UNIVERSITY OF TARTU

Faculty of Science and Technology

Institute of Bioengineering

Blessing Ambang Ekwebe

Removal of Nitrogen and Pharmaceutical Compounds in a Biofilm System

Master's Thesis (30 ECTS)

Curriculum Bioengineering

Supervisor(s):

Ivar Zekker (PhD)

Faysal-Al Mamun (MSc.)

Tartu 2026

Abstract

Removal of Nitrogen and Pharmaceutical Compounds in a Biofilm System

As global wastewater generation reaches an estimated 380 billion cubic meters annually, the primary challenge for sanitation infrastructure has shifted from volumetric management to the mitigation of chemical matrices, specifically pharmaceutical active compounds (PhACs). Conventional treatment systems often fail to sequester theses recalcitrant pollutants, which propagate antimicrobial resistance and threaten aquatics ecosystems. This thesis investigates the synergistic potential of an anaerobic ammonium oxidation (anammox) consortium cultivated within a moving bed biofilm reactor (MBBR) for the simultaneous removal of nitrogenous compounds and five target PhACs – marbofloxacin, ofloxacin, enrofloxacin, sulfamethoxazole and carbamazepine.

The experimental results demonstrated that the MBBR system successfully decoupled biomass retention from hydraulic retention time (HRT), allowing for the enrichment of slow-growing anammox bacteria on K1-shaped biofilm carriers. The system achieved nitrogen removals, maintaining stability despite fluctuations in total nitrogen loading. Batch kinetic studies revealed that while nitrogen removal followed a first order model (R^2 up to 0.8299), PhAC attenuation was highly compound specific.

The findings from this project suggest that, while anammox-mediated systems are highly efficient for energy-neutral nitrogen removal, the attenuation of PhACs relies on maintenance of diverse and stratified microbial community. This research provides a fundamental framework for designing next-generation wastewater infrastructure capable of addressing both nutrient and emerging micropollutants ina single-stage process.

Keywords: Anammox, MBBR, pharmaceutical active compounds (PhACs), biofilm, nitrogen removal.

CERCS: P300 Analytical Chemistry

Institute name: Institute of Chemistry

Research group: Environmental and Colloidal Chemistry

Abstrakt

Lämmastiku ja farmatseutiliste ühendite eemaldamine sünergilises süsteemis

Kuna inimeste poolt tekitatud reovee kogus ulatub maailmas hinnanguliselt 380 miljardi kuupmeetrini aastas, on veepuhastusjaamade infrastruktuuri peamine väljakutse nihkunud mahuliselt haldamiselt keemiliste ühendite, täpsemalt farmatseutiliste toimeainete vähendamisele. Tavapärased puhastussüsteemid ei suuda sageli neid raskesti lagundatavaid saasteaineid siduda, lisaks soodustavad nad antimikroobset resistentsust ja ohustavad veeökosüsteeme. See väitekiri uurib liikuvate kandjatega biokilereaktoris (MBBR) kasvatatud anaeroobse ammooniumi oksüdatsiooni protsessi läbiviiva bakteri (anammox) konsortsiumi sünergistlikku potentsiaali lämmastikühendite ja viie ravimi – marbofloksatsiini, ofloksatsiini, enrofloksatsiini, sulfametoksasooli ja karbamasepiini – samaaegseks eemaldamiseks.

Eksperimentaalsed tulemused näitasid, et MBBR-süsteem võimaldas aeglaselt kasvavate anammox bakterite rikastumist K1-tüüpi biokilekandjatel. Süsteem saavutas lämmastiku eemaldamise, säilitades stabiilsuse vaatamata lämmastiku kogukoormuse kõikumistele. Annustestide kineetika uuringud näitasid, et kuigi lämmastiku eemaldamine järgis esimest järku lagunemise mudelit (R^2 kuni 0,8299), oli ravimite lagundamine väga ühendispetsiifiline.

Selle lõputöö tulemused näitavad, et kuigi anammox vahendatud süsteemid on energianeutraalse lämmastiku eemaldamiseks väga tõhusad, sõltub ravimite lagundamine mitmekesise mikroobikoosluse säilimisest. See uuring pakub alusraamistiku järgmise põlvkonna reoveeinfrastruktuuri kavandamiseks, mis on võimeline tegelema nii toitainete kui ka mikroreostusainetega üheetapilise protsessi käigus.

Märksõnad: Anammox, MBBR, farmatseutilised ained , biokile, lämmastiku eemaldamine.

CERCS: P300 Analüütiline keemia

Uurimisrühm: Keskkonna- ja kolloidkeemia

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS

AOB: Ammonia oxidizing bacteria

AS: Activated sludge

BNR: Biological nitrogen removal

CAR: Carbamazepine

COD: Chemical oxygen demand

CWS: Constructed wetlands

DO: Dissolved oxygen

ENR: Enrofloxacin

EPS: Extracellular Polymeric Substances

HAO: Hydroxylamine oxidoreductase

HDH: Hydrazine dehydrogenase

HNAD: Heterotrophic nitrification and aerobic denitrification

HRT: Hydraulic retention time

HZS: Hydrazine synthase

MABR: Membrane aerated biofilm reactor

MBBR: Moving bed biofilm reactor

MBfR: Membrane biofilm reactor

MBR: Membrane bioreactor

MAR: Marbofloxacin

NOB: Nitrite oxidizing bacteria

OFL: Ofloxacin

PhACs: Pharmaceutically active compounds

SBR: Sequencing batch reactor

SMX: Sulfamethoxazole

SND: Simultaneous nitrification and denitrification

UASB: Up flow anaerobic sludge blanket

WWTP: Wastewater treatment plant

INTRODUCTION

Global wastewater generation has reached an estimated 380 billion cubic meters annually, yet the challenge confronting contemporary sanitation infrastructure is no longer volumetric management alone, but rather the escalating complexity of wastewater composition (Afolalu et al., 2024; Kato & Kansha, 2024). Conventional pollutants - organic matter, suspended solids, and nutrients are now accompanied by a suite of emerging contaminants, notably pharmaceutical active compounds (PhACs) and recalcitrant nitrogen species, which evade traditional treatment paradigms and pose documented risks to ecosystem integrity and public health (Crini & Lichtfouse, 2019). Nitrogen contamination alone contributes to eutrophication, and nitrite causes methemoglobinemia in infants, and chronic diseases, while pharmaceutical residues exert sub-lethal pressures on aquatic organisms and propagate antimicrobial resistance (Choudhary et al., 2022; Usman et al., 2022).

Biological nitrogen removal has historically relied upon sequential nitrification-denitrification, a process demanding substantial aeration energy and exogenous organic carbon supplementation (Di Capua et al., 2022; Lin et al., 2009). The discovery of anammox bacteria, chemolithoautotrophic members of the phylum Planctomycetes, transformed wastewater treatment by revealing that ammonium can be directly converted to dinitrogen gas using nitrite under strictly anoxic conditions (Jetten et al., 2009; Li et al., 2018). This metabolic innovation affords 60% reduction in aeration requirements, eliminates dependence on external carbon sources, and generates approximately 75% less residual sludge compared to conventional processes (Fu et al., 2021; Ronan et al., 2021; Usman et al., 2022).

Nevertheless, anammox bacteria are notoriously slow growing microorganisms, characterized by doubling times of 7–20 days, high susceptibility to environmental perturbations, and a propensity for aggregation into biofilms or granules mediated by quorum sensing and extracellular polymeric substance production (Ma et al., 2016). Their obligate anaerobic chemolithoautotrophic lifestyle, while energetically efficient, renders them vulnerable to inhibition by PhACs routinely detected in municipal and hospital wastewaters at concentrations ranging from nanograms to micrograms per liter (Fu et al., 2021; Zhang et al., 2019). Recent surveillance studies have identified 51 PhACs in hospital effluents alone, with removal efficiencies varying from 0% to 100% across conventional treatment configurations, underscoring the inadequacy of existing infrastructure for micropollutant attenuation (Fu et al., 2021).

This thesis addresses the fundamental question: can an anammox-enriched consortium cultivated within an MBBR configuration achieve simultaneous removal of nitrogenous compounds and PhACs in a synergistic system? By interrogating the synergetic potential of biofilm-mediated nitrogen and PhACs removal, this work contributes fundamental knowledge toward the design of next-generation wastewater treatment infrastructure capable of addressing both conventional nutrient pollutants and emerging micropollutants in a single-stage, energy-efficient process.

1 LITERATURE REVIEW

1.1 Wastewater Overview

Wastewater is a growing global concern and has reached the point where rigorous measures must be employed to prevent severe damage to world growth, progress, and civilization (Afolalu et al., 2024). Global wastewater generation is estimated at 380 billion m^3 annually. However, the primary challenge is no longer just the volume, but the increasing complexity of the composition, which now includes emerging contaminants such as pharmaceuticals and recalcitrant nitrogen compounds (Kato & Kansha, 2024). Wastewater has two major categories: point sources with easily identifiable outlets, such as wastewater treatment plants (WWTPs), and non-point sources whose sources cannot be easily identified, like agricultural and urban runoffs (Crini & Lichtfouse, 2019). Wastewater is estimated to contain approximately 99.90% water and 0.10% suspended and dissolved solids, which are significantly bioorganic, thereby altering the physicochemical and biological properties of water (Afolalu et al., 2024). Some common pollutants include organic compounds, inorganic compounds, phosphorous, nitrogen and heavy metals (Kato & Kansha, 2024).

1.1.1 Global and Health Risks

With the increasing amount of wastewater produced, it can cause enormous global and health risks if not properly managed. The use of water inevitably leads to pollution with about 2 million tons of wastewater discharged into waterways annually (Afolalu et al., 2024; Crini & Lichtfouse, 2019).

Excessive levels of phosphates and nitrates lead to eutrophication in water bodies, causing unusual growth of algae, which depletes dissolved oxygen levels and leads to hypoxic conditions (Choudhary et al., 2022; Kailas & Shibu, 2013). This process degrades water quality, impairs the ecosystem of freshwater and contributes to the loss of biological diversity.

Photosynthesis can also be inhibited in water bodies as sunlight transmission is blocked by untreated dye effluents, as seen in textile wastewater (Kato & Kansha, 2024).

Nitrate is categorised among the most ubiquitous water contaminants, frequently exceeding safety levels in groundwater due to its high water solubility (Usman et al., 2022). Nitrite pollution poses clinical risks, notably methemoglobinemia (blue baby syndrome) in infants, affecting those up to 6 months of age (Choudhary et al., 2022). Other complications reported include diabetes, cardiovascular disorder and genetic mutation (Choudhary et al., 2022).

Long-term exposure to contaminated water can lead to chronic diseases such as stomach ulcer, tumours, cancer, Alzheimer's, psychological disorders, hypersensitive reactions, degenerative heart disease and immune system diseases (Afolalu et al., 2024).

It has been estimated that, annually, 1.8 million children under the age of five either die or suffer from water-related ailments. Acute diseases linked to wastewater exposure include typhoid and cholera (Afolalu et al., 2024). High levels of total coliform in wastewater above 300 MPN/100 mL indicate the potential presence of pathogens detrimental to human health if the water is consumed or used domestically (Paikun et al., 2023).

Exposure to heavy metals and pharmaceuticals in wastewater can be detrimental to plants, animals and humans (Kato & Kansha, 2024). The presence of thiocyanate in industrial wastewater is a threat to organisms due to its high affinity towards proteins and inhibitory effect on enzyme systems (Chen et al., 2024).

1.2 Wastewater Treatment

Wastewater treatment is a multi-stage process designed to remove harmful contaminants – pollutants, chemicals, pathogens, from water before reuse or its discharge into the environment. The end goal is to ensure this treated water meets safety for discharge to protect the ecosystem, human health and aquatic organisms inclusive.

Wastewater treatment methods can be broadly categorised into physical, chemical and biological processes, and are often combined in various sequential stages (preliminary, primary, secondary and tertiary). The technologies used are usually selected based on contaminants, their concentration, desired water quality, energy consumption, waste disposal choices, technological availability and economic viability (Afolalu et al., 2024; Kato & Kansha, 2024).

Initial screening helps to protect the other equipment used in the following stages and improve the effectiveness of the subsequent phases (Kato & Kansha, 2024). It eliminates all objects larger than 20 mm in diameter through screening, comminution and removal of grits (Kato & Kansha, 2024).

During the primary treatment, physical and chemical methods are employed (Kato & Kansha, 2024). The solids settle out of the wastewater through sedimentation and are subsequently removed, fats and oils are also removed in the process.

The secondary treatment involves biological processes to further purify the water. Organic matter, nitrogen and phosphorous are removed. Microorganisms present convert the pollutants into biomass via biochemical reactions (Kato & Kansha, 2024).

In the tertiary treatment stage, oxygen levels are increased, and unpleasant odours are eliminated. Persistent pollutants are eliminated and water quality is expected to meet specific standards for discharge or reuse.

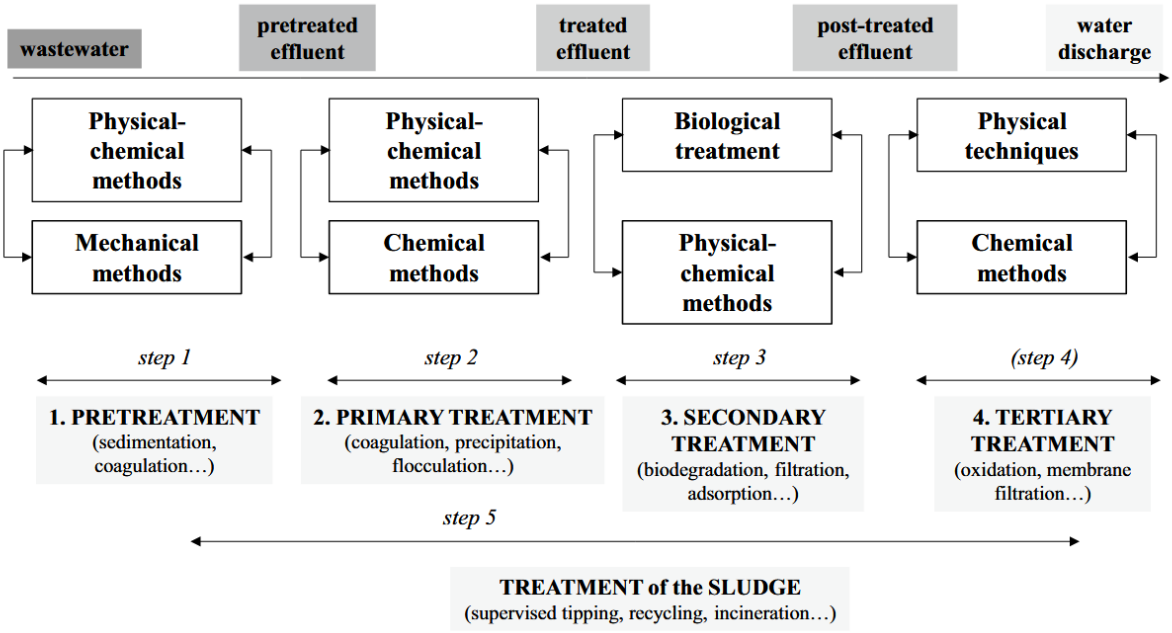


Fig. 1. Summary of main processes for the decontamination of industrial wastewater. Adapted from (Crini & Lichtfouse, 2019).

Categorically, the physical methods rely on the physical properties of the contaminants to separate them from the water. The chemical methods use organic and inorganic polymers or salts, such as soluble iron and aluminum salts) to remove pollutants, achieve disinfection and pH adjustment (Kato & Kansha, 2024). Chemical treatment is often combined with the physical and biological treatments, cutting across the preliminary, primary, secondary, tertiary and established recovery processes (Crini & Lichtfouse, 2019; Kato & Kansha, 2024). The biological methods utilise microorganisms such as bacteria, fungi, yeast, and algae to metabolise contaminants, primarily organic compounds, and occasionally nutrients and inorganic substances (Kato & Kansha, 2024). Biological methods are both cost-effective and environmentally friendly (Muduli et al., 2022; Usman et al., 2022). To categorise biological treatment methods, microorganisms, including bacteria, fungi, yeast, algae and protozoa, are used to decompose organic matter and remove pollutants (Kato & Kansha, 2024).

2.1 Biological Methods for Water Treatment

In the biological treatment method, we consider the treatment can be either aerobic, anoxic or anaerobic (Kato & Kansha, 2024). Microbes can be cultivated in two ways: either suspended form (like in activated sludge systems) or attached to solid media (such as in Moving Bed Biofilm Reactors, or MBBR) (Muduli et al., 2022; Rajesh Banu et al., 2021).

Aerobic suspended growth processes involve the use of aerobic bacteria suspended in aeration tanks to break down organic pollutants into CO_2, water and new biomass (Kato & Kansha, 2024). Well known methods are the activated sludge (AS) processes, which result in flocculated biomass separated in sedimentation tanks (also known as clarifiers), with a portion of the biomass being recycled back to the aeration tank (Kato & Kansha, 2024; Pool, 2014). Sequencing Batch Reactor (SBR), a more flexible system which uses a single tank to

sequentially carry out several phases – fill, mix, aerate, settle, draw, idle (Banu et al., 2021; Zhang et al., 2010). Due to its proficiency, this method can be modified to include anaerobic, anoxic and oxic zones (Rajesh Banu et al., 2021).

Anaerobic processes, for example in up flow anaerobic sludge blanket (UASB) reactors, break down pollutants in the absence of oxygen, leading to the production of biogas – methane and carbon dioxide (Kato & Kansha, 2024; Pool, 2014). Anaerobic processes have a low energy demand and biomass production compared to the aerobic processes (Kato & Kansha, 2024).

2.2 Nitrification and Denitrification

Nitrification and denitrification are biological processes used for nitrogen removal in wastewater treatment.

The traditional biological nitrogen removal (BNR) process consists of two stages that convert ammonium into dinitrogen gas (N_2) (Lin et al., 2009). In the first stage, aerobic nitrification occurs, where ammonium (NH_4^+) is oxidized to nitrate (NO_3^-) by autotrophic microorganisms. The microorganisms involved are ammonia-oxidizing bacteria (AOB) such as *Nitrosomonas* carrying out the nitritation step, and nitrite oxidizing bacteria (NOB) such as Nitrobacter and *Nitrospira* carrying out the nitratation step (Di Capua et al., 2022; Rahimi et al., 2020). In a recent study, it was discovered that complete ammonia oxidizers like *Nitrospira* are able to perform both - the nitritation and nitratation steps in a single organism (Wu et al., 2021).

