SULPHUR-DRIVEN ANAMMOX SYSTEMS FOR NITROGEN REMOVAL FROM WASTEWATER

By

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LIST OF JOURNAL PUBLICATIONS AND CONFERENCES

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3. Hassan, M.O., Gani, K.M., Kumari, S., Awolusi, O., Gumede, L., Seyam, M. and Bux, F., 2022. Start-up of partial nitrification by intermittent aeration, pH shocks, and sulfide addition in a sequential batch reactor. Journal of Chemical Technology & Biotechnology, 97(8), pp.2186-2195
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Conference papers and technical reports

6. Owaes, M., Gani, K.M., Kumari, S., Seyam, M. and Bux, F., 2021. Nitrogen removal from anaerobic effluents by Anaerobic ammonia oxidation coupled with sulphide driven-denitration. *International conference on sustainable development 07– 09 December 2021 Durban, South Africa (Virtual conference).*
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ABSTRACT

This thesis explored sulphur-driven Anammox systems as a potential solution for removing nitrogen from wastewater. It focused on using sulphur compounds as electron donors to drive the Anammox process, aiming to reduce energy requirements and costs compared to conventional methods. The thesis provided an overview of existing nitrogen removal techniques in wastewater treatment plants, highlighting the limitations of conventional methods and the potential of Anammox as an alternative. The theoretical framework of sulphur-driven Anammox systems was discussed, emphasizing their advantages in terms of energy efficiency, carbon footprint reduction, and cost-effectiveness. The study examined the principles and significance of the Anammox process in wastewater treatment, where anaerobic microorganisms convert ammonium and nitrite into nitrogen gas, thereby eliminating nitrogen pollution. By harnessing the power of sulphur-driven Anammox processes, this study aimed to address these environmental challenges and develop innovative and sustainable wastewater treatment technologies.

The research investigated the potential of sulphur compounds, such as sulphide (S^{2-}), sulphate (SO_4^{2-}) and elemental sulphur (S^0), as electron donors in Anammox systems. These compounds serve as alternative sources of reducing equivalents, enabling the Anammox bacteria to carry out the conversion process efficiently. The performance and microbial dynamics of sulphur-driven Anammox systems were extensively analyzed. Various parameters, such as nitrogen removal efficiency, sulphur compound dosage, pH and temperature, are evaluated to optimize system performance. The study also investigated the microbial community composition and metabolic pathways involved in sulphur-driven Anammox, shedding light on the key micro-organisms and their functional roles.

The combination of partial nitrification (PN) and anaerobic ammonium oxidation was investigated as a promising technology for nitrogen removal from wastewater. Strategies such as intermittent aeration, pH shocks and sulfide addition were studied for PN start-up and suppression of nitrite-oxidizing bacteria (NOB). Intermittent aeration with low dissolved oxygen (<5 mg/L) suppressed NOB activity, resulting in a 93% nitrite accumulation rate (NAR). Low pH (5) reduced both ammonia oxidising bacteria (AOB) and NOB activity while raising it to pH 7.5 increased AOB activity (84% NAR) but kept NOB suppressed. Adding sulfide (up to 25 mg/L) without pH control raised NAR from 63% to 85%. These factors affect nitrite accumulation in our system. The study provided insights into establishing PN in a sequential batch system and highlighted the sensitivity of nitrite oxidation to sulfide.

The incorporation of sulphur into carbon and nitrogen removal processes in a wastewater treatment plant was explored in this study. A dosage of 15 mgS/L of sodium salt of sulphide combined with 2-3 mg/L dissolved oxygen, established PN effectively in synthetic and real wastewater. PN was established when pH naturally rose due to sulfide hydrolysis, suppressing NOB activity. Long-term operation achieved a nitrite accumulation ratio of 70 ± 19%, with ammonia and nitrite concentrations of 19 ± 4 mgN/L and 18 ± 4 mgN/L, respectively. NOB communities diminished during stable PN but returned when sulfide dosing stopped, indicating the need for continuous sulfide dosing.The study emphasized the continuous adoption of this strategy for sustained PN and its potential application in nitrogen removal from domestic wastewater.

This study explores the intriguing sensitivity of Anammox bacteria to sulfide, revealing captivating insights into their unique response to this compound. The short-term negative effect of sulfide on Anammox performance was observed but quickly recovered under low sulfide stress. Reducing or removing sulfide in the influent is suggested to accelerate the recovery of Anammox performance. The stoichiometric ratio was identified as an indicator of Anammox performance and consortium development. The study highlighted the importance of environmental conditions and sulfide concentration control in optimizing Anammox processes and nitrogen removal in wastewater treatment systems.

The study extensively examines the performance of the sulfide-driven Anammox (SPDA) process, specifically focusing on the impact of various hydraulic retention times (HRTs) and external sulfide dosing. The objective was to understand how variations in HRTs and the addition of external sulfide influence the efficiency and effectiveness of the SPDA process in removing nitrogen from wastewater. To gain a deeper understanding of the microbial communities involved in the SPDA process, next-generation high-throughput sequencing techniques were employed. These advanced sequencing methods allow for a comprehensive analysis of the structural and functional dynamics of the microbial communities present in the SPDA system. By analyzing the genetic material of these micro-organisms, it becomes possible to identify and quantify their abundance, diversity and potential functional roles in the nitrogen removal process. The findings from this study are expected to provide valuable insights into optimizing the performance of the SPDA process. By examining the effects of different HRTs and external sulfide dosing, researchers can determine the most favorable conditions for achieving efficient nitrogen removal. Additionally, by evaluating the microbial communities' dynamics, the study aimed to uncover the relationships between specific microorganisms and their contributions to the SPDA process.

Ultimately, the results obtained from this investigation can contribute to the development of improved strategies for nitrogen removal from wastewater using the SPDA process. This knowledge can inform the design and operation of wastewater treatment systems, leading to enhanced performance, reduced energy consumption and increased cost-effectiveness in the treatment of nitrogen-rich wastewater.

Keywords: Anammox, Partial Nitrification, Nitrogen removal, Partial nitrification, sulphide, Sulfide autotrophic denitrification.

DEDICATION

I dedicate this thesis to my beloved parents, who have always been my source of inspiration and motivation throughout my academic journey. Their unwavering love, support and sacrifices have been my driving force in pursuing higher education and achieving academic success.

To my siblings, extended family and friends, thank you for your endless support, encouragement and prayers. Your unwavering belief in me has been a constant source of motivation, and I am forever grateful for your presence in my life.

Finally, I dedicate this thesis to all those who are striving to achieve their dreams and pursue their passion. May this thesis serve as a testament that with hard work, perseverance and determination, anything is possible.

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Magray Owaes Hassan

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LIST OF ABBREVIATIONS

Abbreviation	Description
Anammox	Anaerobic Ammonium Oxidation
WWTPs	Wastewater treatment plants
BNR	Biological nitrogen removal
MLE	Modified Ludzack-Ettinger
HRT	Hydraulic retention time
SRT	Sludge retention time
MLSS	Mixed liquor suspended solids.
SBR	Sequencing batch reactors
UASB	Upflow anaerobic sludge blanket
MBR	Membrane bioreactors
MBBR	Moving bed biofilm reactors.
DO	Dissolved oxygen.
PCR	Polymerase Chain Reaction
qPCR	Quantitative real-time polymerase chain reaction
FISH	Fluorescence in situ hybridization
PN	Partial nitritation
CANON	Completely autotrophic nitrogen removal over nitrite
SHARON	Single Reactor High Ammonia Removal Over Nitrite
DEAMOX	DEnitrifying AMmonium OXidation.

PNA	Partial nitritation-Anammox
AOB	Ammonia oxidising bacteria
NOB	Nitrite oxidising bacteria
CSTR	Completely Stirred tank reactor.
FBR	Fluidized bed reactor
SRB	sulfate-reducing bacteria
SOB	sulfur-oxidizing bacteria
TDDA	Thiosulfate-driven denitratation and Anammox
SAD	Sulfur autotrophic denitrification
SDD/Anammox	Sulfur-driven autotrophic denitrification and Anammox
SPDA	Sulfide-driven partial denitrification and Anammox
COD	Chemical oxygen demand
NRR	Nitrogen Removal Rate
NLR	Nitrogen Loading Rate
FNA	Free Nitrous acid
FA	Free ammonia
SAA	Specific Anammox activity
PVA	Polyvinyl alcohol

Thesis structure

The thesis is organized into seven chapters, each serving a distinct purpose within the research framework. Chapter 1 serves as the introduction, setting the stage for the study. In Chapter 2, the literature review is presented, providing a comprehensive overview of the relevant background information.

The subsequent chapters, numbered 3 through 6, correspond to the fulfillment of specific objectives. Chapter 3 addresses the first objective, Chapter 4 delves into the second objective, Chapter 5 focuses on the third objective, and Chapter 6 addresses the fourth objective. Each of these chapters contains detailed discussions on the methodologies employed, the results obtained, and the subsequent discussions drawn from those results.

A set of general conclusions is drawn upon the completion of Chapter 6, providing an overarching summary of the research findings. The references used throughout the thesis are consolidated and presented in a list after these general conclusions.

For supplementary information, appendices A and B have been included, providing additional data, details, or supplementary materials to enhance the comprehensiveness of the research.

CHAPTER ONE

1.0 BACKGROUND TO THE STUDY AND RESEARCH MOTIVATION

1.1 Introduction

Wastewater treatment processes play an important role in reducing the environmental impact caused by anthropogenic activities, particularly in eliminating nitrogenous compounds that pose a significant threat to ecosystems (Hreiz et al., 2015; Rittmann et al., 2001). Various innovative and sustainable treatment strategies have emerged, including partial nitrification, Anammox (Anaerobic Ammonium Oxidation), and integrated sulfide-driven partial denitrification and Anammox processes (Lackner et al., 2014; Chen et al., 2022; Deng et al., 2022). However, the influence of sulfide (S^{2-}) on these processes is a critical factor that demands careful consideration for their successful implementation.

Partial nitrification is a biological process that selectively converts ammonium (NH_4^+) to nitrite (NO_2^-) while preventing further oxidation to nitrate (NO_3^-) (Lackner et al., 2014). This controlled conversion reduces energy consumption and alkalinity requirements, making it an attractive option for wastewater treatment (Chen et al., 2022). Nonetheless, sulfide present in wastewater significantly affects the stability and performance of partial nitrification (Kouba et al., 2017). Sulfide, a common pollutant in industrial wastewater and anaerobic environments, can either inhibit or stimulate partial nitrification, depending on its concentration and environmental conditions (Vela et al., 2015). Understanding sulfide's impact on partial nitrification is crucial for optimizing treatment systems and ensuring long-term stability and efficiency (Ortiz et al., 2013).

The Anammox process represents a ground-breaking approach to nitrogen removal (Qin et al., 2019; Wu et al., 2020). It is an anaerobic process that directly converts ammonium and nitrite into nitrogen gas under specific environmental conditions (Zhang et al., 2020). Anammox offers advantages over conventional nitrogen removal methods, such as lower energy requirements; reduced sludge production; and a decreased carbon footprint. However, sulfide in wastewater can have both positive and negative effects on Anammox performance. Research indicates that sulfide concentrations within specific ranges can enhance the activity of Anammox bacteria, leading to improved process efficiency (Van de Graaf et al., 1996; Zhang et al., 2023). Conversely, higher sulfide concentrations can be toxic and inhibit the Anammox process (Jin et al., 2013). Therefore, a comprehensive understanding of the complex relationship between sulfide and Anammox is essential for optimizing and ensuring the reliability of Anammox-based wastewater treatment systems (Chen et al., 2023).

Recognizing the potential benefits of both partial nitrification and Anammox, researchers have explored the integration of these processes to achieve the simultaneous removal of nitrogen and sulfide. The integrated sulfide-driven partial denitrification and Anammox process capitalizes on sulfide's ability to serve as an electron donor for denitrifying bacteria during autotrophic denitrification (Kalyuzhnyi et al., 2006; Deng et al., 2022). This integration offers significant advantages, including improved resource recovery; reduced operational costs; and simplified processes (Cui et al., 2019b). However, the influence of sulfide on this integrated system is still not fully understood. Investigating the sulfide-mediated interactions between partial denitrification and Anammox is crucial to optimize this combined approach and ensure its successful implementation in wastewater treatment.

The successful implementation of wastewater treatment processes requires careful consideration of sulfide's influence on partial nitrification, the Anammox process, and the integrated sulfide-driven partial denitrification and Anammox process. Through comprehensive research on the effects of sulfide on these processes, strategies can be developed to mitigate any adverse effects, optimize performance, and promote sustainable nitrogen removal in wastewater treatment systems (Deng et al., 2022). Such knowledge and advancements will contribute to the effective management of nitrogenous compounds, safeguarding environmental health and water resources.

Despite the considerable research conducted on partial nitrification, Anammox and the integrated sulfide-driven partial denitrification and Anammox processes, there exists a notable research gap regarding the influence of sulfide on these systems. Although it is acknowledged that sulfide can affect the stability and performance of these processes, a comprehensive understanding of the mechanisms underlying sulfide-mediated interactions and their impact on process optimization is lacking.

In the case of partial nitrification, while the inhibitory or stimulatory effects of sulfide have been observed, the specific mechanisms governing these effects remain unclear. Factors such as sulfide concentration, pH, temperature, and co-existing compounds may interact to influence the overall performance and stability of the partial nitrification process. Additionally, the long-term effects of sulfide exposure and the potential for process recovery after sulfide perturbations have not been extensively studied. Addressing these knowledge gaps is key for developing strategies to mitigate the negative effects of sulfide and enhance the efficiency and stability of partial nitrification in wastewater treatment.

Similarly, in the context of the Anammox process, the complex relationship between sulfide concentration and Anammox performance requires further investigation. While it has been observed that sulfide concentrations within specific ranges can enhance Anammox activity, the underlying mechanisms are not well understood. The threshold at which sulfide becomes toxic to Anammox bacteria and inhibits the process needs to be determined. Furthermore, the influence of sulfide on the long-term stability and reliability of Anammox-based treatment systems requires comprehensive exploration. Understanding these sulfide-mediated interactions is vital for optimizing the Anammox process and ensuring its successful implementation in wastewater treatment.

In the case of the integrated sulfide-driven partial denitrification and Anammox process, the impact of sulfide on this combined approach is still not fully elucidated. The synergistic effects of sulfide on the performance and stability of both partial denitrification and Anammox need to be investigated in order to optimize the integrated system. Furthermore, the influence of sulfide on the recovery and resilience of the integrated process after perturbations remains unexplored. Studying the sulfide-mediated interactions in this integrated system will contribute to its optimization and effective implementation in wastewater treatment.

Thus, the research gap lies in comprehensively understanding the sulfide-mediated interactions in partial nitrification, the Anammox process, and the integrated sulfide-driven partial denitrification and the Anammox process. Addressing this research gap will provide valuable insights into optimizing these treatment processes, ensuring their long-term stability and efficiency, and promoting sustainable nitrogen removal in wastewater treatment systems.

1.2 Aim

To improve the feasibility of the Anammox process as a sustainable nitrogen removal process from anaerobic effluent

1.3 Objectives

1. To determine the long-term effects of sulphide on partial nitrification.

- To evaluate the role of different strategies such as controlling DO intermittent aeration, low pH shocks, and sulfide addition in the start-up of PN using activated sludge as inoculum. *(Chapter 3)*
- To investigate the dynamics of the key nitrifying microbial population under different operational strategies. *(Chapter 3)*
- To investigate the approaches in which the basic hydrolysis of the sulphide salt can be harnessed to establish PN. *(Chapter 4)*

2. To ascertain the effect effect of Sulfide stress on the activity, performance, and recovery of the Anammox process *(Chapter 5)*
    - To evaluate the long-term effects of sulfide on Anammox activity or to evaluate the long-term nitrogen removal of Anammox performance in an up-flow anaerobic sludge blanket bioreactor (UASB) under increasing sulfide concentrations.
    - To identify the inhibitory concentration level of sulfides to the Anammox process.
    - To reveal and compare the microbial characteristics of sludge before and after the complete inhibition by using the 16S rRNA and gene sequence and qPCR analysis.

3. To evaluate the feasibility of a combined Anammox and sulfur-driven partial denitrification process for nitrogen removal from domestic anaerobic effluents. *(Chapter 6)*

- To investigate the impact of varying hydraulic retention times (HRTs) and external sulfide dosing on the performance of the SPDA process.
- To examine the microbial community structure of the reactor under different operational conditions to verify the presence of AOB, Anammox bacteria and SOB in the reactor.

1.4 Scope of the study

The scope of this study encompasses the investigation of sulfide-mediated interactions in nitrogen removal processes, specifically focusing on partial nitrification, the Anammox process, and the integrated sulfide-driven partial denitrification and Anammox process. The study aims to gain a comprehensive understanding of the effects of sulfide on these processes, including its influence on stability, performance, and optimization. The research involves examining the underlying mechanisms of sulfide-mediated interactions and their impact on operational parameters, reactor design, and control strategies. The study also explores the environmental and health implications of sulfide in nitrogen removal processes, addressing the risks associated with nitrogen pollution and the release of harmful nitrogen by-products. By conducting a comprehensive investigation, this study seeks to provide valuable insights, practical guidelines and design criteria for optimizing nitrogen removal processes and promoting sustainable wastewater treatment practices.

1.5 Concluding summary.

In conclusion, the investigation of sulfide-mediated interactions in partial nitrification, the Anammox process, and the integrated sulfide-driven partial denitrification and Anammox process is justified based on the critical research gap, the need for process optimization, the environmental and health implications, the advancements in sustainable wastewater treatment, and the contribution to knowledge and practice. This comprehensive study will provide valuable insights to optimize nitrogen removal processes, ensure their long-term stability and efficiency, and promote sustainable wastewater treatment practices. By comprehensively studying the effects of sulfide on these processes, including the underlying mechanisms and their impact on operational parameters, reactor design and control strategies, strategies can be developed to mitigate any adverse effects and enhance process performance. This research will not only bridge the existing research gap but also contribute to the body of knowledge in the field of wastewater treatment, leading to advancements in sustainable nitrogen removal technologies. Ultimately, the findings from this study will have practical implications, guiding the development of guidelines, operational strategies and design criteria for treatment systems, thus enabling the implementation of more efficient and sustainable nitrogen removal processes in real-world applications. By safeguarding environmental health and water resources, this research will contribute to the effective management of nitrogenous compounds, promoting a more sustainable and resilient approach to wastewater treatment.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of the Nitrogen Removal Process

Nitrogen removal from wastewater is of paramount importance due to its adverse effects on the environment and human health (Zhang et al., 2019; Sandip et al., 2017). Excessive nitrogen in wastewater, primarily in the form of ammonium, can lead to eutrophication, harmful algal blooms, and oxygen depletion in receiving water bodies (Gruber et al., 2008; Johnson et al., 2015). Traditional nitrogen removal methods, such as nitrification and denitrification, have been widely employed in wastewater treatment plants (Rittmann and McCarty 2001). However, these processes require substantial energy inputs and often produce greenhouse gases, thereby contributing to environmental concerns (Berner et al., 2006).

The Anammox (anaerobic ammonium oxidation) process has emerged as an innovative and sustainable alternative for nitrogen removal from wastewater (Lackner et al., 2014; Deng et al., 2022). Anammox is a microbiological process that converts ammonium and nitrite directly into harmless nitrogen gas, significantly reducing energy requirements and carbon footprint compared to conventional methods (Wu et al., 2020; Deng et al., 2022). The Anammox process offers several advantages, including high nitrogen removal efficiency, reduced sludge production, and potential cost savings (Cui et al., 2019a).

In recent years, the role of sulphur compounds in driving Anammox systems has gained attention (Russ et al., 2015; Deng et al., 2022). Sulfur-driven Anammox systems involve the utilization of various sulphur compounds to facilitate and enhance the Anammox process (Shao et al., 2020). These sulphur compounds act as electron acceptors and energy sources for the Anammox bacteria, promoting their activity and overall nitrogen removal efficiency.

This literature review aims to explore the application of sulphur-driven Anammox systems for nitrogen removal from wastewater. The chapter provides an overview of the Anammox process and its mechanisms, highlighting the importance of sulphur in driving Anammox systems. Additionally, the chapter focuses on the performance evaluation, technological challenges and limitations associated with sulphur-driven Anammox systems. The feasibility and applicability of sulphur-driven Anammox systems in full-scale wastewater treatment plants are also be discussed, along with potential economic and environmental benefits.

2.2 Nitrogen Removal Process

Nitrogen is a crucial component of proteins, nucleic acids, and other biological components, and is required by all living organisms (Zhang et al., 2019; Sandip et al., 2017). Most organisms, however, cannot directly utilise atmospheric nitrogen (N2), which must be transformed into more accessible forms by a variety of biological and chemical processes (Erisman et al., 2013; Gruber et al., 2008). The nitrogen cycle involves a series of reactions that convert nitrogen from one form to another and transport it through different environmental compartments (Zhang et al., 2019; Zhang et al., 2016). In Figure 2.1, the major processes and reactions of the nitrogen cycle are illustrated, including those which have recently been identified.

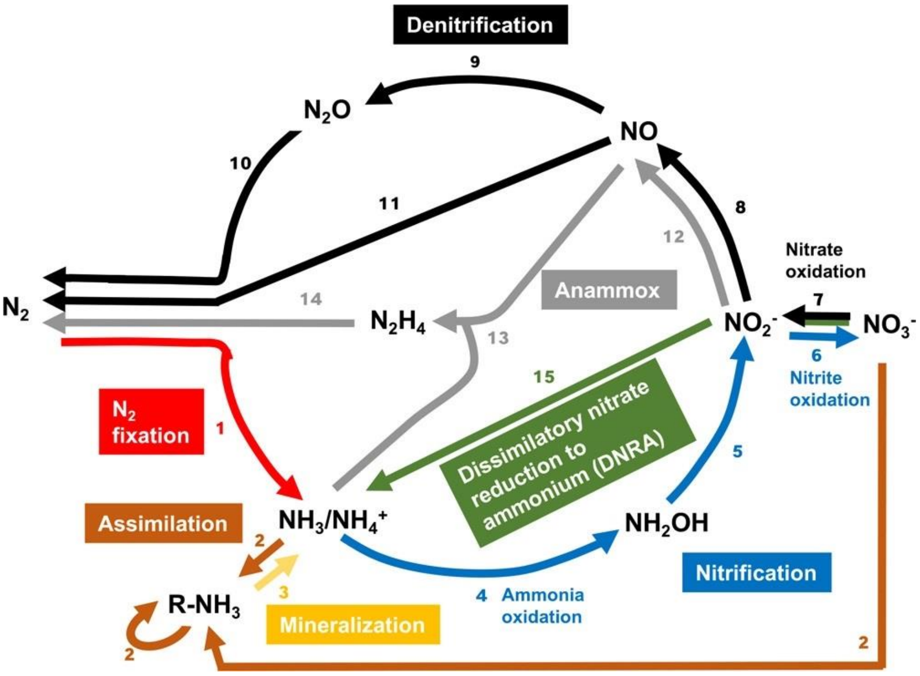

Figure 2.1: Biologically driven nitrogen transformations

The biological driven Nitrogen transformation that takes place in natural and human-influenced terrestrial and marine environments follow a cycle. The cycle consists of various steps, including nitrogen (N_2) fixation (step 1) and N assimilation (step 2), which are anabolic processes. Conversely, mineralization (step 3), nitrification (steps 4−6), DNRA (steps 7, 15), denitrification (steps 7−11), and Anammox (anaerobic ammonium oxidation, steps 12−14), which are catabolic processes or are a consequence of them. Fixed N from abiotic sources enters the biological N cycle as ammonium and nitrate. (Zheng et al., 2019)

Nitrogen Fixation: The nitrogen cycle begins with nitrogen fixation, which is the conversion of atmospheric nitrogen (N_2) into ammonium (NH_4^+). Nitrogen-fixing bacteria carry out this process, which can be free-living or symbiotic (Fowler et al., 2015). The most well-known nitrogen-fixing bacteria are rhizobia, which form symbiotic associations with leguminous plants

(Johnson et al., 2015; Fowler et al., 2015). Nitrogen fixation can also occur through lightning strikes and industrial processes.

$N_2 + 8H^+ + 8e- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$ (2.1)

Nitrification: Nitrification is the process by which ammonium is transformed into nitrite (NO_2^-) and subsequently nitrate (NO_3^-) (Rittmann et al., 2001; McCarty et al., 2018). Ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) are responsible for this process.

$NH_4^+ + 2O_2 \rightarrow NO_2^- + 2H^+ + 2H_2O$ (by AOB) $NO_2^- + 0.5O_2 \rightarrow NO_3^-$ (by NOB) (2.2)

Assimilation: Plants and other organisms take up nitrogen from the environment in the form of ammonium, nitrate or organic nitrogen compounds (Gruber et al., 2008; Berner et al., 2006). This process is called assimilation, and it is essential for the synthesis of proteins, nucleic acids and other cellular components (Berner et al., 2006).

Mineralization: Organic nitrogen compounds, such as amino acids and proteins, are broken down into ammonium through the process of mineralization (Batjes et al., 2014). This process is carried out by microbial decomposers, such as bacteria and fungi.

Denitrification: Denitrification is the process by which nitrate is turned back into nitrogen (N2) in the air (Rittmann et al., 2001; McCarty et al., 2018). Denitrifying bacteria work in anaerobic circumstances to accomplish this task. Denitrification is a crucial step in wastewater treatment plants because it removes nitrogen.

$NO_3- \rightarrow NO_2- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (2.3)

Ammonification: The final step in the nitrogen cycle is ammonification, which is the conversion of organic nitrogen compounds into ammonium (Du et al., 2020). This process is carried out by a variety of micro-organisms, including bacteria and fungi.

In conclusion, the nitrogen cycle is a complex and dynamic process that plays a critical role in the functioning of ecosystems. The cycle involves a series of biological and chemical reactions that transform nitrogen from one form to another and move it through different environmental compartments. Understanding the nitrogen cycle is essential for managing nitrogen in agricultural, industrial and wastewater treatment systems, as well as for preserving the health and productivity of ecosystems.

2.3 Removal of nitrogen in wastewater treatment plants

Nitrogen pollution is a significant environmental concern that has gained increasing attention in recent years (McCarty, 2018). Excessive nitrogen in ecosystems can have detrimental effects on water bodies, air quality and overall ecosystem health (Zhang et al., 2019). One of the primary contributors to nitrogen pollution is human activities, particularly from agricultural practices and wastewater discharge (Fowler et al., 2015; Johnson et al., 2015). Nitrogen is an essential nutrient for plant growth, and farmers often apply fertilizers containing nitrogen to enhance crop yields (Du et al., 2020). However, excessive fertilizer use, improper application techniques and inefficient nutrient management can result in nitrogen runoff from agricultural fields into nearby water bodies (Gruber et al., 2008). Once in the water, nitrogen can cause eutrophication, a process in which excessive nutrient enrichment leads to the rapid growth of algae and other aquatic plants. The subsequent decomposition of this biomass depletes oxygen levels, negatively impacting fish and other aquatic organisms, and creating dead zones in water bodies.

Apart from agricultural sources, wastewater treatment plants (WWTPs) play a crucial role in nitrogen pollution due to the discharge of treated sewage into water bodies (Du et al., 2020; Ahn et al., 2006). Human and industrial activities produce large quantities of nitrogen-rich organic waste, which, if not adequately treated, can contribute to nitrogen pollution. WWTPs serve as a critical line of defence in removing nitrogen compounds from wastewater before it is released into the environment. WWTPs employ various treatment processes to remove nitrogen from wastewater, primarily through biological processes (Pochana et al., 1999). One commonly used method is the activated sludge process, where micro-organisms break down organic matter and convert nitrogen compounds into nitrogen gas, a process known as nitrification-denitrification. During nitrification, ammonia (NH_3) is converted into nitrate (NO_3-), and then denitrification converts nitrate into nitrogen gas (N_2), which is released into the atmosphere harmlessly.

Another approach used in WWTPs is the incorporation of constructed wetlands, which leverage natural processes to remove nitrogen (Eckenfelder et al., 1998; Ren et al., 2020). Wetland plants and micro-organisms in these systems can take up nitrogen and convert it into biomass or release it back into the atmosphere through microbial transformations. In recent years, there have been advancements in nitrogen removal technologies, such as the development of innovative treatment systems and the optimization of existing processes in WWTPs (Madan et al., 2022; Liu et al., 2019). These advancements aim to enhance nitrogen removal efficiency and minimize the release of nitrogen compounds into the environment. However, despite the efforts made by

WWTPs, challenges remain. Ageing infrastructure, limited funding and increasing population growth can strain the capacity of existing treatment facilities, leading to sub-optimal nitrogen removal (Liu et al., 2019). Moreover, agricultural practices continue to contribute to nitrogen pollution, requiring comprehensive strategies involving improved fertilizer management and enhanced nutrient utilization in farming systems (Ren et al., 2020; Wang et al., 2020).

2.3.1 Biological Nitrogen Removal from wastewater

In the field of wastewater treatment, biological nitrogen removal (BNR) plays a crucial role in mitigating the environmental impact of nitrogen compounds present in wastewater (Johnson et al., 2015; Berner et al., 2006). Nitrogen, primarily in the form of ammonium (NH_4^+) and organic nitrogen, is a common pollutant that can lead to eutrophication and harmful algal blooms when discharged into receiving bodies of water (Metcalf & Eddy 2014; Rittmann et al., 2001). BNR processes aim to convert nitrogen compounds into less harmful forms, such as nitrogen gas (N_2), through microbial-mediated transformations (Zhang et al., 2016). Understanding the existing processes of BNR is essential for optimizing wastewater treatment systems and ensuring efficient nitrogen removal (Fowler et al., 2015; Johnson et al., 2015). The effectiveness of BNR relies on harnessing two primary mechanisms: nitrification and denitrification (Ren et al. 2020; Wang et al. 2020).

2.4 Nitrification and Denitrification

Nitrification and denitrification are two essential processes that play a crucial role in the global nitrogen cycle (Metcalf & Eddy 2014). Nitrification is a two-step process that involves two different groups of bacteria. AOB converts ammonia to nitrite, while NOB converts nitrite to nitrate (Metcalf & Eddy 2014; Rittmann et al., 2001). The first step is carried out by AOB, which are mainly members of the genus *Nitrosomonas* and *Nitrospira* (Rittmann et al., 2001; Zhang et al., 2019). These bacteria use the enzyme ammonia *monooxygenase* to oxidize ammonia to nitrite. The second step is carried out by NOB, which are mainly members of the genus *Nitrobacter* and *Nitrospira* (Ahn et al., 2012; Metcalf & Eddy 2014). These bacteria use the enzyme nitrite oxidoreductase to convert nitrite to nitrate. The overall reaction can be represented as follows:

$NH_4^+ + 2O_2 \rightarrow NO_2- + 2H^+ + H_2O$ (2.4)

$NO_2^- + 0.5O_2 \rightarrow NO_3^-$ (2.5)

Denitrification is a process in which bacteria convert nitrate to nitrogen gas, which is released into the atmosphere (Metcalf & Eddy 2014; Rittmann et al., 2001). Denitrification is carried out by facultative anaerobic bacteria that use nitrate as an electron acceptor in the absence of oxygen (Ahn et al., 2006; Cao et al., 2011). The process involves the sequential reduction of nitrate to nitrite, nitric oxide, nitrous oxide, and finally to nitrogen gas. The enzymes involved in denitrification are nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase (Chen et al., 2013). The overall reaction can be represented as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (2.6)$$

Nitrification and denitrification are influenced by various environmental factors such as temperature, pH, dissolved oxygen, and the availability of nutrients (Chen et al., 2013; Metcalf & Eddy 2014; Rittmann et al., 2001). Nitrification is an aerobic process that is favored by high dissolved oxygen levels, a neutral pH and a temperature range of 20-30°C (Ahn et al., 2006). However, nitrification is inhibited by low oxygen levels, low pH and the presence of toxic substances such as heavy metals and pesticides (Chen et al., 2013). Denitrification, on the other hand, is an anaerobic process that is favored by low dissolved oxygen levels, a neutral to slightly acidic pH, and a temperature range of 25-35°C (Chen et al., 2013; Ahn et al., 2006). However, denitrification is inhibited by high oxygen levels, low pH, and the absence of organic matter. Denitrification also requires a source of organic carbon as an electron donor (Ahn et al., 2006).

2.5 Conventional BNR Processes

Conventional biological nitrogen removal (BNR) processes, including activated sludge and modified Ludzack-Ettinger (MLE) processes, have been widely employed in wastewater treatment plants for nitrogen removal (Liu et al., 2017). Activated sludge is a commonly used BNR process that involves the continuous aeration of wastewater in the presence of suspended microbial flocs (Chrispim et al., 2019). The process operates on the principle of creating an environment conducive to nitrification and denitrification within the activated sludge system (Eckenfelder et al., 1998; Ren et al., 2020). In the aeration tank, the incoming wastewater is mixed with a population of micro-organisms, which include nitrifying bacteria and denitrifying bacteria (Wang et al., 2013). These micro-organisms utilize the available oxygen and organic carbon in the wastewater to carry out the respective processes (Vaiopoulou et al., 2008; Lee et al., 2003). The aeration provides the necessary oxygen for the growth and activity of nitrifying bacteria, which oxidize ammonium to nitrate (Madan et al., 2022; Liu et al., 2019). After the aeration phase, the wastewater is directed to a settling tank, where the biomass (activated sludge) is allowed to settle, and the treated effluent is separated from the biomass (Metcalf & Eddy 2014; Rittmann et al., 2001). Some of the settled biomass is recycled back to the aeration tank to maintain a sufficient population of micro-organisms for nitrogen removal (Ren et al., 2020; Lee et al., 2003).

The modified Ludzack-Ettinger (MLE) process is a variant of the activated sludge process that incorporates separate anoxic and aerobic zones to enhance denitrification efficiency (Liu et al., 2017; Ren et al., 2020). This process is designed to create favorable conditions for both nitrification and denitrification. The wastewater flows sequentially through the anoxic zone where nitrate is converted to nitrogen gas through denitrification, and the aerobic zone where ammonium is oxidized to nitrate through nitrification (Wang et al., 2013; Ren et al., 2020). By providing anoxic conditions in the denitrification zone, the process ensures that nitrate is used as an electron acceptor by denitrifying bacteria instead of dissolved oxygen. This allows for the effective removal of both nitrogen compounds in a single treatment system. The design considerations for conventional BNR processes involve optimizing various operational parameters (Metcalf & Eddy 2014; Rittmann et al., 2001). Key considerations include controlling the mixed liquor suspended solids (MLSS) concentration, the sludge retention time (SRT), and the hydraulic retention time (HRT) in order to maintain a stable and efficient process (Ren et al., 2020). The MLSS concentration is crucial for providing a sufficient biomass concentration to achieve effective nitrogen removal (Liu et al., 2019). The SRT influences the microbial population and their activity, allowing for proper nitrification and denitrification (Wang et al., 2013). The HRT determines the time available for micro-organisms to perform nitrogen removal reactions. Additionally, monitoring and controlling parameters such as dissolved oxygen, pH and temperature are essential for maintaining optimal microbial activity and process efficiency (Ren et al., 2020). Conventional BNR processes offer several advantages (Wang et al., 2013; Wang et al., 2020). They have a proven track record of successful nitrogen removal in wastewater treatment plants. These processes can be implemented in both small-scale and large-scale treatment systems. They are relatively simple to operate and require conventional treatment infrastructure, making them cost-effective options for many facilities (Pochana et al., 1999; Wang et al., 2020). Moreover, the experience and knowledge accumulated over the years in operating conventional BNR processes contribute to their widespread application and troubleshooting capabilities.

However, conventional BNR processes also have some limitations. One challenge is their sensitivity to fluctuations in influent wastewater characteristics, such as nitrogen loading rates and composition (Bhattacharya et al., 2021). Sudden changes in these parameters can impact the stability and performance of the process, leading to decreased nitrogen removal efficiency (Wang et al., 2020). Additionally, conventional BNR processes often require significant land area and energy consumption due to the extensive infrastructure needed for aeration, settling and sludge treatment (He et al., 2020). The accumulation of excess biomass (sludge) and its disposal can also pose challenges in terms of solids handling and disposal costs.

2.5.1 Advanced BNR Processes

In recent years, advanced BNR processes have emerged as innovative solutions to enhance the efficiency and performance of nitrogen removal in wastewater treatment. These processes, including sequencing batch reactors (SBR), membrane bioreactors (MBR) and moving bed biofilm reactors (MBBR), offer unique features and benefits that differentiate them from conventional BNR processes (Metcalf & Eddy 2014; Rittmann et al., 2001).

Sequencing batch reactors (SBR) represent a flexible and versatile BNR technology that operates in a cyclic batch mode. The SBR process involves the sequential filling, reaction, settling and decanting of wastewater within a single reactor (Metcalf & Eddy 2014; Rittmann et al., 2001). This cyclic approach allows for precise control of treatment phases and provides inherent flexibility in managing variations in influent characteristics and flow rates (Hreiz et al., 2015). SBRs can achieve high nitrogen removal efficiencies by optimizing the duration and sequence of anoxic, aerobic and settling periods within each cycle. The controlled environment enables the cultivation of specific microbial communities tailored for efficient nitrification and denitrification. Furthermore, SBR systems can easily adapt to variations in hydraulic and organic loading, making them suitable for both small and large wastewater treatment plants.

Membrane bioreactors (MBR) combine the principles of activated sludge treatment with membrane filtration technology. In MBR systems, the suspended growth biomass is separated from the treated effluent using semi-permeable membranes, creating a barrier that retains the biomass while allowing the permeation of high-quality effluent (Mishra et al., 2022). This physical separation provides several advantages for nitrogen removal. Firstly, it enables a high biomass concentration within the bioreactor, enhancing the treatment efficiency (Tang et al., 2022). Secondly, membrane filtration acts as a barrier to suspended solids and pathogens, resulting in superior effluent quality. Additionally, the sludge retention within the bioreactor enhances the retention of nitrifying bacteria and promotes stable nitrogen removal performance. MBRs offer a compact footprint, reduced sludge production and enhanced process control, making them suitable for space-constrained areas and stringent effluent quality requirements (Tang et al., 2022; Mishra et al., 2022).

Moving bed biofilm reactors (MBBR) utilize a suspended biofilm carrier media to create a biofilm reactor environment within the treatment system. The carrier media provides a surface for biofilm formation, where microorganisms attach and develop a biofilm layer (Liu et al., 2023). The wastewater passes through the biofilm reactor, allowing for simultaneous nitrification and denitrification within the biofilm. The key advantage of MBBR systems is their ability to support a high biomass concentration, leading to enhanced treatment efficiency and nitrogen removal rates (Madan et al., 2022; Liu et al., 2023). The carrier media also improves biomass retention and allows for biomass growth in a controlled manner. MBBRs offer operational flexibility, robustness against shock loads, and resistance to biomass washout (Madan et al., 2022). These features make MBBR systems suitable for various wastewater applications, including industrial wastewater and decentralized treatment plants.

When compared to conventional BNR processes, advanced BNR processes demonstrate several performance advantages. Firstly, the enhanced process control and flexibility provided by SBRs enable more precise and efficient nitrogen removal. SBRs can achieve comparable or even higher nitrogen removal efficiencies than conventional processes, whilst also adapting well to changing influent conditions (Hreiz et al., 2015; Rittmann et al., 2001). MBRs, with their membrane filtration, offer superior effluent quality in terms of suspended solids and pathogen removal, ensuring the protection of receiving water bodies (Eckenfelder et al., 1998). MBRs also exhibit higher biomass concentration, resulting in improved nitrification and denitrification rates. Additionally, MBBRs, with their biofilm carrier media, provide a greater surface area for biofilm growth, allowing for higher biomass retention and improved treatment efficiency (Hreiz et al., 2015). MBBR systems have demonstrated robustness in handling shock loads and maintaining stable performance. By harnessing innovative technologies, advanced BNR processes contribute to the sustainable management of nitrogen compounds in wastewater and play a significant role in environmental protection and water resource conservation (Metcalf & Eddy 2014).

2.6 Anaerobic Ammonium Oxidation (Anammox)

The Anammox process is a cutting-edge wastewater treatment technology that has revolutionized the field of environmental engineering. This process is based on the unique metabolic pathway of Anammox bacteria, which allows them to convert ammonium and nitrite directly into nitrogen gas, without the need for external carbon sources (Chen et al., 2022; Ali et al., 2015; Adams et al., 2022). This not only makes the Anammox process highly efficient, but also significantly reduces the cost and environmental impact of traditional wastewater treatment methods (Abma et al., 2010; Ali et al., 2015). With its remarkable ability to remove nitrogen from wastewater in a sustainable and energy-efficient manner, the Anammox process is rapidly gaining popularity worldwide and is expected to play a key role in shaping the future of wastewater treatment (Lackner et al., 2014; Chen et al., 2022). The Anammox process was first discovered in the 1990s by Dutch researchers who observed the anaerobic conversion of ammonium and nitrite to dinitrogen gas by a group of bacteria called Planctomycetes (van de Graff et al., 1996; Strous et al., 1998). These bacteria have unique cell structures, such as intracellular membranes and compartments that allow them to perform complex metabolic reactions without the need for oxygen (Liang et al., 2014; Ali et al., 2015). The Anammox process has since been recognized as a key player in the global nitrogen cycle, as well as being a potential source of renewable energy.

Anammox bacteria belong to the phylum *Planctomycetes*, which is a diverse group of micro-organisms that inhabit various environments, including aquatic, soil and sediment (Jin et al., 2012; Liang et al., 2014). The dominant genera of Anammox bacteria are *Candidatus Brocadia, Candidatus Kuenenia*, and *Candidatus Scalindua*, which have different physiological and ecological characteristics (Cheng et al., 2022). Anammox bacteria, employ a unique metabolic pathway known as the anaerobic ammonium oxidation (Anammox) pathway. This pathway allows them to convert ammonium (NH_4^+) and nitrite (NO_2^-) into nitrogen gas (N_2) under anaerobic conditions. The Anammox pathway involves several key reactions:Ammonium (NH_4^+) oxidation: Anammox bacteria use the enzyme ammonium monooxygenase to oxidize ammonium into hydroxylamine (NH_2OH).

Hydroxylamine (NH_2OH) conversion: Hydroxylamine is further converted to nitric oxide (NO) through the enzyme hydrazine synthase. This step involves the reduction of NH_2OH with a hydrazine molecule (N_2H4) to form hydrazine ($N_2H_5^+$). Nitric oxide (NO) production: Hydrazine is oxidized by hydrazine dehydrogenase, producing nitric oxide (NO) and hydrogen gas (H_2).Nitric oxide (NO) reduction: The produced nitric oxide (NO) is then reduced to nitrous oxide (N2O) by nitric oxide reductase.Nitrous oxide (N_2O) reduction: Finally, nitrous oxide (N_2O) is reduced to nitrogen gas (N_2) by nitrous oxide reductase.By utilizing this pathway, Anammox bacteria can effectively remove nitrogen from various sources, such as wastewater treatment plants and natural environments. The Anammox process plays a crucial role in the nitrogen cycle, contributing to the overall balance of nitrogen in ecosystems. (Cheng et al., 2022; Liang et al., 2014). The reaction can be represented as follows:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- \rightarrow 1.05N_2 + 0.264H_2O + 0.132H^+ + 0.066CO_2 \quad (2.7)$$

The Anammox process is a biological nitrogen removal process that has attracted significant attention in recent years due to its potential for the cost-effective and environmentally friendly treatment of wastewater (Lackner et al., 2014; Cheng et al., 2022). However, the efficiency of the Anammox process can be affected by various environmental factors, such as temperature, pH, dissolved oxygen, Hydraulic retention time (HRT) and substrate concentration.

Temperature: is one of the most important factors that affect the Anammox process. Anammox bacteria have been shown to be able to grow at temperatures ranging from 5 to 43°C, with optimal growth occurring between 30 and 40°C (Wang et al., 2018). At low temperatures, the Anammox process is inhibited, and the conversion rates of ammonium and nitrite to nitrogen gas are reduced. At high temperatures, the Anammox bacteria may become thermally inactivated, leading to reduced conversion rates. Several studies have investigated the effect of temperature on the Anammox process. Liu et al. (2022) studied the effect of temperature on the Anammox process using a sequencing batch reactor (SBR). The results showed that the nitrogen removal efficiency decreased from 85% to 51% as the temperature decreased from 30 to 15°C. At temperatures below 15°C, the Anammox process was completely inhibited. Another study by Gao et al. (2022) investigated the effect of temperature on the performance of Anammox in a membrane bioreactor (MBR). The results showed that the nitrogen removal efficiency increased from 86% to 93% as the temperature increased from 25 to 35°C. However, at temperatures above 35°C, the nitrogen removal efficiency decreased, possibly due to to thermal inactivation of the Anammox bacteria.

In addition to the effect on nitrogen removal efficiency, temperature also affects the microbial community structure of Anammox bacteria. Zhao et al. (2021) studied the effect of temperature on the microbial community structure of Anammox bacteria using high-throughput sequencing.

The results showed that the relative abundance of *Candidatus Kuenenia*, the dominant Anammox bacteria, increased as the temperature increased from 20 to 35°C.

pH: The pH can have a significant impact on the activity and growth of the Anammox bacteria as they are sensitive to changes in the environment. Several studies have investigated the effect of pH on the Anammox process (Liu et al., 2008; Tang et al., 2010). Generally, Anammox bacteria are known to be sensitive to pH changes, and the optimum pH for the Anammox process is reported to be between 7.3 and 8.3 (Van de Graaf et al., 1996). pH values outside this range can affect the activity and growth of Anammox bacteria, leading to a decrease in the performance of the Anammox process. Several studies have reported that low pH values can inhibit the Anammox process. For example, Li et al. (2012) found that Anammox bacteria were significantly inhibited at pH 6.5, resulting in a decrease in nitrogen removal efficiency. Similarly, Tang et al. (2009) reported that Anammox activity was reduced by 50% at pH 6.5 compared to pH 7.5. These results suggest that maintaining the pH of the Anammox reactor within the optimum range is critical for the stable operation of the Anammox process.

While low pH values can inhibit the Anammox process, high pH values can also have a negative effect on the process. For example, Wang et al. (2014) reported that Anammox activity was reduced by 50% at pH 9 compared to pH 8. Similarly, Zhu et al. (2015) found that Anammox activity was reduced by 60% at pH 9 compared to pH 7.5. These studies suggest that maintaining the pH of the Anammox reactor within the optimum range is essential for achieving high nitrogen removal efficiency. The buffering capacity of the Anammox reactor can affect the pH of the reactor, and hence the Anammox process. For example, Karasuta et al. (2021) reported that adding a bicarbonate buffer to the Anammox reactor could enhance the buffering capacity of the reactor and stabilize the pH, resulting in an increase in nitrogen removal efficiency. Similarly, Liu et al. (2008) found that adding calcium carbonate to the Anammox reactor could also enhance the buffering capacity and improve the performance of the Anammox process.

Substrate concentration: The concentration of substrate is a critical factor that affects the efficiency and rate of the Anammox process. Substrate concentration refers to the concentration of ammonium present in wastewater. Inadequate substrate concentration may lead to insufficient ammonium for the Anammox bacteria, while excess concentration may lead to the inhibition of Anammox bacteria. Therefore, the optimal concentration of substrate is crucial for the optimal performance of the Anammox process.

Several studies have investigated the effect of substrate concentration on the Anammox process. For instance, Strous et al. (1998) studied the effect of ammonium concentration on the Anammox process using an upflow anaerobic sludge blanket (UASB) reactor. The study found that the maximum nitrogen removal rate occurred at an ammonium concentration of 60 mg/L, which was consistent with previous studies. At higher concentrations of ammonium, the rate of nitrogen removal decreased, likely due to toxicity effects on the Anammox bacteria. Similarly, Wang et al. (2023) investigated the effect of substrate concentration on the Anammox process in UASB. They found that the optimal substrate concentration for the Anammox process was 50-100 mg/L. Higher concentrations of substrate led to a decrease in nitrogen removal efficiency, likely due to the inhibition of the Anammox bacteria. In addition, Ma et al. (2017) examined the effect of substrate concentration on the microbial community structure in an Anammox reactor. They found that increasing the substrate concentration led to a shift in the microbial community, with an increase in the relative abundance of denitrifying bacteria and a decrease in the relative abundance of Anammox bacteria. This shift in the microbial community may explain the decrease in nitrogen removal efficiency at higher substrate concentrations. In contrast, Fernandez et al. (2012) investigated the effect of substrate concentration on the Anammox process using a continuous-flow reactor. They found that increasing the substrate concentration led to an increase in nitrogen removal efficiency, up to a concentration of 500 mg/L. At higher concentrations, the nitrogen removal efficiency decreased, likely due to toxicity effects on the Anammox bacteria.

Dissolved Oxygen: Anammox bacteria are obligate anaerobes and can only survive and function under anoxic conditions. Therefore, the presence of dissolved oxygen can significantly affect the Anammox process. High concentrations of dissolved oxygen can inhibit the growth and metabolism of Anammox bacteria, resulting in reduced nitrogen removal efficiency. Several studies have investigated the effect of dissolved oxygen on the Anammox process. Cui et al. (2021) studied the effect of dissolved oxygen on the performance of Anammox in an upflow anaerobic sludge blanket (UASB) reactor. The results showed that at a dissolved oxygen concentration of 0.5 mg/L, the nitrogen removal efficiency was over 90%. However, at a dissolved oxygen concentration of 2.0 mg/L, the nitrogen removal efficiency decreased to less than 60%. Another study by Zhang et al. (2021b) investigated the effect of dissolved oxygen on the microbial community structure of Anammox bacteria in a sequencing batch reactor (SBR). The results showed that the relative abundance of Anammox bacteria decreased significantly as the dissolved oxygen concentration increased from 0.5 to 2.0 mg/L. Furthermore, the study revealed that the presence of dissolved oxygen led to a shift in the microbial community structure of the Anammox bacteria, with an increase in the abundance of denitrifying bacteria.

In addition to the effect on nitrogen removal efficiency and microbial community structure, dissolved oxygen can also affect the activity and performance of Anammox bacteria at the gene level. Zhao et al. (2022) studied the effect of dissolved oxygen on the gene expression of Anammox bacteria using the quantitative real-time polymerase chain reaction (qPCR). The results showed that the expression of key Anammox genes decreased significantly under aerobic conditions, indicating the downregulation of Anammox metabolism.

Hydraulic retention time (HRT) refers to the amount of time that wastewater remains in the reactor (Wang et al., 2019). It is an important operational parameter that affects the Anammox process. HRT influences the rate of nitrogen removal and the efficiency of the Anammox process (Lu et al., 2014). An insufficient HRT may not provide enough time for the Anammox bacteria to oxidize ammonium, while an excessive HRT may lead to the accumulation of nitrite, reducing the nitrogen removal efficiency.

Several studies have investigated the effect of HRT on the Anammox process. For instance, Zhang et al. (2022) studied the effect of HRT on the Anammox process using a continuous-flow reactor. The study found that the optimal HRT for the Anammox process was 10 hours. At shorter HRTs, the nitrogen removal efficiency decreased due to insufficient time for the Anammox bacteria to oxidize ammonium. At longer HRTs, the nitrogen removal efficiency decreased due to the accumulation of nitrite. Similarly, Wang et al. (2019) investigated the effect of HRT on the Anammox process using an SBR. The study found that the optimal HRT for the Anammox process was 12 hours. At shorter HRTs, the nitrogen removal efficiency decreased due to insufficient time for the Anammox bacteria to oxidize ammonium. At longer HRTs, the nitrogen removal efficiency decreased due to the accumulation of nitrite. In contrast, Lu et al. (2014) investigated the effect of HRT on the Anammox process using a UASB reactor. The study found that the optimal HRT for the Anammox process was 24 hours. The longer HRT provided enough time for the Anammox bacteria to oxidize ammonium and reduce the accumulation of nitrite. Overall, these studies suggest that the optimal HRT for the Anammox process is between 6-24 hours. Shorter HRTs may not provide enough time for the Anammox bacteria to oxidize ammonium, while longer HRTs may reduce the nitrogen removal efficiency due to the accumulation of nitrite. However, the optimal HRT may vary depending on the type of reactor used, the characteristics of the wastewater, and other operational parameters. Therefore, it is important to carefully optimize the HRT based on the specific conditions of the wastewater treatment system.

The Anammox process has several advantages over traditional nitrogen removal processes, such as lower energy consumption, reduced sludge production and lower carbon footprint (Adams et al., 2022; Ali et al., 2015). However, the Anammox process has some limitations, namely slow growth rates, sensitivity to environmental changes, and limited tolerance to high ammonia and nitrite concentrations (Ali et al., 215; Jin et al., 2012). These limitations restrict its application in large-scale wastewater treatment plants, where high flow rates and varying wastewater characteristics can make it challenging to maintain stable Anammox bacteria populations.

2.7 Partial Nitrification

Partial nitrification (PN) is a biological process that has gained attention in recent years as a sustainable method for treating wastewater (Ge et al., 2015; Zheng et al., 2019). This process has garnered significant attention in recent years due to its potential for reducing energy consumption and carbon footprint in wastewater treatment plants (Rittmann and McCarty. 2001). PN involves the oxidation of ammonia to nitrite, without complete conversion to nitrate (Cao et al., 2017; Zheng et al., 2019). Partial nitrification can be achieved using different reactor configurations, including sequencing batch reactors (SBRs), membrane bioreactors (MBRs), and moving bed biofilm reactors (MBBRs) (Liu et al. 2020; Cao et al., 2017). This process involves specific microorganisms and a series of reactions. The reactions involved in partial nitrification are:

(i) Ammonia oxidation:

$$NH_3 + 1.5\ O_2 \rightarrow NH_2OH + H_2O \qquad (2.8)$$

Ammonia-oxidizing bacteria (AOB) play a crucial role in the partial nitrification process. These bacteria utilize the enzyme ammonia monooxygenase to oxidize ammonia (NH_3) to hydroxylamine (NH_2OH). This reaction consumes oxygen and generates hydroxylamine as an intermediate (Ge et al., 2015; Liu et al., 2020; Cao et al., 2017).

(ii) Hydroxylamine oxidation:

$$NH_2OH + 0.5\ O_2 \rightarrow NO_2^- + H_2O \qquad (2.9)$$

Hydroxylamine, produced in the previous step, is further oxidized to nitrite (NO_2^-) through the activity of either AOB or other microorganisms, such as Nitrospira bacteria. The oxidation of hydroxylamine is typically mediated by enzymes like hydroxylamine oxidoreductase. This reaction results in the formation of nitrite and water (H_2O) (Zhang et al., 2019).

By carefully controlling the environmental conditions, microbial population dynamics, and nutrient availability, partial nitrification can be achieved. Different factors can affect the efficiency of partial nitrification, including temperature (Guo et al., 2010), pH (He et al., 2012), dissolved oxygen (DO) (Guo et al., 2015), and influent nitrogen concentration.

