The network analysis further unveiled significant interactions between the abundance of specific taxa and the efficiency of N and S removal. Many of the identified bacteria are commonly found in activated sludge microbiome. Notably, in SBR1, the dominant *Ignavibacteriaceae* functioned as facultative heterotrophs, playing an important role in N removal (Park et al., 2021), whereas *Acidimicrobiales* were linked to N conversions in an anammox reactor (de Almeida Fernandes et al., 2018). In SBR2, the predominance of the bacteria from *Bdellovibrionales* order, including the *Bdellovibrionota* phylum, known as specialized predators of a wide spectrum of gram-negative bacteria, indicated their influence on shaping the activated sludge microbial community (Lovering and Sockett, 2021).

However, the relationship between these dominant bacteria and SO_4^{2-} conversion remains unclear. It is hypothesized that in the present study, *Thermomonas* benefitted from the addition of SO_4^{2-}, thereby fostering the development of SDAD. A similar observation was made by Huang et al. (2019), who attributed the dominance of *Thermomonas* cultures to the SDAD process under S^0 and NO_2^- amendment conditions. In fact, existing literature suggests that the sequential transformations of S through SDAD and SRAO may occur in the presence of denitrifying heterotrophs (Grubba et al., 2022).

4.2. Effect of alternating COD on/off conditions on the microbial community performing integrated C-N-S removal

The present study shows a strong correlation between SO_4^{2-} production and COD utilization ($R^2 = 0.98$), implicitly due to mixotrophic denitrification by SDAD and increased activity of autotrophic denitrifiers using S^{2-}/S^0 and heterotrophs using COD. The investigation into the effect of alternating COD on/off conditions on the microbial community in the integrated C-N-S system revealed significant insights, showing similarities and differences with other studies. The study of Z. Liu et al. (2021) evaluated the influence of organic C sources in a combined SDAD-anammox system and underscored the predominance of *Proteobacteria*, which is consistent with our findings (>30 %) (Fig. S2). Small abundances of *Armatimonadetes, Planctomycetota, Acidobacteria*, and *Planctomycetes*, detected at <3 %, were similar to our results. In contrast, *Bacteroidetes* exhibited a higher representation (24–42 %) and *Chloroflexi* a lower share (7–11 %) compared to our study.

Proteobacteria have been shown to play an important role in heterotrophic denitrification systems, while *Chloroflexi* and *Bacteroidetes* have widely been detected in both heterotrophic and chemolithotrophic denitrification systems (Li et al., 2023). Metagenomic studies by Liu et al. (2022) revealed that *Chloroflexi* members primarily exhibited a heterotrophic metabolism, indicating their dependence on external C sources. Existing literature suggests that *Chloroflexi* are commonly found in anammox-based systems (Wang et al., 2016). Zhao et al. (2019) showed that *Anaerolineae*, which is a specific representative of *Chloroflexi*, could facilitate the aggregation of microbial consortia in those systems. Moreover, the authors found a synergistic relationship between these bacteria and hydrogenotrophic methanogenic archaea, which supported the growth of methanotrophic denitrifiers and enhanced N removal processes (Zhao et al., 2019). In the present study, a relatively high abundance of *Anaerolineae* in SBR2 may imply potential involvement of these bacteria in the S-related metabolism beyond N removal.

A prominent member of *Anaerolineae* family, RBG-13–54-9, representing a high abundance (6.24–10.99 %) in the present study, was identified by Xin et al. (2021) in biological aerated filters. The authors suggested that this genus was involved in N removal in the studied reactors. Furthermore, other dominant genera in the present study, such as PHOS-HE36 (7.23 % in SBR1 and 5.35 % in SBR2), *Denitratisoma* sp. (6.08 % in SBR1 and 5.07 % in SBR2), were detected by Hu et al. (2022) in an anammox-derived Feammox process. In their study, the abundances of these genera were 7.13 % (PHOS-HE36) and 5.06 % (*Denitratisoma* sp.), showing a high similarity to the results of the present study.

The impact of COD extended beyond conventional heterotrophic transformations and also stimulated autotrophic processes. In the presence of COD, a substantially increased activity of AnAOB was observed, revealing the adaptability of these bacteria to heterotrophic conditions. Organotrophic AnAOB can utilize acetate and propionate as C sources, facilitated by relevant genes in *Candidatus Kuenenia* and *Candidatus* Brocadia (Feng et al., 2019) and *Candidatus Jettenia* (Tao et al., 2019). Zhang et al. (2021) combined autotrophic and mixotrophic conditions for efficient N removal (>85 %). Periodic acetate and propionate additions, in the absence of inorganic C, enhanced C utilization by *Candidatus Kuenenia.* Moreover, *Planctomycetota, Proteobacteria*, and *Chloroflexi* emerged as the predominant phyla, accounting for 91 % of the bacterial population under mixotrophic conditions. *Planctomycetota*, which include AnAOB, had a relative abundance of 49 %, whereas in our study, it was <10 %.

4.3. Benefits of combining the C-N-S cycles in single systems

The N-S-C cycles can be integrated in either single-stage systems, as demonstrated in the present study, or multi-stage systems within a single two-column reactor under anaerobic and anoxic conditions (Kosugi et al., 2019). They can also be applied across multiple reactors, such as an anaerobic internal circulation reactor, an anoxic reactor, and a biofilm aerobic reactor (Wei et al., 2017). Additional configurations include a SO_4^{2-}/SO_3^{2-} reducing upflow sludge bed (SRUSB) for SO_4^{2-}/SO_3^{2-} reduction and an anoxic upflow sludge bed (AnUSB) for mixotrophic denitrification (Qian et al., 2015), as well as reactors designed for organic removal through biological SO_4^{2-} reduction (SRUSB), a moving bed biofilm reactor (MBBR) for N removal via aerobic autotrophic nitrification and anoxic S-oxidizing autotrophic denitrification (Wu et al., 2016).

These various approaches underline the adaptability of N-S-C systems, enabling their implementation across diverse environmental and operational conditions. Depending on the wastewater laden with either NO_3^- or NO_2^-, the processes can be combined in two different configurations, including heterotrophic denitrification and SDAD (NO_3^-) or anammox and mixotrophic denitrification (NO_2^-) (Kosugi et al., 2019). Moreover, both SO_4^{2-} and SO_3^{2-} can serve as sources for their respective reduced forms in SDAD and mixotrophic denitrification, broadening the range of wastewater types to be treated (Qian et al., 2015). The use of SO_4^{2-} present in the treated wastewater is a significant source of S, driving an integrated N and C removal cycle through continuous reduction and oxidation (Wei et al., 2017).

The combined processes within the integrated system exhibit high efficiency in removing N compounds (~100 % in this study), while concurrently reducing S and C compounds without the need for multiple treatment stages. The removal efficiencies for SO_4^{2-}, total nitrogen (TN) and COD ranged between 72 and 98 %, 55–74 %, and 82–97 %, respectively, as reported by Hao et al. (2015). This is highlighted by the work of Kosugi et al. (2019), where various common pollutants, such as NH_4^+, NO_2^-, NO_3^-, SO_4^{2-}, and S^{2-}, were removed within a single system. This comprehensive removal underscores the versatility of the integrated system in eliminating diverse pollutants. A few studies (Wei et al., 2017; Wu et al., 2016; Qian et al., 2015) also demonstrated that the integrated system follows a closed-loop approach, enhancing resource utilization and reducing waste generation, which aligns with the principles of circular economy and sustainable development.

The incorporation of N-S-C cycles in single systems offers a range of other significant advantages, which have been reported in several studies. These benefits encompass operational efficiency, cost effectiveness and environmental impact, making this approach a promising sustainable wastewater treatment strategy. One of the most notable advantages is the substantial reduction in energy consumption and greenhouse gas (GHG) emissions. The study of Lu et al. (2012) indicated an estimated 35 % decrease for both factors compared to conventional full nitrification-denitrification processes. These reductions not only lead to cost savings but also align with sustainable development goals by minimizing the carbon footprint of WWTPs. The same authors emphasized that the integrated system could provide remarkable (up to 90 %) sludge reduction compared to the traditional approach (Lu et al., 2012), potentially mitigating challenges related to sludge handling and disposal. Integrated N-S-C cycle systems also lead to a reduction in the required operational space as compared to the conventional methods (Wu et al., 2016).

Another significant operational benefit lies in the potential reduction of external COD dosing. Ma et al. (2022) indicated that mixotrophic denitrification could reduce the need for COD supplementation compared to the heterotrophic process. This simplifies operational complexity and has positive economic implications concerning C sourcing.

These systems are suitable for treatment of wastewater derived from mining activities, fermentation processes (e.g., breweries, wineries, and biofuel production), landfill leachate, and the pulp and paper production industry. With the integrated approach to manage N-S-C content, the environmental impact of these industries can be mitigated.