Ammonium oxidation (AOB):

$NH^+_4 + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$

Nitrite oxidation (NOB):

$NO_2^- + 0.5O_2 \rightarrow NO_3^-$

Overall reaction:

$NH^+_4 + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$

The second stage is anoxic/anaerobic denitrification, during which facultative heterotrophic bacteria such as Pseudomonas and Bacillus reduce nitrate (NO_3^-) to dinitrogen gas (N_2) (Rajesh Banu et al., 2021; Ronan et al., 2021). This methodology requires energy and often necessitates the addition of an external organic carbon source (electron donor such as methanol or acetate) to aid in the denitrification process (Rajesh Banu et al., 2021; Usman et al., 2022), and also the production of alkalinity to help stabilize the pH lowered during nitrification (Di Capua et al., 2022; Thakur & Medhi, 2019)

$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

Conventional biological nitrogen removal involves nitrification, where ammonium is oxidized to nitrate, followed by denitrification, in which nitrate is sequentially reduced to nitrogen gas through nitrite, nitric oxide, and nitrous oxide intermediates.

Modern biological processes now shift from a separate-stage system towards an integrated pathway which addresses the high oxygen and carbon demands of the traditional/conventional

methods (Lin et al., 2009). Majorly, simultaneous nitrification and denitrification (SND) shortcut pathways, heterotrophic nitrification and aerobic denitrification (HNAD) and anammox-mediated systems.

Simultaneous Nitrification and Denitrification (SND) occur in a single reactor maintaining dissolved oxygen (DO) gradients in biofilms or flocs (Di Capua et al., 2022; Rahimi et al., 2020), where the nitrifiers thrive in aerobic outer layers and denitrifiers reside in the deeper anoxic zones (Loh et al., 2023). The nitrite shunt in the shortcut pathway saves approximately 25% in oxygen and 40% in organic carbon requirements (Di Capua et al., 2022). By inhibiting NOB, wastewater can be treated by converting ammonium only to nitrite, followed by denitrification (partial nitrification-denitrification). With HNAD, the heterotrophic and aerobic microbes perform both nitrification and denitrification simultaneously under aerobic conditions; they grow rapidly and remove both nitrogen and COD efficiently (Loh et al., 2023).

2.3 Anaerobic Ammonium Oxidation (anammox) Bacteria

The anaerobic ammonium oxidation (anammox) bacteria are characterised by a unique phylogeny, a complex compartmentalised cell plan and specialised membrane lipids that facilitate their unusual metabolism. Anammox belong to the phylum *Planctomycetes*; within the monophyletic order *Brocadiales* (Jetten et al., 2009). Currently, six genera have been identified: *Brocadia*, *Kuenenia, Anammoxoglobus, Jettenia, Scalindua* and *Anammoximicrobium* (J. Li et al., 2018; Ren et al., 2022).

Under the microscope, the bacteria appear as small coccoid or slightly irregular cells with diameter ranging 0.8 – 1.1 μm. In cell cultures containing anammox, they appear to be bright red due to high concentration of cytochrome (heme c protein) pigmentation making up 2-% - 30% of the total cellular protein mass (Jetten et al., 2009).

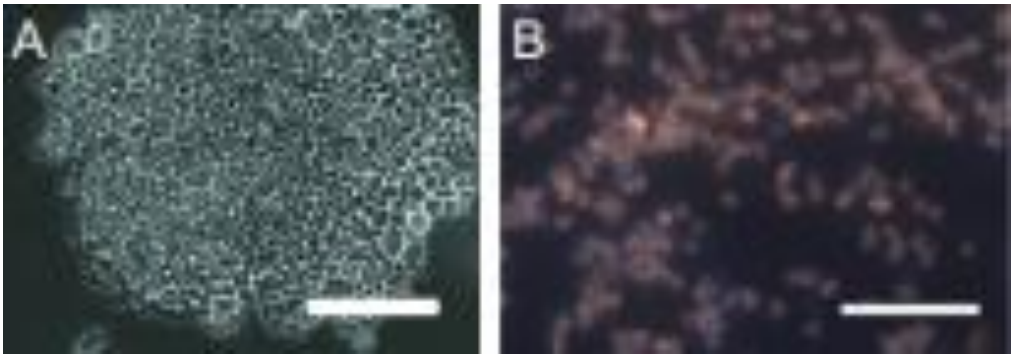


Fig 2. *Kuenenia stuttgartiensis* grown as (A) biofilm and (B) as planktonic single cells. Scale bars: (A) 50 mm, (B) 5 mm. Adapted from (Kartal, B. et Al., 2012).

Their structure comprise of an outermost compartment between the cytoplasmic and intracytoplasmic membranes (paryphoplasm), a middle compartment with nucleoid and ribosomes; where transcription and translation machinery reside (riboplasm) and a large membrane-bound compartment (anammoxosome) vertically inherited by daughter cells during division and occupies 50% to 70% of the total cell volume (Ahmad et al., 2020; Jetten et al., 2009).

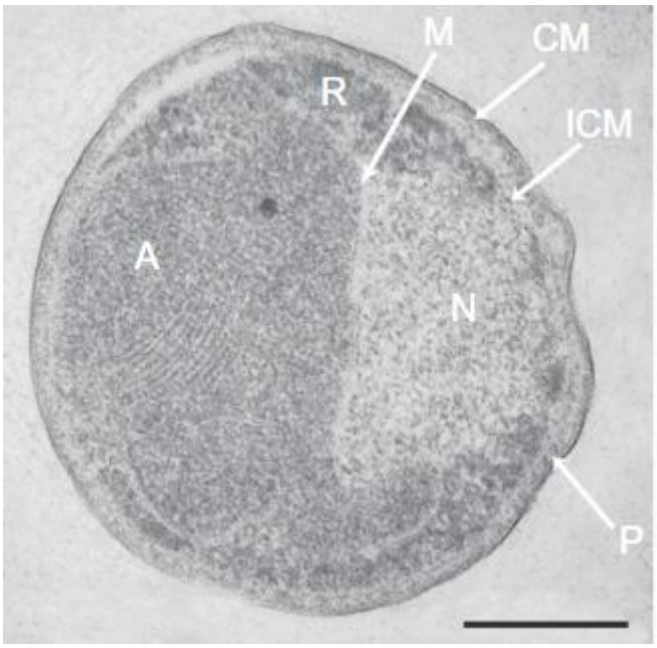


Fig 3. Transmission electron microscopy of *Anammoxoglobus propionicus* bacterium. A, anammoxosome containing tubule-like structures; R, riboplasm containing the nucleoid (N) opposed to the anammoxosome membrane (M); P, paryphoplasm separated from the riboplasm by an intracytoplasmic membrane (ICM); CM, cytoplasmic membrane. Scale bar, 200 nm. (Kartal, B. et Al., 2012)

The anammoxosome is considered the "bioenergetic heart" of the cell because it houses the essential catabolic machinery. Localised in this organelle or associated with its membrane are key enzymes such as hydrazine synthase (HZS), hydrazine dehydrogenase (HDH) and hydroxylamines (Kartal, B et Al., 2012)

Anammox are notoriously slow growing with doubling times ranging from 7-20 days, and attributed to the low metabolic activity of their key enzymes (Kartal, B. et Al., 2012). They are usually found in environments ranging from -30°C to 80°C, but optimal growth is between 30°C and 37°C. Due to quorum sensing mechanisms (using signaling molecules like N-acyl homoserine lactones) and the production of extracellular polymeric substances which provide physical protection and facilitate metabolic interactions (Ma et al., 2016; Wang et al., 2023), anammox bacteria tend to naturally aggregate into biofilms or granules.

Anammox bacteria are considered obligately anaerobic chemolithoautotrophs that derive energy from inorganic nitrogen and fix carbon dioxide. They utilize nitrite as the final electron acceptor in ammonium oxidation by producing dinitrogen gas as the end product. This central catabolism involves oxidation of ammonium using nitrite as the terminal electron acceptor to produce N_2. The pathway starts from reduction of nitric oxide (NO) to synthesis of the reactive intermediate hydrazine (N_2H_4) from NO and ammonium, then the oxidation of hydrazine to N_2 (Li et al., 2018). Bicarbonate or carbon dioxide are the key agents in fulfilling the carbon requirements of anammox bacteria (Weralupitiya et al., 2021). Carbon dioxide is fixed into biomass via the acetyl=CoA (Wood-Ljungdahl) pathway (Weralupitiya et al., 2021). Some bacteria species can utilise organic acids (such as acetate, propionate and formate) as alternative

electron donors or use Fe (III), Mn (IV), nitrate and sulfate as alternative electron acceptors (Ahmad et al., 2020; Weralupitiya et al., 2021). They can even "disguise" themselves as denitrifiers by reducing nitrate all the way to N_2 through a multi -step process (Jetten et al., 2009).

2.3.1 Anammox-Mediated Systems

The anaerobic ammonia oxidation (anammox) process is an innovative wastewater treatment technology that effectively removes nitrogen (Fu et al., 2021). In this process, nitrite (NO_2^-) and ammonia (NH_4^+) are transformed into nitrogen gas (N_2) under anoxic conditions, by anammox bacteria (Fu et al., 2021). This method offers advantages over traditional biological nitrogen removal (BNR) techniques, especially in systems designed for biomass retention like Sequencing Batch Reactors (SBRs) and Moving Bed Biofilm Reactors (MBBRs) (Kallistova et al., 2016; Xiao et al., 2011).

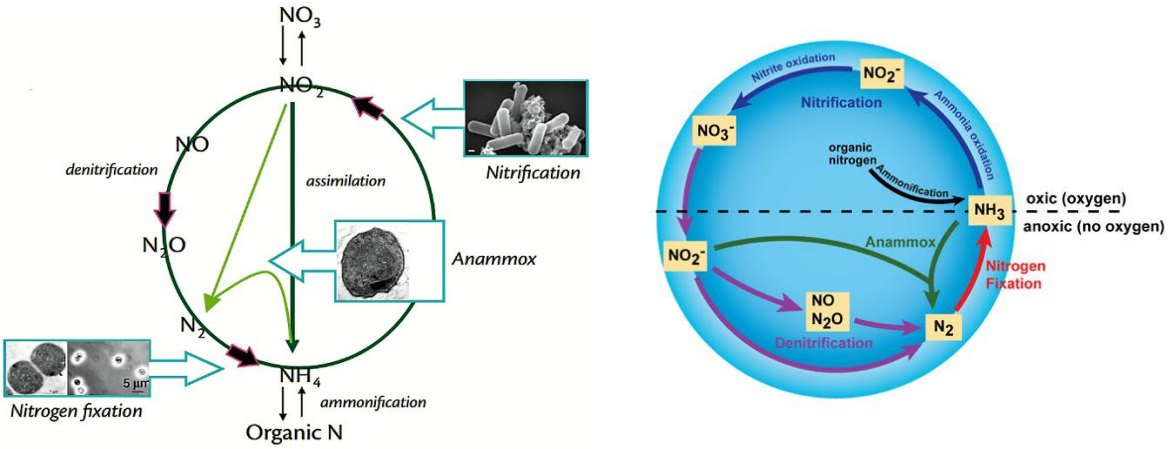


Fig 4. Involvement of the Anammox process in the biological nitrogen cycle including metabolic pathway for anaerobic ammonium oxidation. Fig 5b. Major transformations in the nitrogen cycle (Boishali D. et Al., 2024).

The Anammox process utilises nitrite ions (NO_2^-) as the terminal electron acceptor under anaerobic environmental conditions (Kallistova et al., 2016; Kangwannarakul et al., 2018; Usman et al., 2022; Xiao et al., 2011). Compared to traditional nitrification/denitrification processes, anammox offers significant advantages:

- **Cost and Energy Savings:** anammox process requires 60% less energy for aeration compared to conventional nitrification-denitrification (Ronan et al., 2021; Usman et al., 2022; Xudong et al., 2010) and no exogenous carbon sources, which can save operating costs, reduce energy demand, and lower greenhouse gas production by more than 60% and 25%, respectively. (Fu et al., 2021). Anammox bacteria are autotrophic, hence do not require external organic carbon sources implying a 100% savings in carbon for denitrification (Kangwannarakul et al., 2018; Lu et al., 2009; Tian et al., 2010).
- **Sludge Management:** anammox bacteria grow slowly and have a low cell yield, resulting in less residual sludge, approximately **75%** l**ess** than conventional processes,

thereby saving treatment and disposal costs. (Fu et al., 2021; Kangwannarakul et al., 2018; Ronan et al., 2021; Usman et al., 2022).

The implementation and study of the anammox process are often conducted using reactor configurations designed to retain biomass and accommodate slow-growing bacteria, such as the SBR and MBBR systems. Among various sludge morphologies, granular sludge possesses the strongest resistance to external disturbances, followed by biofilms, with floc sludge having the weakest resistance. However, biofilms are generally the recommended form for anammox because if granular sludge disintegrates under antibiotic shock, recovery can be highly time-consuming (Fu et al., 2021).

Anammox bacteria are typically present in the **anoxic and anaerobic zones** of stratified biofilms. Biofilm systems like MBBRs naturally form stratified layers (aerobic, anoxic, and anaerobic) depending on oxygen availability, facilitating the required conditions for anammox (Xu et al., 2023). The carrier material in MBBRs provides an attachment surface for slow-growing bacteria, which is crucial for maximizing the retention time of anammox bacteria (Wang et al., 2021; Xu et al., 2023).

Partial Nitration/Ammonia (PN/A) AND Partial Denitrification/Ammonia (PD/A)

Partial Nitration/Anammox (PN/A) is one of the most extensively studied methods in which ammonia is partially oxidized to nitrite. This nitrite then reacts with the remaining ammonia to produce nitrogen gas (N_2) (Rajesh Banu et al., 2021; Usman et al., 2022). The PN/A process significantly reduces oxygen consumption, carbon utilization, and sludge generation compared to traditional nitrification/denitrification (N/DN) methods (Guo et al., 2021).

Partial Denitrification/Anammox (PD/A) involves process where nitrate is partially denitrified to nitrite, which anammox bacteria then utilize for nitrogen removal.

2.4 Moving Bed Biofilm Reactors

MBBRs and their derivative configurations, including Membrane Biofilm Reactors (MBfRs) and Membrane Aerated Biofilm Reactors (MABRs), employ attached growth methodologies to mitigate the inherently slow biomass development rate characteristic of anammox bacteria (Fu et al., 2021; Kangwannarakul et al., 2018; Wang et al., 2021).

Biofilm systems are generally more tolerant to toxic substances than activated sludge. Biofilms can enhance the biotransformation of contaminants. For instance, exposure of anammox systems to antibiotics showed that biofilm reactors (like upflow anaerobic biological filters) and integrated fixed-biofilm and activated sludge reactors were tested due to the importance of reactor configuration and sludge properties.

In MBBR, microbial consortia develop as a biofilm attached to freely moving plastic carriers (biocarriers) suspended within the reactor vessel (Kato & Kansha, 2024; Madan et al., 2022; Muduli et al., 2022). This configuration facilitates the decoupling of solids retention time (SRT) from hydraulic retention time (HRT), thereby enabling the retention of slow-growing

autotrophic microorganisms such as ammonia-oxidising bacteria (AOB) and anammox bacteria (Kato & Kansha, 2024; Wang et al., 2021).

In Europe, the first full-scale ASTRASAND moving bed biofiltration process for biological post denitrification has been reported (Lin et al., 2009).

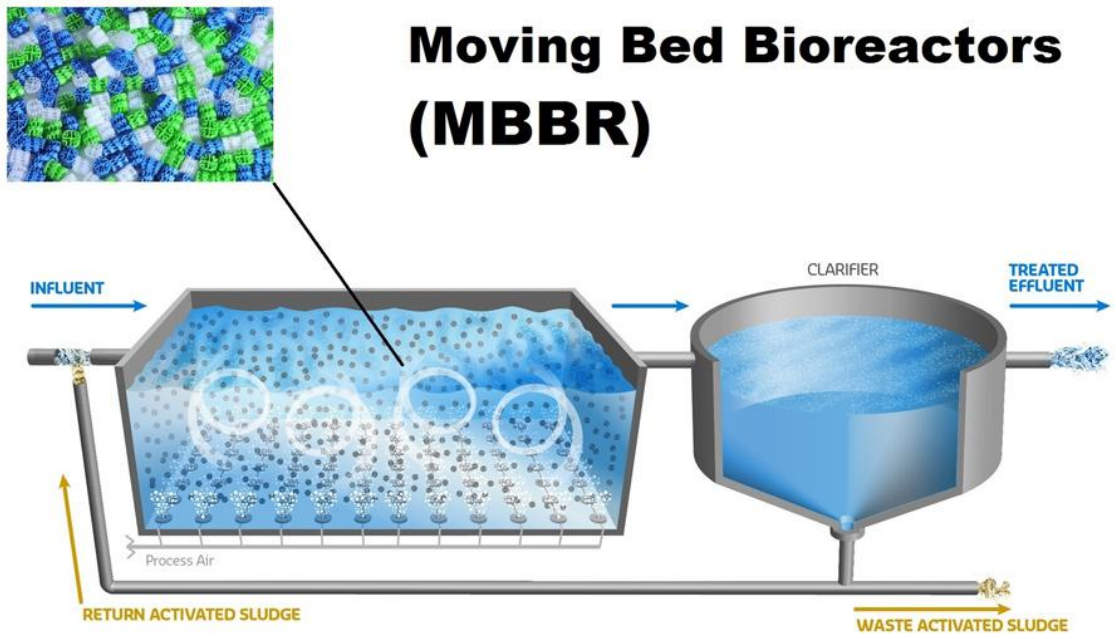


Fig 5. Conventional moving bed biofilm reactor (MBBR) system (D. R. Ravi & M. N. Asha., 2026)

Media utilised in MBBRs feature small pore sizes and substantial effective surface areas enhance the support and proliferation of slow-growing autotrophic biofilms essential for Anammox-mediated nitrogen removal processes (Madan et al., 2022).

MBBRs are prevalently employed within Partial Nitritation/Anammox (PN/A) treatment schemes (Al-Hazmi et al., 2023); commercial full-scale installations frequently incorporate MBBRs with either granulated sludge or floating carriers (Kallistova et al., 2016). Such configurations have demonstrated capacity for achieving elevated nitrogen removal efficiencies (Rajesh Banu et al., 2021). Additionally, MBBRs have shown efficacy in tertiary ammonia treatment, particularly for high-temperature industrial wastewater streams (Madan et al., 2022).

3.1 Pharmaceutically Active Compounds (PhACs)

The PhACs that are of high interest in terms of treatment in current research, are marbofloxacin, ofloxacin, enrofloxacin, sulfamethoxazole and carbamazepine. These can be categorised into three common groups: flouroquinolone antibiotics, sulfonamide antibiotics and psychiatric treatments drugs (specifically anticonvulsants).

Flouroquinolone Antibiotics

Flouroquinolone antibiotics include marbofloxacin, ofloxacin and enrofloxacin. They are characterised by their rapid bactericidal action and are active against many pathogenic species (Frade et al., 2014).