Temperature: is one of the critical factors that can affect the efficiency of partial nitrification (Guo et al., 2010; Ge et al., 2015). A higher temperature can increase the rate of ammonia oxidation by AOB, leading to an increase in the production of nitrite (Guo et al., 2010; Gu et al., 2012). However, a high temperature can also lead to the inhibition of NOB, resulting in the accumulation of nitrite and the loss of partial nitrification (He et al., 2012). Several studies have shown that the optimum temperature range for partial nitrification is between 25-30°C (Guo et al., 2010; He et al., 2012).

pH: is another critical factor that can affect the efficiency of partial nitrification (Gu et al., 2012; Ge et al., 2015). The pH of the wastewater affects the activity of AOB and NOB, with AOB preferring a slightly alkaline pH, and NOB preferring a slightly acidic pH (Liu et al., 2020). A pH range of 7.5-8.5 is considered optimal for partial nitrification (Gu et al., 2012). However, a pH outside this range can lead to a shift in the microbial community, resulting in a loss of partial nitrification.

Dissolved Oxygen (DO): this is an essential factor that can affect the efficiency of partial nitrification (Liu et al.2020; Guo et al. 2015). A higher DO can increase the rate of ammonia oxidation by AOB, leading to an increase in the production of nitrite (Cui et al., 2020). However, a high DO can also lead to the inhibition of NOB, resulting in the accumulation of nitrite and the loss of partial nitrification (Cui et al., 2020; Ge et al., 2015). Several studies have shown that the optimum DO range for partial nitrification is between 0.5-2.0 mg/L (Cui et al., 2020).

Influent Nitrogen Concentration: This is another critical factor that can affect the efficiency of partial nitrification (Li et al., 2020; Cao et al., 2017). A higher nitrogen concentration can increase the rate of ammonia oxidation by AOB, leading to an increase in the production of nitrite (Guo et al., 2010). However, a high nitrogen concentration can also lead to the inhibition of NOB, resulting in the accumulation of nitrite and the loss of partial nitrification (Ge et al., 2015). Several studies have shown that the optimum nitrogen concentration range for partial nitrification is between 100-500 mg/L (Cao et al., 2017; Liu et al., 2020).

Process Control of Partial Nitrification: The control of partial nitrification can be achieved through process parameters, such as pH, dissolved oxygen and temperature, as mentioned earlier (He et al., 2012). However, the use of molecular tools, such as fluorescence in situ hybridization (FISH), quantitative polymerase chain reaction (qPCR) and high-throughput sequencing, has proven to be useful for monitoring the microbial community and identifying the key functional groups involved in partial nitrification (He et al., 2012; Liu et al., 2020). Moreover, mathematical modelling has been used to simulate and optimize partial nitrification processes (Li et al., 2020). The development of mathematical models has helped to predict the performance of partial nitrification under different operating conditions, as well as to optimize the design of partial nitrification reactors (Cui et al., 2020).

2.8 Hybrid Anammox processes

Hybrid processes were developed to overcome the limitations of Anammox process and improve nitrogen removal efficiency. By combining Anammox with other nitrogen removal technologies, such as nitrification, denitrification or partial nitritation (PN), the benefits of both processes can be maximized, resulting in a more robust and efficient nitrogen removal system. These processes have been developed to overcome the limitations of the Anammox process, namely sensitivity to changes in pH, temperature, and organic carbon concentration, as well as to achieve higher nitrogen removal efficiencies. Hybrid Anammox processes have been shown to be more stable, cost-effective and environmentally sustainable than conventional nitrogen removal methods, making them a promising alternative for wastewater treatment. Several hybrid Anammox processes have been developed, such as the CANON (completely autotrophic nitrogen removal over nitrite) process; the DEAMOX (dual-endogenous ammonium removal using Anammox) process; and the PN-ANAMMOX (partial nitritation-Anammox) process (Figure 2.3).

Completely Autotrophic Nitrogen Removal over Nitrite (CANON) is a hybrid Anammox process that was first developed in the early 2000s. CANON is a two-step process that involves the nitrification of ammonium to nitrite by AOB, followed by the conversion of nitrite to nitrogen gas by Anammox bacteria (Kartal et al., 2010; Liu et al., 2021). The process takes place in a single reactor, which eliminates the need for a separate nitrification tank and an Anammox reactor, thereby reducing capital and operational costs. The CANON process is based on the principle that AOB can be used to produce nitrite as an electron acceptor for Anammox bacteria. The process is completely autotrophic, as it does not require external carbon sources for the growth of bacteria. Instead, the process uses the energy derived from ammonium oxidation to drive the Anammox reaction. The process is particularly suitable for treating high-strength ammonia wastewater, such as anaerobically digested sludge liquor (Cao et al., 2017). In the CANON process, the reactor is operated under conditions of low DO concentrations, typically less than 0.5 mg/L, to promote the growth of Anammox bacteria (Reino et al., 2018). The AOB and Anammox bacteria co-exist in the reactor, and the process is self-sustaining as the Anammox bacteria provide the AOB with the necessary nitrite for ammonium oxidation, while the AOB provide the Anammox bacteria with nitrite as an electron acceptor for the production of nitrogen gas (Lotti et al., 2014a; Kartal et al., 2010).

The CANON process has several advantages over conventional wastewater treatment processes. Firstly, it is a cost-effective process as it eliminates the need for a separate nitrification tank and an Anammox reactor, thereby reducing capital and operational costs (Cao et al., 2017). Secondly, the process is energy-efficient as it does not require external carbon sources, which are typically the largest energy consumers in wastewater treatment processes (Liu et al., 2021). Thirdly, the process is environmentally friendly as it produces nitrogen gas, which is a harmless and inert gas. However, the CANON process has some limitations, including the potential for nitrous oxide (N_2O) emissions. N_2O is a potent greenhouse gas that can be produced during the Anammox reaction. Therefore, the optimization of the process parameters, such as pH, temperature and DO concentrations, is critical to minimize N_2O emissions.

Single reactor High Ammonia Removal Over Nitrite (SHARON) is a hybrid Anammox process that was developed in the early 1990s. The process is designed to remove high-strength ammonia from wastewater using a single reactor. The SHARON process is a two-step process that involves the partial nitrification of ammonium to nitrite, followed by the conversion of nitrite to nitrogen gas by Anammox bacteria (Hellinga et al., 1998). In the SHARON process, a single reactor is used to promote the growth of both AOB and Anammox bacteria (Hwang et al., 2005; Fux et al., 2003). The reactor is operated under low DO concentrations, typically less than 1 mg/L, to promote the growth of Anammox bacteria (Fux et al., 2003). The process uses a combination of biofilm and suspended growth systems, which provides a suitable environment for the growth of both types of bacteria. The SHARON process is designed to remove high-strength ammonia wastewater, such as those generated from the anaerobic digestion of organic matter (Fux et al., 2003). In addition, the process is particularly suitable for treating wastewater with an ammonia concentration of up to 3000 mg/L. The process is also effective in reducing the amount of sludge produced during wastewater treatment as it uses less biomass compared to conventional processes (Hwang et al., 2005; Khin et al., 2004). SHARON outperforms traditional wastewater treatment techniques. It reduces capital and operational expenses by eliminating the requirement for a nitrification tank and Anammox reactor. Second, it uses no external carbon sources, which are wastewater treatment's biggest energy users. Thirdly, the procedure creates harmless nitrogen gas (Khin et al., 2004; Hwang, 2005). SHARON's drawbacks include N2O emissions. Anammox produces N2O, a greenhouse gas (Khin et al., 2004). To reduce N2O emissions, pH, temperature, and DO concentrations must be optimized.

DEAMOX (DEnitrifying AMmonium OXidation) is a process for removing nitrogen from wastewater using a single reactor. The process combines the Anammox and denitrification processes, enabling the simultaneous removal of ammonia and nitrate from wastewater under anaerobic conditions (Kalyuzhnyi et al., 2006). DEAMOX is a three-step process that involves the conversion of ammonium to nitrite by AOB, followed by the conversion of ammonium and nitrite to nitrogen gas by Anammox bacteria, and finally, the denitrification of nitrate to nitrogen gas by denitrifying bacteria (Kalyuzhnyi et al., 2006; Du et al., 2017). The process takes place in a single reactor, which is operated under low DO conditions to promote the growth of Anammox bacteria. DEAMOX has several advantages over conventional nitrogen removal processes. Firstly, it eliminates the need for external carbon sources for denitrification, which can account for up to 50% of the operating costs in conventional nitrogen removal processes. Secondly, it is an energy-efficient process, as it does not require external aeration for nitrification, which is typically the largest energy consumer in conventional processes. Thirdly, it produces less sludge than conventional processes, thereby reducing the costs of sludge disposal (Cao et al., 2016; Kalyuzhnyi et al., 2006). However, DEAMOX has some limitations, including the requirement for a well-established and stable microbial community, which can be difficult to achieve and maintain in practice (Du et al., 2017). The process is also sensitive to changes in operating conditions, such as pH, temperature and loading rates, which can affect the performance of the process (Cao et al., 2016). In addition, the process is susceptible to nitrous oxide (N_2O) emissions, which can have a negative impact on the environment (Du et al., 2017). To overcome these limitations, several studies have been conducted to optimize the DEAMOX process. For example, the use of biofilm-based reactors and membrane bioreactors has been investigated to improve the stability of the microbial community. The optimization of the process parameters, such as pH, temperature and DO concentrations, has also been studied to reduce N_2O emissions.

Overall, hybrid Anammox processes offer a sustainable and cost-effective solution for nitrogen removal from wastewater. These processes have the potential to achieve higher nitrogen removal efficiencies and overcome the limitations of the Anammox process. However, careful control of the operating conditions is essential to ensure the stable and efficient performance of these processes. Further research and development are needed to optimize and scale up these hybrid Anammox processes for practical application in wastewater treatment. Figure 2.2 illustrates the process diagrams for advanced biological nitrogen removal processes.

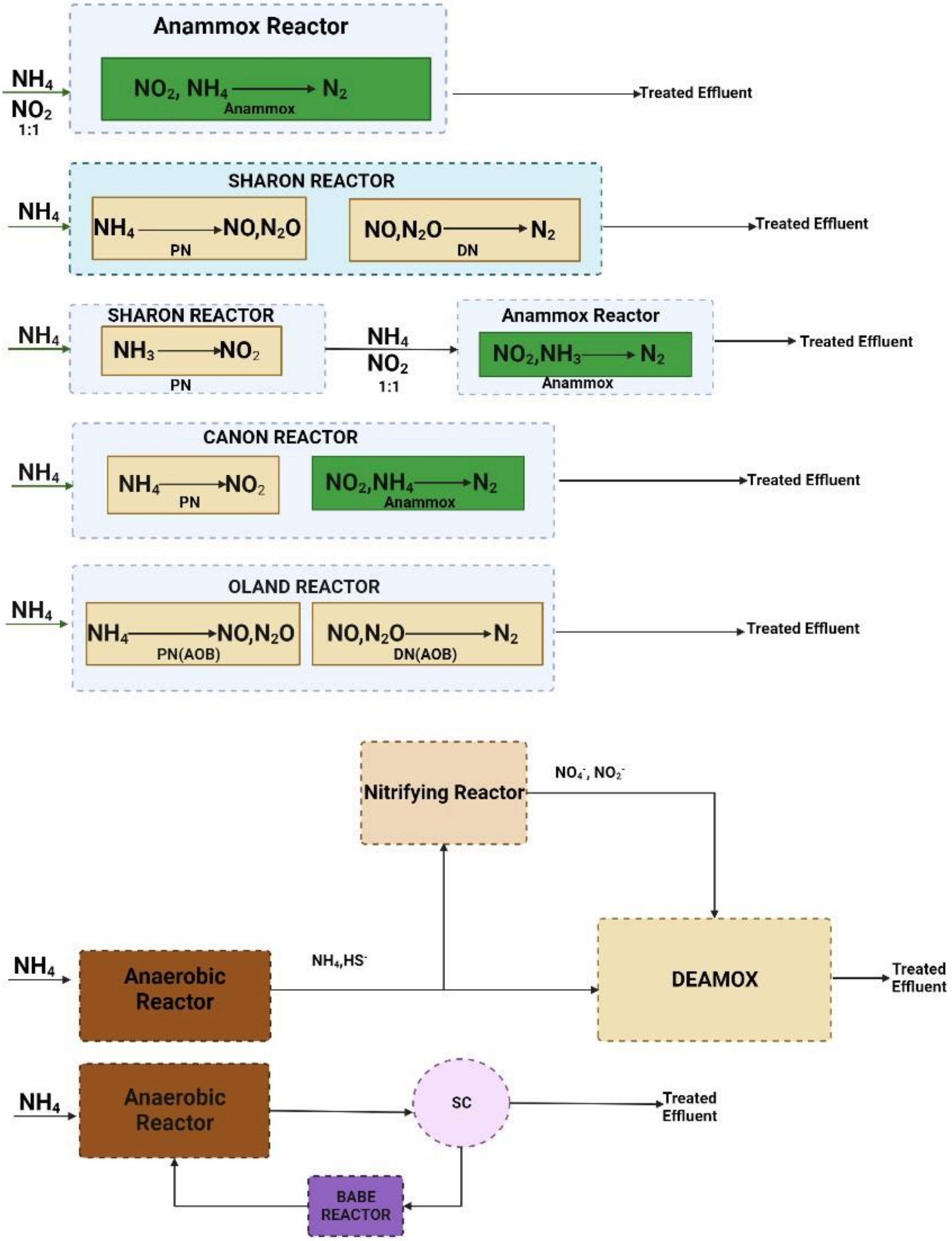

Figure 2.2: Process diagrams of advanced biological nitrogen removal processes

2.9 Sulfur-Driven Denitrification

Sulfur-driven denitrification is a process that has gained much interest in recent years due to its potential to remove nitrate and other oxidized nitrogen compounds from wastewater and industrial effluent (Guo et al., 2016; Cui et al., 2019b). This process is different from traditional denitrification, which relies on carbon as an electron donor, as it utilizes sulfur compounds as the electron donor (Cui et al., 2019a; Wang et al., 2020). The process can be carried out under anaerobic or microaerophilic conditions, with the reduction of nitrate to nitrogen gas occurring through a series of intermediate steps (Wang et al., 2020). The mechanism of sulfur-driven denitrification involves the oxidation of sulfur compounds, such as sulfide, thiosulfate or elemental sulfur, to sulfate (Cui et al., 2019; Di Capua et al., 2016). During this process, electrons are released, which can be used for the reduction of nitrate to nitrogen gas. The overall reaction can be represented as follows:

Sulfur compounds + Nitrate → Sulfate + Nitrogen gas

The use of sulfur as an electron donor for denitrification was first proposed in the 1980s, and since then, various researchers have investigated the effectiveness and efficiency of the process (Cui et al., 2019a). One of the most promising approaches to sulfur-driven denitrification is the use of micro-organisms that belong to the group of sulfur-oxidizing bacteria (SOB), which can oxidize elemental sulfur to sulfate while reducing nitrate to nitrogen gas (Shao et al., 2020). Sulfur-driven denitrification can be classified into two types: heterotrophic sulfur-driven denitrification and autotrophic sulfur-driven denitrification (Wang et al., 2020; Qui et al., 2020). Heterotrophic sulfur-driven denitrification utilizes organic matter as a carbon source and sulfur compounds as an electron donor, while autotrophic sulfur-driven denitrification utilizes carbon dioxide as a carbon source and sulfur compounds as an electron donor (Shao et al., 2010; Cui et al., 2019b).

Elemental sulfur (S^0) is one of the most extensively studied sulfur compounds in the context of sulfur-driven denitrification (Liu et al., 2018; Wang et al., 2020). It is an attractive electron donor for denitrification because it is readily available and relatively inexpensive (Shao et al., 2010). In the presence of oxygen, sulfur-oxidizing bacteria (SOB) oxidize elemental sulfur to sulfate (SO_4^{2-}), releasing electrons that can be used for the reduction of nitrate or nitrite to nitrogen gas (Lu et al., 2018). This process is known as aerobic sulfur-driven denitrification. In the absence of oxygen, SOB can still utilize elemental sulfur as an electron donor for denitrification (Lu et al., 2018). In this case, SOB can use nitrate as an electron acceptor, reducing it to nitrogen gas while oxidizing elemental sulfur to sulfate (Liu et al., 2017). This process is known as anaerobic sulfur-driven denitrification.

Apart from elemental sulfur, other sulfur compounds have also been studied for their potential use as electron donors in sulfur-driven denitrification processes. Thiosulfate (SO_4^{2-}), sulfide (S^{2-}) and elemental sulfur-polysulfides (Sn^{2-}) are amongst the sulfur compounds that have been investigated for their ability to serve as electron donors in the process (Liu et al., 2017). Thiosulfate is a commonly used sulfur compound in sulfur-driven denitrification. It can be readily oxidized by SOB to sulfate (SO_4^{2-}), releasing electrons that can be used for the reduction of nitrate or nitrite to nitrogen gas (Cui et al., 2019a). In addition to thiosulfate, sulfide can also be used as an electron donor for denitrification. SOB can oxidize sulfide to sulfate, generating electrons that can be utilized for the reduction of nitrate or nitrite to nitrogen gas (Deng et al., 2022). Elemental sulfur-polysulfides are a group of sulfur compounds that contain chains of sulfur atoms linked by oxygen atoms. These compounds can also serve as electron donors in sulfur-driven denitrification (Lu et al., 2018). SOB can oxidize elemental sulfur-polysulfides to sulfate, releasing electrons that can be utilized for denitrification. Sulfur-driven denitrification using these sulfur compounds as electron donors has several advantages over traditional denitrification methods that use organic carbon sources as electron donors (Wang et al., 2020). Sulfur compounds are generally less expensive and more readily available than organic carbon sources. Furthermore, sulfur-driven denitrification produces less sludge and has a lower carbon footprint than traditional denitrification methods (Qui et al., 2020).

Sulfur-driven denitrification is a complex process that can be influenced by a variety of factors, one of the most important of which is the type and concentration of sulfur compounds used as electron donors (Wang et al., 2020). As mentioned earlier, elemental sulfur, thiosulfate, sulfide and elemental sulfur-polysulfides are amongst the sulfur compounds that have been investigated for sulfur-driven denitrification. The choice of the optimal sulfur compound(s) depends on the specific conditions of the denitrification process (Deng et al., 2009). The concentration of nitrate is another important factor that affects sulfur-driven denitrification (Zhang et al., 2023). Studies have shown that the rate of denitrification increases with increasing nitrate concentration up to a certain point, beyond which the rate levels off (Shao et al., 2010). The optimal nitrate concentration for sulfur-driven denitrification depends on the type and concentration of sulfur compounds used as electron donors. pH is also an important factor that can affect sulfur-driven denitrification (Lu et al., 2018). The optimal pH for denitrification using sulfur compounds as electron donors ranges from 6.5 to 8.5 (Cui et al., 2019a). Above or below this pH range, denitrification rates decrease. Temperature is another important factor that can influence sulfur-driven denitrification (Du et al., 2017a). Studies have shown that the rate of denitrification increases with increasing temperature up to a certain point, beyond which the rate decreases. The optimal temperature for sulfur-driven denitrification ranges from 25 to 35°C (Guo et al., 2016).

Optimizing these factors can improve the rate and efficiency of sulfur-driven denitrification. For example, increasing the concentration of nitrate and sulfur compounds, maintaining an optimal pH and temperature, and using an appropriate combination of sulfur compounds can improve the rate of denitrification (Liu et al., 2017; Lu et al., 2018). Therefore, it is important to consider these factors when designing and operating a sulfur-driven denitrification system. Indeed, sulfur-driven denitrification has emerged as a promising alternative to traditional denitrification methods that rely on carbon sources. One of the main advantages of sulfur-driven denitrification is that it can utilize sulfur compounds that are abundant and inexpensive as electron donors, compared to carbon sources that are often limited and costly (Guo et al., 2016). This makes it an attractive option for wastewater treatment in areas where carbon sources are scarce or expensive.

Despite its potential benefits, sulfur-driven denitrification is still a relatively new and complex process that requires further optimization and research. For instance, the mechanisms underlying the process need to be better understood to improve its efficiency and reliability. In addition, the selection and optimization of sulfur compounds, pH, temperature and other process parameters need to be studied in order to identify the most effective combination for each specific wastewater treatment setting (Deng et al., 2021b). Another area that requires further exploration is the potential for sulfur-driven denitrification to treat wastewater containing high concentrations of sulfide. While sulfide is an effective electron donor for denitrification, it can also be toxic to micro-organisms and pose challenges to the process (Cui et al., 2019b). Therefore, more research is needed to assess the feasibility of using sulfur-driven denitrification for the treatment of sulfide-rich wastewater.

In summary, sulfur-driven denitrification holds great promise as an alternative to traditional denitrification methods in wastewater treatment. However, further research is necessary to optimize the process and explore its potential applications in different wastewater treatment settings.

2.10 Combined sulfur-driven denitrification and Anammox

Over the last two decades, the combined partial nitrification and Anammox (PNA) process has been intensively explored and implemented for nitrogen removal from rejected water, landfill leachate, and food processing wastewater (Lackner et al., 2014). However, the PNA process is difficult to sustain over time due to the diverse microbial population and the necessity to prevent nitrite oxidation, resulting in an efficiency of less than 89% (Zhang et al., 2019; Ahn et al., 2006). Another strategy is to combine heterotrophic denitrification with Anammox, which is easier to sustain because both processes occur in anoxic environments (Deng et al., 2022). Anammox can utilise nitrite as a common intermediate in denitrification, making it a more effective form of nitrogen removal (Zhang et al., 2019). Du et al. (2017) devised a successful coupled process employing diverse organics, displaying outstanding total nitrogen (TN) removal efficiencies (93.6-96.7%) for both low- and high-strength nitrogen wastewaters. While the combination of heterotrophic denitrification and Anammox is efficient, heterotrophic denitrification is more thermodynamically viable than Anammox, with a greater growth rate and biomass production (Kalyuzhnyi et al., 2006; Du et al., 2017a). Excessive organic input might cause heterotrophic denitrifiers to overgrow (i.e., COD/N>2.5), decreasing nitrogen removal efficiency in this combined process (Takekawa et al., 2014; Du et al., 2014). The combination of heterotrophic denitrification and Anammox might theoretically decrease nitrate, but an external carbon supply would be required, increasing the plant footprint. There is also the possibility of nitrite competition between heterotrophic denitrifying bacteria and Anammox bacteria (Du et al., 2017a; Kumar et al., 2010).

New nitrogen metabolic pathways, such as DEAMOX, which combines the Anammox reaction with autotrophic denitrifying conditions, employing sulfide as an electron donor for the synthesis of nitrite from nitrate inside an anaerobic biofilm (Kalyuzhnyi et al., 2006), have recently been identified. Reduced sulfur compounds (e.g., S^{2-}, S^0, $S_2O_3^{2-}$) can be used by autotrophic denitrifying sulfur oxidising bacteria for chemolithotrophic growth and concomitant nitrate reduction (Lin et al., 2018; Pokorna et al., 2015). Nitrite is created by partial denitratation in this process, which provides a chance to provide nitrite to Anammox bacteria. This approach also has the benefit of lowering the chance of Anammox bacteria being overtaken by a SOB due to the lower biomass output of SOB, and denitrification rates are quicker using sulphide as the electron donor (Deng et al., 2022; Mora et al., 2014). In addition, sulphide can be partly oxidized to elemental sulfur or sulphite, both of which can be employed as electron donors in denitrification

(Deng et al., 2022). Furthermore, the sulphide found in anaerobic effluents is a recognized NOB inhibitor, which might give a potential solution to the PN-Anammox reactor's difficulties. As a result, the Anammox process, which combines partial nitrification and sulfide-driven denitratation, is proposed for total high-level nitrogen removal from anaerobic effluent.

Table 2.1 showcases the extensive exploration of various influent characteristics, including sulfur source, sulfur-to-nitrogen (S/N) ratio, reactor configurations, and operational strategies. In this discussion, the focus is initially on the factors that contribute to nitrite build-up in sulfur-based denitrification. Specifically, the study examines the effects of S^{2-}, S^0, and $S_2O_3^{2-}$.

Table 2.1: Extensive exploration of various influent characteristics

Type of Reactor	Volume (L)	NH_4-N (mg/L)	NO_2-N (mg/L)	NO_3-N (mg/L)	Sulfur (mgS/L)	S/N	Temperature (ºC)	HRT	NLR (kgN/m^{-3}d^{-1})	NRR (kgN/m^{-3}d^{-1})	TN-Removal efficiency (%)	TN removal by AMX	Functional bacteria/Abundance (%)	Reference
UASB	2	80	N.A.	100	S^{2-}, 20-80	0.2-0.8	30 ± 1	12	N.A.	0.29	80	87	*Sulfurimonas* (0.1-1.7%)*Ca.Kuenenia* (1.0-3.0%) *Thiobacillus* (8.4-12.7%)	(Deng et al. 2021b)
UBF	2.1	50-280	N.A.	50-280	S_{2-}, 50-427	1-1.5	32-35	8 to 4	1.52	N.A.	87	78-94	*Thiobacillus* (3.0-55.4%) *Sulfurimonas* (0.32-25.4%) *Ca Kuenenia* (0.1-0.05%)	(Wu et al. 2020)
Biofilm reactor	36.9	45-58	N.A.	N.A.	S^{2-},50-60	N.A.	20-25	6.7-8.2	N.A.	0.15	83.5	N.A.	*Nitrosomonas* (1-3.5%) *Thiobacillus* (2.3%)*Ca. Brocadia* (3-3.1%)	(Yuan et al. 2020)
UBF	2.1	16-136	N.A.	19-146	S^{2-},76-89	0.5-4.5	30 ± 2	9	N.A.	0.107	>90	>53	*Sulfurimonas* (0-3.9%)*Ca.Kuenenia* (4.6-19.7 %)*Thiobacillus*(6.1-21%)	(Qin et al. 2019)
UASB	2.5	41-294	55-388	N.A.	S^{2-},62-330	1.0-1.1	32 ± 1	10-3.3	N.A.	4	88	63-75	*Ca.Brocadia Thiobacillus denirificans* (5.5-27.1%)	(Guo et al. 2016)
EGSB	1.6	45	N.A.	45	S^{2-},135	2.4-3.8	30 ± 1	12 to 8	N.A.	0.27	>97	N.A.	*Ca.Anammoximicrobium* (12.9%) *Alishewanella* (34.2%)	(Liu et al. 2015)
Fermentor	1.5	70	N.A.	112	S^{2-},70-267 μM/d		Room tem	NA	N.A.	N.A.	N.A.	6-75	*Ca.Kuenenia* (>18.2%) *Sedimenticola sp.* (>70%)	(Russ et al. 2014)
UASB	2.5	302 ± 50	36 ± 30	104 ± 53	S^{2-},144 ± 39	NA	35 ± 1	25 to 5	1	N.A.	>90	N.A	N.A.	(kalyuzhnyi et al. 2006)
UASB	1	300	N.A.	350	$S_2O_3^{2-}$, 500-900	1.4	35	8 to 4	2.6	N.A.	84	54	*Ca.kuenenia (18.8%) Thiobacillus (8.6%)*	(Liu et al. 2022)
SBR	5.3	10 to 20	N.A.	20	$S_2O_3^{2-}$, 64-136	1.8-3.9	30 ± 1	16	0.02	N.A.	99		*Thiobacillus (1%) Ca.Brocadia (1%)*	(Yang et al. 2020)

UASB	2	100	N.A.	100	$S_2O_3^{2-}$, 200	2	30 ± 1	12	N.A.	0.33	>90	90	*Ca.Kuenenia (1.3%) Ca.Anammoxoglobus (1.2%) Thiobacillus (3.5%)*	(Deng et al. 2019)
EGSB	2	160-208	210-245	24-27	N.A.	1.2-1.8	32	3	5.3-5.9	N.A.	98	85	*Ca.Brocadia (4.35-5.4%) Thiobacillus (9.1%-20.7%) Ca.Jettenia (1.5%-2.1%)*	(Sun et al. 2018)
UASB	1.2	48	25-50	N.A.	$S_2O_3^{2-}$,150	3 to 6	35	4	N.A.	0.28	>85	27	N.A.	(Qian et. 2018)
UASB	1.2	50-201	70-276	N.A.	S^{o}	N.A.	35 ± 1	24	N.A.	0.48	90	80	*Ca.Brocadia (28.3%) Thiobacillus 94.6%)*	(Huo et al. 2022)
PBR	10	22	45	145	S^{o}	N.A.	15-28	9 to 1	N.A.	1.74	97		*Thiobacillus (47.7%) Sulfurovum (5.5%) Sulfurimonas (4.5%)*	(Zeng et al. 2021)
UBR	1.5	600	N.A.	N.A.	S^{o}	N.A.	32 ± 1	10	N.A.	1.38	98	87	*Ca. Kuenenia (2.6-29.3) Thiobacillus (1.7-63.8) Nitrosomonas (0.1-9.6%)*	Zhang et al. 2020a)
Anaerobic tank	3	410	N.A.	640	S^{o}	N.A.	33 ± 1	6	N.A.	4.11	98	76	*Ca.Brocadia (1.4%-5%) Ca. Kuenenia (0.2-10.1%) sulfurimonas (4.5-5.6%) Thiobacillus (3.1-11.1%)*	Li et al. 2019

N.A.: Not available, T: Temperature, NRR: Nitrogen removal rate, NLR: Nitrogen loading rate, UASB: Upflow anaerobic sludge blanket, UBF: Up-flow blanket filter, PBR: Packed bed-reactor, SBR: Sequencing batch reactor, PBR: Packed bed reactor, EGSB: expanded granular sludge bed

2.11 Sulfide-based denitrification and Anammox process

Sulfide is a common contaminant found in wastewater, particularly industrial wastewater (Pokorna et al., 2015; Vela et al., 2015). It is a compound composed of sulfur and hydrogen and is produced by the breakdown of organic matter by anaerobic micro-organisms in the absence of oxygen (Vela et al., 2015). Sulfide-driven autotrophic denitrification is a process that involves the conversion of sulfide and nitrate/nitrite to nitrogen gas (N_2) in anoxic environments, such as sediments or wastewater. The process of Sulfide-driven autotrophic denitrification can be divided into two stages, with different reactions occurring in each stage (Xu et al., 2016; Deng et al., 2022).

The first stage involves the oxidation of sulfide (S^{2-}) to zero-valent sulfur (BPS^0) by the reduction of nitrate (NO_3-) or nitrite (NO_2-), as shown in equations (2.10) and (2.11):

$S^{2-} + 2NO_3- + 2H^+ \rightarrow BPS^0 + 2NO_2- + H_2O \; \Delta G^O = -211.8 kJ/mol$ (2.10)

$S^{2-} + 2NO_2- + 4H^+ \rightarrow BPS^0 + 2NO + 2H_2O \; \Delta G^O = -523.2 kJ/mol$ (2.11)

In these reactions, sulfide is oxidized to BPS^0, and nitrate or nitrite is reduced to nitrite or nitric oxide respectively. The hydrogen ions (H^+) released during these reactions increase the acidity of the environment. After the depletion of sulfide in the first stage, the second stage of Sulfide-driven autotrophic denitrification begins. In this stage, the produced BPS^0 acts as an electron donor for the reduction of nitrate or nitrite, as shown in equations (2.12) and (2.13):

$BPS^0 + 2NO_3- + 2H^+ \rightarrow SO_4^{2-} + 2NO_2- + 2H_2O \; \Delta G^O = -93.3 kJ/mol$ (2.12)

$BPS^0 + 2NO_2- + 2H^+ \rightarrow SO_4^{2-} + 2NO + H_2O \; \Delta G^O = -335.1 kJ/mol$ (2.13)

In these reactions, BPS^0 acts as an electron donor to reduce nitrate or nitrite, resulting in the production of sulfate (SO_4^{2-}) and nitrite or nitric oxide, respectively (Cui et al., 2019b; Deng et al., 2021). The stoichiometry of the reactions is complex and depends on the specific conditions of the environment in which Sulfide-driven autotrophic denitrification occurs.

For the implementation of the sulfide-based denitrification and Anammox process, stable nitrite accumulation is a prerequisite. However, studies have reported contradictory findings on nitrite accumulation in sulfide-based denitrification. Nitrite accumulation was detected in the presence of sulfide and BPS^0 as electron donors. The study conducted by Gadekar et al. (2006)

investigated the feasibility of the sulfide-based denitrification process using sulfide as an electron donor and nitrate as an electron acceptor. The study found that the accumulation of nitrite in the sulfide-based denitrification process was dependent on the initial nitrate concentration, sulfide concentration and pH of the system. The authors reported that the maximum nitrite accumulation was observed at pH 8.0, with an initial nitrate concentration of 100 mg/L. The study also found that the addition of iron and magnesium improved the nitrite accumulation in the system. Additionally, the study suggested that the use of sulfide as an electron donor for denitrification could potentially reduce the cost of the process and increase the efficiency of nitrogen removal, while other studies did not detect nitrite accumulation with sulfide alone (Xu et al., 2016; Cui et al., 2019b). Authors have also reported that BPS^0 is used as an electron donor after sulfide depletion, resulting in significant nitrite accumulation in the reactor.

Several research studies have been conducted to evaluate the possibility of sulfide-based denitrification and Anammox for the simultaneous removal of nitrate, ammonium and sulfide from wastewater. The sulfide-to-nitrogen ratio in the influent is an important parameter that affects the end-product of sulfide oxidation, as well as the contributions of denitrification and Anammox to nitrogen removal. Initially, sulfide-based denitrification and Anammox co-existed in a fluidized bed reactor, where the presence of 32-160 mgS/L of sulfide increased Anammox activity (Van de Graaf et al., 1996). Later, the study by Kalyuzhnyi et al. (2006) aimed to evaluate the tolerance of Anammox bacteria to sulfide and to determine the optimal sulfide concentration for Anammox activity. The authors found that Anammox bacteria were able to tolerate sulfide concentrations up to 400 mg/L without significant inhibition of their activity. However, at higher concentrations, sulfide inhibited the Anammox process, indicating that sulfide can be a limiting factor for Anammox activity. Kalyuzhnyi et al. (2006) also observed that the inhibition of Anammox activity by sulfide was reversible, and the Anammox process could recover after the removal of sulfide.

The authors speculate that the return of Anammox activity may be related to Anammox bacteria's adaptability to sulfide. In the sulfide-based denitrification and Anammox processes, influent NOX/NH4 + and S/N ratios greater than 1.2 gN/gN and 0.57 gS/gN respectively were excellent operating conditions. However, because the sulfide-based denitrification and Anammox reactor used around 30% of the organics, heterotrophic denitrification may also be responsible for nitrogen removal. Russ et al. (2014) began sulfide-based denitrification and

Anammox by implementing sulfide-based denitrification and then introducing Anammox biomass into the reactor. The results of the experiments revealed that sulfide-based denitrification supplied nitrite for Anammox, and the Anammox process accounted for 65%-75% of nitrogen removal. Despite the fact that Anammox is the primary nitrogen removal pathway in sulfide-based denitrification and Anammox, the linked process occurs via two separate processes. According to Liu et al. (2015), sulfur-based denitrification in sulfide-based denitrification and Anammox is a two-step process that occurs under nitrate-limiting circumstances. Nitrate is first converted to nitrite, which is then coupled with sulfide oxidation to produce BPS^0. The majority of the nitrite generated is used by Anammox, while a tiny quantity is slowly reduced to nitrogen gas by BPS0 oxidation to sulfate. Under nitrate-limiting circumstances, Figure 3a depicts the nitrogen removal route of the sulfide-based denitrification and Anammox process.

The authors claimed that an excess of sulfide was required to make nitrite for Anammox feeding. Deng et al. (2021b) studied the kinetics of sulfide-based denitrification and Anammox under sulfide-limiting circumstances and discovered that no nitrite build-up occurred during nitrate reduction using sulfide as the electron donor. Only when the generated BPS0 was employed as an electron donor for nitrate reduction did nitrite build-up occur, leading to Anammox becoming the primary nitrogen removal route. Under sulfide-limiting circumstances, Figure 2.3 displays the nitrogen removal mechanism of the sulfide-based denitrification and Anammox process. Du et al. (2017) recently developed DEAMOX in SBR for nitrogen removal from household wastewater and nitrate sewage. The study revealed the possibility of producing nitrite from nitrate by partial denitrification. When treating low-strength wastewater, the integrated DEAMOX process achieved easy-to-control and steady nitrite generation when compared to the PN/AMX method. After 500 days of operation, the reactor achieved a total nitrogen removal efficiency of 95% with influent ammonium of 63.5 mg/L and nitrate of 69.2 mg/L. According to the findings of this investigation, frequent washing of surface sludge was required to ensure stable performance in the long-term operation of DEAMOX. *Candidatus Brocadia* was the most common Anammox bacterium, and the dominating species *Thauera* was involved in nitrite build-up during partial denitrification.

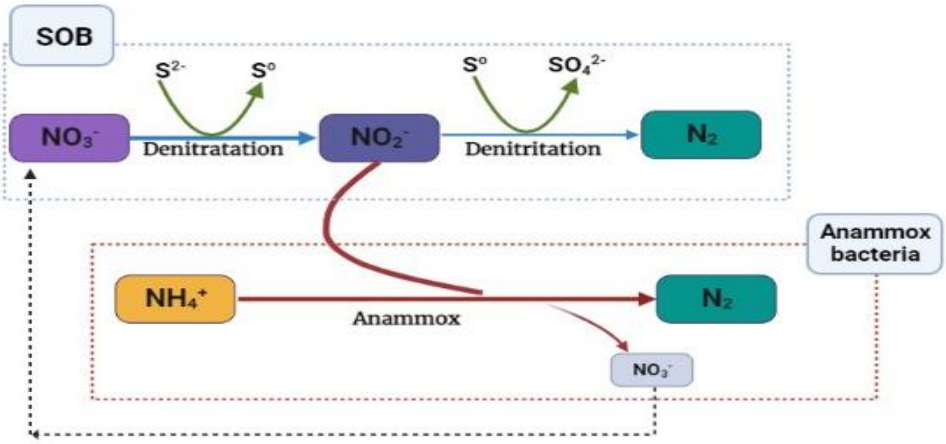

Figure 2.3: Nitrogen removal pathways in sulfide-driven denitrification and Anammox process

2.12 Effect of Sulfide on Partial Nitrification

Sulfide is a common contaminant in wastewater that can have a significant impact on the performance of biological treatment processes, particularly partial nitrification. The effect of sulfide on partial nitrification has been studied extensively in recent years, and several research studies have provided valuable insights into the mechanisms underlying the inhibition of AOB and NOB by sulfide. Being present in anaerobic environments with concentrations from 0.03 to 25 mg/L, it can interfere with the microbiology and chemistry of PN systems. Sulfide inhibits AOB and NOB differently (Ortiz et al., 2013). The study by Ortiz et al. (2013) investigates the impact of sulfide on the nitrification process, specifically on the respiratory activity of AOB and NOB. The main conclusions drawn from the study were that sulfide has a significant inhibitory effect on the respiratory activity of AOB and NOB, which are essential components of the nitrification process. The degree of inhibition of the respiratory activity of AOB and NOB is dependent on the concentration of sulfide, with higher concentrations of sulfide resulting in greater inhibition. Sulfide inhibits the nitrification process by interfering with the electron transport system of AOB and NOB, which is crucial for their respiratory activity. The findings of the study have important implications for the operation and management of wastewater treatment systems, particularly those that rely on the nitrification process, as the presence of sulfide in wastewater can lead to decreased nitrification efficiency and potentially cause process failure.

The NOB is generally considered to be the most sensitive to variations of environmental conditions leading to the accumulation of nitrite in bioreactors for wastewater treatment. Vela et al. (2018) suggested that sulfide may be a useful selective inhibitor for NOB, depending on the composition and diversity of the NOB population. The study concludes that the effect of sulfide on nitrite oxidation in activated sludge depends on the composition of the microbial community. Nitrite oxidation was significantly inhibited in mixed culture batch tests with low sulfide concentrations (≤10 mg S/L), but not in batch tests with high sulfide concentrations (>10 mg S/L). The inhibition of nitrite oxidation was also found to depend on the relative abundance of *Nitrospira, Nitrobacter* and *Nitrotoga* species in the microbial community. *Nitrobacte*r and *Nitrotoga* species were found to be more resistant to sulfide inhibition than Nitrospira. The study suggests that the composition of the microbial community should be considered when designing and operating wastewater treatment systems to minimize the negative effects of sulfide on nitrite oxidation. Sulfide has been used in a few studies to establish PN, such as in the investigation by Erguder et al. (2008) in which the results showed that a 45 mg-S/L of pulse dosing of sulfide can achieve a nitrite accumulation ratio of up to 0.75 under pH-controlled conditions. The study concludes that pulse dosing of sulfide can be an effective strategy for achieving partial nitrification in a sequential batch reactor. The results show that a single sulfide dose of 45 mg-S/L can achieve up to 75% nitrite accumulation ratio while maintaining an ammonium oxidation efficiency of above 83%. However, the study also highlights the potential negative impacts of sulfide on the microbiology and chemistry of partial nitrification systems, including the inhibition of both AOB and NOB, and the increase in pH and free ammonia concentrations. Kouba et al. (2017) also investigated the effect of sulphide dosing on the PN process and concluded that the sulphide dosage should be avoided in the established PN systems as it can inhibit AOB as well as NOB. The study reported a concentration of 10 mg S/L for the effective inhibition of NOB under mixing nitrifying conditions. Other studies reported by Ortiz et al. (2013) do not specifically investigate the effect of sulphide on the PN process but on the overall nitrification process. The study by Ortiz et al. (2013) found that the concentration of sulphur ranged from 3.1 to 112 mg-S/L, the conversion of ammonia to nitrate dropped from 0.9 to 0.3 gNO3-N/gNH4-N, causing nitrite to build up, and the specific rates of nitrate production fell by 77-97%.

Similarly, Sekina et al. (2018) present a study that aimed to investigate the possibility of stable nitrification under sulfide supply in an SBR with a long fill period. The inhibition occurs due to the fact that sulfide can act as an electron donor for nitrite reduction, leading to a decrease in the oxidation of ammonia to nitrite, and nitrite to nitrate (Vela et al., 2018; Kouba et al., 2017). The degree of sulfide inhibition on nitrification depends on various factors such as the concentration of sulfide, pH, temperature, and oxygen concentration. Different methods have been proposed to mitigate the inhibitory effect of sulfide on nitrification, including pH adjustment, aeration, and the addition of alternative electron acceptors such as nitrate or nitrite (Hassan et al., 2022; Kouba et al., 2017). The addition of nitrite as an alternative electron acceptor has been shown to be particularly effective in mitigating sulfide inhibition (Erguder et al., 2008). This is because nitrite is a stronger electron acceptor than sulfide, and the addition of nitrite can shift the electron flow away from sulfide and towards nitrite.

Partial nitrification has several potential applications in wastewater treatment (Hassan et al., 2022; Sekina et al., 2018). One of the most significant benefits of partial nitrification is its ability to reduce energy consumption and carbon footprint in wastewater treatment plants (Liu et al., 2020; Gu et al., 2012). This is achieved by reducing the demand for oxygen and carbon sources required for complete nitrification (Cui et al., 2020). Moreover, partial nitrification can also be used in the treatment of high-strength nitrogen wastewater, such as landfill leachate and industrial effluent, where conventional nitrification is not feasible due to the high ammonia concentration (Hassan et al., 2022; Sekine et al., 2018).

Table 2.2: Research on the effect of sulfide on partial nitrification under different operating conditions

Reactor/Volume	Influent concentration (mg-N/L, mg-S/L)	Temperature(°C)	HRT(d)	NAR (%)	Remarks	Reference
SBR/7.5L	NH_4^+-N=40, S^{2-}=0-55	30 ± 1	N. A	N. A	The results portrayed that simultaneous nitrification-denitrification coupled with sulfide oxidation in a single SBR intermittently aerated was not good for post-treatment of domestic effluents coming from UASB.	Moraes et al., 2013
SBR/2.1L	NH_4^+-N=973 ± 123, S^{2-}= 96	30 ± 1	3	N. A	The effective sludge retention in the SBR having a long fill period perhaps promoted the acclimatization of the microbes to the sulfide allowing the nitrification process to remain stable under sulfide supply.	Sekine et al., 2018
CSTR/5.0L	NH_4^+-N=396, S^{2-}= 65-137	24 ± 1.5	1.8	N. A	When CSTR was used as a multipurpose bioreactor, the efficiency, yield, and production rate of the nitrification process was not affected by sulfide feeding.	Cardoso et al., 2011
SBR/1.5L	NH_4^+-N=700, S^{2-}= 1.3-80, 4-45	23-28	1.5-3	75	The study showed that pulse sulfide doses resulted in partial nitrification during aerobic days. There was significant nitrite accumulation seen after the dosage of sulfide (in anoxic days).	Erguder et al., 2008
SBR/40L, Vials/0.7L	TAN= 450, S^{2-}= 13-137	22-28, 15± 0.5	N. A	N. A	In a mixed nitrifying culture, sulfide explicitly inhibited NOB which concluded that partial nitrification could be established with the help of sulfide.	Kouba et al., 2017
CSTR/6.0, Serum Bottles/160mL	NH_4^+= 100±5, S^{2-}= 3.1±0.2-112±2.5	30	3.5	N. A	The presence of sulfide in effluents even at lower concentrations might affect the nitrifying activity of activated sludge and increase nitrite accumulation.	Ortiz et al., 2012

SBR/1.8L	NH_4^+= 70, S^{2-}= 5-25	20-25	0.5	85	It was seen that NAR increased (63 to 85) due to sulfide addition. Nitrite oxidation is more sensitive to sulfide than ammonium oxidation.	Magray et al., 2021

2.13 Effect of sulphide on Anammox Process

The Anammox process is a microbial process that converts ammonium and nitrite into nitrogen gas under anaerobic conditions (Wu et al., 2020; Jin et al., 2013). The process is widely used for the removal of nitrogen from wastewater, and it has been extensively studied in recent years. However, the presence of sulfide in wastewater can have a significant impact on the Anammox process, affecting both the process rate and the microbial community structure (Jin et al., 2013; Zhang et al., 2020). It has been shown to have both positive and negative effects on Anammox. On the one hand, sulfide can act as a reducing agent and provide electron donors for the Anammox process, increasing the nitrogen removal rate. On the other hand, sulfide can also inhibit the Anammox bacteria and reduce the efficiency of the process (Deng et al., 2021; Zhang et al., 2020). In this literature review, the researcher summarizes the current understanding of the effect of sulfide on the Anammox process.

Sulfide is known to inhibit the Anammox process at concentrations as low as 0.1 mM, with the inhibition increasing as sulfide concentration increases (Qin et al., 2019). The inhibition is believed to occur due to the toxicity of sulfide to the Anammox bacteria, which are highly sensitive to changes in the redox potential of the environment (Wu et al., 2020). The exact mechanism of sulfide inhibition is still not fully understood, but it is thought to involve the binding of sulfide to the heme groups of the Anammox enzymes, which disrupts their function. The exact effect of sulfide on Anammox depends on several factors, such as the concentration of sulfide, the pH of the environment, and the type of Anammox bacteria present (Jin et al., 2013; Wu et al., 2020). Some studies have shown that low concentrations of sulfide (less than 50 mg/L) can stimulate the Anammox process and improve nitrogen removal (Jin et al., 2013). This is because sulfide can act as an electron donor for the Anammox bacteria, which can increase their activity and growth. Several other studies have investigated the effect of sulfide on the microbial community structure of Anammox reactors (Zhang et al., 2020). It has been shown that the presence of sulfide can lead to a shift in the microbial community from Anammox bacteria to sulfate-reducing bacteria (SRB). SRB can out-compete Anammox bacteria for substrates, and their presence can significantly reduce the Anammox process rate (Qin et al., 2019). This shift in the microbial community has been observed even at low sulfide concentrations, suggesting that even trace amounts of sulfide can have a significant impact on the Anammox process (Qin et al., 2019). However, higher concentrations of sulfide (above 50 mg/L) can have a negative effect on Anammox. Sulfide can inhibit the activity of the Anammox bacteria by blocking their respiratory chain and reducing their energy supply (Jin et al., 2013; Zhang et al., 2023). In addition, sulfide can also react with nitrite to form nitrous oxide, which is a greenhouse gas and can contribute to climate change.

One study by Zhang et al. (2023) investigated the effects of sulfide on Anammox activity in a sequencing batch reactor. The researchers found that sulfide could stimulate Anammox activity at low concentrations (<0.2 mg/L) by providing electrons and promoting the growth and activity of Anammox bacteria. However, at higher concentrations (>0.2 mg/L), sulfide inhibited Anammox by inducing oxidative stress and damaging the cell membranes of Anammox bacteria. Chen et al. (2023) looked at how sulfide affected the efficacy of the low substrate Anammox process and the microbial community it supported. The results of the study show that sulfide can directly and indirectly hinder the Anammox process by interfering with the activity and metabolism of the Anammox bacteria and the nitrite oxidation process.However, the inhibitory effect of sulfide can be mitigated by maintaining a low sulfide concentration and short exposure time, as well as implementing certain strategies such as controlling pH and using a hybrid Anammox-sulfide oxidation system. The study also found that sulfide inhibition can alter the microbial community structure of the Anammox process. High sulfide concentrations can reduce the abundance and diversity of Anammox bacteria and promote the growth of sulfide-oxidizing bacteria, which can out-compete Anammox bacteria for nitrite and inhibit the Anammox process. Additionally, a study by Geng et al. (2023) investigated the effect of Na_2SO_3 on the Anammox process and microbial community structure. The findings of the study indicate that Na_2SO_3 can inhibit the Anammox process at high concentrations, with a 50% inhibition observed at a Na_2SO_3 concentration of 15 mM. However, the inhibition was reversible, and the Anammox process could recover after the removal of Na_2SO_3. The study also found that the addition of Na_2SO3 altered the microbial community structure of the Anammox process, with a decrease in the abundance of Anammox bacteria and an increase in the abundance of denitrifying bacteria. The recovery of the Anammox process was associated with the restoration of the Anammox bacterial community. The study suggests that Na_2SO_3 can be used as an effective inhibitor of the Anammox process, which can be useful for controlling the Anammox process in wastewater treatment plants. However, the study also highlighted the importance of managing the concentration of Na_2SO_3 to avoid irreversible damage to the Anammox bacterial community.

Another study by Wang et al. (2017) investigated the effects of sulfide on Anammox activity in a continuous-flow reactor. The researchers found that sulfide could enhance Anammox activity by improving the availability of electron donors and maintaining a low redox potential. They also observed that sulfide could increase the diversity of Anammox bacteria, promoting the growth of diverse Anammox populations and improving the stability of the Anammox system. Wisniewski et al. (2019) looked at the influence of hydrogen sulfide (H_2S) on the Anammox process, which is a key biological nitrogen removal mechanism in wastewater treatment. The research investigates the kinetics of H_2S inhibition in suspended Anammox biomass from a membrane bioreactor. The results of the study showed that H_2S significantly inhibited the Anammox process. The inhibition was concentration-dependent and followed a non-competitive inhibition model. The study also found that the Anammox bacteria had a higher affinity for H_2S than for nitrite, which is one of the substrates for the Anammox process. The researchers concluded that H_2S can be a potent inhibitor of the Anammox process and that its presence in wastewater should be monitored and controlled to ensure effective nitrogen removal. The article provides important insights into the factors that can impact the Anammox process in wastewater treatment plants and underscores the need for the effective monitoring and control of wastewater conditions to optimize nitrogen removal. The findings of this study can be useful for wastewater treatment plant operators and researchers working on improving nitrogen removal efficiency in wastewater treatment systems.

Anammox sludge's microbial population and functional gene response to sulfide stress was initially studied by Xu et al. (2020). Specific Anammox activity was shown to decrease by 61.7% when exposed to 20 mg/L sulfide. The experiment concluded with the quenching of the protein-like molecules found in extracellular polymeric compounds. In addition, Candidatus Kuenenia saw a decline in relative abundance from 28.7% to 6.4%; whereas Thiobacillus saw an increase in relative abundance from 0% to 7.2% as a result of sulfide stress. Sulfide concentrations over 20 mg/L were also associated with a considerable drop in the relative abundance of functional genes (*hzsA, hdh, nirK,* and *nirS*). These results strengthen the theoretical underpinnings of the Anammox process for eliminating nitrogen from wastewater containing sulfides. Anammox substrates and common wastewater elements were studied by Arroyo et al. (2012) for their inhibitory effects on the microbial activity of two distinct Anammox enrichment cultures: suspended and granular. Low to moderate inhibition was shown with ammonium and nitrate, whereas total inhibition was seen with nitrite at concentrations greater than 15mM. Hydrogen sulfide, another ubiquitous wastewater element, was the most potent inhibitor, with an IC50 of just 0.03mM for undissociated H_2S.Ammonium consumption was shown to be increased when 2mM sulfide was applied in pulses to a fluidized bed Anammox reactor (Van de Graaf et al., 1996). Nitrate was reduced by sulfide, creating nitrite, the Anammox process's preferred electron acceptor, which explained the observed event. A study by Peng et al. (2019) investigated the mechanisms behind the positive effects of sulfide on Anammox activity. The researchers found that sulfide could react with nitrite to form ammonium and elemental sulfur, increasing the availability of ammonium and providing electron donors for Anammox bacteria. They also observed that sulfide could reduce the redox potential by reducing oxidized compounds, maintaining a reducing environment for Anammox activity. Table 2.3 shows that the concentration of sulfide that produces inhibitory effects varies across different studies, which can be attributed to variations in seed sludge and operational conditions.

Several strategies have been proposed to mitigate the impact of sulfide on the Anammox process. One strategy is to control the sulfide concentration in the wastewater by removing sulfide through chemical precipitation or biological oxidation (Zheng et al., 2023). Another strategy is to acclimate the Anammox bacteria to sulfide by gradually increasing the sulfide concentration in the reactor over time (Zheng et al., 2023). This can allow the Anammox bacteria to adapt to the presence of sulfide and maintain their activity in the presence of sulfide.

Table 2.3: Research on the effect of sulfide on Anammox activity under different conditions

Reactor	Influent	Inoculum	S^{2-} influent (mgS/L)	pH	Effect on AMX activity	References
Vial batch tests	Batch tests	Enriched AMX biomass	160	7.8	100%	Dapena-Mora et al., 2007
			32–64	7.8	60%	
UASB reactor	Synthetic wastewater	AMX granular biomass	8	8.1–8.5	No inhibition	Jin RC et al., 2013a
			32		50%	
			40		17.2%	
Serum bottles batch tests	Synthetic wastewater	Biomass from a PN/A reactor	5	7.5–8.0	40%	Wu S et al., 2016
Serum bottles batch tests	Synthetic wastewater	Co-culture of AMX and Su Denitrificans.	9	7.1	No inhibition	Russ et al., 2014
Fluidized bed reactor	Wastewater from a baker yeast production plant	Denitrifying sludge	90–130	7	No inhibition	Mulder A at al., 1995
EGSB reactor	Synthetic wastewater	Autotrophic denitrifying sludge and AMX biomass	85	7.5	No inhibition	Liu C et al., 2015

			105		No inhibition	
			135		No inhibition	
UASB reactor	Synthetic wastewater	AMX biomass and methanogenic granules	330	7.5–8.0	No inhibition	Guo Q et al., 2016
Fluidized bed reactor	Batch tests	Autotrophic denitrifying biomass	32–160	7	Improved	Van De Graaf et al., 1996

From the literature, the study concludes that the possible reactions between sulfide and Anammox can be summarized as:

I. Sulfide inhibition of Anammox process:

The presence of sulfide in wastewater can inhibit the Anammox process, leading to a decrease in the process rate. Sulfide is believed to bind to the heme groups of the Anammox enzymes, which disrupts their function and inhibits the Anammox process. The inhibition of the Anammox process by sulfide is dose-dependent, with even low concentrations of sulfide leading to significant inhibition.

