BERNARDO NASCIMBENI DE BRITO

EFFECT OF SULFIDE, pH AND TEMPERATURE ON ANAMMOX ACTIVITY IN A CONTINUOS FLOW STIRRED-TANK REACTOR

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Engenharia Civil, para obtenção do título de *Magister Scientiae*

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ABBREVIATIONS AND ACRONYMS

AMR	Arithmetic mean ratios
AMX	Ammonium-oxidizing bacteria
AOB	Ammonium Oxidizing Bacteria
CANON	Completely Autotrophic Nitrogen Removal Over Nitrite
CEW	Centre of Expertise Water Technology
CONAMA	Conselho Nacional do Meio Ambiente
CSTR	Continuous-Flow Stirred Tank Reactor
Ea	Apparent activation energy
EGSB	Expanded granular sludge bed
FA	Free ammonia
FNA	Free nitrous acid
GLM	Generalized linear model
HH	Hydrazine-hydrolysis
HRT	Hydraulic Retention Time
HZO	Hydrazine-oxidase
MBBR	Moving Bed Biofilm Reactor
NRR	Nitrogen Removal Rate
NOB	Nitrite Oxidizing Bacteria
ORP	Oxidation reduction potential
OLAND	Oxygen Limitant Autotrophic Nitrification Denitrification
RBC	Rotating Biological Contactor
SAA	Specific Anammox activity
SBR	Sequenced batch reactor
SHARON	Single reactor system for High activity Ammonium Removal Over Nitrite
SNAP	Single stage Nitrogen removal using Anammox and Partial nitritation
TNRR	Total Nitrogen Removal Rate
UASB	Upflow anaerobic sludge blanket
UBF	Upflow anaerobic bed-filter

VLR	Volume Loading Rate
WAC	Water Application Center
WHSD	Waterboard Hollandse Delta

RESUMO

BRITO, Bernardo Nascimbeni de, M.Sc., Universidade Federal de Viçosa, setembro de 2015. **Avaliação do efeito do sulfeto, pH e temperatura em um reator contínuo de mistura completa com atividade anammox.** Orientadora: Ana Augusta Passos Rezende. Coorientadores: Cláudio Mudadu Silva e Ann Honor Mounteer.

Um promissor processo biológico para a remoção de elevadas concentrações de nitrogênio de águas residuárias vem sendo estudado nos últimos 20 anos. O processo possibilita uma redução substancial nos requisitos de aeração, fontes de carbono e baixa produção de biomassa. Durante 9 meses, um reator continuo de mistura completa com nitrificação parcial e processo anammox ocorrendo simultaneamente em único estágio foi operado com medições e análises regulares. Foram avaliados o desempenho do reator e sua capacidade de recuperação após exposição a curto-prazo da adição de sulfeto, pH em condições ácidas e temperaturas abaixo dos valores ótimos. O reator demonstrou ser sensível a pequenas variações de pH. Observou-se que o reator operando com valores de pH inferiores a 6,71 ± 0,01, apresentou perda na eficiência de remoção de amônia. As concentrações de 0,1 e 0,25 mg S L^{-1} de sulfeto testados apresentaram efeito inibitório. A faixa de temperatura de 36 – 25°C testada, não comprometeu a performance do reator.

ABSTRACT

BRITO, Bernardo Nascimbeni de, M.Sc., Universidade Federal de Viçosa, September, 2015. **Effect of the sulfide, pH and temperature on continuous flow stirred tank reactor with anammox activity.** Adviser: Ana Augusta Passos Rezende. Co-advisers: Cláudio Mudadu Silva and Ann Honor Mounteer.

A new biological process that removes high concentrations of nitrogen from residual waters has been studied for the last twenty years. Such process allows for a substantial reduction on aeration and carbon sources requirements while also producing low levels of biomass. For nine months, a continuum reactor composed of a complete mixture with partial nitrification and the anammox process occurring simultaneously was operated with regular mediation and analysis. An evaluation was conducted on short-term effects of sulfide's addition, pH in acidic conditions and the temperature below optimum values of reactor's functioning, as well as its capacity to recover after exposed. The reactor showed sensitivity to small variations on pH. Losses in ammonia removal efficiency were observed when the reactor operated at pH values lower than 6.71 ± 0.01. The tested sulfide concentrations, i.e. 0.1 and 0.25 mg S L^{-1}, have caused inhibitory effect. The temperature range of 36 - 25ºC did not compromised the reactor's performance.

1. GENERAL INTRODUCTION

Nitrogen is a vital element on Earth and represents about 79% of atmospheric air in its inert form (N_2). Before the development of Haber-Bosch process for industrial production of ammonia, the transformation routes of N_2 to reactive forms (Nr) were restricted mainly to biological nitrogen fixation, burning of coal as fuel and the action of atmospheric electrical discharges. However, late 19^{th} century development in agriculture techniques increased the demand for nitrogen given its important rate as a fertilizer. The rates of transformations of Nr by anthropogenic activities, as ammonia, urea, nitrite, nitrate, nitrous oxide, proteins, and amino acids, increased to values comparable to natural processes rates. In his studies, Galloway (2004) predicted that in 2050 the generation of nitrogen from anthropogenic sources will overcome its availability via natural routes. This excess of nutrients has a direct effect on the deterioration of water bodies.

Nitrogen removal from effluents is conventionally performed by autotrophic nitrification followed by heterotrophic denitrification. The nitrification step occurs in aerated reactors, while the denitrification happens in an anoxic environment and, in some cases, via addition of a carbon source to supply for the microbiological demand. This configuration implies high operating costs, making it difficult for application in wastewater treatment plants that include the removal of nitrogen, especially in developing countries.

For the last two decades, the anammox technology has been developed as an alternative to conventional systems (nitrification/denitrification). It consists in the oxidation of ammonium ion directly to nitrogen gas using nitrite as a final electron acceptor under anaerobic conditions (STROUS *et al.*, 1997). The main advantages of this process are the reduction of operating costs with aeration and the absence of carbon addition from external sources. However, the slow bacterial growth is a disadvantage, which compromises the startup of reactors and becomes more susceptible to environmental variations.

Anammox reactors have already been employed at full-scale worldwide. Nevertheless, the technology is still in development within a wide field of research, especially regarding industrial effluents, where variations in the characteristics of the wastewater and complexity of chemical compounds can be potentially toxic to the anammox bacteria.

In this sense, the study is justified by relevance that the anammox has as a sustainable alternative in biological nitrogen removal processes. The aim of this study was the evaluation of ANAMMOX®'s reactor performance in the short-term for sulfide, pH and temperature parameters.

This research was conducted at partnership between Universidade Federal de Viçosa (UFV), NHL University of Applied Sciences (The Netherlands) and Paques BV. The experimental setup was located at the laboratory Water Application Center (WAC) Leeuwarden, The Netherlands.