4.4. Future perspectives, challenges and limitations of anammox/mixotrophic denitrification integrated with SRAO

The combined implementation of N-S-C cycles is more complex to handle compared to the independent utilization of specific processes. This is brings numerous challenges and limitations as outlined in Table 1. The challenges and limitations of anammox and mixotrophic denitrification processes revolve around the need to carefully control and maintain optimal operating conditions, including factors like pH, temperature, nutrient concentrations, and COD availability. Achieving the right balance of C/N ratios for anammox can be challenging in some wastewater streams, and competition from other microorganisms in mixed microbial communities may interfere with these processes. These technologies may not be suitable for all types of wastewater, especially when sudden spikes in N or COD concentrations disrupt the delicate balance of microbial communities, leading to process instability. Additionally, the start-up periods for achieving stable and efficient operation can be extended. Furthermore, these processes are best suited for specific wastewater characteristics, primarily high N and S content but low COD, and are limited by potential NO_3^- accumulation. Temperature dependence restricts their use in cold climates, and sensitivity to oxygen and certain contaminants like heavy metals and antibiotics may limit their application in certain wastewater scenarios.

Further research should particularly focus on investigating the role of SRB, SOB, AnAOB and denitrifiers while simultaneously creating optimal conditions for their development in a single-stage system. The integration of anammox/mixotrophic denitrification with SRAO is a new and evolving approach, which needs advancements in optimizing process configurations, enhancing microbial community stability, and addressing challenges related to the scalability and long-term operational reliability.

Table 1
Anammox/mixotrophic denitrification opportunities, challenges and opportunities.

Topic	Anammox/mixotrophic denitrification
Opportunities	1) Improved TN removal efficiency compared to separate processes and anammox/SDAD. 2) Synergistic interactions between different microbial groups. 3) Promising for the treatment of complex wastewaters that contain many pollutants such as high N, S and C compounds. Wastewater environments are rarely exclusively heterotrophic or autotrophic. 4) Compared to traditional denitrification methods, anammox and mixotrophic denitrification require less external carbon sources, leading to lower energy consumption. 5) The use of alternating COD on/off conditions may increase the activity of AnAOB (COD off) and denitrifiers (COD on). 6) Reduced need for chemicals as the products of one process are the substrate for the other. 7) These processes produce less sludge, leading to lower waste generation and environmental impact. 8) Anammox and mixotrophic denitrification can mitigate the production of nitrous oxide, a potent greenhouse gas associated with conventional denitrification processes. 9) Supplementation of COD compounds may increase anammox activity.
Challenges	1) Achieving and maintaining optimal operating conditions for both mixotrophic denitrification and anammox can be challenging. Parameters such as pH, temperature, nutrient concentration and COD availability must be carefully controlled to ensure efficient and stable operation of both processes. 2) Anammox bacteria require specific ratios of C/N and maintaining the right balance can be challenging in some wastewater streams. 3) In mixed microbial communities, other microorganisms may outcompete AnAOB or interfere with their activities, affecting process efficiency. 4) Anammox and mixotrophic denitrification may not be suitable for all types of wastewater. Sudden spikes in N or COD concentrations in the influent can disrupt the delicate balance of AnAOB and mixotrophic denitrification, leading to process instability. 5) Achieving stable and efficient anammox/mixotrophic denitrification has extended start-up periods.
Limitations	1) These processes are best suited for high-N, high-S, low-COD wastewaters, limiting their application range. 2) There is potential for NO_3^- accumulation if the denitrification step is not effectively balanced with anammox. Increased levels of NO_3^- may inhibit the activity of anammox bacteria and result in a decrease in overall N removal efficiency. 3) Both processes are temperature-dependent, and their efficiency may decrease significantly at low temperatures, restricting their use in cold climates. 4) AnAOB are sensitive to oxygen, which may limit the use of these processes in systems where oxygen control is challenging. 5) Some studies suggest that AnAOB might be sensitive to certain heavy metals and antibiotics and S^{2-}, which could limit their application in some types of wastewater.

5. Conclusions

This study reveals the potential of integrating N-S-C cycles to efficiently treat complex wastewater streams. The results confirmed the hypothesis that the enhanced N removal rates (increase by 15 %) can be attained in the presence of SO_4^{2-}, which underscores the importance of diversifying electron acceptors beyond NO_2^- when optimizing the treatment efficiency of NH_4^+-rich wastewater. It was demonstrated that wastewater rich in N, S and organic compounds can effectively be treated through a synergistic combination of anammox and mixotrophic denitrification, complemented by SRAO and reduction of SO_4^{2-} mediated by heterotrophic SRB. To ensure the successful performance of this integrated approach, maintaining low C/N ratios (ranging from 0.4 to 1.6) is critical to prevent AnAOB inhibition. In addition, the introduction of alternating COD conditions is essential for regeneration of AnAOB during the COD-off periods and activation of heterotrophic denitrifiers during the COD-on periods.

The presence of supplementary SO_4^{2-} in the influent wastewater acted as a selective factor, inducing significant shifts in the microbial composition. While the overall bacterial diversity decreased, the notable increases in *Candidatus* Brocadia abundance and emergence of *Chloroflexi – Anaerolineae* in the SO_4^{2-} - supplied reactor. These changes imply a potential involvement of these bacteria in S-associated metabolic processes, further highlighting the complexity and adaptability of the microbial community in response to varying wastewater compositions.

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CRediT authorship contribution statement

Dominika Derwis: Writing – original draft, Visualization, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Hussein E. Al-Hazmi:** Writing – original draft, Visualization, Methodology, Investigation. **Joanna Majtacz:** Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Przemysław Kowal:** Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Sławomir Ciesielski:** Writing – original draft. **Jacek Mąkinia:** Writing – review & editing, Validation, Supervision.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170477.

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Paper IV

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Research article

Enhancing nitrogen removal in the partial denitrification/anammox processes for SO_4^- - Rich wastewater treatment: Insights into autotrophic and mixotrophic strategies

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ABSTRACT

The investigation of partial denitrification/anammox (PD/anammox) processes was conducted under autotrophic (N–S cycle) and mixotrophic (N–S–C cycle) conditions over 180 days. Key findings revealed the remarkable capability of SO_4^{2-}-dependent systems to produce NO_2^- effectively, supporting anaerobic NH_4^+ oxidation. Additionally, SO_4^{2-} served as an additional electron acceptor in sulfate reduction ammonium oxidation (SRAO). Increasing influent SO_4^{2-} concentrations notably improved ammonia utilization rates (AUR) and NH_4^+ and total nitrogen (TN) utilization efficiencies, peaking at 57% for SBR1 and nearly 100% for SBR2. Stoichiometric analysis showed a 7.5-fold increase in AUR (SRAO and anammox) in SBR1 following SO_4^{2-} supplementation. However, the analysis for SBR2 indicated a shift towards SRAO and mixotrophic denitrification, with anammox disappearing entirely by the end of the study. Comparative assessments between SBR1 and SBR2 emphasized the impact of organic compounds (CH_3COONa) on transformations within the N–S–C cycle. SBR1 performance primarily involved anammox, SRAO and other SO_4^{2-} utilization pathways, with minimal S-dependent autotrophic denitrification (SDAD) involvement. In contrast, SBR2 performance encompassed SRAO, mixotrophic denitrification, and other pathways for SO_4^{2-} production. The SRAO process involved two dominant genera, such as *Candidatus* Brocadia and PHOS-HE36.

1. Introduction

Conventional nitrification-denitrification is the most common method of nitrogen (N) removal in municipal wastewater treatment plants (WWTPs). Despite a high utilization efficiency of the process, its sustainability has been questioned due to high energy demand, carbon (C) supplementation and potential for significant nitrous oxide (N_2O) emissions (Zhou et al., 2020). An alternative approach to N removal, based on anaerobic ammonia (NH_4^+) oxidation (anammox), can overcome these disadvantages. This process converts NH_4^+ into nitrogen gas (N_2), using nitrite (NO_2^-) as an electron acceptor (Zekker et al., 2015, 2023). However, anammox is challenged by a stable supply of NO_2^- to ensure the optimum ratio of NH_4^+/NO_2^-. Partial nitritation (PN) was initially proposed as sole solution, but the competition from nitrite-oxidizing bacteria (NOB) for NO_2^- can reduce the overall N utilization efficiency in anammox-based systems (Wu et al., 2022). More recently, partial denitrification (PD), involving reduction of nitrate (NO_3^-) to NO_2^-, has emerged as a promising alternative pathway of generating NO_2^- for anammox (Al-Hazmi et al., 2023). The advantages of PD include reduced energy demand and reduced N_2O emissions with a small need for supplemental C.

Exploring denitrification processes in wastewater treatment requires a thorough understanding the dynamic interactions between heterotrophic and autotrophic mechanisms. Heterotrophic denitrification is predominantly carried out by bacteria utilizing organic C as their energy source. In contrast to the utilization of organic substances, autotrophic

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denitrification involves the use of inorganic compounds such as hydrogen (H_2), sulfide (S^{2-}), elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$), iron (Fe^{2+}), and manganese (Mn^{2+}) as electron donors (Zhang et al., 2022). This innovative approach to denitrification relies on non-organic sources to supply electrons for the reduction of N compounds.

One notable denitrification process that utilizes sulfur (S) compounds (S^{2-}, S^0, and $S_2O_3^{2-}$) as electron donors is S-dependent autotrophic denitrification (SDAD) (Grubba et al., 2022). Although the N utilization rate of SDAD is lower compared to heterotrophic denitrification (Qian et al., 2018), the advantages of SDAD comprise low sludge production and no need for external C (Cao et al., 2019; Di Capua et al., 2019) as well as lower N_2O emissions (Huang et al., 2019).