Marbofloxacin (MAR) – $C_{17}H_{19}FN_4O_4$ is primarily used in veterinary medicine and often released into agricultural wastewater. Initially, it is non-biodegradable but advanced mechanisms like electro-Fenton can enhance it biodegradability over time (Hakami et al., 2024).

Ofloxacin (OFL) – $C_{18}H_{20}FN_3O_4$ is highly water soluble with a low octanol water partition coefficient. Due to these unique properties, it is largely in the liquid phase of wastewater rather than adsorbed into solids, leading to high concentrations on treated effluents (Shigei et al., 2021).

Enrofloxacin (ENR) – $C_{19}H_{22}FN_3O_3$ used in veterinary medicine, it has a strong tendency to adsorb to mineral and organic matter in soil and sediments (Frade et al., 2014). It can be removed in some constructed wetlands, however, its presence in wastewater is a major source of concern in antimicrobial resistance (Shigei et al., 2021).

Sulfonamide Antibiotics

Sulfamethoxazole (SMX) – $C_{10}H_{11}N_3O_3S$ is one of the most frequently detected pharmaceuticals in surface waters globally due to incomplete removal in treatment plants. It is highly polar and water soluble, making it difficult to eliminate using standard adsorption methods. A significant property is its ability to retransform from metabolites (such as N4-acetylsulfamethoxazole) back into its original active form during biological treatment process (Radjenović et al., 2008; Shigei et al., 2021).

Psychiatric Treatment Drugs

Carbamazepine (CAR) – $C_{15}H_{12}N_2O$, frequently used as a model for persistent pollutants is notoriously refractory to biodegradation and often resistant to conventional wastewater treatment (Alsubih & Khan, 2024). It does not readily adsorb to sludge, hence, its concentration int treatment plant effluents are frequently equal to or even higher than its concentration in the influent (Montemurro et al., 2018). Carbamazepine has moderate hydrophobicity, which allows it to be taken up by plant roots and translocated or accumulated within plant tissues in systems like constructed wetlands (Y. Li et al., 2014).

3.1.1 Pharmaceutical Removal Mechanisms

Pharmaceutical removal from wastewater is achieved through a combination of physical, chemical, and biological mechanisms. Conventional WWTP are not originally designed to eliminate these complex organic micropollutants, hence, their removal is often incomplete, necessitating the need for advanced technologies and natural treatment systems (Li et al., 2014). Conventional treatment processes were primarily developed to remove suspended solids, organic matter, nutrients, and pathogens rather than trace organic contaminants. Pharmaceutical compounds are typically present at low concentrations and often possess complex, persistent chemical structures that resist biodegradation. In addition, variations in their physicochemical

properties, such as hydrophobicity, molecular size, and biodegradability, influence their removal efficiency in conventional treatment systems, resulting in incomplete elimination and discharge into aquatic environments.

Sorption (Adsorption)

Many pharmaceuticals are removed from the liquid phase by adsorbing onto sewage sludge (Montemurro et al., 2018). This process is governed by the compound's hydrophobicity (Log K_{ow}) and the sludge-water distribution coefficient (K_d) (Montemurro et al., 2018). While this removes the drug from the water, it often transfers the contaminant to the solid sludge, which may later be used as fertiliser. In constructed wetlands (CWS), the substrate (gravel, sand, clay or biochar) serves as a major sink for pollutants (Y. Li et al., 2014). Non-polar pollutants preferentially adsorb to organic-rich materials through hydrophobic interactions, while polar or ionic pollutants are captured through electrostatic interactions or ion exchange (Li et al., 2014). Technologies such as activated carbon (GAC and PAC), biochar and specialised clays (like LECA or montmorillonite) provide massive surface areas for molecular attachment (Montemurro et al., 2018). This is effective for hydrophobic drugs like carbamazepine.

Biodegradation and biotransformation

Microorganisms in activated sludge or wetland rhizospheres use enzymes (oxidases, esterases, hydrolases) to break down organic compounds. This can occur through aerobic and anaerobic biodegradation (Montemurro et al., 2018; Radjenović et al., 2008). Some pharmaceuticals, particularly persistent xenobiotic compounds such as carbamazepine, sulfamethoxazole, and certain fluoroquinolone antibiotics including ofloxacin and enrofloxacin, are not easily consumed by microbes. In these cases, they may be degraded slowly through co-metabolism, where non-specific enzymes degrade the drug while microbes feed on a different, easily degradable carbon source (Radjenović et al., 2008). A significant challenge in biological treatment is the enzymatic cleavage of metabolites (like glucuronides) back into their active parent compounds which can lead to higher pharmaceutical concentrations in effluents than in influents (Li et al., 2014).

Advanced oxidation processes (AOPs)

These processes are often considered effective for eliminating persistent pollutants that resist biological treatment including fluoroquinolones such as ofloxacin and enrofloxacin (Frade et al., 2014). Processes like ozonation, UV/H_2O_2, and electro-Fenton generate highly reactive hydroxyl radicals (.OH) (Hakami et al., 2024). These radicals non-selectively attack and break specific chemical bonds, and eventually mineralise the organic compounds into CO_2, water and inorganic ions.

2. THE AIM OF THE THESIS

While the anammox process is established as a cost-effective method for nitrogen removal, its stability in the presence of several emerging contaminants at same time was investigated. Existing literature indicates that antibiotics can inhibit anammox activity, but inhibitory levels are not clear.

However, there is a knowledge gap regarding the synergetic potential of biofilm systems (MBBR) to mitigate this toxicity and enhance removal.

Specifically, it is unclear if the stratified structure of a biofilm offers a protective mechanism against pharmaceutical shock loads compared to granular sludge.

Furthermore, the capacity of an anammox-enriched consortium to simultaneously degrade these pharmaceutical compounds via co-metabolism in a single-stage synergetic system has not been fully elucidated.

In this study, we aim to investigate the removal of nitrogen compounds, including nitrites, nitrates, and ammonium, and 5 different PhACs from real reject wastewater to ensure the effectiveness of biofilm carriers extracted from MBBR systems.

3. EXPERIMENT

3.1 Materials and Methods

3.1.1 Moving Bed Biofilm Reactor (MBBR) system

A continuous-flow reactor system was employed to investigate the nitrogen transformation process and short-term batch test for pharmaceutical removal.

The Moving Bed Biofilm Reactors (MBBR), operating at a volume of 20 L, was fed with reject water sourced from the Tartu Municipal Wastewater Treatment Plant (WWTP), Estonia. The reactor was a cylindrical plexiglass column (height of 52 cm and diameter of 25 cm). To support biofilm growth, the reactor was filled with 50% of volume approximately 10,000 Kaldnes K1-shaped biofilm carriers (specific surface area ≈ 800 m^2/m^3).

The feed for the reactor consisted solely fresh reject water. Mixing was provided by a mechanical stirrer. The hydraulic retention time (HRT) was set for 3 days. The influent pH ranged from 6-8.5 and was manually adjusted with 1 M HCl or 1 M NaOH when necessary.

Fig. 6. View of two parallel MBBR reactors of 20 L volume based on anammox process operated at 22 ºC. This project utilized just one of these reactors for PhACs removal testing (the reactor to the right).

3.1.2 Batch Tests

Specific nitrogen conversion pathways were investigated under controlled conditions using biofilm carriers harvested from the MBBR system. The batch tests were performed in 1 L two-neck glass bottles with a total liquid volume of 1000 mL. Each bottle contained 100-200 mL of biofilm carriers.

Synthetic Wastewater Composition

The synthetic wastewater medium used in these tests was prepared with the following composition per litre:

- 2 mL $NaNO_2$ stock solution
- 2 mL NH_4Cl stock solution
- 0.4 g H_2CO_3
- 1 mL Phosphate buffer solution – typically contained KH_2PO_4 and K_2HPO_4 to maintain pH
- 1 mL $MgSO_4 \cdot 7H_2O$ solution – prepared at a concentration of 200 g/L
- 1 mL $CaCl_2$ solution - prepared at a concentration of 300 g/L
- 1 mL $FeCl_3 \cdot 6H_2O$ solution - prepared at a concentration of 5 g/L
- 1 mL alkaline trace element solution – prepared in 100 mM HCl.
    - $NaSeO_3.5H_2O$: 0.01 g/L
    - $Na_2WO_4.2H_2O$: 0.01 g/L
    - $Na_2MoO_4.2H_2O$: 0.01 g/L
- 1 mL acidic micronutrient solution
    - $FeSO_4.7H_2O$: 5.0 g/L
    - $ZnSO_4$. $7H_2O$: 0.05 g/L
    - $MnCl_2$. $4H_2O$: 0.5 g/L
    - $CuSO_4$. $5H_2O$: 0.05 g/L
    - $CoCL_2$. $6H_2O$: 0.05 g/L
    - $NiCL_2$. $6H_2O$: 0.01 g/L
    - H_3BO_3: 0.01 g/L

Test Conditions

All batch tests were conducted in a temperature-controlled oven at 25.0 ± 0.2 °C with continuous mixing provided by a magnetic stirrer at 500 rpm. Two replicate bottles were prepared for each condition. The following conditions were investigated:

- **Aerobic conditions:** continuous aeration was supplied to the bottles using an air pump.
    - **Aerobic Standard:** Prepared synthetic wastewater
    - **Aerobic + Additive:** Synthetic wastewater with the addition of 10 and 20 mL of hydrazinium sulphate
- **Anoxic Conditions:** The wastewater solution was first sparged with nitrogen gas (N_2) for 15 minutes to remove dissolved oxygen prior to sealing the bottles.
    - **Anoxic Standard:** Prepared synthetic wastewater
    - **Anoxic + Additive:** Synthetic wastewater with the addition of 10 and 20 mL of hydrazinium sulphate

- **Anoxic-Aerobic Conditions**: The batch test cells were subject to alternating cycles of deaeration and aeration, beginning with 1 hour of deaeration with N_2 gas, followed by 2 hours of aeration using the air pump.
    - **Anoxic-Aerobic Standard:** Prepared synthetic wastewater
    - **Anoxic-Aerobic + Additive:** Synthetic wastewater with the addition of 10 and 20 mL of hydrazinium sulphate

3.1.3 Nitrogen and chemical analysis

Samples from the reactor were collected monthly. The influent and effluent samples were collected one day after the monthly feed change.

From each batch test bottle, 30-40 mL samples were taken at specific time intervals: 0, 3, 6, 24 and 48 hours. The 0-hour samples were taken immediately after preparation and prior to establishing the specific test conditions.

Total nitrogen concentrations in mg N/L, including NO_2-N, NO_3-N, and NH_4-N, were recorded for each condition. They were quantified using a Hach Lange DR 2800 spectrophotometer. The pH was also measured for these samples.

- **Ammonium nitrogen (NH_4−N):** Ammonium concentration was determined using Nessler's colorimetric method. To a 25 mL volumetric flask, 5 mL of the sample was added. Three drops of mineral stabiliser and two drops of polyvinyl alcohol dispersing agent were added, followed by dilution to the mark. Finally, 1 mL of Nessler reagent was added. After a 10-minutes reaction time, the absorbance was measured. The final concentration was calculated based on a 5-fold dilution.
- **Nitrite nitrogen (NO_2−N):** Nitrite concentration was determined using the sulfanilamide colorimetric method. A sample aliquot (0.1-5 mL) was placed in a 25 mL volumetric flask and diluted to the mark with deionized water. Then, 0.5 mL of sulfanilamide solution and 0.5 mL of N-(1-Naphthyl) ethylenediamine dihydrochloride solution were added. The absorbance of the resulting pink-purple solution was measured after a 10-minute reaction period.
- **Nitrate nitrogen (NO_3−N):** Nitrate concentration was determined using the sodium salicylate method. One mL of the sample was mixed with 1 mL of sodium salicylate in a crucible and evaporated to dryness in a water bath. After cooling, 1 mL of concentrated sulfuric acid (H_2SO_4) was added. Following a 15-minute reaction, 8 mL of deionized water and 10 mL of a sodium hydroxide and EDTA solution (NaOH + EDTA) were added to develop the yellow color. The solution was then quantitatively transferred to a suitable volumetric flask (25, 50, or 100 mL, depending on color intensity) and filled to the mark for spectrophotometric measurement.
- **pH:** The pH of the samples was measured using an Evikon E6115 pH meter. The instrument was calibrated daily using standard buffer solutions of pH 4.0 and 7.0. For the batch experiments, the initial pH of the synthetic wastewater was adjusted to approximately **7.5 (± 0.5)** using 1 M HCl or 1 M NaOH to ensure optimal conditions for microbial activity.

3.1.4 Batch Studies for Pharmaceutical Compounds Removal

To evaluate the removal of specific pharmaceutical compounds, batch tests were conducted in three-neck plexiglass reactors of 1 L working volume. Polyethylene biofilm carriers (≈ 200 units) were transferred from the pre-acclimatized MBBR to the batch reactors. The carriers were gently washed three times in Milli-Q water prior to transfer to removed detached biomass.

Target Compounds

The study investigated the following pharmaceutical active compounds (PhACs) simultaneous degradation:

1. Marbofloxacin
2. Ofloxacin
3. Sulfamethoxazole
4. Enrofloxacin
5. Carbamazepine

Stock solutions were prepared to reach desired initial concentrations (high = 0.80 mg/L and low = 0.06 mg/L).

Experimental Procedure

The synthetic wastewater (pH 7.5-8.0) used was identical to the nitrogen batch tests ($NaNO_2$, NH_4Cl, minerals, and trace elements). Experiments were run for a duration of 48 hours at 25 ± 1°C under two conditions – strictly anoxic and combined anoxic/aerobic (fluctuating for one hour each).

Samples were collected every 2 hours (0, 2, 4, 6, 24, 48 h) to monitor degradation kinetics. Experiments were conducted in duplicates.

3.1.5 PhACs Concentration Determination (HPLC-MS)

Pharmaceutical concentrations were estimated using High-Performance Liquid Chromatography – Mass Spectrometry (HPLC – MS) following Solid Phase Extraction (SPE).

5 mL of sample was mixed with 100 mL Milli-Q water and 1 ± 0.1 mg/L Na_2EDTA. pH was adjusted to ≈ 4 using formic acid. The SPE was carried out using HLB cartridges (6 cm^3, 500 mg LP, 60 µm). Cartridges were conditioned with methanol, milli-Q and Na_2EDTA. Samples were loaded at 3 mL/min, washed with a formic acid solution, and eluted with 12 mL methanol. Extracts were evaporated in a vacuum centrifuge (40°C, 70 mbar) and reconstituted in 0.5-1 mL of solvent (0.1% formic acid: methanol = 9:1) for HPLC-MS analysis.

3.1.6 Biomass Dry Weight

Biomass was determined by weighing 20 carriers before and after biofilm removal. The twenty biofilm carriers were placed in a centrifuge tube. The mixture was agitated vigorously for 5 min to ensure the separation of biomass from the carriers. Carriers were dried at 105°C for 24 h and weighed. Thereafter, further heating step to remove organic compounds (furnaced at 450 ◦C), and ensure complete combustion of the organic matter was performed. The dry biomass weight was calculated by subtracting the weight after organic matter removal from the weight of the

dried biomass. treated with concentrated chromic acid to remove biomass, washed and dried again. The difference in weight represented the attached biomass. The biomass dry weight per carrier was determined by dividing the final dry biomass weight by the total number of carriers used (Mamun et al., 2026).

3.1.7 Microbiological Community Analysis (16S rRNA)

The V4 hypervariable region of the 16S rRNA gene was amplified using universal F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′- GGACTACHVGGGTWTCTAAT-3′) primers, as described in Caporaso et al., 201. The amplified products were sequenced using the Illumina iSeq 100 platform following the procedures outlined in Kazantseva et al., 2021. Taxonomic classification of the obtained sequences was performed using the BION-meta software (https://github.com/nielsl/mcdonald-et-al), in accordance with the protocol detailed by Mandel et al., 2019.

3.2 RESULTS AND DISCUSSION

3.2.1 Bioreactor Performance for Nitrogen Removal

The moving bed biofilm reactor (MBBR) was monitored over 308 days to evaluate its nitrogen removal performance when treating reject water from Tartu municipality WWTP. The figure below shows the time course of influent and effluent total nitrogen concentrations, along with the corresponding removal efficiencies.

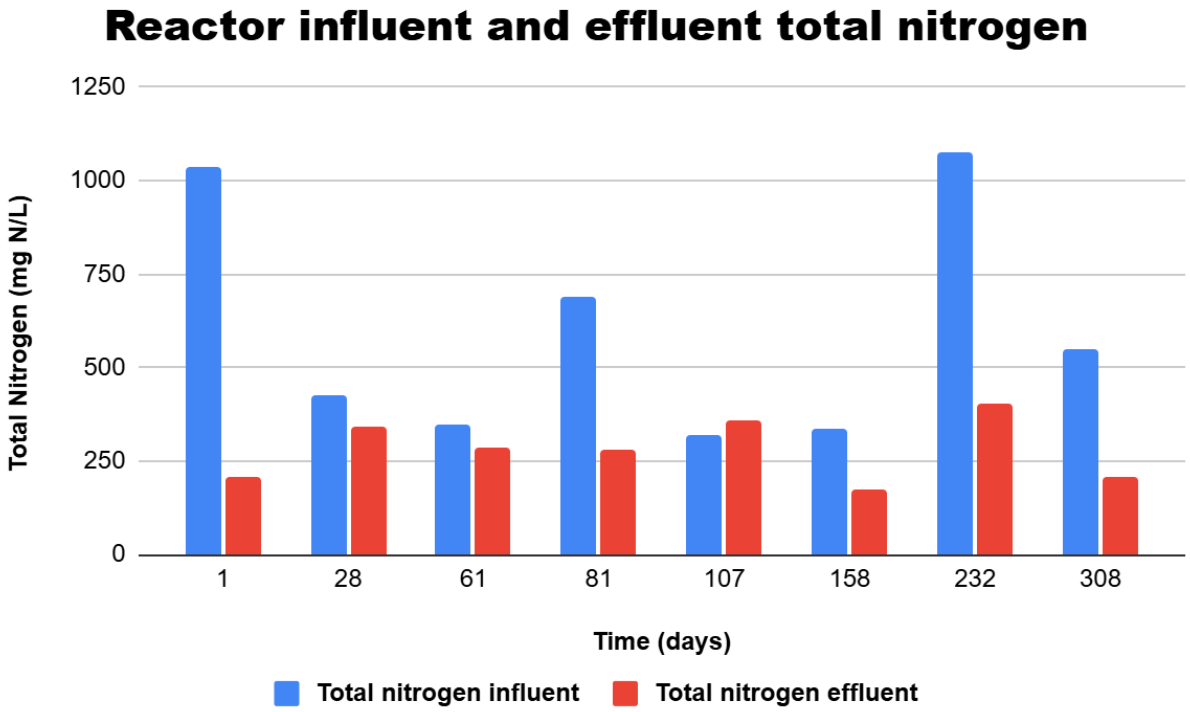


Fig 7. Relative amount of total nitrogen concentrations fed into the influent tank and total nitrogen concentration realised in the effluent.