This thesis is divided in four chapters, where the first is a historical review combined with a short analysis about possible perspectives for the Brazilian market of anammox technology.

The second chapter describes the effects of sulfide in the short term in a Continuous Stirred Tank-Flow Reactor (CSTR). It was considered short-term observation period less than 7 days. Chapter three discusses the rector’s performance under acidic conditions and the effect of these pH changes on sludge's granules size. The last experimental chapter presents results about the effect of reducing temperature in the short term.

2. CHAPTER I - ANAMMOX'S PROCCESS POTENCIAL IN REMOVING PRESENT NITROGEN ON EFFLUENTS.

2.1. Abstract

Brazilian's effluents treatment process are still restricted to the removal of the organic carbonaceous matter. Nevertheless, it is known that nitrogen's presence cause problems to the water's quality through eutrophication. Anammox process (Anaerobic Ammonium Oxidation) has been presenting itself as a relatively new way to remove high concentrations of nitrogenous compounds with less energy consumption and small sludge production. The process is yet not extensively commercially explored, even though there are several publications about its functioning and characteristics. This chapter carries out a literature review of the process and compares it to traditional nitrification/denitrification processes in wastewater treatment and a short analysis about its potential application on the country.

2.2. Introduction

Recent updates on the Brazilian environment legislation, i.e. Resolution number 430/2011 of the Conselho Nacional de Meio Ambiente (CONAMA, 2011), as well as crescent deterioration of the country's hydric bodies, have revealed the necessity of searching for wastewater treatment technologies. In this sense, the anammox (Anaerobic Ammonium Oxidation) technology present itself as a good alternative to conventional (nitrification/denitrification) nutrients biological removal systems.

In this process, bacteria oxidize ammonia directly to nitrogen gas using nitrite as an electron acceptor and CO_2 as the only carbon source (STROUS *et al.*, 1998). Beside the advantages of removing high rates of nitrogen and small relation of used area per volume of treated effluent, anammox can also reduce 90% on the operational costs once it replaces the conventional step of denitrification and saves half of the aeration costs (JETTEN *et al.*, 2005; VAN DER STAR *et al.*, 2007; JOSS *et al.*, 2009; PEREIRA, 2013).

Application of the technology in full-scale have been reported (LACKNER *et al.*, 2014). In lab scale, it is already possible to reach nitrogen removal rates (NRR) up to 77 kg $m^{-3}d^{-1}$, as reported by Tang *et al.* (2011). When dealing with industrial effluents, the ANAMMOX® technology has been largely applied by Paques B.V., a company responsible for the development and manufacturing of this system (KUENEN, 2008).

ANAMMOX® reactor consists of a continuous stirred-tank reactor with granular biomass retention in which the anammox process and partial nitrification occur simultaneously in the same compartment.

This chapter discusses the perspectives and challenges of implementing such technology in Brazil, as well as an overview about the application of the anammox process worldwide. Moreover historical of the technology and its developments is addressed.

2.3. Historical research of anammox

Up until 1997, it was believed that the conversion of ammonia to gas nitrogen would only occur in the presence of oxygen. However, Broda (1977), through thermodynamic concepts, showed it was possible to predict the existence of lithochemicalautotrophy bacteria capable of realizing such conversion using nitrite (NO_2) as oxidant agent (Figure 2.1). Only twenty years later, the first anammox activity evidence was observed and reported in a fluidized bed reactor in order to treat effluent for methane production (MULDER *et al.*, 1995).

This discovery motivated some studies to advance in order to better understand which microorganisms are involved in such process and ultimately proved some bacteria would prefer NO_2 over nitrite (NO_3) (MULDER *et al.*, 1995; VAN DE GRAAF *et al.*, 1995). Besides, it was verified that such anammox bacteria are known to having a slow growth rate. At optimum conditions its doubling time is eleven days and at real conditions are about two to three weeks (JETTEN *et al.*, 2005). Another characteristic is its low biomass production, *i.e.* 11g SSV g^{-1} $NH4^{+}$-N (ISAKA *et al.*, 2006).

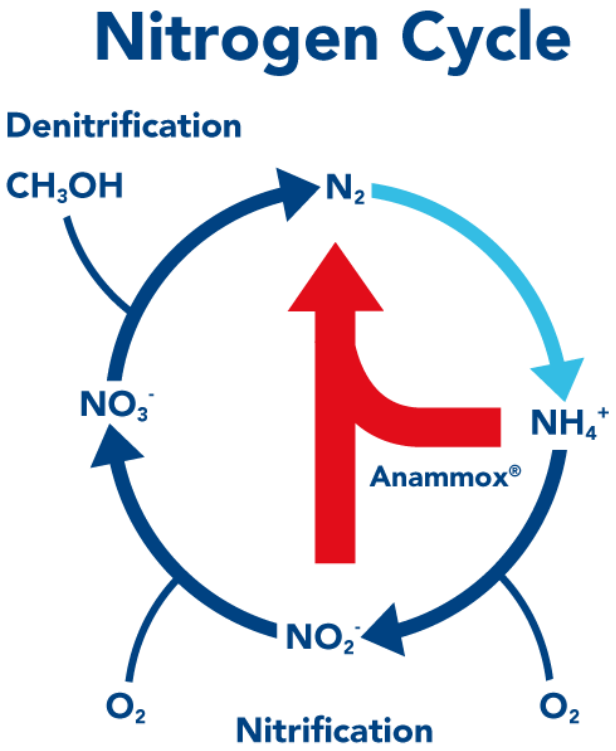

Figure 2.1 -Scheme of anammox bacteria metabolic route (source: Paques B.V.)

The next step on the technology's development was the proposition of a metabolic route by Van de Graaf *et al.* (1995). The authors used the masked nitrogen technic to discover that hydroxylamine (NH_2OH), intermediary on the reduction of NO_2, reacts with ammonia (NH_3) to produce hydrazine (N_2H_4). Apparently, NO_2 is reduced by the hydrazine-hydrolysis (HH) enzyme on NH_3 presence. Posteriorly, the hydrazine-oxidase (HZO) enzyme converts the hydrazine to N_2, transferring four electrons to the NIR enzyme, closing the cycle (Figure 2.2) (ZHU *et al.*, 2008).

