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**AUTOTROPHIC NITROGEN REMOVAL FOR LOW ORGANIC WASTEWATER  
TREATMENT**

Master's Thesis (30 ECTS)

Bioengineering

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## **Abstract**

### **Autotrophic Nitrogen Removal for Low Organic Wastewater Treatment**

Municipal wastewater can be directly anaerobically treated to recover energy in the form of biogas. By using an autotrophic process, such as anaerobic ammonium oxidation (anammox) process, to remove the ammonium from the anaerobic effluent, one can further reduce the energy needed for wastewater treatment. Up until now, anammox has primarily been utilized to treat waste streams at elevated temperatures ( $>25^{\circ}\text{C}$ ) for concentrated ( $>500\text{ mg N/L}$ ) flows. The challenges of anammox process are its application of the water line of municipal wastewater treatment include lower nitrogen concentrations ( $100\text{ mg N/L}$ ), high organic carbon (pharmaceutical presence) and lower temperatures ( $20^{\circ}\text{C}$ ). Two 20-liter moving bed biofilm reactors were used for this study, and they were run at  $22^{\circ}\text{C}$  for 918 days after being injected with a significant amount of anammox bacteria coming from anaerobic digester suspended biomass. The specific nitrogen removal rate (NRR) and removal efficiency increased significantly, reaching values of  $0.642\text{ g N/L/d}$  and 92% volume, respectively.

Additionally, five pharmaceuticals—ciprofloxacin, norfloxacin, ofloxacin, sulfamethaxazole, and sulfadimethoxine were employed in this investigation to assess their impact on anammox activity. The diversity of anammox bacteria was impacted by the presence of the targeted pharmaceutical chemicals, but these microorganisms were able to withstand and effectively eliminate nitrogenous substances. Throughout the course of the experiment, several conditions, including anoxic and aerobic conditions, starvation and non-starvation phases, and other conditions, were observed.

**Keywords:** aerobic, anoxic, anammox activity, pharmaceuticals, nitrogen removal, starvation, non-starvation.

**CERCS:** P300 analytical chemistry

**Autotroofne lämmastiku eemaldamine madala orgaanilise süsinikuga reovee puhastamiseks**

Olmereovee käitlus toimub traditsiooniliselt aeroobsete/anaeroobsete nitrifikatsiooni/denitrifikatsiooni protsesside abil, mille korral reoveest eemaldatakse lämmastiku- ning süsinikuühendid. Olmereovett saab ka anaeroobselt puhastada, et sellest saada energiat biogaasi kujul. Kasutades heitveest ammooniumi eemaldamiseks autotroofset protsessi, näiteks anaeroobset ammooniumi oksüdatsiooni (anammox) protsessi, saab reovee puhastamiseks vajalikku energiat veelgi vähendada ca 50% võrra, kuna näiteks ammooniumi oksüdeerimiseks nitritiks kulub vähe elektrienergiat. Seni on anammoxi kasutatud peamiselt kõrgemate temperatuuridega (>25 °C) ja kontsentreeritud (>500 mg N/L) reoveevoogude töötlemiseks. Olmereoveepuhastuse reovee kasutamisel on väljakutseteks madalamad lämmastiku kontsentratsioonid (100 mg N/L), kontsentratsioonide ebaühtlus (nälgimisfaasid), ravimijääkide olemasolu reovees ja madalamad temperatuurid (20 °C). Antud faktorite uurimiseks kasutati kahte 20-liitrist liikuvate kandjatega biokilreaktorit, 1 L annussüsteemis mis töötasid pärast märkimisväärse koguse anammoxi bakterite kasvatamist 841 päeva temperatuuril 22°C. Spetsiifiline lämmastiku eemaldamise kiirus ja efektiivsus suurenesid oluliselt, jõudes vastavalt väärtuseni 0,642 g N/L/päevas ja 92%.

Lisaks kasutati selles uuringus viit ravimit – tsiprofloksatsiini, norfloksatsiini, ofloksatsiini, sulfametaksasooli ja sulfadimetoksiini, et hinnata nende mõju anammox protsessi aktiivsusele. Anammox bakterite poolt läbiviidud lämmastikuärastust mõjutas märkimisväärselt farmatseutiliste kemikaalide olemasolu, mis näitab, et need mikroorganismid suudavad samaaegselt tõhusalt kõrvaldada lämmastikku ja ravimijääke sisaldavaid aineid. Kogu katse käigus katsetati ravimijääkide eemaldamist kasutades erinevaid tingimusi, sealhulgas anoksilisi ja aeroobseid tingimusi, nälgimise ja mittenälgimise faase ning muid tingimusi.

**Märksõnad:** aeroobne, anoksiline, anammox aktiivsus, ravimid, lämmastiku eemaldamine, nälgimine, mittenälgimine.

**CERCS:** P300 analüütiline keemia

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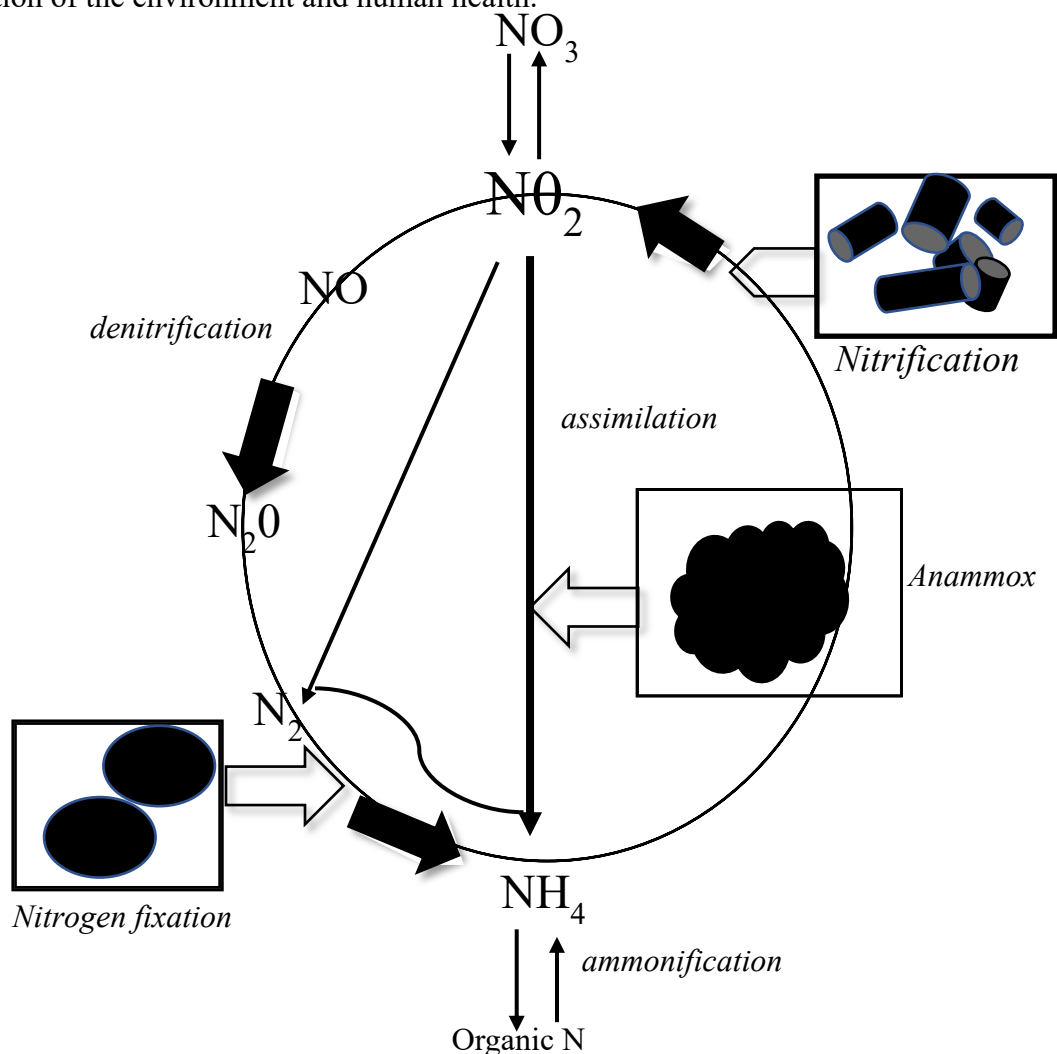
## TERMS, ABBREVIATIONS AND NOTATIONS

AMO	ammonia monooxygenase
AMR	antimicrobial resistance
ANAMMOX	anaerobic ammonium oxidation
AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
ASSs	activated sludge systems
AnMBR	anaerobic membrane bioreactor
CANON	complete autotrophic nitrogen removal over nitrite
COD	chemical oxygen demand
CIP	ciprofloxacin
DEMON	aerobic/anaerobic deammonification
DO	dissolved oxygen
FBR	fluidized bed reactor
FISH	fluorescent in-situ hybridization
HAO	hydroxylamine oxidoreductase
HDH	hydrazine dehydrogenase
MLSS	mixed liquor suspended solid
MBBR	moving bed biofilm reactor
MBRs	membrane bioreactors
MS	mass spectrometry
NLR	nitrogen loading rate

NOR	norfloxacin
NLR	nitrogen loading rate
NRR	nitrogen removal rate
OFL	ofloxacin
OLAND	oxygen limited autotrophic nitrification denitrification
PCR	polymerase chain reaction
RBC	rotating biological contractor
SAs	sulfonamides
SBR	sequencing batch reactor
SD	standard deviation
SDM	sulfadimidine
SRT	Sludge retention time
TN	total nitrogen
TNRR	total nitrogen removal rate
UASB	upward flow anaerobic sludge blanket
UBF	upflow blanket filter
WAS	waste activated sludge
WWTP	wastewater treatment plant

## INTRODUCTION

In the presence of organic material, nitrifying and denitrifying bacteria convert ammonium ( $\text{NH}_4^+$ ) into nitrogen gas ( $\text{N}_2$ ) in a biological process known as heterotrophic nitrogen removal. For the treatment of nitrogen, autotrophic nitrogen removal is a more affordable option than traditional nitrification-denitrification procedures. Partial nitrification through oxidation of ammonium ( $\text{NH}_4^+$ ) to nitrite ( $\text{NO}_2^-$ ) over intermediate hydroxylamine ( $\text{NH}_2\text{OH}$ ) (Irisa et al., 2014), coupled with the anaerobic ammonium oxidation (anammox) process, enables 60% saving from aeration and 100% from organic carbon addition compared to nitrification-denitrification process (Hu et al., 2013). Because nitrogen ions in wastewater can promote eutrophication and the growth of hazardous algae blooms, which can result in fish kills and other ecological imbalances, the autotrophic nitrogen removal process is crucial for the preservation of the environment and human health.



**Figure 1.** A diagram that highlights the nitrogen cycle. Anammox is seen as a cycle shortcut in the middle. Anaerobic ammonium oxidation is a metabolic activity that is involved in the biological nitrogen cycle and is part of the Anammox process (Ward et al., 2007).

Partial nitrification and anammox are the first and second steps of the autotrophic nitrogen removal process. These processes occur under two different sets of environmental conditions. First, a partial nitrification that requires aerobic conditions, which is followed by the anammox process under anoxic conditions and without requirements of organic carbon source. The partial nitrification process involves the oxidation of half of the influent ammonium to nitrite by metabolism of autotrophic ammonium-oxidizing bacteria (AOB) (Castellano-Hinojosa et al., 2018)

Due to the lack of additional organic carbon sources and excess sludge production, the autotrophic nitrogen removal technique is an effective way to treat wastewater with low levels of organic matter. As contrast to other nitrogen removal technologies, such as biological nitrogen removal using external organic carbon sources or chemical nitrogen removal using substances like potassium permanganate and chlorine, anammox procedure also requires less energy ( Li et al., 2021).

## **1 LITERATURE REVIEW**

### **1.1 Anammox Bacteria**

Anammox bacteria, also known as anaerobic ammonium oxidizing bacteria, are a group of microorganisms that can convert ammonium ( $\text{NH}_4^+$ ) and nitrite ( $\text{NO}_2^-$ ) into nitrogen gas ( $\text{N}_2$ ) under anaerobic conditions. This process is called anaerobic ammonium oxidation (anammox) and it plays an important role in the nitrogen cycle of natural ecosystems (van Niftrik & Jetten, 2012).