II. Sulfide as an electron donor for Anammox:

While sulfide can inhibit the Anammox process, it can also act as an electron donor for Anammox bacteria under certain conditions. Anammox bacteria can use sulfide as an alternative electron donor when the availability of nitrite is limited. This reaction can occur via the sulfide oxidation reaction, which produces sulfate and protons, and the reduction of nitrate to nitrogen gas.

III. Sulfide as a sulfur source for Anammox:

Anammox bacteria require sulfur for their growth and metabolism, and sulfide can act as a sulfur source for these bacteria. Sulfide can be oxidized by Anammox bacteria to elemental sulfur, which can be used as a sulfur source.

IV. Competition between Anammox and sulfate-reducing bacteria:

The presence of sulfide in wastewater can lead to competition between Anammox bacteria and sulfate-reducing bacteria (SRB) for substrates. SRB can out-compete Anammox bacteria for substrates, leading to a decrease in the Anammox process rate. The competition between

Anammox and SRB can be influenced by several factors, namely pH, temperature and nutrient availability.

Overall, these studies suggest that sulfide can have both positive and negative effects on Anammox activity, depending on the concentration and the mechanisms involved. While high concentrations of sulfide can be inhibitory to Anammox bacteria, low concentrations of sulfide can enhance Anammox activity by providing electron donors; improving the availability of nitrite and ammonium; maintaining a low redox potential; and promoting the growth of diverse Anammox populations. Further research is needed to better understand the interactions between Anammox and sulfide, and to optimize the use of these processes in wastewater treatment.

2.14 Thiosulfate-based denitrification and Anammox process

Thiosulfate can be generated in wastewater through a variety of sources. One common source is the use of sodium hydrosulfite as a reducing agent in the textile and paper industries (Cui et al., 2019a). Sodium hydrosulfite can be used to remove colour from dye and pulp, and it generates thiosulfate as a by-product. Other sources of thiosulfate in wastewater can include the discharge of photographic processing solutions; the use of certain chemical reagents in the pharmaceutical industry; and the breakdown of thiosulfate-containing compounds in the environment. Thiosulfate can be problematic in wastewater treatment because it can interfere with certain treatment processes, such as biological nutrient removal. Moreover, thiosulfate can act as a competing electron donor for denitrification, which can reduce the efficiency of nitrate removal.

Thiosulfate-based denitrification is a process that can remove nitrate (NO_3-) from wastewater by using thiosulfate ($S_2O_3^{2-}$) as an electron donor. This process occurs in two steps and involves the production of nitrite (NO_2-) as an intermediate. The first step of thiosulfate-based denitrification involves the reduction of nitrate to nitrite by thiosulfate (Mora et al., 2014; Cui et al., 2019a). This reaction can be represented by the following equation (2.14):

$$NO_3- + S_2O_3^{2-} \rightarrow NO_2- + SO_4^{2-} \qquad (2.14)$$

In this reaction, nitrate is reduced to nitrite, and thiosulfate is oxidized to sulfate. Nitrite is produced as an intermediate product in this step. The second step of thiosulfate-based denitrification involves the reduction of nitrite to nitrogen gas (N_2) by thiosulfate. This reaction can be represented by the following equation (2.15):

$$2NO_2- + 5\ S_2O_3^{2-} + 4H^+ \rightarrow N2 + 5SO_4^{2-} + 4H_2O \qquad (2.15)$$

In this reaction, nitrite and thiosulfate react to form nitrogen gas, sulfate and water. This step consumes nitrite produced in the first step and completes the removal of nitrate from the wastewater. Overall, thiosulfate-based denitrification is an effective method for removing nitrate from wastewater, and the use of nitrite as an intermediate allows for the efficient conversion of nitrate to nitrogen gas. The process can be optimized by controlling the ratio of thiosulfate to nitrate/nitrite, as well as the pH and temperature of the system. The intermediate production of nitrite allows for better control of the process and can be advantageous over other denitrification processes.

Deng et al. (2019) investigated the viability of a novel biological nitrogen removal method that combines thiosulfate-driven autotrophic denitratation (NO_3-N/NO_2-N) and anaerobic Anammox for simultaneous nitrate and ammonium removal from industrial wastewater. The suggested thiosulfate-driven denitratation and Anammox method was developed in two stages: Consider the thiosulfate-driven denitratation that was established in an activated sludge-inoculated UASB that was fed ammonium, nitrate, and thiosulfate for 52 days until the effluent nitrite level reached 32.1 mg N/L. Second, enhanced Anammox biomass was supplied to the UASB in order to establish the integrated thiosulfate-driven denitratation and Anammox (TDDA) bioprocess. With influent NH4-N of 101.2 mgN/L, NO3-N of 101 mgN/L, and thiosulfate of 202.5mgS/L, the integrated TDDA system successfully removed nitrate and ammonium from synthetic wastewater with a total nitrogen (TN) removal efficiency of 82.5%. Sulfur-oxidizing bacteria (e.g., *Thiobacillus* and *Sulfurimonas*) co-existed with Anammox bacteria (e.g., *Ca. Kuenenia* and *Ca. Anammoxoglobus*) in this syntrophic biocenosis, according to a high-throughput sequencing study. Guo et al. (2016) presented a novel approach for biological nitrogen sulfur removal that combines Anammox and autotrophic desulfurization-denitrification (AADD). The reactor (UASB) was run for 245 days in order to study the co-treatment of nitrogen and sulfur-containing wastewater.

The reactor performed well in terms of nitrogen and sulphide removal. Qian et al. (2018) presented a unique biological nitrogen removal technique based on a combination of Anammox and thiosulfate-driven denitratation to capitalize on the benefits of less electron donor use in partial denitrification. The Anammox and high-rate thiosulfate denitratation were carried out in a lab-scale SBR and a UASB reactor respectively. The thiosulfate-to-nitrogen ratio and pH are two essential parameters influencing thiosulfate-driven denitratation activity and nitrite accumulation, and nitrite build-up was seen in this study at an S/N ratio of 1.5:1 and pH of 8 high denitratation activity. At 35 degrees Celsius, the overall nitrogen removal efficiency was 73%. Yeng et al. (2020) examined the development of an electron buffer in a single-stage sequencing batch reactor by linking thiosulfate-dependent denitratation with Anammox. The researchers discovered that combining these two processes resulted in a steady and effective nitrogen removal, with a removal rate of up to 2.6 kg N/m3/day. Furthermore, the electron buffer formed by the combination of thiosulfate-dependent denitratation and Anammox was able to continue the denitratation process even at low electron donor concentrations. Overall, the research indicates that this technology might be a potential strategy for boosting the efficacy of nitrogen removal operations in wastewater treatment.

2.15 Elemental Sulfur-based denitrification and Anammox process

Sulfur is a common contaminant found in wastewater from various industrial and domestic sources (Yuan et al., 2020). It can come from a variety of sources, including industrial processes, mining activities and agricultural operations (Sun et al., 2018). Sulfur can be present in wastewater in various forms, including sulfates, sulfides and elemental sulfur (Wu et al., 2020). Sulfates are the most common form of sulfur in wastewater and are primarily derived from sources such as detergents, fertilizers and other chemical products (Wu et al., 2020). Sulfates are not toxic to humans, but they can cause scaling and corrosion in wastewater treatment equipment, reducing their lifespan and efficiency (Xu et al., 2016).

Elemental sulfur-based denitrification is a biological process by which certain microrganisms use elemental sulfur (S^0) as an electron donor to reduce nitrate (NO_3-) to nitrogen gas (N_2). This process is important for the global nitrogen cycle, as it plays a role in removing excess nitrogen from ecosystems. The process of elemental sulfur-based denitrification occurs in two steps, with nitrite (NO_2-) as an intermediate. In the first step, nitrate is reduced to nitrite by a nitrate reductase enzyme, using S^0 as the electron donor. This produces elemental sulfur and nitrite:

$$NO_3\text{-}+ S^0 \rightarrow NO_2\text{-}+ S^0+ H_2O \qquad (2.16)$$

In the second step, nitrite is further reduced to nitrogen gas by a nitrite reductase enzyme, using S^0 as the electron donor. This produces elemental sulfur and nitrogen gas:

$$NO_2\text{-}+ S^0+ H_2O \rightarrow N_2 + H_2SO_4 \qquad (2.17)$$

Therefore, denitrification based on elemental sulfur is a two-step process that involves nitrite as an intermediate and plays a significant role in the natural removal of excess nitrogen from ecosystems. Guo et al. (2016) conducted a study on a promising innovative technology called the combined Anammox and autotrophic desulfurization-denitrification (AADD) process, which can remove nitrogen and sulfur biologically. The reactor demonstrated excellent performance in terms of nitrogen and sulfide removal, with the highest nitrogen removal rate and sulfide removal rate of 4 kgN/m3/d and 2.39 kgS m3/d respectively. During the operation of this process, the specific Anammox activity and autotrophic desulfurization-denitrification, as well as extracellular polymeric substance, were investigated since they are considered important factors for high efficiency. *Brocadiacea* and *Hydrogenophilaceae* were found to be the dominant bacteria. The results of the study indicate that this process is particularly effective in removing ammonia, nitrite and sulfide, while utilizing an unusual mechanism involving Anammox and autotrophic desulfurization-denitrification. The treatment of high ammonium-containing wastewater is challenging due to the high concentration of nitrogen compounds that are difficult to remove through conventional methods. To address this issue, a novel process of coupling partial nitritation-Anammox (PN-AN) with short-cut sulfur autotrophic denitrification (SAD) in a single reactor has been proposed by Zhang et al. (2020a). The article discusses a novel process of coupling partial nitritation-Anammox (PN-AN) with short-cut sulfur autotrophic denitrification (SAD) in a single reactor for the treatment of high ammonium-containing wastewater. The study found that the PN-AN-SAD process was effective in removing high concentrations of ammonium from wastewater, achieving ammonium removal rates of 92.7%, 98.9% and 99.6% for PN, AN and SAD respectively, at an HRT of 8 hours and a temperature of 30°C. The study also found that the PN-AN-SAD process was more effective than the traditional PN-AN process in terms of nitrogen removal efficiency. The PN-AN-SAD process is a promising alternative for the treatment of high ammonium-containing wastewater, with advantages including lower energy consumption, higher nitrogen removal efficiency, and reduced sludge production. Wang et al. (2019) established a separate coupled system of Anammox and sulfur autotrophic denitrification in order to achieve efficient nitrogen removal.

The article discusses efficient nitrogen removal in a separate coupled system of Anammox and sulfur autotrophic denitrification with a nitrification side branch under substrate fluctuation. The study found that the separate coupled system was effective in achieving stable nitrogen removal, with total nitrogen removal efficiency exceeding 80% under different substrate fluctuations. The nitrification side branch effectively removed ammonia during high ammonia concentration periods and helped stabilize the pH. The sulfur autotrophic denitrification process also helped remove nitrate during low ammonia concentration periods. The study highlights the potential of a separate coupled system of Anammox and sulfur autotrophic denitrification with a nitrification side branch for efficient nitrogen removal under substrate fluctuation, providing a new approach for the treatment of wastewater with fluctuating nutrient concentrations.

2.16 Factors influencing Sulfur-driven autotrophic denitrification and Anammox.

Sulfur-driven autotrophic denitrification and Anammox (SDD/Anammox) are biological nitrogen removal processes that have received increasing attention in recent years due to their potential for energy-efficient and cost-effective wastewater treatment (Zheng et al., 2021; Deng et al., 2022). However, the performance of these processes is influenced by several factors that need to be carefully controlled to ensure their efficient operation. The factors affecting SDD/Anammox processes are discussed below:

pH:

The pH of the wastewater plays a critical role in the performance of SDD/Anammox processes (Xu et al. 2016). Studies have shown that a pH range of 6.5-8.5 is optimal for both processes, with denitrification rates decreasing at lower pH values and Anammox rates decreasing at higher pH values (Wang et al., 2019). The optimal pH range can vary depending on the electron donor used, with thiosulfate-driven denitrification performing best at a pH of 7.5-8.0 and sulfide-driven denitrification performing best at a pH of 6.5-7.0 (Deng et al., 2022; Yeng et al., 2019).

Temperature:

Temperature is another important factor affecting SDD/Anammox processes. Optimal temperatures for denitrification and Anammox processes range from 25-35°C, with denitrification rates decreasing at lower temperatures and Anammox rates decreasing at higher temperatures (Cui et al., 2019b; Deng et al., 2021a). However, some studies have suggested that the performance of Anammox can be enhanced at lower temperatures with the use of low-temperature-adapted Anammox bacteria (Guo et al., 2022).

Dissolved oxygen (DO):

The presence of oxygen can inhibit both SDD/Anammox processes, as it can compete with nitrate and nitrite for the attention of denitrifying and Anammox bacteria (Kumar et al., 2010). Therefore, low DO concentrations (less than 0.5 mg/L) are required for the optimal performance of both processes (Huo et al., 2022). However, some studies have also suggested that the presence of a small amount of DO (0.5-1.0 mg/L) can enhance Anammox performance due to the oxygen-dependent oxidation of sulfide to elemental sulfur, which provides additional reducing power to the system (Guo et al., 2016).

Sulfide concentration:

Sulfide is a common intermediate product of the anaerobic degradation of organic matter in wastewater treatment systems, and it has a significant effect on the performance of SDD/Anammox processes (Qin et al., 2019). High concentrations of sulfide can inhibit both processes, while low concentrations may enhance denitrification rates (Russ et al., 2014). The optimal sulfide concentration can vary depending on the electron donor used, with thiosulfate-driven denitrification performing best at a sulfide concentration of 5-10 mg/L, while sulfide-driven denitrification and Anammox perform best at sulfide concentrations less than 1 mg/L (Qian et al., 2018; Qin et al., 2019).

Inhibitory compounds:

The presence of inhibitory compounds, such as volatile fatty acids (VFAs), phenolic compounds and heavy metals, can also have a significant effect on the performance of SDD/Anammox processes (Li et al., 2019). These compounds can compete with sulfur compounds for the attention of denitrifying and Anammox bacteria, and can inhibit their activity at high concentrations (Huo et al., 2022). Therefore, it is important to control the concentration of inhibitory compounds in the wastewater to ensure the optimal performance of these processes.

2.17 Applications of Integrated Sulfur-driven autotrophic denitrification and Anammox

Sulfur-based denitrification and Anammox are two biological processes that can be used together to remove nitrogen compounds from wastewater. SDD/Anammox has several applications in wastewater treatment, including:

I. *Treatment of industrial wastewater*: SDD/Anammox is effective in treating industrial wastewater that contains high levels of nitrogen compounds. Industries such as chemical, petrochemical and pharmaceuticals produce wastewater with high nitrogen concentrations that are difficult to treat using conventional biological treatment methods. SDD/Anammox is an efficient and cost-effective method for treating these wastewater types (Qian et al., 2018; Zeng et al., 2021; Wang et al., 2019).

II. *Treatment of municipal wastewater*: SDD/Anammox can be used in the treatment of municipal wastewater to remove nitrogen compounds. This helps to prevent the eutrophication of natural waterways and reduce the risk of algal blooms. The process is particularly useful in areas where space and energy are limited (Deng et al., 2021b; Du et al., 2017a).

III. *Treatment of agricultural wastewater*: Wastewater from livestock farming contains high levels of nitrogen compounds from animal waste, which can pollute nearby waterways if not treated. SDD/Anammox provides an effective way to remove these compounds, reducing the environmental impact of livestock farming (Yuan et al., 2020).

IV. *Treatment of landfill leachate*: Leachate from landfills contains high levels of nitrogen compounds, which can contaminate groundwater if not properly treated. SDD/Anammox is an effective way to treat landfill leachate, reducing the environmental impact of landfills (Zeng et al., 2021; Huo et al., 2022).

V. The SDD/Anammox process has also been applied in the *treatment of saline wastewater*, such as those produced in the oil and gas industry. Saline wastewater has high levels of dissolved salts, which can inhibit traditional biological nitrogen removal processes. The SDA process can effectively remove nitrogen from saline wastewater, making it a promising technology for the treatment of wastewater in the oil and gas industry. A study by Li et al. (2021) demonstrated that the SDA process achieved a total nitrogen removal efficiency of up to 80% in treating saline wastewater from the oil and gas industry.

VI. *Treatment of aquaculture wastewater*: Wastewater from aquaculture contains high levels of nitrogen compounds from fish waste and uneaten feed, which can cause environmental problems if not properly treated. SDD/Anammox can be used to remove these compounds, reducing the environmental impact of aquaculture (Wu et al., 2020).

Overall, SDD/Anammox is an effective and efficient method for removing nitrogen compounds from wastewater in a variety of applications. It can be used to treat wastewater from various industries and activities, reducing the environmental impact and improving water quality.

2.18 Advantages of Sulfur-driven autotrophic denitrification and Anammox

SDD/Anammox is a biological process that has several advantages over traditional methods of nitrogen removal in wastewater treatment. Some of the advantages of SDD/Anammox include:

2. *High efficiency*: SDD/Anammox is a highly efficient process that can remove up to 90% of nitrogen compounds from wastewater. This high efficiency makes it ideal for treating wastewater with high nitrogen concentrations, such as industrial and agricultural wastewater.
3. *Low energy consumption*: SDD/Anammox requires less energy than traditional methods of nitrogen removal, such as nitrification-denitrification. This is because the process does not require oxygen, which is typically supplied by energy-intensive aeration systems. As a result, SDD/Anammox can save energy and reduce operating costs.
4. *Reduced sludge production*: The SDD/Anammox produces less sludge than traditional methods of nitrogen removal. This is because the process converts nitrogen compounds directly into gas, rather than producing sludge that requires disposal. As a result, SDD/Anammox can save on sludge disposal costs and reduce the environmental impact of wastewater treatment.
5. *Compact footprint*: SDD/Anammox has a compact footprint compared to traditional nitrogen removal methods, making it ideal for applications where space is limited. This is because the process combines two biological processes into one, reducing the amount of space required for treatment.
6. *Low chemical requirements*: SDD/Anammox requires fewer chemicals than traditional nitrogen removal methods because the process relies on natural biological processes to remove nitrogen compounds, rather than adding chemicals to the wastewater. As a result, SDD/Anammox can reduce chemical costs and the environmental impact of chemical usage.
7. *Reduced greenhouse gas emissions*: SDD/Anammox produces less greenhouse gas emissions than traditional nitrogen removal methods because the process converts nitrogen compounds into gas, which is primarily nitrogen gas, rather than producing greenhouse gases such as nitrous oxide. As a result, SDD/Anammox can reduce the environmental impact of wastewater treatment on climate change.

Overall, SDD/Anammox is an efficient, cost-effective and environmentally friendly method of nitrogen removal in wastewater treatment. Its advantages make it an ideal solution for treating various types of wastewaters and reducing the environmental impact of wastewater treatment on both a local and global scale.

2.19 Concluding summary.

In conclusion, this chapter examined nitrogen removal processes in wastewater treatment plants (WWTPs), with a particular focus on sulfur-driven autotrophic denitrification and Anammox. The literature review provided a comprehensive overview of the nitrogen removal process, emphasizing the environmental significance of nitrogen removal and the role of WWTPs in addressing this issue. This highlighted various existing processes of biological nitrogen removal, including nitrification and denitrification, as well as conventional and advanced BNR processes. Additionally, alternative nitrogen removal pathways such as Anammox and partial nitrification were explored in detail.

Furthermore, the study examined the effects of sulfide on partial nitrification, the Anammox process, and sulfur-driven denitrification. It also investigated the potential of combined sulfur-driven denitrification and Anammox, as well as sulfur, thiosulfate and elemental sulfur-based denitrification and Anammox processes. Factors influencing sulfur-driven autotrophic denitrification and Anammox were thoroughly analyzed, along with the applications and advantages of integrated sulfur-driven autotrophic denitrification and Anammox. Overall, this study contributes to the understanding of nitrogen removal processes in WWTPs, particularly focusing on the novel approach of sulfur-driven autotrophic denitrification and Anammox. The findings provide valuable insights into the potential application and advantages of this integrated process, paving the way for further research and development in the field of wastewater treatment. By implementing sulfur-driven autotrophic denitrification and Anammox, WWTPs can enhance their nitrogen removal efficiency and contribute to the preservation and protection of the environment.

CHAPTER THREE

3.0 START-UP OF PARTIAL NITRIFICATION BY INTERMITTENT AERATION, PH SHOCKS AND SULFIDE ADDITION IN A SEQUENTIAL BATCH REACTOR

This chapter present the Start-up of partial nitrification by intermittent aeration, pH shocks and sulfide addition in a sequential batch reactor. The chapter represent an abridge version of the publication reference: Hassan, M.O., Gani, K.M., Kumari, S., Awolusi, O., Gumede, L., Seyam, M. and Bux, F., 2022. Start-up of partial nitrification by intermittent aeration, pH shocks, and sulfide addition in a sequential batch reactor. *Journal of Chemical Technology & Biotechnology*, *97*(8), pp.2186-2195.

3.1 Abstract

The combination of partial nitrification (PN) and anaerobic ammonium oxidation has been identified as a promising technology for the removal of nitrogenous contaminants from wastewater. The inhibition or wash-out of nitrite-oxidizing bacteria (NOB) while keeping the ammonia-oxidizing bacteria (AOB) active is key for PN. Multiple startegies, including intermittent aeration, pH shocks and sulfide addition, were investigated in this study for the start-up of PN and stable suppression of NOB. Intermittent aeration with a dissolved oxygen (DO) level of less than 5 mg/L effectively suppressed NOB activity and resulted in an average nitrite accumulation (NAR) of 93%, implying that the initial DO level during the aeration phase may influence the establishment of successful PN. Similarly, when the reactor was run at a low (pH 5), both AOB and NOB activity was reduced. When the pH was raised to neutral (pH 7.5), there was an increase in AOB activity with an average NAR of 84%. However, NOB activity remained suppressed. Likewise, when the reactor was fed with sulfide (up to 25 mg/L) without pH control, the average NAR increased from 63% to 85%. This study proposed a successful approach to achieving PN by controlling DO level in the aeration phase during intermittent aeration and pH control. The findings also show that the nitrite oxidation process is more sensitive to sulfide than the ammonium oxidation process. As a result, the findings of this study may contribute to a better understanding of establishing PN in a sequential batch system.

Keywords: Anammox; Ammonia-oxidizing bacteria; Nitrite-oxidizing bacteria; Partial Nitrification; Sulfide addition.

3.2 Introduction

Nitrogen removal from wastewater has attracted widespread attention due to the negative impact associated with its discharge into the aquatic environment. Nitrification, as the initial step of the biological nitrogen removal (BNR) process, is carried out in two stages. Firstly, ammonia is oxidized to nitrite by ammonia-oxidizing bacteria (AOB); and in the second step, the nitrite is further oxidized to nitrate by nitrite-oxidizing bacteria (NOB) (Metcalf and Eddy. 2014). The nitrate thus formed is then converted to dinitrogen gas by a group of heterotrophic denitrifying bacteria in the absence of oxygen. However, a combination of partial nitrification (PN) and Anammox processes (PN/A) has recently emerged as a promising sustainable alternative to the traditional nitrification-denitrification process (Liu et al., 2020) due to low energy demand; no organic carbon requirement for denitrification; and reduced sludge production of up to 90% (Zhang et al., 2019). However, one of the main challenges for its implementation is the difficulty in maintaining a stable PN reactor by suppressing the NOBs (i.e., reducing the production of nitrate and facilitating nitrite accumulation), which is a key factor in the success of a PN/A process (Law et al., 2019; Ma et al., 2010; Zhang et al., 2019).

Different studies have reported strategies to achieve PN and nitrite accumulation by controlling one or more operational and environmental parameters such as low dissolved (DO) (Cui et al., 2020); pH (Zhou et al., 2011); elevated temperatures (Kim et al., 2008); intermittent aeration (Miao et al., 2017); free ammonia (FA) (Zhang et al., 2018); free nitrous acid (FNA) (Li et al., 2019); and sulfide addition (Kauba et al ., 2018; Erguder et al., 2008).

Intermittent aeration is considered one of the simplest strategies implemented for achieving stable PN whilst also potentially saving energy in comparison to continuous aeration (Jiang et al., 2018; Chen et al., 2020). Previous studies have shown that intermittent aeration promotes PN because AOB recovers more quickly after anoxic disturbance than NOB (Xu et al., 2020; Chen et al., 2020). Similarly, control of DO level, aeration time and aeration regimen contributed to AOB selection over NOB in the oxygen competition (Chen et al., 2020). Intermittent aeration (8 minutes on and 18 minutes off) with a DO level of 1.0 mg/L was reportedly effective in suppressing NOB (Jardin and Hennerkes, 2012). Optimizing the intermittent aeration approach could reduce the operational complexity related to this approach (Zhang et al., 2020). Despite this, there are few studies demonstrating the effectiveness of intermittent aeration in inhibiting NOB in conventional PN systems.

Another strategy for the quick start-up of PN can be shocks of reduced pH. At low pH (< 6), there will be production of FNA, which has an inhibitory effect on both AOB and NOB. FNA would inhibit NOB at concentrations between 0.22 and 2.8 mg/L (Li et al., 2019). AOB growth was inhibited by approximately 0.10 mg HNO_2-N/L and completely stopped by approximately 0.40 mg HNO_2-N/L, whereas NOB growth was inhibited by approximately 0.011 mg HNO_2–N/L and completely stopped by approximately 0.023 mg HNO_2-N/L. Based on this, it is evident that NOB growth is selectively inhibited within a range of approximately 0.011–0.10 mg HNO_2-N/L FNA. Therefore, compared to AOB, the NOB is selectively suppressed under FNA treatment (Jiang et al., 2018), which may favour the PN start-up. However, maintaining a stable PN process for a longer period is still a challenge owing to the highly adaptive nature of NOBs to the reactor conditions (Peng et al., 2006). A pH shock can be effective for facilitating AOB in nitrifying bioreactors where alkalinity is limited relative to total ammonium. However, PN under acidic conditions has not yet been demonstrated.

Sulfide is a known nitrification inhibitor and has specifically been shown to inhibit ammonium oxidation and nitrite oxidation differently (Bejarano Ortiz et al., 2013). Vela et al. (2018) suggested that sulfide may be a useful selective inhibitor for NOB, depending on the composition and diversity of the NOB population. The addition of sulfide to wastewater could cause a rise in pH level, which in turn might increase the FA concentration in the system. This means that AOB and NOB are both exposed to higher FA concentrations, an order of magnitude higher than the inhibitory threshold caused due to sulfide addition. However, the effect of sulfide addition to FA and its subsequent effect on AOB and NOB has not been investigated for the start-up of PN.

From the available literature data, the establishment of effective strategies for stable PN performance still needs further investigation, especially with the startup of PN. Therefore, the objective of this study was to evaluate the role of different strategies, namely controlling DO intermittent aeration, low pH shocks and sulfide addition, in the start-up of PN using activated sludge as inoculum. All the strategies were investigated in a sequential batch reactor (SBR). The role of sulfide addition was investigated under controlled pH conditions as well as without pH control. The dynamics of the key nitrifying microbial population under different operational strategies were also investigated.

3.3 Material and Methods

3.3.1. Reactor set-up and operations

A laboratory-scale SBR with a working volume of 1.8 L and headspace of 200 mL was used in this study. The schematics of the reactor, together with its instrumentation and control systems, are shown in Figure 3.1. The reactor was operated at an ambient temperature (20–25 °C). The operational cycle in the SBR consisted of a one hour (1h) feeding period, 1h aeration period, 30 minutes of settling and 30 minutes of decanting, resulting in a total number of 8 cycles in 24 hours. The volumetric exchange ratio was fixed at 40%. Oxygen was provided by diffusers placed inside the reactor, and the aeration time was controlled with the use of timers. The hydraulic residence time (HRT) in the reactor was 12 hours and the flow rate was 5.2 L/day. The pH of the reactor was controlled by dosing 1N sodium hydroxide (NaOH) and 1N hydrochloric acid (HCL). The pH and DO were monitored using portable Vernier LabQuest2 USA. For each experiment, the reactor was inoculated with activated sludge samples obtained from the aeration tank of the nearby wastewater treatment plant (WWTP) treating domestic wastewater. The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) of the inoculated sludge were 6072 mg/L and 4480 mg/L, respectively.

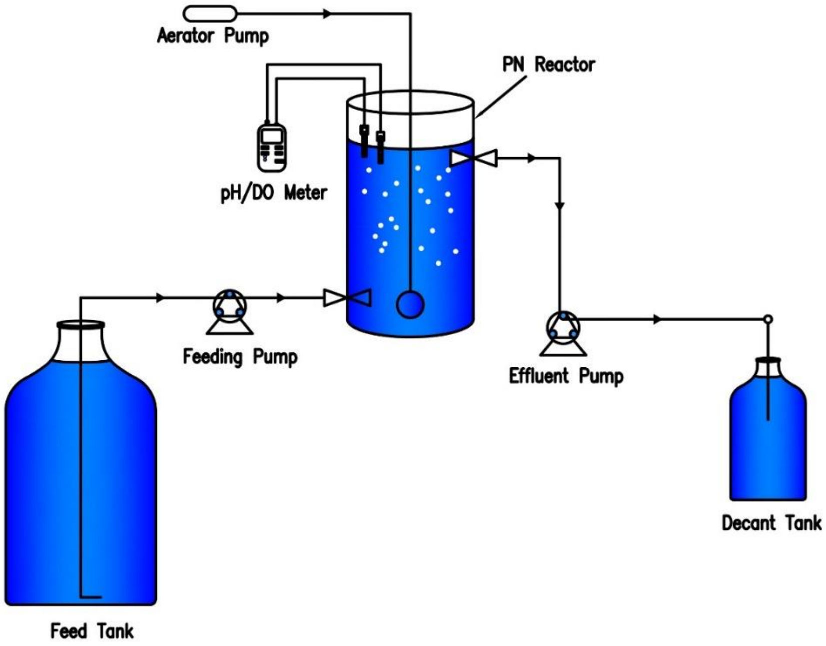

Figure 3.1 Schematic diagram of partial nitrification process in a sequential batch reactor

3.3.2. Synthetic wastewater

The reactor was fed with a synthetic ammonium feed containing: $NaHCO_3$ (900 mg/L), NH_4^+-N (from NH_4Cl) as needed, NaCl (70 mg/L), KH_2PO_4 (12 mg/L) $CaCl_2.2H_2O$ (5 mg/L) $FeSO_4.7H_2O$ (2 mg/L). One mL/L of each of the trace element solutions I and II (Van de graff et al., 1995) were added to the synthetic feed. The amount of NH_4Cl added to the synthetic wastewater was adjusted to ensure the initial influent ammonium (NH_4^+-N) concentration summarized in Table 3.1. In experiments with sulfide, the solution containing Na_2S (25 mgS/L) was added to the synthetic medium. To avoid aerobic oxidation of sulfide, distilled water was used to prepare the sulfide-S solution and was flushed with high-purity argon to remove the DO.

3.4 Experimental Plan

3.4.1. Effect of DO

The first set of experiments was to investigate the effect of DO in intermittent aeration for the start-up of PN. Intermittent aeration was achieved from the normal operation of the SBR.[14,15,16] Each cycle included four operational stages, namely 60 minutes of feeding, variable-length intermittent aeration (30 min aeration/30 min anoxic), 30 minutes of settling, and 30 minutes of discharging of 5 L supernatant. Three sets of experiments were performed with different DO levels maintained in the aeration phase. The average DO levels maintained in the three sets of experiments were 6.7 mg/L, 5.5 mg/L, and 4.3 mg/L. Other operational parameters during these experiments were kept constant. The pH of the reactor was maintained at 7.5 and the average NH_4-N concentration in the synthetic feed was 70 mg/L.

3.4.2. Low pH shocks

In the second experimental set-up, pH in the reactor was adjusted by dosing with 0.5M HCL, keeping all other parameters constant. The pH in the reactor was first set to (7-7.5) for the first 25 days and then reduced to an acidic level (pH 5) for 10 days for inducing pH shock to the biomass in the reactor. After 10 days of operation, the hydrocholoric acid (HCL) dosing was suspended and the pH in the reactor was resumed to the normal level (pH of 7-7.5) until the end of the experiment. The average NH_4-N concentration during this experiment in the synthetic feed was 120 mg/L.

3.4.3. Sulfide addition

The experiments of PN by sulfide addition were conducted in four phases with similar operating conditions. Phase I (0-10 days) was performed to confirm the activities of AOB and NOB in the reactor. During Phase II (10-30 days), a sulfide concentration of (25 mg/L) was added to the reactor, keeping all the parameters constant; and Phase III (30-40 days) was the recovery phase where the addition of sulfide (25 mg/L) was suspended. In Phase IV (40-50 days), once again the addition of sulfide to the reactor was initiated, but with pH control. The pH value was increased to 8-9.5 after the addition of sulfide, which was manually adjusted to 7.5 using 1M HCL (in sulfide with pH-controlled experiments). The average NH_4-N concentration during this experiment in the synthetic feed was 70 mg/L.

Table 3.1: Performance of PN reactor during different experiments

Experiment	**PN by controlling DO levels in the aeration phase**			**PN by low pH shock**			**PN by Sulphide addition**	
	DO(6.7mg/L)	**DO (5.5 mg/L)**	**At DO (4.3 mg/L)**	**At pH (7-7.5)**	**At pH (5-6.5)**	**At pH (7-7.5)**	**Without pH control**	**With pH control**
Influent NH_4^+-N(mg/L)	70	70	70	120	120	120	70	70

NAR (%)	20	45	93	0-40	53	84	84.98	69.90
FNA (mg/L)	--	---	----	<0.01	0.02-0.05	0.01	0.004	0.00
FA (mg/L)	--	--	---	---	--	---	4.85	0.73
Average (NH_4-N, NO_2-N, NO_3-N) mg/L in the effluent	12.6,7.0,7.0	23,7,24	30.0,38.0,0.5	0.5,24,60	99.6,9.6,12	45,36.0,6	30.1,23.1,2.8	68.6,5.6,7.7

3.5 Analytical methods

The influent and effluent samples from the SBR were filtered through 0.45 µm syringe filters before the measurements of nitrogenous compounds. Concentrations of NH_4^+-N, nitrite (NO_2^--N), nitrate (NO_3^--N), and sulfate were analyzed using the Gallery TM Automated Photometric Analyser (Thermo Scientific; USA). The sulfide concentrations in the influent and effluent of the SBR were analyzed by the methylene blue spectrophotometric method (DR 6000, Hach USA) according to standard Methods (APHA). Analysis of sludge properties, i.e., Mixed-Liquor suspended solids (MLSS) and mixed-liquor volatile suspended solids (MLVSS), was carried out according to standard methods (APHA, 2005).

3.5.1 Analysis of sludge Calculations

The NH_4-$N_{\text{Removal Efficiency}}$ (%), Nitrogen Loading Rate (NLR; [kg N/ (m³.d)] and Nitrite Accumulation Ratio (NAR%) were calculated with the equations (1)-(3), respectively:

$$NH_4\text{-}N_{\text{Removal Efficiency}}\ (\%) = \frac{(NH_4 - Ninf) - (NH_4 - Neff)}{NH_4 - Ninf} X\ 100 \qquad (1)$$

$$\text{NLR [kgN/ (m}^3\text{.d)]} = \frac{TNinf \times 24}{\text{HRT} \times 1000} \qquad (2)$$

$$\text{NAR (\%)} = \frac{NO_2{-}N\,eff}{(\mathrm{NO_2}{-}Neff + NO_3{-}Neff)}\ X\ 100 \qquad (3)$$

The concentrations of free ammonia (FA) and free nitrous acid (FNA) inside the SBR were estimated using the equations (4) and (5) proposed by Li et al. (2019).

$$\text{FNA (HNO}_2\text{ mg N/L)} = \frac{\text{total nitrite as N (mg/L)}}{10^{pH} e^{-2300/(273+\text{temp (°C)})}} \qquad (4)$$

$$\text{FA (NH}_3\text{ mg N/L)} = \frac{\text{Total ammonia as N (mg N/L)} * 10^{pH}}{e^{6344/(273+\text{temp (°C)})} + 10^{pH}} \qquad (5)$$

3.5.2 Sample collection and DNA extraction

The dynamics of the nitrifying community within the SBRs during different experimental runs were investigated using the quantitative PCR technique (q-PCR). Six sludge samples were collected from the SBR for DNA extraction. Samples 1 and 2 were collected on days 5 and 45 respectively when the reactor was operated under intermittent aeration, and samples 3 and 4 were collected when the reactor was operated under pH shocks. Similarly, samples 5 and 6 were collected on days 10 and 29 respectively when the reactor was operated under sulfide addition without pH control (Phase II). The sludge samples of 10 mL mixed liquor were collected from SBR using sterile centrifuge tubes and then concentrated at 5000 rpm for 20 min. Finally, the supernatant was removed, and the pellet was used for the genomic DNA extraction. The total DNA from the pellet was extracted using a DNeasy Powersoil kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The concentration and purity of the extracted DNA were checked spectrophotometrically with NanoPhotometer® NP80 (IMPLEN, Munich, Germany). The extracted DNA was stored at -20 °C until further use in TE buffer.

3.5.3 Real-Time Quantitative-PCR

The Q-PCR assays were performed to investigate the dominant nitrifiers, such as AOB and NOB (*Nitrospira* and *Nitrobacter* spp.) in the reactors under different operational strategies. Standard curves were generated using purified 16S rRNA gene fragments (target DNA) obtained from PCR-amplified AOB (amoA-1F/amoA-2R), *Nitrospira* spp. (NSR1113F/NSR1264R), and *Nitrobacter* spp. (Nitro1198f/Nitro1423r) as described by Orschler et al. (2019) and Awolusi et al. (2017). Ten-fold serial dilutions of the target DNA were prepared from 10^8 to 10^1 copy numbers and used as a positive standard. The reaction mixture was made up of 5 μL of 2X PowerUp™ SYBR® Green Master Mix, 0.4 μM of each primer, 2 μL of template DNA (final concentration of 1 ng), and molecular grade water to a final volume of 10 μL. The real-time qPCR was conducted using QuantStudio 3 Real-Time PCR System (Applied Biosystems). A no template control (NTC) without DNA was included in all reactions. All the qPCR assays were conducted in compliance following the MIQE guideline.[25] Primer specificity and correct product-size amplification was confirmed by gel-electrophoresis of the amplicon in 1.2 % (w/v) agarose gel.

3.6 Results and Discussion

3.6.1 PN by controlling DO levels during intermittent aeration.

Three phases of intermittent aeration were carried out to understand the role of DO value in initiating PN in a reactor (Figure 3.2a). The average DO in the first experiment was 6.7 mg/L in the aeration phase, which was decreased to 1 mg/L in the decanting phase. During this phase, NAR fluctuated between 8-25%, with an average value of 20% (Figure 3.2b). A DO drop of 5.8 mg/L from the aeration to decanting phase was noted. In the second experiment, the DO in the aeration phase was reduced to an average of 5.5 mg/L, which resulted in a DO drop of 3.7 mg/L from the aeration phase till decanting phase. During this experimental phase, an increase in NAR up to 45% was observed (Figure 3.2b). In the third experiment, after 33 days of operation, the DO in the aeration phase was further lowered to less than 5 mg/L with an average value of 4.3 mg/L in the aeration phase. This resulted in the drop of DO to 1 mg/L in decanting phase. During this phase, an increase in NAR (average of 93%) (Figure 3.2b) with the effluent of NO_2:NH_4 ratio of 1.26 was observed. These results indicate that initial DO in the aeration phase is an influencing factor for establishing successful PN by intermittent aeration and a DO level of less than 5 mg/L and an average of 4.3 mg/L proved effective in this study. The average concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N in the effluent during all three sets of experiments are summarized in Table 3.1. Similar results were noted by Wang et al. (2020) where NAR during DO (4 mg/L) conditions increased from 44.8 and 66.7% in 20 days. At high DO, the activity difference between AOB and NOB is maintained due to the high AOB rate and stable microbial community structure, resulting in a steady PN process (Cui et al., 2020). PN was reported in some intermittent reactors that treated low-ammonium wastewater, such as domestic wastewater or synthetic wastewater (Miao et al., 2017; Li et al., 2013). This could be because the studies maintained a higher DO concentration in the system for example to maintain a prolonged nitrite accumulation. Regmi et al. (2014) used a high-DO concentration (>1.5 mg/L) in their intermittently aerated pilot-scale process. Similarly, during the high-DO phase in this investigation, PN was obtained gradually. As a result, intermittent aeration under high-DO conditions could be a contributing factor to PN in the reactor.

Compared to it controlling low-DO conditions in wastewater treatment systems has been extensively researched in order to establish PN. For example, Bao et al. (2017) observed that PN was successfully achieved at DO (1.8 ± 0.32) with intermittent aeration in the reactor. However, the effect of DO in the intermittent aeration phase on PN has not been well investigated. Moreover, because AOB has a lower oxygen half-saturation constant than NOB, low DO concentration is considered the main factor responsible for PN (Wang et al., 2020). However, other studies have reported that after a long-term operation of the reactor at a DO of 0.16 mg/L, a shift from Nitrobacter-like NOB to Nitrospira-like NOB occurred (Liu and Wang et al., 2013). As a result, NOB could not be washed out under low DO concentrations. Thus, the low-DO strategy for achieving PN should be reconsidered on a case-by-case basis. This study has shown that the control of DO level in the aeration phase during intermittent aeration has an impact on the initiation of the PN. The results from this study showed that nitrite can still be accumulated at concentrations higher than reported in a few previous studies agreeing with Ciudad et al. (2005), in that DO value can be a specific case due to the variation of oxygen mass transfer efficiency in the reactor.

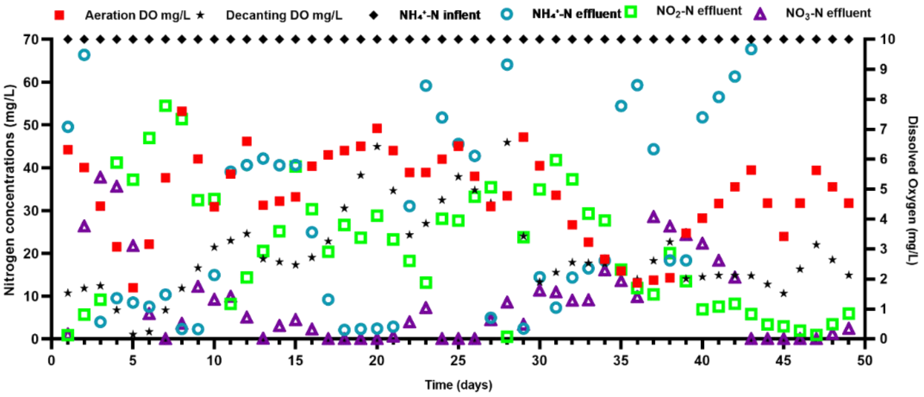

Figure 3.2a: The effect of DO on the nitrogen concentrations in the PN-SBR

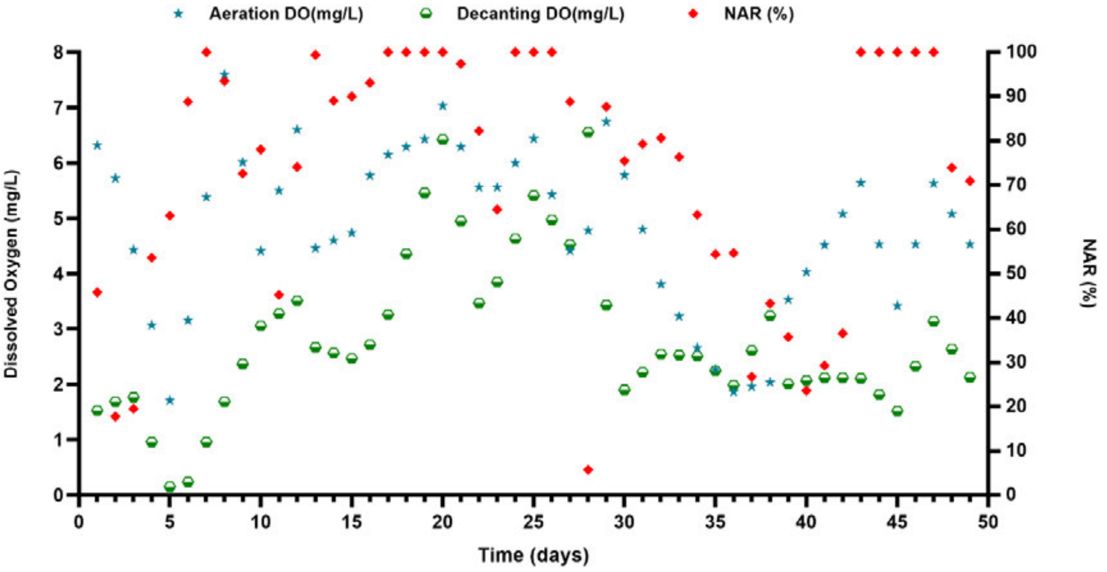

Figure 3.2b: Effect of DO in aeration and decanting vs NAR

3.6.2 PN by inducing pH shock to the biomass

Figure 3.3a represents the effect of pH on the concentrations of the nitrogen species in the PN reactor. At pH 7–7.3, the NO_2^--N in the effluent was below 24 mg/L and the NO_3^--N concentration was high, ranging from 36–60 mg/L, signifying the activities of both AOB and NOB in the reactor. The NAR was between 0 – 40% (Figure 3.3b). After 25 days of operation, the pH in the reactor was lowered to 5 by dosing it with 0.5 M HCl. This has also resulted in the reduction of NO_3^--N concentration to 12 mg/L with no significant increase in NO_2^--N production for the initial 10 days of operation at this pH, indicating potential inhibition of both AOB and NOB at this condition. The average NO_2^--N and NH_4^+-N concentration in the effluent during this period was 9.6 mg/L and 99.6 mg/L, respectively (Figure 3.3a) and the average NAR was 53% (Figure 3.3b), indicating a suppression of AOB. After 10 days of operation, the HCL dosing was suspended and the pH in the reactor was resumed to its normal level (pH of 7-7.5). Shortly after stopping the HCL dosage in the reactor, the NO_2^--N concentration in the effluent increased to an average of 36 mg/L. The maximum NAR obtained at this stage was 100%, with an average NAR of 84%, showing satisfactory PN. The NO_3^--N concentration in the effluent was however still low (6 mg/L), indicating a continuous suppression of NOB even after the pH was resumed at the normal level. These results may suggest an operational strategy for the start-up of PN in activated sludge by lowering pH until NOB is suppressed significantly and then resuming the pH to normal conditions for AOB growth. The results agreed with Li et al.'s (2020) findings that in addition to the successful suppression of NOB activity, the acidic operation (at a pH 5-6) partially inhibited AOB activity. Some other studies have also reported that NOB activity is severely inhibited when the reactor is operated at an acidic operation (pH 2.2) (Tarre et al., 2004; Gieseke et al., 2006). Regulation of pH is commonly used to achieve PN due to its different inhibition effects on AOB and NOB (Peng and Zhu, 2006).

To investigate the cause of NOB suppression at lower pH, FNA measurements were carried out simultaneously, as shown in Figure. 3.4a. At a pH value of 7-7.5, the FNA concentration was less than 0.01 mg/L in the PN reactor. With an influent NH_4^+-N concentration of 120 mg/L, the average effluent NH_4^+-N, NO_2^--N, and NO_3^--N concentrations were 77.8 mg/L, 7.2 mg/L, and 39.7 mg/L, respectively. When the pH was reduced to 5-6.5, the FNA concentration in the PN reactor was increased to a range of 0.02–0.05 mg/L the effluent NO_3^--N concentration decreased from 39.7 mg/L to 9.1 mg/L, indicating suppression in NOB activity. The results agreed with Anthonisen et al.'s (1976) findings that the FNA concentration required to suppress the NOB activity is between 0.02 and 0.4 mg/L. Zhou et al. (2011) reported that lower concentrations of 0.011–0.07 mg/L start to inhibit NOB and completely inhibit NOB at 0.026–0.22 mg/L. These findings indicate that the NOB inhibition in the reactor was mainly mediated by the increased production of FNA under acidic conditions. When the HCl dosing was terminated in the third phase, an increase in pH to neutral (pH 7) was observed, which resulted in a drop in FNA levels to less than 0.01 mg/L (Figure 3.4a). However, the reactor responded positively to the production of NO_2^--N at this phase, and the concentration of NO_2^--N in the effluent increased to 37.8 mg/L, indicating the possible resumption of AOB activity. However, the NO_3^--N concentration at this stage remained the same (8 mg/L). This indicated that the NOB suppression that occurred at lower pH continued even after resuming the pH to the initial level (pH 7). This study therefore proposes a successful approach to achieve PN using pH control, as indicated by the increased NAR in the effluent (exceeding 65%) over the start-up period.

In this study, however, the achievement of the in-situ FNA-mediated NOB inhibition was due to the acidic condition (i.e. at pH 5). FNA concentration at the ppm level (i.e., up to 0.05 mg/L) was obtained with an accumulated NO_2^--N of approx. 40 mg/L at pH 5-6.5. Based on this study's findings by using chemical-based pH adjustment at the start-up of the reactor, efficient NOB suppression can be achieved without a significant impact on AOBs.

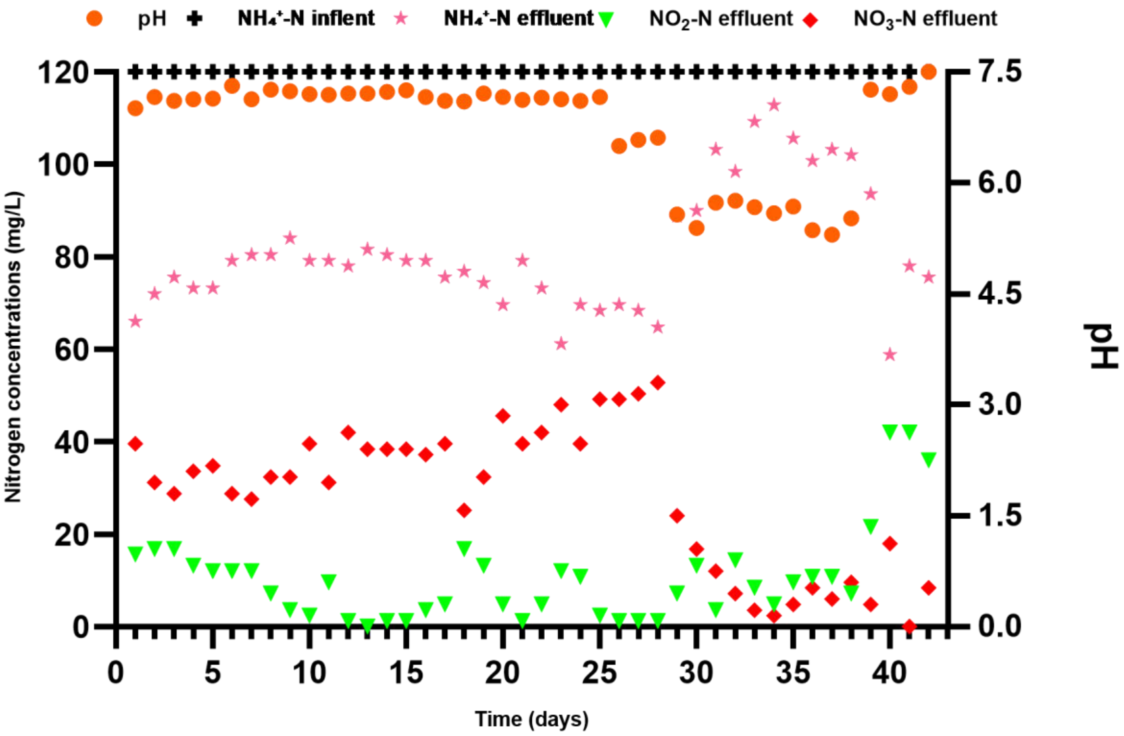

Figure 3.3a: The effect of pH on the nitrogen concentrations in the PN-SBR

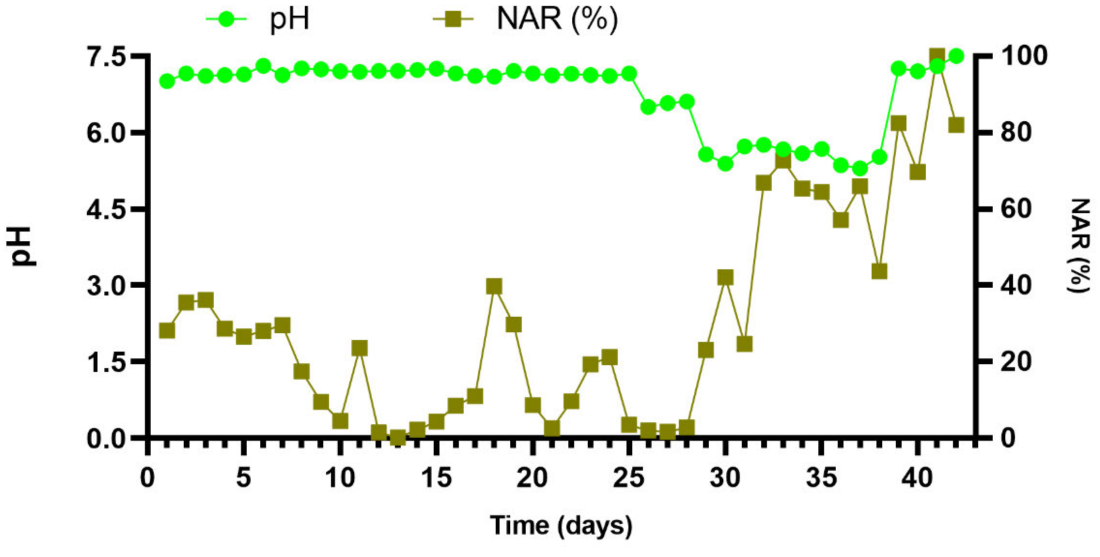

Figure 3.3b: The effect of pH on the nitrite accumulation rate in the PN-SBR

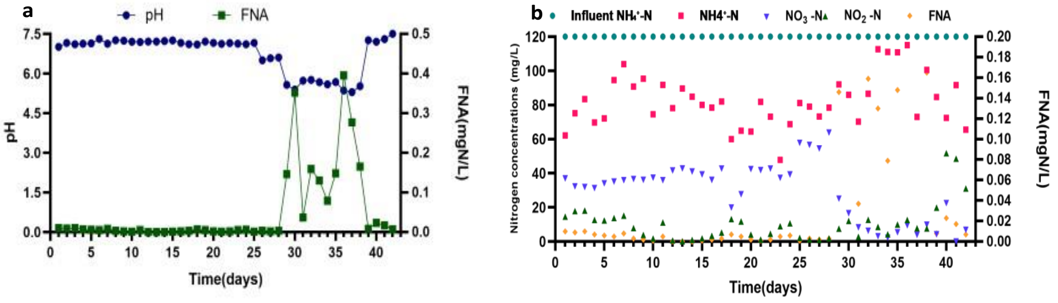

Figure 3.4: (a)The effect of pH on the production of free nitrous acid and (b) The effect of free nitrous acid on the nitrogen concentrations in the PN-SBR

3.6.3 PN by Sulfide addition without pH control

Figure. 3.5a shows the time course and concentrations of nitrogenous concentrations in the effluents of the SBR fed with an initial sulfide concentration of 25 mg/L. The influent NH_4^+-N concentration during the whole experiment was kept constant at 70 mg/L (Figure 3.5a). During the experimental phase 1 in which no sulfide was added, the average concentrations of NH_4^+-N, NO_2^--N and NO_3^--N in the effluent were 20.8 mg/L, 18.41mg/L and 13.38 mg/L respectively, and NAR was around 62.32%. During the experimental phase II, when the sulphide was added to the reactor, the average NAR increased from 63% to 85% and the concentration of NH_4^+-N, NO_2^--N and NO_3^--N in the effluent were observed to be 30.1 mg/L, 23.1 mg/L and 2.76 mg/L respectively. The average NO_3^--N in the effluent was decreased from 13.38 mg/L to 2.76 mg/L, indicating suppression of NOB activity. The addition of sulfide caused a sudden increase in the pH of the reactor (from 7.06-8.12), resulting in the average FA and FNA concentrations of 4.85 mg/L and 0.004 mg/L. FNA and FA are known to inhibit nitrification, particularly nitrite oxidation (Ma et al., 2010; Peng et al., 2006). Although several researchers have documented FA and FNA concentrations that could inhibit NOB growth and cause the accumulation of nitrite, the critical values reported in those studies varied (Chai et al., 2015; Kim et al., 2008). NOB inhibition was reported at FA concentrations ranging from 1-70 mg/L, whereas AOB inhibition was recorded at 150 mg/L (Anthonisen et al., 1976).