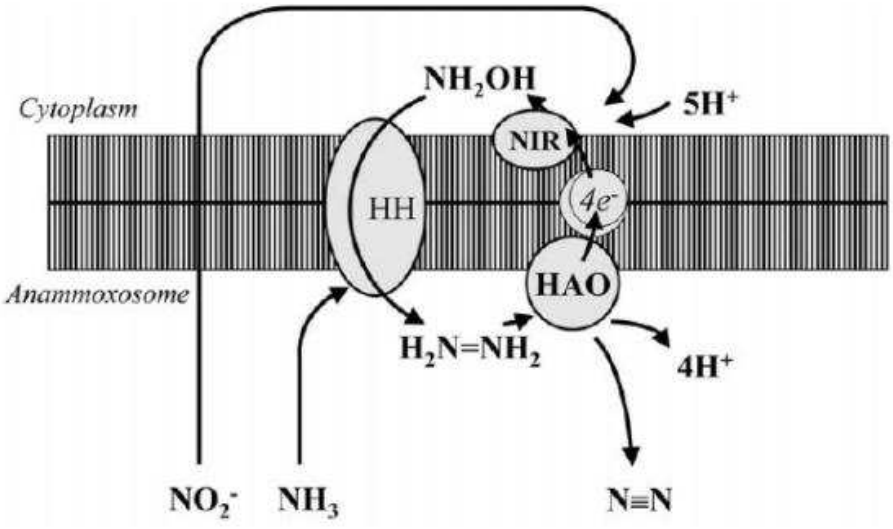

Figure 2.2 -Intermediate products of the anammox process (KUENEN and JETTEN, 2001)

The stoichiometric relation of the anammox process was proposed by Strous *et al.* (1998) (Equation 1). In the same work, it was also concluded that the anammox bacteria uses CO_2 as a source for biomass production.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15} \quad (1)$$

Despite the proofs of the process' biological nature, it was only through the experiments of Strous *et al.* (1999) that the identification of bacteria involved in the anammox process was possible. Anammox's microorganisms belongs to the phylum *Planctomycete* and the five genders were described: *Candidatus Brocadiaanammoxidans, Ca. Kuenenia, Ca. Anammoxoglobus,* and *Ca. Jettenia* (JETTEN *et al.,* 2001; SCHMID *et al.,* 2003; KARTAL *et al.,* 2007).

Several research groups and companies have reported work done with the technology. Mostly, in the case of research groups, looking into improvements and in the case of companies generating patents with different names, which causes confusion in relation to the conceptions of such reactors. Despite the generated confusion, two different configurations of the reactor are known: 1) The two stages configuration, where ammonia conversion to nitrite and the anammox process occur separately and 2) in one stage, where configuration, partial nitrification and the anammox process occurs simultaneously (SULTANA, 2014).

The first full-scale reactors implemented were the two-staged ones, adapting already

existing nitrifying reactors in some wastewater treatment plant (LACKNER *et al.*, 2014). A good example is the SHARON®-ANAMMOX® system (HELLINGA *et al.*, 1998). This reactor was the result of a public-private partnership between *Delft* University of technology and Paques B.V. company, which created the first wide full-scale reactor (80 m^3 of volume) in the city of Rotterdam. The objective was to operate the treatment's plant sewer effluent on the cities of Dokhaven and Sluisjesdijk, in The Netherlands. Due to the low growth rate of the anammox bacteria and other chemical issues, the system took about two years to reach stable conditions (KUENEN, 2008). As to 2006, the system presented a removal of 8-10 kg $N.m^{-3}d^{-1}$, the double of its original capacity (VAN DER STAR *et al.*, 2007).

SHARON® (*Single Reactor System for High Ammonium Removal over Nitrite*) represents the first stage, when 50% of the ammonia is converted to nitrite (SULTANA, 2014). To convert ammonia to nitrite is necessary to specify the biota with Nitrosomonas bacteria. Therefore, the reactor is operated with a temperature between 30 to 40°C, oxygen limitation and no biomass retention (VAN DONGEN *et al.*, 2001). At elevated temperatures, the Nitrosomonas growth rate is higher than the Nitrobacter ones (Figure 2.3) which, without the sludge retention and hydraulic retention time of one day, is possible to control the partial nitrification. The produced nitrite in the first reactor is then reduced to nitrogen gas at the ANAMMOX® reactor.

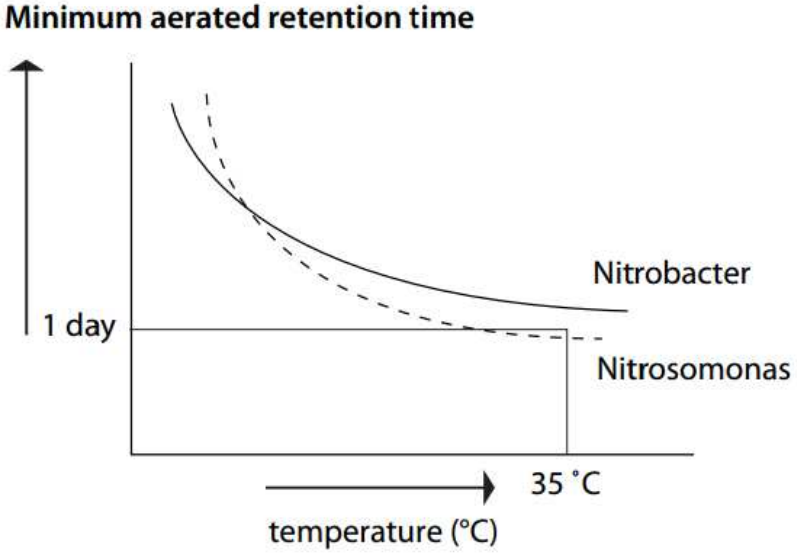

Figure 2.3 -Different growth rates of Nitrosomonas and Nitrobacter (BERTINO, 2010; GRONTMIJ, 2015).

The buildup knowledge about reactors ideal condition for partial nitrification provided

conditions for one-stage operation system, which became predominant later. The main one-stage reactors used are the biological ones with moving-bed biofilm reactor (MBBR), sequencing batch reactor (SBR) and granular sludge (LACKNER *et al.*, 2014). At current reviews, such systems can be found with different names such as OLAND (Oxygen Limitant Autotrophic Nitrification Denitrification) (KUAI and VERSTRAETE, 1998) and CANON (Complete Autotrophic Nitrogen Removal over Nitrite) (THIRD *et al.*, 2001; VAZQUEZ-PADÍN *et al.*, 2009a), SNAP (Single-stage Nitrogen Removal using the Anammox and Partial Nitrification) (BERTINO, 2010), or yet, commercially registered ones as DEMON®, TERRANA®, ANAMMOX®, amongst others.

The anammox process has been successfully applied on treatment of various effluents, such as sludge's liquid digestion, landfill leachate, coke-ovens wastewater, monosodium glutamate, pharmaceutical effluents and swine wastewater (JIN *et. al.*, 2012). Tang *et al.* (2011), using an upflow anaerobic sludge blanket (UASB) inoculated with anammox bacteria, were able to achieve a NRR of 77 kg m^{-3} day^{-3}. Furukawa *et al.* (2006) used biogas production plant's effluent and a load of 5 kg N m^{-3} d^{-1}, being able to hit over more than 80% nitrogen removal efficiency.