The discovery of anammox bacteria is a relatively recent development, with the first description of these bacteria dating back to the 1990s. Since then, they have been found in a variety of environments, including wastewater treatment plants, marine sediments, and soil (Kartal et al., 2007). Anammox bacteria have been found in new species: '*Candidatus Brocadia changi*' is the name of a new species of anammox bacterium that was discovered in 2021, according to a study that was published. This species, which can use both nitrite and nitrate as electron acceptors, was discovered in the wastewater treatment system of a textile dyeing facility in China development of anammox (Liu et al., 2021). The invention of a new form of bioreactor termed the "anaerobic dynamic membrane bioreactor" (ADM), which is intended to maximize the growth and activity of anammox bacteria, was reported in a paper that was published (Liu et al., 2020). Anammox bacteria from a wastewater treatment facility in Spain had their genome sequenced and analyzed, according to a study published (Sonthiphand et al., 2014). The

investigation revealed information about the metabolic pathways of these bacteria and numerous genes involved in the anammox process (Bovio Winkler et al., 2023).

### **1.1.1 Anammox Bacteria and Nitrogen Removal**

Ammonium and nitrite are converted into nitrogen gas in two steps by the anammox process, the first stage where nitrite is formed, which takes place in aerobic environments and the second stage, where both nitrite and ammonium are consumed to form nitrogen gas, is anoxic. With this method, up to 90% of the nitrogen in wastewater can be removed with great efficiency. Several habitats, including freshwater sediments, marine sediments, and wastewater treatment facilities, have been shown to contain anammox bacteria. Anammox bacteria have shown as promising solution in several studies that have investigated its usage in wastewater treatment.

Studies from 2021 have tested the efficacy of anammox bacteria in treating fish farm effluent (Yang et al., 2021). According to the study, nitrogen compounds were removed by the anammox process at an efficiency of above 80%. Anammox bacteria were also found to be able to adapt to changes in the wastewater's composition, which suggests that the method might be used with a variety of wastewater types.

Another study by Li et al. (2019) investigated the use of anammox bacteria in a sequencing batch reactor (SBR) for treating industrial wastewater. The study found that the SBR system achieved a nitrogen removal efficiency of 93%, with a maximum removal efficiency of 99.6%. The study also found that the anammox bacteria were able to adapt to changes in the wastewater composition, indicating that the process could be applied to a range of industrial wastewater types.

### **1.1.2 Challenges and Limitations:**

The removal of nitrogen from wastewater, which is a significant environmental issue, has been suggested as a potential remedy and is called anammox. Unfortunately, using anammox bacteria in wastewater treatment comes with several difficulties and restrictions, such as:

- i. High sensitivity to toxins: anammox bacteria can be negatively impacted by toxins such as heavy metals (copper (Cu), cadmium (Cd), lead (Pb), and mercury (Hg)) and chemical compounds (polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and phenolic compounds), which can impede their development and activity (Kartal et al., 2010).

- ii. Limited diversity: When compared to other microbial groups, anammox bacteria are less diverse, which may limit their capacity to adapt to various environmental factors and compete with other microbes (Strous et al., 1999).
- iii. Substrate inhibition: Any bacteria on the substrate concentration have a certain range of adaptation, low concentration of bacteria is difficult to protect the growth rate, nitrite and ammonium concentration being too high will inhibit the bacteria and interfere with the metabolism of bacteria (Edwards, 1970).
- iv. Temperature inhibition: Temperature affects cell growth and metabolic activity. In general, cell growth is faster at high temperature, so anammox technology can be used for the treatment of wastewater having temperature higher than 15°C. The activation energy of anammox bacteria is very similar to that of ammonia-oxidizing bacteria (Hendrickx et al., 2012).

### **1.1.3 Wastewater Anaerobic Digester Effluent Treatment**

Nitrogen-rich wastewater is generated by various human activities such as domestic, agricultural, and industrial operations. The effluent from these activities may contain organic and inorganic pollutants such as nitrogen, phosphorus, and heavy metals that have adverse effects on the environment if discharged without treatment. The raw wastewater sludge contains huge amount of water (more than 90%) along with organic solids which causes problems in its transportation, treatment, and disposal. Therefore, sludge volume reduction is important to minimize the operating, treatment, and disposal costs. Sludge settling, dewatering and degradation are important processes for wastewater sludge recycling and disposal to reduce the volume of sludge generated. Nitrogen removal is a critical process in wastewater treatment, and various methods have been developed to achieve this goal. Autotrophic nitrogen removal has gained significant attention in recent years due to its cost-effectiveness and sustainability. This literature review aims to provide a comprehensive and detailed analysis of autotrophic nitrogen removal for lower organic wastewater treatment.

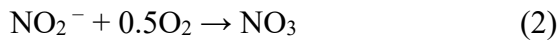
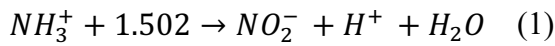
### **1.1.4 Lower Organic and Municipal Wastewater Treatment**

Several studies have investigated the autotrophic nitrogen removal process for lower organic wastewater treatment. In a study by Xu et al. (2021), a laboratory-scale sequencing batch reactor (SBR) was used to treat synthetic wastewater with an ammonium concentration of 100 mg/L. The results showed that autotrophic nitrogen removal was achieved with a total nitrogen removal efficiency of 93.4% and a nitrogen removal rate of 1.2 g N/(m<sup>3</sup>·d). Municipal waste landfills, wastewater treatment plants and other plants where air pollution is probable are used

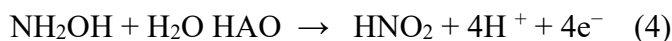
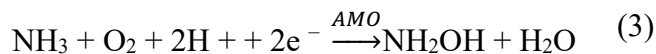
to be built on the outskirts of cities. The distance between residential areas and such plants has been decreasing as urbanization has advanced. The issue of odors and other air pollution is getting worse, and it affects society as well as the environment (Hendrickx et al., 2012).

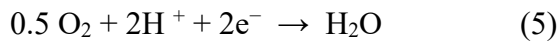
### 1.1.5 Nitrifying Bacteria

Nitrification is the two-step process by which microorganisms convert  $\text{NH}_4^+$  to nitrate ( $\text{NO}_3^-$ ). Oxygen is used as an electron acceptor in this process. The first step involves oxidizing  $\text{NH}_4^+$  to  $\text{NO}_2^-$  (**Nitration**) (Eq.1), which is followed by the oxidation of  $\text{NO}_2^-$  to  $\text{NO}_3^-$  (**Nitritation**) (Eq.2).

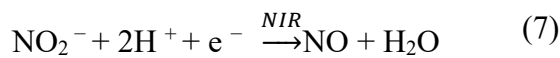
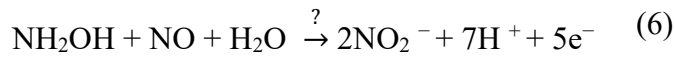


The oxidation of  $\text{NH}_4^+$  to  $\text{NO}_2^-$  is a function of ammonium oxidizing microorganisms (AOM). There are bacteria that can oxidize ammonium as well as archaea that can oxidize ammonium. The *betaproteobacteria* class (*Nitrosomonas* and *Nitrospira*) and the *gammaproteobacteria* class (*Nitrosococcus*) both contain known AOM. Members of the phylum *Taumarcheota* make up all known AOA (Stahl & de la Torre, 2012). *Nitrosomonas* appears to be the predominant AOM in wastewater treatment facilities. In both AOB and AOA, the enzyme ammonia monooxygenase (AMO) first converts ammonia to the intermediary hydroxylamine ( $\text{NH}_2\text{OH}$ ) (Eq. 3). The formation of  $\text{NO}_2^-$  requires the enzyme hydroxylamine oxidoreductase (HAO) in AOB (Wagner et al., 2006), however AOA appear to lack this enzyme (Stahl & de la Torre, 2012). According to the conventional model for nitrification in AOB, nitrous acid ( $\text{HNO}_2$ ) is produced when hydroxylamine is transformed by HAO, (Wagner et al., 2006). The respiratory chain would use two of the reaction 4's electrons, with oxygen serving as the chain's last electron acceptors (Eq 5). However, nitric oxide (NO) rather than  $\text{NO}_2^-$  may be the end product of hydroxylamine oxidation by HAO (Caranto & Lancaster, 2017).

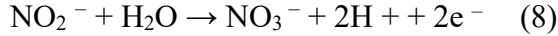




In AOA, NO generation is a crucial step for ammonia oxidation. A proposed mechanism for  $\text{NO}_2^-$  generation in AOA involves an unidentified enzyme reacting with hydroxylamine and NO (Eq. 6). Acopper nitrite reductase (NirK) (Eq 7) would create the NO (Kozlowski, Kits, et al., 2016).



Nitrite oxidizing bacteria (NOB) can further oxidize the  $\text{NO}_2^-$  generated by AOM to  $\text{NO}_3^-$ . The enzyme nitrite oxydoreductase (NOR) (Eq. 8) catalyzes this reaction. The respiratory chain uses the two electrons from reaction 8 along with oxygen as an electron acceptors.



The two classic NOB in WWTTPs are *Nitrobacter* (*Alphaproteobacteria*) and *Nitrospira* (*Nitrospirae*). The *Betaproteobacterium Nitrotoga*, a cold-tolerant NOB that appears to be prevalent in many WWTTPs, and two other NOB have recently been identified (van Kessel et al., 2015) and *Nitrolancea hollandicus* belonging to the *phylum Chloroflexi* (Sorokin et al., 2012).

The presence of AOM providing  $\text{NO}_2^-$  is necessary for NOB to convert  $\text{NO}_2^-$  to  $\text{NO}_3^-$ . The elimination of the hazardous  $\text{NO}_2^-$  most likely aids AOM.

Additionally, some *Nitrospira* can convert urea to ammonium and supply it to urease negative AOM (Koch et al., 2015) which provide  $\text{NO}_2^-$  to *Nitrospira* in turn. The cyanase enzyme, used by *Nitrospira*, can also produce cyanase-negative AOM with ammonium from cyanate (Palatinszky et al., 2015). Some members of the genus *Nitrospira* (comammox) are capable of completely oxidizing  $\text{NH}_4^+$  to  $\text{NO}_3^-$  (comammox), Comammox bacteria have been found to be ubiquitous.

Although little is known about its relevance to partial nitrification anammox (PNA) and nitrifying biofilms in WWTTPs. Because *Nitrospira* are a diverse group of microorganisms with

metabolic processes that are not limited to nitrification, the distinction between function and identity is difficult to draw (Daims et al., 2016).

### **1.2.1 Denitrifying Bacteria**

Denitrifiers are a class of heterotrophic microorganisms that can convert nitrate and  $\text{NO}_2$  to nitrogen. In anaerobic environments, these bacteria often utilise organic carbon as an electron donor and  $\text{NO}_3^-$  or  $\text{NO}_2^-$  as an electron acceptor. Denitrification is a widespread phylogenetic process that occurs in a variety of species throughout all three domains of life (Kozłowski, Stieglmeier, et al., 2016).

Although denitrification is a multi-step process that calls for numerous enzymes, not all denitrifiers possess the full complement of enzymes required for full denitrification. Nitrous oxide ( $\text{N}_2\text{O}$ ) emissions are linked to this inadequate denitrification (Stein & Klotz, 2016).

### **1.2.2 Cultivation of Anammox Bacteria**

Anammox bacteria are slow-growing and have substantially longer doubling times than other bacteria. Because of their lengthy start-up times, bioreactors can be used to successfully begin cultivation and growth. A bioreactor that provides stability and effective retention is needed to grow biomass that is viable and active (van der Star et al., 2008). According to studies, the type and configuration of a bioreactor have an impact on how anammox bacteria are enriched. To guarantee a sufficient nitrogen removal process, a bioreactor with a high surface area that will allow optimal substrate circulation (nitrite), is important. Using carrier materials for the attachment of biofilms increases the bioreactor's overall surface area, ensuring good biomass retention (Van Der Star et al., 2007). According to studies, the following bioreactor configurations can be used to cultivate bacterial biomass in granular suspended growth: anaerobic membrane bioreactors (AnMBR), gas-lift bioreactors, moving bed bioreactors (MBR), up-flow anaerobic sludge blankets (UASB), and sequential batch reactors (SBR). However, the fluidized bed reactor (FBR), rotating biological contractor (RBC), and up-flow blanket filter (UBF) reactor configurations have been utilized to successfully produce and cultivate bacteria that are attached to the surface of a carrier material (Sri Shalini S & Joseph, 2014). Other examples of moving bed biofilm reactors include a hybrid system called AccuFAS MBBR that combines the MBBR and activated sludge processes (Malovanyy et al., 2015), The Biocell MBBR is another example of an MBBR system that employs plastic media carriers for biofilm (Boavida-Dias et al., 2022).