The above phase was followed by a recovery phase (phase-III) in which sulfide addition to the reactor was terminated. This operational change has resulted in a decrease in NAR from 85.98% to 64.2% and a gradual increase in the effluent NO_3^--N from 2.7 mg/L-13.8 mg/L, indicating resumption of NOB activity. The average pH in the reactor during this phase was dropped to 7.44, which also led to a decrease in FA concentration from 4.85 mg/L to 0.46 mg/L. A drop in NH_4^+-N removal efficiency was observed in phase IV when the sulfide was re-introduced into the reactor with pH control. An increase in pH to 8-9.5 was noted just after the addition of sulfide, which was manually adjusted to 7.5 using 1M HCL. As the experiment continued, the average NH_4^+-N concentration in the effluent was 67.82 mg/L, which corresponded to a gradual decrease in NH_4^+-N removal efficiency to 3.12%. The average NAR was only 69.90% and, the average concentration of nitrogenous concentrations NH_4^+-N, NO_2^--N, and NO_3^--N in the effluents during this phase were 67.82 mg/L, 5.46 mg/L and 8.05 mg/L respectively.

In this experiment, FA increased (average 4.85 mg/L) with the addition of sulfide without pH control in phase II (Figure 3.5b) as a result of an increase in pH. In addition, the FNA concentration was found to be 0.004 mg/L, the valid inhibition value; therefore, only the effect of the FA was considered. FA concentration up to 4 mg/L did not suppress the NOB activity in this study. However, when the FA concentration increased to above 4 mg/L, a significant decrease in NO_3^--N production was observed, which corresponded to an increased nitrite accumulation (Figure 3.5c).

Therefore, the PN observed during phase-II (after the addition of sulphide) may be because of increased pH, which led to an increase in FA concentration. Subsequently, in the following days of the experiment, a lower FA concentration was observed in Phase III and Phase IV i.e., 0.46 mg/L and 0.73 mg/L respectively, which is lower than the reported inhibitory threshold for NOB. The obtained results indicate that nitrite oxidation is a process more sensitive to sulfide than ammonium oxidation. Therefore, the result from this study indicates that the presence of sulfide in the wastewater at an optimum level can be beneficial in inhibiting nitrite oxidation and imposing partial nitrification.

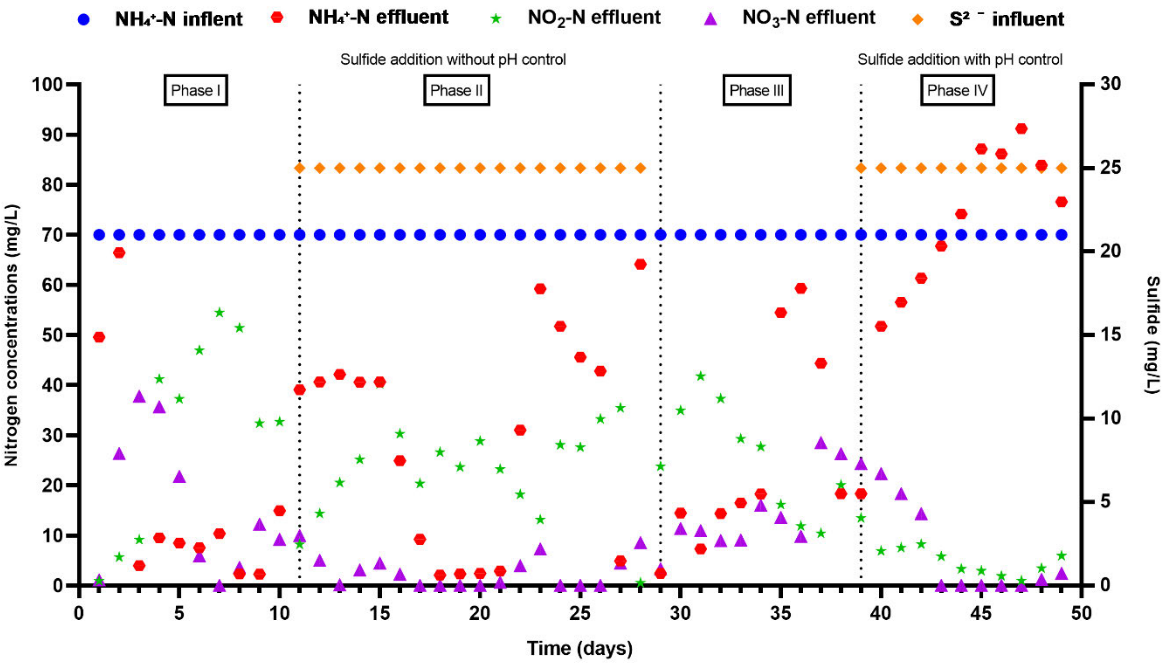

Figure 3.5a: Effect of sulfide on the nitrogen concentrations in the PN-SBR

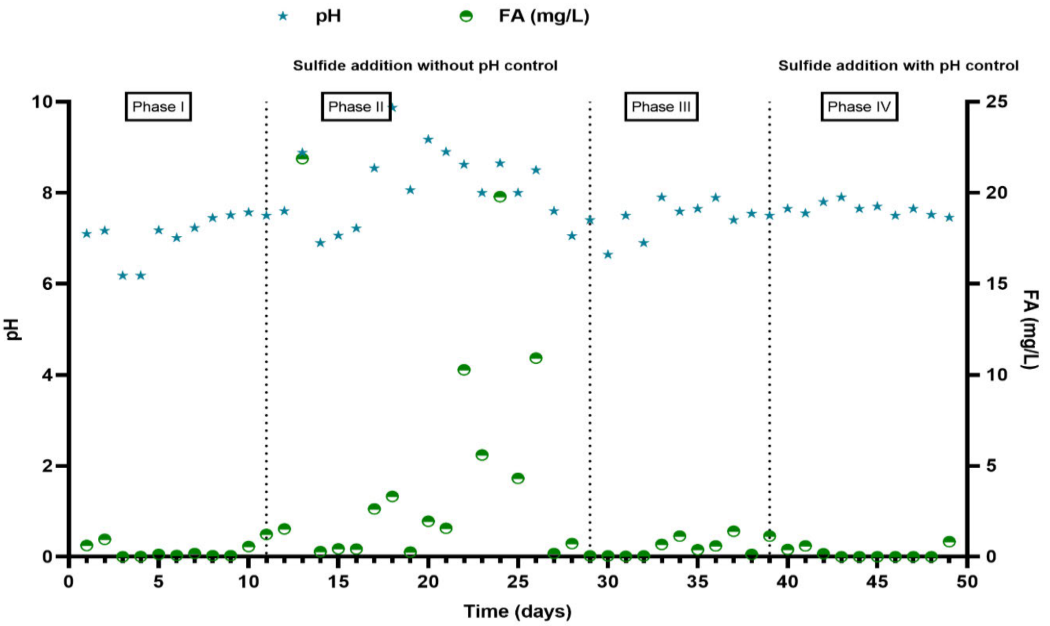

Figure.3.5b: pH and FA concentration in the effluent

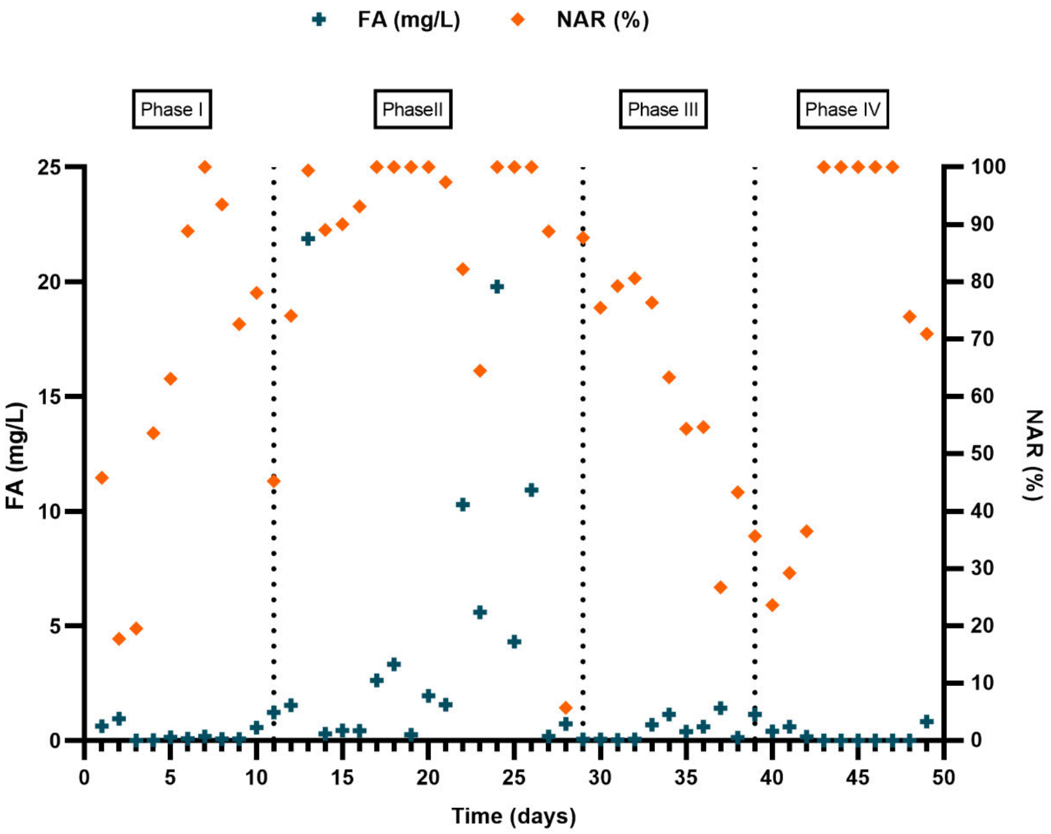

Figure. 3.5c: NAR in the effluent and FA concentration in the PN-SBR

3.7 Real-Time Quantitative-PCR Analysis

Figure. 3.6 shows the functional gene (amoA) copies for AOB, and 16SrRNA copies for *Nitrospira* and *Nitrobacter* quantified using qPCR in the reactor during the experiment. The overall AOB abundance based on *amoA* gene copies was within the range of (1.2 ± 0.01) x 10^6 to (3.4 ± 0.01) x 10^7 copies/mL and *Nitrospira* (5.8E ± 0.3) x 10^5-(5.9 ± 0.7) x 10^6 copies/mL and *Nitrobacter* (4.5 ± 0.2) x 10^6-(1.1 ± 0.01) x 10^7 copies/mL (Figure 3.6). The results from the first experiment, i.e., when the aeration strategy was applied for PN indicated that the AOB and NOB co-existed during this stage of the experiment. However, there was a different effect of the DO regulation on them. During this period, the average DO concentration of 6.7 mg/L was reduced to 4.3 mg/L with a resultant 8-fold increase of the AOB concentration (from 3.4 x 10^6 - 2.7 x 10^7 copies/mL), whilst *Nitrospira* spp. and *Nitrobacter* spp. had a 1.3-fold (increase) and 1.1-fold (decrease) respectively in their population densities. This suggests that the AOB population was favored by the prevailing DO concentrations during this period. This in turn encouraged an increased NAR with an average of 93% (Figure 3.2b). The NOB population was reduced when DO concentration was increased to 4.5 mg/L, and they were eventually washed away over a lengthy period of reactor operation at this condition, according to previous studies (Law et al., 2019).

In the second stage of this experiment, pH was used as the PN control strategy. The initial pH during this period was pH 7–7.3. However, due to the HCl dosing, the pH dropped to 5. This resulted in the AOB community experiencing a 15-fold drop in its population density, whilst there was a 4- and 2-fold increase in the *Nitrospira* and *Nitrobacter* spp. respectively. This in turn resulted in the fluctuation of the NAR from an initial 0 to 40 %, and later 53%. However, when the HCl dosing was stopped during the last part of this phase, it moved up to a maximum of 98%. Earlier on, He et al. (2009) reported an inhibitory effect of acidic pH (4.8) on AOB, with about 50% reduction in its NH_4^+-N removal capacity.

The last stage of the experiment involved the application of sulfide addition without pH control. The findings from this study showed that AOB and *Nitrospira* spp. had a slight reduction of about 1.5-2.1-fold respectively; whilst *Nitrobacter* spp. on the other hand increased by 1.4-fold. This may be due to the addition of sulfide, which caused a sudden increase in pH (from 7.06–8.12) and gave rise to FA concentrations of 4.85 mg/L in the reactor. Although it has been reported that FA can exhibit an inhibitory effect on nitrifiers generally, AOB (at 10–150 mg/L) is known to be more resistant to FA inhibition than NOB (at 0.1–1 mg/L) (Zhang et al., 2018). However, among NOB, *Nitrobacter* spp. only exhibits limited FA inhibition compared to *Nitrospira* spp. (Hawkins et al., 2010) as evidenced in this study.

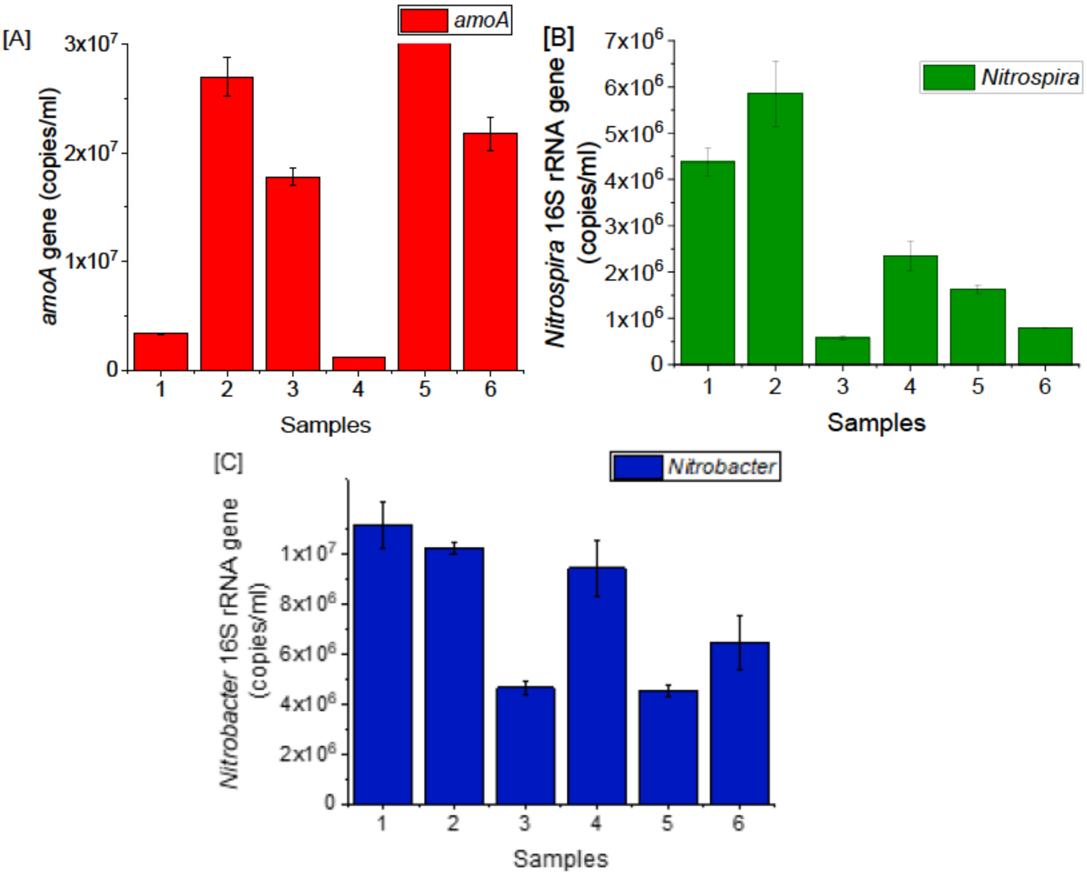

Figure 3.6: Changes in copy number variations of nitrifiers in the PN-SBR

3.8 Conclusion

This research demonstrates an innovative approach for the start-up of the partial nitrification process. In an SBR system, the strategies for producing nitrites, such as intermittent aeration, pH shocks and sulfide addition, were experimentally investigated. The following are the main conclusions:

- Successful PN with an average nitrite accumulation of 93% was observed by controlling DO levels in intermittent aeration in the reactor. The effluent NH_4^+-N and NO_2^--N concentration ratio was 1:1 at an average DO level of 4.3 mg/L in the aeration phase of intermittent aeration.
- Another strategy for the start-up of PN identified in this study was lowering pH until NOB is suppressed significantly, and then resuming the pH to normal conditions for AOB growth.
- Nitrite oxidation is a process more sensitive to sulfide than ammonium oxidation and the sulfide concentration of (25 mg/L) in the wastewater can be beneficial in inhibiting nitrite

oxidation and imposing partial nitrification. This research is important as it shows that even modest concentrations of sulfide in effluents can significantly change the nitrifying activity of activated sludge and cause nitrite accumulation.

- This work is significant as it makes it easier to startup PN and ensures the operation's stability.

CHAPTER FOUR

4.0 ACHIEVING PARTIAL NITRIFICATION BY HARNESSING BASIC HYDROLYSIS OF SULPHIDE SALTS AMID HIGH DISSOLVED OXYGEN CONDITIONS

4.1 Abstract

Attempts are going on to incorporate sulphur into the carbon and nitrogen removal processes at a biological wastewater treatment plant by external dosing or its intrinsic presence. A possible form of dosing sulphide at the field scale is the external dosing as the sodium salt of sulphide which under basic hydrolysis increases the pH. We demonstrated that the sodium salt dosage of sulphide can also be useful in achieving partial nitrification (PN) which can further augment the ANAMMOX stability. The results showed that a dose of 15 mgS/L from the sodium salt of sulphide was sufficient to establish PN in the synthetic and real wastewater and that too under the adequate concentration of dissolved oxygen (2-3 mg/L). PN was achieved only during the operational phases when the pH rise due to the basic hydrolysis of sodium sulphide was not controlled. The dosage of sulphide as its sodium salt has dual advantages as an inhibitor to nitrite oxidising bacteria (NOB). The activity of NOBs was 10-20 mgN/gVSS/d during the operational phases when PN was effective. The long-term operation showed a nitrite accumulation ratio of 70 ± 19% with an effluent ammonia concentration of 19 ± 4 mgN/L and nitrite concentrations of 18 ± 4 mgN/L. At the genus level, the NOB communities *Nitrospira* and *Nitrobacter* diminished in the long-term phases of stable PN with real wastewater. NOB communities evolved again when sulphide dosage was stopped indicating this strategy needs to be adopted continuously to achieve PN not time-based. The study demonstrated a strategy to achieve the feasibility of sulfide- driven Anammox systems and has a potential application in nitrogen removal from domestic wastewater. Figure 4.1 depicts the graphical abstract.

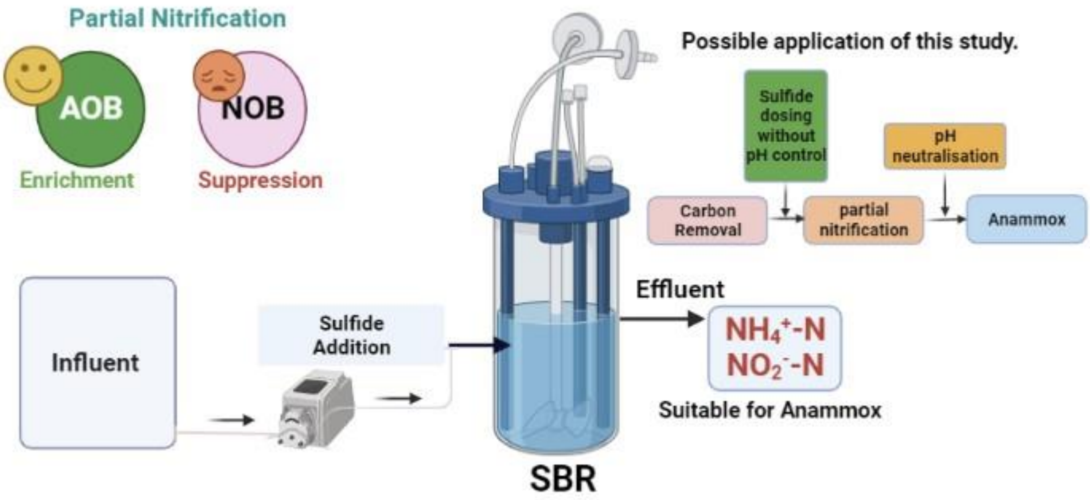

Figure 4.1: Graphical Abstract

4.2 Introduction

Currently, there is a growing interest in the study of nitrogen removal technologies due to the increasing incidences of eutrophication. Anammox (anaerobic ammonium oxidation) is a promising biotechnological process of nitrogen removal, which can directly convert ammonium and nitrite into nitrogen gas under anoxic conditions (Van de Graaf et al., 1996). The nitrite supply for Anammox in wastewater can be derived from the partial nitrification ($NH_4 \rightarrow NO_2$) process through combined partial nitrification and Anammox (PN/A) (Lackner et al., 2014). The PN/A process is regarded as one of the sustainable alternatives to the conventional nitrification-denitrification nitrogen removal process due to its possible economic benefits and high nitrogen removal capacity (Hassan et al., 2022). However, putting such a concept into action needs an overwhelming a number of obstacles, the most significant of which is the suppression of undesirable nitrite-oxidizing bacteria (NOB) in a partial nitritation (PN) reactor. NOB could be suppressed by controlling factors like high temperature (Kim et al., 2008), low dissolved oxygen (DO) (Wang et al., 2020), Free ammonia (FA), and free nitrous acid (FNA) concentration (Wei et al., 2018), high-salinity (Garcia-Ruiz et al., 2018), intermittent aeration mode and real-time control (Miao et al., 2017; Regmi et al., 2014). The current NOB suppression strategies in PN reactors are mostly based on kinetic selection, but they all have specific limitations (Kouba et al., 2017).

Owing to the high sensitivity of the bacterial species involved, the PN process is influenced by the operating environment (Kouba et al., 2017; Hassan et al., 2022).

In addition to the inhibition of NOB by operating conditions, inhibitors such as metals, organic compounds, and inorganic compounds can adversely impact biomass activity and process performance (McCarty, 1999). Hydrogen sulphide (H_2S), a toxic form of sulfur that denatures native proteins could also inhibit a wide range of microorganisms (Chen et al., 2008). Sulphide is often present in combination with ammonium in industrial wastewater (Beristain-Cardoso et al., 2011). Similarly, both industrial and municipal wastewater contain ammonium and sulfate, and sulfate can be reduced to sulphide by anaerobic pre-treatment or under anaerobic conditions in sewers (Vela et al., 2018). Sulphide inhibits ammonia-oxidizing bacteria (AOB) and NOB differently (Ortiz et al., 2013). The presence of sulphide has a negative impact on nitrification in general, reducing the efficiency of ammonium consumption, nitrate yield, or volumetric and specific rates of nitrification (Ortiz et al.,2013). The NOB is commonly regarded as highly susceptible to changes in environmental factors, which can cause nitrite buildup in bioreactors utilized for treating wastewater.

Vela et al., (2018) suggested that sulphide may be a useful selective inhibitor for NOB, depending on the composition and diversity of the NOB population. To the best of our knowledge, sulphide has been used in a few studies to establish PN, such as the investigation by (Erguder et al., 2008), in which a single sulphide dose of 45 or 80 mg-S/L accumulated 75 and 100 % of nitrite, respectively, while maintaining an ammonium oxidation efficiency of above 83%. Since the hydrolysis of sulphide in water is basic in nature releasing hydroxide ions, the increase in pH is obvious after dosing with a su sulphide salt such as Sodium Sulphide (Na_2S). Consequently, the pH peaks induced by the addition of sulphide to a PN reactor will have the influence of increased pH as well as sulphide inhibition. The rise in pH level due to the addition of sulphide to wastewater might increase the free ammonia (FA) concentration in the system, which in turn affects PN. Previous studies reported the inhibition and establishment of the PN with the addition of sulphide (Joye and Hollibaugh, 1995; Erguder et al., 2008; Ortiz et al., 2012; Kouba et al., 2017; Vela et al., 2018). Pulse dosing was reported by Erguder et al. (2008) in which the results showed that a 45 mg-S/L of pulse dosing of sulphide can achieve upto nitrite accumulation ratio of 0.75 under pH-controlled conditions.

Kouba et al. (2017) also investigated the effect of sulphide dosing on the PN process and concluded that the sulphide dosage should be avoided in the established PN systems as it can inhibit AOB as well as NOB. The study reported a concentration of 10 mg-S/L for the effective inhibition of NOB under mixing nitrifying conditions. Supporting this, Vela et al. (2018)

investigated how different microbial communities belonging to NOB respond to sulphide inhibition. The findings support that NOB communities rich in *Nitrotoga and Nitrobacter* are more resistant to sulphide inhibition than *Nitrospira*. Other studies reported by Ortiz et al. (2012) do not specifically investigate the effect of sulphide on the PN process but on the overall nitrification process. The study reported a sulphide concentration of 3.1 - 112 mg-S/L, the ammonia to nitrate conversion lowered from 0.9 gNO_3-N/gNH_4-N to 0.3 gNO_3-N/gNH_4-N leading to nitrite accumulation the specific rates of nitrate production decreased by 77-97%.

However, as discussed previously, being basic in nature, the dosage of sulphide in the form of salt will have an impact on the pH of the medium which will influence the concentration required for initiating PN. At the field scale, this property of increase in pH can be harnessed for the suppression of NOB and thereby supplementing the stability of PN. This can have economic benefits compared to the dosage of sulphide gas. Cui et al. (2019) concluded that the dosage of sulphide can be cheaper than methanol with respect to achieving autotrophic denitrification of the produced nitrate in the nitrification process. An approximate $0.19 – 0.72 of sulphide species is required to denitrify 1 kg of nitrate compared to methanol which costs around $1.05 for the same removal (Cui et al., 2019). However, there are not enough findings on how an increase in pH due to the dosage of sulphide can be more advantageous in achieving PN. Further, a long-term adaptation of the PN process with the help of sulphide dosage in real wastewater is not reported so far. Erguder et al. (2008) and Kouba et al. (2017) reported their experiments and findings on the effect of sulphide dosing on PN only with synthetic wastewater.

The objective of this study is therefore to investigate the approaches in which the basic hydrolysis of the sulphide salt can be harnessed to establish PN. Also, the effect of sulphide concentration on the establishment of PN was investigated to decide on the least concentration required for the PN establishment with a dosing of sulphide salts. The conclusions were further complemented by investigations with real wastewater and shifts in nitrifying microbial communities.

4.3 Material and Methods

4.3.1 Lab scale Reactor setup and operations

A laboratory-scale sequential batch reactor (SBR) with a working volume of 1.8 L and a headspace of 200 mL was used in this study (Fig S1). The reactor was operated in the SBR mode at an ambient temperature (20 to 25°C). The operational cycle in the SBR consisted of 1 hour of feeding, 1 hour of aeration, 30 minutes of settling, and 30 minutes of decanting. Two peristaltic pumps were used in the reactor operation, one to provide feed to the reactor from the feed tank, and the other to extract the effluent from the reactor. An aerator pump connected to the diffuser was placed inside the reactor and the aeration phase was controlled by timers. The volumetric exchange ratio was set at 40%. The reactor's hydraulic residence time (HRT) was 12 hours, and the flow rate was 5.2 liters per day. The pH and DO were monitored using portable Vernier LabQuest2 USA. The pH of the reactor was controlled using 1N NaOH and 1N HCL and a regularly calibrated pH probe. The DO concentration during the aeration phase was maintained between 2- 3 mg O_2/L by regulating the airflow rate. An airflow meter was used to measure the volume of air being delivered to the system and regulate the airflow rate by adjusting a control valve.

The reactor was inoculated with activated sludge obtained from the aeration unit of a domestic wastewater treatment plant in Durban South Africa. The initial mixed liquor suspended solids (MLSS) concentration and mixed liquor volatile suspended solids (MLVSS) concentration of the seed sludge in the PN reactor were 5272 mg/L and 4010 mg/L, respectively. The SBR was fed with synthetic wastewater during Phase 1, Phase 2, and Phase 3 containing: $NaHCO_3$ (900 mg/L), NH_4^+-N (from NH_4Cl) (50-70 mg/L), NaCl (70 mg/L), KH_2PO_4 (12mg/L) $CaCl_2.2H_2O$ (5mg/L) $FeSO_4.7H_2O$ (2mg/L). The trace element solutions were prepared according to Van de Graaf et al. (1996) and added to the synthetic wastewater at 1mL/L. In experiments with su sulphide dosing, the solution was prepared from the sodium salt of sulphur, sodium sulphide (Na_2S), and the concentration of 25 mg S/L, 5 mg S/L, and 15 mg S/L was dosed respectively during phases 1, 2, and 3. To avoid aerobic oxidation of sulphide, distilled water was used to prepare the sulphide -S solution and was flushed with high-purity argon to remove the DO.

To simulate municipal wastewater effluent, the effluent from the wastewater treatment plant (WWTP) treating municipal wastewater was spiked with NH_4Cl (NH_4^+-N= 50mg/L) and Na_2S (S^{2-} =25mg/L) during Phase IV. The composition of the effluents from the WWTP used as the feed during phases 4a and 4b is mentioned in Table S2. The treated wastewater treatment plant effluents containing 50 mg/L NH_4^+-N, 45 mg/L of COD, and 25 mg/L of sulphide are also typical concentrations approximated to anaerobically treated effluents (Vela et al., 2018).

4.3.2 Experimental Procedure

The experiments of PN by s sulphide addition were conducted in four phases which took approximately 326 days (Table 1). Each phase was divided into two sub-phases that varied based on the sulphide dosage without pH control and with pH control (Table 1). Start-up (days 1-10) consisted of inoculation and acclimation stages to confirm the activity of nitrifying (AOB and NOB) bacteria. In phase 1a (days 11-38), sulphide concentrations of 25mg-S/L were added to the reactor keeping all other operating conditions constant. After adding sulphide, the increase in pH value was not controlled until day 38. From days 38-58 of phase 1b, the pH value was controlled to 7.5 using 1M HCL (Table 1). The period between day 58 and day 66 served as a recovery period in which sulphide addition was stopped. The sulphide concentration in the reactor was lowered to 5mg-S/L during Phase 2a (days 67-94) without pH control. From days 94-114, which was phase 2b, the pH value was controlled to 7.5 using 1M HCL (Table 1). During Phase 3a (days 126-148), sulphide dosage was increased to 15 mg-S/L without pH control. In phase 3b (days 148 -176), the pH was controlled to 7.7 by 1M HCL. During Phase 4 (days 176-326), the effluent from the wastewater treatment plant (WWTP) was used in the experiments. The characteristics of the effluent of WWTP have been discussed in the previous section. Phase 4 was to verify the long-term performance of PN with sulphide addition with real municipal wastewater.

Table 4.1depicts the operational phases and the description that was adopted in this study to investigate the feasibility of using sulphide salts for the establishment of PN under uncontrolled DO conditions by harnessing the basic hydrolysis of sulphide salts.

Table 4.1: The operational phases and methodology adopted.

Phase	Operational focus	pH (Range) (Mean)	Influent NH_4^+-N (mg/L)	Influent S^{2-} mgS/L	N (days)
Startup		7.1	70	0	1-10
Phase 1	1a: Without pH control	8.5	70	25	11-38
	1b: Controlling pH	7.6	70	25	39-58
Phase 2	2a: Without pH control	8.3	70	5	67-93
	2b: Controlling pH	7.6	70	5	94-114

Phase 3	3a: Without pH control	8.5	70	15	126-148
	3b: Controlling pH	7.5	70	15	149-175
Phase 4	4a: WWTP effluents with S^{2-} addition	8.6	50	25	176-308
	4b: WWTP effluents without S^{2-} addition	7.6	50	0	309-326

4.3.3 Analytical Methods

The influent and effluent samples from the reactor were filtered through 0.45 µm syringe filters prior to the measurements of nitrogenous compounds. Concentrations of ammonium, nitrite, nitrate, and sulfate were analyzed using the Gallery TM Automated Photometric Analyser (Thermo Scientific; USA). The sulphide concentration in the influent and effluent was analysed by the methylene blue spectrophotometric method (DR 6000, Hach USA) according to the standard Method (APHA 2005). Analysis of sludge properties i.e., Mixed-Liquor suspended solids (MLSS) and mixed-liquor volatile suspended solids (MLVSS) was carried out according to the standard method (APHA 2005).

4.4 Calculations

The NH_4-N Removal Efficiency (%), Nitrogen Loading Rate (NLR; [kg N/ (m^3. d)] and Nitrite Accumulation Ratio (NAR; %) were calculated using equations (1) -(3), respectively (Hassan et al., 2022) as shown:

$$NH_4\text{-N}_{\text{ Removal Efficincy}}\ (\%) = \frac{(NH_4{-}Ninf)-(NH_4{-}Neff)}{NH_4{-}Ninf}\ X\ 100 \qquad (1)$$

$$\text{NLR [kg N/ (m}^3\text{.d)]} = \frac{TNinf \times 24}{\text{HRT} \times 1000} \qquad (2)$$

$$\text{NAR (\%)} = \frac{NO_2{-}N\ eff}{(\text{NO}_2{-}Neff + NO_3{-}Neff)}\ X\ 100 \qquad (3)$$

The values of the Free Nitrous Acid (HNO_2; mg N/L) concentrations were calculated as a function of pH and total nitrite as shown in equation (4) below:

$$\text{FNA (HNO}_2\text{ mg N/L)} = \frac{\text{total nitrite as N (mg/L)}}{10^{pH} e^{-2300/(273+\text{temp (°C)})}} \qquad (4)$$

$$\text{FA } (\text{NH}_3 \text{ mg N/L}) = \frac{\text{Total ammonia as N (mg N/L)} * 10^{pH}}{e^{6344/(273+\text{temp (°C)})} + 10^{pH}} \quad (5)$$

where NH_4-N_{inf} is the input ammonium concentration (mg-N/L); and NH_4-N_{eff}, NO_2-N_{eff}, and NO_3-N_{eff} are ammonium, nitrite, and nitrate concentration, respectively in the effluent (mg-N/L). TN_{inf} is the total nitrogen concentration in the influent (mg-N/L). HRT is the hydraulic retention time in days. Total ammonia represents the (NH_3-N +NH_4-N) concentrations in the effluent.

4.4.1 NOB inhibition parameters

The activities of NOB were assessed by measuring the nitrate generation rates specific to the biomass. The inhibition of NOB was calculated using the non-competitive inhibition model (Vela et al., 2015). The rate of growth under inhibited conditions (μ_{inh}) under the non-competitive inhibition model is :

$$Relative\ NOB\ activity\ (\%) = \frac{\mu_{inh}}{\mu_{\text{control}}} = \frac{100}{1 + [i]/Ki}$$

where: $\mu_{control}$ is the uninhibited specific growth rate which was assumed as the growth rate under no sulphide addition; [i] is the concentration of inhibitor (sulphide in this case): Ki is the inhibition constant.

Ki was calculated using non-linear regression fit in Origin Version 18 ($p < 0.05$) by calculating the average (μ_{inh}) in various operational phases at different dosages of sulphide.

4.5 DNA extraction and Polymerase Chain Reaction (PCR)

Approximately 10 mL of sludge samples were collected from the bioreactor at the end of each phase during the reactor operation. DNA was extracted using the DNeasy Powersoil extraction kit (Qiagen, USA) as per the manufacturers' instructions. The quantity and quality of the DNA were determined using the IMPLEN Nanophotometer NP 80 (Implen GmbH, Munich, Germany), as well as electrophoresis in 1% (w/v) agarose gel electrophoresis.

The PCR was carried out using the AOB and NOB 16S rRNA primers sets (Table S1, supplementary material). Each PCR reaction consisted of a total reaction volume of 25 µL containing 20 ng of DNA template (2 µL), PCR Master Mix (2×) (12.5 2 µL) (Thermo Scientific™), and 0.5 µM of each primer in a final volume of 25 µL. To confirm the positive amplification, the amplicons were run on 1.2 % (w/v) agarose gel (pre-stained with ethidium bromide) electrophoresis, and the products were visualized using a gel documentation unit. Moreover, sludge samples were used for microbial community analysis by high-throughput sequencing. The purified PCR amplicons were submitted to Inqaba Biotechnical Industries (Pty), South Africa, for further confirmation using sequencing and analysis.

4.5.1 Quantitative real-time PCR (qPCR) and community analysis

The quantitative PCR was carried out with the primer sets targeting different nitrifying bacteria (Table S1, supplementary material). A Bio-Rad C1000 Touch Thermal Cycler-CFX96 Real-Time System (BIO-RAD, USA) was employed for the qPCR reactions. The reaction mixture consisted of 4 µL of PowerUp SYBR Green PCR Master Mix (Applied Biosystems), 0.4 µL of each primer (final concentration of 0.4 µM), 2 µL of template DNA (final concentration of 1 ng), and molecular grade water to a final volume of 10 µL. For each experimental setup, appropriate negative controls containing no genomic DNA were subjected to the same amplification condition. The specificity of each qPCR assay was confirmed by using both melting curve analysis and agarose gel electrophoresis.

4.6 Results

The nitrite accumulation rate (NAR) in the reactor during various operational phases of the experiment is shown in Figure 1. For the initial 15 days, no sodium sulphide was added to the reactor in order to allow the biomass to adapt to the synthetic feed. The NAR fluctuated from 4% to 46% with an average of 22% in this phase. Nitrate was the main species in the effluent of the reactor during this period (maximum concentration of 50.5 mg N/L) (Figure 4.2). Sulphide dosing (25 mg S/L, 5 mg S/L, and 15 mg S/L) was initiated in different phases. Each dosing phase compromised of two sub-phases to investigate the effect of basic hydrolysis of sodium sulphide. One sub-phase was without pH control and the other was with pH control.

4.6.1 Phase 1a (25mg/L sulphide dosing without pH control): **-** The PN was efficiently achieved during phase 1a when sulphide was dosed at 25 mg S/L as sodium sulphide and the pH rise with the basic hydrolysis of sodium sulphide was not controlled. The average NAR was 86 ± 11% with a maximum NAR of 99.4% on the third day of operation (Figure 4.1). The average nitrite-to-ammonia concentration ratio in the effluent was 0.98 ± 0.4 and the maximum value was 1.5. The nitrate in the effluent was minimal at 4 ± 2 mgN/L (Figure 4.2) suggesting stable partial nitrification in the reactor. The pH due to the addition of sulphide during this phase was 6.9 – 9.2. Free ammonia concentration in the reactor was 9.9 ± 1.2 mg N/L with a maximum concentration of 16.7 mg N/L on the 30th day of operation (Figure 4.3). The AOB activity was not influenced during this phase while NOB activity was less than 20 mgN/gVSS/d (Figure 4.4).

4.6.2 Phase 1b (25mg/L sulphide dosing with pH control):- In the second part of this experiment (phase 1b), the concentration of the sulphide was the same as in phase 1a (25 mg S/L). However, the pH increase due to the basic hydrolysis of sodium sulphide was controlled to an average of 7.6 ± 0.2. The NAR lowered to 27% within five days, and thereafter the NAR started to increase until the end of the operational phase reaching 89.4% on the 19th day of reactor operation. The average NAR was 71.5 ± 3.8% in the last 15 days of this phase (Figure 4.1). However, it was noted that the increase in NAR was not due to the lower production of nitrate, but due to the lower production of nitrite. Compared to phase 1a in which the average nitrite was 26.5mg N/L, nitrite was reduced to 6.7 mg N/L in this phase. The average concentration of nitrite in the effluents during this phase was 6 ± 4 mgN/L (Figure 4.2). This lower production of nitrite indicated the possible effect of sulphide dosing on AOB which was also apparent in the oxidation of ammonia. In phase 1a, the average ammonia decrease was 70 mgN/L to 30 mgN/L while during this phase, it was 70 mgN/L to 43 mgN/L. The ratio of nitrite to ammonia concentration in the effluent also lowered to 0.08 on the fifth day of controlling pH conditions with an average ratio was 0.17. Free ammonia concentration in the reactor got decreased to 2.2 ± 0.7 mgN/L and further decreased to 1.38 ± 0.5 mgN/L on the 56th day of operation (Figure 4.3). The AOB and NOB activity decreased to less than 10 mgN/gVSS/d in this phase (Figure 4.4).

4.6.3 Phase 2a (5mg/L sulphide dosing without pH control):- To determine the effect of a lower sulphide concentration on PN, another experimental phase (phase 2a and 2b) was carried out which was similar to previous experiments (phase 1a and phase 1b respectively), except the sulphide dosage was lowered to 5 mg-S/L. There was no significant nitrite accumulation in this phase. The average NAR during this phase was 6.62% and the nitrite-to-ammonia ratio in the effluents of the reactor was 0.36 (Figure 4.1). Nitrate in the effluent of the reactor during this phase was significantly higher 34.3 ± 7.3 mgN/L (Figure 4.2) than in the preceding operational phases. In addition, the nitrite in the effluent was not detected on most of the sampling days (12 out of 28 days), suggesting the poor establishment of PN at a sulphide dosage of 5 mg S/L without pH control. The ammonia reduction was fairly good than the preceding operational phases (from 70 mg N/L to 16 mgN/L).

4.6.4 Phase 2b (5mg/L sulphide dosing with pH control):- This operational phase was started to gauge whether the sole inhibition effect of sulphide dosing of 5 mg S/L has any impact on nitrite accumulation and establishment of PN. PN was poor in this phase when the pH was controlled at 7.6 with a 5 mgS/L dosage of sulphide (phase 2b). The average NAR was 17.8% with a maximum of 46% (Figure 4.1). The ratio of nitrite to ammonia concentration in the effluent during this phase was 0.22 (Figure 4.1) and the concentration of nitrate in the effluent was higher (33.7 mgN/L) along with nitrite (7.4 mgN/L) and ammonia (30 mgN/L) (Figure 4.2). Non-establishment of the PN process in this phase also demonstrated that the inhibition of NOB achieved in phase 1a is reversible which may demand continuous dosage of sulphide as an inhibitor for achieving PN.

4.6.5 Phase 3a (15mg/L sulphide dosing without pH control):- Phase 3a and 3b experiments were designed to investigate the effect of an intermediate concentration of sulphide (15 mgS/L) and investigate recovery of the biomass. During this phase, when pH got increased due to the dosage of sulphide (pH ranged from 7.9-8.9), PN was achieved efficiently with an average NAR of 80.4 ± 14% and an average nitrite to ammonia ratio was 1.1 ± 0.3 (Figure 4.1). The effluent ammonia and nitrite concentration were 19.4 ± 4 mg N/L and 20.8 ± 4 mg N/L respectively. The nitrate concentration was reduced to 5.4 ± 1.5 mg N/L (Figure 4.2) which was lower than in the preceding phases. Free ammonia concentration in the reactor during this phase varied from 1.4 mgN/L to 10.3 mgN/L with an average of 5.5 mgN/L (Figure 4.3). The results also confirmed the re-inhibition of the NOB under the influence of sulphide dosage and its consequent pH increase. The activity of AOB and NOB and its variation with respect to NAR was almost similar to phase 1a (25 mgS/L without pH control) (Figure 4.4C).

4.6.6 Phase 3b (15mg/L sulphide dosing with pH control):- Next sub-phase of this experiment, was to control pH (pH =7.5) which increased due to sulphide dosage. The purpose of this trial was to figure out which effect (sulphide inhibition or pH increase) is dominant during sulphide dosage. The efficiency of PN in the reactor decreased significantly with an average NAR of 38.4 ± 12% and an average nitrite-to-ammonia ratio of 0.27 ± 0.04 (Figure 4.1). The ammonia concentration in the effluent remained the same as it was during phase 3a, (20.9mgN/L). However, nitrite concentration decreased to 4.6 ± 1.9 mgN/L and nitrate concentration increased to 9.6 ± 7.2 mgN/L (Figure 4.2). This indicated that the AOB was not inhibited and decreased NAR was not due to ammonia inhibition. Instead, NOB has recovered from the inhibition leading to the oxidation of nitrite and lowering of NAR. Free ammonia concentration in the reactor also decreased to 0.7 ± 0.2mgN/L during this phase. Unlike phase 1b, when the sulphide concentration was 25 mgS/L, there was no decrease in the activity of the AOBs in this phase (Figure 4.4D) suggesting that a sulphide concentration of 15 mgS/L with pH control may not be sufficient for inducing PN and a lower impact on the activity of AOBs than 25 mg S/L.

4.6.7 Phase 4a (Long term adaptation with real WWTP effluents)

The inferences relevant to the establishment of PN with sulphide dosage without pH control in phase 1a and phase 3a were verified further by changing influent to treated effluents of a municipal wastewater treatment plant (phases 4.4a and 4.4b). External dosing of sulphide will increase pH so this phase with WWTP effluents was carried out without pH control. The effluents were spiked with typical concentrations for pre-gritted sewage, NH_4-N (50 mgN/L), and sulphide (25 mgS/L) (Vela et al., 2015). During phase 4a, when the pH was not controlled (7.9 – 9.5), stable PN was maintained for over 100 days with an average NAR of 70 ± 19% (Figure 1). The average nitrite to ammonia ratio during this phase was 1 ± 0.4 and the concentration of ammonia and nitrite in the effluent was 19 ± 4 mgN/L and 18 ± 4 mgN/L respectively (Figure 4.2). The nitrate in the effluent was 7.6 ± 4 mgN/L with a minimum value of 1.2 mgN/L (Figure 4.2). Free ammonia in the reactor during this phase was 7.6 ± 1.2 mgN/L (Figure 3). The AOB activity increased to the maximum value of 81 mg N/gVSS/d and the maximum NAR was 90-100% (Figure 4.4E). The duration of this phase was 91 days demonstrating the impact of the sulphide dosage without controlled pH for the establishment of PN. This indicated that the NOB inhibition with a sulphide dosage of 25 mgS/L and its consequent pH increase is unadaptable, and the effect can be useful in practice for achieving fast and steady PN.

4.6.8 Phase 4b (Inhibition stability)

During phase 4b, external sulphide dosing was stopped to investigate if the PN established by NOB inhibition with sulphide dosing in previous phase 4a is permanent or sulphide dependent. As a result of discontinuing the sulphide dosing during phase 4a, the established PN became disrupted. The nitrate concentration increased from 7.6 ± 4 mgN/L (phase 4a) to 17 ± 7 mgN/L (Figure 4.2) indicating a recovery/increase in the activity of NOB. This increase in nitrate production resulted in the decrease of NAR to an average of 34 ± 6% (Figure 4.1). The NAR decreased from 70% to 30% in the first five days of operation. However, the nitrite to ammonia ratio also lowered from 1 (phase 4a) to 0.7 with average ammonia and nitrite concentration in the effluent as 11.6 ± 4.7 mgN/L and 7.7 ± 3.2 mgN/L respectively. However, the activity of AOBs was not affected (Figure 4.4 F). The average ammonia oxidation rate was still 77 ± 12% in this phase.

Free ammonia in the reactor during phase 4b was also lowered to 0.4 ± 0.2 mgN/L (Figure 4.3) indicating that AOB was inhibited at this concentration.

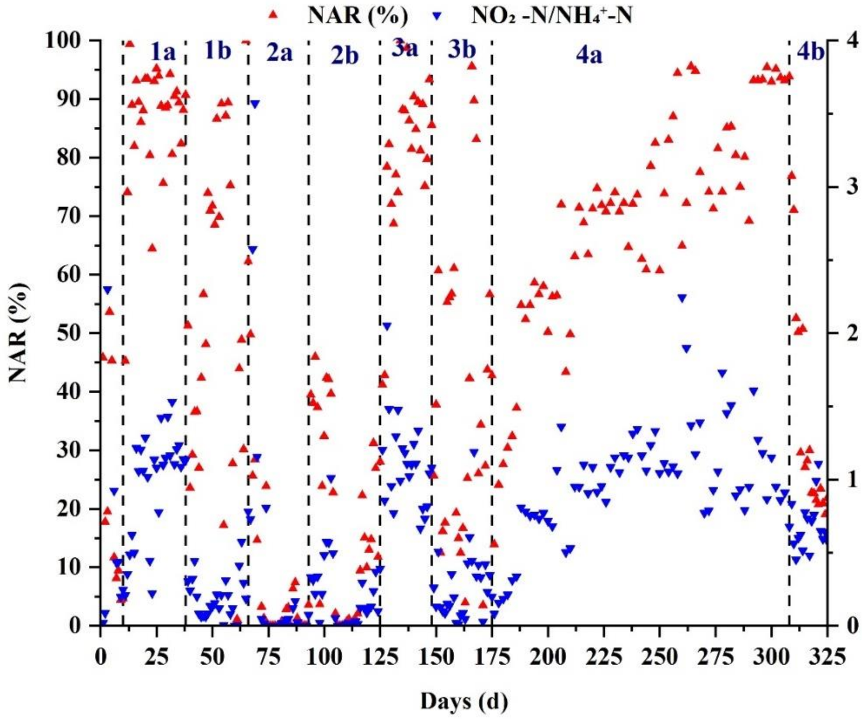

Figure 4.1: Nitrite accumulation rate (NAR) and the ratio of nitrite to ammonia concentration

The nitrite accumulation ratio and the ratio of nitrite to ammonia concentration in the effluent of the reactor using influent as synthetic wastewater and treated effluents from wastewater treatment plant spiked with hydrogen sulphide and ammonia.

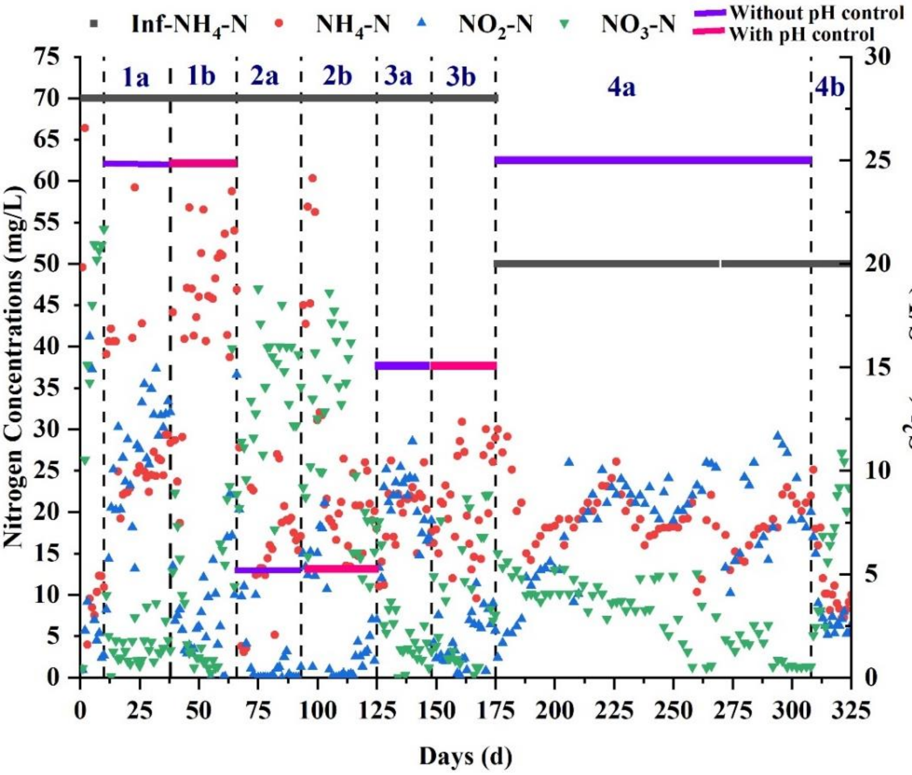

Figure 4.2: Variations in the concentration of ammonia, nitrite, and nitrate in the effluent of the PN reactor under designed experiments.

Influent as synthetic wastewater and treated effluents from wastewater treatment plants spiked with hydrogen sulphide and ammonia. Figure 4.3 depicts the pH and free ammonia concentrations in the reactor during different experimental phases. Influent as synthetic wastewater and treated effluents from wastewater treatment plant spiked with hydrogen sulphide and ammonia.