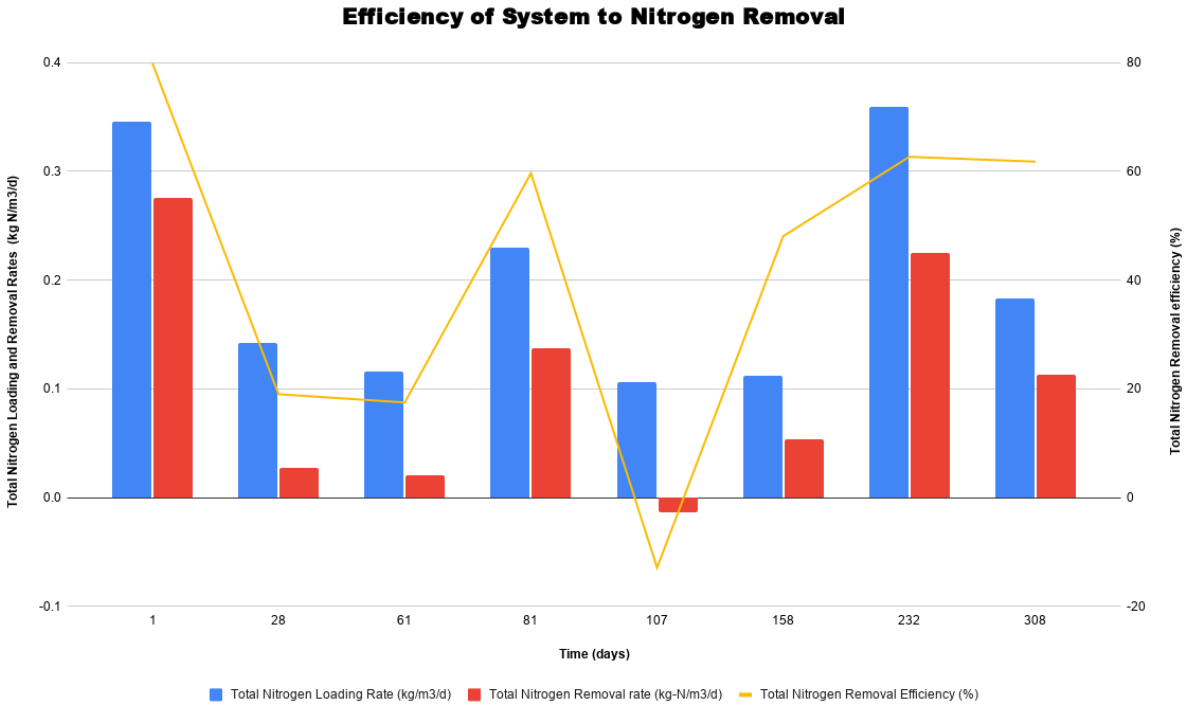


Fig 8. Time course of nitrogen removal after being loaded and an indication of the overall removal efficiency of the MBBR system.

The nitrogen removal efficiency was calculated based on the changes in NH_4^+-N, NO_2^--N, and NO_3^--N concentrations in the effluent compared to the influent. Eq. (1) was used to estimate the total nitrogen removal efficiency (TNRE).

$$TNRE = \frac{total\ influent\ nitrogen - total\ effluent\ nitrogen}{total\ influent\ nitrogen} x\ 100\%$$

The system demonstrated robust nitrogen removal, with efficiencies in the range of 17.5% to 79.8% across this operational period. The removal corresponds to effluent total nitrogen concentrations between 175.2 and 401.74 mg N/L from the influent concentrations of 319.1 to 1076.0 mg/L. The highest removal efficiency was recorded on day 1, while the lowest occurred on day 61, and a notable anomaly on day 107 with a negative removal (-12.9%).

On day 107, the effluent total nitrogen (360.2 mg N/L) exceeded the influent (319.1 mg N/L), driven by an increase in effluent nitrate (342.5 mg N/L) from a relatively low influent nitrate concentration (21.1 mg N/L). This observation can be attributed to a temporary imbalance between nitrification and anammox activities, possible oxygen intrusion into anoxic zones (which stimulates NOB), detachment of nitrifier-rich biomass into the bulk liquid and transient inhibition of anammox activity. However, the system recovered, returning to 48.1% removal efficiency by day 158, demonstrating the resilience of the biofilm consortium.

Fate of Individual Nitrogen Species

Ammonium removal remained consistently high throughout the test period with efficiencies exceeding 96% at all time points. For the most part, the effluent NH_4^+-N concentrations were

below 5 mg N/L despite influent concentrations ranging from 107.5 to 400 mg N/L. This ammonium oxidation process confirms the presence and sustained activity of AOB within the biofilm consortium.

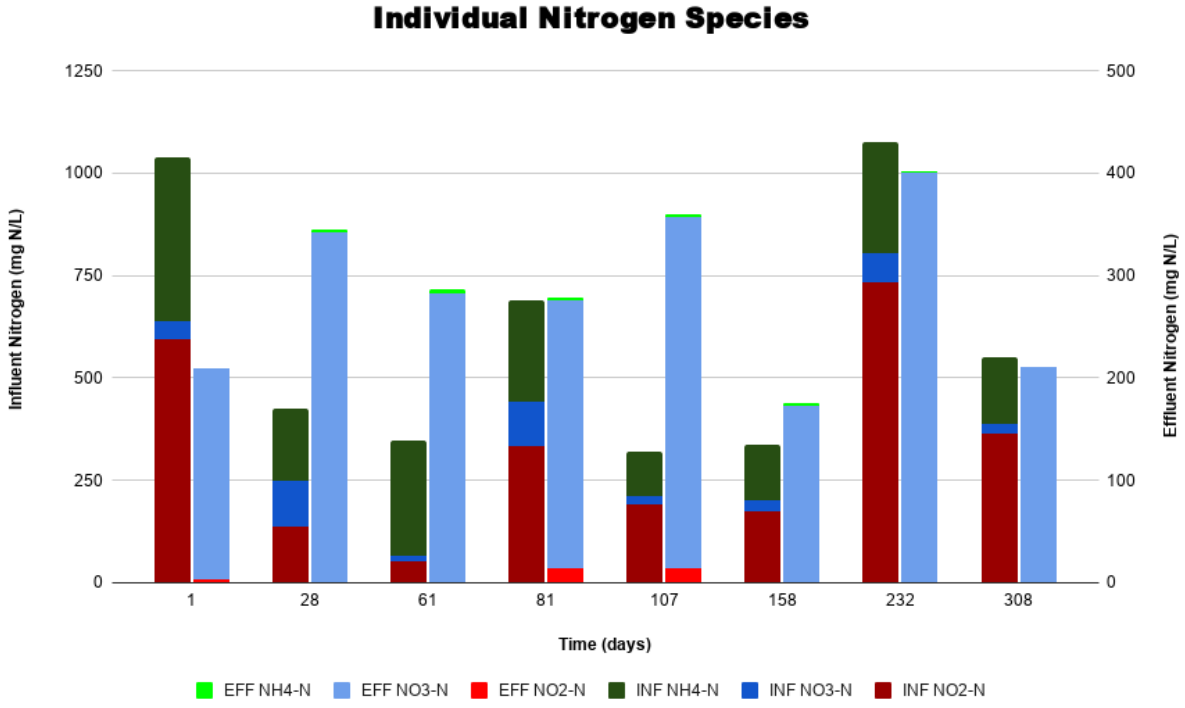


Fig 9. Observation of the nitrogen species indicating ammonium removal, nitrite consumption and nitrate accumulation

Evidence of anammox-mediated nitrogen removal was also observed as nitrite removal efficiencies exceeded 92% with most timepoints being >99%. The influent NO_2^--N concentration was varied (52.0 to 735.0 mg N/L), yet effluent concentrations remained below 14 mg N/L (typically below 1 mg N/L). The efficient nitrite consumption coupled with ammonium removal is indicative of anaerobic ammonium oxidation activity.

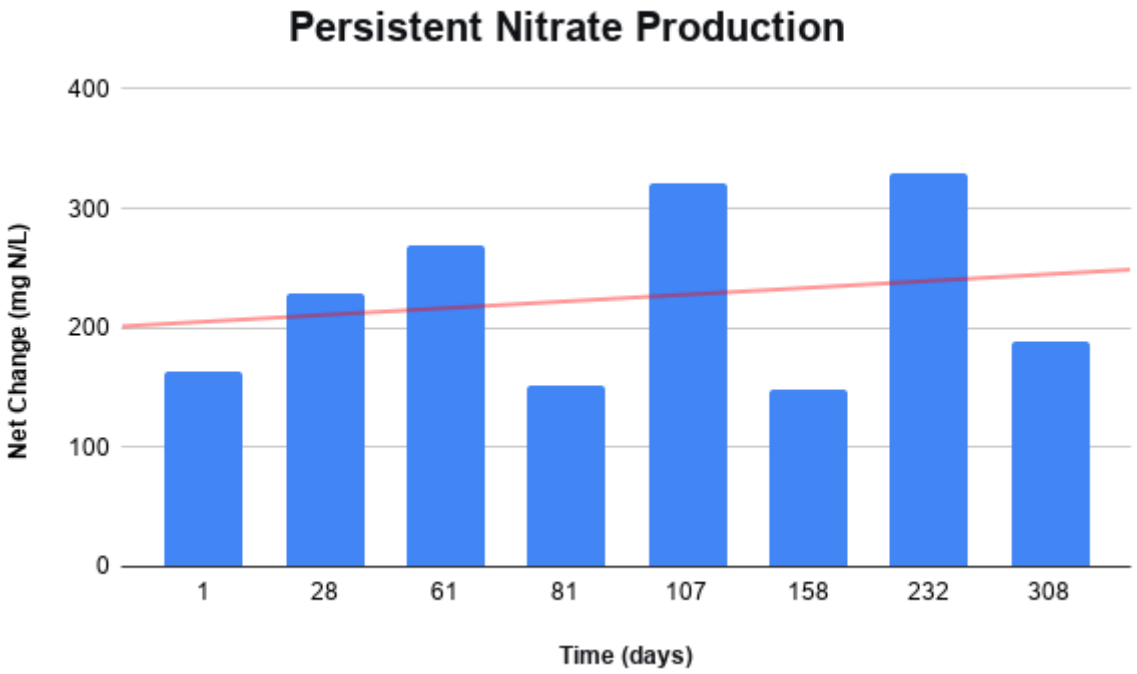


Fig 10. Persistent nitrate accumulation observed at every sampling timepoint.

Concurrent nitrification activity within the biofilm – specifically NOB converting NO_2^- to NO_3^-, is observed as nitrate effluent concentrations constantly exceeded the influent values by 148.1 to 329.0 mg N/L as seen in Figure 10. The nitrate production suggests that while anammox effectively consumed nitrite and ammonium, the system does not achieve complete nitrogen transfer to dinitrogen gas due to NOB competition for nitrite and/or limited denitrification capacity.

Overall, the reactor showed stability when considering the extreme variations in influent loading. Influent total nitrogen ranged nearly four-fold (319.1–1076.0 mg N/L), yet effluent quality remained within a relatively narrow band (175.2–401.7 mg N/L). Notably, the highest loading events (day 1: 1037 mg N/L; day 232: 1076 mg N/L) corresponded with the highest removal efficiencies (79.8% and 62.7%, respectively), suggesting that the anammox consortium was not substrate-limited and could respond to increased nitrogen availability.

3.2.2 Batch Tests Analysis

Nitrogen Transformations under Aerobic Conditions

Under continuous aeration, ammonium removal proceeded steadily, with the aerobic/100 biofilm carriers condition achieving a 25.57% removal over 48 hours. Nitrite accumulation and moderate nitrate production indicated active AOB with partially inhibited NOB. Total nitrogen removal remained limited at 19.15% suggesting a correlation with the absence of anoxic zones necessary for denitrification or anammox activity.

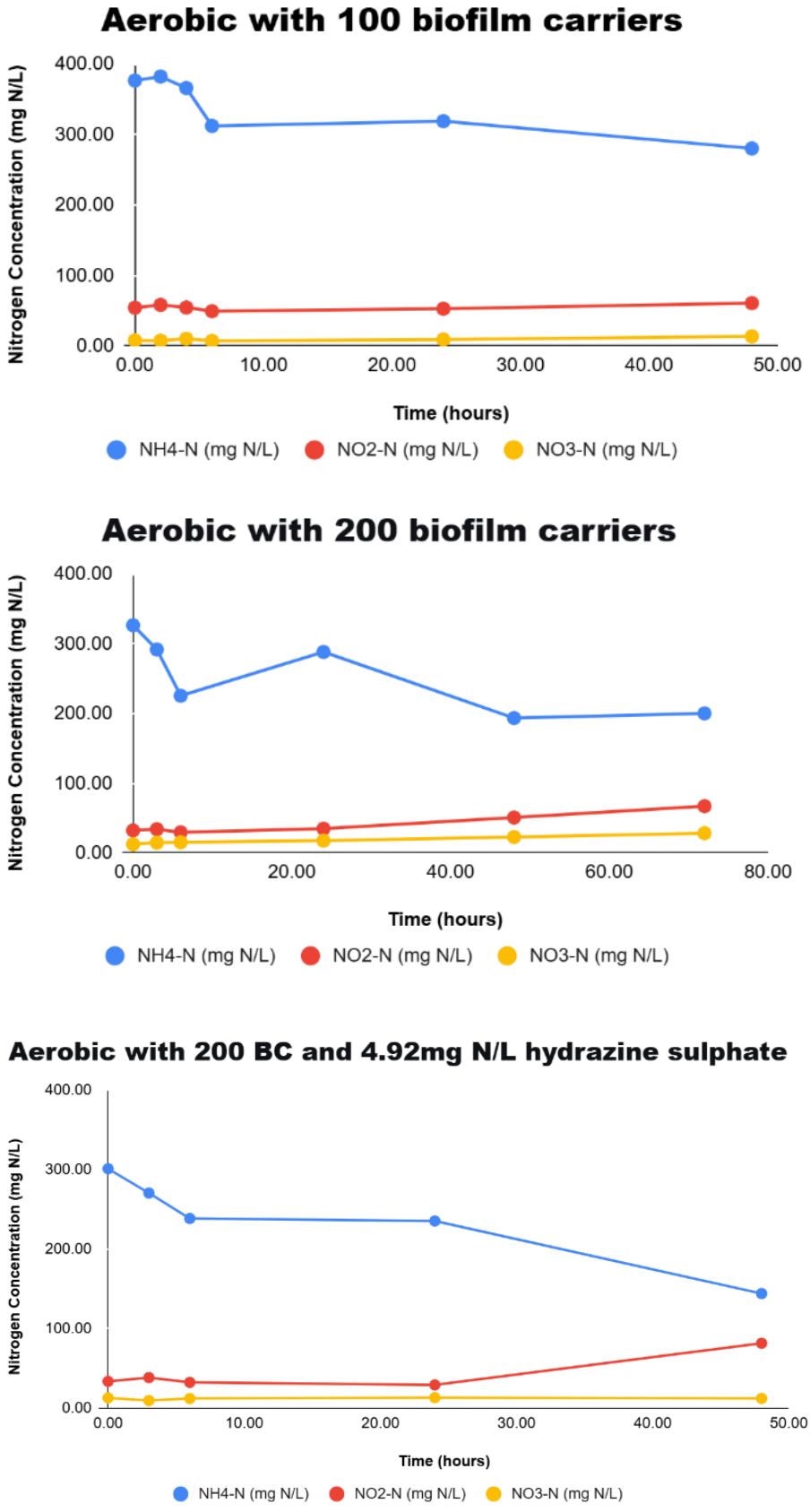


Fig 11. Individual nitrogen species removal under aerobic conditions: a) with 100 biofilm carriers, b) 200 biofilm carriers, and c) 200 biofilm carriers and 4.92 mg/L hydrazine added.

The addition of 4.92 mg N/L hydrazine sulphate in the aerobic/200 biofilm carriers condition enhanced the ammonium oxidation process: achieving 52.14% NH_4^+-N removal. However, the nitrite accumulation at 48 hours suggests that hydrazine sulphate, while stimulating AOB activity, may cause some inhibitory effects on NOB leading to incomplete nitrification. The total nitrogen removal improved to 31.54%, but it is worth noting that enhanced ammonium oxidation alone does not guarantee complete nitrogen removal.

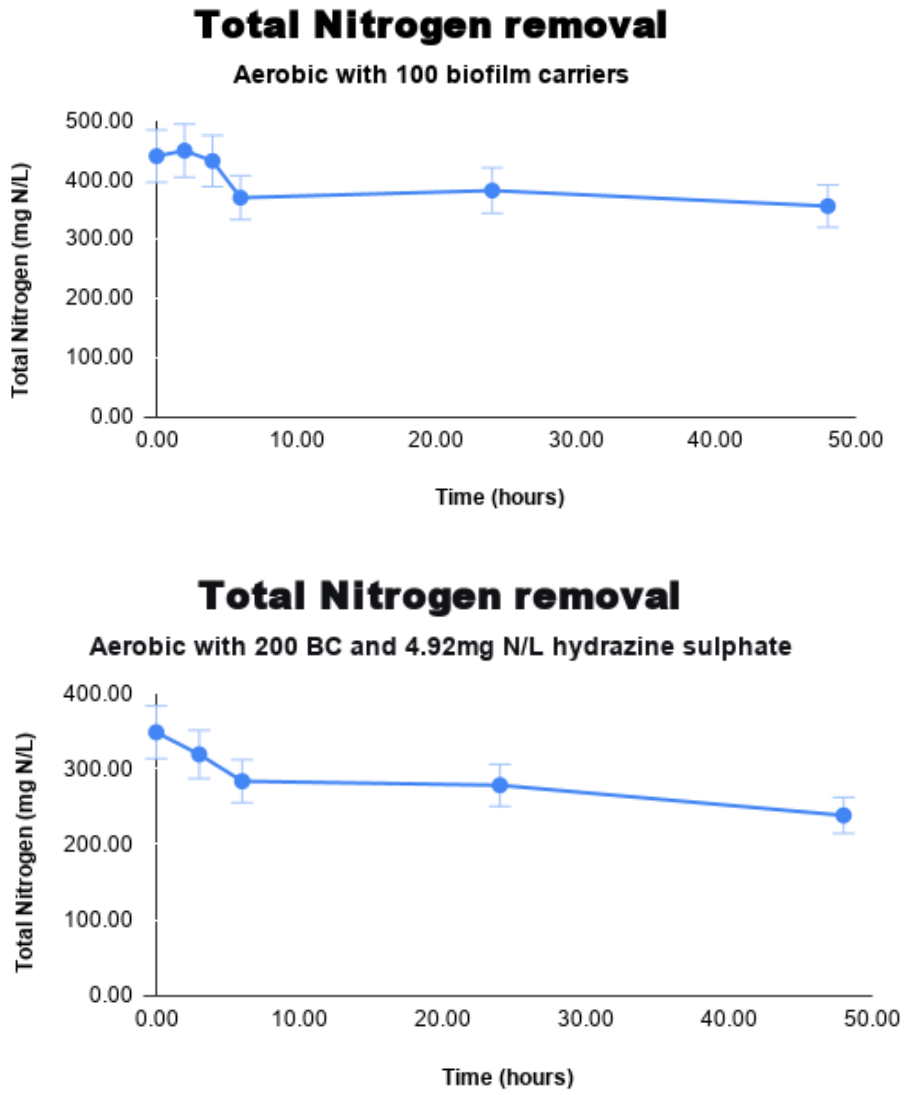


Fig 12. Total nitrogen concentrations decrease under aerobic conditions: a) removal of nitrogen with 100 biofilm carriers, b) removal of nitrogen from enhanced process with 200 biofilm carriers and addition of 4.92 mg N/L hydrazine sulphate.