### **1.2.3 Starving, Non-starving Effects and Aeration Process**

Anammox bacteria can be affected by the by microbial ecology processes in both starving and non-starving ways. Amongst anammox bacteria lack of nitrite as a substrate during anammox activity may result in nitrogen limitation for microbes, creating a starvation effect. These outcomes could cause reduction in anammox bacteria growth. Research has demonstrated that anammox bacteria outcompete other microorganisms for nitrite. Nitrite could also inhibit anammox ability to develop and produce biomass (Yin et al., 2015) and community structure change can cause nitrogen limitation and microbial community structures to change. This has been seen in a variety of habitats, including marine sediments and wastewater treatment systems ( Tang et al., 2018). Anammox activity can be limited at starving conditions, but at these conditions, the microbial population might use other sources of carbon (pharmaceuticals) or nutrient compounds. This may also promote the development of ammonium-oxidizing bacteria (AOB), which will make it easier to remove ammonium from the water environment (Lee et al., 2001). Anammox bacteria can also interact syntrophically with other microbes. Anammox bacteria, for instance, can interact with heterotrophic bacteria in wastewater treatment systems, using the heterotrophs' released carbon molecules or pharmaceuticals residues as a source of carbon (Xu et al., 2013).

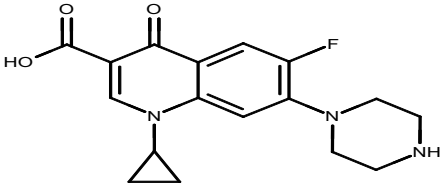
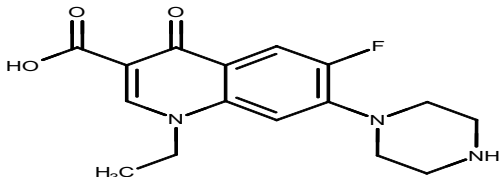
Since, the anammox process is anoxic and the anammox bacteria need anoxic conditions to survive, aeration is typically not used during the anammox process. Aeration, however, might occasionally be utilized to keep the conditions suitable for the anammox process (for producing nitrite) (Liu et al., 2021). The mainstream deammonification process, which is based on anammox and nitrification processes has been shown to be effective in removing nitrogen from municipal wastewater; in latter case intermittent aeration is used. The anammox and nitrification processes are combined in a single reactor during this process, and intermittent aeration is used to give the nitrifying bacteria oxygen during the aerobic phase while also generating anoxic conditions for the anammox bacteria to carry out their metabolic process during the anoxic phase (Corbalá-Robles et al., 2016).

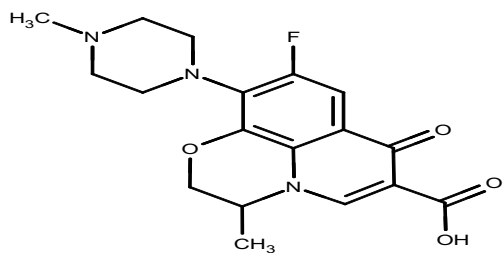
The use of anammox biofilm reactors, which have been demonstrated to be efficient in treating high-strength wastewater with low carbon-to-nitrogen ratios, is another example of aeration being used in the anammox process. Aeration is utilized in these reactors to both provide ammonium oxidizing conditions for the ammonium oxidizing bacteria to carry out their metabolic process and to supply oxygen to the heterotrophic bacteria that are in charge of degrading organic materials (X. Li et al., 2016).

### 1.2.4 Selected Antibiotics for Monitoring

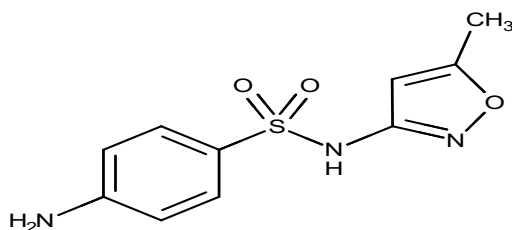
When analyzing the wide range of pharmaceuticals that are widely available in the wastewater, certain groups have gained special consideration due to their high usage in both humans and animals as well as their persistence qualities. Ciprofloxacin, norfloxacin, ofloxacin belonging to the fluoroquinolone class while sulfamethaxazole and sulfadimethoxine belonging to sulfonamides are the two special categories that would gain special focus on this research campaign. This is owing to the fact that they are highly prescribed pharmaceuticals making these substances ecologically relevant since they can be potentially occurring in high concentrations within different environmental media. The structures and applications of these antibiotics can be captured in (Fig. 2).

Several antibiotics chemical compositions and some of their characteristics. The molecular weights and classification values were taken from *drugbank.ca* and the work of (Kipper et al., 2011).

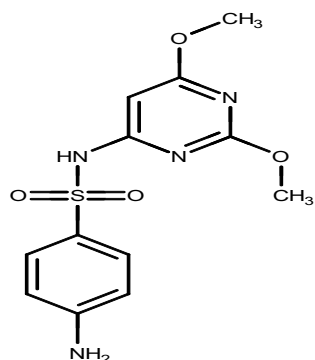
<i>Compound structure</i>	<i>Classification</i>
	Ciprofloxacin Human medicine (331.34 g/mol)
	Norfloxacin Human/veterinary medicine (391.33 g/mol)



Ofloxacin  
Human/veterinary medicine  
(316.37 g/mol)



Sulfamethaxazole  
Human/veterinary medicine  
(253.278 g/mol)



Sulfadimethoxine  
Human/veterinary medicine  
(253.278 g/mol)

**Figure 2. Pharmaceuticals: ciprofloxacin, norfloxacin, ofloxacin, sulfamethaxazole, sulfadimethoxine .**

## 2 THE AIMS OF THE THESIS

**2.1** Due to the numerous problems brought on by the rising nitrogen and pharmaceuticals levels in the marine environment this work aims to create a practical, sustainable wastewater treatment method that can efficiently remove nitrogen from wastewater with low organic content conditions from the Estonia anaerobic tanks reject wastewater. With the use of starvation phases, external organic carbon sources utilization could be enhanced by autotrophic nitrogen removal mechanisms relying on the biofilm microbial transformation.

**2.2** To achieve effective nitrogen removal in lower organic wastewater treatment, the study seeks to comprehend the underlying mechanisms of autotrophic nitrogen removal and optimize the process parameters (nitrogen loading rates, starvation, non-starvation, and levels of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ , and pH, respectively). Investigating how variables like starvation and non-starvation affects pharmaceutical removals (ciprofloxacin, norfloxacin, ofloxacin, sulfamethaxazole, sulfadimethoxine). Thereby, offering a more environmentally responsible and long-lasting substitute for current nitrogen removal techniques, which frequently rely on organic carbon sources and can increase greenhouse gas emissions.

**2.3** To compare aerobic and anoxic phases differences in pharmaceutical and nitrogen compounds removal. In general, the anoxic phase of anammox predominantly relies on anammox bacteria and their related activities, such as sorption, biotransformation, and microbial reduction processes, whereas the aerobic phase of anammox system could depend on the activity of aerobic bacteria for pharmaceutical removal. The anammox technique effectively removes pharmaceutical residues from wastewater by combining both the aerobic and anoxic phases.

### **3. EXPERIMENTAL PART**

#### **3.1 MATERIALS AND METHODS**

##### **3.1.1 Materials**

Two moving bed biofilm reactors (MBBR; working volume 20 liters) were used for the cultivation of anaerobic ammonium oxidizing bacteria (anammox) and nitrification-anammox biomass. Intermittent aeration was used in periods of 45 min aeration/15 non-aeration keeping dissolved oxygen concentration below 1 mg/L. The value of pH was between 7.5-8.5.

The regulation of growth-promoting conditions and the assessment of biomass activity through batch testing which helps gauge the influence of certain kinetic parameters and the impact of potentially harmful compounds are necessary for the successful implementation of the anammox process. By keeping track of the concentration of important substrates like  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{N}$ , and  $\text{NO}_2^-\text{N}$  as they are used up over the shortest amount of time. Monitoring and evaluating how various substances affect and contribute to the activity of the anammox bacteria is also required.

In this study, it was attempted to start the anammox bacteria process using the process for pharmaceuticals removal under favorable circumstances. The environment was made to be more favorable for anammox biomass growth and activity.

### **3.1.2 Experimental Set-up**

This project's goal was to establish anammox bacteria cultivation in a moving bed biofilm reactor, in a 20 L liquid volume capable MBBR reactor with a 52 cm height and 25 cm diameter plexiglass reactors. The bioreactor was connected to a dissolved oxygen measurement and a controlled stirrer from the top maintained system mechanically mixed. An additional liquid feeding and draining input and exit are located on the reactor's lid. To continuously mix the biomass and ensure homogeneity of the feed into the reactor for analysis during the first stage of the cultivation process, the reactor had to be operated at moderate stirring rates of  $200 \pm 5$  rotations per minute (rpm).

### **3.1.3 Conditions in Bioreactor**

The Tartu municipal wastewater treatment plant's (WWTP) anaerobic digestion stage produced the reject water used in tests. Reject wastewater obtained from Tartu municipal wastewater sludge, Estonia was used for feeding solution of MBBRs operated at 22°C. Using reject water that has moderate levels of micronutrients and large ammonium concentrations (1400 mg/L) was done for reactor feeding provision. The Tartu WWTP facility uses a conventional nitrogen removal procedure, and efforts in future to treat rejected water using a full-scale deammonification bioreactor. The hydraulic retention time (HRT) ranged between two and three days. To encourage simultaneous activity of oxic, anoxic and anaerobic bacteria, the bioreactor was initially opened on top. Afterwards, a plastic cover was placed over the bioreactor to prevent the oxidation of nitrite to nitrate. Reject water, which has the right balance of minerals and trace elements, was used as a source of nitrogen.



*Figure 3. Schematic view showing two lab-scale MBBR reactor (20 L) set-up for anammox process at 22<sup>0</sup>C. Left side reactor was used for most of batch tests with pharmaceuticals.*

### 3.1.4 Sample Collection

Reactor samples were taken weekly. After centrifuging at 4500 rpm, the mixtures of influent and effluent sample samples were examined for nitrogen characteristics. In the beginning of the culture phase and then at various stages after that, samples were collected for microbiological analysis.

### 3.1.5 Batch Testing

The total nitrogen removal rate was tested in batches to determine how different pharmaceuticals concentrations of 0.206 mg/L, 0.432 mg/L, 1.000 mg/L, 1.753 mg/L, 2.506 mg/L, 3.506 mg/L would affect it. The examination of the impact of added pharmaceuticals and synthetic wastewater on the effectiveness of nitrogen removal was also covered by batch testing. In tests conducted at room temperature in a 1 L test cell over a 6-hour period, nitrogen compounds (ammonium, nitrite, nitrate) were measured every two hours.

Batch assays were conducted to examine the combined effects of spiking with pharmaceuticals on anammox bacteria.

Analytical-grade chemicals and solvents were used throughout. Antibiotic stock solutions (pharmaceuticals added) were used ciprofloxacin (50.05 mg/L), norfloxacin (40.09 mg/L), ofloxacin (20.09 mg/L), sulfamethaxazole (10.07 mg/L), and sulfadimethoxine (5.03 mg/L).

From the following, nitrogen species  $\text{NO}_2^-$ -N and  $\text{NH}_4^+$ -N were respectively added. From the beginning of cultivation and then as start-up progressed, the amount of biomass in the reactor was calculated as total suspended solid (TSS). TSS concentration on one carrier was 3.74 mg.

### 3.1.6 Sample Analysis

Once per week, the bioreactor's effluent and influent combination was tested for total nitrogen in mg N/L concentrations of  $\text{NO}_3^-$ -N,  $\text{NO}_2^-$ -N, and  $\text{NH}_4^+$ -N, pH and temperature were recorded weekly. Total nitrogen was quantified using ion chromatography and spectrophotometric procedures done at the chair of Colloidal and Environmental Chemistry, at the institute of Chemistry, University of Tartu, Estonia. Ammonium was analysed by Nessler's method while nitrite and nitrate by ion chromatograph or using colorimetry in Hach Lange colorimeter.

### **3.1.7 HPLC Pharmaceuticals Measurements, Recovery Experiment and Pharmaceuticals Degradation Estimation**

For recovery experiment, 2 ml of 20 ppb solution was spiked in 5 ml sample that almost doesn't contain antibiotics and diluted with 100 ml Milli-Q water, later 20 times dilution was performed. Obtained data present in the table (appendix 2)

The results of this experiment indicate sulfadimethoxine and sulfamethaxazole were recovered more effectively, correspondingly, at 86% and 86% recovery processes. However, ciprofloxacin, norfloxacin, and ofloxacin had recovery rates of 54%, 46%, and 68%, respectively. The range for the overall recovery rate is (54% to 86%). Comparable results for fluoroquinolones using the same extraction solvents were reported (Ezzariai et al., 2018). Estimation of recovery was carried out by a collaborative laboratory at the Institute of Analytics; University of Tartu.