Nevertheless, the reduced forms of S may not always be present in wastewater, except for SO_4^{2-}. The resolution to this issue involves either executing the autotrophic sulfate reduction ammonium oxidation (SRAO) process, which reduces SO_4^{2-} to reduced S forms, or opting for the second solution, which entails the heterotrophic reduction of SO_4^{2-} by sulfate-reducing bacteria (SRB) before initiating S-dependent PD. Moreover, SRAO can also be a source of NO_2^- for the anammox reaction (Grubba et al., 2022).

Wastewater streams frequently exhibit complexity, comprising a blend of N, S and C compounds. This is evident in scenarios, such as landfill leachates or industrial wastewater originating from mining operations or paper production (Ma et al., 2022b). Consequently, wastewater seldom aligns exclusively with either heterotrophic or autotrophic conditions. To address this complexity, extensive research has focused on combining autotrophic and heterotrophic denitrification in mixotrophic systems (Huang et al., 2022). These systems enable the coexistence of autotrophic and heterotrophic organisms, which can lead to improving the stability and overall N removal performance of wastewater treatment systems. Mixotrophic systems facilitate the simultaneous removal of N, S and C, thereby reducing the need for supplementary organic electron donors (Xu et al., 2015).

This study addresses a critical research gap by examining the integration of partial S-dependent denitrification (N–S cycle in SBR1) or mixotrophic denitrification with the anammox process (N–S–C cycle in SBR2) in a single reactor for wastewater treatment, particularly focusing on streams rich in NH_4^+, NO_3^-, SO_4^{2-}, and COD. Additionally, our objective was to utilize the SRAO process to reduce SO_4^{2-} to its reduced forms for SDAD, a novel approach not previously investigated in literature. Expanding upon our previous research in the same SBRs configurations, which demonstrated the positive effects of SO_4^{2-} supplementation on NH_4^+ and SO_4^{2-} utilization rates (AUR and SUR) in both autotrophic (Derwis et al., 2023) and mixotrophic conditions (Derwis et al., 2024), we now investigate the combined impact of SO_4^{2-} supplementation and the integration of the N–S (SBR1) or N–S–C (SBR2) cycles on PD/anammox performance. An innovative aspect of this study is the absence of NO_2^- in the influent, which was present in both previous studies.

The primary objective of this study was to operate two parallel SBRs for evaluating and comparing the performance of S-dependent denitrification (SBR1) versus mixotrophic denitrification (SBR2) processes alongside anammox, with and without COD supplementation. It was hypothesized that integrating autotrophic (N–S) and mixotrophic (N–S–C) processes could generate sufficient amounts of NO_2^- to effectively support anammox, while the SRAO process would enhance NH_4^+ utilization. Furthermore, a positive influence of additional SO_4^{2-} on N–S compounds removal under autotrophic conditions in SBR1 and N–S–C compounds under mixotrophic conditions in SBR2 was expected. Moreover, the production of reduced S compounds for SDAD would occur through SO_4^{2-} reduction in SRAO (N–S) and additionally by SRB (N–S–C). In addition to evaluating process performance, the contributions of individual metabolic pathways and microbial communities in the reactors were elucidated. By integrating stoichiometric reactions with observed behavior of N compounds (NH_4^+, NO_2^-, NO_3^-), SO_4^{2-}, and COD, a comprehensive understanding of integrated N–S and N–S–C metabolism was sought. Furthermore, key functional bacterial groups necessary for these processes were identified. The results of this study have significant implications for wastewater treatment practices, offering insights into optimizing PD/anammox processes for S-rich streams. By emphasizing the innovative aspects of the proposed approach and clearly defining the hypotheses and research objectives, a contribution was made to the advancement of both scientific knowledge and practical applications in environmental engineering. The study emphasizes the importance of ensuring mixotrophic conditions and obtaining reduced S compounds from SO_4^{2-} while treating the complex real wastewater, including that from various industrial sources like chemical plants, petroleum refineries, dyeing plants, tanneries, paper mills, molasses wineries, food processing plants, landfills and mining plants (Li and Tabassum, 2022).

2. Materials and methods

2.1. *Laboratory setup*

The laboratory setup comprised two SBRs, each with a working volume of 15 L, connected to a control box for regulating the influent/effluent flowrates and mixing. These reactors were constructed from plexiglass and placed in a water jacket to control the process temperature. A Julabo F32 (Germany) water bath was employed to achieve a precise temperature control with an accuracy of ±0.1 °C. Additionally, both reactors were equipped with a variable-speed Heidolph RZR 2041 (Germany) mechanical stirrer. For monitoring, Endress + Hauser (Switzerland) probes were employed to measure pH (EH CPS 471D-7211) and dissolved oxygen (DO) (COS22D-10P3/O) were installed and connected to two Hach Lange HQ40D (Switzerland) multimeters. Further details on the setup design and operational methodology can be found in Al-Hazmi et al. (2020). A diagram of the reactor setup is presented in the Supplementary Material (Fig. S1). To ensure data quality control, consistent measurement practices were employed, incorporating routine calibration of probes and the maintenance of uniform operating conditions.

2.2. *Inoculum biomass and operational conditions*

The inoculum biomass (30 L), used in the experiments, was obtained from a sidestream granular deammonification system at a large municipal wastewater treatment plant (200,000 PE) in Słupsk, Poland. The seed sludge used had a mixed liquor suspended solids (MLSS) concentration of 4500 mg/L.

Throughout a 180-day period, the reactors were consistently operated at a constant process temperature of 30 ± 1 °C without aeration. The measured DO concentration was maintained below 0.02 mg O_2/L. pH levels were controlled within the range of 7.5–7.8 by the automated addition of 6M hydrochloric acid (HCl). The synthetic medium supplied to the SBRs contained microelements based on the composition outlined in a previous study by Al-Hazmi et al. (2020). Key feed components, such as NO_3^-, NH_4^+, COD, and SO_4^{2-}, were introduced in the form of KNO_3, NH_4Cl, CH_3COONa, and $MgSO_4$, respectively.

The dynamic behavior of influent and initial concentrations in the reaction phase in both bioreactors, including NH_4^+, NO_3^-, COD and SO_4^{2-}, are illustrated in Supplementary Material (Fig. S2, Fig. S3). Influent flow rates in SBR1 and SBR2 are illustrated in Fig. S2e. NH_4^+, NO_3^-, and SO_4^{2-} were introduced into both reactors, while COD was specifically added to SBR2 to facilitate a comparison between N–S cycle (SBR1) and N–S–C (SBR2) conditions.

Influent NH_4^+ concentrations varied between 10 and 50 mg N/L, and 20–40 mg N/L for SBR1 and SBR2, respectively. Simultaneously, NO_3^- concentrations ranged from 0 to 20 mg N/L for SBR1 and 10–40 mg N/L for SBR2. Variations in NH_4^+ and NO_3^- concentrations were observed due to the accumulation of these compounds during the process, prompting periodic adjustments in dosage. Influent SO_4^{2-} concentration was kept the same in both reactors and ranged from 150 to 800 mg S/L. The experiment comprised two stages: the first stage conducted before elevating the influent SO$_4^{2-}$ concentration, followed by the subsequent stage after the concentration increase. The transition occurred around day 100. During the first stage, the maximum influent SO$_4^{2-}$ concentration reached 300 mg S/L, while in the second stage, it rose to 800 mg S/L. SO$_4^{2-}$ was introduced to simulate a SO$_4^{2-}$-rich wastewater scenario, anticipating its reduction to reduced S forms (S^{2-}, S^0, and S$_2$O$_3^{2-}$) in SRAO (N–S) and even by SRB (N–S–C). In SBR2, the influent COD concentration ranged from 100 to 200 mg COD/L, maintaining the initial higher concentration, but ultimately remaining at 100 mg COD/L for most of the period.

2.3. *Microbiological and chemical analytical methods*

DNA extraction from soil samples utilized the FastDNA Spin kit for soil (MP Biomedicals, USA). After thawing at room temperature, 200 mg of semi-dry biomass was bead-beaten in an Uniequip device for 5 min. The extraction, conducted in duplicates, underwent agarose gel electrophoresis for DNA quality and Quant-iT BR DNA Assay (Thermo Fisher Scientific, USA) for concentration measurement.

Taxonomic composition was determined via Illumina sequencing of the 16S rRNA gene V3–V4 regions. Raw reads were processed using Cutadapt and dada2 in QIIME2. Taxonomic assignments were made against the Silva (138 release) 16S rRNA reference database. Further analysis on EzBioCloud included computing Operational Taxonomic Units (OTU) numbers, rarefaction curves, and the Shannon alpha-diversity index. Good's coverage is calculated as 1 minus the ratio of singleton OTUs (F1) to the total sum of counts for all Operational Taxonomic Units (OTUs), where F1 represents the number of OTUs that are uniquely represented by a single sequence, and N is the cumulative count of all OTUs in the sample. Statistical significance was assessed with the Wilcoxon rank-sum test. Phylogenetic distances utilized generalized UniFrac distances, and PERMANOVA assessed genetic distance significance.