Anammox Activity Under Anoxic Conditions

Anoxic conditions revealed evidence of anaerobic ammonium oxidation activity. In the anoxic/100 biofilm carriers' condition, a sharp decline in both ammonium and nitrate occurred within 6 hours (with stable nitrite concentrations). The simultaneous consumption of NH_4^+ and NO_2^- is characteristic of anammox metabolism. The subsequent increase in nitrate after 23 hours

suggests either nitrifier activity in microaerophilic zones within the biofilm or partial disruption of anoxic conditions during sampling.

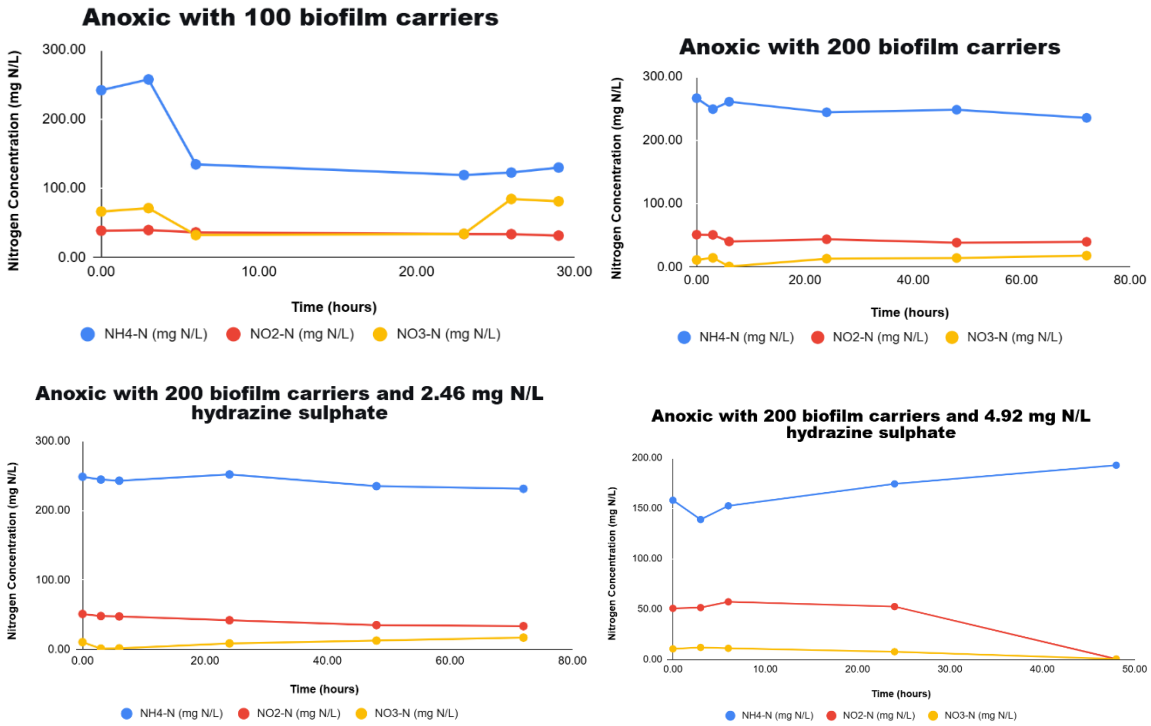


Fig 13. Individual nitrogen species removal under different anoxic conditions: a) with 100 biofilm carriers, b) with 200 biofilm carriers, c) 200 biofilm carriers and 2.46 mg N/L hydrazine sulphate, and d) with 200 biofilm carriers and 4.92 mg N/L

Under the anoxic/200 biofilm carriers' condition, the performance was not enhanced, yielding only 10.76% total nitrogen removal over 72 hours. This counterintuitive result may reflect mass transfer limitation within denser biofilm aggregates where substrate diffusion to inner anammox cells become restricted, or competition for nitrite between anammox and other denitrifiers.

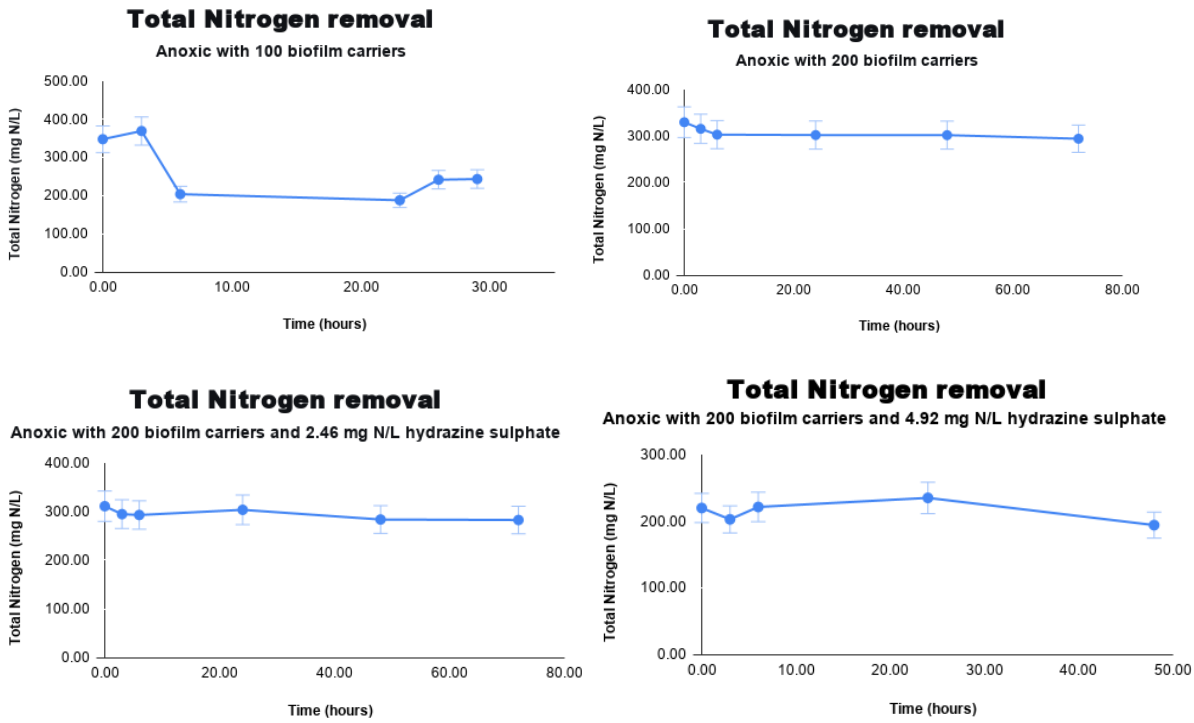


Fig 14. Total nitrogen concentrations realized under different anoxic conditions: a) total nitrogen removal with 100 biofilm carriers, b) total nitrogen removal with 200 biofilm carriers, c) total nitrogen removal in enhanced condition bearing 200 biofilm carriers and 2.46 mg N/L hydrazine sulphate, d) total nitrogen removal with 200 biofilm carriers and 4.92 mg N/L

Effects Under Alternating Anoxic/Aerobic Conditions

The alternating anoxic/aerobic condition was designed to simulate the stratified redox condition prevalent in MBBR biofilms. The anoxic/aerobic condition with 200 biofilm carriers and 2.96 mg N/L hydrazine sulphate exhibited remarkable nitrogen removal performance over the first 48 hours of incubation.

Ammonium concentrations decreased from 258.8 to 67.1 mg N/L representing a 74% removal (the highest across all batches). Nitrite accumulated progressively while nitrate remained below 10 mg N/L till the 48-hour time point, indicating suppression of complete nitrification.

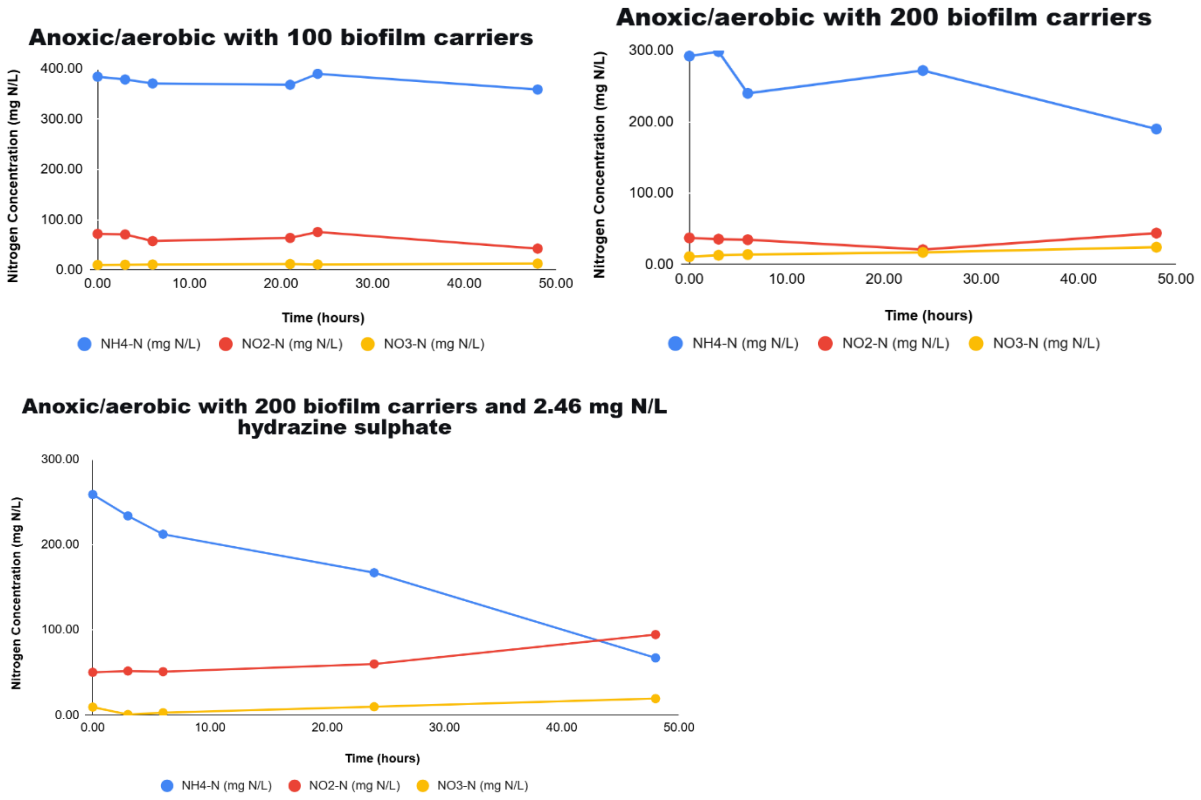
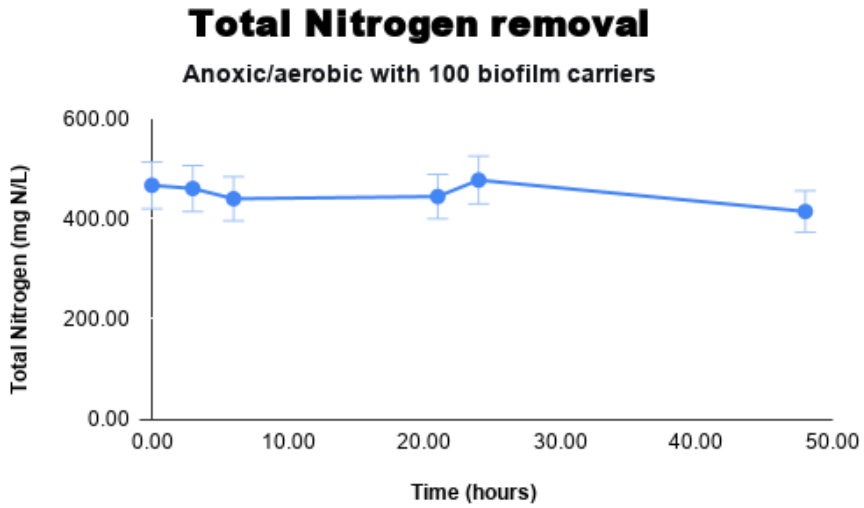


Fig 15. Individual nitrogen species removal under different anoxic/aerobic conditions: a) with 100 biofilm carriers, b) with 200 biofilm carriers, c) 200 biofilm carriers and 2.46 mg N/L hydrazine sulphate, and d) with 200 biofilm carriers and 4.92 mg N/L

The performance surpassed both standard aerobic and anoxic conditions, suggesting coupling between aerobic phases (AOB-mediated partial nitrification – $NH_4^+ \rightarrow NO_2^-$) and anoxic phases (anammox-mediated conversion - $NH_4^+ + NO_2^- \rightarrow N_2$).



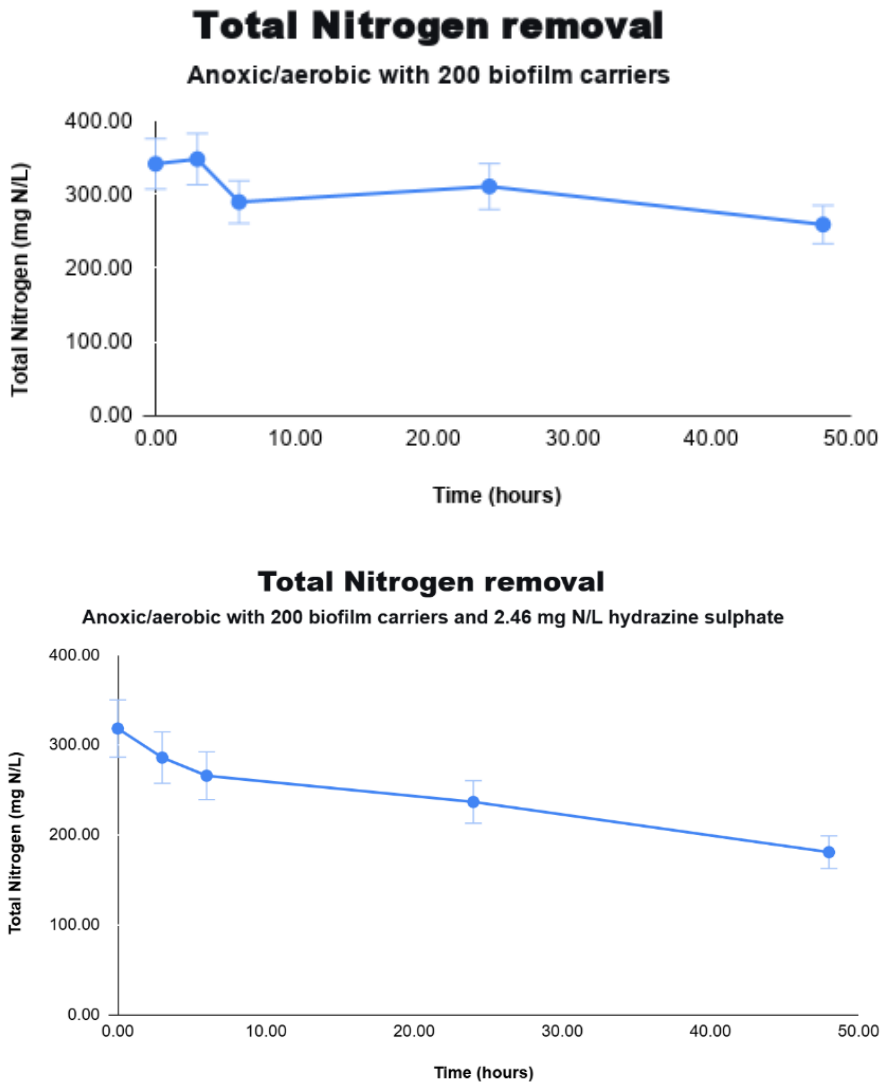


Fig 16. Total nitrogen concentrations change under anoxic/aerobic conditions with and without hydrazine addition: a and b) total nitrogen removal with 100 and 200 biofilm carriers respectively but without hydrazine sulphate added, c) total nitrogen removal from enhanced condition with 200 biofilm carriers and 2.96 mg N/L hydrazine sulphate.

3.2.3 Kinetic Analysis of Nitrogen Removal from Batch Tests

To investigate the rate-limiting mechanisms for nitrogen removal under different operational conditions, kinetic analysis was performed on the test data. Zero-order (concentration-independent) and first-order (concentration-dependent) models were fitted to the nitrogen decline profiles with the coefficient of determination (R^2) used to assess the model's suitability.