2.4. Challenges of the anammox technology and applications

A good performance of the anammox reactor is mainly conditioned to a synergistically of the ammonia-oxidizing bacteria (AOB), the anammox bacteria and the nitrite-oxidizing (NOB). Whereas more important parameters that strongly influence its stability are: pH, temperature, substrate concentration, dissolved oxygen (DO) and the reactor's configuration (YANG *et al.*, 2007; VAN HULLES *et al.*, 2010; CHEN *et al.*, 2011; JIN *et al.*, 2012; JIN *et al.*, 2013b).

Ammonium and nitrite substrate can cause inhibition process, depending on their concentrations (SCHEEREN *et al.*, 2011). Even though studies have shown that the anammox activity is not affected by ammonium concentration smaller than 1 g N L^{-1} (STROUS *et al.*, 1999). Dapena-Mora *et al.* (2006) observed a loss of 50% of microorganisms activity (IC_{50}) with a concentration as low as 770 mg L^{-1}. The difference on the results can be attributed to the influence of NH_3/NH_4^+ and HNO_2/NO_2 balance. The same authors reported that free ammonia (NH_3) and nitrous acid (HNO_2) would be the potentially toxic forms and not the ammonium ions and nitrite itself. Fernandez *et al.* (2012) observed

that the IC_{50} to the HNO_2 toxic ratio of 11µgL^{-1}, but a concentration of 1.5µgL^{-1} would be enough to destabilize the anammox process and significantly reduce the nitrogen removal efficiency. Tang *et al.* (2010a) found inhibition of the anammox bacteria from NH_3 between 57 and 187 mg L^{-1}. Anthonisen *et al.,* (1976) related that the inhibition to AOB of free ammonia occurred at concentrations from 8 to 120 mg L^{-1} and of HNO_2 between 0.2 and 2.8 mg N L^{-1}.

According to Jin *et al.* (2012), the pH control close to neutrality can avoid the inhibition by substrate. However, the appropriated pH for anammox bacteria is slightly alkaline, i.e. between 7.8 and 8.0 (STROUS *et al.*, 1999b; LANGONE *et al.,* 2014). Too acidic or very basic environments can lead to hydrolysis of the cellular membrane, cutting the vital metabolic process and causing complete inhibition of the process (SCHEEREN *et al.,* 2011). There is a divergence in relation to the effects of the pH regarding AOB and NOB. However, it is a consensus that the optimum pH is between 7.0 and 8.0 (GRUNDITZ and DALHAMMAR, 2001; VAN HULLES *et al.*, 2010).

Temperature is an important operational parameter because it affects the metabolism and cellular growth. Once the anammox bacteria have a slow growth rate it is very important that temperature is well controlled. The anammox bacteria are found in several natural environments, such as marine, terrestrial and glacial ecosystems (KUYPERS *et al.*, 2003; RYSGAARD *et al.,* 2004; BURGIN and HAMILTON, 2007; ZHANG *et al*., 2007; HUMBERT *et al.,* 2010). Such variety indicates that anammox activity happens in temperatures between -2 to 85°C (RYSGAARD *et al.,* 2004; BYRNE *et al.,* 2009; JETTEN *et al.,* 2009; GAO and TAO, 2011). Scientific literature mainly report anammox application in wastewater treatment at an optimum temperature from 30 to 40°C (STROUS *et al.,* 1999b; EGLI *et al.,* 2001; JIN *et al.,* 2013a). Grunditz and Dalhammar (2001) studied pure cultures and observed optimum temperature of 35°C for AOB and 38°C for NOB. The authors also demonstrate (Figure 2.4) that at temperatures below 20°C, the NOB activity is superior to the AOB. This represents limitation to the anammox technology on effluents with low temperature. However, several authors have reported reasonable efficiencies within one-stage system with partial nitritation/anammox process (SZATKOWSKA *et al.,* 2007; DOSTA *et al.,* 2008; VALQUEZ-PADÍN *et al.,* 2011; WINKLER *et al.,* 2011; DE CLIPPELEIR *et al.,* 2013; HU *et al.,* 2013; LOTTI *et al.,* 2014). For example, Hu *et al.*

(2013), operating a SBR on lab-scale, reached 90% of nitrogen removal at temperature as low as 12°C. Dosta *et al.* (2008) observed that the anammox process can have satisfactory activity operating at temperatures of 18°C. Rysgaard *et al.* (2004) reported denitrification and anammox activity on marine sediments at temperatures about 12°C. All mentioned works demonstrate the possibility of long period of acclimatization to establish lower temperature for the operation of anammox reactors.

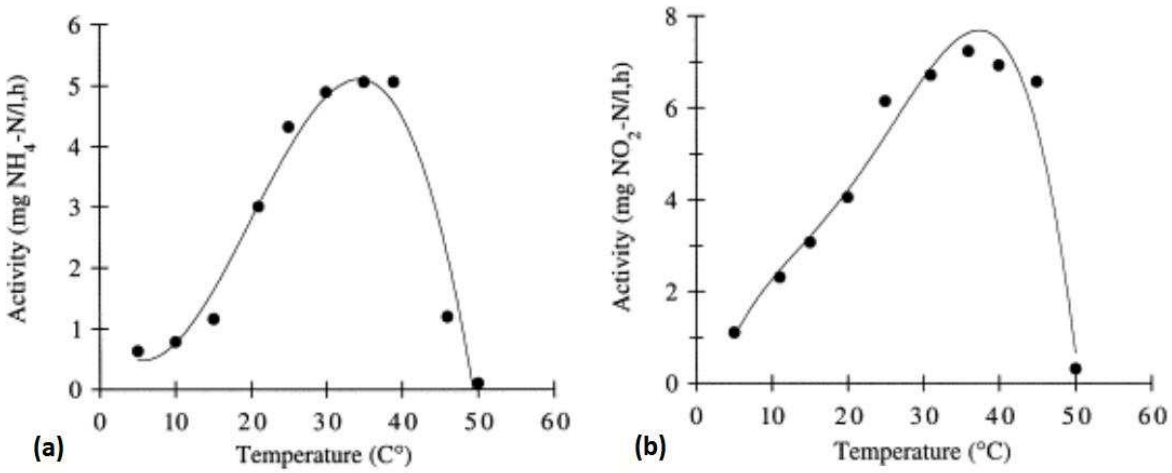

Figure 2.4 - Effect of temperature on AOB (a) and NOB (b) activity (GRUNDITZ and DALHAMMAR, 2001)