Mixed liquor samples were promptly filtered using disposable glass microfiber filters (1.2 μm) MFV-3 (47 mm diameter) sourced from Sartorius (Germany). Concentrations of NO$_3^-$, NO$_2^-$, NH$_4^+$, COD, and SO$_4^{2-}$ were assessed using the DR 3900 spectrophotometer, employing dedicated cuvette tests from Hach Lange GmbH (Dusseldorf, Germany). Table S1 in Supplementary Material contains methods numbers according to Hach-Lange standards. We selected particular analysis days to encompass a crucial observation window and align with our prior investigations. The absence of sample testing and analysis between days 44–94 was attributed to operational issues with the system. Determination of MLSS and MLVSS concentrations was performed according to the Standard Method 2540 G as per the American Public Health Association (APHA) (Greenberg et al., 2005).

Cuvette measurements underwent vigilant monitoring for deviations, and if required, measurements were repeated to uphold precision. Additionally, certified cuvette tests were employed, providing certificates that encompass standard deviation, coefficient of variation, and the correlation between nominal and actual values. The standard deviation for each measured and calculated value can be found in the Supplementary Material (Table S2).

2.4. *Determination of specific process rates based on stoichiometric calculations*

The determination of specific process utilization/production rates was based on the maximum slopes of several variables, including NH$_4^+$ utilization rate (AUR), NO$_3^-$ production/utilization rate (NPR/NUR), COD utilization rate (CUR), and SO$_4^{2-}$ production/utilization rate$^-$ (SPR/SUR). All calculated process rates underwent rigorous statistical analysis and were subjected to N–S–C mass balances (Table S3). Furthermore, the efficiency of N, SO$_4^{2-}$, and COD utilization was determined by assessing the differences between influent and effluent concentrations, where negative values denoted the production of SO$_4^{2-}$.

The stoichiometric analysis was carried using the SOLVER tool in MS Excel assuming the same reaction stoichiometries as described in Derwis et al. (2024). The stoichiometric calculations were performed based on reactions described in the literature (Fdz-Polanco et al., 2001; Huang et al., 2019; Sun et al., 2018). This series of reaction equations facilitated the dissection of total rates, attributing them to specific processes, guided by the following assumptions.

1. Anammox, SDAD, SRAO, and SO$_4^{2-}$ utilization other than SRAO (SUR_other) were considered in SBR1,
2. Anammox, mixotrophic denitrification, SRAO, heterotrophic SO$_4^{2-}$ reduction, other SO$_4^{2-}$ utilization (SUR_other) and other SO$_4^{2-}$ production (SPR_other) were considered in SBR2,
3. Other SO$_4^{2-}$ production (SPR_other) and utilization (SUR_other) processes operate independently of the N cycle. SPR_other could involve the oxidation of reduced S species using trace oxygen, while SUR_other might include SO$_4^{2-}$ utilization using bacterially produced organic matter.

Analyses were conducted on the days when microbiological sampling took place (1d, 94d, 114d, 164d) to assess patterns in process rates and variations in microbial composition. The selected analysis periods corresponded to key stages in the study: at the outset (1d), prior to heightened SO$_4^{2-}$ addition in both reactors (94d), immediately following the increase in SO$_4^{2-}$ addition (114d), and finally, at the end of the study (164d). This strategic selection facilitated a comprehensive examination of microbial structural changes in response to varying conditions over the study duration. The outcomes were visualized through Sankey diagrams created in the simulation platform GPS-X ver. 7.0 (Hydromantis, Canada).

3. Results

3.1. *Sulfate-dependent anaerobic ammonium oxidation indicators (AUR, SPR/SUR)*

During the study period, MLSS concentrations varied from 3000 to 3300 mg/L, while the volatile fraction (MLVSS) concentrations ranged from 2300 to 2500 mg/L, representing approximately 76% of MLSS.

The dynamics of AUR and SPR/SUR throughout the entire study period are presented in Fig. 1. Initially, in both reactors, the AUR was at low values of 0.5–2 mg N/(g VSS • h) until day 118 in SBR1, and 1.1–2 mg N/(g VSS • h) until day 103 in SBR2. Following the initial period, a significant increase in AUR to a maximum value of 6.1 mg N/(g VSS • h) on day 180 and 4.8 mg N/(g VSS • h) on day 148 was observed in SBR1 and SBR2, respectively. After 100 days, the SO$_4^{2-}$ supplementation began to increase, coinciding with the rising AUR in both reactors (Fig. 1a–b).

In contrast, SO$_4^{2-}$ was utilized in SBR1 with a gradual increase in the SUR from 1.6 mg S/(g VSS • h) to 12.7 mg S/(g VSS • h) until day 118, followed by a rapid increase to a level >20 mg S/(g VSS • h) with a maximum value of 38.6 mg S/(g VSS • h) achieved on day 129 (Fig. 1a). In SBR2, until day 103, SO$_4^{2-}$ production was observed with the SPR in the range of 6.8–10 mg S/(g VSS • h). Subsequently, the S metabolism switched to utilization, a gradual increase in the SUR was observed from 1.5 to 10.6 mg S/(g VSS • h) until day 121 (Fig. 1b). After this period, similar to SBR1, there was a rapid increase in the SUR to a level >30 mg S/(g VSS • h) with a maximum value of 44.9 mg S/(g VSS • h) achieved on day 125. After day 120, SO$_4^{2-}$ began to accumulate in both reactors, and the initial of SO$_4^{2-}$ concentrations reached their highest values, fluctuating in the range of 1400–1600 mg S/L.

3.2. *Sulfur-dependent autotrophic and heterotrophic denitrification indicators (NPR/NUR, CUR)*

The dynamics of CUR and NPR/NUR throughout the entire study

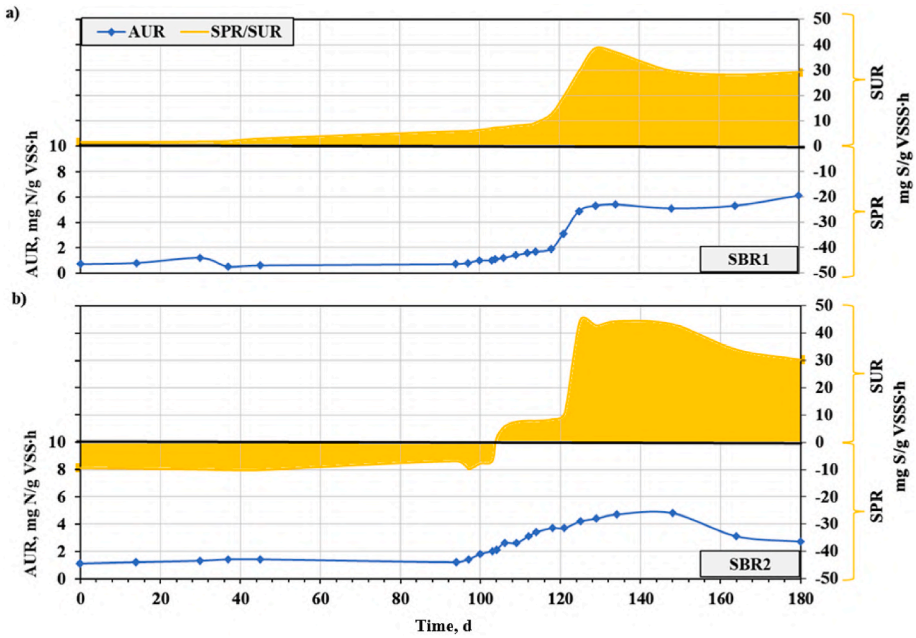

Fig. 1. The dynamics of AUR and SPR/SUR in SBR1 (a) and SBR2 (b).

period are presented in Fig. 2. In SBR1, initially (0–45 days), the NUR ranged from 0.2 to 0.6 mg N/(g VSS • h). Subsequently, the N metabolism switched to production, with the NPR ranging from 0.05 to 0.4 mg N/(g VSS • h) until the end of the study (Fig. 2a). In the contrary, in SBR2, only NO_3^- utilization was observed from the beginning of the study. The NURs increased from the initial level of approximately 1.2 mg N/(g VSS • h) to >2 mg N/(g VSS • h) after 30 days of operation and then remained relatively constant. The CURs were more variable during the study period and fluctuated in the range of 5.4–13.6 mg COD/(g VSS • h) (Fig. 2b).

3.3. *The efficiency of process utilization when combining the N–S/N–S–C cycles*

The efficiency of N utilization (NH_4^+, NO_3^- and TN) in both reactors is illustrated in Fig. 3a (SBR1) and Fig. 3b (SBR2). The efficiency was notably higher in SBR2, performing the N–S–C cycle, compared to SBR1, performing the N–S cycle. With the elevation of influent SO_4^{2-} concentration after day 100, NH_4^+ utilization efficiency increased, reaching a maximum of 64% and 100% almost in SBR1 and SBR2, respectively.

Regarding NO_3^- metabolism in SBR1, NO_3^- production switched to accumulation on day 100, reaching a maximum of 64% on day 129. In SBR2, NO_3^- utilization occurred throughout the entire experiment, with the efficiencies ranging from 54% to 100%. The efficiency of TN utilization, involving the change in the sum of NH_4^+ and NO_3^- concentrations, reached a maximum of 57% and almost 100% in SBR1 and SBR2, respectively.

The efficiency of SO_4^{2-} and COD utilization/production are presented in Fig. 4. The efficiency of SO_4^{2-} utilization in SBR1 ranged from 3% to 31% with the maximum value on day 104. In contrast, in SBR2, initially SO_4^{2-} was produced and then utilized from day 104, reaching a maximum (40%) on day 125. Meanwhile, the efficiency of COD utilization in SBR2 ranged from 40% to 66%, with the maximum value on day 118.