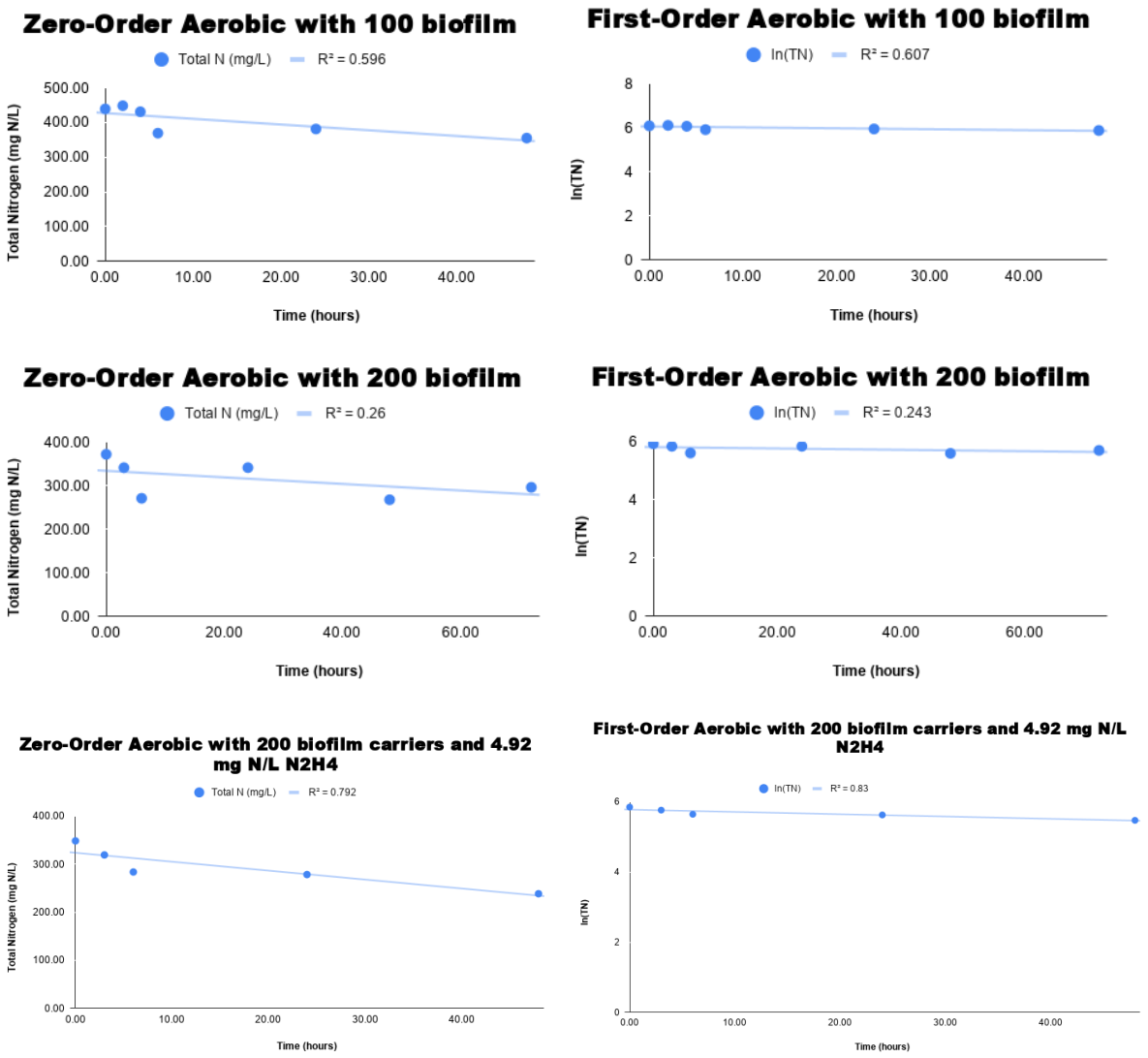
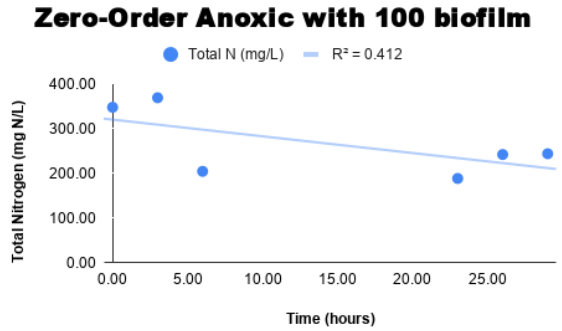


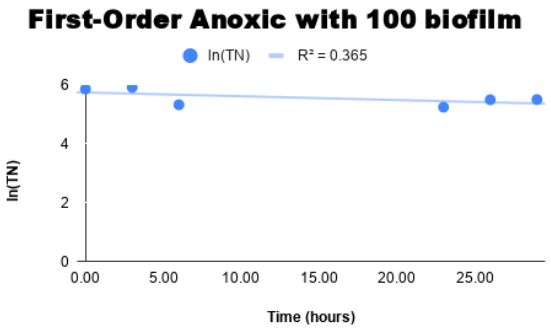
Fig 17. Kinetic analysis of anammox rection for aerobic conditions: a) zero-order reaction with 100 biofilm carriers, b) first-order reaction with 100 biofilm carriers, c) zero-order reaction with 200 biofilm carriers, d) first-order reaction with 200 biofilm carriers, e) zero-order reaction with 200 biofilm carriers and added hydrazine sulphate, f) first.-order reaction with 200 biofilm carriers and added hydrazine sulphate

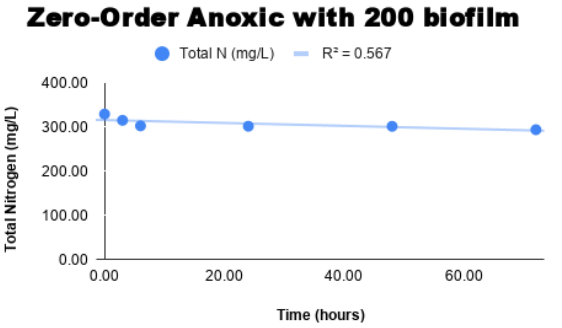
Under aerobic conditions with 100 biofilm carriers, the first-order model provided a superior fit (R^2 = 0.607) compared to zero-order kinetics (R^2 = 0.508). This indicates that nitrogen removal was substrate-limited - the rate of removal depended on the available nitrogen concentration, decreasing as substrates were consumed. Such behavior is characteristic of nitrifying biofilms where ammonia-oxidizing bacteria (AOB) activity responds proportionally to ammonium availability.

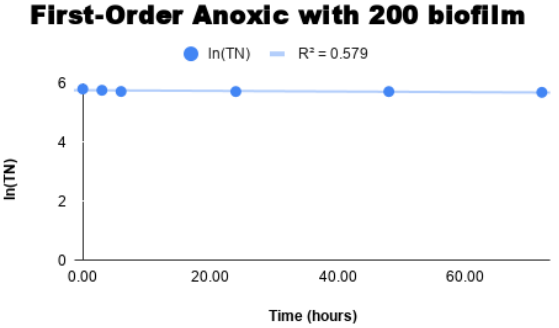
Increasing the biofilm carrier concentration to 200 biofilm carriers without hydrazinium amendment resulted in poor fits for both kinetic models ($R^2 < 0.3$). The low coefficients reflect the non-monotonic behavior observed in this condition, where total nitrogen decreased initially, increased at 24 hours, and decreased again thereafter. This pattern suggests dynamic biofilm processes such as transient detachment or changes in metabolic activity.

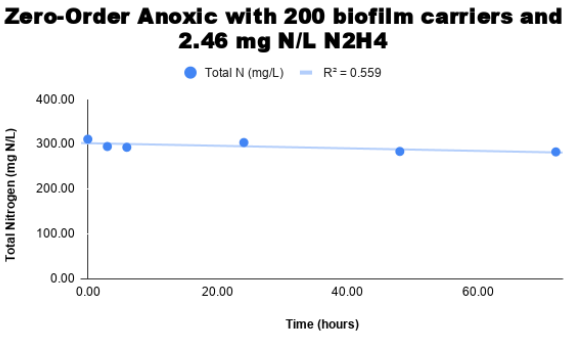
Addition of 4.92 mg/L hydrazinium sulphate to the Aerobic with 200 biofilm carriers condition dramatically improved kinetic predictability, yielding excellent fits for both zero-order (R^2 = 0.792) and first-order (R^2 = 0.83) models. The superior first-order fit (R^2 = 0.83) confirms that hydrazine-enhanced aerobic removal remains substrate-limited while achieving greater overall stability. The high R^2 values indicate that hydrazine regularizes the nitrogen removal process, eliminating the erratic behavior observed without amendment.

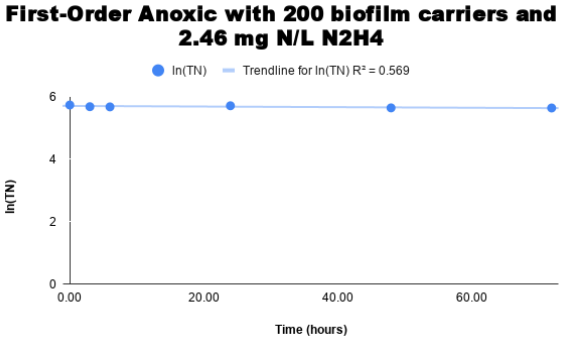


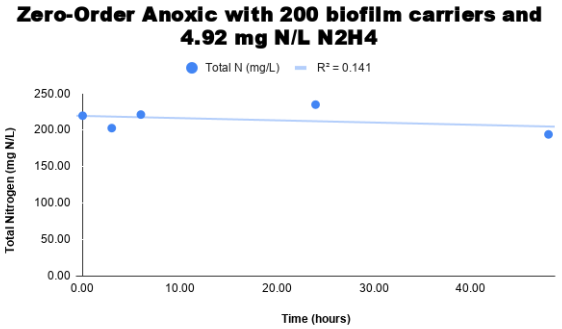












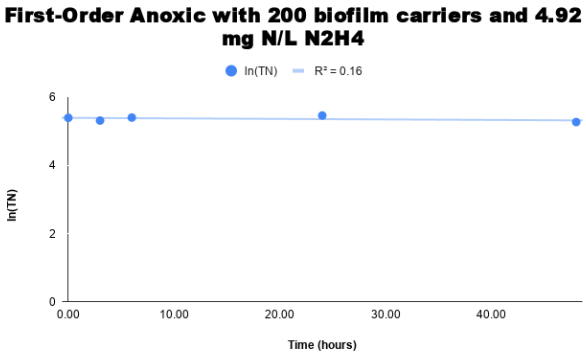
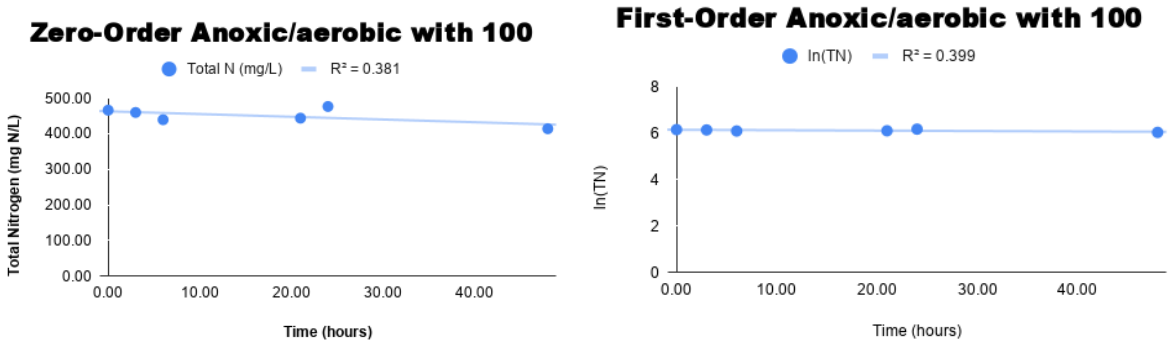


Fig 18. Kinetic analysis for biofilm tests under anoxic conditions with different amount of biocarriers and without hydrazine addition: a) zero-order reaction with 100 biofilm carriers, b) first-order reaction with 100 biofilm carriers, c) zero-order reaction with 200 biofilm carriers, d) first-order reaction with 200 biofilm carriers, e) zero-order reaction with 200 biofilm carriers and added 2.46 mg/L hydrazine sulphate, f) first.-order reaction with 200 biofilm carriers and added 2.46 mg/L hydrazine sulphate, g) zero-order reaction with 200 biofilm carriers and added 4.92 mg/L hydrazine sulphate, h) first.-order reaction with 200 biofilm carriers and added 4.92 mg/L hydrazine sulphate.

Kinetic analysis of anoxic conditions revealed the characteristic complexity of anammox-mediated removal. For Anoxic condition with 100 biofilm carriers, both zero-order ($R^2 = 0.412$) and first-order ($R^2 = 0.365$) models showed only moderate fits. This reflects the biphasic nature of anammox activity observed in this condition - rapid removal between 0-6 hours followed by partial rebound between 23-29 hours. The relatively low R^2 values are not indicative of poor experimental quality but rather demonstrate that anammox systems operating in batch mode exhibit temporal dynamics that simple kinetic models cannot capture.

Increasing biofilm carriers to 200 under anoxic conditions improved model fits modestly (zero-order $R^2 = 0.587$; first-order $R^2 = 0.579$). The slightly better zero-order fit suggests that at higher biofilm densities, mass transfer limitations may begin to constrain removal, making the rate less dependent on bulk liquid concentration. This interpretation aligns with the lower absolute removal rates observed in anoxic conditions with 200 biofilm carriers compared to anoxic conditions with 100 biofilm carriers.

Hydrazine amendment under anoxic conditions (anoxic conditions with 200 biofilm carriers + 2.46 mg/L hydrazine sulphate) produced similar kinetic behavior (zero-order $R^2 = 0.559$; first-order $R^2 = 0.569$), confirming that hydrazine does not substantially alter anammox kinetics and that its primary effect is on aerobic ammonia-oxidizing activity.



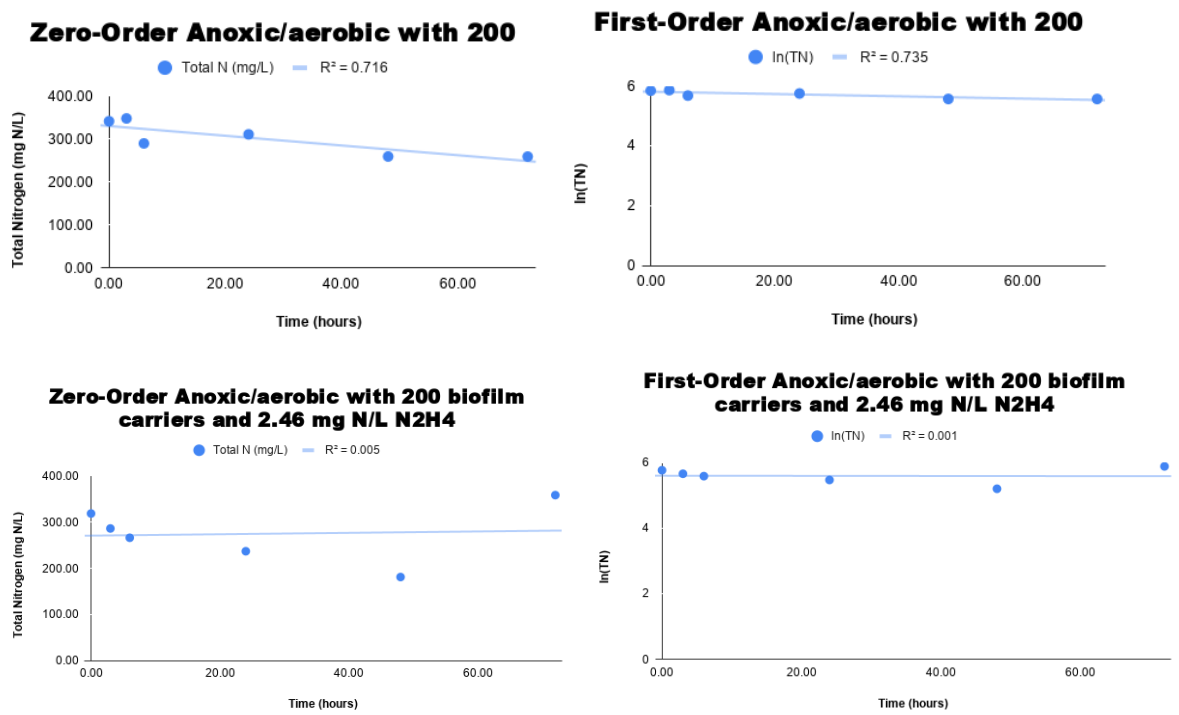


Fig 19. Kinetic analysis for anoxic/aerobic conditions: a) zero-order reaction with 100 biofilm carriers, b) first-order reaction with 100 biofilm carriers, c) zero-order reaction with 200 biofilm carriers, d) first-order reaction with 200 biofilm carriers, e) zero-order reaction with 200 biofilm carriers and added 2.46 mg/L hydrazine sulphate, f) first.-order reaction with 200 biofilm carriers and added 2.46 mg/L hydrazine sulphate.

The alternating anoxic-aerobic condition designed to simulate MBBR biofilm stratification yielded the most informative kinetic results. For anoxic-aerobic conditions performed with 200 biofilm carriers, both kinetic models provided good fits to the experimental data (zero-order R^2 = 0.716; first-order R^2 = 0.735). The high coefficients of determination indicate that the alternating creates stable, predictable nitrogen removal kinetics.

For the Anoxic-Aerobic with 200 biofilm carriers condition amended with 2.46 mg/L hydrazine sulphate, both zero-order (R^2 = 0.005) and first-order (R^2 = 0.001) models exhibited effectively no correlation with the experimental data. This apparent failure of kinetic modeling is itself a significant finding that the near-zero R^2 values result from system perturbation observed between 48 and 72 hours, where total nitrogen concentration increased from 181.03 mg/L to 358.28 mg/L despite previous removal.

While the alternating anoxic-aerobic regime with hydrazine achieves the highest nitrogen removal at 48 hours (74.0% NH_4^+ removal; 43.2% TNRE), it does so at the cost of long-term instability. The kinetic analysis reveals that beyond 48 hours, the system undergoes a fundamental shift, likely biofilm detachment, oxygen intrusion, or hydrazinium toxicity.

Table 1: Summary of kinetics parameters

Condition	Biofilm Carriers	Hydrazine Sulphate (mg N/L)	Zero-order R^2	First-order R^2	Best model
Aerobic	100	0	0.596	0.607	First-order
Aerobic	200	0	0.26	0.243	None
Aerobic	200	4.92	0.792	0.83	First-order
Anoxic	100	0	0.412	0.365	Zero-order
Anoxic	200	0	0.567	0.579	First-order
Anoxic	200	2.46	0.559	0.569	First-order
Anoxic	200	4.92	0.141	0.16	None
Anoxic/Aerobic	100	0	0.381	0.399	First-order
Anoxic/Aerobic	200	0	0.716	0.735	First-order
Anoxic/Aerobic	200	2.46	0.005	0.001	None

Overall, the predominance of first-order kinetics confirms that the MBBR-enriched anammox consortium responds predictably to nitrogen loading, supporting the feasibility of the proposed synergistic system while highlighting the need to balance peak removal rates against operational stability.

3.2.4 Pharmaceutically Active Compounds (PhACs) Attenuation and Analysis

To evaluate the micropollutant removal capacity of the anammox-enriched MBBR, batch kinetic tests were executed under two distinct conditions: a baseline anoxic phase (no hydrazine) and an anoxic phase (with 4.92 mg/L hydrazine sulphate). The target PhACs represented distinct therapeutic classes with varying degrees of biodegradability.

3.2.4.1 Degradation profile and kinetics of fluoroquinolones

The fluoroquinolones – ofloxacin, enrofloxacin and marbofloxacin, exhibited the highest susceptibility to attenuation under standard anoxic conditions.