Another parameter that influences significantly the anammox operation systems is the dissolved oxygen (OD) (SULTANA, 2014). Being anaerobic microorganisms, anammox bacteria activity can be inhibited at a very low oxygen concentration (JETTEN *et al*., 2001; SCHMID *et al.*, 2005; KARTAL *et al.*, 2007). Strous *et al*. (1997) verified complete inhibition at OD concentrations around 0.2 to 1.0 mg O_2 L^{-1}. The authors also noted that for lower concentrations, i.e. 0.2 mg O_2 L^{-1}, reversible partial inhibition occurs. The optimum OD for NOB and AOB was set between 3.0 and 4.0 mg O_2 L^{-1} (BARNES and BLISS, 1983). Low OD concentrations are limiting the growth of NOB (WIESMANN, 1994), while for the AOB some studies (ABELIOVICH, 1987; HANAKI *et al.*, 1990, WYFFELS *et al.*, 2003), showed significant ammonia oxidation rate between 0.05 and 0.5 mg O_2 L^{-1} Mentioned works indicates that microaeration avoids the inhibition of anammox bacteria and provides appropriated conditions for obtaining partial nitrification.

Some interesting combinations for the coexistence of AOB and anammox bacteria, with NOB limitation are MBBR, SBR and the granular sludge process. In the granular sludge case, the presence of anammox bacteria into the sludge reduces exposition to oxygen, making the system more tolerant to elevated OD concentrations.

2.5. Anammox in Brazil

Brazilian law concerning about effluents discharging are established by CONAMA (Conselho Nacional de Meio Ambiente). CONAMA is a consultative and deliberative institution for environmental issues in Brazil. Through resolution 430/2011, article 16, the limit of ammonium nitrogen concentrations for effluents in water bodies was elevated from 5 mg L^{-1} to 20 mg L^{-1} However, the current pattern is applicable for industrial effluents, it has not been required for municipal wastewaters. The flexibility of Brazilian targets, maintaining focus on the removal of carbonaceous material, adding to the high operational costs of conventional nitrification and denitrification systems does not contribute to nitrogen removal in the country, permitting the discharge of effluents with high nitrogen compounds load in water bodies, causing eutrophication processes (CHEIS, 2015). In that sense, the anammox process can be presented as a potential alternative to the nutrients removal.

Although the anammox process is still very recent in Brazil, there are some scientific studies and literature reporting the application on real effluents treatment (SCHEEREN *et al.*, 2011). Reginatto *et al.* (2005) conducted one of the first works in the country, describing nitrogen removal by anammox process of biomass from a nitrification/denitrification reactor to wastewater treatment. Araújo *et al* (2010) used biomass from a conventional activated sludge system to treat domestic wastewater. The reactor was able to remove 95% of ammonium with an initial concentration between 55 and 82 mg NH_4^+ L^{-1}. Recently, Casagrande *et al.* (2013) used upflow reactor to study the start-up time and its maximum NRR. The highest nitrogen removal rate (18.3 g N L^{-1} dia^{-1}) occurred after 120 days, using an initial load of 24 g N L^{-1} day^{-1} and a hydraulic detention time of 0.2 hours.

Most of the published works reported the use of anammox process to treat agro-industrial effluents, especially swine culture, which produces residues with high loads of nitrogen (SHCEEREN *et al.*, 2010). Kunz *et al.* (2007) were able to enrich bacteria with anammox activity through swine dejects, by acclimatization and immobilization with synthetic effluents. In posterior research, the same authors used an anammox reactor

preceded by a partial nitrification reactor. In this work, it was possible to reach a nitrogen removal rate above 80% using a load of 1.6 g N L^{-1} d^{-1}.

In Brazil there is, currently, an ANAMMOX® reactor at full-scale (Paques B.V.). The ANAMMOX® technology is applied worldwide mainly on the treatment of food industry, beverages, textile and pharmaceutical effluents. This model of reactor is set to operate in single stage with granular sludge (Figure 2.5).

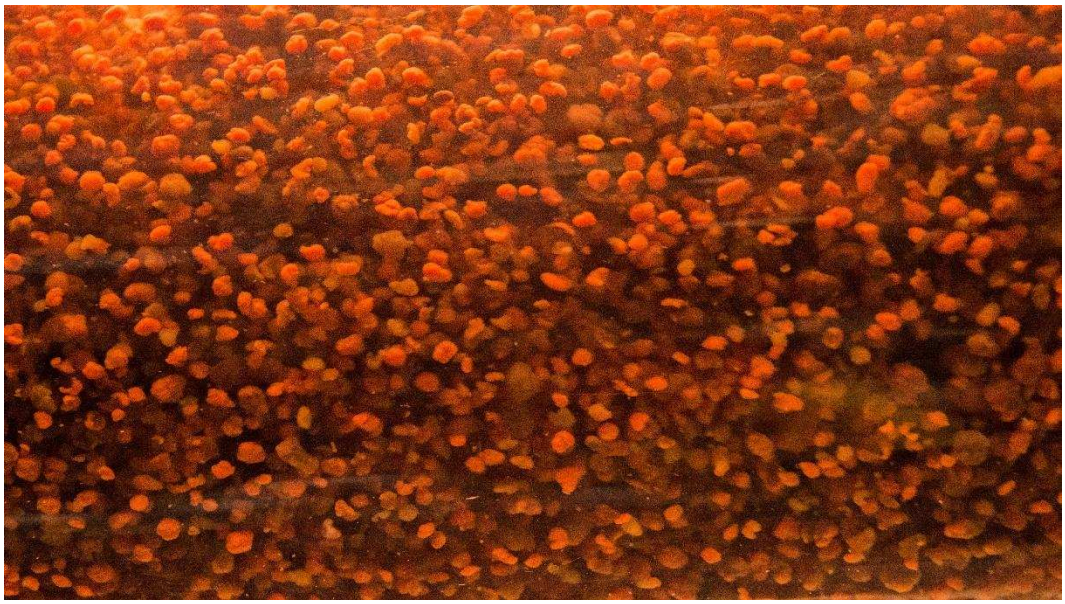

Figure 2.5 - Anammox granular sludge

ANNAMOX® technology stands out mainly because it is the most compact (only one reactor), has high rates of nitrogen removal and smaller operational cost compared to a conventional systems (nitrification/denitrification). Table 2.1 shows the advantages of this system in comparison to conventional nitrogen removal systems.