### **3.1.8 Anammox Experiment Principles**

In every of the test performed, biofilm carriers were employed. One litre total volume in two neck bottles was used for the studies. The trials employed 800 mL of synthetic wastewater inclusion in total.

Each part of the test biomass is pre-washed three times with either 100 mL of settled biomass from the suspended mud system or 100 mL of biofilm carriers from the MBBR system.

The following components were added to the test cells in addition to the biomass:

- 2 mL NaNO<sub>2</sub>
- 2 mL NH<sub>4</sub>Cl
- 0.4 g H<sub>2</sub>CO<sub>3</sub>
- 1mL BHT feed solution for phosphate buffer
- 1mL BHT medium MgSO<sub>4</sub> \* 7H<sub>2</sub>O
- 1mL BHT medium CaCl<sub>2</sub>
- 1mL BHT feed solution FeCl<sub>3</sub> \* 6H<sub>2</sub>O
- 1mL alkaline trace element solution
- 1mL acidic micronutrient solution
- H<sub>2</sub>O

At the start of the suspended biomass test, 2 \* 50 mL of the blended sample is taken from the test bottle and analysed for ammonium, nitrite, nitrate, pharmaceuticals and TSS. The prepared wastewater solution containing biofilm carriers / sludge must be sealed with a cap and deaerated for 15 minutes with N<sub>2</sub>. Nitrogen deaeration is necessary to remove all the oxygen present in the wastewater solution in order to perform efficient anammox process.

At the end of the test, 30 - 40 ml samples were taken from each test bottle. The test bottles were sealed and placed in a temperature oven at  $25.0 \pm 0.2$  ° C. The temperature of the thermocouple is always constant.

To obtain a uniform solution, the magnetic stirrer rod had previously been added to the test bottle and the magnetic stirrers in the thermal cabinet had a rotational speed of 500 rpm.

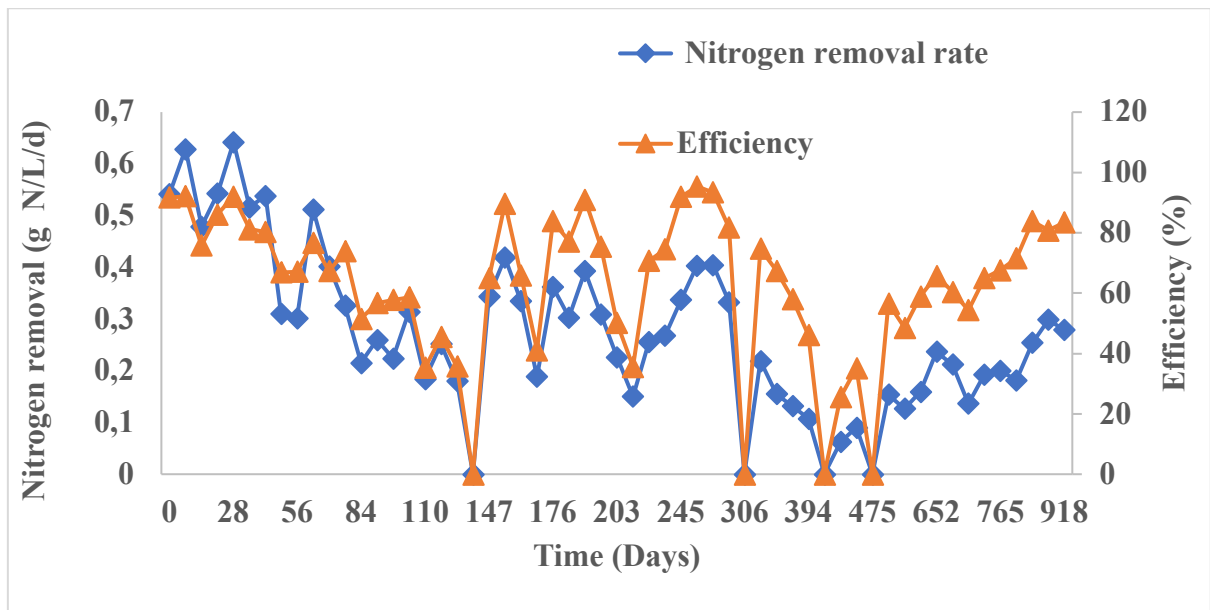
Samples were taken every two hours, with samples from 4 different time points (blank, 2 h sample, 4 h sample, 6 h sample). Two to three replicates were made in each experiment.

Samples of nitrite, ammonium and nitrate nitrogen were determined from the samples taken, together with the pH of the sample. Dry weight of biofilm carriers is calculated from weight differences of carriers with and without biomass. 2-3 x 20 carriers were taken from the reactor, which were rinsed with deionized water and dried in the oven for 24 hours at 105 ° C. After the first drying of the bio-carriers, the carriers were weighed. Biomass is then removed from the carriers using chromic acid. After removal of the biomass, the biofilm carriers are washed with deionized water and re-dried in a drying oven at 105 ° C for 24 hours. After the second drying, the difference between the two weighing results is calculated and the dry weight of the bio-carriers is known.

## **3.2 RESULT AND DISCUSSION**

### **3.2.1 Phase of Bioreactor Performance**

To ensure proper operating conditions for anammox bacteria, influent ammonium concentration at 300- 1443 mg N/L, the bioreactor was operated with temperature set at 22°C and pH maintained at range of 7.5 - 8.5. The Tartu municipal wastewater treatment plant provided the anaerobic sludge digester effluent (reject water) as feeding medium of MBBRs. To maintain the onset of an autotrophic process, no external organic matter was added to the bioreactor. Reject water was added as a source of ammonium during the start-up period. The bioreactor achieved an efficiency of approximately 92% of total nitrogen removal efficiency after 14 days of operation (Fig 4.4).



**Figure 4.1 General performance of bioreactor and occurring Nitrogen removal rate (NRR) and the nitrogen removal efficiency within 918 days.**

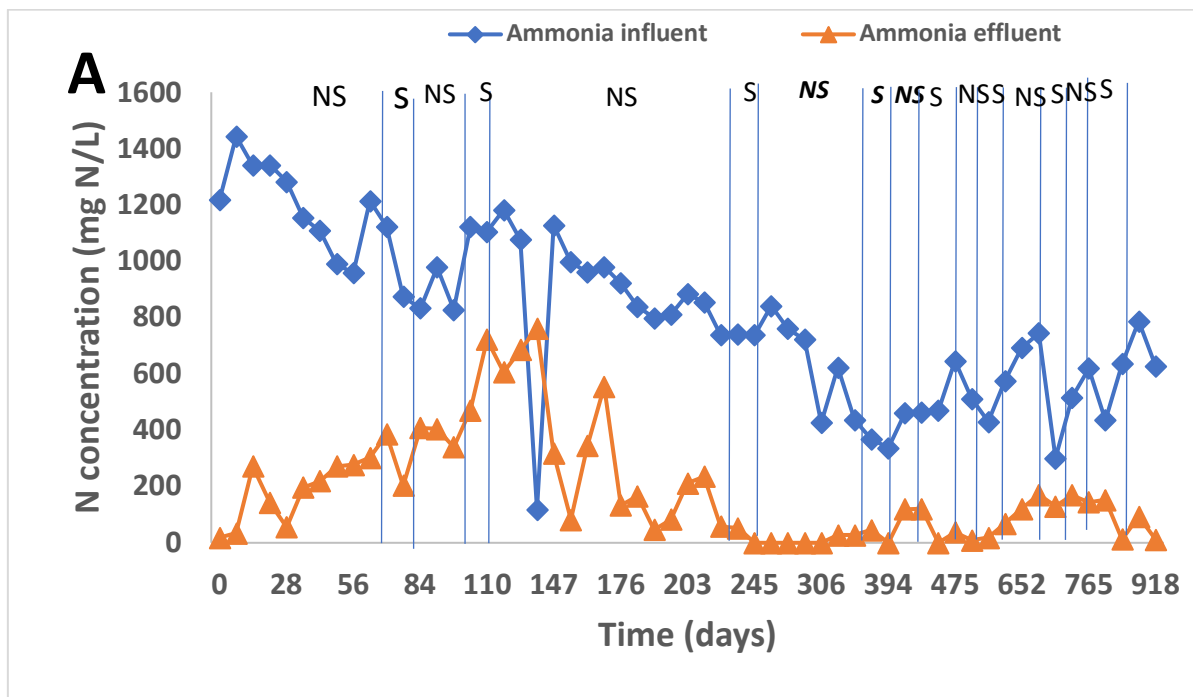
In the total operation procedure, the study of the biomass and anammox activity is broken down into starvation and non-starvation phase recognizable transitional times. The microbial population is continuously supplied with ammonium and nitrite during the non-starvation phase of nitrogen removal by anammox bacteria, allowing for the effective conversion of these substances into nitrogen gas (Watson et al., 1998). This maintains the nitrogen removal process, which benefits the ecosystem by reducing eutrophication and water pollution. The microbial community's stability enhances the operational effectiveness of wastewater treatment systems by ensuring stable nitrogen removal capability throughout time (Watson et al., 1998). For effective and long-lasting wastewater treatment and environmental preservation, it's critical to achieve and maintain a non-starvation phase in anammox-based nitrogen removal.

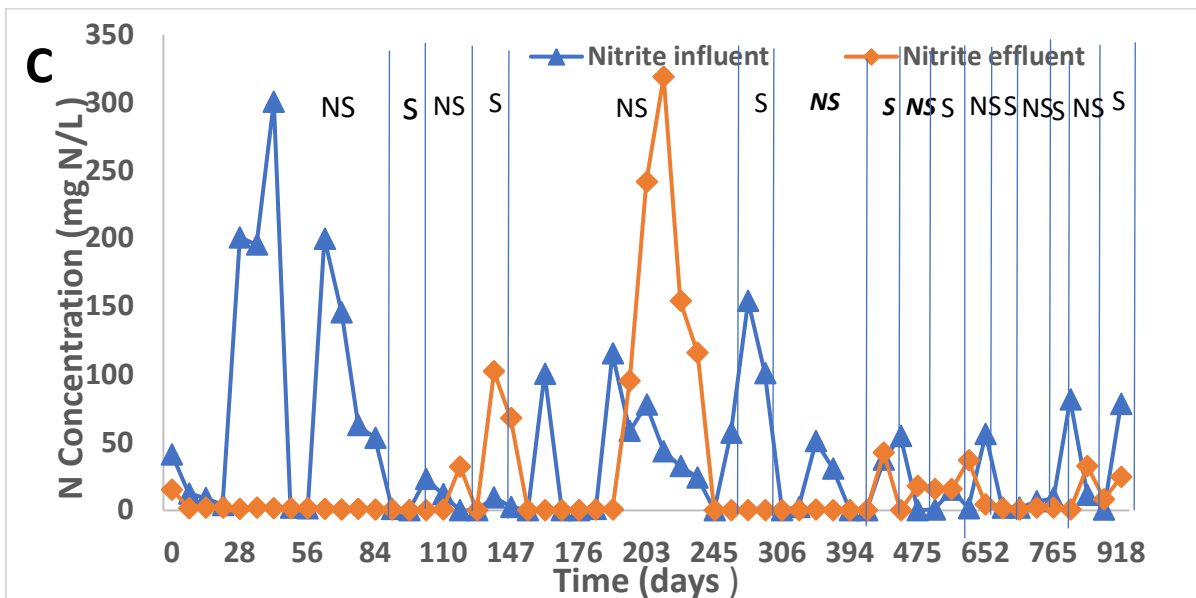
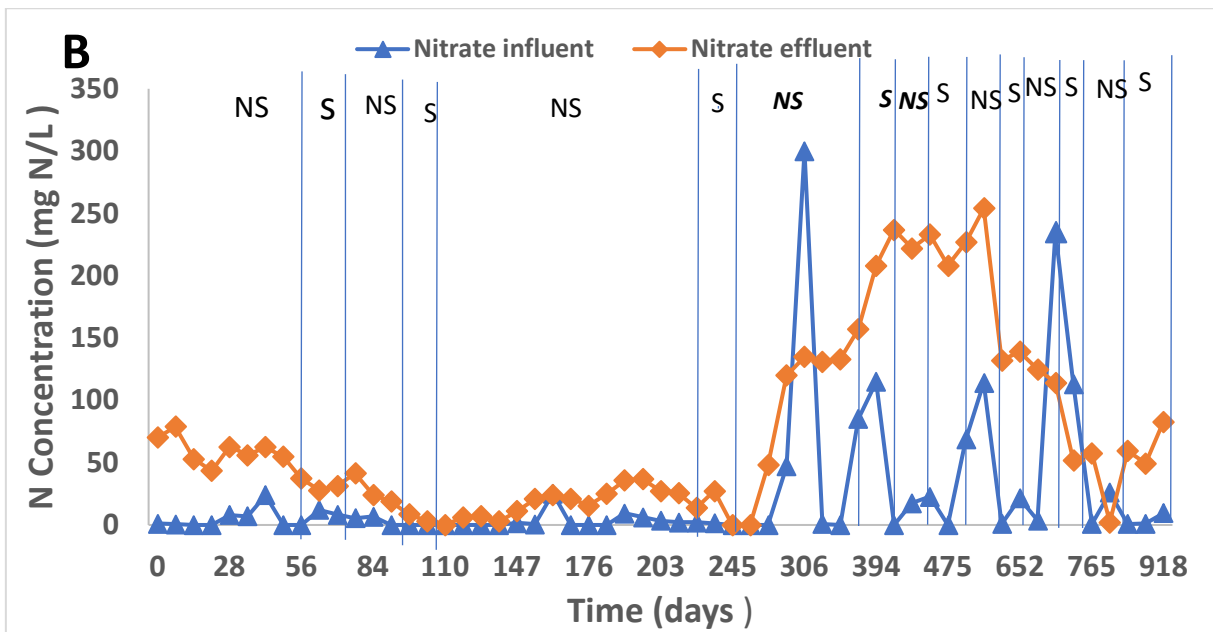
### **3.2.2 Phases of Reactor Substrate Starving/Non-starving**