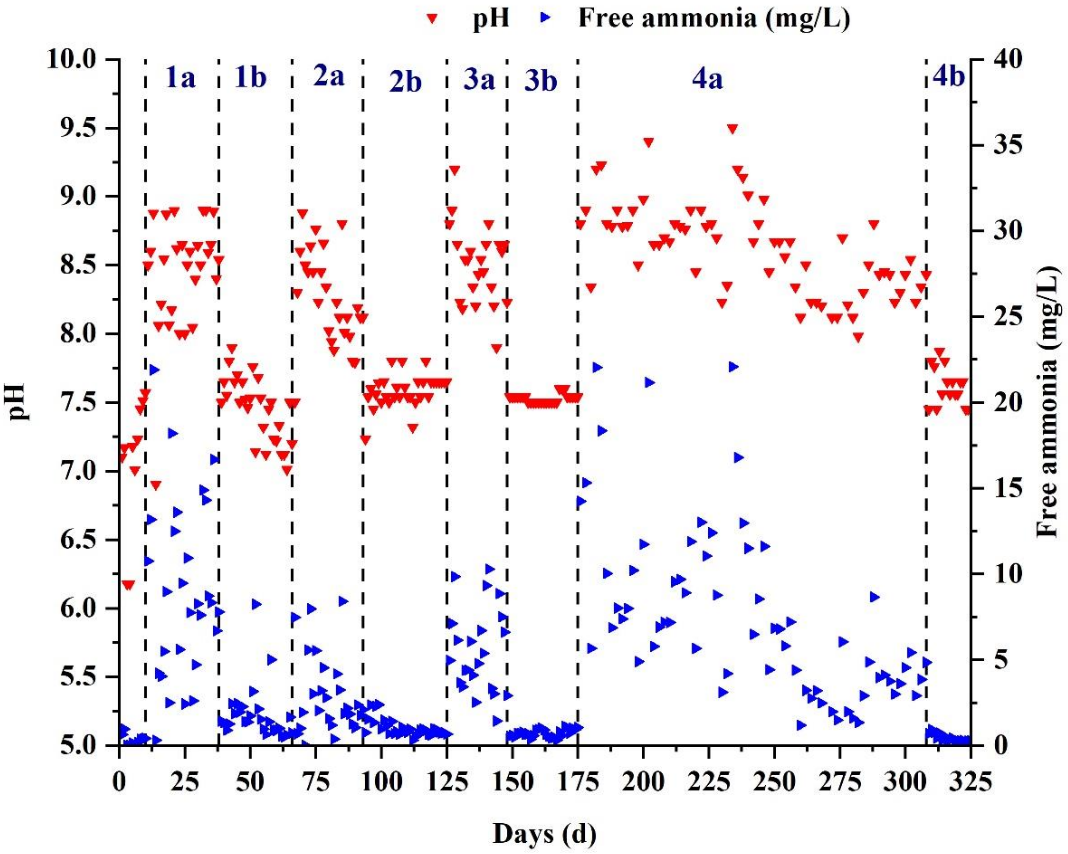

Figure 4.3: The pH and free ammonia concentrations

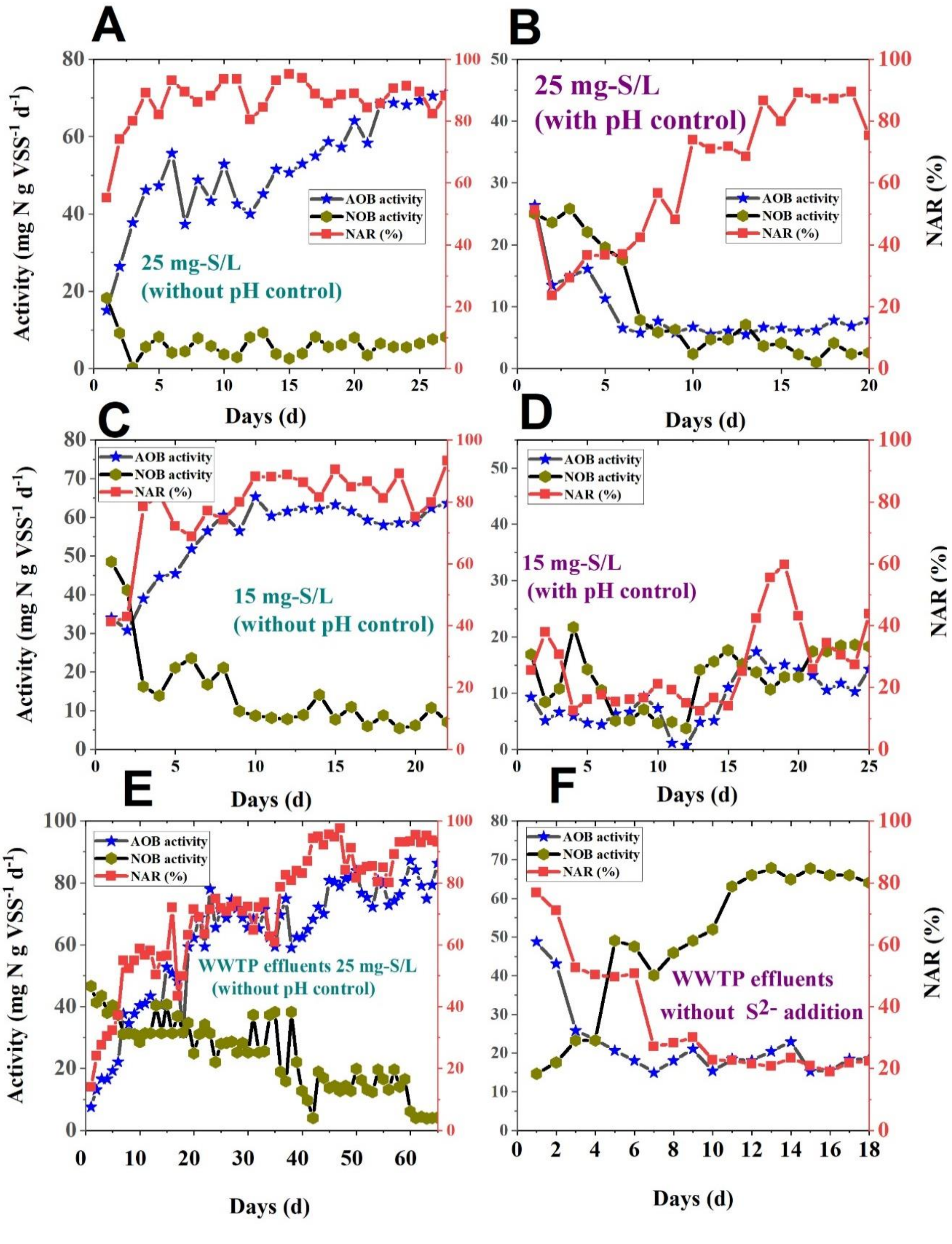

Figure 4.4: AOB and NOB activity and corresponding nitrite accumulation ratio during various operational phases.

A, C, and E show sulphide salt dosage without pH control, and plots B, and D show sulphide salt dosage with pH control. Plot F shows the recovery of NOBs when the long-term sulphide salt dosage was stopped.

4.7 Microbial community dynamics results

The microbial community shift was observed at the class level in which the focus was to investigate which microbial community adapted and proliferates under the sulphide salt dosage without controlling pH. In the initial phase during startup, *Gammaproteo- bacteria* were abundant (36.6%), which increased with sulphide salt exposure to the maximum level (80.2%) in phase 4a when the sulphide salt (25 mg S/L) was added without controlling pH at longer term in real WWTP effluents. This class of microbial community decreased in phase 4b (69%) when the dosage was stopped indicating the community proliferated under the sulphide salt dosage and associated elevated pH. On the contrary, other classes *Bacteroidia* and *Alphaproteobacteria* were present at the relative abundance of 32.8% and 1.7% during startup and showed relative diminishing with the dosage of sulphide resulting in their respective abundance of 8.3% and 3.1% (phase 4a).

This was related to nitrifying genera obtained by sequencing to identify which microbial community is adapting to the elevated pH and sulphide dosage. However, none of the nitrifying species followed the same trend at genera level.

At the genus level, both present NOB communities diminished (*Nitrospira and Nitrobacter)* in phases of stable PN. However, the decrease was not steady for both types of nitrifiers on moving from phase 1a to phase 4a which was expected as there were in-between phases when sulphide was dosed without pH control that increased the NOB communities. However, at the end of long-term operation in phase 4a, both NOB communities decreased significantly with *Nitrospir*a, almost absent in this phase (Figure 4.5). At the beginning of experiments, the NOB community *nitrospira* was present (2.21%), which decreased to 1% when sulphide dosage was 25 mgS/L without controlled pH (phase 1a). This NOB community diminished during the long-term operation of the reactor at a sulphide dosage of 25 mg/L (phase 4a) and evolved again when sulphide dosage was stopped during phase 4b. The sequencing data was further confirmed by qPCR results of the nitrifying species. A plot of NOB species relative to their initial concentration at startup indicated that among the NOB population, *Nitrospira* was dominant in phase 1a when effective PN was achieved at uncontrolled pH and sulphide dosage of 25 mg-

S/L. There was a decrease in of *Nitrospira and Nitrobacter* as well when the sulphide was dosed at uncontrolled pH for the longer term in phase 4a.

That will influence the decision of the practical application of this strategy that the dosage of sulphide salts should not impact microbial communities associated with other wastewater treatment processes. Figure 4.5 depicts the Relative abundance of AOB and NOB communities in operational phases, comparing the presence of Nitrospira and Nitrobacter with respect to initial cultivation.

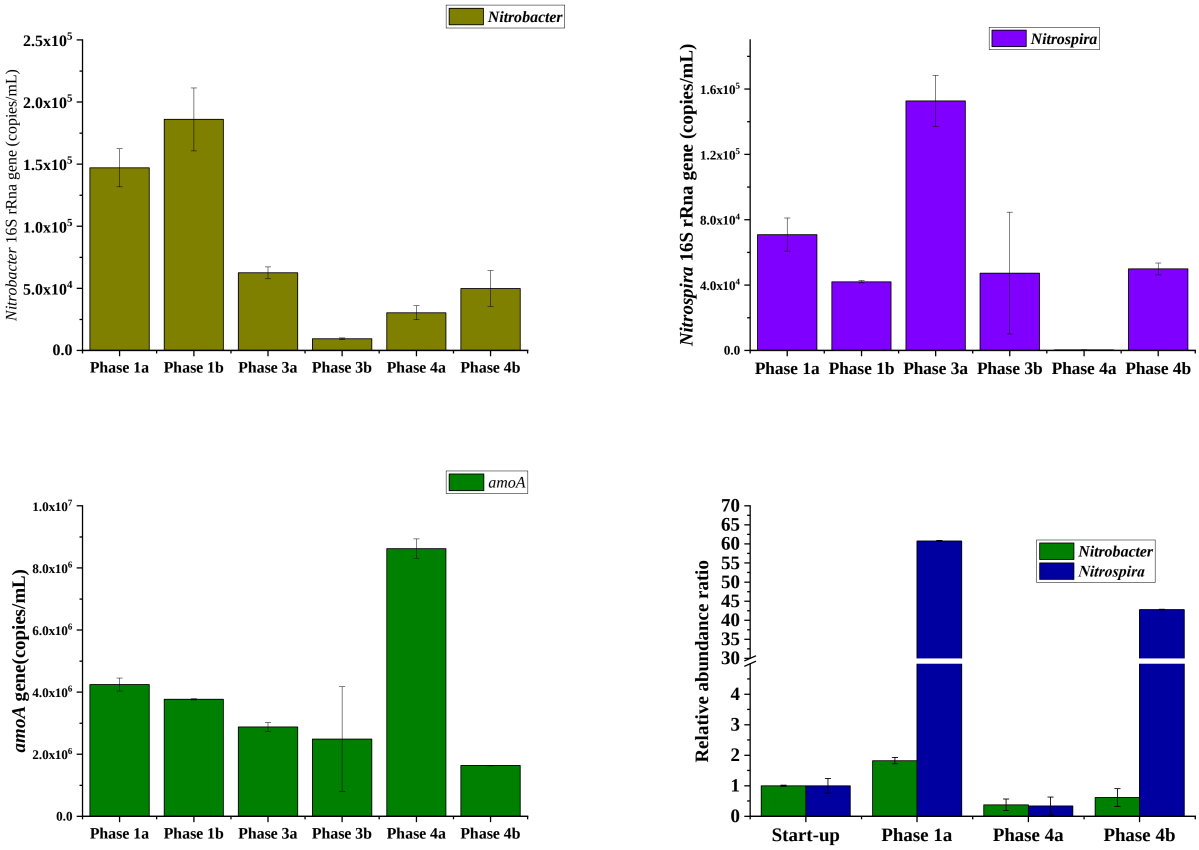

Figure 4.5: Relative abundance of AOB and NOB Communities in operational phases.

4.8 Results Discussion

4.8.1 Dual role of dosing salts of sulphide

The concentration of sulphide addition was changed from 25mg S/L to 5 mg S/L and then again to 15mg S/L to understand whether there is a recovery in the reactor in the activity of NOB after shifting the dosage from higher to lower and back to higher concentration. The results indicated that the inhibition of NOBs was an effect of the inhibitory environment caused by sulphide addition. The inhibiting environment may be the formation of free ammonia at increased pH. As the pH rises, so do the levels of free ammonia. After adding sulphide, the pH rose above 8.5, leading to elevated free ammonia concentrations. However, the pH was lowered to 7.5 with 1 M HCl during phases with controlled pH, decreasing free ammonia's potential to block AOB and NOB. The higher NAR achieved in phase 1a (at a concentration of 25 mg S/L) decreased significantly at the dosing concentration of 5 mg S/L in phase 2a. This may suggest continuous dosing of sulphide at higher concentrations to initiate and maintain PN in the reactor. The PN was achieved again at a concentration of 15 mg S/L in Phase 3a, depicting the minimum concentration of dosing for establishing PN with sulphide dosing as approximately 15 mg S/L. The observed concentration of sulphide for PN establishment is lower than reported by 40 mg S/L by Erguder et al. (2008) and 150 mg S/L by Seuntjens et al. (2018). However, both studies evaluated lower concentrations at short-term periods. Erguder et al. (2008) investigated the pulsed dosing of sulphide increasing the dosage slowly to 40 mg S/L while Seuntjens et al. (2018) investigated the effect of a particular dosing concentration for 2 days. Considering the short-term effects of sulphide dosing only, our results agree with these studies. In our study as well, PN was achieved only after 5-10 days of dosing not after two days of dosing. After 2 days, the NAR was 74% of 25 mg S/L dosing in phase 1a, and 42% in phase 3a with 15 mg S/L of dosing, and 24% in phase 4a with 25 mg S/L dosing in real WWTP effluents. After investigating the literature, our study seems to be the first that investigated the effect of sulphide dosing for a longer period in continuous mode.

The study ascertained the potential role of sulphide in the suppression of the NOB activity in the PN reactor. There can be two possible mechanisms that favor the effective establishment of PN when sulphide was dosed without pH control. The first one can be the development of an inhibiting environment for the NOB, and another can be sulphur-derived partial denitrification (SPD) of nitrate to nitrite (Deng et al., 2021). However, the dissolved oxygen in the reactor was 2-5 mg/L, thus minimizing the probability of autotrophic partial denitrification of nitrate in the reactor. Furthermore, a plot of sulphur oxidizing bacteria (SOBs) and nitrogen loss showed no relation between SOB growth and nitrogen loss (Figure S2), thus neglecting the possibility of SPD in the reactor. The inhibition kinetic parameters were estimated at uncontrolled pH and controlled pH conditions (Figure 4.6). The inhibition coefficient almost doubled under uncontrolled pH conditions although the sulphide dosage was the same. This shows the elevated advantage of harnessing the basic hydrolysis of sulphide salts. The inhibiting environment can be the production of free ammonia at elevated pH when the sulphide was dosed without controlling pH. Free ammonia has an inhibiting effect on NOBs which can lead to nitrite accumulation. Anthonisen et al. (1976) reported inhibition of nitrite oxidation begin at a FA concentration of 0.1 – 1 mg FA/L and ammonium oxidation is inhibited at a FA concentration of 10 – 150 mg FA/L. Selective inhibition of nitrite oxidation is reported at a FA concentration range of 1 – 10 mg FA/L.

There have been reports of the acclimatization of NOBs to FA concentration of around 20 mg/L (Chung et al., 2007). The theoretical average FA concentration in phase 1a, phase 3a, and phase 4a were 9.9 mg/L, 5.5 mg/L, and 7.6 mg/L respectively. These values were within the favorable nitrite inhibition concentration range of 5 – 10 mg/L proposed by Chung et al. (2007). Figure 4.6 depicts the NOB activity corresponds to different sulphide concentrations. The red line indicates the estimated inhibition equation for the shown inhibition coefficients (K_i).

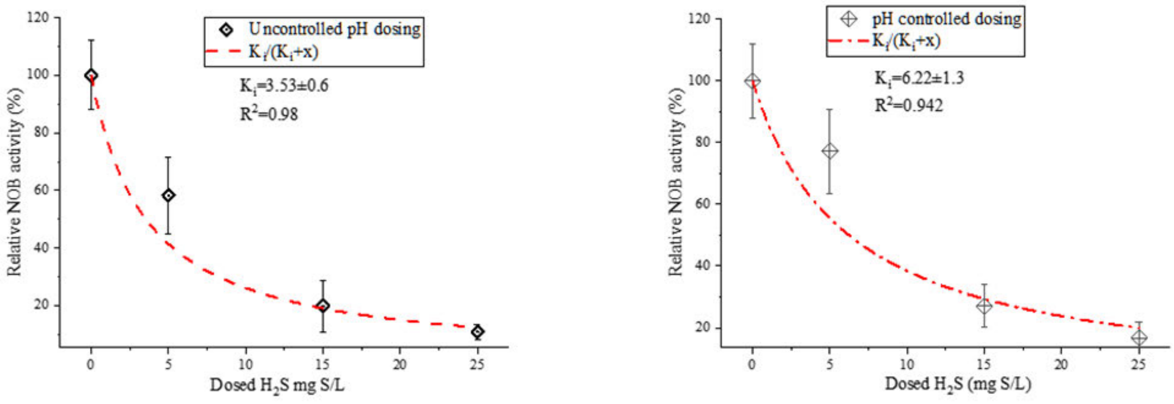

Figure 4.6: NOB activity corresponds to different sulphide concentrations and inhibition model fitted curve.

4.8.2 Microbial adaptation

As reported earlier the inhibition of sulphide is more sensitive to *Nitrospira* due to *Nxr* being periplasmic, this may be the reason for its diminishing in these phases (Vela et al., 2018). However, *Nitrobacter* which is reported to be more resistant to sulphide inhibition does not show dominance when the PN was established (Kouba et al., 2017). This may be the influence of elevated pH increasing FA production and thereby inhibiting *Nitrobacter*. A plot of the *Nitrobacter* population and FA concentration showed the inverse trend with FA increase and *Nitrobacter* population. Sulphide addition and pH control increase the undissociated form of hydrogen sulphide which is more toxic to microbes (Jin et al., 2013). This was in agreement with Vela et al. (2018) who showed that resistance to sulphide inhibition depends on the type of NOB species. Blackburne et al. (2007) also reported that *Nitrospira* is more sensitive to free ammonia. Similar findings were reported by Vela et al. (2018), however, the difference between the dissociated and undissociated forms of hydrogen sulphide is an addition to this study.

Since the ionized form proved inhibitory in our study in phase 1a, phase 3a, and phase 4a, this may be explained by the probability of binding action of the charged HS- ion with the nitrite oxidoreductase enzyme (*Nxr*) which increases with the concentration of sulphide in the bioreactor. These three phases were operated without pH control after sulphide dosing, and an increase in pH increased the ionized form of hydrogen sulphide (HS- ion) more than a unionized form of hydrogen sulphide. Also, between *Nitrobacter* and *Nitrospira*, the inhibition is more sensitive later due to *Nxr* being cytoplasmic and periplasmic respectively. In the case of *Nitrospira*, the HS-ion does not have to pass through the cell membrane to bind with Nxr as compared to *Nitrobacter*.

Nitrotoga, Nitrococcus, Nitrospina, and Nitrolancetus were not present in the reactor from the initial Phases, therefore further investigations may be required to investigate their response to the various sulphide concentrations and associated environmental conditions. Further, DNA is an indication of their presence but not their activity, so RNA-specific investigations are required than DNA specific, to understand the immediate effect of sulphide on enzyme activities. Figure 4.7 illustrates the schematics application of sulphide dosing for the establishment of partial nitrification in aerobic and anaerobic wastewater treatment processes.

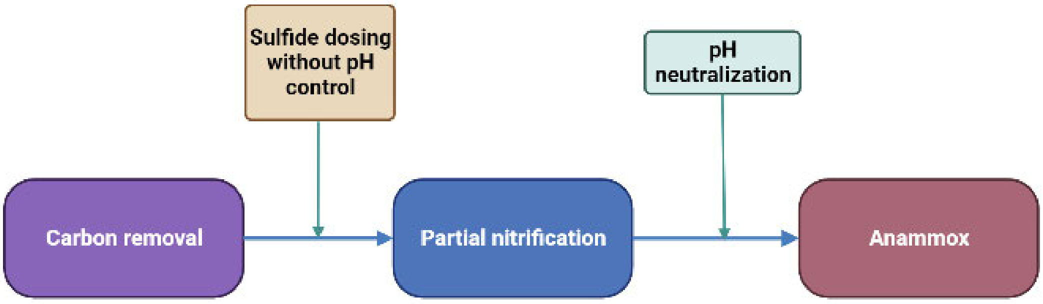

Figure 4.7: Schematics showing the possible application of sulphide dosing.

4.9 Practical implications

The sulphide additions in real practice can be of two types: one is external dosing, and another is dosing from existing streams such as anaerobic effluents. The former can increase, and investigations were required for long-term adaptation of this strategy with minimum sulphide concentration that can enable to initiation of PN on domestic wastewater streams. The supplementary methods contain information on the sourcing of sulfide and the theoretical calculations of the electron/energy consumption costs associated with this approach. Previous studies have limited the sulphide dosing strategies to short-time dosages. However, it was necessary to understand how the inhibition effect sustains after dosing is removed.

After carbon removal, external dosing of sulphide may help to achieve PN. However, the increase in pH due to sulphide addition can also inhibit ammonia oxidation which can be controlled by controlling sulphide concentration up to 25 mgS/L as concluded from this study. After achieving PN, the pH can be balanced for the downstream process Anammox. However, this concentration may need continuous dosing as depicted in this study. In phase 4a, even continuous dosing of sulphide for 90 days could not sustain the PN after the dosing was stopped. Within 5 days of the stoppage of sulphide dosing, nitrate concentration in the effluent increased from 7 mg N/L to 17 mg N/L. With this strategy, the dependence on establishing a microbial consortium of denitrifying sulfur-oxidizing bacteria for autotrophic partial denitrification that can denitrify nitrate to nitrate is not required. The inferences from this study can be further explored in investigating partial nitrification of wastewater streams that are alkaline such as the beverage industry, tannery industry, and paper and pulp industry.

4.10 Conclusions

The study ascertained the potential application of using sodium salt of sulphide for the establishment of partial nitrification under high dissolved oxygen. Basic hydrolysis of the sodium salts of sulphide has a dual effect leading to rising in pH that can establish PN at lower concentrations. The strategy can be effective for real wastewater after the removal of organic carbon as investigated in this study. The NOB inhibition coefficient decreased by 50% after harnessing the basic hydrolysis of sodium sulphide which means a lower concentration of sulphide is required for inhibition and thereby low chemical requirement. Economic analysis is required in the future to evaluate the tradeoffs between using this strategy and conventional PN systems. Future research should be focused on investigate interactions of Anammox and PN with sulphide dosage at uncontrolled pH. The current findings investigated in this study can be combined with sulfide-derived partial denitrification Anammox in a single reactor system that will increase the application prospectus of Anammox in domestic wastewater treatment.

CHAPTER FIVE

5.0 THE EFFECT OF SULFIDE STRESS ON THE ACTIVITY, PERFORMANCE, AND RECOVERY OF THE ANAEROBIC AMMONIUM-OXIDIZING (ANAMMOX) PROCESS

5.1 Abstarct

Anaerobic ammonium oxidation (Anammox) is a vital process in the nitrogen cycle; used in wastewater treatment to remove nitrogen from wastewater. However, Anammox bacteria are highly sensitive to sulfide, which can significantly inhibit the process. The addition of sulfide had a short-term negative effect on Anammox performance, but it was quickly recovered under low sulfide stress. The study concluded that reducing or removing the sulfide in the influent would be beneficial to accelerate the recovery of the Anammox performance. The stoichiometric ratio can be used as an indicator of the performance of Anammox and the development of Anammox consortiums. Anammox was found to be the dominant pathway for nitrogen removal during most phases, but high sulfide concentrations 90 mg-S/L inhibited Anammox activity resulting in poor nitrogen removal ability. The study highlights the importance of considering the effects of environmental conditions on microbial communities and their contributions to nitrogen removal in wastewater treatment systems. Controlling sulfide concentration is crucial to optimize the performance of Anammox processes.

5.2 Introduction

Anaerobic ammonium oxidation (Anammox) was discovered as a novel microbial nitrogen cycle process (Mulder et al., 1995). Under anaerobic environments, the dominated consortia of Anammox bacteria converted ammonium to dinitrogen with nitrite as the electron acceptor. Compared to conventional nitrification and denitrification processes, the Anammox process saves aeration energy, eliminates the requirement for organic carbon, and reduces sludge (Jin et al., 2012). The Anammox process has been used as a side stream application to treat filtrate/centrate from conventional anaerobic digesters (AD) and pre-treated AD from the thermal hydrolysis process (THP-AD) Wang et al., 2022). However, the mainstream applications of Anammox to manage ammonium nitrogen in municipal wastewater treatment plants are increasing (Ma et al., 2020). Nonetheless, Anammox side stream applications are common, with several full-scale applications in Europe and a few pilot and full-scale installations in the United States (Yang et al., 2021; Lackner et al., 2014).

Previous research has found that the Anammox process is vulnerable to external conditions due to the long doubling times (15-30 d) of Anammox bacteria, which is assumed to be the main barrier preventing its practical development (Lotti et al., 2015). Substrate, salinity, organic matter, phosphate, heavy metals, and sulfide are some of the factors that inhibit the Anammox process, according to Jin et al. (2012). Amongst these inhibitors, sulfide, particularly unionized hydrogen sulfide (H2S), has been a major issue in wastewater treatment plants (WWTPs) for decades (Wisniewski et al., 2019).

Sulfate-reducing bacteria in sewers, pumping stations, and anaerobic digesters create odorous, corrosive, and poisonous H2S. Industrial effluent from seafood processing, leather tanning, and oil refining may produce significant sulfide concentrations during anaerobic digestion. In industrial wastewater, sulfide concentrations can reach 100 mg/L (Zhang et al., 2020). Furthermore, sulfide- and nitrogen-containing wastewater have been found in paper manufacturing, landfills, and pharmaceutical discharge (Jensen et al., 2011; Liu et al., 2015). Sulfide is typically present in domestic sewage at values of 0 to 4.3 mg/L and in anaerobic effluent at 0.03 to 25 mg/L (Rodrguez-Gomez et al., 2021; Liang et al., 2022). So, it is important to figure out how sulfide affects the Anammox process.

A number of studies have been published on the inhibition of the Anammox process by sulfide (Carvajal-Arroyo et al., 2013, Dapena-Mora et al., 2007, Jin et al., 2013, Van de Graaf et al., 1996, Yang et al., 2013). For the first time, van de Graaf et al. (1996) investigated this effect and discovered that a sulfide concentration of less than 5 mM could enhance specific Anammox activity (SAA). Studies conducted later disproved this, though scientists are still divided on how much sulfide is required to inhibit growth. Russ et al. (2014) discovered that hydrogen sulfide had a strong inhibitory effect on Anammox bacteria, with μM levels of hydrogen sulfide inhibiting Anammox activity. Carvajal-Arroyo et al. (2013) discovered similar results in an Anammox granular enrichment culture.

Jin et al. (2013a) found that a concentration of 32 mg/L of sulfide inhibited Anammox activity by 50% when treating 200 mg/L TN. In contrast to Carvajal-Arroyo et al.,2013; Jin et al. (2013b) concluded that sulfide was inhibitory rather than toxic to Anammox, implying that the inhibition was reversible. Additionally, the half inhibitory concentration of sulfide on Anammox biomass was calculated to be 4.67 mg/L by Wisniewski et al. (2019). Despite the extensive research conducted on the inhibition of the Anammox process by sulfide, the effect of sulfide on the Anammox process for low substrate remains unclear.

Furthermore, the development of sulfide-dependent autotrophic denitrification (SDAD) provides a new route for simultaneous nitrogen and sulfide removal, which could not only reduce the nitrate produced by Anammox but also supply nitrite for Anammox when the reduction was regulated at the nitrite stage. It has been demonstrated that it is possible to remove both nitrogen and sulfide from a reactor using a combined Anammox and SDAD method. Anammox bacteria were first discovered in a denitrifying reactor, which suggests that sulfide-driven partial denitrification could provide nitrite to Anammox (Mulder et al., 1995). According to Kalyuzhnyi et al., 2006, the sulfur-driven Anammox process has the potential to remove 90% of the nitrogen in wastewater. Liu et al. (2015) started the integrated Anammox and sulfide process with a TN removal efficiency of more than 97% and observed.

The optimal concentration for the interaction between Anammox and the SDAD process is also unclear. Furthermore, the long-term effects of sulfide on Anammox, nitrogen removal mechanisms, and interactions between sulfide oxidising bacteria (SOB) and Anammox bacteria are still unknown. As a result, more research is required to better understand the impact of sulfide on the Anammox process. The aims of this study were: To evaluate the long-term effects of sulfide on Anammox activity or to evaluate the long-term nitrogen removal of Anammox performance in an up-flow anaerobic sludge blanket bioreactor (UASB) under increasing sulfide concentrations. To determine the inhibitory concentration level of sulfides to the Anammox process, as well as to uncover and compare the microbiological features of sludge before and after full inhibition using 16S rRNA and gene sequence analysis and qPCR. The findings are significant and may provide the theoretical foundation for the practical application of the Anammox process in the treatment of sulfur-containing wastewater. This study's findings may also contribute to the optimization of the Anammox process in the presence of sulfide and provide insight into the design of sustainable wastewater treatment systems.

5.3 Materials and methods

5.3.1 Experimental setup and operational conditions

A laboratory-scale up-flow anaerobic sludge blanket bioreactor (UASB) fabricated from plexiglass with an effective volume of 2 L and an internal diameter of 50 mm was used in this experiment (Figure 5.1). The reactor had sensors installed to measure pH, temperature, DO, stirring speed, and antifoam. With the control panel on the fermenters and the Iris control software (used for controlling the fermenters using an external PC), the pH, stirring speed, and temperature could be set and kept stable, using acid and base pumps for the pH and a heating block with cooling for temperature control (Figure 5.1). The fermenters also had a gas sparger to provide gas infusion to the reactor and a rotameter. The reactors were sparged on a regular basis with a mixture of nitrogen (95%) and carbon dioxide (5%), in order to maintain anoxic conditions by keeping DO below the detection limit (0.1 mg O_2/L) and sufficient buffering capacity. The temperature was kept at 35°C, and the hydraulic retention time (HRT) was set at 72-24h throughout all of the experimental periods. Table 5.1 summarizes the reactor operation period, which was divided into seven phases: Phase I (0–21 days) is for steady operation, phase II (22–41 days) and III (42–55 days) are for sulfide addition, phase IV (56–66 days) is for performance recovery, and phases V (67–116 days), VI (117–167 days) and VII (167-181 days) are for the reintroduction of sulfide.

Table 5.1: The experimental conditions during various phases of operation

Phases	Operation period (d)	HRT (d)	Inf. S^{2-}-S (mg-S/L)	SLR (kgm^{-3}d^{-1})	NLR (kgm^{-3}d^{-1})	NRR (kgm^{-3}d^{-1})	NRE %	Rs ^{b}	Rp ^{c}
I	0-21	4	0	0.000	0.039 ± 0.011	0.036 ± 0.01	79.91 ± 10	1.69	0.31
II	22-41	3	8	0.003	0.04 ± 0.01	0.030 ± 0.001	77.97 ± 8	1.72	0.34
III	42-55	3	16	0.005	0.03 ± 0.01	0.027 ± 0.01	68.98 ± 3	2.07	0.51
IV	56-66	1	5	0.005	0.098 ± 0.03	0.07 ± 0.03	82.57 ± 11.8	1.53	0.19
V	67-116	1	25	0.025	0.098 ± 0.00	0.08± 0.02	81.34 ± 9.4	1.43	0.07
VI	117-167	1	30	0.030	0.098 ± 0.02	0.08 ± 0.02	83.86 ± 4.8	1.56	0.06
VII	167-181	1	50	0.050	0.098 ± 0.03	0.040 ± 0.02	39.49± 25	5.37	0.34

SLR=Sulfide loading rate: NLR=Nitrogen Loading rate: NRR=Nitrogen removal efficiency:
b Rs =NO2 -N conversion/NH4 -N depletion:
c Rp=NO3 -N production/NH4 -N depletion.

5.4 Seeding sludge and Synthetic wastewater.

The seed sludge used in the experiments was obtained from a full-scale Anammox reactor operating in continuous mode at a wastewater treatment plant in Zwolle, Netherlands. The reactor contained granular Anammox species and achieved a nitrogen removal efficiency of 74%. The initial concentrations of mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) in the seed sludge were 1.75 g/L and 1.10 g/L, respectively, as determined by standard methods (APHA, 2005). Since Anammox bacteria are autotrophic microorganisms (Strous et al., 1999), an inorganic synthetic wastewater was used as the influent in the experimental setup. This synthetic wastewater contained ammonium, nitrite, bicarbonate, and trace elements, which served as substrates for the Anammox bacteria to consume and produce energy. The nitrogen content of the influent was maintained at a constant level of 98 mg/L, supplied by NH_4Cl and $NaNO_2$ in an equivalent molar ratio.

The composition of the synthetic wastewater was as follows: $NaHCO_3$ (900 mg/L), NH_4^+-N (from NH_4Cl) (42 mg/L), NO2-N (from NaNO2) (55 mg/L), MgSO4.7H20 (200 mg/L), KH_2PO_4 (12 mg/L), $CaCl_2.2H_2O$ (5.6 mg/L), $FeSO_4.7H_2O$ (2 mg/L), and EDTA (2 mg/L). Additionally, trace element solutions I and II (Van de Graff et al., 1996) were added at a concentration of 1 ml/L each to the synthetic wastewater. Dilutions were made using tap water. In experiments involving sulfide, a solution containing Na2S (5-50 mgS/L) was added to the synthetic medium as required. To prevent aerobic oxidation of sulfide, the sulfide-S solution was prepared using distilled water and purged with high-purity argon to remove dissolved oxygen (DO).

5.5 Analytical methods

Before measuring nitrogenous chemicals, influent and effluent samples from the reactors were filtered via 0.45 m syringe filters. The Gallery TM Automated Photometric Analyzer (Thermo Scientific; USA) was used to measure NH_4^+-N, nitrite (NO_2^--N), nitrate (NO_3^--N), and sulfate concentrations. The methylene blue spectrophotometric technique (DR 6000, Hach USA) was used to assess the sulfide contents in the UASB influent and effluent (APHA, 2005). Standard techniques were used to analyze sludge qualities such as MLSS and MLVSS (APHA, 2005).

Sulfide toxicity tests on Anammox activities

To evaluate the immediate impact of sulfide stress on Anammox, a batch test was conducted using 120 ml serum bottles with a working volume of 100 ml. The objective was to measure the extent of sulfide inhibition by determining the maximum bacterial activity at different sulfide concentrations. For the experiment, 60 ml of biomass from the existing UASB reactor was transferred into three sets of 100 ml serum vials. One vial was designated as the control group, where no sulfide was added. The remaining two vials were spiked with different sulfide concentrations: 5 mgS/L (low dosage) and 30 mgS/L (high dosage) of Na2S, respectively.All three batches were run in duplicate and supplemented with ammonium and nitrite at predefined amounts. The initial pH was adjusted to 7.5-8 by adding hydrochloric acid or sodium hydroxide (0.1 moles/L). Anaerobic conditions were achieved by purging the combined liquor in the serum bottles with nitrogen gas for ten minutes. In order to prevent sulfide striping, sulfide was added to the bottles once anaerobic conditions developed. Butyl rubber was used to provide an airtight seal on the bottle caps. To create an optimal environment, the serum bottles were placed on a shaker and incubated at a temperature of 35°C. This temperature was chosen to provide favorable conditions for the experiment. Throughout the batch experiments, 2 mL samples were collected from the bottles at specific time intervals ranging from 5 to 60 minutes. These samples were then filtered appropriately. The purpose of collecting these samples was to analyze the concentrations of various compounds, including sulfide, sulfate, nitrate, nitrite, and ammonium. The measurements were conducted in duplicate, ensuring a level of replication and accuracy in the results.

5.6 Microbial communities and functional gene analysis

5.6.1 DNA extraction

Approximately 10 mL of sludge samples were collected from the bioreactor at the end of each phase. The DNA extraction was carried out in accordance with the manufacturer's instructions for the Power Soil DNA kit (Qiagen, USA). A 1% agarose gel was used to determine the purity of the DNA, and a spectrophotometer was used to determine the yield (NanoDrop NP-80, Implen GmbH, Munich, Germany).

5.6.2 Polymerase Chain Reaction

The PCR was carried out using the AOB NOB and Anammox 16S rRNA primers sets and optimized protocols as described in Table 2. Each PCR reaction consisted of a total reaction volume of 25 µL containing 20 ng of DNA template (2 µL), PCR Master Mix (2×) (12.5 2 µL) (Thermo Scientific™), and 0.5 µM of each primer (Table 5.2) in a final volume of 25 µL (Avrahami *et al.*, 2011). To confirm the positive amplification, the amplicons were run on 1.2 % (w/v) agarose gel (pre-stained with ethidium bromide) electrophoresis, and the products were visualized using a gel documentation unit.

Table 5.2: Primers used for PCR amplification.

Name	Sequence 5’-3’	Specificity	Annealing Temp (°C)	Fragment size (bp)	Reference
PLA46 F Amx667 R	GGATTAGGCATGCAAGTC ACCAGAAGTTCCACTCTC	ANAMMOX	56	691	(Van Der Star et al. 2007)
amoA-1f amoA-2r	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	AOB	58	491	(Jin et al., 2011)
Nitro 1198f Nitro 1423r	ACCCCTAGCAAATCTCAAAAAACCG CTTCACCCCAGTCGCTGACC	*Nitrobacter spp.*	58	229	(Park et al., 2010)
NSR 1113f NSR 1264r	CCTGCTTTCAGTTGCTACCG GTTTGCAGCGCTTTGTACCG	*Nitrospira spp.*	68	151	(Dionisi et al., 2002)

5.6.3 Real-time Quantitative-PCR (qPCR) analysis

ANAMMOX, AOB and NOB (the *Nitrospira* spp. and the *Nitrobacter* spp.) were quantified using quantitative PCR (qPCR) with the Bio-Rad C1000 Touch Thermal Cycler-CFX96 Real-Time System (BIO-RAD, USA) to carry out the qPCR reactions according to specific primer sets in Table 5.2. The qPCR reactions were performed in a total volume of 10 µl containing 4 µl of Sso AdvancedTM Universal SYBR® Green Supermax (BIO-RAD, USA), 0.4 µl of forward primer, 0.4 µl of reverse primer, 2 µl of template DNA, and 3.2 µl sterile distilled water. The qPCR conditions were optimized for each set of primers accordingly. For each experimental setup, appropriate negative controls and reference standard DNA (purified specific PCR product of each primer pair) were subjected to the same amplification condition. The specificity of each qPCR assay was confirmed by using both melting curve analysis and agarose gel electrophoresis.

5.6.4 Calculations of the contributions of Anammox and denitrification to total nitrogen removal

The contributions of Anammox and denitrification to nitrogen removal can be computed using the Anammox reaction equation:

$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O0.5N0.15 + 2.03H_2O$ as follows:

$$\text{Anammox contribution (\%)} = \frac{(1+1.32-0.26)*(NH4-N\ influent-NH4-N\ effluent)}{TN\ influent-TN\ effluent} * 100\% \quad (1)$$

$$\text{Denitrification contribution (\%)} = 100 - \text{Anammox contribution (\%)} \quad (2)$$

5.7 Results and discussion

5.7.1 Performance of Anammox reactor under sulfide stress

The nitrogen removal performance was studied over a period of 181 days. Throughout the operation, the influent NO_2-N and NH_4^+-N concentrations in the UASB reactor were maintained at 55 and 45 mgN/L, respectively (Figure. 5.2a), and the nitrogen loading rate was changed in different phases (Table 5.1). The Anammox reactor was stable without sulfide addition throughout Phase I, according to the Anammox performance (Figure. 5.2a). The average NH_4^+-N, NO_2-N, and NO_3-N effluent concentrations during Phase I was 9 ± 4, 1±1, and 10 ± 7 mg/L, respectively (Figure 5.2a). On the 20th day, the nitrogen removal efficiency (NRE) and nitrogen removal rate (NRR) was 91% and 0.026 ± 0.009 kg-$m^{-3}d^{-1}$, respectively (Figure. 5.2b), indicating outstanding nitrogen removal performance. In addition, the ratios of R_S to R_P were close to the theoretical values of the Anammox reaction, indicating a stable Anammox mechanism.

In Phase II to Phase III, the Anammox reactor was operated under low sulfide concentrations 8 mg/L and 16 mg/L respectively, in order to investigate the short-term effect of sulfide on Anammox. During Phase II (days 22-41), the influent sulfide concentrations of 8mg/L were added to the reactor and the Anammox performance had no negative effects. The quantities of NH_4^+-N and NO_2-N and NO_3-N in the effluent increased abruptly at first, but then gradually decreased until the concentrations stabilized at 10 ± 4, 1±1, and 11± 4 mg/L at the end of Phase II (day 41) (Figure 5.2a). The higher biomass and structure of the granular sludge in the reactor provided conditions for the reactor's nitrogen removal performance to be maintained as the NRE and NRR were stabilized at 78 ± 8% and 0.030 ± 0.003 kg$m^{-3}d^{-1}$ (Figure 5.2b). The influent sulfide concentration was increased to 16 mg/L in Phase III (days 42-55), and the Anammox performance fluctuated noticeably. The NH_4^+-N concentrations increased to 15 ± 3 (Figure 5.2a), while the NRE and NRR decreased to 69 ± 3 % and 0.027 ± 0.009 kg-$m^{-3}d^{-1}$, respectively (Figure 5.2b).

During Phase IV (days 56-66), the Anammox reactor was run under low sulfide stress to recover Anammox performance (Figure. 5.3). The Anammox performance began to improve from day 57 after the NLR and SLR were simultaneously adjusted by regulating the HRT. On day 64, the NRR and NRE recovered to 0.09 kg-$m^{-3}d^{-1}$ and 94% respectively (Figure 5.2b), which was similar to the maximum value obtained in Phase I, indicating that Anammox performance had recovered. The average NH_4^+-N, NO_2-N, and NO_3-N concentrations during this phase were 8.32 ± 4.83, 3.16 ± 2.95, and 5.61 ± 5.06 mg/L, respectively (Figure 5.2a). This result indicates that lowering the sulfide level in the influent aided in the recovery of Anammox performance. When sulfide inhibits the Anammox process, inhibition-relief measures should be implemented immediately; reducing or removing the sulfide in the influent immediately would be beneficial to accelerate the recovery of the Anammox performance.

After the recovery phase, the long-term effect of sulfide on the Anammox process was explored in Phase V (days 67-116) and Phase VI (days 117-167). The influent sulfide-S concentration gradually increased from 5 to 25 mg/L, then to 30 mg/L, and finally to 50 mg/L, as shown in Table 5.1, corresponding to an SLR of 0.025, 0.030, and 0.050 kg-$m^{-3}d^{-1}$ respectively. At the beginning of Phase V, the Anammox performance was slightly disturbed for the first few days. The effluent NH_4^+-N concentration sharply increased to 14 mg/L on day 74 with an undetectable level NO_3-N, resulting in a reduction in the NRE to 70%. Then the effluent quality quickly recovered, and the reactor experienced stable nitrogen performance. As shown in Figure 5.2 (a and b) the average NRR and NRE at the end of Phase V were 0.08 ± 0.02 $kgm^{-3}d^{-1}$ and 81.35 ± 9.41% respectively. The influent sulfide-S concentration was gradually increased from 25 to 30mg/L in Phase VI as shown in Figure 5.1, corresponding to an SLR of 0.030 kg-$m^{-3}d^{-1}$. The average NRR and NRE were maintained at 0.08 ± 0.02 kg-$m^{-3}d^{-1}$ and 83.61 ± 4.6% respectively when the Anammox process was operated at the influent sulfide-S level of 30 mg/L for 50 days.

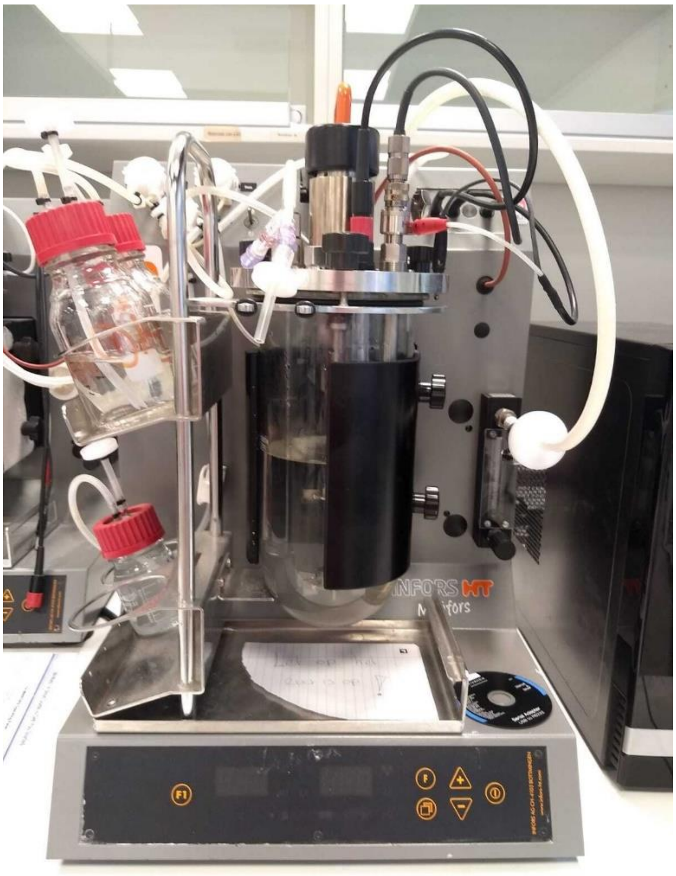

Figure 5.1: Informs Minifors benchtop fermenter front view.

This finding indicates that the sulfide-resistance ability increased after long-term acclimatization in Phase V and Phase VI. Furthermore, as shown in Figure 5.2, the influent sulfide concentration was finally increased to 50 mg/L, which corresponded to an SLR of 0.05 kg-m^{-3}d^{-1} (Figure 5.2b). An inhibiting phenomenon was seen as soon as sulfide-S concentration was raised to 50mg/L. The effluent NH_4-N increased from 5.34 to 30 mg/L on the seventh day of operation decreasing the NRR and NRE to 0.03 ± 0.02 and 31.53± 13.08 % respectively (Figure 5.2b). Meanwhile, the effluent quality deteriorated. The NH_4-N and NO_2-N concentrations increased to 34.25 ± 7.13 and 30.64 ± 9.99 mg/L respectively (Figure 5.2a**).** The granules brilliant red color faded after being exposed to sulfide over an extended period of time. According to reports (Klotz et al., 2008; Schmid et al., 2008), heme c plays a significant role in the enzymes produced by Anammox bacteria. Heme c concentration has also been linked to Anammox activity. As a result, two indicators of the level of Anammox activity can be used: the colour of the sludge and the concentration of heme c. After day 175, however, milky-white solids covered in tiny bubbles adhered to the reactor's inner wall, showing the formation and accumulation of elemental sulfur. Excess sulfide may have accelerated the conversion of S^{2-} to S^0, which could explain this event.

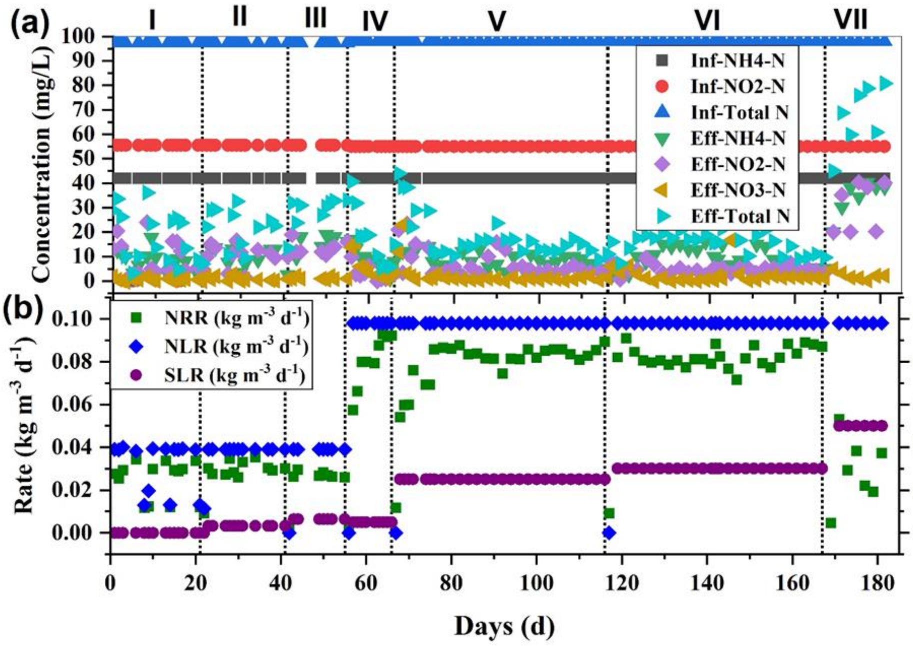

Figure 5.2a: The nitrogen removal performance of the Anammox reactor. (a) Profiles of influent and effluent nitrogen concentrations. (b) the loading rate and removal rate (NLR, NRR and SLR).

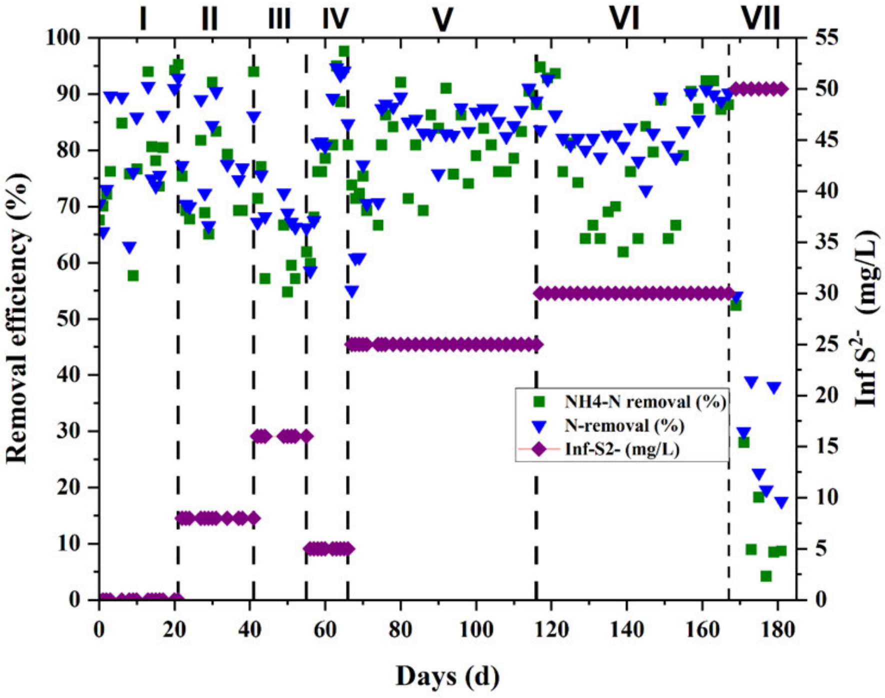

Figure 5.3: Nitrogen removal efficiency along different phases.

5.7.2 Stoichiometric ratio

The stoichiometric ratio is a valuable indicator for assessing the performance and development of Anammox consortia. The theoretical stoichiometric ratio of R_S (removed NO_2-N to removed NH_4^+-N) is 1.32, while the ratio of R_P (produced NO_3-N to removed NH_4^+-N) is 0.26 (Stross et al., 1998). However, it is important to note that actual measured values may deviate from these theoretical values due to variations in reactor design, shape, volume, operating conditions, and culture conditions during the process (Rothrock et al., 2011). In the present study, the Anammox reactor exhibited an increasing trend in R_S and R_P values during the start-up phase, indicating the growing dominance of the Anammox reaction. The stoichiometric ratios RS and RP during start-up were measured as 1.69 ± 0.23 and 0.31 ± 0.24 (Figure 5.4), respectively, which were in close proximity to the theoretical values of 1.32 and 0.26. The specific values of R_S and R_P can be found in Table 5.1.

The stoichiometric ratios of the Anammox reaction, R_S and R_P, were observed to deviate from their theoretical values under the influence of different sulfide concentrations. This observation aligns with the findings of Jin et al. (2013), who demonstrated that the presence of sulfide can modify the stoichiometry of the Anammox reaction. Furthermore, Jin et al. (2013) also showed that distinct sulfide concentrations exerted varying effects on the values of R_S and R_P. The value of R_S increased to around 1.72 and 2.07 (phase VI), while the value of R_P was low at 0.51 (phase III) (Figure 5.3). Under the long-term effects of sulfide on the Anammox process, the Rs value was 1.56 while the Rp value was as low as 0.06 (Figure 5.4). This result could have been caused by bacteria that used sulfide as an electron donor to convert some of the NO_3-N produced by the Anammox process to NO_2-N. Some bacteria, on the other hand, turned nitrogen into NH_4^+-N, which lowered NH_4^+-N while increasing R_S and R_P. It is worth noting that R_S and R_P varied greatly throughout Phase VII, which might explain the considerable shift in bacterial community structure in the Anammox system at the time due to the sulfide impact.

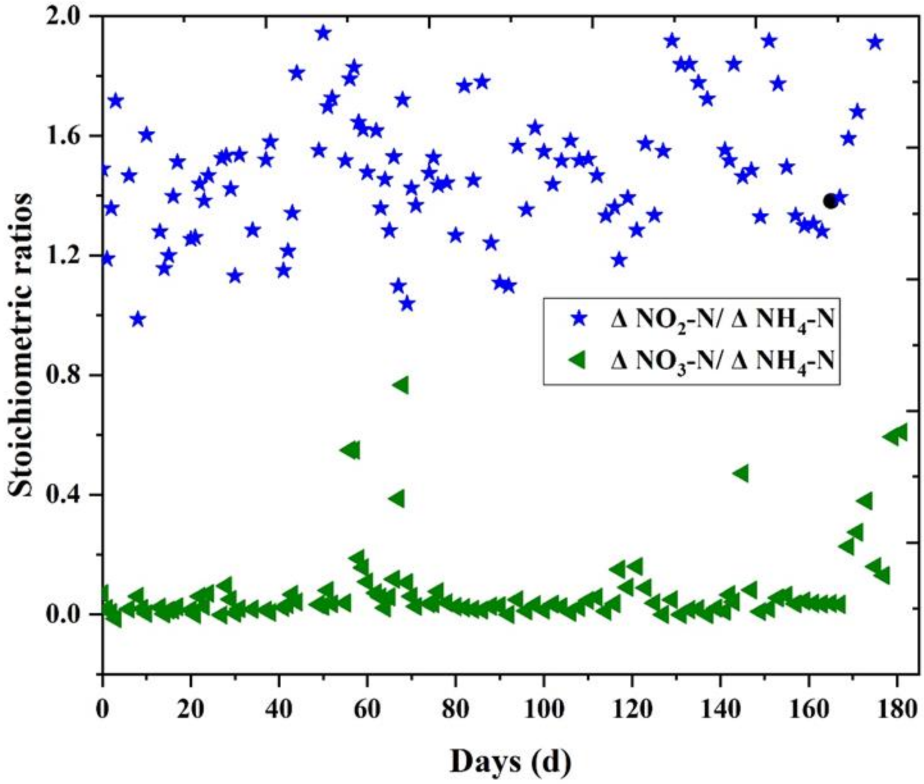

Figure 5.4: Change in the stoichiometric ratios throughout time

5.7.3 Contribution of Anammox and denitrification to total nitrogen removal

In this study, the contributions of Anammox and denitrification to nitrogen removal were evaluated using Equations (1) and (2); respectively. The results of these calculations are presented in Figure 5.5, which shows the percentage contribution of Anammox and denitrification to nitrogen removal at each phase of the experiment. It was assumed that Anammox and denitrification were the two major nitrogen removal pathways, and that ammonium could only be removed through Anammox. At the end of Phase I, the contribution of Anammox to nitrogen removal was found to be 87.21% (Figure 5.5). This indicates that Anammox was the primary pathway for nitrogen removal during this phase.