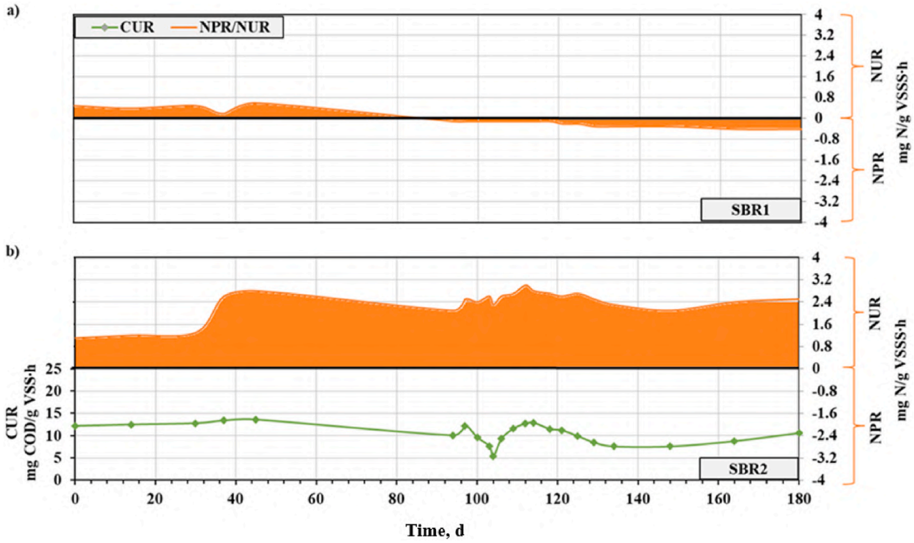

Fig. 2. The dynamics of NPR/NUR and CUR in SBR1 (a) and SBR2 (b).

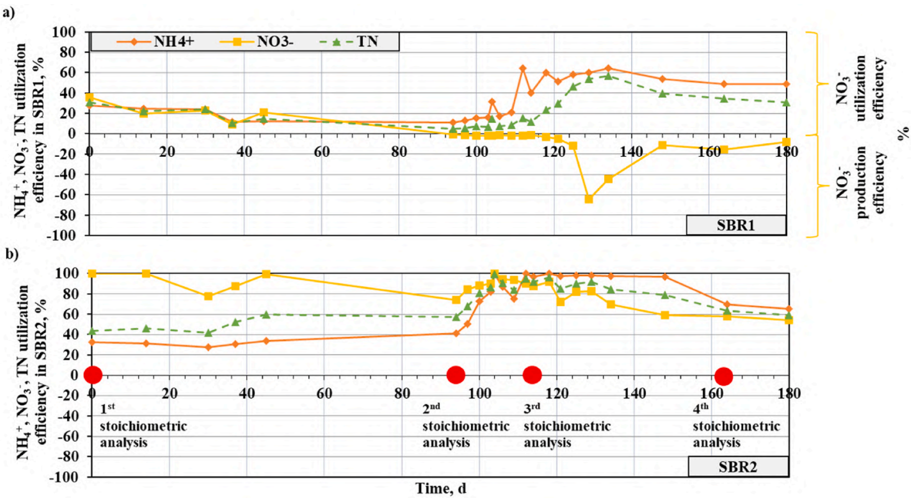

Fig. 3. NH_4^+, NO_3^- and TN utilization efficiencies in SBR1 (a) and SBR2 (b).

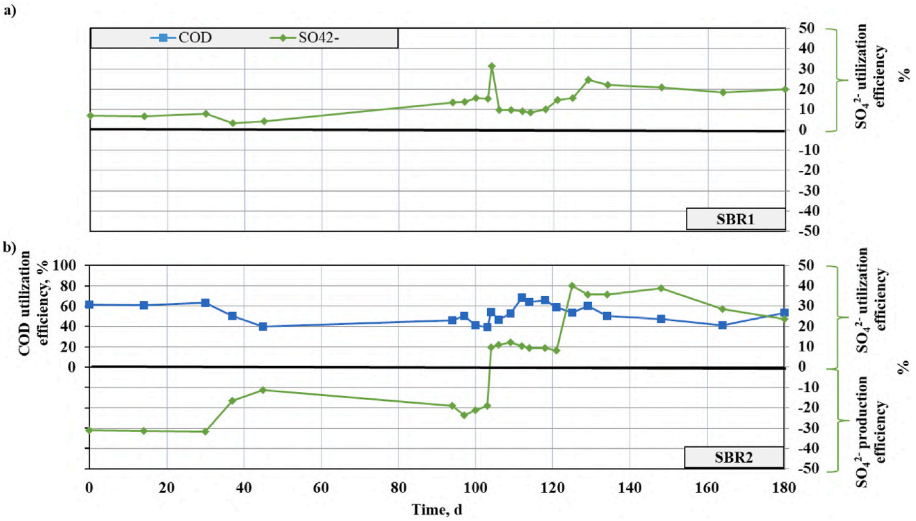

Fig. 4. SO_4^{2-} and COD utilization efficiencies in SBR1 (a) and SBR2 (b).

3.4. *Stoichiometric calculations*

The days selected for stoichiometric analysis (1 d, 94 d, 114 d, 164 d) are presented in Fig. 3. Figs. 5 and 6 illustrate the results of the analysis in the form of Sankey diagrams for the days prior to starting the supplementation of SO_4^{2-} (day 94) and after stabilizing the supplementation (day 164). For further comparison, the results of stoichiometric calculations on days 1 and 114 are shown in Fig. S4. In general, in SBR1, the reactions were primarily associated with anammox, SRAO, and other SO_4^{2-} utilization pathways, with a minor contribution from SDAD. On the other hand, SBR2 involved SRAO, mixotrophic denitrification, and other SO_4^{2-} utilization pathways.

Specifically, in SBR1, the AURs related to SRAO were 0.51 mg N/(g VSS • h) and 3.84 mg N/(g VSS • h) on days 94 and 164, respectively. Concurrently, on the same days, the AURs related to anammox were 0.19 mg N/(g VSS • h) and 1.46 mg N/(g VSS • h). Notably, both AURs exhibited an approximately 7.5-fold increase following the SO_4^{2-} supplementation. It should be emphasized that the AUR related to anammox became evident only after triggering NO_2^- production by SRAO. The NO_2^- generated from SRAO served as a substrate for the activation of anammox, leading to a rise in NiUR_amx from 0.25 mg N/(g VSS • h) to 1.92 mg N/(g VSS • h). This, in turn, influenced the NPR from anammox, increasing from 0.05 mg N/(g VSS • h) to 0.38 mg N/(g VSS • h).

Fig. 5a–b explicitly shows that the S transformations in SBR1 primarily concentrated on the reduction of SO_4^{2-}, while the SPR remained close to 0. The SUR in SBR1 related to both SRAO and non N-related processes (SUR_other). The SUR from SRAO raised from 0.87 mg S/(g VSS • h) to 6.57 mg S/(g VSS • h) between days 94 and 164, while the SUR_other increased from 5.03 mg S/(g VSS • h) to 21.73 mg S/(g VSS • H) during the same period. The SPR remained close to 0 mg S/(g VSS • h).

The results of the analysis for SBR2 (Fig. 6a–b) revealed a gradual prevalence of SRAO and mixotrophic denitrification in the expense of anammox, which completely disappeared at the end of the study period. The AUR related to SRAO increased from 0.05 mg N/(g VSS • h) to 3.1 mg N/(g VSS • h), whereas the AUR related to anammox decreased from 1.14 mg N/(g VSS • h) to 0 mg N/(g VSS • h) between days 94 and 164. Consequently, the contribution of NiUR and NPR from anammox also diminished to 0 mg N/(g VSS • h). Fig. 6b explicitly demonstrates the absence of anammox activity in the final phase of the study. On day 94, the NUR was facilitated by SDAD (2.4 mg N/(g VSS • h)), succeeded by NiUR through heterotrophic denitrification (0.91 mg N/(g VSS • h)). Conversely, on day 164, the dynamics of N transformations shifted (Fig. 6b), with the NUR related to heterotrophic denitrification (2.4 mg N/(g VSS • h)) and NiUR related to SDAD (3.95 mg N/(g VSS • h)).