Now we turn to examine the bacterial apoptosis profiles of the anammox culture under starvation stress and in the non-starvation phase. Anammox bacteria were introduced into non-starving phases during bioreactor operation with reject water. The goal of the starvation phase is to test unfavorable environmental circumstances for the anammox bacteria nutrient removal and to test its effect on pharmaceuticals removal as at starved conditions, anammox organisms are assumed to be capable of removing pharmaceuticals effectively.

The starvation phases regarded as ones without feeding. Depending on the removal of  $\text{NH}_4^+\text{-N}$ , aeration intensity was chosen. According to the Monod model, the concentration of the substrate is directly related to the specific bacteria activity (specific substrate consumption rate) (Guo et al., 2013). A comparison of influent and effluent parameters for  $\text{NH}_4^+\text{-N}$  concentration showed that during the initial days (non-starvation phase) of the bioreactor's performance, effluent  $\text{NH}_4^+\text{-N}$  increased on average of 700 mg N/L, while influent  $\text{NO}_2^-\text{-N}$  fluctuated very little during this time. Cell autolysis and the anaerobically broken-down organic materials from dead cells could cause the rise in  $\text{NH}_4^+\text{-N}$  in anaerobic digester (Gutwiński et al., 2016). The organic material created during this time served as a substrate for denitrifiers, which simultaneously consumed 100%  $\text{NO}_2^-\text{-N}$  and  $\text{NO}_3^-\text{-N}$  during the denitrification process. Although daily effluent results indicated minimal nitrate  $\text{NO}_3^-\text{-N}$  during this phase, batch testing results demonstrated the generation of  $\text{NO}_3^-\text{-N}$ , which clearly indicates the presence and activity of denitrifiers. In the 8<sup>th</sup> week of the starvation phase there was a sharp drop down in the bioreactor performance of ammonia influent and effluent (Fig 4.2A) which could be because of lack of adequate nutrient levels.

The estimation of total nitrogen concentration removed in this phase was contributed by  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$  (Fig. 4.2B&C). The absorption of nitrogen from decaying bacteria was the main factor in the limited drop in nitrate content (Fig. 4.2A).  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$  was low compared to  $\text{NH}_4^+\text{-N}$  removal (Fig. 4.2)





**Fig 4.2. Nitrogen removal profiles with time course during starvation (S) and non-starvation (NS) phase. (A) Ammonia concentration, (B) Nitrate concentration, (C) Nitrite concentration.**

Decreasing the influent concentration of  $\text{NH}_4^+\text{-N}$  was done in accordance with how well the bioreactor was working. Nitrogen removal efficiency of approximately 95% was achieved with the reactor stabilized at temperature  $22^\circ\text{C}$  and pH 7.5–8.3 (Fig 4.1). In the first six month of this work  $\text{NO}_3\text{-N}$  and  $\text{NO}_2\text{-N}$  effluent was considerably low at both phases (starvation/non-starvation), although influent removal rate was high for  $\text{NO}_2\text{-N}$ . The highest N level for these

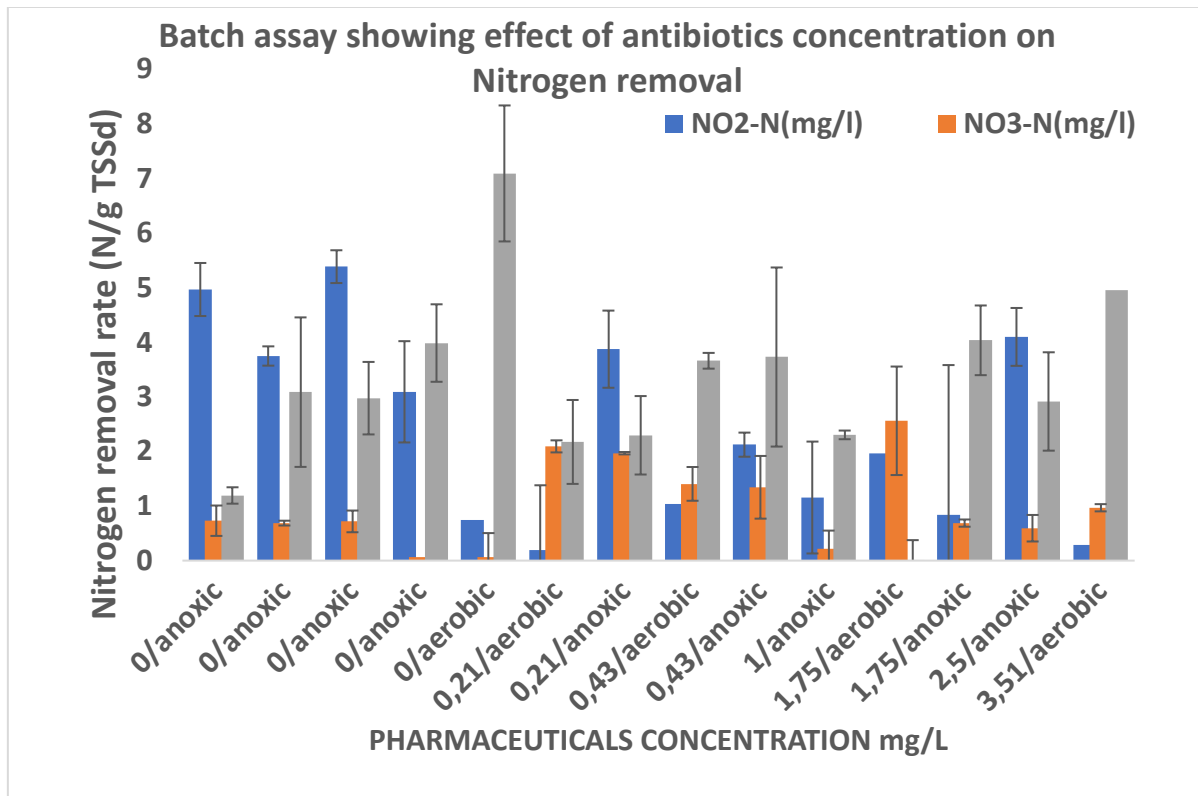
three components were all observed during the non-starvation phase specifically 1480 mg N/L for  $\text{NH}_4^+\text{-N}$  while  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$  was averagely 300 mg N/L.

This study also demonstrates that influent nitrogen was typically removed at a high rate autotrophically. Past study has emphasized the high abundance of concurrent partial denitrification and anammox performing bacteria simultaneously occurring, which can reduce nitrate levels. For some types of anammox bacteria, the use of organic carbon as an electron donor to favor the organotrophic anammox process over the autotrophic anammox process has also been considered (Wu et al., 2018), which could assume that pharmaceuticals could be removed by anammox biofilms. Additionally, anammox bacteria have been related to mediating dissimilatory nitrate reduction to ammonia (DNRA). This process explains how anammox bacteria can partially degrade nitrate and organic carbon in anaerobic conditions (Castro-Barros et al., 2017). Previous research has also suggested that *Candidatus Brocadia sinica* may compete with denitrifiers for the use of organic carbon at low C/N ratios (<0.2) released through DNRA processes (Shu et al., 2016). It is possible to link partial DNRA activity by anammox bacteria and partial denitrification by denitrifying bacteria to some of the  $\text{NO}_3^-\text{-N}$  losses seen in this phase. The concentration of substrates, specifically ammonium and nitrite, is one of the most important factors for the stability of anammox reactions, no matter in the actual industrial wastewater or the natural environment, the fluctuation of substrate concentrations usually occurred (Tang et al., 2010). Insufficient substrates obviously result in lower biomass survival rates, which further impairs reactor efficiency (Cabezas et al., 2009).

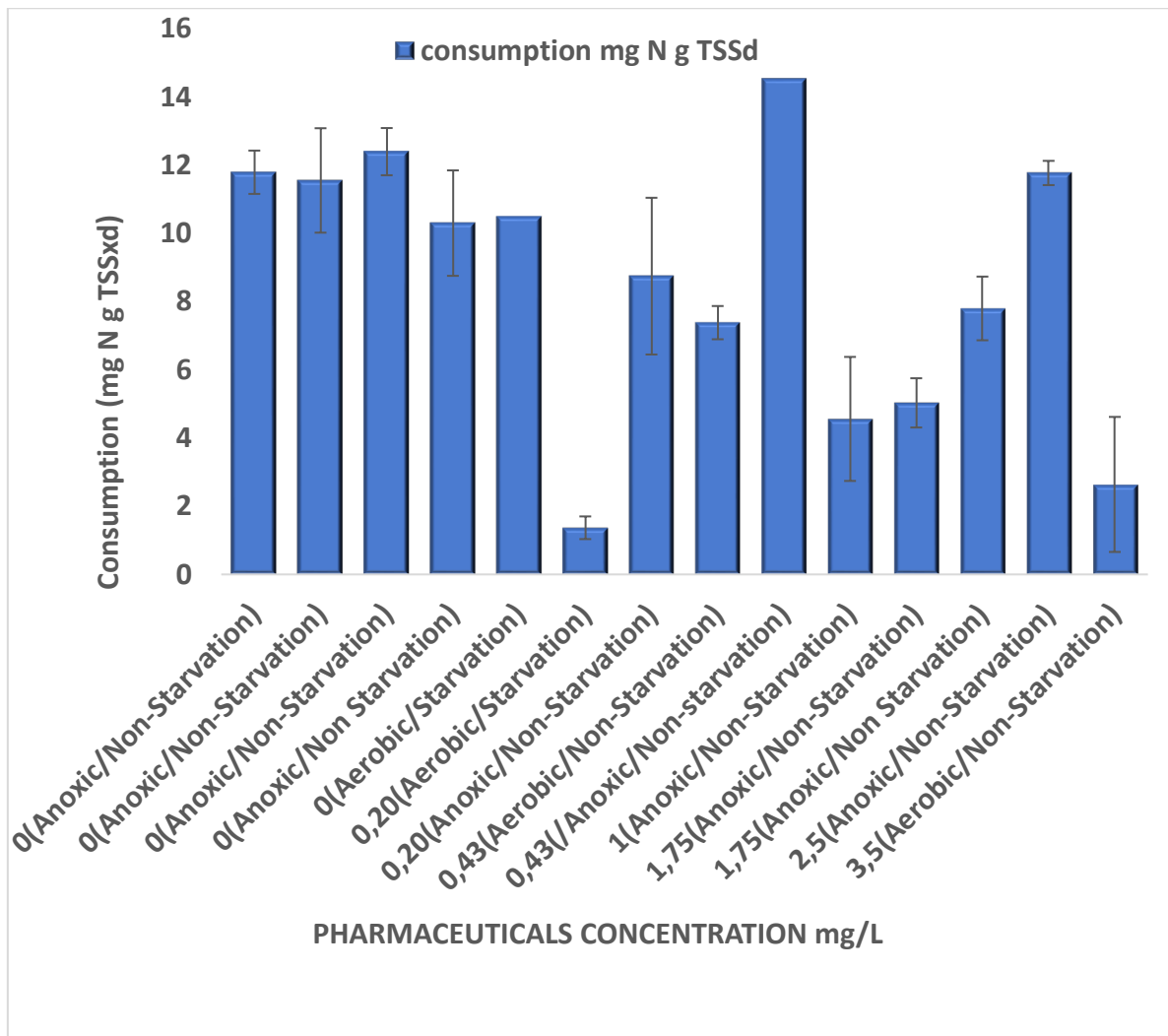
### 3.2.3 Batch Cycle Analyses

In the present study, several indicators such as  $\text{NO}_2^-\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ ,  $\text{NH}_4^+\text{-N}$  and pH were measured at tests done at aerobic/anoxic and anoxic conditions to deepen our understanding of anammox bacteria and aerobic ammonium oxidizers application prospects in biological treatment of wastewater containing pharmaceuticals. In the batch cells, various batches of analyses were run at 8-h interval throughout the 103 days. Antibiotics were not added for the first 36 days of operation (Fig. 4.3). The anammox bacteria were in their adaptation/stagnation phase for the first 36 days. During this time, anammox activity was at stable values, and nitrogen was generally removed successfully with specific anammox activity ( $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ ) at 7.0 mg N/g TSS d, 5.5 mg N/g TSS d and 0.8 mg N/g TSS d, respectively. Low nitrate removal rate depicts that anammox bacteria instead of denitrifiers were dominantly performing in biofilm nitrogen removal. Nitrite and ammonium removal simultaneous manner is characteristic to anammox bacteria while nitrate removal for organotrophic denitrifiers. The