However, when sulfide was added to the system from 8 mg/L (Phase II) to 16 mg/L (Phase III), the contribution of Anammox to nitrogen removal dropped significantly from 86.38% to 80.89% (Figure 5.5). This decrease in Anammox contribution may be attributed to the inhibitory effect of sulfide on the Anammox bacteria. Despite this decrease, Anammox remained the dominant pathway for nitrogen removal during Phases II and III, with contributions ranging from 86% to 80% (Figure 5.5). During Phases IV and V, the contribution of Anammox to nitrogen removal remained relatively stable, at 85.48% and 86.72%, respectively (Figure 5.5). However, in Phase

VI, this contribution decreased to 82.30%. This reduction may be attributed to changes in the microbial community or environmental conditions, which may have favored other nitrogen removal pathways over Anammox.

Interestingly, when the influent sulfide concentration was increased to 50 mg/L in Phase VII, the contribution of Anammox to nitrogen removal decreased significantly to 41.3%, while the contribution of denitrification increased to 58.6% (Figure 5.5). This shift in the dominant nitrogen removal pathway may be explained by the inhibitory effect of high sulfide concentrations on Anammox bacteria, as well as the potential stimulation of denitrifying bacteria. This phenomenon could also explain the increase in Rs observed during Phase VII, as the denitrification pathway produces more nitrous oxide than Anammox.

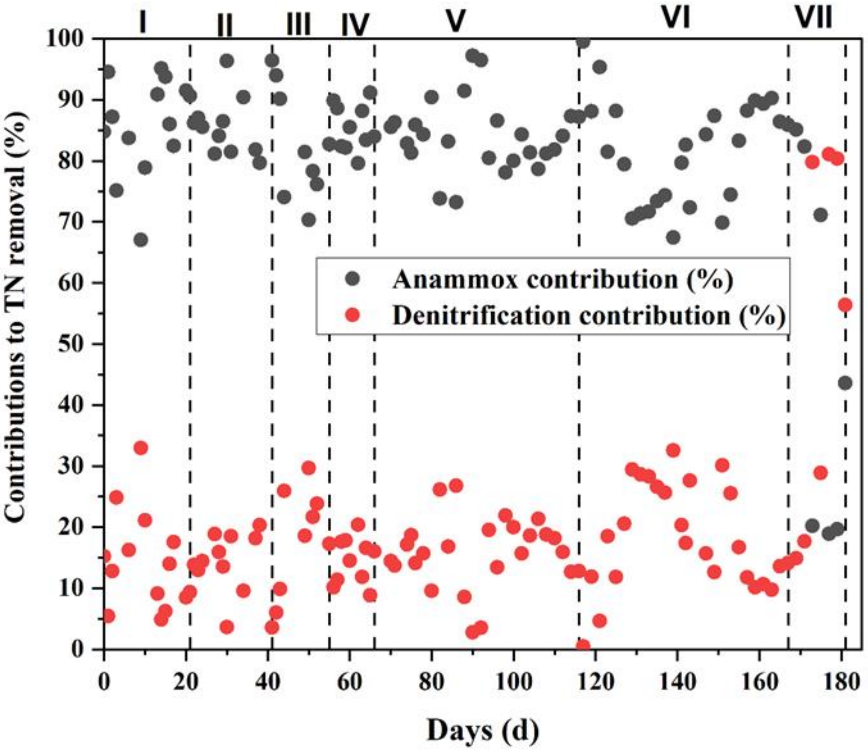

Figure 5.5: Contributions of Anammox and denitrification to total nitrogen removal

5.7.4 Quantification of key functional genes

During Phase I (0-21 days, 0.0 mg/L sulfide), the abundance of Anammox bacteria was highest (2.25 x10^6 copies/mL), followed by AOB (1.73 x10^4 copies/mL), *Nitrobacter* spp. (8.65 x10^2 copies/mL), and *Nitrospira* spp. (3.82 x10^1 copies/mL) (Figure 5.5). This suggests that Anammox bacteria and AOB were the primary contributors to nitrogen removal during this phase. The high abundance of Anammox bacteria and AOB can be attributed to their preference for low-sulfide environments, as sulfide can inhibit the activity of these microbes. As sulfide concentration increased in Phases II (22-41 days, 8 mg/L) and III (42-55 days, 16 mg/L), the abundance of Anammox bacteria decreased (2.46 x10^5 copies/mL and 1.67 x10^6 copies/mL respectively), while the abundance of *Nitrobacter* spp. increased (2.73 x10^2 copies/mL and 8.13 x10^2 copies/mL respectively) (Figure 5.6). This suggests that nitrite oxidation became more important in nitrogen removal during these phases. The higher affinity of *Nitrobacter* spp. for nitrite compared to Anammox bacteria, as well as the inhibitory effect of sulfide on Anammox bacteria, likely contributed to this shift in microbial community composition.

During Phase IV (56-66 days, 5 mg/L sulfide), where sulfide concentration was reduced, the abundance of Anammox bacteria increased (1.33 x10^6 copies/mL), indicating that they were once again able to play a significant role in nitrogen removal (Figure 5.6d). This finding suggests that Anammox bacteria can recover quickly when sulfide levels are reduced, as they have a higher tolerance to low-sulfide environments than *Nitrobacter* spp. These results are consistent with previous studies that have reported a rapid recovery of Anammox activity following a decrease in sulfide concentration (Chen et al., 2023). However, during Phase V (67-116 days, 25 mg/L sulfide), Anammox bacteria were unable to recover under high-sulfide conditions (1.54 x10^6 copies/mL), which can be attributed to the toxic effect of sulfide on these microbes (Figure 5.6d). The high sulfide concentration likely caused a decrease in pH and an increase in redox potential, leading to the inhibition of Anammox bacteria and the promotion of *Nitrobacter* spp. These findings are consistent with previous studies that have reported a negative impact of high sulfide concentrations on Anammox activity (Deng et al., 2022).

In Phases VI (117-167 days, 30 mg/L sulfide) and VII (167-181 days, 50 mg/L sulfide), the abundance of all four microbial groups decreased significantly (5.3 x10^5 copies/mL and 3.77 x10^2 copies/mL for Anammox bacteria, 1.07 x10^3 copies/mL and 8.95 x10^1 copies/mL for *Nitrobacter spp.*, 4.11 x10^1 copies/mL and 7.83 copies/mL for *Nitrospira* spp., respectively) (Figure 5.6). This finding suggests that high sulfide concentrations have a negative impact on the overall performance of the Anammox process, as they can inhibit the activity of all microbial groups responsible for nitrogen removal. Figure 5.6 illustrates the trends in microbial community composition during the different phases of the experiment.

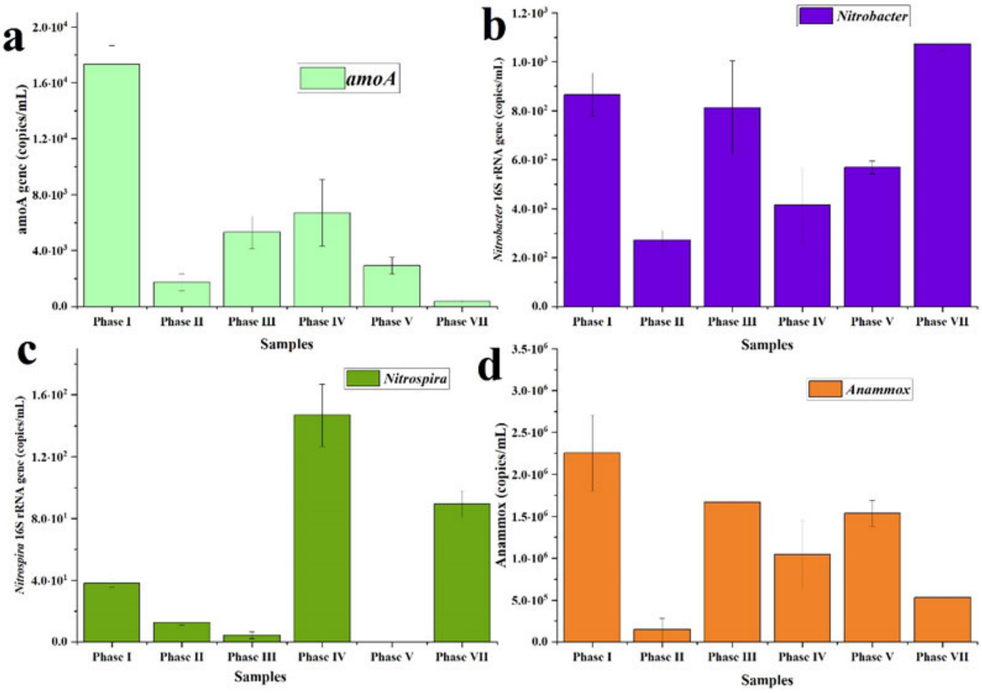

Figure 5.6: Changes in copy number variations of nitrifiers and Anammox bacteria in the UASB.

5.7.5 Batch Test Results

The effect of sulfide toxicity on Anammox activity was investigated over a 16-hour batch test. The specific Anammox activity was calculated based on the ammonium and nitrite concentrations measured at each time interval. At the beginning of the experiment (time=0), the ammonium concentration was 42 mg/L in all samples, indicating a homogenous inoculum. As the experiment progressed, the concentration of ammonium decreased in all samples, but the rate of decrease was slower in samples with higher sulfide concentrations (25,45 and 65mg-S/L) (Figure.5.7a)). The inhibition of Anammox activity by sulfide was evident, as evidenced by the lower specific Anammox activity observed in the samples with higher sulfide concentrations (65mg-S/L).

The concentration of nitrite increased in all samples over time, but the rate of increase was slower in samples with higher sulfide concentrations (Figure. 5.7b). The specific Anammox activity was also lower in these samples, indicating that sulfide inhibited the ability of Anammox bacteria to convert nitrite to nitrogen gas. Based on the results, sulfide concentrations above 25 mg-S/L were found to be significantly inhibitory to Anammox activity.

The specific Anammox activity in the samples with 45 mg-S/L and 65 mg-S/L were reduced by 61.4% and 69.3% respectively, compared to the sample with no sulfide.

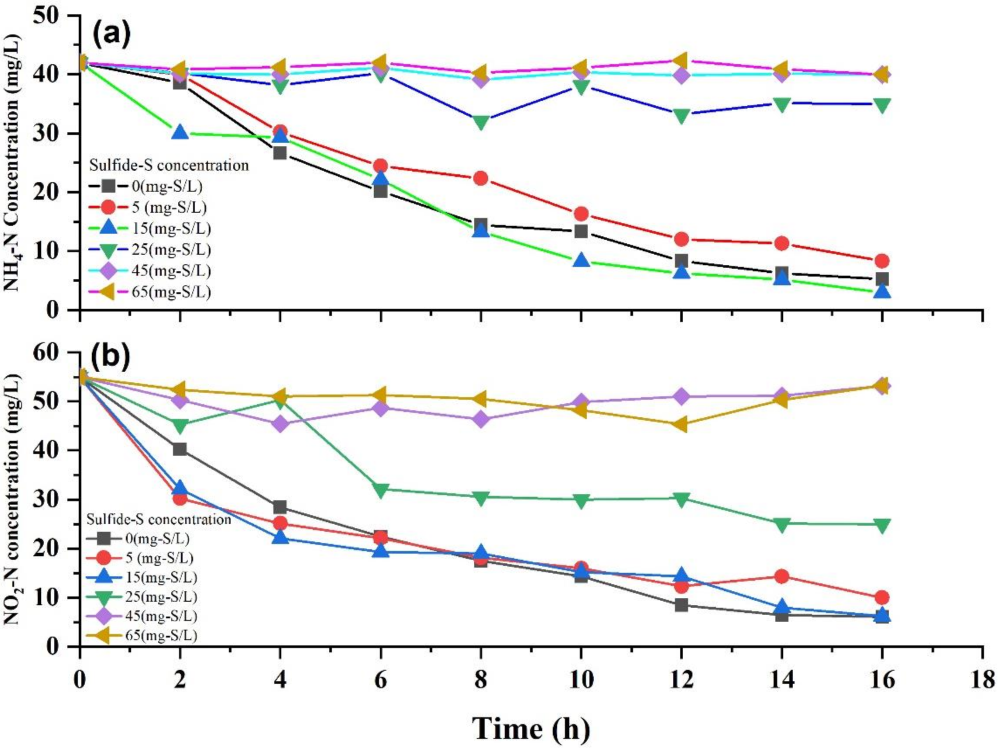

Figure 5.7: Evolution curves of NH4-N (a) and NO2-N (b)at different sulfide concentrations

5.7.6 Discussion

The results showed that the Anammox process could maintain its nitrogen removal efficiency under the long-term effect of sulfide addition. Specifically, sulfide addition up to 30mg-S/L did not negatively impact the Anammox process's performance, as the stoichiometric ratios Rs and Rp were maintained close to their theoretical values, and the microbial community responsible for nitrogen removal was stable, with a high abundance of Anammox bacteria and low levels of *Nitrobacter* spp. and *Nitrospira* spp.

When the influent sulfide concentration was gradually increased due to biomass acclimatization, the NRR could stabilize at a high level. Also, the amount of NO_3-N in the effluent slowly went down when it was exposed to 25 mg/L of sulfide. This result might be because some of the NO_3-

N generated from the Anammox reaction was converted into NO_2-N by denitrification bacteria using sulfide as an electron donor. In other words, there were denitrifiers that had adapted in response to long-term sulfide feeding. On the one hand, the high concentration of sulfide (25 and 30 mg/L) increased Anammox activity, resulting in good nitrogen removal capability; conversely, denitrification bacteria converted some NO_3-N to NO_2-N, resulting in an increase in NO_2-N supply to Anammox bacteria. This implied that 25.0 mg/L and 30 mg/L of sulfide effectively induced denitrification and consumed excess nitrate.

However, the addition of 50mg-S/L of sulfide in Phase VII led to a significant decrease in nitrogen removal performance, highlighting the importance of controlling sulfide concentrations within the range of 30mg-S/L to optimize the performance of Anammox processes in wastewater treatment systems. The negative impact of sulfide addition on the Anammox process's performance in Phase II and III was likely due to the inhibition of Anammox bacteria's activity by sulfide, as indicated by the decrease in Anammox bacteria's abundance and the shift in microbial community composition towards nitrite oxidation. However, the Anammox process's nitrogen removal performance recovered when sulfide concentration was reduced in Phase IV, indicating the potential of Anammox bacteria to adapt to sulfide stress.

Overall, the present study provides valuable insights into the long-term effects of sulfide addition on the performance of the Anammox process and the microbial community present in UASB reactors. The findings can help inform the design and operation of wastewater treatment systems to ensure optimal performance of Anammox processes for effective nitrogen removal. Further studies are warranted to investigate the underlying mechanisms of Anammox bacteria's sulfide resistance and the potential of Anammox processes to recover from sulfide stress.

Sulfide has been shown to severely inhibit Anammox activity; and Dapena-Mora et al. (2007) proposed that sulfide must go through an effective pre-treatment oxidization step to avoid entering the Anammox reactor. The inhibitory effects of undissociated H_2S on Anammox bacteria are believed to be mediated by decreasing and binding the metal centers of hemeprotein-related enzymes (Jin et al., 2013; Zhang et al., 2022). Wisniewski et al. (2019) demonstrated that low H_2S concentrations disrupt Anammox metabolism, inhibiting Anammox biomass. One possible explanation for this effect is the consequences of sulfide are usually associated with its unionized form, but a decrease in cytochrome heme iron due to the interaction of heme centers and cytochrome oxidase could also be responsible (Pietri et al., 2011). According to reports, Anammox metabolism heavily depends on numerous hemeproteins for catalytic and electron transfer (such as *NirS, NirK*, and Rieske-heme b complexes), as can be seen in Anammoxosomes (Kartal and Keltjens 2016).

In other research using total sulfide as the target inhibitor, contradictory findings were observed. At a pH of 7.8, Dapena-Mora et al. (2007) discovered that doses of sulfide ranging from 32 to 64 mg/L were able to bring the SAA down by 60%. In contrast, the Anammox activity remained unchanged up until a concentration of 160 mg sulfide in the study with suspended biomass. Jin et al. (2013) discovered that the half inhibitory effects of total sulfide for Anammox biomass were dependent on the substrate (NH_4-N, NO_2-N), and they reported a concentration of 264 mg TSS/L at pH = 7.5 for granular sludge. This value was determined for granular sludge. On the other hand, Van de Graaf et al. (1996) showed that the addition of 160 mg S/L even boosted the Anammox activity for suspended biomass when the pH was equal to 7.0. The authors defended this stimulation by pointing out that sulfide's ability to degrade nitrate to nitrite, which served as the true electron donor for the Anammox process, was the basis for their argument. It is important to highlight that certain sulfide-oxidizing bacteria, such as those belonging to the genus Sulfurimonas, can use sulfide as an electron donor to reduce NO_3-N to NO_2-N (Oshika et al., 2016).

In addition, sulfide-oxidizing bacteria belonging to the genus *Thioploca* have the ability to convert NO_3-N to NH_4-N by way of NO_2-N through the process of respiratory ammonification (i.e., dissimilatory nitrate reduction to ammonium). These sulfide-oxidizing bacteria would extend the ecological niche of Anammox bacteria by lowering the toxicity of sulfide and providing the necessary substrates for Anammox (NO_2-N and NH_4-N) (Oshika et al., 2016). This would be accomplished by decreasing the toxicity of sulfide and by providing the necessary substrates. The same authors also pointed out that sulfide-dependent denitrification did, in fact, boost Anammox activities in estuary sediments, and that it was shown that sulfide-oxidizing bacteria and Anammox bacteria coexisted in marine sediments as well as laboratory reactors.

5.8 Conclusions

In conclusion, this study provides valuable insights into the effect of sulfide on the nitrogen removal performance of Anammox bacteria in a UASB reactor. The study demonstrated that sulfide has a short-term negative effect on Anammox performance, but it can be quickly recovered under low sulfide stress. Furthermore, the Anammox process showed increased sulfide-resistance ability after long-term acclimatization. This finding is important for practical applications as it emphasizes the importance of implementing inhibition-relief measures immediately when sulfide inhibits the Anammox process.

Additionally, reducing or removing the sulfide in the influent would be beneficial to accelerate the recovery of the Anammox performance. The study further revealed that sulfide concentrations in the Anammox reactor caused a deviation from theoretical values. Under the long-term effects of sulfide on the Anammox process, the RS value was 1.56; while the Rp value was as low as 0.06. This finding indicates that monitoring the stoichiometric ratio is a useful tool for understanding the performance of Anammox bacteria under varying conditions.

The results showed that Anammox was the dominant pathway for nitrogen removal during most phases, with contributions ranging from 80% to 86%. However, at high sulfide concentrations, the contribution of Anammox to nitrogen removal decreased significantly to 41.3%, while the contribution of denitrification increased to 58.6%. This finding highlights the importance of considering the effects of environmental conditions on microbial communities and their contributions to nitrogen removal in wastewater treatment systems.

Furthermore, the study investigated the effects of sulfide concentration on the microbial community responsible for nitrogen removal in an Anammox process. The study revealed that as sulfide concentration increased, the abundance of Anammox bacteria decreased, while Nitrobacter spp. increased, suggesting a shift in microbial community composition towards nitrite oxidation. When sulfide concentration was reduced, the abundance of Anammox bacteria increased, indicating their ability to recover quickly in low-sulfide environments. However, in high-sulfide environments, the Anammox bacteria were unable to recover, leading to a decrease in overall nitrogen removal performance. This finding emphasizes the importance of controlling sulfide concentration to optimize the performance of Anammox processes.

CHAPTER SIX

6.0 FEASIBILITY OF A COMBINED ANAMMOX AND SULFUR-DRIVEN DENITRATATION PROCESS FOR NITROGEN REMOVAL FROM DOMESTIC ANAEROBIC EFFLUENTS

Abstract:

Nitrogen removal from wastewater is a critical environmental challenge, and the development of efficient and sustainable treatment methods is of utmost importance. In this study, we investigate the performance and microbial dynamics of a sulfide-driven partial denitrification and Anammox (SPDA) process for enhanced nitrogen removal. The SPDA process leverages the synergistic capabilities of sulfide-driven partial denitrification and Anammox, offering a promising approach for tackling nitrogen-rich wastewater streams. However, several challenges such as hydrogen sulfide inhibition, competition with high chemical oxygen demand (COD) concentrations, and long-term stability need to be addressed to optimize the SPDA process. To overcome these challenges, we explored the impact of varying hydraulic retention times (HRTs) and external sulfide dosing on the performance of the SPDA process. A comprehensive analysis of the structural and functional dynamics of microbial communities was conducted using next-generation high-throughput sequencing. The study spanned 423 days, divided into four operational phases, and examined the efficiency of nitrogen and carbon removal from the influent. Throughout the study, the SPDA process exhibited remarkable nitrogen removal capabilities. The average ammonium nitrogen (NH_4-N) removal efficiency increased from 31.4% to 95.7% as the process progressed, while nitrite (NO_2-N) and nitrate (NO_3-N) removal efficiencies also improved significantly. Total nitrogen (TN) removal efficiency increased from 6% to 87.9% over the operational phases, demonstrating the effectiveness and long-term stability of the SPDA process. Furthermore, the bioreactor efficiently removed COD, with removal efficiencies ranging from 54.3% to 93.0% over the four phases. The investigation of microbial dynamics revealed the intricate interplay between various microbial communities involved in the SPDA process. The success of the SPDA process was attributed to the collaborative interactions between sulfur-oxidizing bacteria (SOB), denitrifying bacteria, and Anammox bacteria. By utilizing sulfide as an electron donor, the SPDA process achieved stable nitrite production without the need for additional carbon sources, thus inhibiting the overgrowth of heterotrophic biomass and enhancing process efficiency. This study provides valuable insights into optimizing the SPDA process for enhanced nitrogen removal from wastewater. The findings demonstrate the feasibility and effectiveness of utilizing sulfide-driven partial denitrification and Anammox as a combined treatment approach. The investigation of microbial dynamics sheds light on the complex interactions within the microbial communities, contributing to a deeper understanding of the SPDA process and its long-term stability.

Keywords: Sulfide-driven partial denitrification and Anammox, Nitrogen removal, Wastewater treatment, Microbial dynamics, Hydraulic retention time, Sulfur-oxidizing bacteria, Process optimization.

6.1 Introduction

Anammox is a highly regarded biotechnological process for nitrogen removal, with applications in wastewater treatment, bioremediation, and industrial processes (Cao et al., 2017; Qui et al., 2021). It converts ammonium and nitrite directly to nitrogen gas under anoxic conditions, eliminating the need for external carbon sources (Mulder et al., 1995; Du et al., 2017). However, the limited availability of nitrite in wastewater has been a challenge. To overcome this, a method called combined partial nitrification and Anammox (PN/A) has been developed. PN/A involves partial nitrification to produce nitrite under aerobic conditions, which is then used as an electron acceptor for Anammox under anoxic conditions (Miao et al., 2020; Kartal & Kelthens,2016). The PN/A process offers significant cost reductions compared to conventional methods due to the elimination of external carbon sources and reduced energy requirement for aeration (Lackner et al., 2014; Qui et al., 2021). In addition to the PN/A process, another alternative approach for generating nitrite involves partial denitrification, where nitrate is converted to nitrite under anoxic conditions (cho et al., 2011; Mioa et al., 2020). This method shows promise for treating wastewater containing nitrate and ammonium, offering advantages such as simultaneous removal of nitrate and ammonium and lower energy consumption compared to nitrification. Recent studies have focused on developing new partial denitrification and Anammox processes. One explored approach involves utilizing organic carbon as the electron donor for heterotrophic partial denitrification, resulting in nitrite production (du et al., 2014; Kalyuzhnyi et al., 2006). However, the use of heterotrophic denitrifying bacteria in this process has drawbacks. Heterotrophic bacteria have a higher growth rate and biomass yield compared to Anammox bacteria, potentially leading to an overgrowth of heterotrophic biomass (Kumar & Lin,2010). This overgrowth can negatively impact Anammox activity, thereby affecting the efficiency of the partial denitrification and Anammox process (Miao et al., 2020).

Researchers are actively working on addressing the issue of controlling the growth of heterotrophic biomass in the process (Y. Zhang et al., 2023; Delgado Vela et al., 2015). One successful approach involves using sulfur-oxidizing bacteria (SOB) for partial denitrification (Deng et al., 2019). SOB can utilize reduced sulfur compounds as electron donors and has lower biomass yields and growth rates compared to heterotrophic denitrifying bacteria (Cui et al., 2019; Li et al., 2019). By producing stable nitrite through partial denitrification using SOB, it may be possible to reduce the need for additional carbon and inhibit the growth of Anammox bacteria, as Anammox bacteria require nitrite as an electron acceptor for their activity. Recent studies have explored the use of elemental sulfur and thiosulfate as electron donors for partial denitrification, demonstrating their effectiveness in stably producing nitrite without requiring significant process control (Deng et al., 2019; Du et al., 2017; Russ et al., 2014). Various researchers have investigated the use of elemental sulfur and thiosulfate as sulfur sources for the simultaneous removal of nitrate and ammonium from wastewater (li et al., 2019; Deng et al., 2019).The tests conducted using elemental sulfur or thiosulfate as electron donors have successfully demonstrated the ability to achieve Anammox and partial denitrification, effectively removing nitrate and ammonium from wastewater. Another relevant sulfur compound found in wastewater is sulfide, which is commonly produced through the microbial reduction of sulfate under anaerobic conditions (Delgado vela et al., 2015; Kalyuzhnyi et al., 2006). This process involves the conversion of nitrate to nitrite in the presence of sulfide, mediated by nitrate-reducing bacteria using sulfide as an electron donor. The produced nitrite can then be coupled with the Anammox process to form a combined process known as sulfide-driven partial denitrification and Anammox (SPDA). In SPDA, nitrate, ammonium, and sulfide react to produce nitrogen gas, water, and sulfate. This process is highly effective in removing nitrogen from wastewater and offers advantages over traditional denitrification methods, including reduced sludge production, improved nitrogen removal efficiency, and increased stability under low oxygen conditions. It is particularly suitable for treating nitrogen-rich wastewater types such as anaerobic digester effluent, landfill leachate, and agricultural runoff. However, challenges such as hydrogen sulfide inhibition, competition with high COD concentrations, and the need for long-term stability must be addressed through research and development to optimize the SPDA process and enhance its performance and efficiency. Also the application in the treatment of domestic anaerobic effluents remains relatively unexplored.

There is still much that is not fully understood about the process. For example, there have been very few studies that have looked at the long-term stability of the process, which is important to determine if it can be a reliable and effective treatment method over an extended period of time. Furthermore, the effects of sulfide on the Anammox bacteria and the mechanisms by which nitrogen is removed through SPDA are not yet fully understood. Finally, the interactions between SOB and Anammox bacteria in SPDA are not completely known. SOBs are a kind of bacterium that can oxidize sulfur compounds and can be found in wastewater. Interactions between SOB and Anammox bacteria in SPDA may have an effect on process efficiency. As a result, further study is required to better understand the SPDA process, its long-term stability, and the applicaion in treating real domestic wastwater. This will help to ensure that SPDA can be a reliable and effective method for treating wastewater.

This study aims to investigate the impact of varying hydraulic retention times (HRTs) and external sulfide dosing on the performance of the SPDA process. Additionally, the structural and functional dynamics of microbial communities were evaluated using the next-generation high through put sequencing. The results of this study can provide valuable insights into optimizing the performance of the SPDA process for nitrogen removal from wastewater.

6.2 Materials and Methods

6.2.1 Bioreactor setup

A 60 L bioreactor with three reaction tanks, an upflow anaerobic sludge blanket (UASB) reactor, partial/complete nitrification (PN) reactor and SPDA reactor, were used in this study (Figure 6.1). The reaction volume of each tank was 18 L. An 8 L settling tank was installed at the end of the treatment process. The bio-carriers used in each reactor were polyvinyl alcohol gel beads occupying 10% (1.8 L) of the reactor volume. Aeration in the PN reactor was provided with diffusers placed at the bottom to maintain dissolved oxygen (DO) around 1-1.5mg/L. Continuous mixing was carried out in all three reactors with the help of a mechanical stirrer. Peristaltic pumps were used to control the flow rates of feed, returned sludge, and recirculation in the reactors. The bioreactor was operated under ambient temperature conditions (20-25°C), and the UASB reactor was operated under sulfidogenic conditions, degrading organic matter, and reducing sulfate to sulfide. The UASB reactor effluent was fed partially (approximately 50%) to the PN reactor, producing mainly nitrite and a small amount of nitrate, while the remaining 50% was directed to the SPDA reactor where both flows mixed. The bioreactor was equipped with sampling ports, pH, temperature, and dissolved oxygen sensors. The schematic diagram of the bioreactor is shown in Figure 6.1.

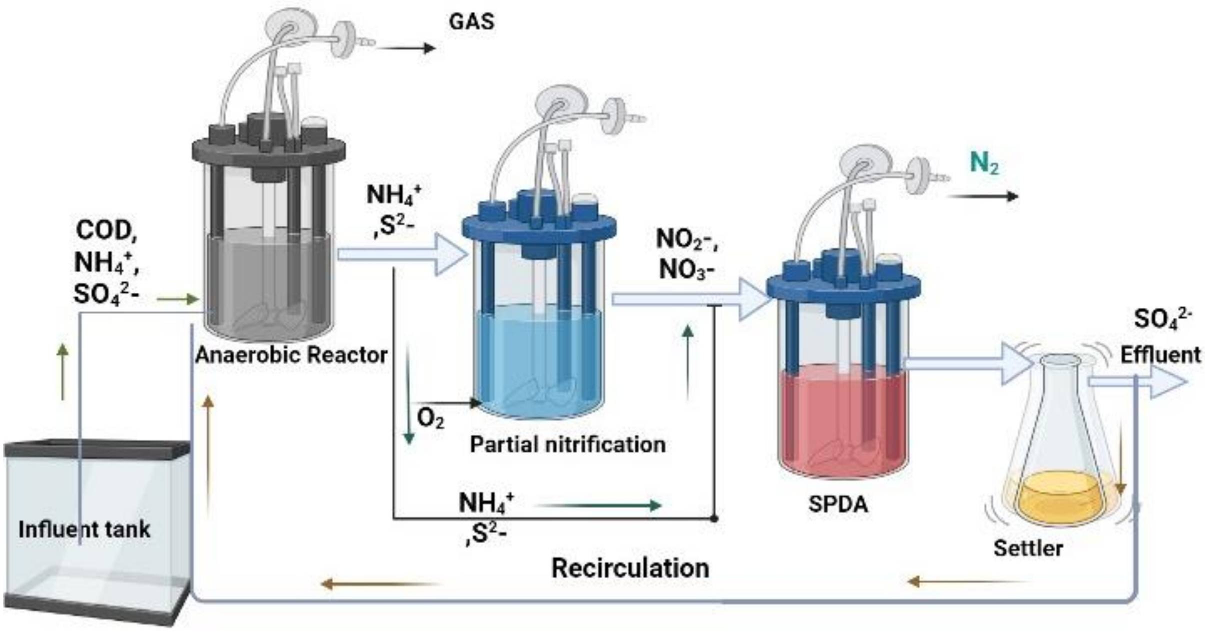

Figure 6.1: Flow diagram showing municipal wastewater treatment containing organics, ammonium, and sulfate.

6.2.2 Domestic wastewater and sludge Inoculum

The inoculum for the UASB reactor was collected from an existing anaerobic digester that treats primary and secondary sludge. The activated sludge for the PN reactor was sourced from an aeration tank collected from a municipal wastewater treatment plant. The activated sludge was acclimatized to the laboratory conditions for a period of two weeks prior to being used in the experiment. Anammox and sulfide-driven autotrophic denitrification (SAD) sludge were used as inoculum for the SPDA reactor. The inoculum used to produce Anammox was collected from an anaerobic reactor that had been fed with a solution of ammonium and nitrite in the lab. An activated sludge-inoculated UASB reactor that had been fed nitrate and sulfide produced the SAD sludge. The mass ratio of the inoculum was 1:1, and it was made up of Anammox sludge and SAD sludge. Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were initially 6 and 7.5 g/L, respectively.

The substrate used in this study was municipal wastewater, which was collected just after the screening process from a nearby wastewater treatment plant. The wastewater was stored at 4°C prior to use in the experiment. The COD of the wastewater was 450 ± 50 mg/L, total nitrogen (TN) and total phosphorus (TP) concentrations were 30 ± 5 mg/L and 5 ± 1 mg/L, respectively. The nitrogen pollution was listed as NH_4^+-N in the range of 25-45 mg/L, NO_2^--N was below 0.5mg/L and NO_3^--N concentrations below 2 mg/L The pH of the wastewater was 7.5 ± 0.2.The average sulfate ($S0_4^{2-}$-S) concentrations were in the range of 40-60 mg-S/L and sulfide (S^{2-}) in the range of 0-2 mg-S/L.

6.2.3 Experimental Procedure

The experiment was conducted for a total of 423 days and divided into four phases (Table 6.1). Phase I lasted for 44 days, while Phase II spanned from day 46 to day 135. Phase III covered day 136 to day 300, and Phase IV included the remaining period between day 300 and day 423. During the experiment, the hydraulic retention time (HRT) was gradually decreased from 24 hours in Phase I to 20 hours in Phase II and further reduced to 12 hours in Phase III and IV. This change was implemented to investigate the effect of varying hydraulic retention times on performance of the system. In Phase IV, an external sulfide dosing of 15 mg-S/L was introduced to the SPDA reactor, which was continued for the remainder of the experiment. This was done to evaluate the impact of external sulfide dosing on the system's performance. The dosing of sulfide commenced at the start of Phase IV and continued throughout this phase until the end of the experiment. Table 6.1 depicts the reactor performance and operational conditions result.

Table 6.1: Reactor performance and operational conditions

Phase	I	II	III	IV
Duration	1- 44	46 -135	136-300	300-423
Inf-NH_4-N (mgN/L)	29±6	32.9±6.4	33.6±7.17	39.93±4.99
Inf-NO_2-N (mgN/L)	0.06±0.09	0.008±0.026	0.06±0.7	0.12±0.24
Inf-NO_3-N (mgN/L)	2±1	0.814±0.86	0.7±0.633	1.57±0.92
HRT(h)	24	20	12	12
Inf-COD (mg/L)	344±54	379.9±70	384.8±85.3	447.6±76.22
Inf-S^{2-}-S (mgS/L)	1±1	2.978±1.84	1.28±0.93	15±1.36
TN removal efficiency (%)	6±32	16±66	51.9±32	87.94±7.8
COD removal efficiency (%)	53±13	64.85±16	89.79±4.45	92.68±2.21
Sulfide removal efficiency (%)	100	100	100	100

6.2.4 Batch experiments

Batch test A: To determine the reaction kinetics of Anammox and sulfide-driven autotrophic denitrification (SAD) and to investigate the possible nitrogen removal methods.

For batch test A, a 120 mL serum bottle with a 100 mL working volume was used. The test was carried out to evaluate the dynamics of nitrogen and sulfur compounds in the SPDA system. The sludge from the SPDA reactor was collected and suspended in a mineral medium before being incubated for one hour in a thermostatic shaker without oxygen. The bottle was then filled with stock solutions of ammonium, nitrate, and sulfide (NH_4Cl, $NaNO_3$, and Na2S, respectively) to obtain initial concentrations of NH_4^+-N (35 mg/L), NO_3-N (32 mg/L), and S^{2-} (20 mg-S/L), and the mixture was incubated to determine the kinetics of nitrogen and sulfur compounds.

In the batch experiments, samples of 2 mL were collected from the bottles at specific time intervals ranging from 5 to 60 minutes. These samples were then filtered to determine the concentrations of sulfide, thiosulfate, sulfate, nitrate, nitrite, and ammonium. All tests were conducted in duplicate.

6.3 Calculations

6.3.1 Anammox and sulfide-driven autotrophic denitrification contribution towards total nitrogen removal in SPDA reactor

In the SPDA process, the reduction of ammonia and nitrite was observed due to the presence of the Anammox in the system, as indicated in Eq. (1). To determine the contribution of the Anammox to the total nitrogen removal, Eq. (2) was used (Chen et al., 2016).

$$NH_4 + 1.32NO_2 + 0.066HCO_3 + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3 + 2.03H_2O + 0.066CH2O0.5N0.5 \quad \textbf{(Eq. 1)}$$

$$A\% = (1+1.32-0.62) * (NH_4^+\text{-N influent - } NH_4^+\text{-N Effluent}) / \text{TN influent-TN Effluent} *100 \quad \textbf{(Eq. 2)}$$

A% represents the percentage contribution of Anammox, NH_4^+-N influent, and NH_4^+-N Effluent represent the influent and effluent NH4-N concentrations, respectively, and TN influent and TN Effluent indicate the total nitrogen concentration of the influent and effluent, respectively.

The denitrification contribution percentage (B%) was calculated as follows:

B% = (1-A%) * 100%

This formula represents the percentage contribution of denitrification to total nitrogen removal, where B% is the denitrification contribution percentage and A% is the Anammox contribution percentage. This method can help in determining the individual contributions of Anammox and denitrification to total nitrogen removal in the SPDA process.

6.3.2 Sampling and Analytical Methods

Samples were collected from each tank of the bioreactor every three days. The influent and effluent samples from the reactor were filtered through 0.45 µm syringe filters prior to the measurements of nitrogenous compounds. Concentrations of ammonium, nitrite, nitrate, and sulfate were analyzed using the Gallery TM Automated Photometric Analyser (Thermo Scientific; USA). The sulfide concentration in the influent and effluent was analysed by the methylene blue spectrophotometric method (DR 6000, Hach USA) according to the standard method. Analysis of sludge properties i.e., Mixed-Liquor suspended solids (MLSS) and mixed-liquor volatile suspended solids (MLVSS) was carried out by following the standard method as mentioned by Eaton et al. (2005). The pH, temperature, and dissolved oxygen (DO) were measured in situ using a pH meter, a temperature sensor and a DO sensor, respectively.

6.3.3 Microbial analysis

Sample collection and DNA extraction: Six sludge samples from the SPDA reactor were collected on days 1, 45, 135, 300, 420 and Anammox sludge (AS). The collected samples were chosen to represent the microbial community structure and function during different phases of SPDA process and Anammox. Total genomic DNA was extracted from the sludge samples (25 ml) using the DNeasy Power Soil Kits (QIAGEN, Denmark) as per the manufacturer's instructions. The quality and concentration of the DNA were then assessed through 1% agar gel and Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, USA), respectively. Shotgun metagenomic sequencing was performed on Illumina's NextSeq 500, at National Institute for Communicable Diseases (NICD), Division of National Health; Modderfontein Road, Sandringham South Africa.

6.3.4 Metagenomics analysis

The taxonomic and functional annotation of the illumina generated raw sequence were analyzed through an open-source web-based server MG-RAST, version 4.0.3. (Keegan; 2016). The low quality reads were discarded by using SolexaQA(Cox et al., 2010;Tang et al., 2013) and the artificial duplicate reads were removed by applying k-mer based approach. Annotations were then made against the SILVA reference database at 97% similarity threshold (Wilke et al., 2013). All the annotations were made with an *E*-value of 10^{-5}, minimum 70% identity cut off and a minimum alignment length of 25 bp. Similarly, the functional prediction was performed using SEED subsystem database with a minimum *E*-value of 10^{-5} identity cut-off of 70%.

6.3.5 Statistical Analysis

The α and β diversity indices were performed at OUT level. One-way ANOVA with Duncan tests was used to test the significance between different samples at $p \leq 0.05$. Box -plot and Principal Coordinate Analysis (PCoA) was also performed at OUT level in the "microeco" packages of R software (version 4.2.2). Furthermore, the comparative analysis for the predicted protein functions were performed using STAMP (Statistical Analysis of Metagenomic Profiles) software package (Parks et al. 2014). The Kruskal-Wallis H-test, followed by Storey's FDR methods were used for multiple test corrections to minimize the false discovery rates during multiple group comparative analysis (Storey, 2002). PCoA analysis was also performed through STAMP for functional profiles in order to determine the distances between different samples.

6.4 Results and discussion

6.4.1 Long-term performance of the bioreactor

The nitrogen removal performance of the system was evaluated over a period of 423 days, which was divided into four phases. During Phase I (1-44 days), Phase II (46-135 days), Phase III (136-300 days), and Phase IV (300-423 days), the influent NH_4-N concentration ranged from 29±6 to 40±4 mgN/L, and the HRT ranged from 24 to 12 hours.

Throughout the study, the system demonstrated a remarkable ability to remove NH_4-N from the influent. The average NH_4-N removal efficiency during each phase was 31.4%, 69.7%, 95.7%, and 91.5%, respectively (Figure. 2a). The highest removal efficiency was achieved during Phase III when the influent NH_4-N concentration was 33.6±7.17 mgN/L and the HRT was 12 hours, with an average effluent NH_4-N concentration of 1.44±1 mgN/L (Figure. 2a). The results also revealed that the system effectively removed NO_2-N and NO_3-N from the influent, with the average effluent concentration decreasing significantly in Phases III and IV. The average effluent concentration for NO_3-N increased during Phase II, indicating the conversion of NO_2-N to NO_3-N through nitrite oxidation. However, in Phase III, the average effluent concentration for NO_2-N and NO_3-N decreased, indicating the successful conversion of NO_2-N to NO_3-N and the subsequent removal of NO_3-N through denitrification. In Phase IV, the average effluent concentration for both NO_2-N and NO_3-N decreased further, indicating the continued successful denitrification process. Furthermore, the system demonstrated a remarkable ability to remove total nitrogen (TN) from the influent, with an average removal efficiency of 6±32%, 16±66%, 51±32%, and 87.9±7% during each phase, respectively (Figure. 2b). The results suggest that the TN removal efficiency increased from Phase I to Phase IV, indicating improved process performance over time (Figure. 6b).

The study also investigated the effectiveness of a bioreactor in removing COD from wastewater. The results revealed that the average Inf-COD (mg/L) was highest in Phase IV, while the Eff-COD (mg/L) was lowest in Phase IV, indicating a significant improvement in the reactor's efficiency over time (Figure. 2c). During Phase I, the average Inf-COD (mg/L) was 344±54, and the Eff-COD (mg/L) was 157±34, resulting in a removal efficiency of 54.3%. In Phase II, the average Inf-COD (mg/L) was 379.9±70, while the Eff-COD (mg/L) decreased to 127±53, with a removal efficiency of around 66.6%. In Phase III, the average Inf-COD (mg/L) was 384.8±85.3, and the Eff-COD (mg/L) decreased significantly to 37.2±13.09, indicating a high COD removal efficiency of 90.3%. Finally, in Phase IV, the average Inf-COD (mg/L) increased to 447.6±76.22, while the Eff-COD (mg/L) decreased even further to 31.6±7.24, with a removal efficiency of approximately 93.0% (Figure. 6c).

The results suggest that the reduction in HRT positively impacted the treatment efficiency, which could be attributed to the optimization of the microbial population and their activities. As the HRT decreased, the hydraulic load on the reactor increased, leading to better contact between the micro-organisms and the organic matter, resulting in more efficient degradation. Additionally, the system's microbial population might have adapted to the reactor conditions and become more effective in degrading the organic matter.

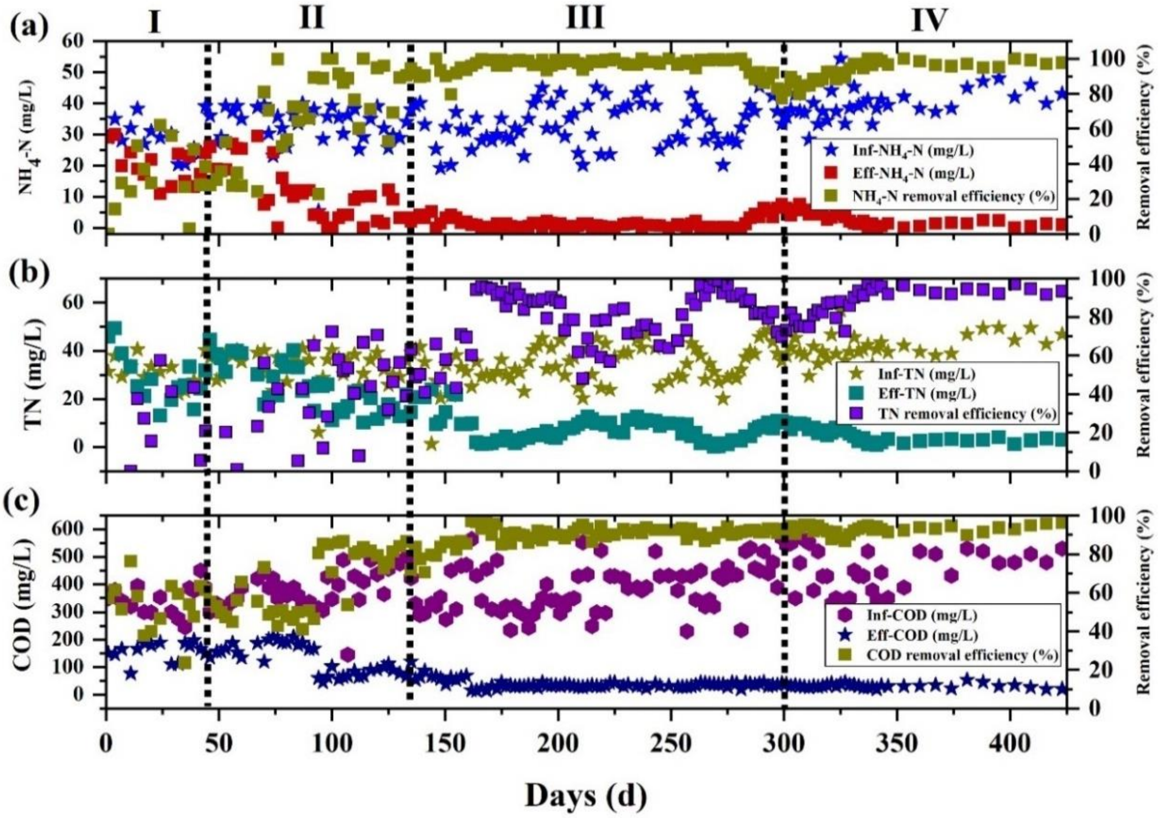

Figure 6.2: Reactor performance (a) NH_4-N Influent, NH_4-N effluent, and NH_4-N removal efficiency (b) TN influent, TN effluent and TN removal efficiency (c) COD influent, COD Effluent and COD removal efficiency

6.4.2 Performance of the UASB (anaerobic) reactor

Organics removal

In this study, an Upflow Anaerobic Sludge Blanket (UASB) reactor was employed to operate in the anaerobic mode. The primary objective of the UASB reactor was to remove excess COD from the influent wastewater while also facilitating the generation of sulfide under sulfidogenic conditions. The sulfide was then utilized in the subsequent SPDA reactor for further treatment.The performance of the UASB reactor showed significant improvement as the reactor progressed through different phases of operation. The reactor was operated in four phases with different HRTs ranging from 8 hours in Phase I to 4 hours in Phase IV.

The results showed that the UASB reactor was highly effective in removing organic matter from the influent wastewater in all four phases. The influent COD concentrations ranged from 344±54 mg/L in Phase I to 447.6±76.22 mg/L in Phase IV, while the effluent COD concentrations were 119±24 mg/L in Phase I and 245±57 mg/L in Phase IV (Figure. 3a). The average COD removal efficiency was ranging from 53.2% in Phase I to 49.1% in Phase IV, indicating the effectiveness of the UASB reactor in removing organic matter in the influent wastewater (Figure. 3a).

HRT varied from 8 hours in Phase I to 4 hours in Phases III and IV. The results indicated that reducing the HRT led to a decrease in COD removal efficiency, with the highest COD removal efficiency observed at an HRT of 8 hours. This finding suggests that longer contact times between the wastewater and the micro-organisms in the reactor may be beneficial in achieving higher COD removal efficiencies. However, longer HRTs may not always be feasible, as they require larger reactor volumes. It is noteworthy that the COD removal efficiency in Phase IV, with an HRT of 4 hours, was at 49.1% (Figure. 3a). This result demonstrates the robustness of the UASB reactor in removing organic matter, even under unstable operating conditions. As the reactor was operated at ambient temperature, a possible reason for the lower COD removal efficiency in the UASB reactor could be the fluctuation or deviation in the reactor temperature. According to a study by Zhang et al. (2018), maintaining a constant temperature within the optimal range is crucial for the stable operation and high efficiency of UASB reactors. They reported that a deviation of only 1-2°C from the optimal temperature (30-35°C) range can significantly affect the performance of the reactor, including COD removal efficiency. Similarly, a study by Li et al. (2017) found that a fluctuation in the reactor temperature can lead to a decrease in the abundance and activity of microorganisms responsible for COD removal in UASB reactors, resulting in lower COD removal efficiency. Therefore, it is important to maintain a stable and optimal temperature range for the UASB reactor to achieve high COD removal efficiency.

The results of this study on organics removal by an anaerobic reactor are consistent with previous literature. The observed trend of decreasing COD removal efficiency with decreasing HRT is consistent with previous studies (Joshi et al., 2019; Zhang et al., 2020). This is because shorter HRTs result in less contact time between the wastewater and the micro-organisms, limiting the extent of organic matter degradation. However, the effect of HRT on COD removal efficiency may depend on the wastewater composition and reactor design (Sayed et al., 2021).

In addition to the organic carbon removal efficiency, the anaerobic process also contributes to the removal of sulfate from the wastewater. The results of the sulfate analysis show that the influent sulfate concentration was relatively constant throughout the different phases of the reactor operation, with an average value of 63±9, 65±18, 54±15, and 70±15 mgS/L for Phase I, II, III and IV respectively (Figure 3c). On the other hand, the effluent sulfate concentration decreased during the operation of the reactor, with average values of 36±11, 46±16, 34±13, and 34±12 mgS/L for Phase I, II, III and IV respectively (Figure 3c).

The decrease in sulfate concentration is an indication of sulfate reduction by the anaerobic micro-organisms in the reactor, which is an important aspect of anaerobic digestion. During sulfate reduction, sulfate is converted to sulfide by sulfate-reducing bacteria, which is then oxidized to elemental sulfur or sulfate by sulfur-oxidizing bacteria or chemically by dissolved oxygen in the effluent. The sulfide concentration in the influent wastewater was low, with an average value of 1±1, 2.9±1.9, 1.2±0.9, and 1.39±0.6 mgS/L for Phase I, II, III and IV respectively (Figure 3d). However, the effluent sulfide concentration increased during the operation of the reactor, with average values of 1±1, 2.35±2, 5.34±1.62, and 6.05±1.3 mgS/L for Phase I, II, III and IV, respectively (Figure 3d).

The increase in sulfide concentration further indicates sulfate reduction by the anaerobic micro-organisms in the reactor. The sulfide generated during sulfate reduction contributes to the removal of organic carbon by reacting with it to produce methane and carbon dioxide. Therefore, the increase in sulfide concentration in the effluent indicates that the anaerobic process is effective in removing organic carbon from wastewater. The results of the present study agree with previous studies that reported on the removal of organic carbon and sulfate by anaerobic reactors. For instance, a study by Wu et al. (2020) reported on the performance of an anaerobic membrane bioreactor (AnMBR) in treating synthetic wastewater containing sulfate. The study found that the AnMBR was effective in removing both organic carbon and sulfate, with removal efficiencies of up to 90% and 70%, respectively. Another study by Zhu et al. (2020) investigated the performance of a sulfidogenic anaerobic baffled reactor (SABR) in treating sulfate-containing wastewater. The study found that the SABR was effective in removing organic carbon and sulfate, with removal efficiencies of up to 80% and 60%, respectively. Overall, the results demonstrate the effectiveness of anaerobic reactor in removing organic carbon and sulfate from wastewater.

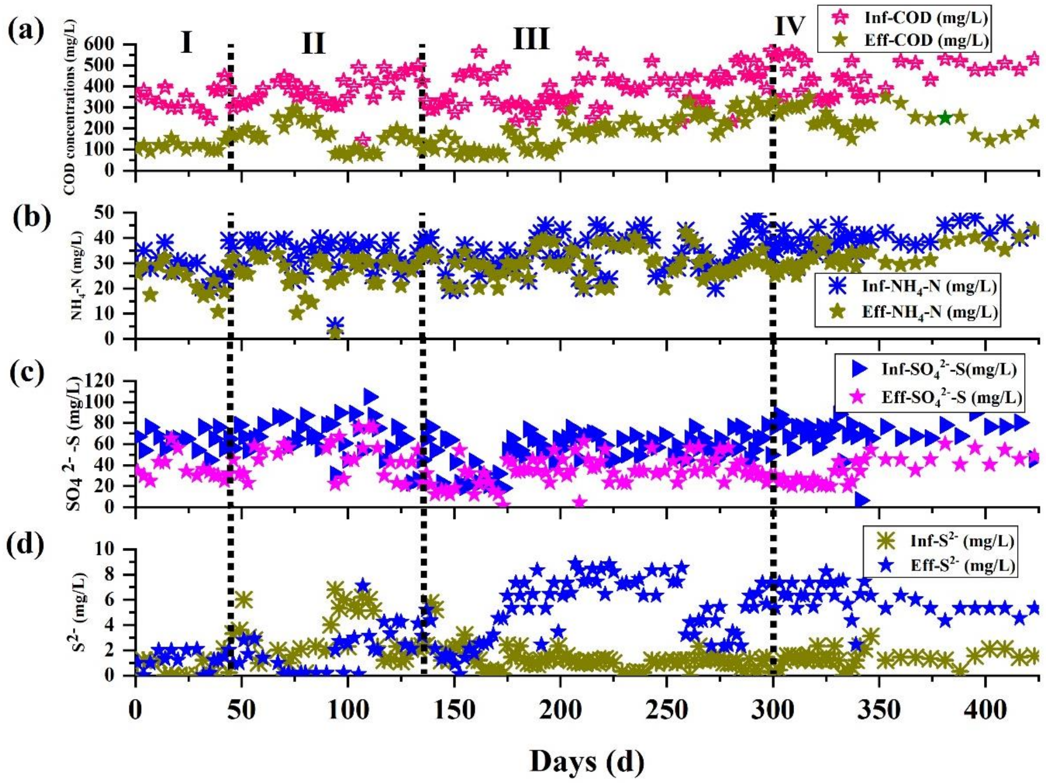

Figure 6.3: Profiles of influent and effluent concentration in UASB reactor (a) Influent and effluent COD concentrations (b) Influent $NH_{4\text{-}N}$ and effluent $NH_{4\text{-}N}$ (c) influent SO_4^{2-} -s and effluent so42--s (d) Influent s^{2-} and effluent s^{2-}

6.4.3 Performance of the Partial/complete nitrification (PN) reactor

Nitrogen removal

The performance of a PN reactor was studied over four different phases, with varying HRTs and DO maintain at 2±1 mg/L throughout the experiment. The focus of this reactor was on the generation of NO_2-N and NO_3-N in the PN reactor, as well as the removal of NH_4-N and COD. The results showed that the PN reactor removed NH_4-N from the wastewater, with average effluent concentrations of 9±5, 3.5±5.5, 1.7±1.6, and 2.78±1.3 mgN/L for Phases I, II, III and IV, respectively (Figure 4a). The reactor also achieved COD removal, with average effluent concentrations of 157±36, 128±77, 41.3±18 and 36.8±10 mg/L for Phases I, II, III and IV, respectively. The reactor generated significant amounts of NO_2-N and NO_3-N, with effluent concentrations of 3±4 and 15±8 mgN/L in Phase I, 8.6±4.47 and 26.2±7 mgN/L in Phase II, 9.1±5 and 8.7±5 mgN/L in Phase III, and 7.1±1.9 and 7.15±2.7 mgN/L in Phase IV (Figure 4b and c).