On day 94, primarily SPR_other occurred alongside SPR in SDAD

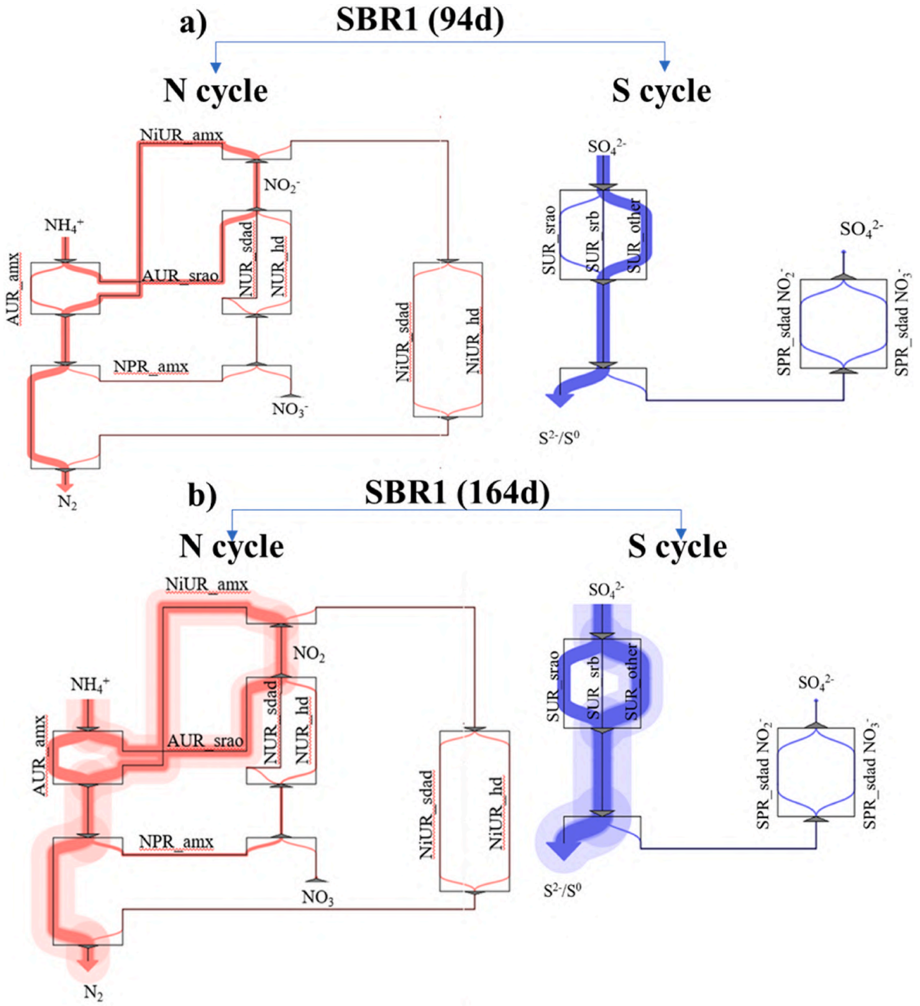

Fig. 5. Results of stoichiometric analysis in SBR1 on day 94 (a) and 164 (b).

involving NO_3^-. The SUR was lower and primarily attributed to SRB. On day 164, mainly SUR_other occurred, surging to 32 mg S/(g VSS • h), with the SUR from SRAO exceeding 5.3 mg S/(g VSS • h). The SO_4^{2-} production was mainly due to SDAD involving NO_2^-. Fig. 6a–b illustrates that the S transformations in SBR2 primarily focused on production on day 94 and the reduction of SO_4^{2-} on day 164.

The CUR in SBR2 related to both SRB activity and mixotrophic denitrification. Towards the end of the study, the SRB contribution diminished, and CUR primarily resulted from heterotrophic denitrification with NO_3^-, as shown in Fig. 6b. The influence of COD on the N–S–C transformations is evident when comparing SBR1 and SBR2 on the same days (i.e., 94 and 164). On day 94, SBR2 exhibited a 10-fold lower AUR_srao and a 6-fold higher AUR_amx compared to SBR1. Conversely, on day 164, SBR2 displayed a 1.2-fold lower AUR_srao and a complete halting AUR_amx, contrasting with SBR1. Additionally, SBR2 demonstrated mixotrophic denitrification, which was absent in SBR1 on days 94 and 164.

When comparing the S transformations in both reactors, different metabolic pathways were explicitly observed on day 94. A net reduction of SO_4^{2-} was observed in SBR1, while SBR2 experienced a net production of SO_4^{2-}. Specifically, in SBR1, the predominant process was SUR_other, whereas in SBR2, the S transformations involved SPR_other and SUR_srb. On the contrary, on day 164, both reactors mainly exhibited SUR_srao and SUR_other, however, a notable distinction was observed in SBR2, where SPR_niur from SDAD also contributed to the S transformations.

3.5. *Microbiology*

The bacterial community was analyzed using sequences of 16S rRNA gene amplicon. The average number of reads in samples representing SBR1 was 72,010. In the case of SBR2, the average number of reads was 61952,25. The Good's coverage values were 99.6% for both reactors. The average OTUs numbers in SBR1 was 114,00, whereas in SBR2 average OTUs number was 999. The average value of the Shannon diversity index was 4.51 for SBR1 and 4.46 for SBR2. The PERMANOVA test showed a lack of significant differences between the microbial community structures representing the studied reactors ($p = 0.752$).

At the phylum level, in SBR 1, the most abundant bacteria belonged to *Chloroflexi* (26.44%), *Proteobacteria* (19.67%), *Bacteroidota* (16.81%), and *Planctomycetota* (14.06%). Similarly, in SBR2, the most abundant phyla were *Chloroflexi* (23.61%), *Proteobacteria* (23.00%), *Bacteroidota* (17.84%), and *Planctomycetota* (14.79%) (Fig. 7). Less common were the phyla *Actinobacteriota* and *Acidobacteriota*, with abundance of 9.19% and 5.43) in SBR1, and 7.89 and 5.05, respectively. Bacteria from other phyla accounted for less than 2% of all bacteria.

At the genus level, the most numerous genera were generally *Candidatus* Brocadia and PHOS-HE36 (*Ignavibacteriales*), with average abundances of 7.73% and 7.95% in SBR1, and 9.38% and 7.89 in SBR2, respectively. A relatively abundant genus was AKYH767 (*Sphingobacteriales*), accounting for 5.45% in SBR1 and 6.02% in SBR2. Common in both reactors were three genera from the *Anaerolineaceae* family (SBR1031, RBG-13-54-9, and one uncultured genus), together

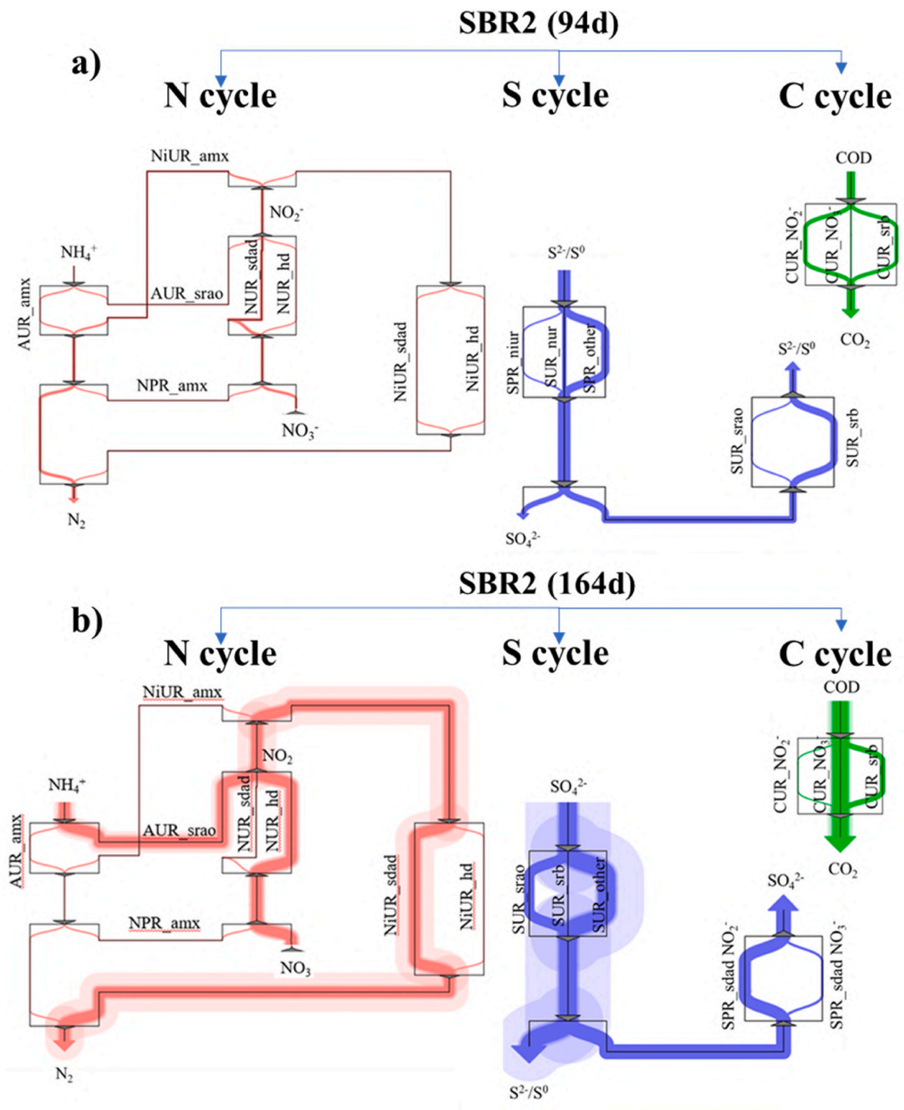

Fig. 6. Results of stoichiometric analysis in SBR2 on day 94 (a) and 164 (b).