effectiveness rate of the anammox bacteria started to significantly decline after being spiked for a total of 6 hours with pharmaceutical components (ciprofloxacin, ofloxacin, norfloxacin, sulfamethoxazole, and sulfadimethoxine) at various concentrations. With a total removal rate of 5 mg N/g TSS d for  $\text{NH}_4^+\text{-N}$ , 3.8 mg N/g TSS d for  $\text{NO}_2^-\text{-N}$  and 2.8 mg N/g TSS d for  $\text{NO}_3^-\text{-N}$ . This was observed to evaluate how varying pharmaceutical concentrations affected the action of anammox. However, the variations in the nitrogen removal rate at various concentrations depend on several other factors as well, including the aerobic or anoxic condition, with the latter tending to promote anammox activities more effectively than the former. Operational oxygen levels within aerobic tests can also affect the variations at various concentrations during nitrogen removal.



**Figure 4.3:** Batch assays performed within 36 days without addition of antibiotics, followed by spiking with antibiotics at different concentrations (Nitrate removal rate, Nitrate removal rate and Ammonia removal rate).



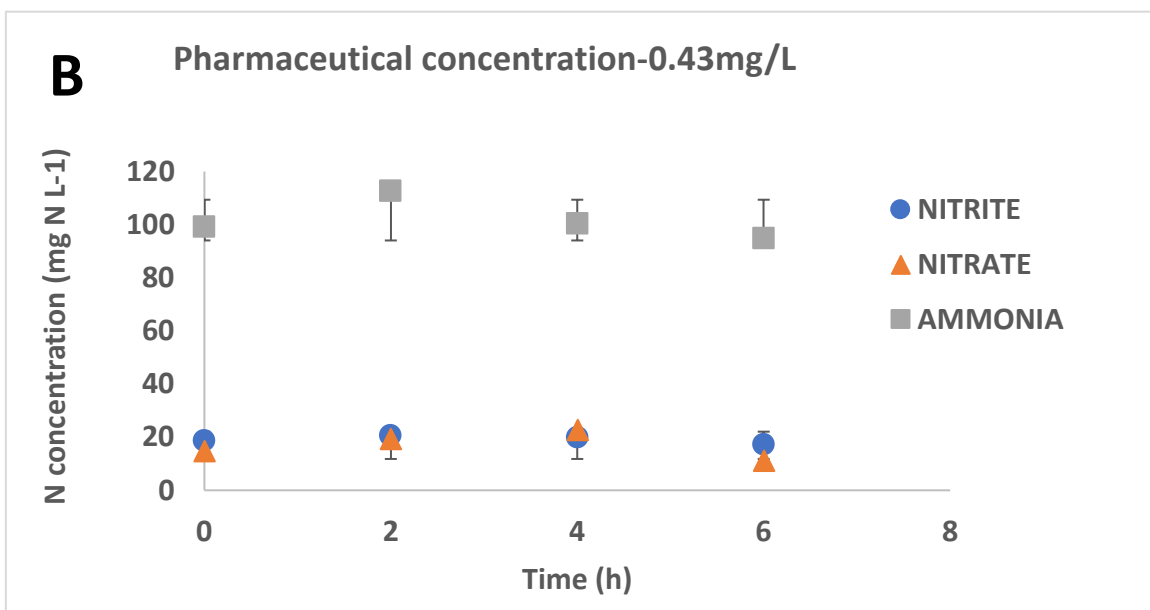
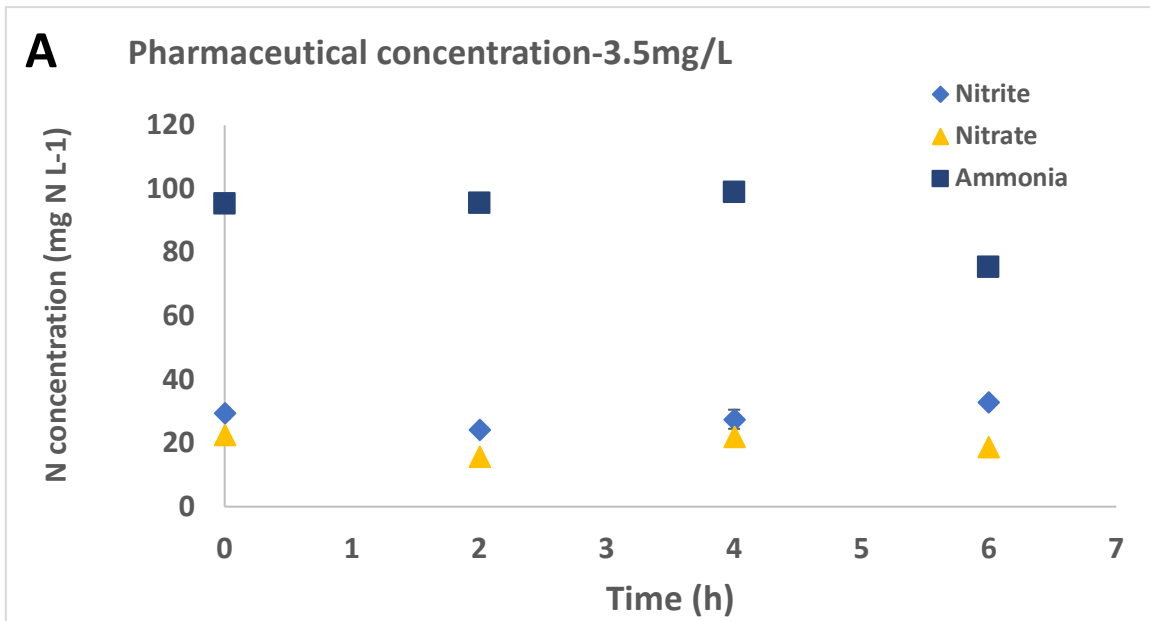
**Figure 4.4: Batch assay for different concentration of antibiotics showing consumption mg N g TSSd**

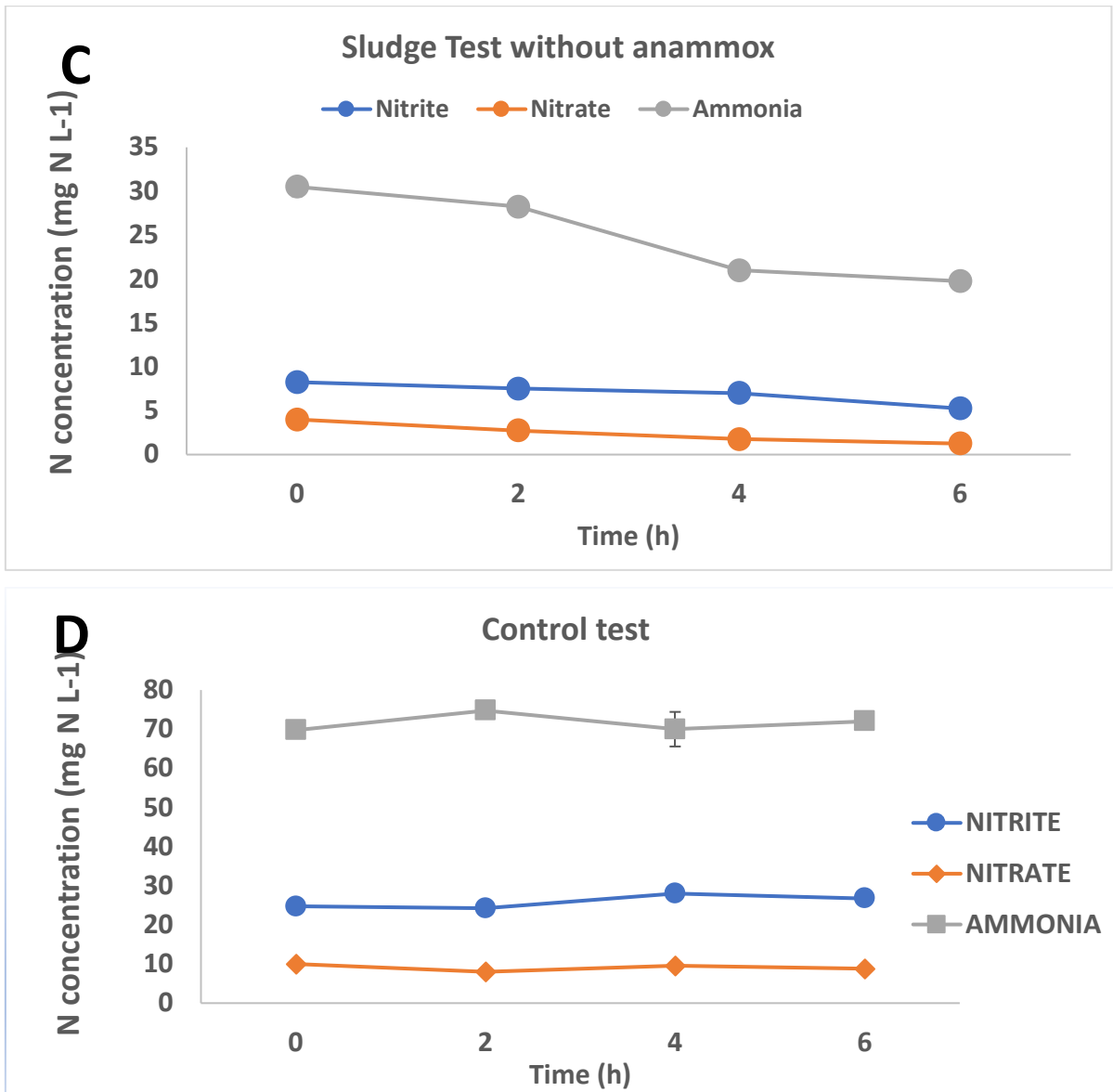
The rate of nitrogen consumption appears to have been 11 mg N g TSS d for the first 36 days without antibiotic spiking, with little variation. However, after spiking with different concentrations (1  $\mu\text{g/l}$  and 0.432  $\mu\text{g/l}$ ), nitrogen consumption increased to 12 mg TSS d and 14.5 mg TSS d, respectively. Compared to aerobic settings, this maximum rise was primarily observed in anoxic conditions.

### 3.2.4 Effects of Antibiotics on Nitrogen Removal

To demonstrate the underlying impact of antibiotics on anammox bacteria, the effect of antibiotics on nitrogen removal activities was investigated. This procedure was carried out by adding pharmaceutical substances at various concentrations to see how effective the anammox

activity was compared to no addition of pharmaceuticals, as well as to see how quickly the anammox bacteria activity was removed.





**Figure 4.5: Comparing the removal of nitrogen using biofilm with two different pharmaceutical concentrations, the control test (without any biomass), and the sludge test. (A) pharmaceutical concentration 3.5 mg/L (B) 0.43 mg/L (C) sludge test (D) control test**

Figure (4.5) in the current study shows the nitrogen removal rate with or without the aid of biofilm. To demonstrate the effectiveness of anammox bacteria and how it facilitates the nitrogen removal process from wastewater, we conducted this test. Two alternative pharmacological concentrations, especially 3.5 mg/l and 0.43 mg/l, are shown in Figures (4.5a and 4.5b).