The PN reactor achieved excellent NH_4-N removal efficiency throughout the four phases, which indicates that the reactor was stable and able to adapt to changes in HRT and constant DO. The consistent DO maintained at 2±1 mg/L throughout the experiment ensured a favorable environment for nitrifying bacteria, which is essential for the PN process. The reduction in HRT from 16 h in Phase I to 8 h in Phases III and IV resulted in an increase in ammonium removal efficiency, which is consistent with previous studies on PN reactors. The reduction in HRT also resulted in a decrease in COD removal efficiency, which is to be expected given that the reactor was designed for nitrogen removal rather than carbon removal. The generation of NO_2-N and NO_3-N in the PN reactor was also notable, with higher NO_2-N production observed in Phases I and II and higher NO_3-N production observed in Phases III and IV (Figure 6.8b and c). The increase in NO_3-N production in Phases III and IV may be attributed to the shorter HRTs, which may have favoured the growth of NOB over AOB. The production of NO_2-N and NO_3-N in the reactor is desirable as it indicates that the PN process was successful, and that the wastewater was being treated effectively.

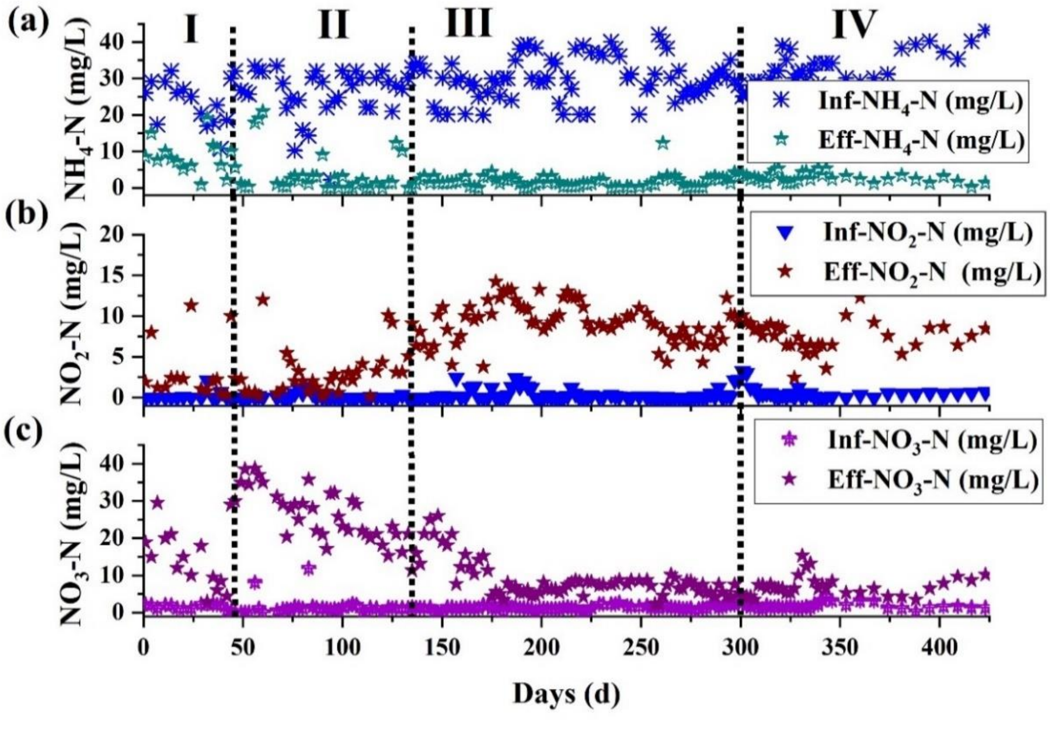

Figure 6.4: Profiles of influent and effluent concentration in PN reactor (a) influent NH_4-N and Effluent NH_4-N (b) influent NO_2-N and Effluent NO_2-N (c) influent NO_3-N and Effluent NO_3-N.

6.5 Performance of the SPDA reactor

6.5.1 Nitrogen removal in the SPDA reactor

The study investigated the nitrogen removal performance of an SPDA reactor. The operational phases were categorized as Phase I (1-44 days), Phase II (46-135 days), Phase III (136-300 days) and Phase IV (300-423 days), and the HRTs were 8, 6.6, 4, and 4 hours respectively. Additionally, a sulfide dosing of 15 mgS/L was started in Phase IV to the SPDA reactor only.

During Phase I, the influent nitrogen concentrations were relatively consistent, with Inf-NH_4-N (mgN/L) averaging at 16±4, Inf-NO_2-N (mgN/L) averaging at 2±2, and Inf-NO_3-N (mgN/L) averaging at 8±4 (Figure 6.5). The SPDA reactor demonstrated moderate NH_4-N removal efficiency with an average of 38±19%, while the NO_2-N and NO_3-N concentrations in the effluent remained relatively consistent with influent concentrations (Figure 6.9).

During Phase II, the influent nitrogen concentrations with Inf-NH_4-N (mgN/L) averaging at 15±5, Inf-NO_2-N (mgN/L) averaging at 4.4±0.5, and Inf-NO_3-N (mgN/L) averaging at 13.8±4 (Figure 6.5). The SPDA reactor demonstrated improved NH_4-N removal efficiency with an average of 74±14% and TN removal efficiency of 20.4±30% (Figure 6.7b). The NO_2-N concentration decreased significantly, while the NO_3-N concentration remained consistent with influent concentrations.

During Phase III, the influent nitrogen concentrations were more variable, with Inf-NH_4-N (mgN/L) averaging at 17.7±15, Inf-NO_2-N (mgN/L) averaging at 4.77±1.3, and Inf-NO_3-N (mgN/L) averaging at 5.07±2.5 (Figure 6.5). The SPDA reactor demonstrated significantly improved NH_4-N removal efficiency with an average of 91±10% and TN removal efficiency of 71±18% (Figure 6.7b). The NO_2-N concentration decreased further, while the NO_3-N concentration remained consistent with influent concentrations.

During Phase IV, a sulfide dosing of 15 mgS/L was started to the SPDA reactor. The influent nitrogen concentrations were consistent, with Inf-NH_4-N (mgN/L) averaging at 18±2, Inf-NO_2-N (mgN/L) averaging at 4.02±1.1, and Inf-NO_3-N (mgN/L) averaging at 4.49±1.3 (Figure 6.5). The SPDA reactor demonstrated continued improvement in TN removal efficiency of 80.7±12% (Figure 6.7b). The NO_2-N concentration decreased further, while the NO_3-N concentration decreased significantly in the effluent.

The data obtained from the SPDA reactor experiments suggest that the reactor facilitated a unique combination of biological processes that are capable of efficient nitrogen removal from wastewater. Specifically, the reactor appears to have harnessed the power of both sulfide-driven partial denitrification and Anammox, which are two distinct pathways that are capable of removing nitrogen species through different mechanisms.

During the early phases of the experiment (Phases I and II), the reactor showed relatively low nitrogen removal efficiency, particularly for NH_4-N and NO_2-N (Figure 6.7). However, during Phases III and IV, nitrogen removal efficiency greatly increased, particularly for NH_4-N, which decreased by over 90%. At the same time, the effluent NO_3-N concentration also decreased substantially. These trends suggest that the reactor was able to shift its biological processes over time, likely in response to changes in influent and operational conditions.

The observed trends in nitrogen removal efficiency are consistent with the known mechanisms of sulfide-driven partial denitrification and Anammox. In the initial phases, partial denitrification may have been dominant, with S^{2-} serving as the electron donor for nitrate reduction to nitrite and ultimately to nitrogen gas. However, in the later phases, Anammox may have become more prominent, with the NH_4-N produced by partial denitrification reacting with NO_2-N to form nitrogen gas. Sullfide has been shown to promote Anammox activity and enhance nitrogen removal efficiency in low-nitrogen concentration systems. The combination of sulfide-driven partial denitrification and Anammox processes is a promising approach for achieving efficient nitrogen removal from wastewater. This is particularly relevant for wastewater with low nitrogen concentrations, where conventional biological processes may not be effective.

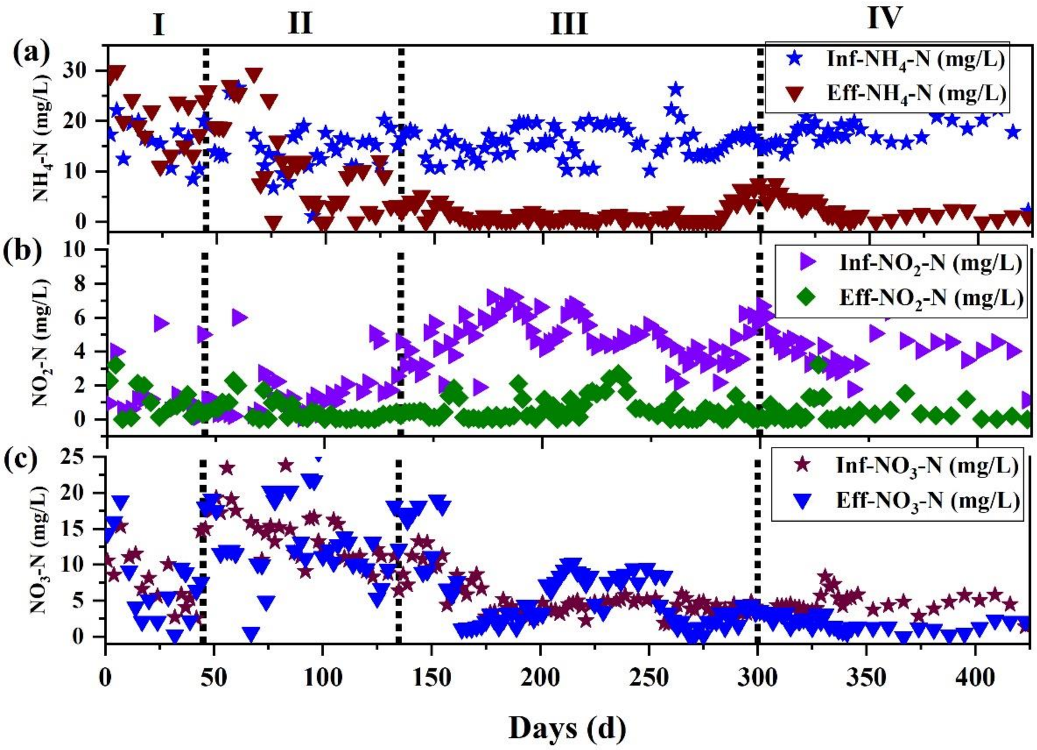

Figure 6.5: Profiles of influent and effluent concentrations (SPDA) reactor (a) NH_4-N Influent and NH_4-N effluent (b) NO_2-N influent and NO_2-N effluent (c) NO_3-N influent and NO_3-N effluent

6.5.2 Sulfide removal in the SPDA reactor

The sulfide removal efficiency of the SPDA reactor can be analyzed based on the influent and effluent concentrations of sulfate (SO_4^{2-}) and sulfide (S^{2-}) in each phase. In **Phase I**, the influent and effluent concentrations of SO_4^{2-} remained relatively constant at 47 ± 6 and 75 ± 15 mgS/L, respectively, with a slight increase in the effluent concentration (Figure 6.6b). The influent S^{2-} concentration was very low at 1 ± 0 mgS/L and was completely removed from the reactor (Figure 6.6b).

In **Phase II**, the influent and effluent concentrations of SO_4^{2-} and S^{2-} remained relatively constant, with little to no removal occurring in the reactor. The influent SO_4^{2-} concentration was 43 ± 16 mgS/L, and the effluent concentration was 44 ± 21 mgS/L (Figure 6.6a), while the influent S^{2-} concentration was 1.45 ± 1.9 mgS/L, and the effluent concentration was 0 mgS/L (Figure 6.6b).

In **Phase III**, the influent and effluent concentrations of SO_4^{2-} increased significantly, with an influent concentration of 45 ± 16 mgS/L and an effluent concentration of 99 ± 36 mgS/L (Figure

6.6a). The influent S^{2-} concentration also increased substantially, reaching 2.78 ± 1.18 mgS/L (Figure 6.6b). Despite the increase in influent S^{2-} concentration, the reactor was able to remove a significant amount of SO_4^{2-} and maintain a relatively constant effluent S^{2-} concentration of 0 mg/L.

In **Phase IV**, S^{2-} dosing was initiated at a concentration of 15 mgS/L (Figure 6.6b). As a result, the influent S^{2-} concentration was significantly higher than in the previous phases, with an influent concentration of 18.9 ± 0.8 mgS/L (Figure 6.6b). Despite an increase in sulfide levels, it is worth noting that no trace of sulfide was detected in the reactor effluent (Figure 6.6b). The reactor was able to remove a significant amount of S^{2-}, with a removal efficiency of approximately 100% (Figure 6.7b). However, effluent SO_4^{2-} concentrations increased during this phase, indicating that the sulfide dosing may have resulted in an increase in sulfate production in the reactor (Figure 6.6a).

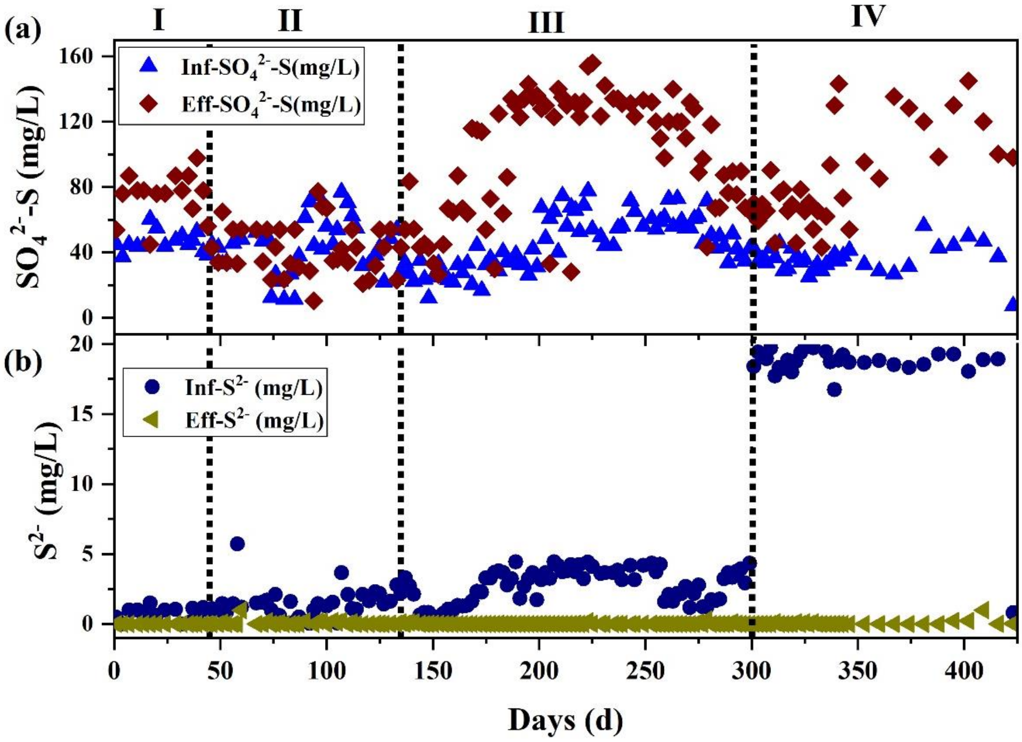

Figure 6.6: Profiles of influent and effluent sulfur concentrations in SPDA (a) influent and effluent SO_4^{2-}-S concentrations (b) influent and effluent S^{2-} concentrations.

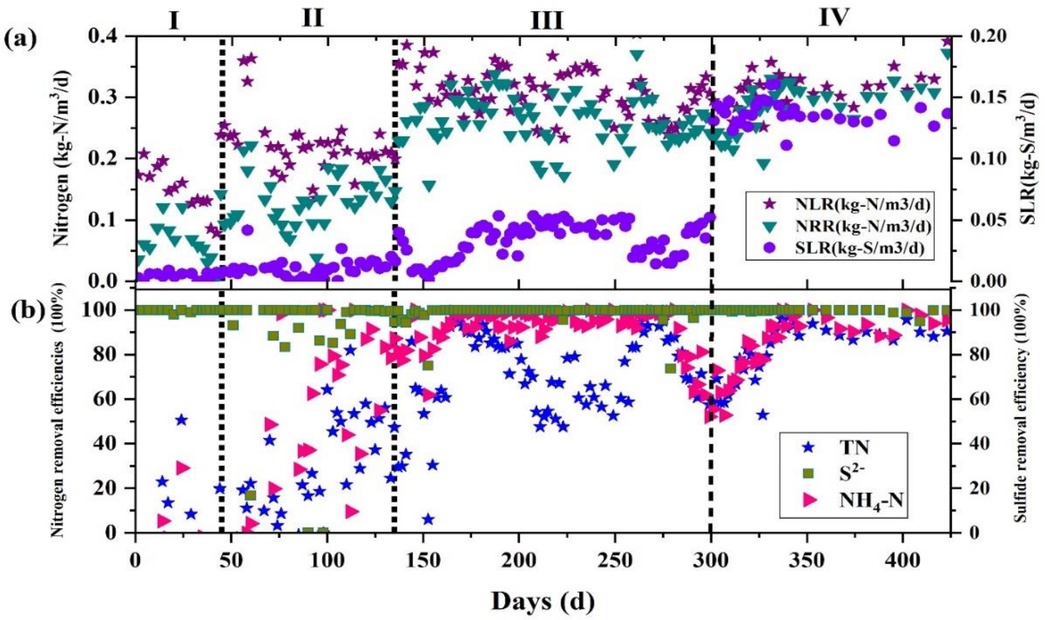

Figure 6.7: (a) Nitrogen and sulfide loading and removal rates in SPDA (b) Nitrogen and sulfide removal efficiencies in SPDA.

6.5.3 Contribution of SAD and Anammox in the SPDA reactor

Nitrogen removal via the coupled autotrophic partial-denitrification and Anammox system was investigated in this study. Using Eqs. (1) and (2), the contributions of Anammox and SAD to nitrogen removal in the SPDA system were evaluated, and the results are shown in Figure 6.8. The results showed that the SPDA reactor efficiently removed nitrogen via Anammox, with the nitrogen removal contribution of Anammox varying across different phases of the experiment. In Phase II (46-135 days), the Anammox contribution was 17.14±20%, which increased to 70±12% in Phase III (136-300 days) and remained stable at 69±10% in Phase IV (300-423 days) (Figure 6.8). On day 400, the Anammox contribution reached 80%, indicating the SPDA reactors capability to remove nitrogen via the Anammox pathway.

On the other hand, SAD played a more significant role in the earlier stages of the experiment. During Phase II, SAD contributed to 60.14±20% of total nitrogen removal, while its contribution decreased to 30±12% during Phase III and 32±10% during Phase IV (Figure 6.8). The decrease in SAD contribution during the later stages of the experiment indicates that Anammox bacteria outcompeted SAD bacteria for substrates.

Moreover, the study suggested that sulfide could be a suitable sulfur source in the coupled autotrophic partial-denitrification and Anammox system, which could enhance the system's performance. This observation was supported by the fact that the SPDA reactor provided an ideal competitive environment to achieve partial denitrification and restrict further nitrite reduction in SAD, resulting in less nitrogen gas production by SAD. The study also observed that nitrite reduction competed both with partial denitrification for electron donors (i.e., reduced sulfur compounds) and with Anammox for electron acceptors (i.e., nitrite) in the SPDA system. Therefore, the system's performance was influenced by the balance between these processes.

In conclusion, the coupled autotrophic partial-denitrification and Anammox system showed promising results for nitrogen removal, with Anammox being the dominant pathway. The system's performance could be further improved by using sulfide as a sulfur source and maintaining the balance between nitrite reduction, partial denitrification, and Anammox.

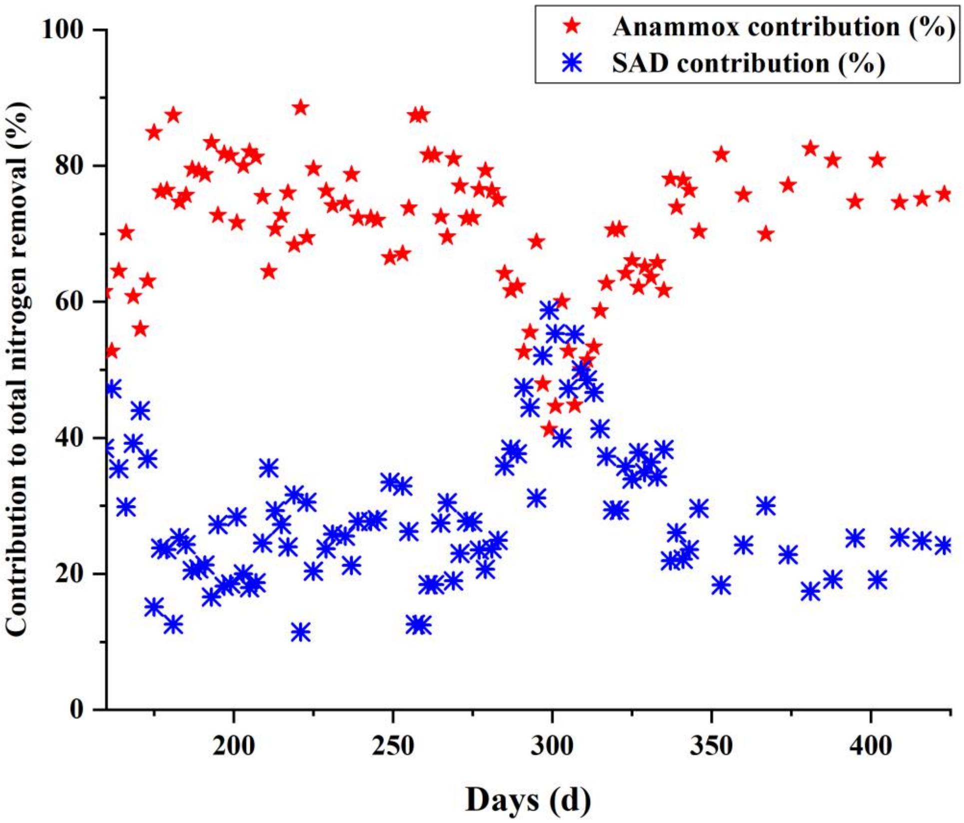

Figure 6.8: Contribution of Anammox and sulphide-driven autotrophic denitrification to total nitrogen removal

6.6 Reconfirmation of nitrogen removal mechanism in SPDA

6.6.1 Batch Experiments

The Batch test A was performed to study the kinetics of two separate processes in the SPDA system: Anammox and sulphur autotrophic denitrification (SAD). Sulphide autotrophic denitrification (SAD) is a complicated two-stage process. Sulfide is oxidized to elemental sulfur (BPS^0) in the first step via nitrate/nitrite reduction, as demonstrated in the following equations: $S^{2-} + 2NO_3^- \rightarrow BPS^0 + 2H^+ + H_2O$ **(3)**

$S^{2-} + 2NO_2^- \rightarrow BPS^0 + H^+ + H_2O$ **(4)**

where S^{2-} represents sulfide, NO_3- represents nitrate, NO_2- represents nitrite, and BPS^0 represents elemental sulfur.

After sulfide depletion, the generated BPS^0 is used as an electron donor for nitrate/nitrite reduction in the second stage of SAD, as stated in the following equations:

$BPS^0 + 2NO_3^- \rightarrow N_2 + SO_4^{2-} + 2H^+$ **(5)**

$BPS^0 + 2NO_2^- \rightarrow N_2 + SO_4^{2-} + H^+ + H_2O$ **(6)**

where N_2 represents nitrogen gas, and SO_4^{2-} represents sulfate.

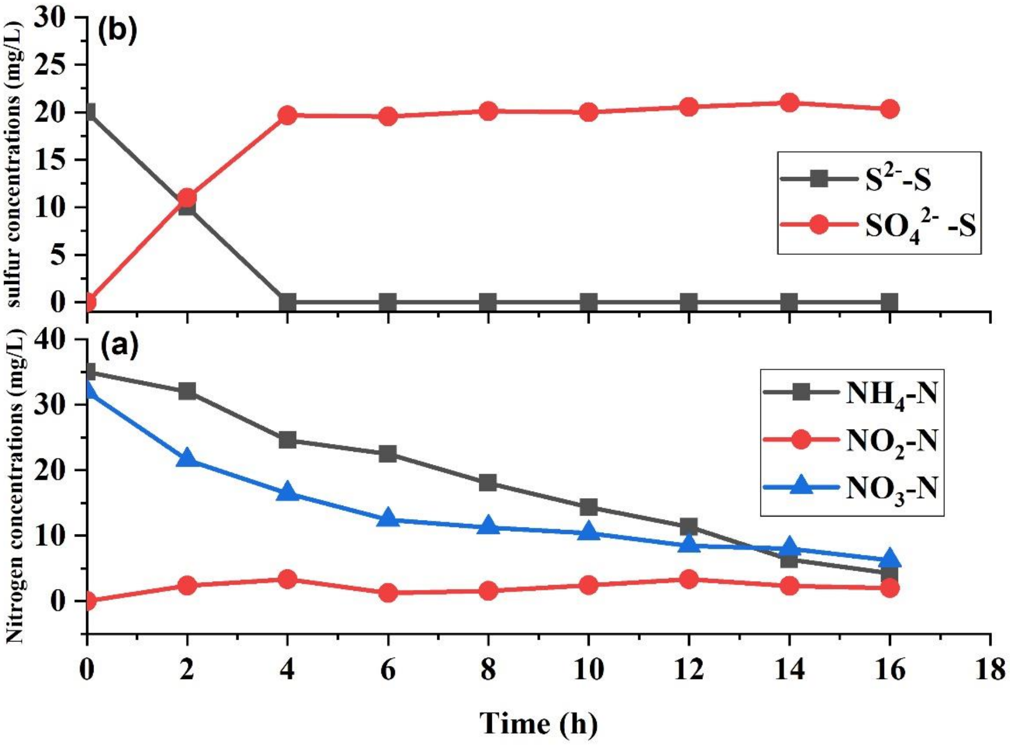

Figure 6.9: Batch test results (a) Nitrogen conversions in the batch experiment (b) Sulfur conversions

According to the results of batch test A, the SPDA process occurred in two different phases, which were distinguished by the employment of sulfide and BPS^0 as electron donors. Stage 1 (0-2h) featured fast sulfide depletion at a rate of 10mgS/(gVSS•h), whereas BPS^0 concentration grew significantly to 10mgS/L. The rise in sulfate concentration remained minimal at this period, which is consistent with prior research on the SAD system (Deng et al., 2022). During Stage 2 (2-16 h), the sulfate concentration steadily grew at a rate of 0.64 mgS/(gVSS•h) to a final concentration of 20.34 mgS/L, whereas the concentration of accumulated BPS0 progressively declined and was completely depleted within 16 h. As previously documented, the starting S/N ratio and sulfide concentration had an effect on the final product of sulfide bio-oxidation. Gadekar et al. (2006) discovered that with a S/N of 0.64, sulfate comprised 93% of the result of sulfide oxidation, but this number reduced to only 9.3% at a S/N of 3.67. Greene et al. (2003) discovered that sulfide was completely oxidized to sulfate at starting sulfide concentrations of 147.2 mgS/L. However, sulfate production was not detected at initial sulfide concentrations greater than 147.2 mgS/L. The sulfide end-product in batch test B was consistent with the previous studies since the starting S/N ratio was 0.8 and the beginning sulfide concentration was only 38.9 mgS/L. At the start of the experiment Stage 1 (0-2h), the liquid contained 35 mg/L of ammonium and 32 mg/L of nitrate. However, after 2 hours, while the bulk liquid remained unchanged, the ammonium concentration had decreased to 32 mg/L, indicating that the presence of sulfide had hindered the activity of Anammox bacteria. On the other hand, the nitrate concentration decreased rapidly to 21 mg/L at a reduction rate of 5.5mgN/(gVSS.h), suggesting that SOB were able to efficiently reduce nitrate to nitrogen gas, completely consuming the nitrite generated in the process. This is evident from the fact that nitrite was not detected during this period, implying that the rate of nitrite consumption was equal to the rate of nitrite generation.

Due to the different electron-donating capabilities of sulfide and BPS0, the nitrate reduction rate in Stage 2 was drastically reduced to 0.5 mgN/(gVSS•h). Previous studies have found a similar pattern when employing sulfide as an electron donor during SAD. Deng et al. (2022) discovered that the electron transfer rate from sulfide oxidation was 3.26 mmol e-/(gVSS•h), but the rate from BPS^0 oxidation was 0.27 mmol e-/(gVSS•h). According to Prange et al. (2002), the decreased electron-donating capability of BPS^0 can be related to its physical features since this product was coupled in a common stable S8 ring structure and was weakly water-soluble. As a result, it is hypothesized that BPS^0 oxidation is restricting the rate of nitrogen removal in Stage 2.

Experiments proved that when BPS^0 was used as the electron donor and nitrate was used as the preferred electron acceptor, the nitrate reduction rate was greater than the nitrite reduction rate, resulting in nitrite build-up (Cui et al., 2019b; Gadekar et al., 2006). It was discovered that nitrite was eliminated only when nitrate was fully depleted. However, when BPS^0 was used as an electron donor in this work, nitrite was taken up by the Anammox process and did not accumulate. The low nitrite content reported at the start of Stage 2 was most likely owing to insufficient Anammox activity recovery after sulfide suppression in Stage 1. Later, the nitrite content rapidly fell below the detection limit, and the Anammox nitrite reduction rate was faster than nitrite reduction using BPS^0 as the electron donor.

In Stage 2, the ammonium concentration fell at a rate of 1.5 mgN/(gVSS•h), reaching a concentration of 4 mgN/L at the end of the experiment. This clearly shows that nitrite produced during Stage 2 partial denitrification can be efficiently utilised by Anammox to oxidize ammonium. As a result, the intermediate BPS^0 in sulfide bio-oxidation is critical in the SPDA system. To our knowledge, this is the first work to emphasize the role of BPS^0 in the coupling of sulfide-driven partial denitrification and Anammox.

To conclude the batch test A studies, the various nitrogen removal processes in the SPDA system were observed. The investigations demonstrated that sulfide bio-oxidation happens in two phases, with sulfide oxidation occurring first and BPS^0 oxidation occurring second. The nitrogen removal route in Stage 1 is dominated by full denitrification mediated by SOB, and the Anammox process is momentarily stalled. All sulfide is transformed to BPS^0 during this cycle. In Stage 2, Anammox dominates the nitrogen removal route, while SOB play an important role in partial denitrification employing BPS^0 as an electron donor. This implies that the SPDA system may successfully remove nitrogen via a combination of SOB-mediated denitrification and Anammox, with sulfide acting as the principal electron donor. Overall, our findings give important insights into the complicated nitrogen removal mechanisms in the SPDA system and can guide future attempts to improve its performance.

6.7 Microbial community analysis in SPDA reactor

6.7.1 Bacterial Community structure and composition

Analyzing the structural and functional dynamics of the microbial communities in SPDA reactors is of paramount importance as the residential microbes have an important role in oxidation, reduction and biodegradation of both organic macromolecules and inorganic chemical species (Balcom et al., 2016). High through sequencing and metagenomics was used to characterize the structure and functional diversity of the SDPA on different days. By analyzing the microbial from the sludge samples, the researcher obtained a total of 258,052,025 high quality, which resulted into 876,447 OTUs at 97% similarity threshold/index. The adequacy of sampling was estimated through rarefaction curve, and the results showed an in-depth sampling. All the measured diversity indices such as Chao1 ($p < 0.0067$), observed OTUs ($p < 0.041$), ACE ($p < 0.00001$) Shannon ($p < 0.00006$), and Simpson ($p < 0.013$) showed a significant difference amongst the samples (Figure 6.10 a-e). Furthermore, the beta diversity amongst different samples was estimated by using Bray-Curtis and Jaccard's similarity index, both of which revealed comparable diversity amongst different samples suggesting that all the samples from different phases of SPDA process harbour diverse type of microbial communities (Table 6.2). Moreover, the principal component analysis revealed that the first two axes explained about 71% variations in microbial community composition, with PC1 explaining 42.3% and PC2 explaining 28.2% of the variations (Figure 6.11).

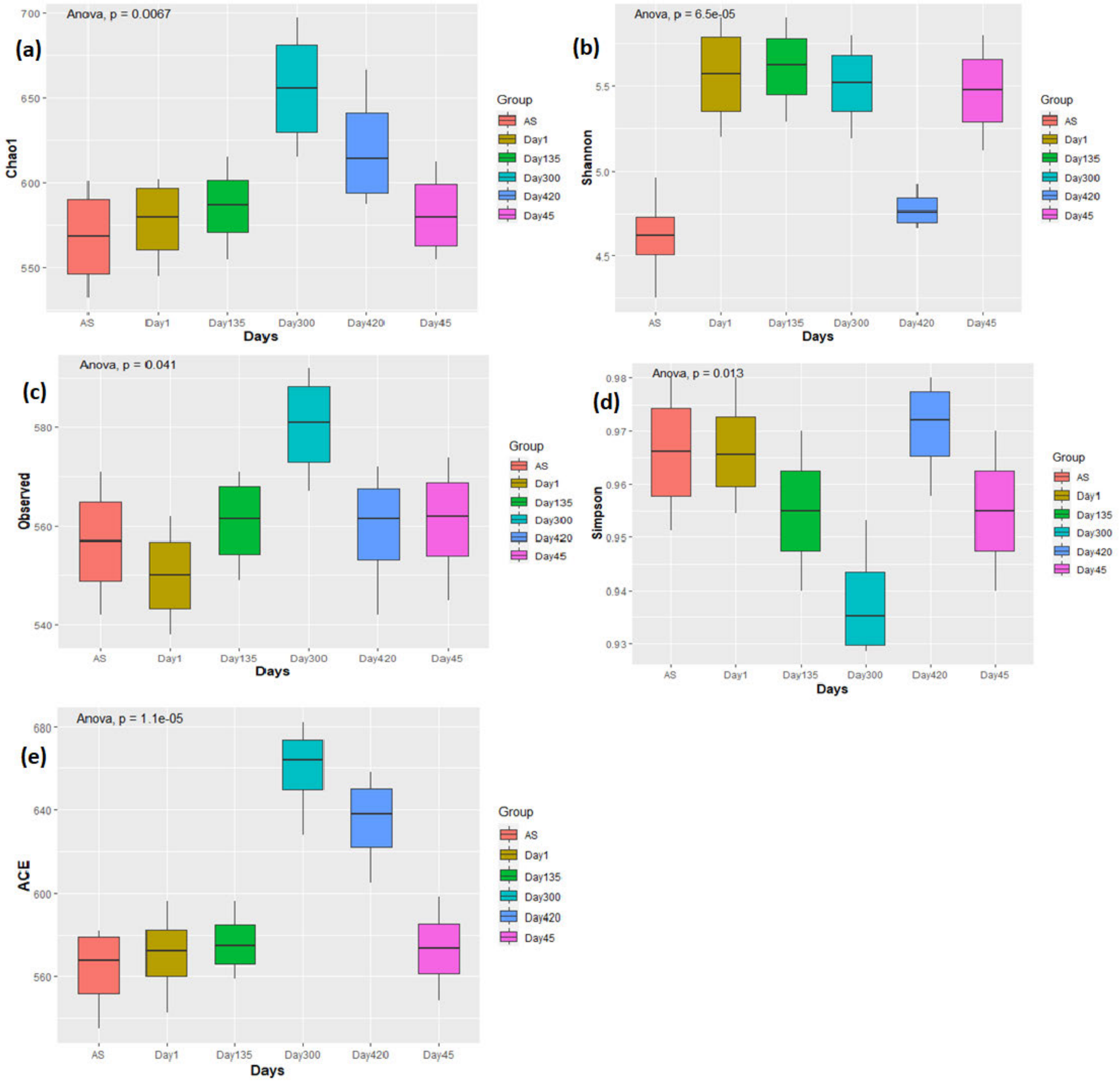

Figure 6.10: Box plot showing the alpha-diversity indices of bacterial communities

The box plot showing the alpha -diversuty indices of bacterial communities collected from SPDA reactor collected at different time intervals (a) Chao1, (b) Shannon, (c) Observed, (d) Simpson and (e) ACE.

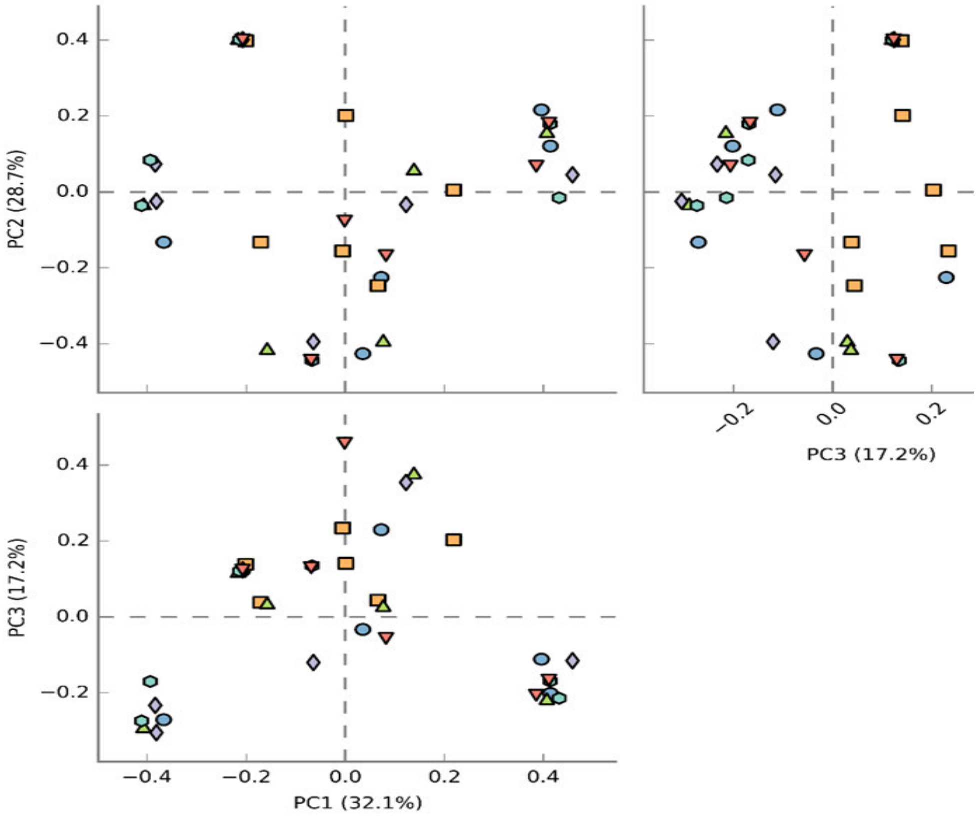

Figure 6.11: Principal Component Analysis Based on the Operational Taxonomic Unit Abundance of Bacterial Communities from Different Samples

Table 6.2: Diversity (bray-curtis) based on genus level organism abundance.

0	Day 1	Day 45	Day 135	Day 300	AS	Day 420
Day 1	0					
Day 45	0.382024	0				
Day 135	0.174088	0.452376	0			
Day 300	0.698104	0.703005	0.673884	0		
AS	0.74531	0.717632	0.712455	0.286767	0	
Day 420	0.737814	0.723841	0.700479	0.569344	0.52354	0

6.7.2 Taxonomic composition of the SPDA reactor

The phylogenetic identification of the bacterial communities was performed up to genus levels. The taxonomic classification and relative abundance of the bacterial communities at the phylum and class levels are given in (Figure 6.12). At the phylum level, a significant difference was observed in the relative abundances of bacterial groups between the samples (Figure 6.12a), with Proteobacteria (~ 61%) being the most abundant phyla, followed by Bacteroidetes (~16.8 %), Firmicutes (~14.5%), Actinobacteria (~8.4%). The other dominant phyla include Chloroflexi (~ 5.9%), Cyanobacteria (~3.6 %) etc. In this study, significant variations were observed in the relative abundance of bacterial groups between different samples, with higher relative abundance of specific bacterial taxa at different days being the most significant finding. The results showed that the microbial communities of SDPA consisted of different bacterial groups, with Proteobacteria being the predominant group, followed by Bacteroidetes, Firmicutes and Actinobacteria, similar results were previously observed by (Deng et al., 2019; Ruan et al., 2019). The relatively higher abundance of Proteobacteria across all the samples indicates the dominance and adaptation of this bacterial taxa to complex environments, as well as their role in role sewage treatment and contaminant degradation (Lin et al., 2022; D. Liu et al., 2021; Zhang et al., 2021).

Similarly, the relatively higher abundance of Firmicutes across all the samples suggest the potential of this bacterial group to thrive under harsh conditions and ensure their role in in aerobic fermentation and pollutant removal (Y. Liu et al., 2021). The most prominent functional bacteria at the genus level include *Methanosaeta* (0.09- 0.9%) and *Syntrophobacter* (1.6 - 4.95%), and both these bacterial groups are the dominant sulfate-reducing bacteria in SDPA (Liu et al., 2018; Shi et al., 2020). Similarly, the higher relative abundance of bacterial strains such as *Desulfohalobium* (0.14 -0.86%), *Thiobacillus* (0.09- 2.17%), *Thermomonas* (0.13 - 3.1%), *Ottowia* (0.06 - 2.6%) and *Sulfurimonas* (0.24-2.07%), suggests their role in denitrification, as reported previously (Deng et al., 2019; Han and Perner, 2015). All the strain have the capability of reducing sulfur and nitrate or nitrite compounds and use them as energy sources (Broman et al., 2017; Moloantoa et al., 2022). Meanwhile, one bacterial group belonging to Anammox genera such as *Kuenenia* (1.8%), was detected in the SDPA system. It has been reported that this bacterial group has high affinity for ammonia and nitrite and has a strong ability to co-oxidise ammonium and propionate in presence of nitrate and nitrite (Deng et al., 2019; Oshiki et al., 2016). Overall, the relatively higher abundances of all these bacterial genera in SDA process significantly influences sulfur metabolism, process dynamics as well as nitrogen removal performance. The findings of this study are similar to those of (Deng et al., 2022; Guo et al., 2022; Li et al., 2022).

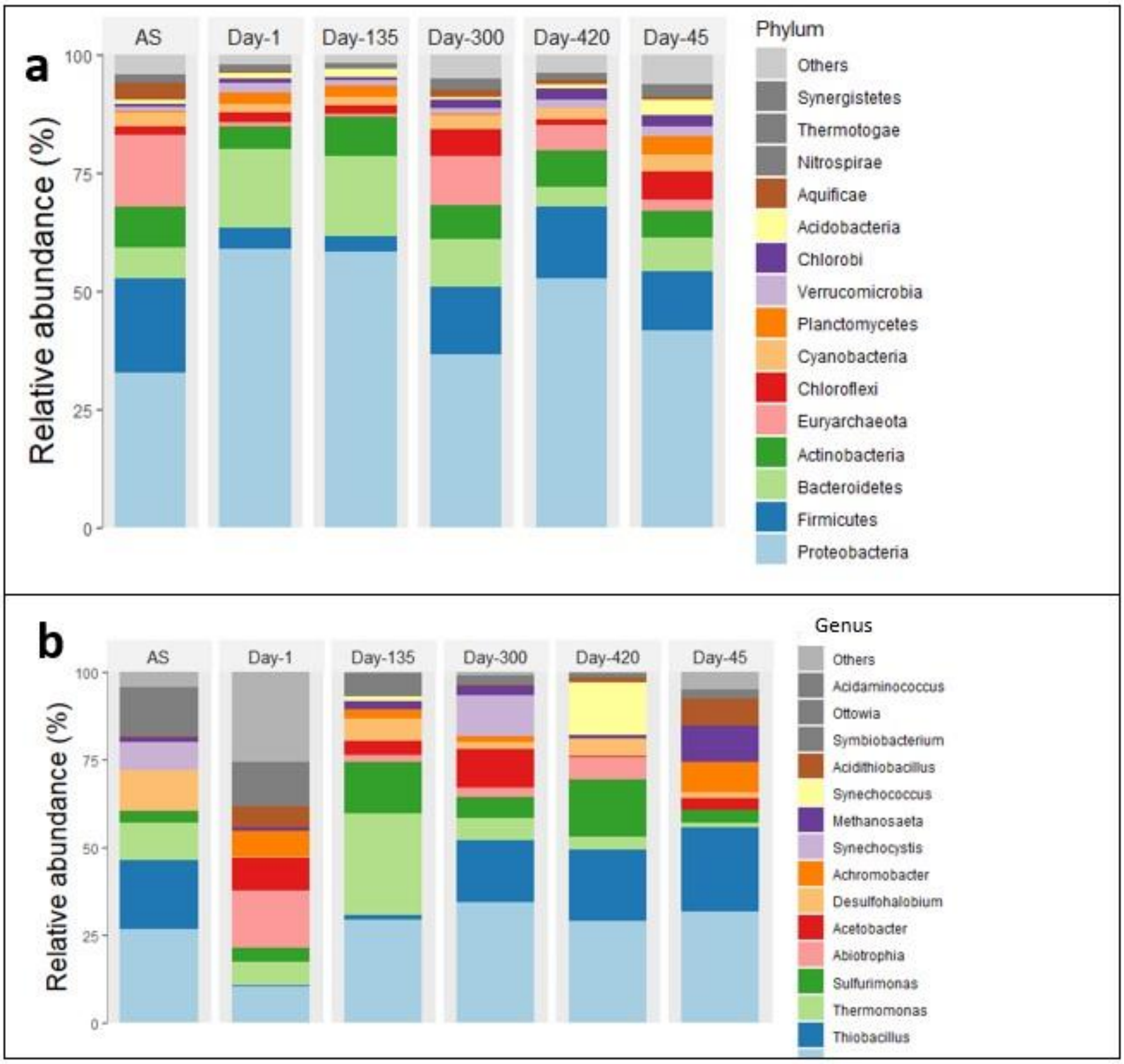

Figure 6.12: Relative abundance (a) phylum and (b) genus of bacterial communities

6.7.3 Functional metagenomics

The functional profiles of different samples were analysed up to 4 levels, starting from the most elementary to the functional level. At the most elementary level of predicted protein functions, all the samples were predominated by proteins associated with metabolism and xenobiotic degradation (60.2%), followed by genetic processing (18.5%), environmental processing (14.9%), cellular processes (4.7%) and others (1.7%) (Figure 6.13). At the functional level, it turned out to be transferases and oxidoreductases enzymes associated with amino acid biosynthesis cycle, two-component system, aminoacyl-tRNA biosynthesis and nitrogen cycle responsible for nitrogen metabolism to be the most prominent predicted protein in all samples (Figure 6.14). The researcher then calculated the principal component analysis to observe the differences in mean proportions of functional genes and the results showed a significant difference between the samples. The PCA showed a clear separation of the functional genes from all the samples with PC1 explaining 86.3%, PC2 10.4% and PC3 2.6 % variance respectively (Figure 6.15.)

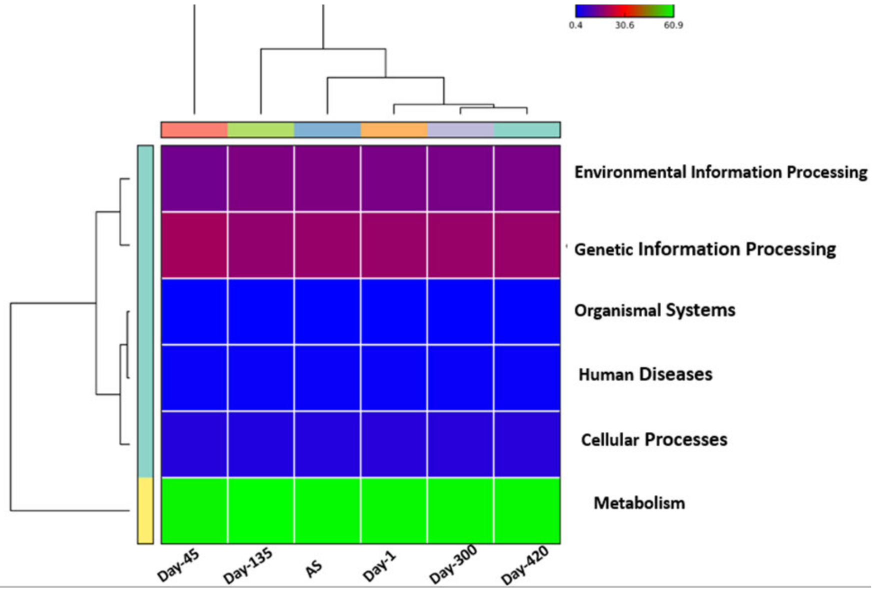

Figure 6.13: Heatmap of COG functional categories of microbial communities

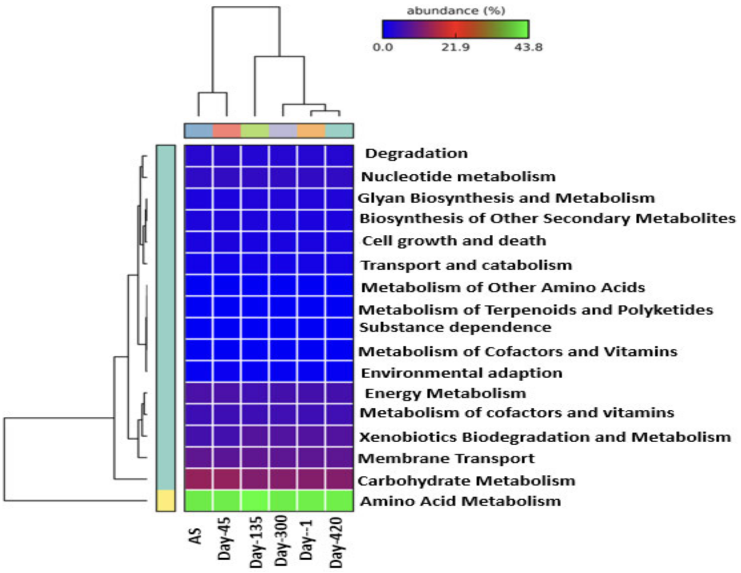

Figure 6.14: Relative abundance of xenobiotic metabolism genes

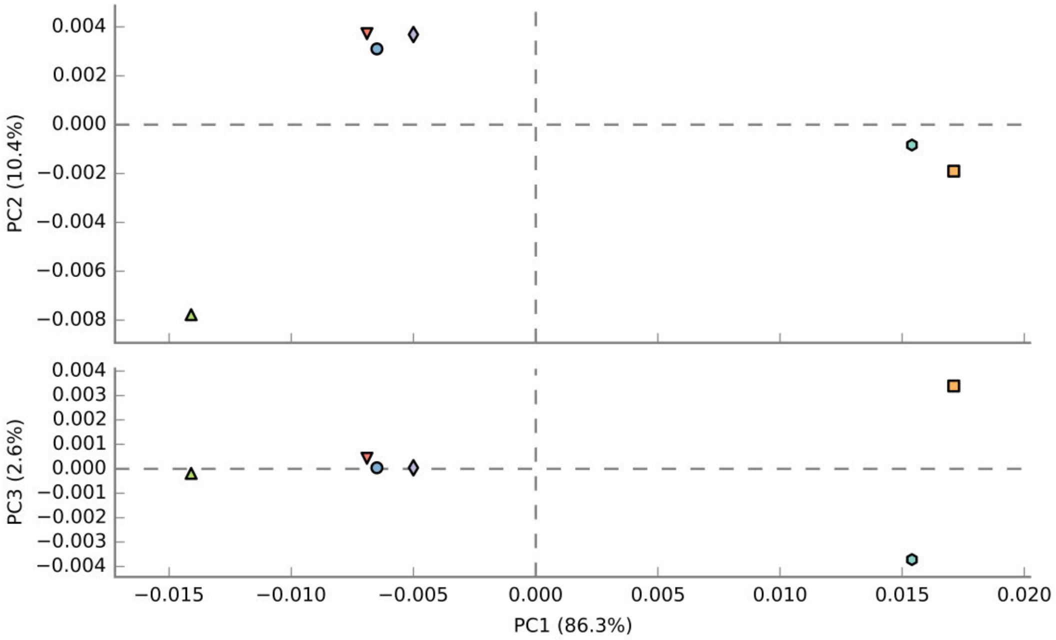

Figure 6.15: Principal component analysis of gene abundance from different samples

6.7.4 KEGG metabolic pathway analysis

6.7.4.1 Nitrogen and Sulfur metabolism Pathways

Figure 6.16 and Figure 6.17 shows different stages of nitrogen and sulphur metabolism pathway. It was observed that the metabolism and conversion of nitrogen in all the samples was closely related to the related abundance of various functional genes. In all the samples, the relative abundances of the genes expressing the enzymes responsible for the reduction, dissimilation and denitrification of nitrogen were 0.025%, 0.011%, 0.016%, 0.020%, 0.010% and 0.0211 % respectively for Day-1, Day-45, Day-135, Day-300, AS and Day 420. Similar results were also reported (Deng et al., 2022; Gao et al., 2022). Generally, the anaerobic systems metabolize sulphates by assimilation and dissimilation processes. The relative abundance of the genes encoding the enzymes responsible for assimilatory and dissimilatory sulfate reduction were found to be 1.35%, 1.33%, 1.32%, 1.29%, 1.39% and 1.36% respectively for Day-1, Day-45, Day-135, Day-300, AS and Day-420. From the perspective of the relative abundance of the key enzymes in the assimilatory and dissimilatory pathways of sulfate reduction, no obvious difference in the amount of sulfate was observed across different sampling days. The results of the present study were more or less similar to the previously conducted studies of Li et al. (2019) and Yuan et al. (2021).