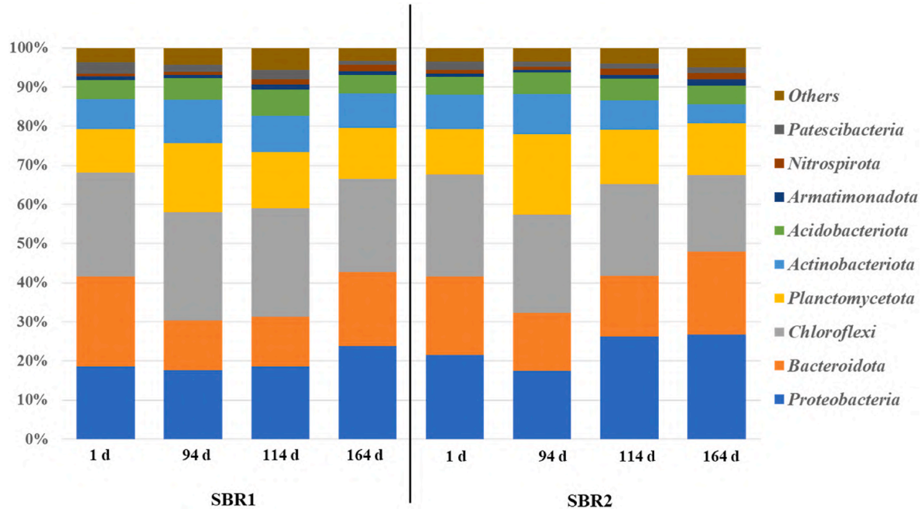

Fig. 7. Relative abundance of bacteria at the phylum level on days 1, 94, 114, and 164 in SBR1 and SBR2.

accounting for 5.83% and 5.21% in SBR1 and SBR2, respectively. Bacteria from *Burkholderiales* (*Thauera*, *Denitratisoma*, B1–7BS, *Limnobacter*) constituted another significant group in both reactors (Fig. S5). Among them, *Thauera* sp. and *Denitratisoma* sp. were more common in SBR2.

The phylogenetic tree, developed based on the genetic distance between samples, shows changes in the microbial community structure during process performance (Fig. S6). Samples from both reactors were very similar on the first day and formed a separate clade. The second group was formed by samples collected from both reactors on day 94 and one sample collected from SBR1 on day 114. The final group consisted of a sample collected from SBR2 on day 114 and samples collected from both reactors on day 164. The tree showed that changes in microbial communities began after 114 days in SBR1 and 94 days in SBR2.

4. Discussion

4.1. *Current insights into coupling of N–S–C cycles in wastewater treatment*

The utilization of SO_4^{2-} alongside NH_4^+ has garnered increasing attention in wastewater treatment due to its potential to enhance N utilization efficiencies. Existing literature, e.g., Fdz-Polanco et al. (2001) and Liu et al. (2008), has highlighted SO_4^{2-} role as an additional electron acceptor for anaerobic NH_4^+ oxidation, augmenting NH_4^+ utilization processes. Liu et al. (2008) observed an NH_4^+/NO_2^- consumption ratio of approximately 1.1:1 when $(NH_4)_2SO_4$ was present in the influent, surpassing a theoretical value of 1:1.34, indicating the significant impact of SO_4^{2-} on NH_4^+ utilization. Yang et al. (2009) and Zhang et al. (2019) demonstrated the synergistic effect of elevated NH_4^+ and SO_4^{2-} concentrations, leading to improved utilization efficiencies for both compounds. For instance, Zhang et al. (2019) observed that increasing influent SO_4^{2-} concentrations from approximately 100 mg S/L to 180 mg S/L, along with higher NH_4^+ concentrations (50 mg N/L vs. 120 mg N/L), improved NH_4^+ utilization efficiency from approximately 40%–90%. Wu et al. (2020) reported remarkable success in achieving an NH_4^+ utilization efficiency of 98%, including 44% removed through sulfammox (SRAO). Moreover, the presence of COD significantly impacts the anammox/mixotrophic denitrification process. Wang et al. (2016) illustrated the dominance of autotrophic AnAOB in low COD environments, while denitrifying heterotrophs thrived in higher COD environments. The coupling of anammox with SDAD can occur in two alternative pathways. When NO_3^- is present in the wastewater, anammox bacteria utilize the NO_2^- generated during partial SDAD, as described by Ma et al. (2022b). The second pathway involves producing NO_3^- through anammox for further denitrification, as in our previous studies (Derwis et al., 2023, 2024).

Studies by Qian et al. (2015) and Du et al. (2020) emphasized the significant impact of COD on denitrification processes, with mixotrophic denitrification showing higher TN utilization efficiencies compared to SDAD alone. Qian et al. (2015) observed a rapid increase in the rate of mixotrophic denitrification when COD was added to the SDAD reactor, approximately 10 times faster compared to studies without COD (Lee et al., 2001; Kim et al., 2002). Furthermore, Du et al. (2020) reported an increase in TN utilization efficiency from 70% in SDAD to 97% in mixotrophic denitrification. Wang et al. (2020) also suggested that SDAD can be applied in the treatment of low C/N wastewater, integrating different biological N removal processes (e.g., anammox) and facilitating the removal of multiple components.

4.2. *Balancing autotrophic and mixotrophic conditions for improved NH_4^+ oxidation, SO_4^{2-} utilization and wastewater treatment efficiency*

The results of this study reveal significant insights into the impact of various factors on the treatment processes within the reactors. Increasing the SO_4^{2-} influent after day 100 led to an increase in both AUR and SUR in both reactors. Notably, the SO_4^{2-} influent had a greater effect on AUR in SBR1, achieving a higher value compared to SBR2. In contrast, it had an inverse effect on SUR, yielding better results in SBR2. Thus, maintaining autotrophic conditions without COD addition is crucial for faster NH_4^+ oxidation, whereas for faster SO_4^{2-} utilization, mixotrophic conditions with COD addition, especially at high SO_4^{2-} concentrations (>300 mg S/L), appear to be more favorable. The efficiency of TN removal also increased in both reactors with increased SO_4^{2-} influent. In SBR2, at low SO_4^{2-} concentrations (<300 mg S/L), SO_4^{2-} production occurred initially, followed by effective SO_4^{2-} utilization after increasing the influent to >300 mg S/L.

The optimal initial N/S and N/S/C ratios were determined based on the observed TN and SO_4^{2-} utilization efficiencies. For SBR1, the most effective N/S ratio for TN utilization efficiency was found to be 1/16, and for SO_4^{2-} utilization efficiency - it was 1/3.5. In contrast, in SBR2, the optimal N/S/C ratio for TN utilization efficiency was identified as 1/16/2.8, while for SO_4^{2-} utilization efficiency - it was 1/24/2.8. In the case of wastewater loaded with NO_3^-, mixotrophic conditions seem to be more advantageous due to faster NO_3^- utilization in SBR2 compared to SBR1, where utilization eventually shifted to production under autotrophic conditions over time. Anammox surpassed SDAD in SBR1, whereas mixotrophic denitrification played a crucial role in SBR2, influencing higher NUR values. Overall, in SBR1, reactions were mainly associated with anammox, SRAO, and other SO_4^{2-} utilization pathways, with minimal SDAD involvement. Conversely, SBR2 involved SRAO, mixotrophic denitrification, and other SO_4^{2-} utilization pathways. In SBR1, both AUR from anammox and SRAO processes increased approximately 7.5 times after increasing SO_4^{2-} supplementation. Anammox utilized NO_2^- generated from the SRAO process, mutually enhancing NH_4^+ utilization efficiency. However, in SBR2, the SRAO process surpassed anammox after increasing SO_4^{2-} supplementation. This could be related to the addition of COD in SBR2, which adversely affected the anammox process, allowing SRAO to oxidize NH_4^+ under these conditions. Nevertheless, comparing AUR values for both processes at the end of the study, both SRAO and anammox processes exhibited lower AUR for SBR2 compared to SBR1. Therefore, for better NH_4^+ utilization results in anammox and SRAO processes, autotrophic conditions without COD are recommended. In both reactors, SRAO primarily accounted for SO_4^{2-} utilization, along with processes unrelated to the N cycle, with a minor additional contribution from SRB in SBR2.

These findings underscore the complexity of microbial interactions and the importance of understanding them for optimizing wastewater treatment processes. Moreover, they highlight the significance of research in elucidating these mechanisms and guiding efficient treatment strategies for environmental protection and resource recovery.

4.3. *Impact of NO_2^- addition on N–S–C cycles under transformations*

Our previous studies (Derwis et al., 2023, 2024) focused on the dynamics of N–S–C transformations, specifically exploring the impact of NO_2^- without concurrent NO_3^- addition under autotrophic (Derwis et al., 2023) and heterotrophic conditions (Derwis et al., 2024). The present study elucidated the differences and similarities arising from the absence of NO_2^- and the inclusion of NO_3^-.

In the study by Derwis et al. (2023) in the reactor receiving both SO_4^{2-} and NO_2^-, the study revealed the concurrent development of SRAO process, AOB, anammox and SDAD. SO_4^{2-} was primarily generated in SDAD through the involvement of NO_2^-. Processes independent of the N cycle also contributed significantly to SO_4^{2-} removal.

In contrast, the addition of SO_4^{2-} and NO_3^- in this study resulted in significantly slower process rates for SRAO (3 times) and anammox (17 times) compared to the autotrophic conditions studied by Derwis et al. (2023). Moreover, the SDAD process only developed to a minimal extent. Therefore, the addition of NO_2^- favored anaerobic NH_4^+ oxidation with SO_4^{2-} addition for both SRAO and anammox. SDAD also demonstrated better performance with the addition of NO_2^- than NO_3^-, indicating a preference of S-dependent autotrophic bacteria for NO_2^- in oxidizing reduced S compounds.