Figures (4.5c and 4.5d) show a sludge test and a control test, both of which did not rely on anammox bacteria to remove nitrogen. Ammonium had the largest removal rate among nitrogen species of the two distinct concentrations, 98 mg N L<sup>-1</sup> at the highest pharmaceutical dose (3.5

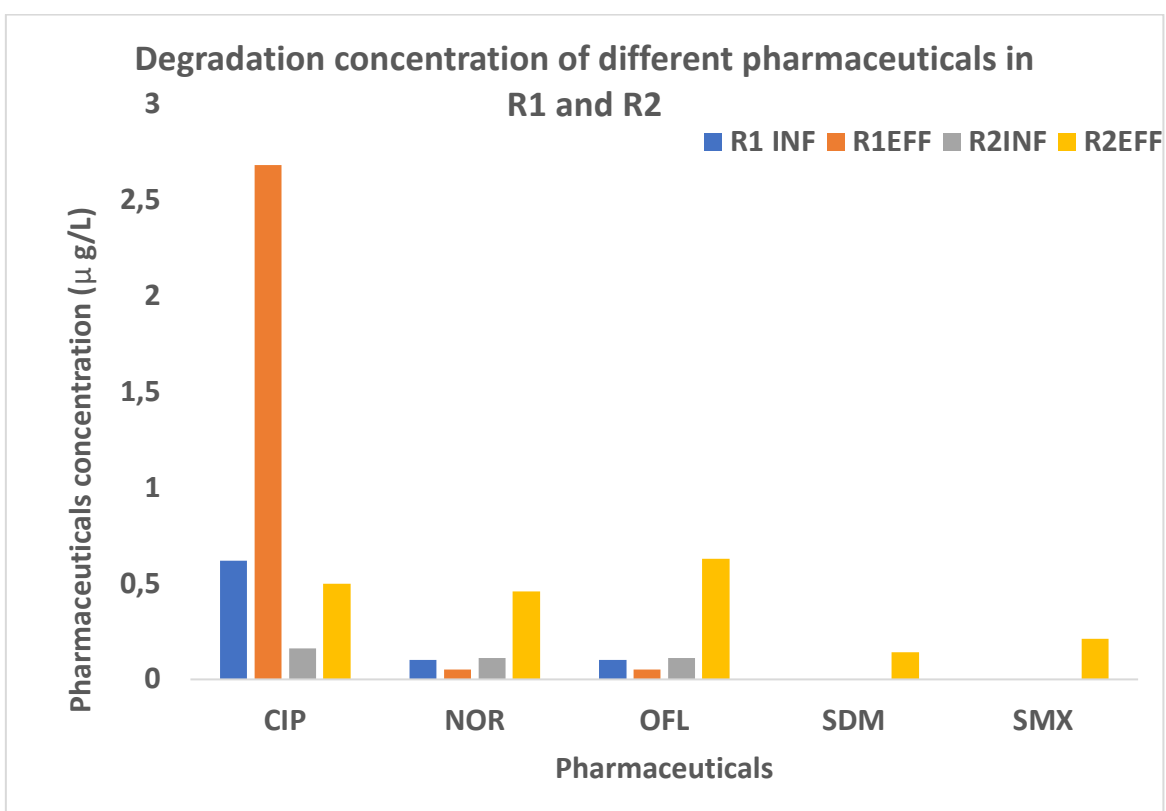
mg/L) compared to 110 mg N L<sup>-1</sup> at the lower antibacterial concentration. The total removal rate for nitrite and nitrate was ranging between 15 mg N L<sup>-1</sup> to 25 mg N L<sup>-1</sup>. According ( Guo et al., 2020) the accumulation of antibiotics in reactors has been observed to have negative or inhibiting effects on the nitrogen removal process. Meanwhile, widespread use and disposal of antibiotics raises environmental health issues and advances research into eliminating these substances (Grandclément et al., 2017). The sludge and control tests, as previously noted, were conducted without the use of biofilm, and the removal rates of the three components (ammonia, nitrate, and nitrite) varied just little and unnoticeably over the course of the 6-hour intervals. The sludge, however, was able to somewhat increase the removal rate. Sludge is determined by dividing the tank volume by the flow rate for volumetric removal of sludge. In slowly growing autotrophic bacteria like nitrifiers, which can also secrete enzymes that may be able to degrade some low degradable compounds with aromatic rings, high sludge retention times allow an enrichment of the biomass (Cirja et al., 2008; Rosenberger et al., 2002).

Ammonium-oxidizing bacteria (AOB) flourish in an aerobic environment, which also makes it easier for ammonium (NH<sub>4</sub><sup>+</sup>) to be converted to nitrite (NO<sub>2</sub><sup>-</sup>) (Liu et al., 2021). These circumstances are influenced by variables like temperature, dissolved oxygen concentrations, pH, and the availability of organic carbon. The effectiveness of the anammox process is determined by tracking performance markers like nitrogen removal efficiency (van de Graaf et al., 1995), nitrite build up, and oxygen absorption rates. It is essential to comprehend these factors and optimize how to manage them if you want nitrogen removal from wastewater treatment systems to be effective. In appendix 2, Figures (A & B) show anoxic and aerobic circumstances with the same pharmacological concentration, although the anoxic condition tends to encourage anammox activities relative to the aerobic condition. According to (Sonthiphand et al., 2014) anaerobic ammonium-oxidizing bacteria (AnAOB) convert nitrite (NO<sub>2</sub><sup>-</sup>) to nitrogen gas (N<sub>2</sub>) in the presence of organic carbon sources, and anoxic conditions are necessary for this reaction.

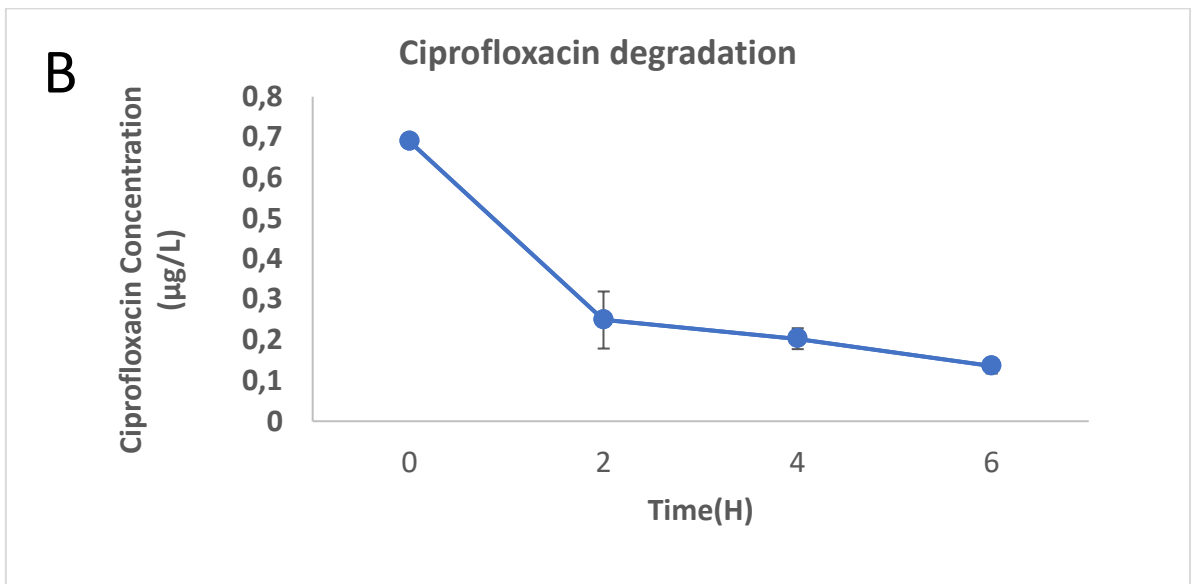
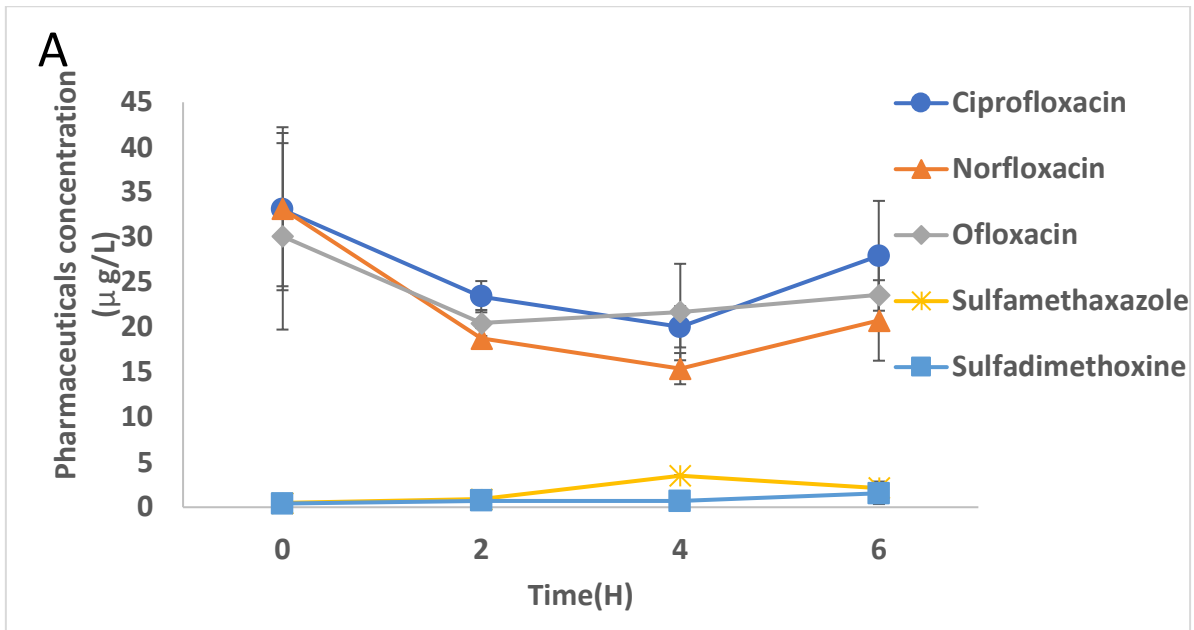
### **3.2.5 Recovery**

Amongst several environmental factors, the susceptibility of anammox bacteria to antibacterial influence was also investigated to simulate what is likely to occur in environmental media. Our current findings suggest that the sulfonamides are comparatively less stable and are more prone to degradation than their fluoroquinolone counterparts (fig. 4.7A). This is strongly affirmed by the inherently robust chemical structures of the fluoroquinolones that makes this class of antibiotics highly stable and less susceptible to degradation facilitated by factors such as

temperatures, pH, hydrolysis, photolysis, and microbial activity. The present results is further supported by the findings of (Accinelli et al., 2007; Golet et al., 2003) which reported that the half live of ciprofloxacin is greater than 60 days, making the fluoroquinolone about three (3) times more stable than reported sulfonamides. Within the fluoroquinolone class, the degradation rate seems to be relatively the same with the exception of ciprofloxacin degrading at a slower rate than norfloxacin and ofloxacin (fig. 4.6). More so, the slow degradation rate of ciprofloxacin is more pronounce in bioreactor 1 effluent, further suggesting that it might have the least degradation susceptibility, and thus more likely to persist within the environment.