- **Baseline anoxic removal:** Under standard anoxic conditions, all three fluoroquinolones demonstrated consistent time-dependent concentration decays. Ofloxacin concentrations fit a first-order decay model with an R^2 value of 0.852, while enrofloxacin and marbofloxacin achieved R^2 values of 0.784 and 0.579 respectively.

Peak removal efficiencies under baseline conditions reached 68.09% for ENR and 51.32% for MAR. This removal is attributed to co-metabolic biodegradation pathways maintained by the diverse, heterotrophic microbial communities residing within the outer layer of the stratified biofilm matrix.

- **The shift under hydrazine stimulation**: The addition of hydrazine heavily disrupted the decay pathways, as evidenced by the drop in kinetics. For OFL, the first-order fit fell to an R^2 value of 0.399, while ENR decay collapsed to a R^2 value of 0.111. As shown in the bar chart (Fig 23.), the overall removal efficiency of ENR dropped from 42.3% to 2.9% when hydrazine was introduced. Conversely, OFL removal was boosted from 29.6% to 73% with hydrazine.

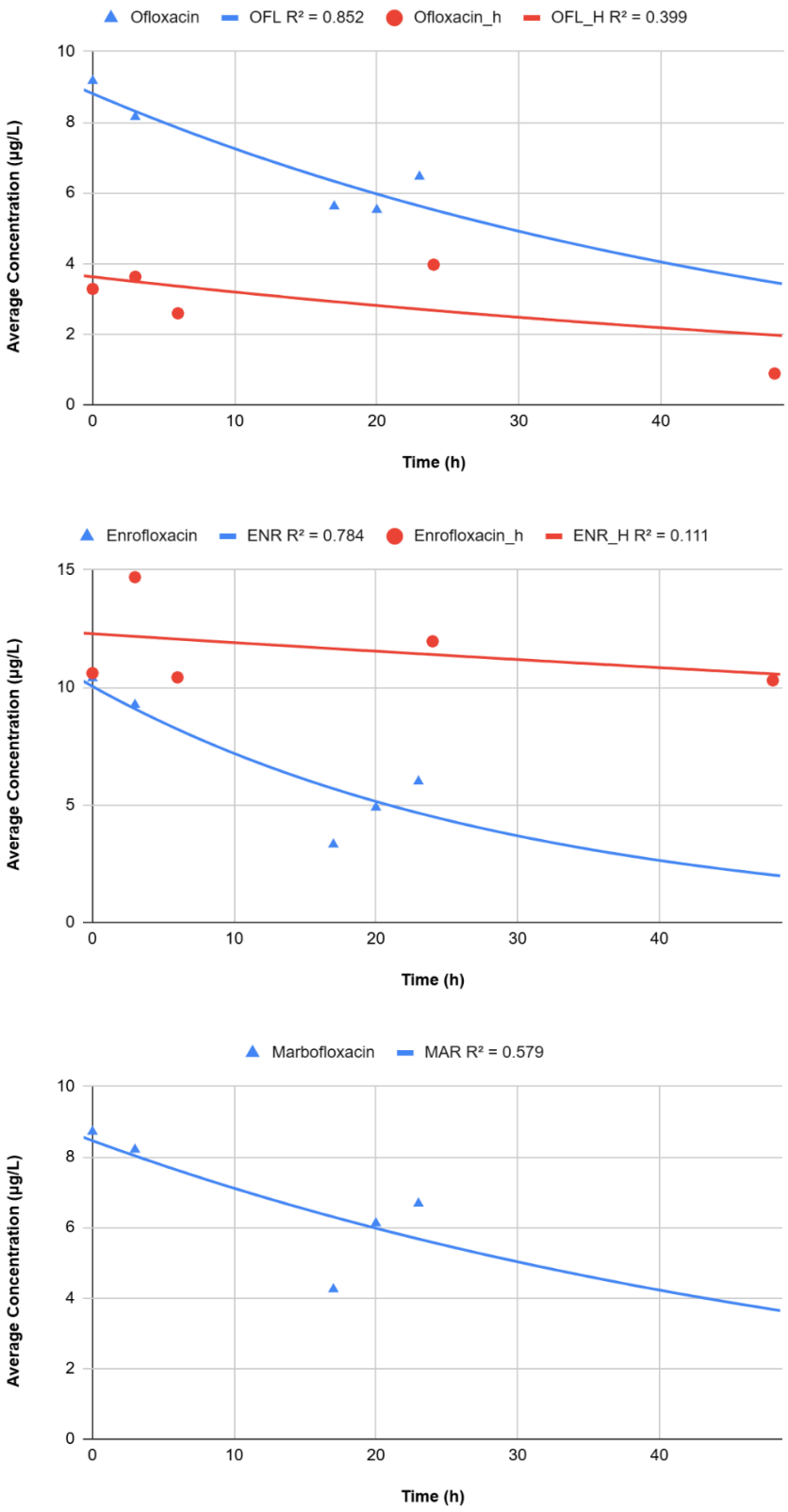


Fig 20. Degradation profiles of selected fluoroquinolones during anoxic batch experiment with (H) and without hydrazine addition: a) ofloxacin, b) enrofloxacin, and c) marbofloxacin

The divergence between hydrazine and non-hydrazine experiments indicates that hydrazine acts as a metabolic modifier. It selectively accelerates the pathways that may co-regulate OFL

degradation, but simultaneously acts as a toxicant or competitive inhibitor to the heterotrophic enzymes responsible for breaking down ENR and MAR.

3.2.4.2 Mechanism of negative removal efficiency in sulfamethoxazole and carbamazepine

The most striking results from the experiment are significant negative removal efficiencies observed for SMX and CAR, particularly during the hydrazine-amended processes.

Sulfamethoxazole dynamics

Under baseline anoxic conditions, SMX removal was poor ($R^2 = 0.066$), exhibiting a net flatline or slight accumulation. Upon the addition of hydrazine, SMX concentrations rose substantially over time ($R^2 = 0.395$ with an upward trend), culminating in a severe negative net removal efficiency (-53.9%). This can be explained by two biochemical actions:

- De-conjugation of human metabolites: real municipal wastewater matrix contains high percentages of pharmaceuticals in conjugated form (such as N4-acetylsulfamethoxazole). These conjugated variations are undetectable during initial standard LC-MS targeted profiling. However, under biological stress or altered metabolic conditions in the reactor, bacterial enzymes like glucuronidases cleave these acetyl groups, converting the hidden metabolites back into the parent SMX compound, causing the measured liquid concentration to rise.
- Hydrazine-induced biosurfactant production: the metabolic spike experienced by anammox bacteria upon receiving hydrazine can stimulate the secretion of extracellular polymeric substances (EPS) or biosurfactants. These compounds lower the interfacial tension within the biofilm pores, forcing loosely bound, previously adsorbed SMX back out into the bulk liquid.

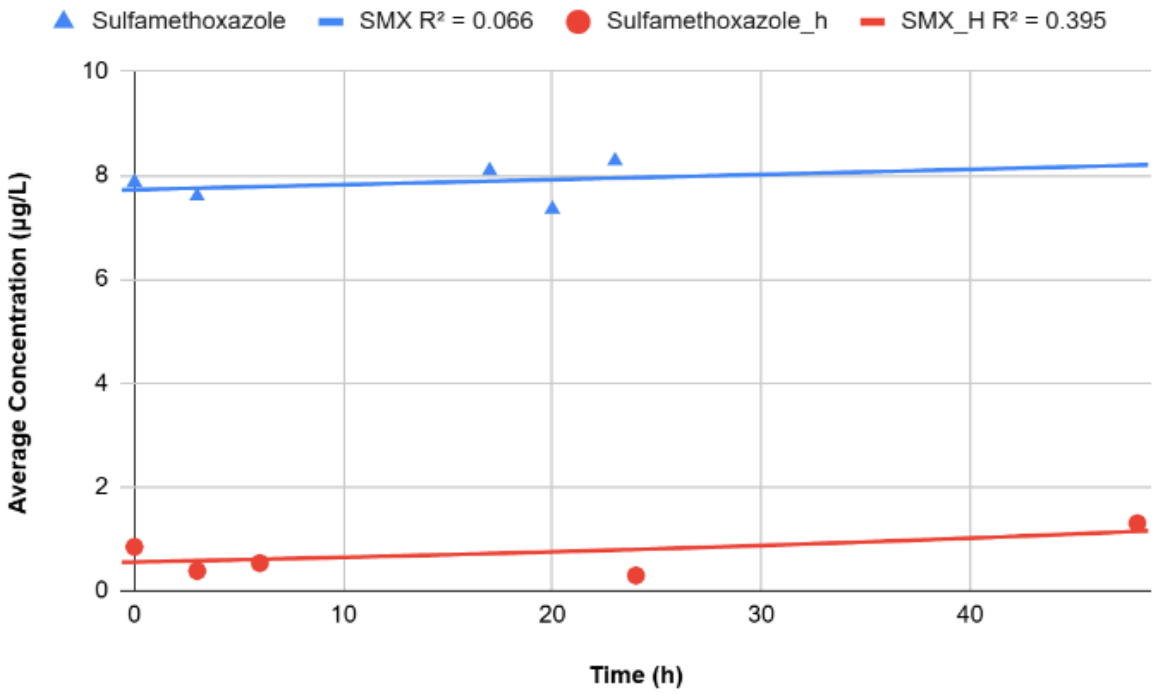


Fig 21. PhACs removal efficiency under anoxic conditions for sulfamethoxazole with (H) and without addition of hydrazine.

Carbamazepine dynamics

Carbamazepine is universally regarded as a highly recalcitrant indicator drug. Under anoxic conditions, CAR displayed absolute stability with a flatline kinetic model ($R^2 = 0.004$) and a near-zero removal.

However, when hydrazine was introduced, CAR performance plummeted to a negative removal efficiency of -32.3%. Because CAR is highly hydrophobic, it accumulates over months inside the interior plastic carrier matrix and deep within dead biofilm layers during continuous-flow pilot runs. Since anammox bacteria lacks the metabolic pathways to break down CAR, the addition of hydrazine caused zero biological degradation. Instead, the sudden shift in fluid chemistry, cellular energy changes or localized gas production (N_2) caused a physical structural shift in the biofilm, forcing old, accumulated CAR to desorb into the clean batch test liquid.

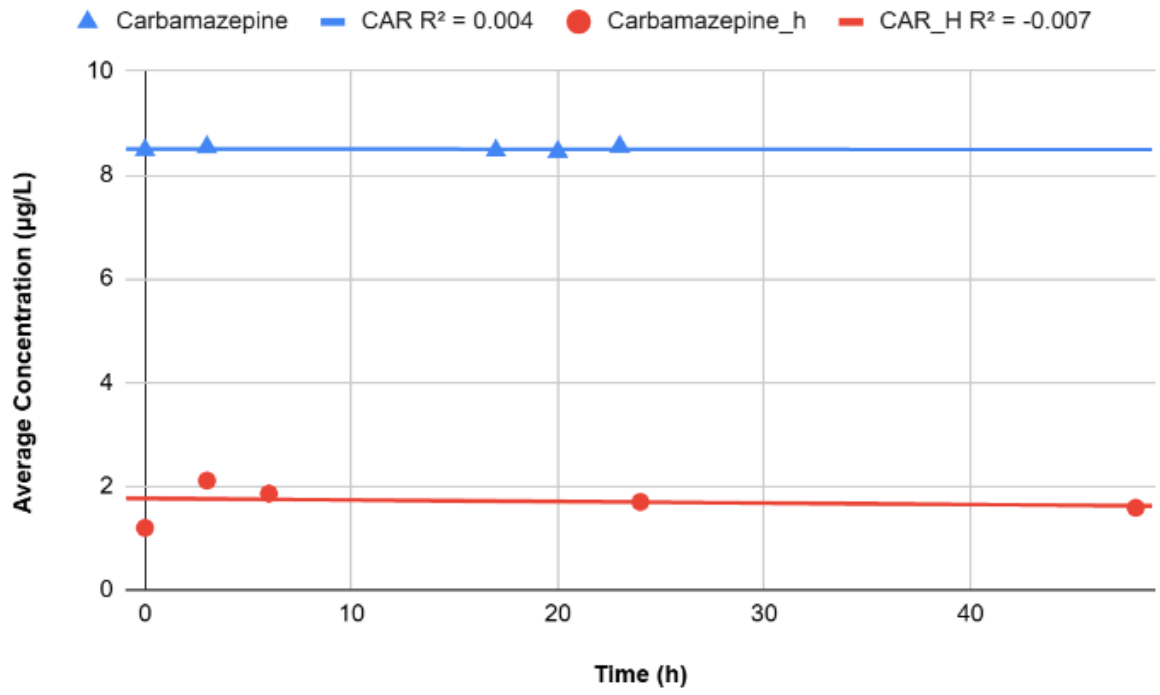


Fig 22. PhACs removal efficiency under anoxic conditions for carbamazepine with (H) and without addition of hydrazine.

The removal efficiency of the selected PhACs in anoxic batch tests, both with and without N_2H_4 is presented in Fig 20. Different groups of PhACs showed varied efficiencies with OFL showing the most efficient removal.

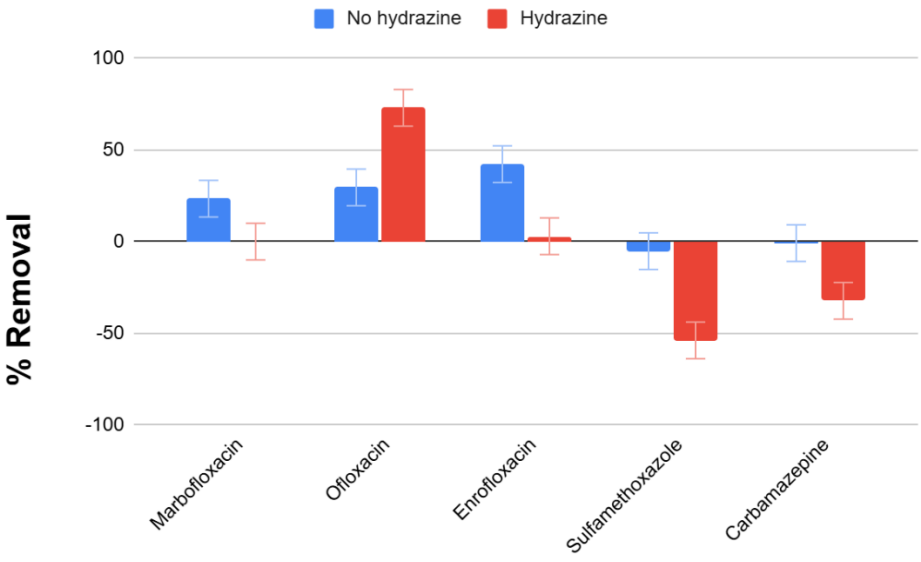


Fig 23. Removal efficiency (%) of PhACs in conditions with and without hydrazine addition.

Detection of native pharmaceuticals in samples

During the LC-MS analysis, three additional PhACs – Clarithromycin, Diclofenac and Trimethoprim were qualitatively identified in the samples. Since these compounds were not

part of the PhACs of interest for this study, those added to the samples, their presence is attributed to the background matrix of the reject water sourced from the Tartu Municipal WWTP used for this project.

Though these compounds were not quantified, their detection is significant. They confirm that the reactor biomass was operating within a complex chemical environment where multiple antibiotics (Clarithromycin and Trimethoprim) and non-steroidal anti-inflammatory drugs (Diclofenac) were already present. The presence of these pharmaceuticals may have exerted a selective pressure on the anammox-enriched biofilm, potentially influencing the baseline removal efficiencies observed for the PhACs of interest.

SUMMARY

The investigation of the anammox-enriched MBBR successfully demonstrated a robust capacity for high-rate nitrogen removal (maximum removal rate of 276 mg N/L/d with an efficiency of 79.85%) and selective pharmaceutical attenuation. The reactor maintained stable performance across the operational period, effectively decoupling biomass retention from HRT using K1-shaped biofilm carriers.

The system achieved efficient nitrogen removal, primarily driven by the anammox pathway. Stoichiometric analysis of NH_4^+-N and NO_3^--N production confirmed that the biofilm maintained a dominant anammox consortium, even when subjected to varying nitrogen loading rates (TNLR). While a temporary performance dip occurred around day 107, the system showed high resilience with removal efficiencies quickly rebounding to steady-state levels.

The baseline anoxic batch tests revealed a highly selective degradation profile for the target pharmaceuticals. Fluoroquinolones exhibited significant removal, with enrofloxacin achieving a peak efficiency at 68.09%, indicating active co-metabolism within the biofilm. Carbamazepine remained entirely recalcitrant, serving as benchmark for the biological limits of the system. And sulfamethoxazole showed unstable removal patters, suggesting complex transformation or desorption dynamics.

The addition of hydrazinium sulphate, while a known stimulant for anammox metabolism resulted in erratic PhAC removal rates and evidence of compound desorption. This suggests that anammox may outcompete with heterotrophic species for micropollutant attenuation. The detection of other pharmaceutical in the samples highlight the real-world complexity of the treatment matrix.

In conclusion, the anammox-MBBR configuration proves to be highly effective technology for mainstream nitrogen removal, though its role in pharmaceutical removal is dependent on the maintenance of diverse, stratified biofilm community that supports specialised anammox and co-metabolic heterotrophic microorganisms.