Table 2.1 -Comparison between conventional systems and ANAMMOX®

Characteristics	Conventional	Partial nitritation -Anammox process (ANAMMOX®)
Sludge type	Flocs	Granules
Numbers of reactors	2	1
Conditions	Aerobic/anoxic	Oxygen limited
Oxygen required	4.6 kg O_2 / kg N	1.9 kg O_2 / kg N
Main bacteria involved	Nitrifiers (AOB, NOB) and denitrifiers	AOB/Anammox
Biomass retention	No	Yes
Sludge production	High	Low
Sludge growth	1.9 kg VSS / kg N	0.12 kg VSS / kg N
VLR (kg N/m³.d)	0.05 to 0.2	0.5 to 2.5
BOD requirement	2.9 kg BOD / kg N	0 kg BOD / kg N
Operation complexity	Separate aerobic and anoxic compartments or periods; methanol dosing; need for pH control.	Keep tuned populations of AOB, NOB and anammox; no need for significant pH control cost if wastewater has enough buffer capacity.
Application status	Established	Full-scale plants and lab-scale

(PAQUES B.V. 2015; JETTEN *et al.* 2001; AHN, 2006; BERTINO, 2010; CUI, 2012).

Low biomass production stems from slow growth of the anammox bacteria. The doubling time at optimum temperature conditions ranges from 10 to 14 days (STROUS *et al.,* 1998; VAN DER STAR *et al.,* 2007, PAQUES B.V., 2015). The biomass treatment becomes cheaper but the low rate of bacteria growth demands an efficient retention of biomass and compromise the inoculation on the reactors. Therefore, the startup turned more sensible to variations on effluent conditions (JIN *et al.,* 2012). Some studies are looking into

optimize the operational conditions to improve the bacteria doubling time. Tang *et al.* (2011) reported lab scale experiments doubling time of 4.8 days and Isaka *et al.* (2006) managed to reduce to 1.8 days.

The 40% reduction on demanded oxygen is due to the occurrence of partial nitrification and the anammox process simultaneously. In this case, only microaeration is necessary to avoid inhibition of anammox bacteria activity and control NOB activity.

As mentioned previously, the anammox bacteria are lithochemicalautotrophy, not being necessary to add methanol as a source of carbon. ANAMMOX® reactors have been operating with satisfactory efficiency with a volumetric load 10 times bigger than conventional systems and COD/NH_4-N relations smaller than 2:1 (LACKNER *et al.,* 2014). Such characteristic have been observed when the system was out in operation with different industrial effluents. However, a diversity of potentially toxic compound found in industrial effluents might inhibit the anammox activity (PEREIRA, 2013). Research work about the effects of compounds such as heavy metals, sulfide, phosphate, alcohols and phenols are still not well known.

3. CHAPTER II - THE EFFECT OF SULFIDE IN A ANAMMOX® REACTOR

3.1. Abstract

Anaerobic ammonium oxidation (anammox) is a cost-effective biotechnological process capable of removing high concentrations of ammonium from wastewater. This study evaluated the performance of ANAMMOX® reactor under the presence of sulfide and its recoverability after inhibition. Experimental work was conducted in a Continuous-Flow Stirred Tank Reactor (CSTR). The highest total nitrogen removal rate (TNRR) was observed during addition of 0.10 mg S L^{-1} of sulfide. The TNRR over that period was 1.52 kg m^{-3} d^{-1}. The combination of high pH (7.30 ± 0.19) and sulfide (0.25 mg S L^{-1}) caused an irreversible decrease of removal efficiency. While pH was at 6.88 ± 0.4 and 0.1 mg S L^{-1}, the reactor had loss of activity. However, it was able to recover after interrupting the addition of sulfide.

3.2. Introduction

Anaerobic ammonium oxidation (anammox) is a cost-effective biotechnological process capable of removing ammonium from wastewater. Compared to a conventional nitrification and denitrification process, it has lower demand of oxygen (JIN *et al*., 2013b). As compounds found in wastewater are various and can inhibit the growth rate of anammox bacteria (STROUS *et al.,* 1998), its application could not be recommended for certain types of industrial effluents. Several industrial activities generate wastewater presenting sulfide. Generally, wastewater from coke oven, chemical and petrochemical industry presents combinations of sulfide and high concentration of ammonium (JIN *et al.,* 2013b). Such wastewater characteristics are also detected on the outflows of anaerobic reactors of sulfate and sulfate-reducing bacteria can reduce sulfur-based compounds.

Anammox's process has been extensively analyzed in laboratory, pilot and/or full scale as a prominent way to remove nitrogen while studies investigating the presence of sulfide as inhibitor of the anammox bacteria are scarce (DAPENA-MORA *et al.*, 2007; JIN *et al*., 2013b).

Sulfide's most toxic form is the hydrogen sulfide (H_2S). It can easily permeate the cell membrane, and denature its proteins (TURSMAN *et al.*, 1988; CHEN *et al.*, 2010). Also, sulfide is corrosive and can compromise the reactor's operation. However, Van de Graaf *et al*. (1996) and Jung *et al.* (2007) noted some increase of anammox activity in the presence of sulfide. Diversification of methodologies and designs of reactors in question added up to such

contradictory findings. In some cases, for example, evaluation of anammox activity was made only with specific bacteria.

Thus, the present study aims to evaluate the anammox activity by determining which sulfide concentrations affect the efficiency in removing nitrogen in short-term on ANAMMOX® reactor while also evaluating the capacity of recovery after reduction in biomass activity. The research established that short-term refers to the period of less than 8 days.

3.3. Material and Methods

The experimental work was conducted in Continuous-Flow Stirred Tank Reactor (CSTR) for a period of 36 days using synthetic wastewater. Further installations, procedures analysis and mathematical modeling of the experiment are described below.

3.3.1. Synthetic wastewater

Wastewater used in the experiments were prepared according to Jin *et al*. (2012). They were composed by ammonium as a substrate in the form of ammonium chloride (NH_4Cl) and sodium bicarbonate ($NaHCO_3$), providing alkalinity and mineral elements, as showed in Table 3.1. Additionally, trace micronutrients diluted 1000 times in demi-water were added to the wastewater in order to support the growth of anammox bacteria. Those traces are compounds for Fe, Zn, K, Co, Mn, Cu, Mo, Ni, Se, B, W.

Table 3.1 -Concentration of chemicals in the influent wastewater.

Component	Target compound	g L^{-1}
NH_4Cl	NH_4-N	2.293
KH_2PO_4	PO_4	0.220
$NaHCO_3$	Alkalinity	4.380
$CaCl_2 \cdot 2\ H_2O$	Ca	0.300
$MgCl_2 \cdot 6\ H_2O$	Mg	0.165

The wastewater was kept in a tank and acidified in order to maintain the pH approximately at 7.0 to reduce losses of ammonium by volatilization and facilitate dissolution of reagents.

The sulfide was introduced directly into the wastewater tank by $Na_2S.9H_2O$ as source of sulfur. This reagent was always added after acidification of the influent to minimize losses in the form of gases.