**Fig 4.6 Degradation concentration of different pharmaceuticals in reactor one (R1) and reactor two (R2).**

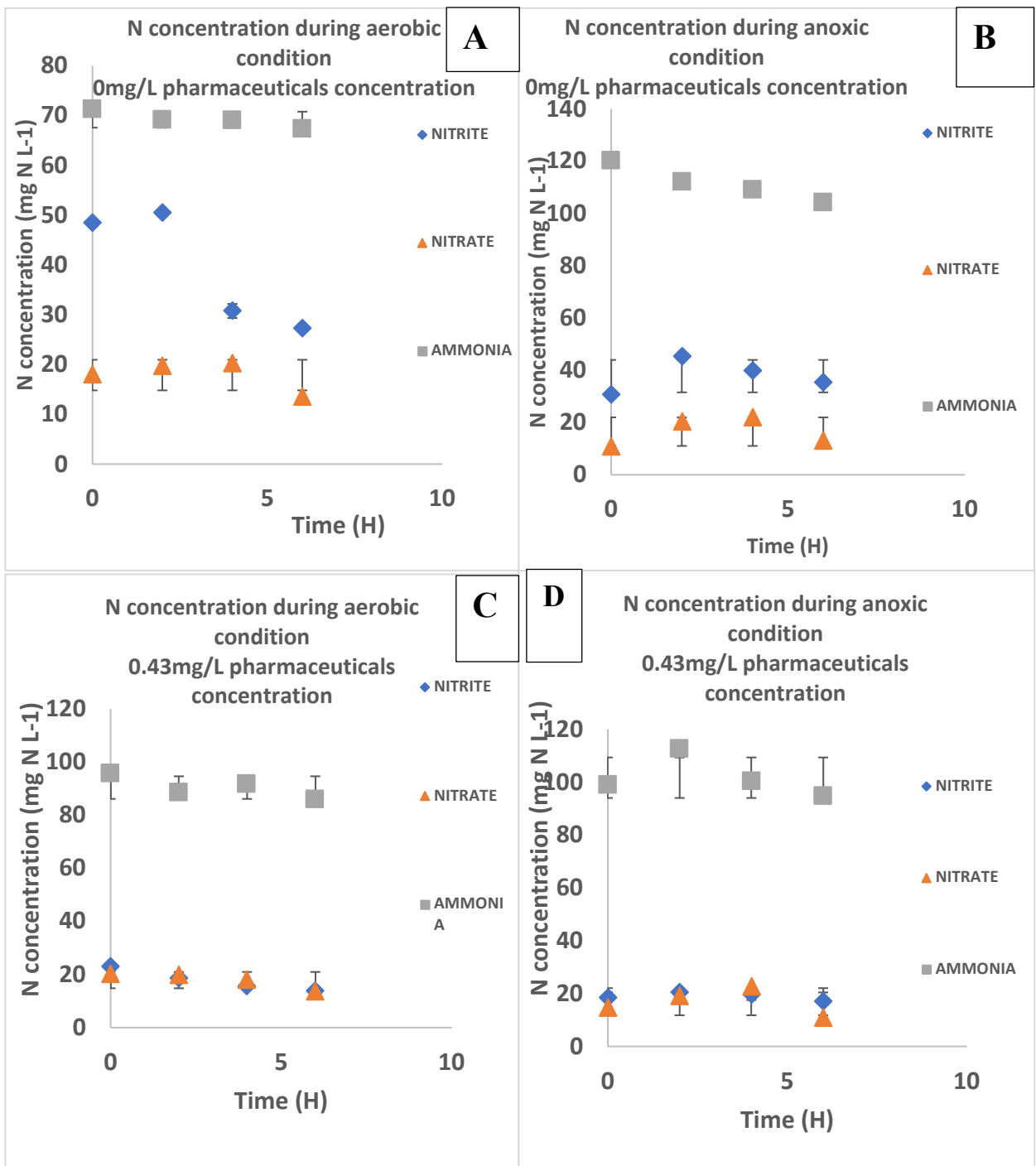


*Fig 4.7 Batch assay showing different pharmaceuticals (ciprofloxacin, norfloxacin, ofloxacin, sulfamethoxazole and sulfadimidine) degradation. (A) pharmaceutical degradation (B) ciprofloxacin degradation.*

### 3.2.4 Comparing Nitrogen Removal Rate During Anoxic and Aerobic Conditions.

To compare the elimination of pharmaceutical and nitrogen compounds in the aerobic and anoxic phases. The anoxic phase of the anammox system typically depends on anammox bacteria and their associated activities, such as sorption, biotransformation, and microbial

reduction processes, while the aerobic phase of the anammox system may be dependent on the activity of aerobic bacteria for the removal of pharmaceuticals. By mixing the aerobic and anoxic phases, the anammox approach efficiently eliminates pharmaceutical residues from wastewater. Figure (4.8) while I keep track of the nitrogen removal rate over the course of the 6 hours of operation, exhibits various pharmaceutical concentrations and operating parameters ( $\text{NO}_2\text{-N}$ ,  $\text{NO}_3\text{-N}$ ,  $\text{NH}_4^+$ ). For the two differing quantities, nitrogen tends to be removed more quickly under anoxic conditions for all nitrogen components, whether or not there is pharmacological inhibition. The maximum elimination of ammonia occurred at 0 mg/L in anoxic conditions (Fig. 4.8B) and at 70 mg N L<sup>-1</sup> in aerobic conditions (Fig. 4.8A). As a result, the nitrogen removal rate for all nitrogen compounds significantly decreased after being spiked with pharmaceuticals at a concentration of 0.43 mg N L<sup>-1</sup>, and during this phase, nitrate and nitrite were removed concurrently.



### 3.3 SUMMARY

In the current work, nitrogen removal by anammox bacteria was accomplished by monitoring the starvation and non-starvation phases in a simple SBR design setup. This process was operational for total period of 918 days. The nitrogen removal efficiency and specific NRR of the treatment system in this investigation were both 95% and 0.642 g N/L/d, respectively. During this cause, the starving and non-starvation phases were also observed. A stable anammox bacteria biomass was established through the process of managing and optimizing circumstances, pH 7.5 – 8.3, temperature at 22°C, controlled circulation and mixing.

Additionally, five (5) pharmaceuticals from two distinct families—flouroquinolones and sulfonamides—were used to examine their effects on the removal of nitrogen utilizing anammox bacteria as a biofilm and the rate at which these pharmaceuticals degraded. The impact of these pharmaceuticals on anammox bacteria's ability to remove nitrogen from wastewater emphasizes the need for efficient pharmaceutical residue removal from wastewater as well as the necessity for proper wastewater treatment techniques. Essentially because they are more readily available and present in the Estonia WWTP, pharmaceuticals were chosen to be employed in this investigation. Although the various antibiotics, such as ciprofloxacin, norfloxacin, ofloxacin, sulfamethaxazole, and sulfadimethoxine, have varying effects on nitrogen removal, they have all been shown to inhibit the activity of anammox; the higher the pharmaceutical concentration, the less effectively anammox bacteria remove nitrogen from the environment. The rate of recovery for these medications was high despite subsequent degradation.

The overall outcome indicates that the use of anammox bacteria for nitrogen removal may be hampered by the presence of ciprofloxacin, norfloxacin, ofloxacin, sulfamethaxazole, and sulfadimethoxine in wastewater. Suspended granular biomass was successfully used in the treatment system design and operation in this study, which resulted in a stable nitrogen removal efficiency and an increase in specific NRR.

Further research is recommended to optimize the performance of anammox bacteria under varying concentrations of pharmaceutical compounds and to evaluate the long-term stability and efficiency of autotrophic nitrogen removal using anammox-based systems in real world wastewater treatment applications.

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## Appendix 1

Sample/Dates	CIP (mg/L)	NOR (mg/L)	OFL (mg/L)	SMX (mg/L)	SDM (mg/L)	Condition
6.4.23	0.125	0.08	0.04	0.02	0.01	Anox
23.3.23	0.05	0.004	0.002	0.001	0.0005	Aer/Anox
30.3.23	0.05	0.004	0.002	0.001	0.0005	Anox
2.03/9.03.23	0.0625	0.04	0.02	0.01	0.05	Anox

**Table S1: Showing concentration of pharmaceutical concentration during**

Bioreactors	Pharmaceuticals concentration				
	CIP	NOR	OFL	SDM	SMX
R1 INF	0,62	0,1	0,1	0	0
R1EFF	2,68	0,05	0,05	0	0
R2INF	0,16	0,11	0,111	0	0
R2EFF	0,5	0,46	0,63	0,14	0,21

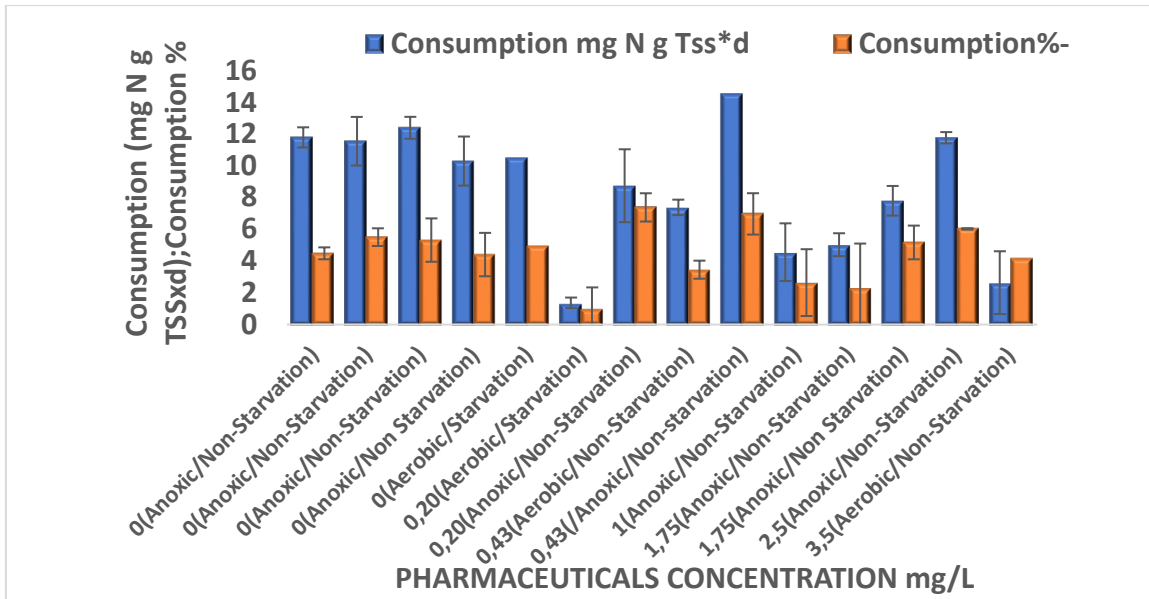
**Table S2: Concentration of pharmaceuticals in R1 and R2 bioreactor**

Pharmaceutical Conc. (mg/L)	Conditions	Nitrite compared to Ammonia (mg N L-1)	Total Nitrogen Removal Rate (mg N L-1)
0	Anoxic/ Non-Starvation	6.346	126.29
0	Anoxic/ Non-Starvation	5.363	129.81
0	Anoxic/ Non-Starvation	1.786	200.86
0	Anoxic/Starvation	1.669	171.01
0	Aerobic/Starvation	0.151	165.58
0.21	Aerobic/Starvation	0.856	130.76
0.21	Anoxic/ Non-Starvation	0.816	125.40
0.43	Aerobic/Non-Starvation	0.386	123.13
0.43	Anoxic/ Non-Starvation	0.643	125.34
1.00	Anoxic/ Non-Starvation	2.144	132.44
1.75	Anoxic/ Non-Starvation	0.115	143.37

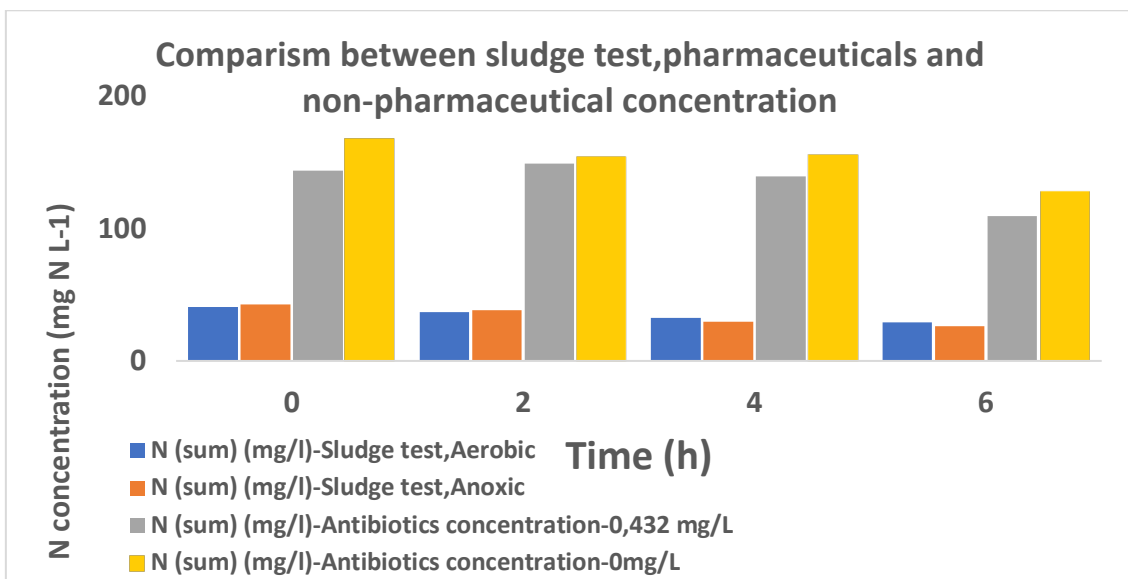
1.75	Anoxic/ Starvation	Non-	0.154	111.52
2.51			0.343	143.37
3.51	Aerobic		0.321	143.45

**Table S3: Showing TNRR and Nitrite compared to Ammonia values at different pharmaceuticals concentration and Conditions**

**Appendix 2**



**Figure S1: The figure shows the consumption (mg N g TSSd) and the Consumption % of nitrogen removal**



**Figure S2: shows comparism between sludge test (aerobic and anoxic), pharmaceutical and non-pharmaceutical concentration.**

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