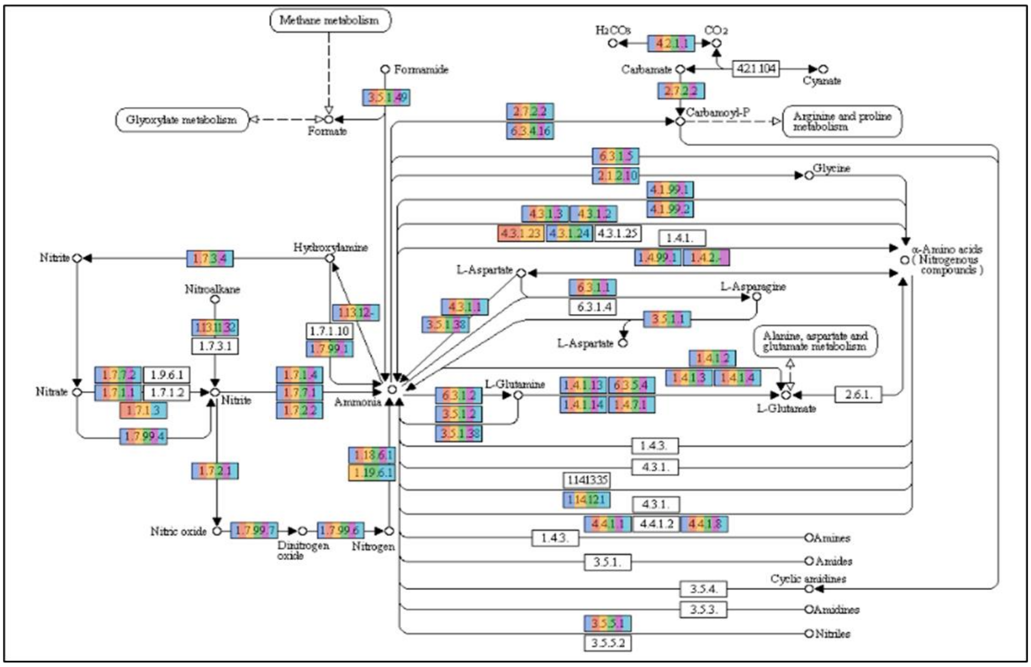

Figure 6.16a: The nitrogen metabolism cycle KEGG map of different samples

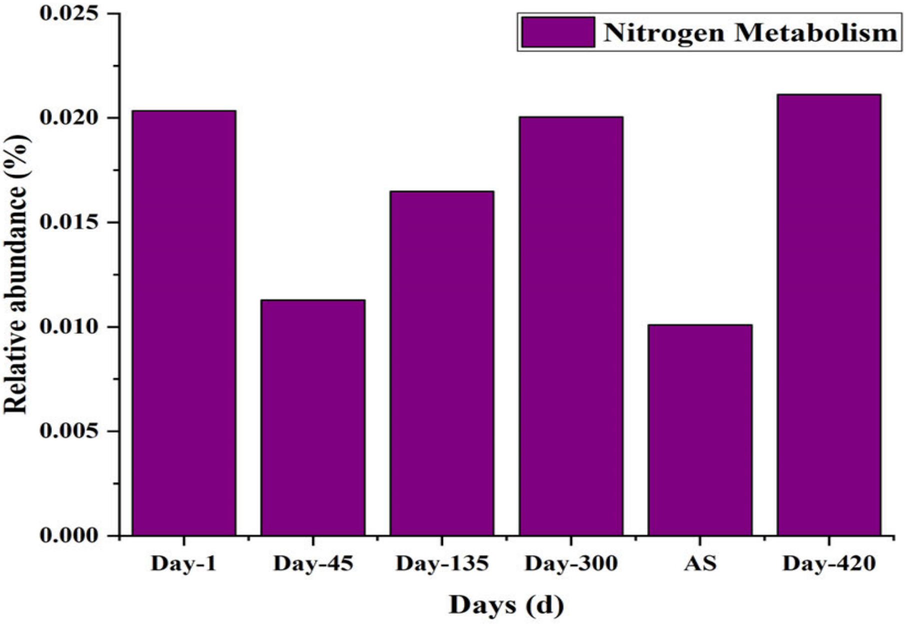

Figure 6.16b: Abundance of functional genes involved in nitrogen metabolism.

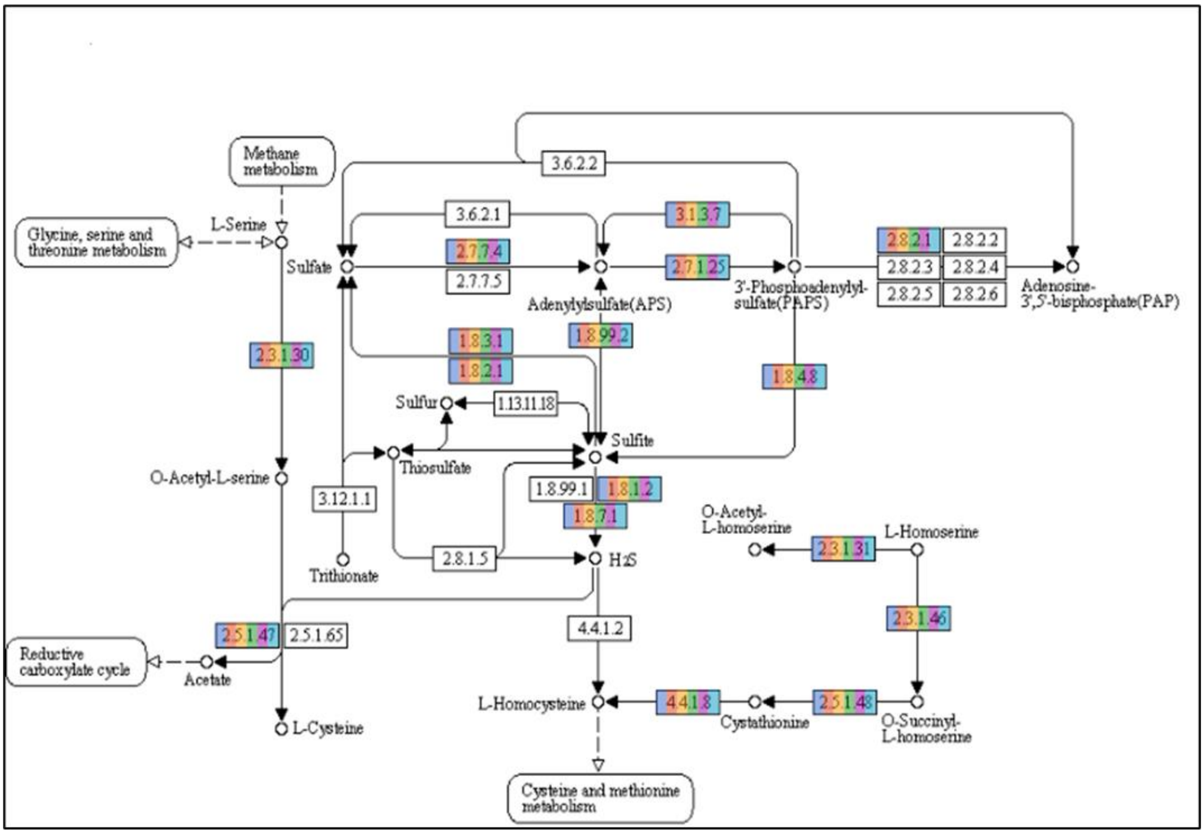

Figure 6.17a: Sulfur metabolism cycle KEGG map of different samples

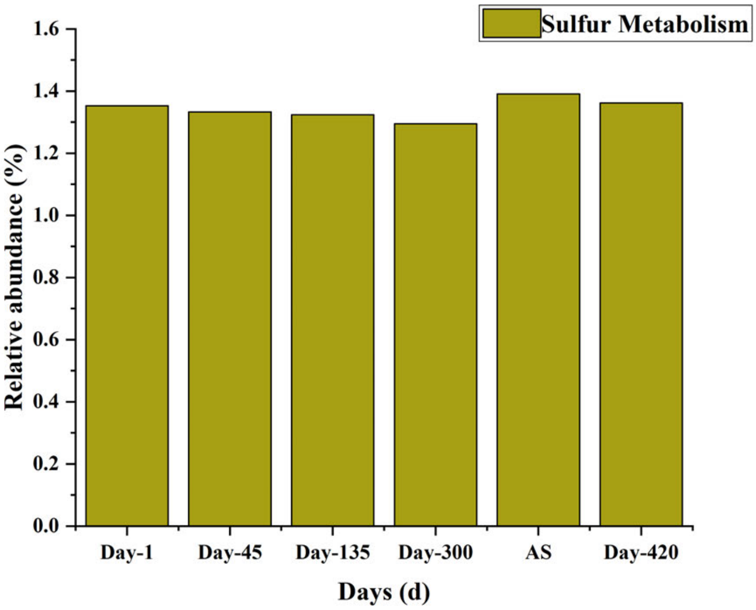

Figure 6.17b: Relative abundance of the functional genes involved in sulfur metabolism across the samples based on SEED sub-system.

6.8 Applications implications

The findings of this study have significant applications and implications for the treatment of wastewater in various industries, including pharmaceuticals, petrochemicals, and food processing. The coupled autotrophic partial-denitrification and Anammox system demonstrated high nitrogen removal efficiency, particularly during Phase III and IV, indicating its potential as a viable treatment option for nitrogen-rich wastewater.

The study provides important insights into the potential application of the SPDA reactor for wastewater treatment, particularly in terms of nitrogen and sulfide removal. The results suggest that the SPDA reactor can effectively remove NH_4-N and TN from wastewater, with significant improvements in removal efficiency observed as the study progressed. Furthermore, the addition of sulfide dosing in Phase IV was found to further improve nitrogen removal efficiency, particularly in regard to NO_3-N removal.

The potential applications of the SPDA reactor in wastewater treatment are significant. Nitrogen removal is a critical step in wastewater treatment, as excess nitrogen can cause eutrophication and other environmental problems. The SPDA reactor's ability to effectively remove nitrogen from wastewater makes it a promising candidate for use in wastewater treatment plants.

Moreover, the use of sulfide dosing in the SPDA reactor can provide additional benefits beyond nitrogen removal. Sulfide is a common contaminant in wastewater, and its removal can be challenging. The SPDA reactor's ability to effectively remove sulfide from wastewater can improve the overall quality of the treated wastewater and reduce the potential for environmental problems.

The results of this study also have implications for the development of new wastewater treatment technologies. The SPDA reactor's performance highlights the potential benefits of combining different biological processes in a single reactor to achieve optimal wastewater treatment. The mechanisms driving the observed improvements in nitrogen and sulfide removal efficiency in the SPDA reactor require further research, and this information can be used to develop more effective and efficient wastewater treatment technologies.

Furthermore, the use of the SPDA reactor can also have economic implications. The reactor's ability to effectively remove nitrogen and sulfide from wastewater can reduce the need for expensive chemical treatments and energy-intensive processes, leading to lower operating costs and potentially lower overall treatment costs. This can make the treatment of wastewater more accessible and affordable, particularly in regions where the development of new wastewater treatment technologies is critical.

6.9 Conclusions

The study investigated the nitrogen removal performance of an SPDA reactor operated at different phases and HRTs. The reactor demonstrated varying levels of nitrogen removal efficiency throughout the experiment. During the early phases, the reactor showed relatively low nitrogen removal efficiency, but as the experiment progressed, nitrogen removal efficiency greatly increased. The results suggest that the SPDA reactor harnessed the power of both sulfide-driven partial denitrification and Anammox, which are two distinct pathways capable of removing nitrogen species through different mechanisms. The reactor was able to shift its biological processes over time in response to changes in influent and operational conditions.

Sulfide dosing in Phase IV contributed to the observed increase in nitrogen removal efficiency. The combination of sulfide-driven partial denitrification and Anammox processes is a promising approach for achieving efficient nitrogen removal, particularly in low-nitrogen concentration wastewater. The sulfide dosing also had an impact on the sulfide removal efficiency of the SPDA reactor. In earlier phases, sulfide removal was minimal, but in Phase IV, the reactor efficiently removed sulfide from the influent, maintaining a sulfide-free effluent. The study also highlighted the importance of maintaining the balance between nitrite reduction, partial denitrification, and Anammox in the SPDA system. Anammox was the dominant pathway for nitrogen removal, while partial denitrification played a more significant role in the earlier stages of the experiment.

The results revealed significant variations in the relative abundance of bacterial groups at different sampling days. Proteobacteria was the predominant group, followed by Bacteroidetes, Firmicutes, and Actinobacteria. This indicates the dominance and adaptation of Proteobacteria to complex environments and its role in sewage treatment and contaminant degradation. Firmicutes also showed relatively higher abundance, suggesting their potential to thrive in harsh conditions and contribute to aerobic fermentation and pollutant removal. At the genus level, prominent functional bacteria included *Methanosaeta, Syntrophobacter, Desulfohalobium*, *Thiobacillus, Thermomonas, Ottowia, and Sulfurimonas*. These bacteria are known for their involvement in sulfate reduction, denitrification, and the ability to utilize sulfur and nitrate/nitrite compounds as energy sources. The presence of the Anammox genus Kuenenia was also observed, indicating its role in co-oxidizing ammonium and propionate in the presence of nitrate and nitrite. Figures 6.17a, b depict the proposed treatment process for wastewater containing organic compounds, ammonium, and sulfate.

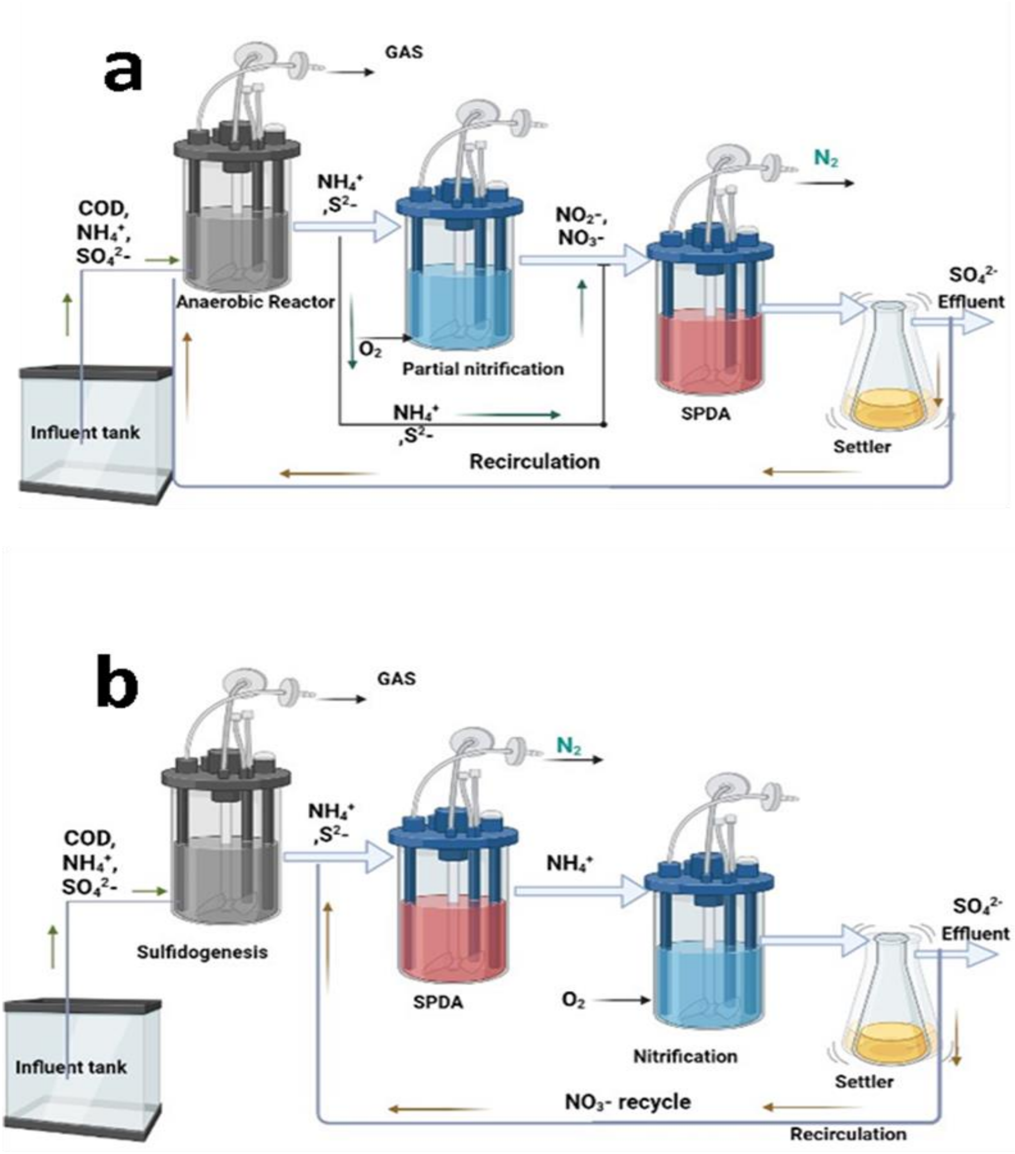

Figure 6.18: The proposed treatment process for wastewater (a,b) containing organic compounds, ammonium, and sulfate

CHAPTER SEVEN

7.0 CONCLUSIONS AND RECOMMENDATIONS

7.1 General conclusion

Each objective has led to important conclusions, which can be summarized as follows:

Controlling dissolved oxygen (DO) levels below 5 mg/L proved crucial in achieving successful partial nitrification (PN) with high nitrite accumulation. This highlights the importance of DO control for efficient nitrogen removal. Lowering the pH to suppress nitrite-oxidizing bacteria (NOB) activity and subsequently returning it to normal conditions for ammonia-oxidizing bacteria (AOB) growth was an effective strategy for PN start-up. This selective promotion of AOB while inhibiting NOB resulted in improved nitrogen removal efficiency. The addition of sulfide at a concentration of 25 mg/L in the wastewater had a beneficial effect on promoting partial nitrification. Sulfide acted as an inhibitor to NOB, specifically targeting nitrite oxidation. This emphasizes the potential of sulfide as a tool for achieving stable PN processes.

The use of the sodium salt of sulphide holds the potential for establishing partial nitrification (PN) under high dissolved oxygen (DO) conditions. The basic hydrolysis of the sodium salt results in a pH increase, facilitating PN at lower sulphide concentrations. This strategy shows promise for real wastewater treatment after the removal of organic carbon. Harnessing the basic hydrolysis of sodium sulphide led to a 50% decrease in the inhibition coefficient of nitrite-oxidizing bacteria (NOB). This indicates that lower sulphide concentrations are required to inhibit NOB activity, resulting in reduced chemical requirements. Future economic analysis is needed to evaluate the trade-offs between this strategy and conventional PN systems, considering cost-effectiveness and feasibility.

The effect of sulfide on partial nitrification can have both positive and negative outcomes depending on the specific conditions and concentrations involved. One of the main effects of sulfide on partial nitrification is its potential to inhibit the growth and activity of nitrite-oxidizing bacteria (NOB). Sulfide can act as a competitive inhibitor for the enzyme responsible for nitrite oxidation, thus reducing the nitrite conversion to nitrate. This inhibition can lead to the accumulation of nitrite and the establishment of a partial nitrification process, where ammonia is converted to nitrite but not further to nitrate.

This partial nitrification can be desirable in certain wastewater treatment scenarios where a controlled nitrite accumulation is beneficial, such as for shortcut nitrogen removal or for the promotion of Anammox processes. However, the presence of sulfide can also have negative effects on partial nitrification if the concentrations exceed certain thresholds. High sulfide levels can inhibit both ammonia-oxidizing bacteria (AOB) and NOB, leading to a decline in the overall nitrification activity. This inhibition can disrupt the balance between AOB and NOB, resulting in reduced ammonia removal efficiency and the accumulation of both ammonia and nitrite in the system. Therefore, it is crucial to carefully manage sulfide concentrations to maintain an optimal range for partial nitrification to occur effectively. The specific effects of sulfide on partial nitrification depend on factors such as the sulfide concentration, the duration of exposure, and the microbial community composition. It is important to monitor and control sulfide levels to ensure the stability and performance of partial nitrification processes in wastewater treatment systems. Proper process design, operational control, and optimization strategies can help mitigate the negative effects of sulfide and harness its potential benefits for achieving efficient nitrogen removal.

Sulfide has a short-term negative impact on the nitrogen removal performance of Anammox bacteria in a UASB reactor. However, the Anammox process can quickly recover under low sulfide stress, indicating its resilience. Long-term acclimatization to sulfide leads to an increased sulfide-resistance ability in the Anammox process, highlighting the importance of implementing inhibition-relief measures when sulfide inhibits the process. Monitoring the stoichiometric ratio is a useful tool for understanding the performance of Anammox bacteria under varying sulfide concentrations. High sulfide concentrations deviate the Anammox process from theoretical values, with a decreased RS value and a significantly low Rp value. This highlights the importance of considering sulfide effects on the performance of Anammox bacteria. The contribution of Anammox to nitrogen removal decreases significantly at high sulfide concentrations, while denitrification becomes more dominant. Environmental conditions play a crucial role in microbial community composition and their contributions to nitrogen removal in wastewater treatment systems. Sulfide concentration affects the microbial community responsible for nitrogen removal in an Anammox process. Higher sulfide concentrations lead to a decrease in Anammox bacteria abundance and an increase in Nitrobacter spp., indicating a shift towards nitrite oxidation. Controlling sulfide concentration is vital to optimize the performance of Anammox processes.

The combined sulfide-driven partial denitrification and Anammox process offers promising potential for efficient nitrogen removal in wastewater treatment. By harnessing the synergistic interactions between sulfide inhibition of nitrite-oxidizing bacteria (NOB) and the simultaneous Anammox reaction, this process can achieve simultaneous nitrogen removal through nitrite reduction and ammonia oxidation. The addition of sulfide to the system acts as an inhibitor for NOB, suppressing their activity and allowing for the accumulation of nitrite. This controlled nitrite accumulation is beneficial for partial denitrification, where nitrite is reduced to nitrogen gas through Anammox bacteria. This process provides a shortcut in the nitrogen removal pathway, bypassing the traditional nitrate production step, resulting in enhanced nitrogen removal efficiency and reduced energy requirements.

Moreover, the combined sulfide-driven partial denitrification and Anammox process offers advantages such as lower sludge production, reduced carbon requirements, and improved resilience to fluctuations in influent conditions. The Anammox bacteria involved in the process are known for their slow growth rates and low biomass production, making them suitable for achieving stable and sustainable nitrogen removal. However, it is important to note that the success of this process is dependent on careful control of sulfide concentrations. Sulfide levels must be maintained within an optimal range to inhibit NOB effectively without inhibiting Anammox bacteria or other key microorganisms involved in the process. Close monitoring and precise dosing of sulfide are crucial to ensure process stability and prevent potential negative effects. In conclusion, the combined sulfide-driven partial denitrification and Anammox process shows promise as an innovative and sustainable approach for efficient nitrogen removal in wastewater treatment. With further research and development, this process has the potential to contribute to the advancement of environmentally friendly and energy-efficient nitrogen removal technologies.

7.2 Recommendations

- Further investigation of the optimal DO levels during intermittent aeration for PN is required, considering different wastewater characteristics and reactor configurations. This could help refine the operational parameters for enhanced nitrogen removal. Exploration of pH control strategies for sustained PN operation, including the determination of the optimal pH range and the impact of different pH shock durations. This would contribute to improving process stability and promoting AOB activity. Continued research on the effects of sulfide concentration on PN performance and the associated microbial community dynamics. Understanding the mechanisms of sulfide inhibition and its long-term effects will help optimize the use of sulfide as an inhibitor for NOB and improve nitrogen removal efficiency. Further investigation into the application of these findings in full-scale wastewater treatment plants, considering the scalability, cost-effectiveness, and practical implementation of PN strategies. Field trials and long-term monitoring could provide valuable insights into the real-world performance and operational challenges of implementing PN.

- Future research should focus on investigating the interactions between Anammox and PN with sulphide dosage under uncontrolled pH conditions. Understanding these interactions will contribute to optimizing the simultaneous application of Anammox and PN in a single reactor system. This integration has the potential to enhance the application prospects of Anammox in domestic wastewater treatment.

- Exploring and developing effective inhibition-relief measures to mitigate the short-term negative effects of sulfide on the Anammox process should be done. Furthermore,an investigation strategies for reducing or removing sulfide in the influent to accelerate the recovery of Anammox performance and minimize its deviation from theoretical values should be carried out. A study on the long-term effects of sulfide on the microbial community dynamics and metabolic pathways involved in nitrogen removal to better understand their responses and optimize process conditions can be carried out. Investigating the potential interactions between sulfide and other environmental factors, such as pH, temperature, and organic loading, to assess their combined effects on the performance of Anammox bacteria is needed. In addition, assessing the feasibility of incorporating sulfide-monitoring systems and control strategies into wastewater treatment plants to optimize the performance and stability of Anammox processes is required. Exploring the application of advanced molecular techniques, such as metagenomics and metatranscriptomics, to gain a deeper understanding of the microbial community composition and functional gene expression in sulfide-driven Anammox systems should also be done.

- Further optimization of reactor design: Future research should focus on optimizing the reactor design for integrated sulfide-driven partial denitrification and Anammox processes. Investigating various reactor configurations, such as sequencing batch reactors

(SBRs), moving bed biofilm reactors (MBBRs), or membrane bioreactors (MBRs), could provide valuable insights into enhancing process efficiency, minimizing operational costs, and maximizing nitrogen removal rates.

- Scaling-up and field implementation: While sulfide-driven partial denitrification and Anammox have shown promise at laboratory and pilot scales, further research is needed to evaluate their feasibility and performance under full-scale operating conditions. Scaling-up studies and field trials in real wastewater treatment plants can provide valuable data on process stability, robustness, and economic viability, ultimately paving the way for the widespread implementation of this integrated approach.

- Integration with other treatment technologies: Exploring the integration of sulfide-driven partial denitrification and Anammox with other treatment technologies can lead to synergistic effects and improved overall nitrogen removal efficiency. Investigating potential synergies with processes like anaerobic ammonium oxidation (Anammox), enhanced biological phosphorus removal (EBPR), or membrane filtration can optimize resource utilization, enhance process stability, and improve the overall environmental sustainability of wastewater treatment systems.

- Life cycle assessment and techno-economic analysis: Conducting comprehensive life cycle assessments and techno-economic analyses of the sulfide-driven partial denitrification and Anammox process is crucial for evaluating its environmental impact and economic viability. Assessing the energy requirements, carbon footprint, and cost-effectiveness of the process can guide decision-making processes and facilitate its integration into existing wastewater treatment infrastructure.

- Application in niche environments: While the integrated sulfide-driven partial denitrification and Anammox process has primarily been explored for wastewater treatment, its potential application in niche environments should also be investigated. For example, examining its feasibility for nitrogen removal in aquaculture systems, industrial effluents, or agricultural runoff can provide sustainable solutions for nitrogen management in these specific contexts.

7.3 Findings and Implication of this work

The findings of this study reveal the immense potential of the Sulfide-Driven Partial Denitrification and Anammox (SPDA) process for the simultaneous removal of nitrate, ammonium, and sulfide from wastewater. Notably, the SPDA process can be seamlessly integrated with anaerobic and aerobic treatment methods, enabling the simultaneous removal of organics and nitrogen compounds. The proposed process flow, depicted in Figure 6.18, outlines the sequential steps for efficient treatment of wastewater containing organics, ammonium, and sulfate.

The initial stage involves an anaerobic reactor operated under sulfidogenic conditions, where organic compounds are degraded, and sulfate is reduced to sulfide. This step facilitates the conversion of complex organic matter into simpler forms, promoting subsequent treatment processes. Following the anaerobic reactor, a second anoxic reactor is employed to execute the SPDA process for nitrogen removal. Within this reactor, the synergistic interactions of microorganisms lead to the removal of sulfide, ammonium, and recirculated nitrate. The SPDA process demonstrates its capability to effectively remove multiple contaminants, enhancing the overall efficiency of the wastewater treatment system. Subsequently, the wastewater flows into a third aerobic reactor, primarily dedicated to nitrification reactions. In this aerobic environment, specific microorganisms facilitate the oxidation of ammonium to nitrate, further reducing the nitrogen load in the wastewater. By incorporating this aerobic stage, the proposed process achieves comprehensive nitrogen removal, ensuring compliance with stringent effluent quality standards. The integrated SPDA process holds tremendous potential for various industrial wastewater treatment settings. Industries such as pulp and paper, food processing, and pharmaceuticals can benefit from its application. Additionally, the process's versatility extends to the treatment of saline sewage, where high sulfate concentrations resulting from seawater toilet flushing and seawater intrusion can be effectively addressed. This versatility highlights the adaptability and broad applicability of the SPDA process in diverse wastewater treatment scenarios.

In conclusion, the SPDA process emerges as a promising solution for the simultaneous removal of nitrate, ammonium, and sulfide from wastewater. Its seamless integration with anaerobic/aerobic treatment methods, coupled with its potential applications in various industrial and saline sewage treatment settings, positions the SPDA process as a highly effective and versatile approach for sustainable wastewater treatment.

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Appendix A: Achieving partial nitrification by harnessing basic hydrolysis of sulphide salts amid high dissolved oxygen conditions.

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Table S1. Primers used for PCR amplification of nitrifiers and their functional genes.

Gene target	Primer name	Primer sequence (5′→3′)	Base pair	Reference
AOB 16S rRNA	CTO 189fA/B CTO 189fC RT1r	GGAGRAAAGCAGGGGATCG GGAGGAAAGTAGGGGATCG CGTCCTCTCAGACCARCTACTG	467	(Hermansson and Lindgren, 2001)
***Nitrospira* 16S rRNA**	NSR1113F NSR1264R	CCTGCTTTCAGTTGCTACCG GTTTGCAGCGCTTTGTACCG	151	(Wang *et al.*, 2014)
***Nitrobacter* 16S rRNA**	Nitro 1198f Nitro 1423r	ACCCCTAGCAAATCTCAAAAAACCG CTTCACCCCAGTCGCTGACC	229	(Graham *et al.*, 2007)
Sulfide-oxidizing bacteria	RP2060F DSR4R	CAACATCGTYCAYACCCAGGG GTGTAGCAGTTACCGCA	350	(Giloteaux *et al.*, 2010)
Sulfide-oxidizing	DSR1F RH3-DSR-	CSCACTGGAAGCACG GGTGGAGCCGTGCATGTT	222	(Giloteaux *et al.*, 2010)

bacteria	R

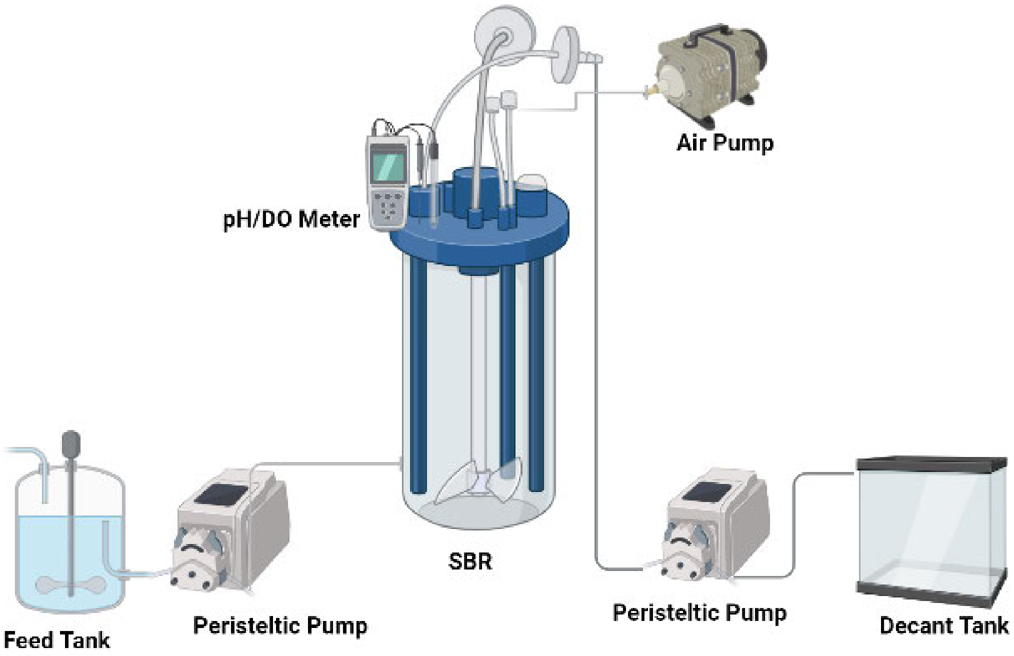

Figure S1. Schematic diagram of partial nitrification process in a sequential batch reactor.

Table S2. Characteristics of the effluent of the wastewater treatment plant used as a feed in phase 4a and phase 4b (Mean ± SD). Ammonia and nitrite were spiked in the effluents to characterize anaerobic effluent concentrations.

Parameter	Value
pH	7.57 ± 0.18
COD (mg/L)	75.17 ± 6.71
NH_4-N (mg N/L)	5.24 ± 2.35
NO_2-N (mg N/L)	0.23 ± 0.23

NO_3-N (mg N/L)	1.04 ± 0.61

Table S3. Concentrations of the biomass in the reactor used for experimental study. (Mean ± SD)

	Phase 1a	Phase 1b	Phase 2a	Phase 2b	Phase 3a	Phase 3b	Phase 4a	Phase 4b
MLSS (mg/L)	4644 ± 888	4848 ± 586	4465.2 ± 829	4157 ± 539	4328 ± 629	4547.25 ± 1173	4041 ± 948	4195 ± 740
MLVSS (mg/L)	3542.4 ± 594	3324 ± 401	2704 ± 731	2136 ± 341	2533 ± 748	2953.5 ± 497	2093 ± 445	2266 ± 581

Calculations for TN loss:

We calculated the total nitrogen loss due to sulfide addition (Table S4). These calculations were done based on stoichiometric demands, given (1 mole of S^{2-} needs 1.6 moles of NO_3 to convert into 2 moles of SO_4^{2-} The purpose of the calculation was to determine the total nitrogen loss due to sulfide addition in partial nitrification. Calculations were done as follows.

1. **Added mM of S^{2-}**= Added sulfide /Molecular weight of sulfide = S^{2-}/32

[$S^{2-} + 1.6\ NO_3 + 0.2\ H_20 \rightarrow 2SO_4^{2-} + 0.8\ N_2 + 0.4H^+$] (Equation 1).

According to above reaction 1 mole of S^{2-} needs 1.6 moles of NO_3 to convert into 2 moles of SO_4^2 Therefore:

2. **mM NO_3 by SND Loss** = 1.6 * mM of S^{2-} {For 25mg-S/L it will be1.6 * 0.788mM of S^{2-}}

3. **NO_3 Loss by SND (mg/L) =** mM $NO_{3\ \mathrm{by\ SND}}$ Loss *Molecular weight of NO_3

4. **NO_3-N loss by SND (mg/L) =** NO_3 Loss by SND (mg/L)/4,4268

5. **Observed NO_3-N loss (mg/L) =** TN influent- TN effluent

6. NO+N2O losses = Observed NO_3-N loss (mg/L) - NO_3-N loss by SND (mg/L).

7. **Average TN Loss (mg/L) = (**NO+ N2O losses) – FA (mg/L)

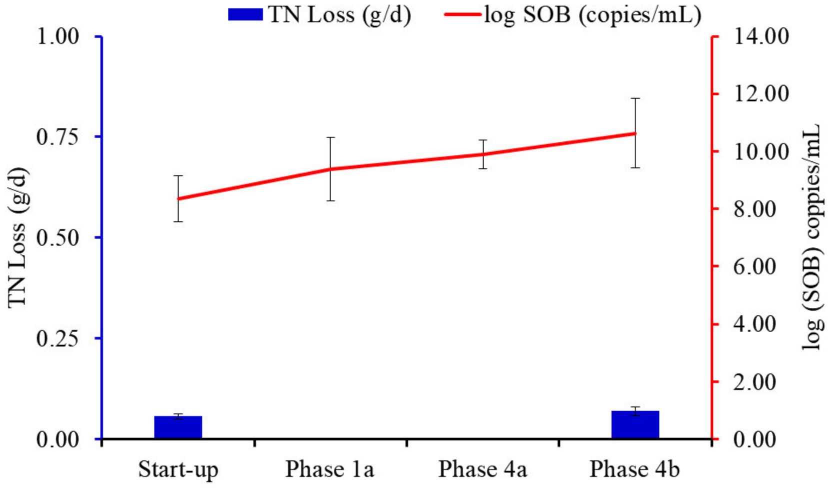

Figure S2 Sulphide oxidizing communities and corresponding nitrogen losses in different operational phases of the reactor, to check loss of nitrate by sulphide derived denitrification. Nitrogen loss was negligible in phase 1a (25 mg S/L at uncontrolled pH with synthetic wastewater) and phase 4a (25 mg S/L at uncontrolled pH with real wastewater), when PN was effectively established.

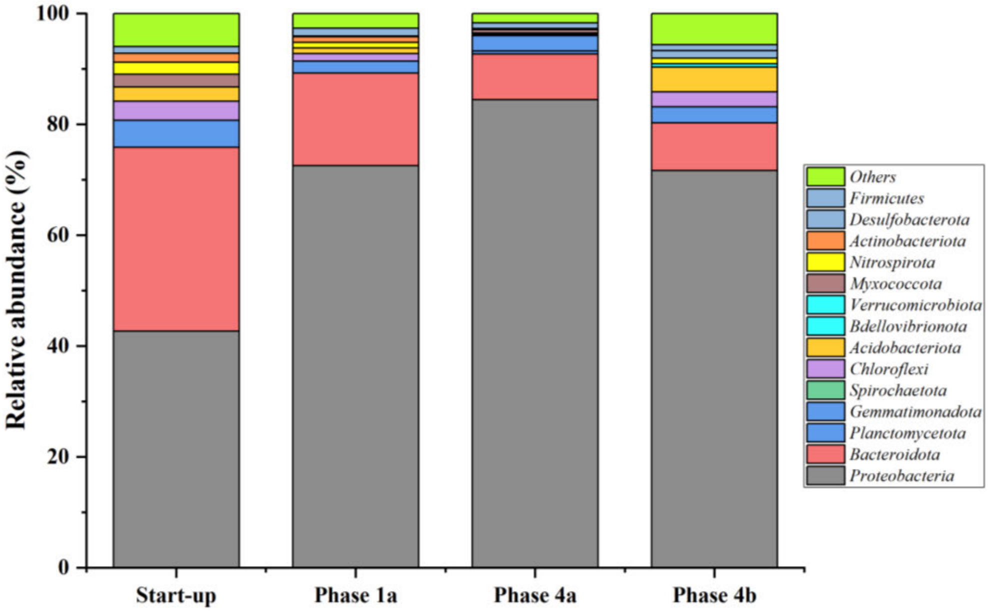

Figure S3 Phylum level abundance of microbial communities in the reactor.

Sourcing of sulphide

The choice of sourcing sulphide for biological wastewater treatment would depend on various factors, including the type of wastewater being treated, the availability of resources, and the specific treatment goals. Chemicals such as sodium sulphide or sodium hydrosulphide can be a reliable source of sulphide for wastewater treatment and are readily available from chemical suppliers. However, the cost of purchasing chemicals may be higher compared to producing sulphide using sulfate-reducing bacteria.

On the other hand, producing sulphide with sulfate-reducing bacteria can be a cost-effective and sustainable method of sourcing sulphide for wastewater treatment. This method involves cultivating and maintaining the bacteria in a laboratory setting, which can be resource intensive. However, the use of sulfate-reducing bacteria may provide other benefits, such as the production of valuable by-products like methane.

Ultimately, the choice of sulphide sourcing method would depend on the specific needs and constraints of the wastewater treatment plant. A combination of both chemical and biological methods may also be used to achieve the desired treatment goals.

Theoretical calculation of the electron/energy consumption costs of this approach.

Assuming a sulphide dosing rate of 25 mgS/L and a wastewater flow rate of 1000 L/day, the total amount of sulphide dosed per day would be:

25 mgS/L x 1000 L/day = 25,000 mgS/day

To convert this amount of sulphide to the number of electrons transferred, we can use the following equation:

$2e- + S2O_3^{2-} + 2H_2O \rightarrow 2HS^{-} + 2SO_4^{2-} + 4H^{+}$

This equation shows that each sulphide molecule ($S2O_3^{2}$-) accepts two electrons (2e-) and produces two hydrogen sulphide molecules ($2HS^{-}$). Therefore, the total number of electrons transferred per day would be:

25,000 mgS/day / 248.18 mgS/mmol / 2 electrons per sulphide molecule = 50.43 mol electrons/day

To calculate the energy consumption costs of this approach, we need to consider the amount of energy required to produce the sulphide. The energy required to produce sulphide depends on the method of sulphide sourcing. For example, producing sulphide through the use of sulfate-reducing bacteria would require energy inputs for the cultivation and maintenance of the bacteria.

Assuming a typical energy consumption rate of 0.1 kWh/m^3 for biological wastewater treatment processes, the energy consumption costs of the sulphide dosing approach would be:

50.43 mol electrons/day x 96,485 C/mol x 1.6 x 10^{-19} J/C x 0.1 kWh/J / 1000 L/day = 0.773 kWh/day

This calculation assumes that all of the electrons transferred are used for wastewater treatment, which may not be entirely accurate due to losses in the system. Additionally, the actual energy consumption costs would depend on various factors, such as the efficiency of the treatment process and the cost of electricity.

Appendix B: Pictures plate

Plate 1. Photographic image of Anammox enrichment reactor.

Plate 2: Photographic image of start-up of Partial Nitrification Reactor.

Plate 3: Phtographic image of effect of sulfide on PN reactor

Plate 4: Photographic image of effect of sulfide on Anammox process

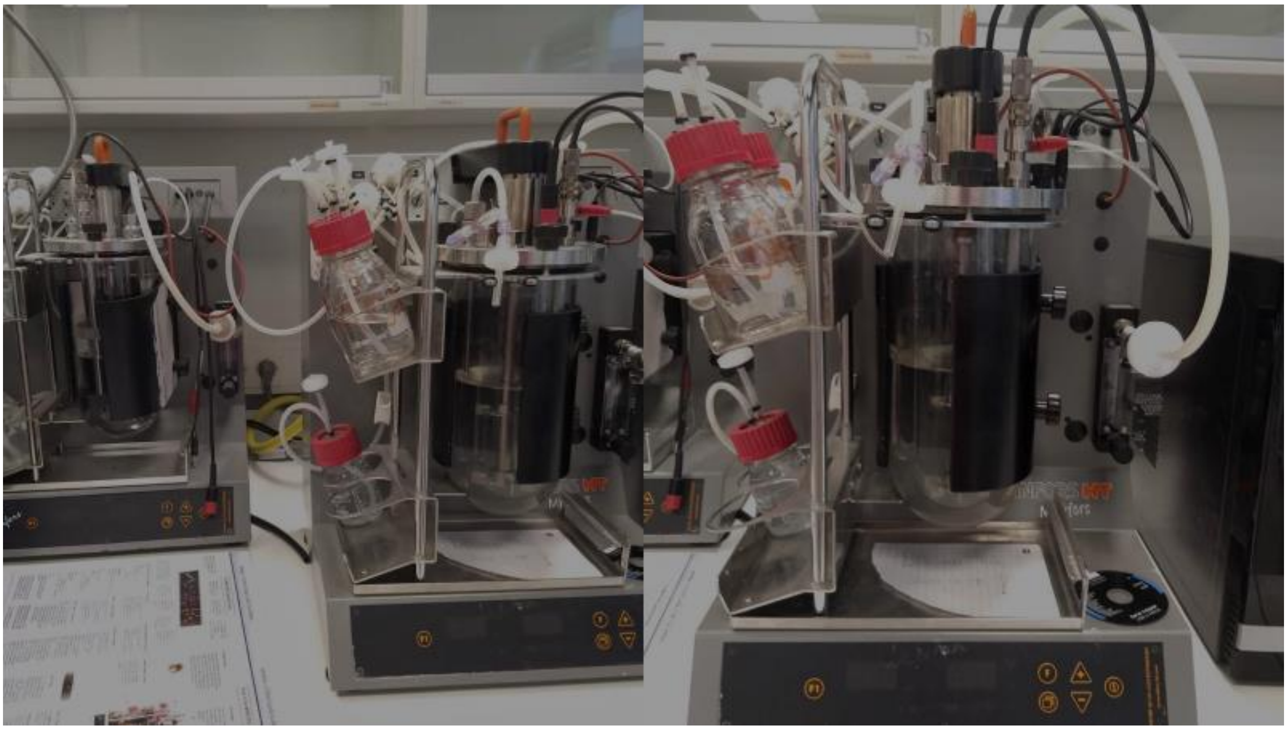

Plate 5: Photographic image of Anammox reactor after the addition of sulphide.

Plate 6: Photographic image of Anammox Bacteria

Plate 7: Photographic image of Enriched Anammox bacteria

Plate 8: Phtographic image of PN reactor studying the long term effects of sulfide on partial nitrification process

Plate 9: Sewage collection at Kawa Mashu WWTP

Plate 10: Reactor set-up

Plate 11: Photographic image of SPDA Reactor treating real muncipal wastewater

Plate 12: Photographic image of the reactor during the study

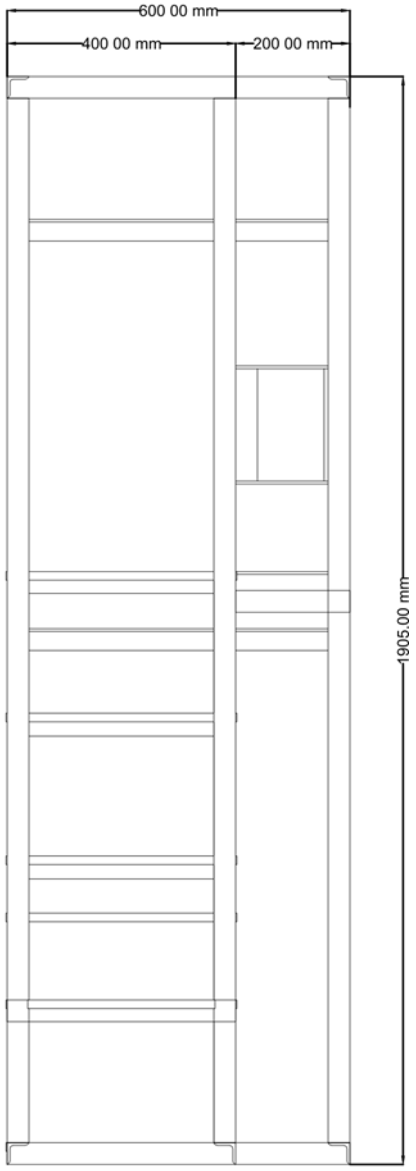

Plate 13: Designing the frame of SPDA Reactor

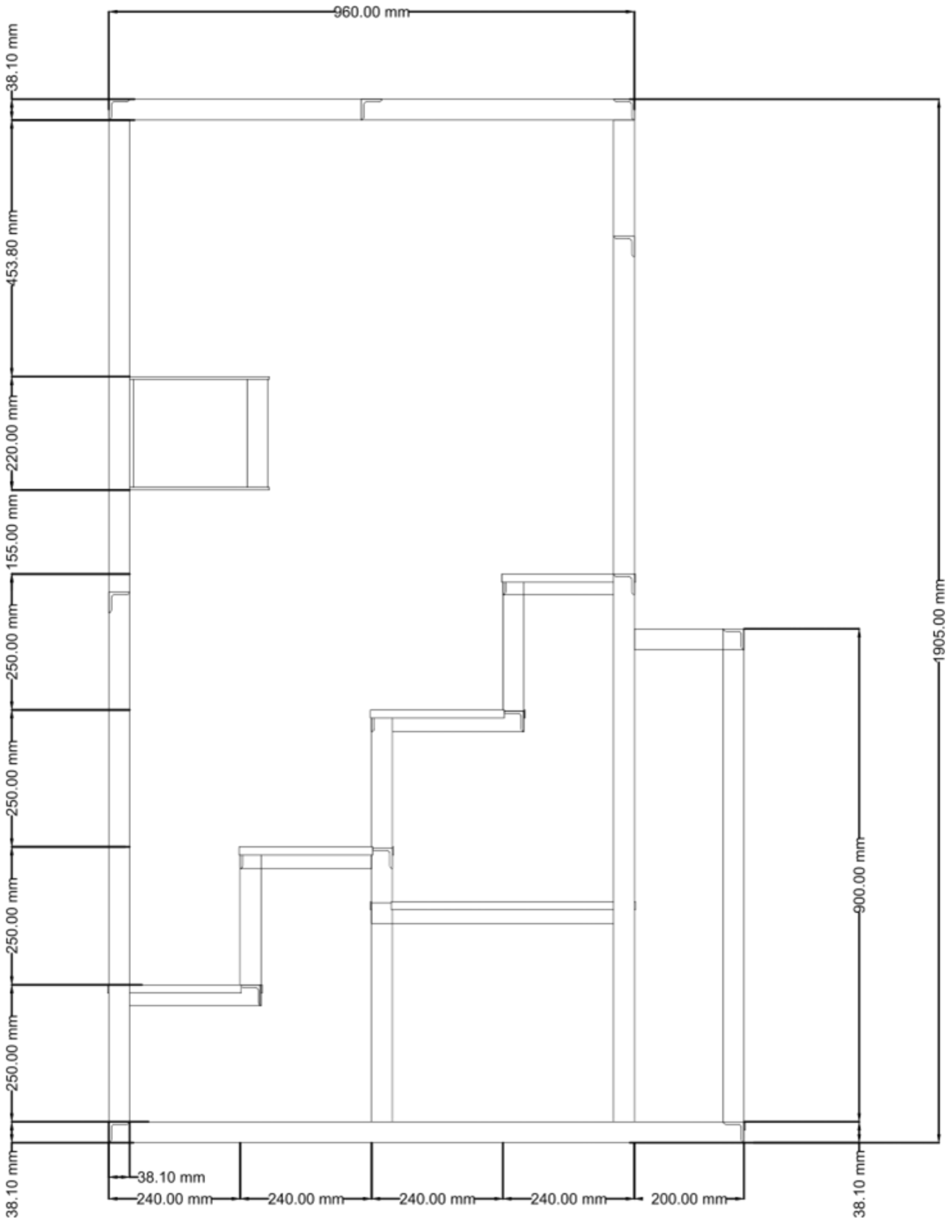

Plate 14: Design of the reactor

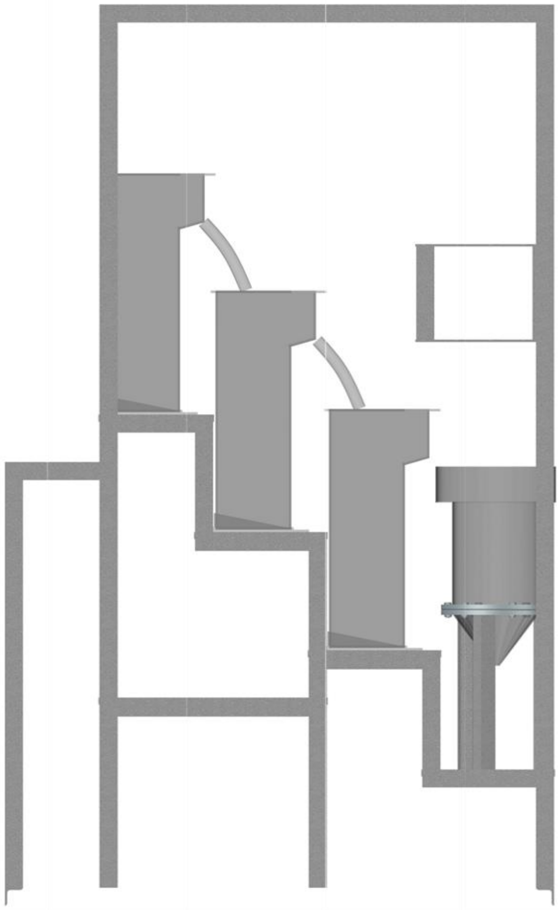

Plate 15: Schematic diagram of the reactor

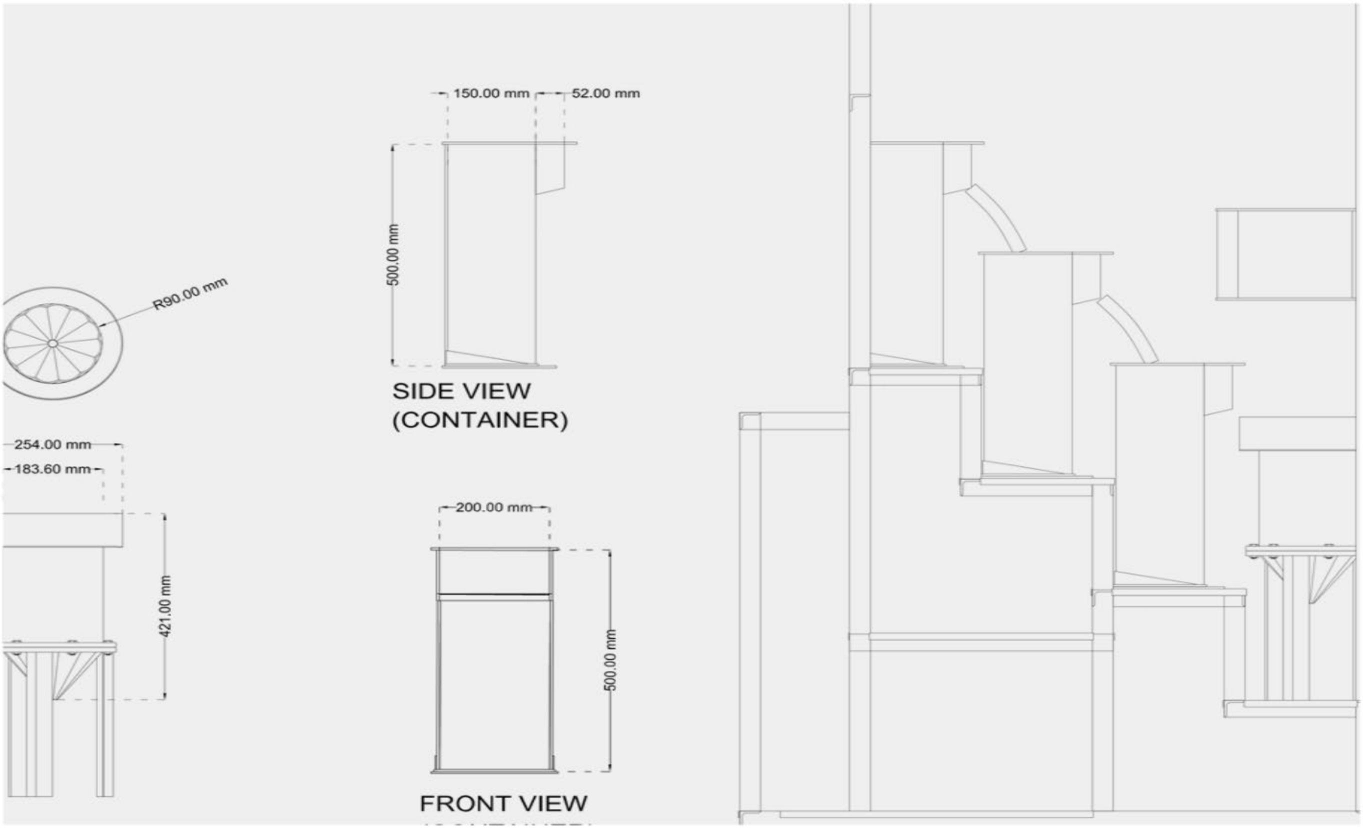

Plate 16: Schematic diagram of the reactor comprising of UASB, PN and SPDA reactors