3.3.2. ANAMMOX® reactor setup

The experimental setup consisted of a continuous flow stirred-tank reactor (CSTR) of about 9 liters with an one-stage partial nitritation/anammox process of granular sludge. Three different types of mass flow controllers (Bronkhorst) for the gases were used in the system, as Figure 3.1 illustrates. Air was added to the reactor in order to reach the appropriate dissolved oxygen (DO) concentration (between 0.5 and 2.0 mg L^{-1}). Nitrogen gas was added to ensure full mixture of liquor inside the reactor. Carbon dioxide (CO_2) was used to control pH surges and also as carbon supplementation source for the activity of aerobic microorganisms. A dose pump with 1.5 M NaOH and flow of 2.0 mL min^{-1} was installed in order to control pH reduction. The pH was maintained between 7.00 and 7.10.

Temperature was regulated by water bath at 36°C. DO, temperature and pH were measured by sensors (Endress + Hauser) whose data were saved every hour by a transmitter (Liquiline, Endress + Hauser).

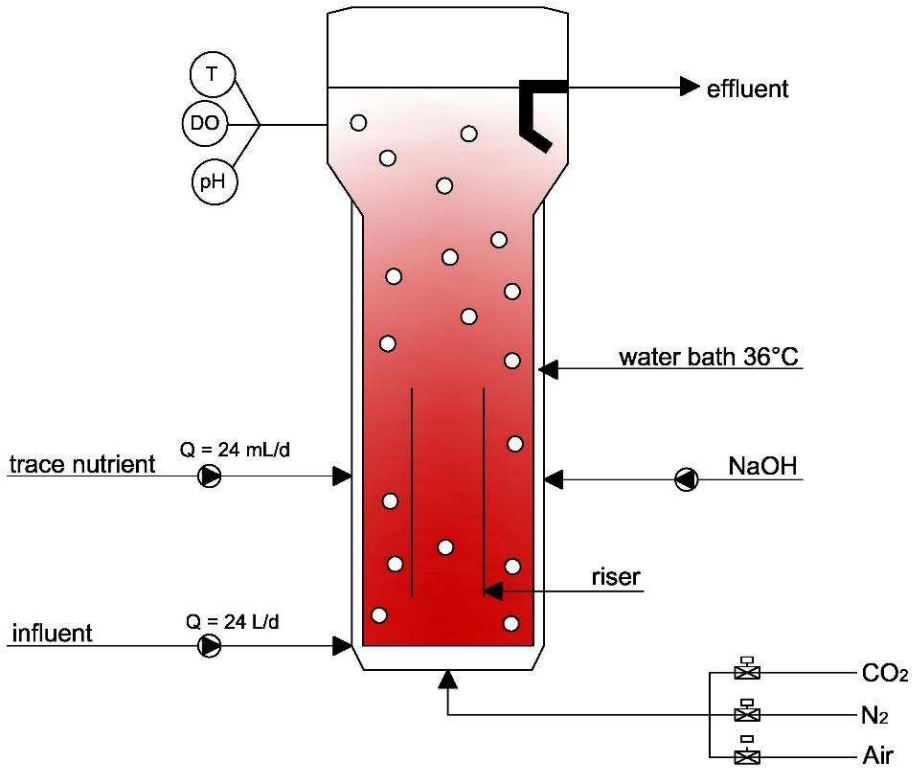

Figure 3.1 - The CSTR setup scheme, compound by three mass flow controllers for gases (N_2, O_2, and CO_2); DO, pH and temperature sensors and three pumps for addition of influent, trace nutrients and NaOH.

The reactor was fed with synthetic wastewater with a flow rate of 24 L d^{-1} and hydraulic retention time (HRT) of 9 hours. The reactor was also connected to a dose pump with a flow of 24 ml d^{-1} to provide necessary trace micronutrients. This flow rate was lower than the minimum pumping capacity of the dose pump; therefore, it was necessary to install a timer, programmed to activate the pump every hour for 30 seconds at the flow of 2 ml min^{-1}.

Sulfide's impact on reactor was evaluated in two different phases. Firstly, the reactor's response under presence of sulfide was analyzed and then its ability to recover the nitrogen removal efficiency after ceasing the addition of sulfide.

During the experimental work, it was possible to evaluate sulfide's concentrations of 0.10 and 0.25 mg S L^{-1}. The concentrations of sulfide were adjusted according to the efficiency of TNRR (Total Nitrogen Rate Removal) and accumulation of nitrite (NO_2^--N) and nitrate (NO_3^--N) in the reactor. Initially, the reactor was stabilized and the optimum parameters of operation were determined.

After this phase, a concentration of 0.10 mg S L^{-1} was added to the wastewater. After three days, if the reactor performance was not affected by the new condition, the sulfide's concentrations were increased to 0.25 mg S L^{-1}. The addition of sulfide was interrupted if there were significant losses on the anammox activity. During this period, the capacity of the reactor to recover its initial conditions were evaluated. The reactor recovery was defined according to the initial stabilization period which optimum parameters of operation were set. To maintain the balance of ammonia-oxidizing bacteria (AOB), anammox bacteria and nitrite-oxidizing (NOB), through of Paques BV experience and developing of this research, NO_2^--N concentrations were set to below 10 mg L^{-1} and NO_3^--N concentration less than 100 mg L^{-1} are important to the reactor's stability of these three groups of microorganism. Besides nitrogen removal efficiency, it was also considered nitrite and nitrate concentrations in the recovery evaluation, after a toxic event. If the reactor was stabilized after recovering, sulfide was again added. In such way, the tests were made continuously until the moment that the inhibition was complete and the reactor was unable to recover. Figure 3.2 presents a flowchart of the decision-making within the phases of the experiment.