REFERENCES

Afolalu, S. A., Ogedengbe, T. S., Ikumapayi, O. M., Adesoji Adediran, A., & Yussouff, A. A. (2024). Wastewater and its Management – A Review. *2024 International Conference on Science, Engineering and Business for Driving Sustainable Development Goals (SEB4SDG)*, 1–7. https://doi.org/10.1109/SEB4SDG60871.2024.10630096

Ahmad, H. A., Ni, S.-Q., Ahmad, S., Zhang, J., Ali, M., Ngo, H. H., Guo, W., Tan, Z., & Wang, Q. (2020). Gel immobilization: A strategy to improve the performance of anaerobic ammonium oxidation (anammox) bacteria for nitrogen-rich wastewater treatment. *Bioresource Technology*, *313*, 123642. https://doi.org/10.1016/j.biortech.2020.123642

Al-Hazmi, H. E., Lu, X., Grubba, D., Majtacz, J., Badawi, M., & Mąkinia, J. (2023). Sustainable nitrogen removal in anammox-mediated systems: Microbial metabolic pathways, operational conditions and mathematical modelling. *Science of The Total Environment*, *868*, 161633. https://doi.org/10.1016/j.scitotenv.2023.161633

Alsubih, M., & Khan, R. A. (2024). Pharmaceutical Active Compounds Contamination and Seasonal-Based Ecotoxicity Assessment of the Yamuna River for the Delhi stretch. *Rocznik Ochrona Środowiska*, *26*, 587–602. https://doi.org/10.54740/ros.2024.052

Anammox—Growth Physiology, Cell Biology, and Metabolism. (2012). In *Advances in Microbial Physiology* (Vol. 60, pp. 211–262). Academic Press. https://doi.org/10.1016/B978-0-12-398264-3.00003-6

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, *108*(supplement_1), 4516–4522. https://doi.org/10.1073/pnas.1000080107

Chen, X., Duan, F., Yu, X., Xie, Y., Wang, Z., & Ni, S.-Q. (2024). Uncovering pathway and mechanism of simultaneous thiocyanate detoxicity and nitrate removal through anammox and denitrification. *Npj Clean Water*, *7*(1), 109. https://doi.org/10.1038/s41545-024-00402-w

Choudhary, M., Muduli, M., & Ray, S. (2022). A comprehensive review on nitrate pollution and its remediation: Conventional and recent approaches. *Sustainable Water Resources Management*, *8*(4), 113. https://doi.org/10.1007/s40899-022-00708-y

Crini, G., & Lichtfouse, E. (2019). Advantages and disadvantages of techniques used for wastewater treatment. *Environmental Chemistry Letters*, *17*(1), 145–155. https://doi.org/10.1007/s10311-018-0785-9

Di Capua, F., Iannacone, F., Sabba, F., & Esposito, G. (2022). Simultaneous nitrification–denitrification in biofilm systems for wastewater treatment: Key factors, potential routes, and engineered applications. *Bioresource Technology*, *361*, 127702. https://doi.org/10.1016/j.biortech.2022.127702

Frade, V. M. F., Dias, M., Teixeira, A. C. S. C., & Palma, M. S. A. (2014). Environmental contamination by fluoroquinolones. *Brazilian Journal of Pharmaceutical Sciences*, *50*(1), 41–54. https://doi.org/10.1590/S1984-82502011000100004

Fu, J., Zhang, Q., Huang, B., Fan, N., & Jin, R. (2021). A review on anammox process for the treatment of antibiotic-containing wastewater: Linking effects with corresponding

mechanisms. *Frontiers of Environmental Science & Engineering*, *15*(1), 17. https://doi.org/10.1007/s11783-020-1309-y

Guo, Y., Luo, Z., Shen, J., & Li, Y.-Y. (2021). The main anammox-based processes, the involved microbes and the novel process concept from the application perspective. *Frontiers of Environmental Science & Engineering*, *16*(7), 84. https://doi.org/10.1007/s11783-021-1487-1

Hakami, R. A., Yahya, M. S., Bakkali, M. E., & Hakami, A. A. (2024). Comparative Analysis and Kinetic Modeling of Marbofloxacin Degradation via Electro-Fenton and Biodegradation Optimization. *Rocznik Ochrona Środowiska*, *26*, 638–657. https://doi.org/10.54740/ros.2024.056

Jetten, M. S. M., Niftrik, L. van, Strous, M., Kartal, B., Keltjens, J. T., & Op den Camp, H. J. M. (2009). Biochemistry and molecular biology of anammox bacteria. *Critical Reviews in Biochemistry and Molecular Biology*, *44*(2–3), 65–84. https://doi.org/10.1080/10409230902722783

Kailas, S., & Shibu, K. (2013). Nitrogen removal from eutrophic water using Biological Reactors. *2013 International Conference on Energy Efficient Technologies for Sustainability*, 458–462. https://doi.org/10.1109/ICEETS.2013.6533427

Kallistova, A. Yu., Dorofeev, A. G., Nikolaev, Yu. A., Kozlov, M. N., Kevbrina, M. V., & Pimenov, N. V. (2016). Role of anammox bacteria in removal of nitrogen compounds from wastewater. *Microbiology*, *85*(2), 140–156. https://doi.org/10.1134/S0026261716020089

Kangwannarakul, N., Wantawin, C., & Noophan, P. (2018). Anammox bacteria with attached-growth media for nitrogen removal in wastewater. *Clean Technologies*

and Environmental Policy, *20*(1), 219–226. https://doi.org/10.1007/s10098-017-1458-2

Kato, S., & Kansha, Y. (2024). Comprehensive review of industrial wastewater treatment techniques. *Environmental Science and Pollution Research*, *31*(39), 51064–51097. https://doi.org/10.1007/s11356-024-34584-0

Kazantseva, J., Malv, E., Kaleda, A., Kallastu, A., & Meikas, A. (2021). Optimisation of sample storage and DNA extraction for human gut microbiota studies. *BMC Microbiology*, *21*(1), 158. https://doi.org/10.1186/s12866-021-02233-y

Li, J., Li, J., Gao, R., Wang, M., Yang, L., Wang, X., Zhang, L., & Peng, Y. (2018). A critical review of one-stage anammox processes for treating industrial wastewater: Optimization strategies based on key functional microorganisms. *Bioresource Technology*, *265*, 498–505. https://doi.org/10.1016/j.biortech.2018.07.013

Li, Y., Zhu, G., Ng, W. J., & Tan, S. K. (2014). A review on removing pharmaceutical contaminants from wastewater by constructed wetlands: Design, performance and mechanism. *Science of The Total Environment*, *468–469*, 908–932. https://doi.org/10.1016/j.scitotenv.2013.09.018

Lin, Y.-M., Tay, J.-H., Liu, Y., & Hung, Y.-T. (2009). Biological Nitrification and Denitrification Processes. In L. K. Wang, N. C. Pereira, & Y.-T. Hung (Eds.), *Biological Treatment Processes* (pp. 539–588). Humana Press. https://doi.org/10.1007/978-1-60327-156-1_13

Loh, Z. Z., Zaidi, N. S., Syafiuddin, A., Yong, E. L., Bahrodin, M. B., Aris, A., & Boopathy, R. (2023). Current status and future prospects of simultaneous nitrification and denitrification in wastewater treatment: A bibliometric review. *Bioresource Technology Reports*, *23*, 101505. https://doi.org/10.1016/j.biteb.2023.101505

Lu, Y., Wang, L., Wang, X., Sun, T., Yang, R., & Wang, Z. (2009). Shortcut Nitrification and ANAMMOX Combined Process for Autotrophic Ammonia Removal. *2009 3rd International Conference on Bioinformatics and Biomedical Engineering*, 1–4. https://doi.org/10.1109/ICBBE.2009.5163383

Ma, B., Wang, S., Cao, S., Miao, Y., Jia, F., Du, R., & Peng, Y. (2016). Biological nitrogen removal from sewage via anammox: Recent advances. *Bioresource Technology*, *200*, 981–990. https://doi.org/10.1016/j.biortech.2015.10.074

Madan, S., Madan, R., & Hussain, A. (2022). Advancement in biological wastewater treatment using hybrid moving bed biofilm reactor (MBBR): A review. *Applied Water Science*, *12*(6), 141. https://doi.org/10.1007/s13201-022-01662-y

Mamun, F.-A., Kumar, R., Männisalu, L. B., Zhong, J., Bester, K., Espenberg, M., Rao, A., Appels, L., Dewil, R., & Zekker, I. (2026). Enhanced anammox biofilm-mediated nitrogen and pharmaceuticals removal under anoxic conditions. *Journal of Water Process Engineering*, *82*, 109387. https://doi.org/10.1016/j.jwpe.2025.109387

Mandel, A., Zekker, I., Jaagura, M., & Tenno, T. (2019). Enhancement of anoxic phosphorus uptake of denitrifying phosphorus removal process by biomass adaption. *International Journal of Environmental Science and Technology*, *16*(10), 5965–5978. https://doi.org/10.1007/s13762-018-02194-2

Montemurro, N., García-Vara, M., Peña-Herrera, J. M., Lladó, J., Barceló, D., & Pérez, S. (2018). Conventional and Advanced Processes for the Removal of Pharmaceuticals and Their Human Metabolites from Wastewater. In M. Cledon, S. K. Brar, R. Galvez, & V. Oyanedel-Craver (Eds.), *ACS Symposium Series* (Vol. 1302, pp. 15–67). American Chemical Society. https://doi.org/10.1021/bk-2018-1302.ch002

Muduli, M., Chanchpara, A., Choudhary, M., Saravaia, H., Haldar, S., & Ray, S. (2022). Critical review on sustainable bioreactors for wastewater treatment and water reuse. *Sustainable Water Resources Management*, *8*(5), 159. https://doi.org/10.1007/s40899-022-00747-5

Paikun, Nurjaman, D., Utami, M., Alam, V. N., Sunhadji, R. R., & Barkah. (2023). Analysis of the quality of domestic wastewater before and after treatment using WWTP. *2023 IEEE 9th International Conference on Computing, Engineering and Design (ICCED)*, 1–6. https://doi.org/10.1109/ICCED60214.2023.10425404

(PDF) Wastewater Treatment Plants: Operations and Characteristics. (n.d.). *ResearchGate*. https://doi.org/10.36647/TTRRAMB/01.02.A003

Pool, R. (2014). Coming clean the future of sewage treatment. *Engineering & Technology*, *9*(12), 80–83. https://doi.org/10.1049/et.2014.1211

Radjenović, J., Matošić, M., Mijatović, I., Petrović, M., & Barceló, D. (2008). Membrane Bioreactor (MBR) as an Advanced Wastewater Treatment Technology. In D. Barceló & M. Petrovic (Eds.), *Emerging Contaminants from Industrial and Municipal Waste: 5S/2* (pp. 37–101). Springer Berlin Heidelberg. https://doi.org/10.1007/698_5_093

Rahimi, S., Modin, O., & Mijakovic, I. (2020). Technologies for biological removal and recovery of nitrogen from wastewater. *Biotechnology Advances*, *43*, 107570. https://doi.org/10.1016/j.biotechadv.2020.107570

Rajesh Banu, J., Merrylin, J., Kavitha, S., Yukesh Kannah, R., Selvakumar, P., Gopikumar, S., Sivashanmugam, P., Do, K.-U., & Kumar, G. (2021). Trends in Biological Nutrient Removal for the Treatment of Low Strength Organic Wastewaters. *Current Pollution Reports*, *7*(1), 1–30. https://doi.org/10.1007/s40726-020-00169-x

Removal of pharmaceuticals from wastewater: A review of different adsorptive approaches. (2024). *Global NEST Journal*. https://doi.org/10.30955/gnj.005300

Ren, Z.-Q., Wang, H., Zhang, L.-G., Du, X.-N., Huang, B.-C., & Jin, R.-C. (2022). A review of anammox-based nitrogen removal technology: From microbial diversity to engineering applications. *Bioresource Technology*, *363*, 127896. https://doi.org/10.1016/j.biortech.2022.127896

Ronan, E., Aqeel, H., Wolfaardt, G. M., & Liss, S. N. (2021). Recent advancements in the biological treatment of high strength ammonia wastewater. *World Journal of Microbiology and Biotechnology*, *37*(9), 158. https://doi.org/10.1007/s11274-021-03124-0

Shigei, M., Assayed, A., Hazaymeh, A., & Dalahmeh, S. S. (2021). Pharmaceutical and Antibiotic Pollutant Levels in Wastewater and the Waters of the Zarqa River, Jordan. *Applied Sciences*, *11*(18), 8638. https://doi.org/10.3390/app11188638

Thakur, I. S., & Medhi, K. (2019). Nitrification and denitrification processes for mitigation of nitrous oxide from waste water treatment plants for biovalorization: Challenges and opportunities. *Bioresource Technology*, *282*, 502–513. https://doi.org/10.1016/j.biortech.2019.03.069

The Ultimate Guide to MBBR (Moving Bed Biofilm Reactor)—Gustawater. (n.d.). Retrieved February 17, 2026, from https://www.gustawater.com/blog/mbbr.html

Tian, Z., Zeng, P., Song, Y., Li, D., & Zhang, J. (2010). Nitrogen Removal Potential and Biofilm Characteristics in the Anaerobic Ammonium Oxidation ('ANAMMOX') Biofilter Reactor. *2010 4th International Conference on Bioinformatics and Biomedical Engineering*, 1–5. https://doi.org/10.1109/ICBBE.2010.5514910

Usman, M., Sanaullah, M., Ullah, A., Li, S., & Farooq, M. (2022). Nitrogen Pollution Originating from Wastewater and Agriculture: Advances in Treatment and Management. *Reviews of Environmental Contamination and Toxicology*, *260*(1), 9. https://doi.org/10.1007/s44169-022-00010-0

Verma, S., Kuila, A., & Jacob, S. (2023). Role of Biofilms in Waste Water Treatment. *Applied Biochemistry and Biotechnology*, *195*(9), 5618–5642. https://doi.org/10.1007/s12010-022-04163-5

Wang, B., Li, H., Liu, T., & Guo, J. (2021). Enhanced removal of cephalexin and sulfadiazine in nitrifying membrane-aerated biofilm reactors. *Chemosphere*, *263*, 128224. https://doi.org/10.1016/j.chemosphere.2020.128224

Wang, X.-P., Wu, Q., Wang, X., Fan, N.-S., & Jin, R.-C. (2023). Research advances in application of mainstream anammox processes: Roles of quorum sensing and microbial metabolism. *Chemosphere*, *333*, 138947. https://doi.org/10.1016/j.chemosphere.2023.138947

Weralupitiya, C., Wanigatunge, R., Joseph, S., Athapattu, B. C. L., Lee, T.-H., Kumar Biswas, J., Ginige, M. P., Shiung Lam, S., Senthil Kumar, P., & Vithanage, M. (2021). Anammox bacteria in treating ammonium rich wastewater: Recent perspective and appraisal. *Bioresource Technology*, *334*, 125240. https://doi.org/10.1016/j.biortech.2021.125240

Wu, G., Geng, J., Xu, K., & Ren, H. (2021). Removal of pharmaceuticals by ammonia oxidizers during nitrification. *Applied Microbiology and Biotechnology*, *105*(3), 909–921. https://doi.org/10.1007/s00253-020-11032-w

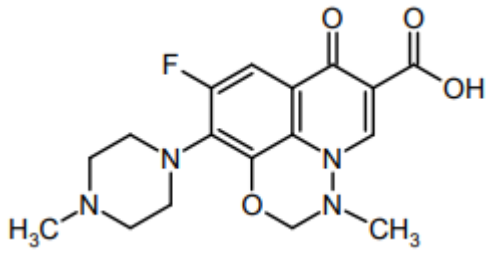
Xiao, J., Xu, G., & Tang, J. (2011). Advance in microbiology and application of anammox bacteria. *2011 International Conference on Electronics, Communications and Control (ICECC)*, 3531–3534. https://doi.org/10.1109/ICECC.2011.6068039

Xu, Y., Gu, Y., Peng, L., Wang, N., Chen, S., Liang, C., Liu, Y., & Ni, B.-J. (2023). Unravelling ciprofloxacin removal in a nitrifying moving bed biofilm reactor: Biodegradation mechanisms and pathways. *Chemosphere*, *320*, 138099. https://doi.org/10.1016/j.chemosphere.2023.138099

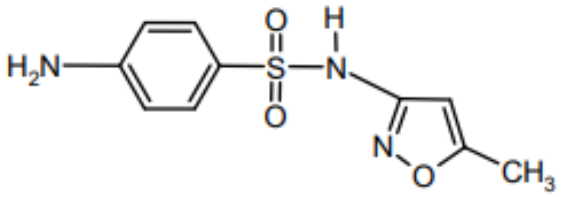
Xudong, Z., Zongsheng, Z., Weijing, J., Jing, D., & Jing, J. (2010). Mathematical simulations of nitrogen removal via a partial nitrification-anaerobic ammonium oxidation in a membrane-aerated biofilm reactor. *2010 International Conference on Mechanic Automation and Control Engineering*, 2018–2025. https://doi.org/10.1109/MACE.2010.5536335

Zhang, Z., Wang, Y., Geng, J., & Zhao, W. (2010). N2O production during nitrogen removal in sequence batch reactor wastewater treatment systems. *2010 International Conference on Mechanic Automation and Control Engineering*, 1846–1849. https://doi.org/10.1109/MACE.2010.5536726

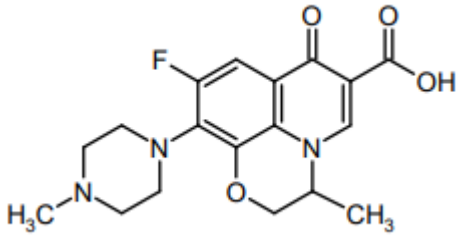
Appendix: Structure of pharmaceutically active compounds



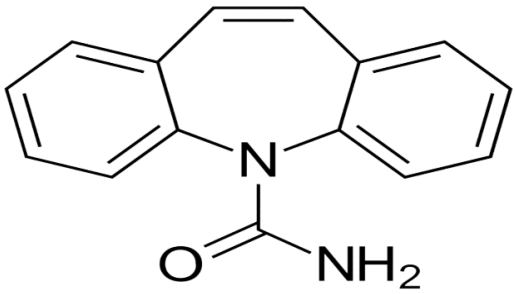
Marbofloxacin (MAR)



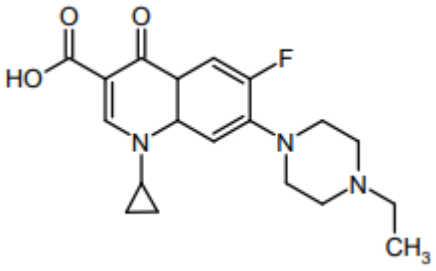
Sulfamethoxazole (SMX)



Ofloxacin (OFL)



Carbamazepine (CAR)



Enrofloxacin (ENR)

Acknowledgement

I would like to express my sincere gratitude to my supervisors, Dr Ivar Zekker and Faysal-Al Mamun, for their continuous guidance, support, and valuable feedback throughout the course of this project. Their expertise, patience, and encouragement greatly contributed to the completion of this work.

I am also thankful to Dr Ernesto Zapata and Assoc. Prof. Koit Herodes for providing the resources, facilities, and stimulating research environment necessary for this study. I appreciate the support and collaboration of all members of the laboratory and research group.

Special thanks go to my brother, Phelim A. Ekwebe for his financial and personal support during my studies. His encouragement and belief in me made this journey possible.

Finally, I would like to thank my family and friends for their unwavering support, understanding, and motivation throughout this process.

NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC

I, **Blessing Ambang Ekwebe,**

1. grant the University of Tartu a free permit (non-exclusive licence) to

   reproduce, for the purpose of preservation, including for adding to the digital archives of the University of Tartu until the expiry of the term of copyright, my thesis

   Removal of Nitrogen and Pharmaceutical compounds in a Synergistic System

   Supervised by **Ivar Zekker (PhD) and Faysal-Al Mamun (MSc.)**

2. grant the University of Tartu a permit to make the thesis specified in point 1 available to the public via the web environment of the University of Tartu, including via the digital archives, under the Creative Commons licence CC BY NC ND 4.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work until the expiry of the term of copyright;

3. am aware of the fact that the author retains the rights specified in points 1 and 2;

4. confirm that granting the non-exclusive licence does not infringe other persons' intellectual property rights or rights arising from the personal data protection legislation.

Blessing Ambang Ekwebe

20/05/2026