Under heterotrophic conditions with the addition of SO_4^{2-}, COD and NO_2^- (Derwis et al., 2024), the development of SRAO, anammox, mixotrophic denitrification was observed. When NO_3^- was added instead of NO_2^- in the present study, SRAO developed to a similar extent, while anammox disappeared. Mixotrophic denitrification emerged, where NO_3^- and NO_2^- were primarily reduced by heterotrophs and S-dependent autotrophs, respectively, aligning with the findings of Derwis et al. (2024). One of the key findings was that anammox thrived under COD conditions, but the presence of NO_2^- was essential. In the absence of NO_2^- under heterotrophic conditions, anammox disappeared.

4.4. Microbial community dynamics and functional roles in S-based systems

Taxonomic analyses revealed that the microbial communities were predominantly composed of members from the genera *Candidatus*_Brocadia and PHOS -HE36 with the average abundances in both reactors of 8.55% and 7.92%, respectively. While the former taxon is commonly known as conventional anammox bacteria, it could potentially be responsible for performing the entire SRAO reaction (Liu et al., 2015; Grubba et al., 2021). The role of PHOS -HE36 remains less understood, but it was identified as the dominant bacterium (9.59%) in a system performing S-based autotrophic denitrification (Fu et al., 2023). It has also been speculated that these obligate heterotrophic bacteria might be involved in the denitrification process (Koenig et al., 2005; Fu et al., 2023).

The hypothesis suggested PHOS -HE36's role as a symbiotic provider of necessary NO_2^- to *Candidatus* Brocadia. However, results reveal no significant correlation despite the relatively high frequency of both types. The complexity of the symbiosis, potentially heightened by the presence of numerous denitrifiers, may account for this observation. The current challenge lies in discerning whether alternative metabolic pathways might influence this association.

Other dominant genera, such as AKYH767 (average abundance of 5.73% in both reactors 5.73%) and SBR1031 (average abundance of 5.34% in both reactors), were common. Notably, all three genera (PHOS -HE36, AKYH767, and SBR1031) were also found in similar abundances by Fu et al. (2023). AKYH767 likely belongs to aerobic organotrophs (Kim et al., 2020), while SBR1031 belongs to anaerobic bacteria capable of fermenting carbohydrates, and its metabolic function is related to NH_4^+ conversion (Wang et al., 2018; Sun et al., 2020; Fu et al., 2023).

In the work of Hu et al. (2022), investigating a Feammox-based process, PHOS-HE36 (7.13%), SBR1031 (4.41%), and *Denitratisoma* (5.06%) were found in the seed anammox sludge. Following the process start-up, these genera enriched their abundances. The authors suggested that they all were heterotrophic denitrifiers and played a similar role in the Feammox bioreactor. In the present study, PLTA13 (Ma et al., 2022a), *Limnobacter*, and uncultured bacteria from the *Anaerolineaceae* family were involved in heterotrophic denitrification, in addition to PHOS -HE36, SBR1031, *Denitratisoma*. In the work of Deng et al. (2024), the performance of N removal by PD/anammox process with Fe(II) addition was assessed. The authors identified *Denitratisoma*, Ignavibacterium, *Thauera* and *Limnobacter* as key genera providing NO_2^- substrate for the subsequent anammox reaction, primarily facilitated by *Candidatus* Brocadia. In our study, the abundance of *Thauera* was significantly higher in SBR2 than in SBR1 (Fig. S5). The substantial increase in *Thauera* abundance after 100 days suggests a potential correlation between its activity and the enhanced NH_4^+ utilization observed in SBR2.

In addition, members of the genus *Thauera* may be responsible for the reduction of SO_4^{2-} as an electron donor (Huang et al., 2015). Members of the B1–7BS family, with an average abundance of 3.65% in our reactors, have previously been retrieved from various environments, including an alpine cave (Jurado et al., 2020), an S^{2-} mineral deposit (Jones et al., 2017), and a lava tube cave (Gonzalez-Pimentel et al., 2021). Hence, it is reasonable to infer that these genera, along with *Thauera*, contribute to S utilization. Moreover, the oxidation of NH_4^+ utilizing SO_4^{2-} as an electron acceptor appears to be a multifaceted process that entails multiple sequential steps executed by a consortium of species.

4.5. Future perspectives and practical applications of S-dependent systems of anaerobic NH_4^+ oxidation combined with denitrification

Future perspectives of SO_4^{2-}-dependent systems in wastewater treatment encompass the continuous improvement and optimization of technologies that integrate SRAO in combination with SDAD/anammox (forming the N–S cycle) or mixotrophic denitrification/anammox (forming the N–S–C cycle). These systems exhibit high effectiveness in treating wastewater with elevated concentrations of NH_4^+, NO_3^-, SO_4^{2-} and COD, especially in scenarios with low NO_2^- content, i.e. those that can be used in the PD/anammox process, which is the first key insight. SO_4^{2-}-dependent systems demonstrate the effective removal of NH_4^+ by utilizing SO_4^{2-} in the case of lacking an electron acceptor for anammox in the form of NO_2^-, which is the second key insight. This resilience makes these systems versatile and reliable in a variety of applications to diverse wastewater compositions.

The integration of the S cycle plays a key role in SO_4^{2-}-dependent systems, serving as a driving force for N and C transformations, i.e. those present in PD/anammox, which is the third key insight. This complex N–S–C cycle involves the reduction of SO_4^{2-} in the SRAO process (N–S cycle) or by SRB (N–S–C cycle), and the subsequent re-oxidation of reduced S species in the SDAD process. In the presence of COD, mixotrophic denitrification becomes the dominant pathway of N transformations.

In the field of SO_4^{2-}-dependent anaerobic NH_4^+ oxidation systems combined with denitrification, an additional research direction involves interactions between the microbial groups. The complex interactions among microorganisms responsible for SRAO, SDAD and mixotrophic denitrification pose challenges for a comprehensive understanding, hindering the unlocking of the full potential of SO_4^{2-}-dependent systems, which is the fourth key insight. Further research efforts should focus on elucidating these microbial interactions to uncover hidden synergies, improve system performance and potentially discover untapped microbial functionalities.

Another study (Jia et al., 2017) explored the integration of N–S–C cycles through the Sulfate-reduction Autotrophic-denitrification and Nitrification Integrated (SANI). That process employs SO_4^{2-} as an electron acceptor for organic matter removal, while utilizing S^{2-} as an electron donor for N removal via autotrophic denitrification. This differs from our study, where SO_4^{2-} primarily enhances NH_4^+ utilization efficiency in SRAO and mixotrophic denitrification is responsible for COD reduction. Additionally, our approach does not involve oxygenation, thereby reducing operational costs.

In the study of Li et al. (2022), autotrophic-heterotrophic denitrification was investigated, emphasizing two primary biological pathways: simultaneous nitrification and denitrification (SNAD), and PD/anammox. SNAD operates in oxygen-limited environments, involving concurrent processes of nitrification, denitrification, and anammox. In contrast, PD/anammox pathways occur under anaerobic conditions, combining partial denitritation with anammox. Both pathways yield NO_2^- for anammox. However, our research indicates that NO_2^- is not essential for NH_4^+ oxidation, and the N–C cycles can be extended via the S cycle, where SO_4^{2-} serves as an adequate electron acceptor for NH_4^+. Furthermore, NO_2^- generated in SRAO can be utilized in anammox, enhancing NH_4^+ oxidation under anaerobic conditions.

In practical applications, SO_4^{2-}-dependent systems offer a sustainable and flexible solution for the treatment of complex wastewater. Ongoing studies should concentrate on improving the efficiency, cost-effectiveness, and environmental sustainability of SO_4^{2-}-dependent systems to make them integral components of future wastewater treatment strategies.

5. Conclusions

The study demonstrated the viability of integrating either SDAD or mixotrophic denitrification with the anammox process in a single reactor, specifically designed for treating streams rich in NH_4^+, NO_3^-, SO_4^{2-} and COD. It confirmed that SO_4^{2-} - dependent systems are capable of generating NO_2^- to facilitate NH_4^+ oxidation. Additionally, the SRAO process served as an extra electron acceptor for NH_4^+ oxidation while reducing SO_4^{2-} to produce S compounds for SDAD. Increasing the influent SO_4^{2-} concentrations enhanced the utilization rates and efficiencies of NH_4^+ and SO_4^{2-}. Optimal N/S and N/S/C ratios were determined to maximize TN and SO_4^{2-} utilization efficiencies in SBR1 and SBR2. Essential pathways included anammox, SRAO, and SO_4^{2-} utilization (SBR1) compared to SRAO, mixotrophic denitrification, and SO_4^{2-} production (SBR2). The study emphasized the capacity of anammox bacteria to maintain high activity and abundance within a microbial consortium over prolonged periods, contingent upon balanced concentrations of N, S and COD compounds. The SRAO process was likely facilitated by dominant genera, such as *Candidatus*_Brocadia and PHOS-HE36.

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CRediT authorship contribution statement

Dominika Derwis: Writing – original draft, Visualization, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Hussein E. Al-Hazmi:** Writing – original draft, Visualization, Methodology, Investigation. **Joanna Majtacz:** Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Sławomir Ciesielski:** Writing – original draft. **Jacek Mąkinia:** Writing – review & editing, Validation, Supervision.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2024.120908.

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