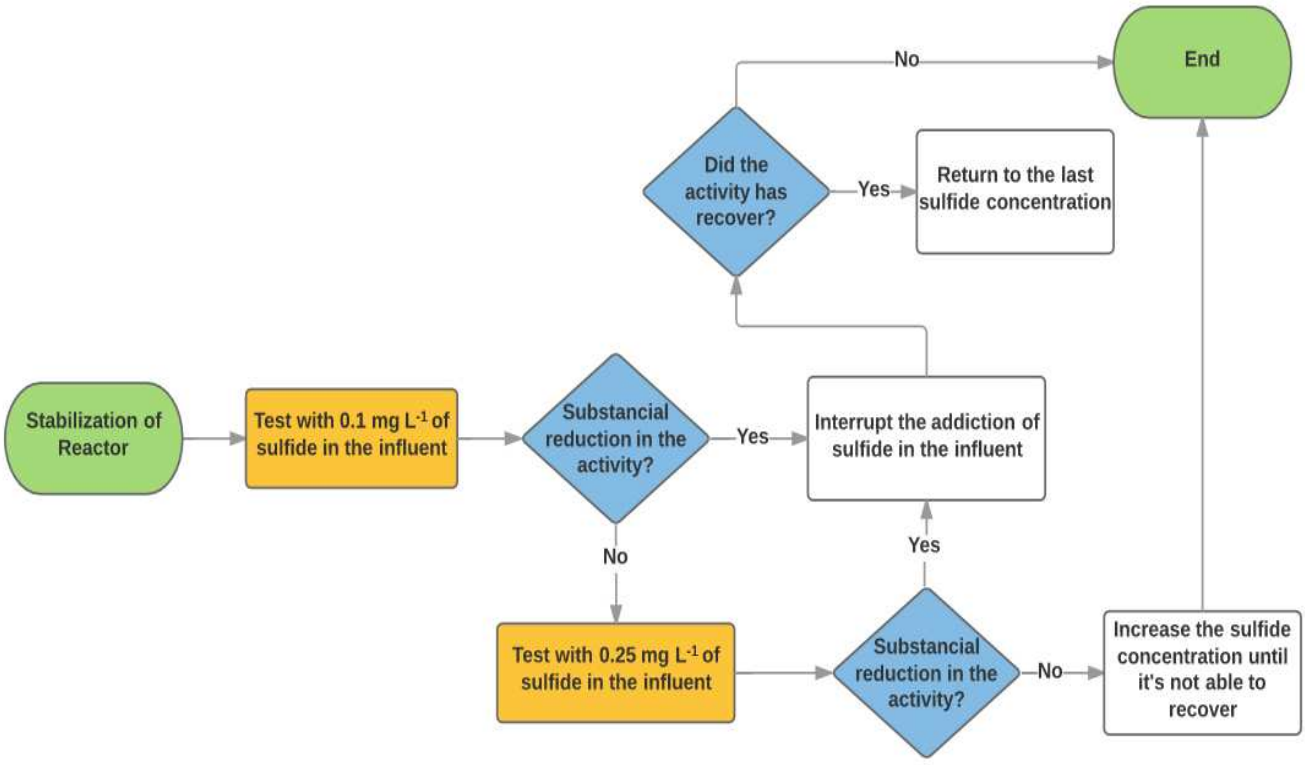

Figure 3.2 - Flowchart of the experiment procedure

3.3.3. Analytical procedure

The anammox activity was being monitored by the concentrations (mg L^{-1}) of total nitrogen (N_t), chemical oxygen demand (COD), ammonium (NH_4-N), nitrite (NO_2-N) and nitrate (NO_3-N). Such parameters were measured daily in the reactor's influent and effluent. Ammonium and nitrate effluent's sample were obtained inside the reactor while the other parameters were obtained in the output. Samples were filtered through a filter diameter of 110 mm with 11 µm porosity. Analyses were carried out by spectrophotometer Dr. Lange XION 500, using the cuvette tests (Hach Lange) LCK 303, LCK 342, LCK 339, LCK 238 and LCK 314. The procedures were followed accordingly to the manufacturer's instructions.

3.3.4. Mathematical Models

Equations 2, 3 and 4, proposed by Cui (2012), were used to estimate the biomass activity in the CSTR reactor among aerobic nitrite-oxidizers (NOB), aerobic ammonium-oxidizing bacteria (AOB) and anaerobic ammonium-oxidizing (AMX) bacteria, expressed in mg N L^{-1} d^{-1}.

$$\Delta N = \left(\left(NH_4^+ - N_{inf}\right) + \left(NO_2^- - N_{inf}\right) + \left(NO_3^- - N_{inf}\right)\right) - \left(\left(NH_4^+ - N_{eff}\right) + \left(NO_2^- - N_{eff}\right) + \left(NO_3^- - N_{eff}\right)\right) \quad (1)$$

$$AOB\ activity = \frac{\left(NH_4^+ - N_{inf}\right) - \left(NH_4^+ - N_{eff}\right) - \left(\frac{\Delta N}{2.04}\right)}{HRT} \quad (2)$$

$$NOB\ activity = \frac{\left(NO_3^- - N_{inf}\right) - \left(\frac{0.26\Delta N}{2.04}\right)}{HRT} \quad (3)$$

$$Anammox\ activity = \frac{\Delta N}{HRT} \quad (4)$$

Where NH_4^+-N_{inf}, NO_2^--N_{inf}, NO_3^--N_{inf} are ammonium, nitrite and nitrate concentrations in the influent (mg N L^{-1}), NH_4^+-N_{eff}, NO_2^--N_{eff}, NO_3^--N_{eff} are respectively ammonium, nitrite and nitrate concentrations in the effluent (mg N L^{-1}). HRT is hydraulic retention time expressed in days.

Sludge anammox activity (SAA) is calculated from maximum slope of the curve of nitrogen gas production rate divided by the biomass concentration (equation 5).

$$SAA = \frac{n \times M_{N_2}}{\Delta t \times VSS}\ , g\ N_2 (g\ VSS\ d)^{-1} \quad (5)$$

Where M_{N_2} is 28 g mol^{-1}, Δt is time interval corresponding to Δp, VSS is the Volatile Suspended Solids concentration in grams.

$$n = \frac{V \times \Delta p}{R \times T} \quad (6)$$

Where V is volume of N_2 in the headspace of the batch flask; Δp is the pressure increase in the steepest part of the curve; R is the Gas constant (83,145 N m^{-2} L mol^{-1} K^{-1}) and T is the temperature (K).

The H_2S concentration in the solution was estimated from the equation 7 (Ma *et al.*, 2005).

$$[H_2S] = [TS] \times \frac{1}{1 + \frac{K}{10^{-pH}}} \quad (7)$$

Where [H_2S] is the concentration of H_2S in the solution, mg L^{-1}, [TS] is the concentration of sulfur-S in the solution., mg L^{-1}, K is the first step ionization equilibrium constant, K = 1.49 x 10^{-9}, when the temperature was 36 °C.

3.4. Results and Discussion

3.4.1. ANAMMOX® reactor performance

ANAMMOX® reactor performance during the sulfide test is represented in Figure 3.3, considering NH_4-N, NO_2-N, NO_3-N effluents, pH, TNRR and sulfide concentration factors. The variations in pH and presence of sulfide affected TNRR throughout the experiment.

During period A, pH dropped to 6.49 between the 9th and 11th day which resulted in concentration increase on NH_4-N effluent of 187 to 197 mg L^{-1}, reduction of 4.08 to 1.04 mg L^{-1}of NO_2-N effluent's concentrations and increase of 63.75 to 73.50 mg L^{-1} of NO_3-N effluent's concentrations. Also, concentration of dissolved oxygen (DO) in the reactor

increased. Nevertheless, TNRR increased about 0.96 kg m^{-3} d^{-1} to 1.25 kg m^{-3} d^{-1} over the period A. Therefore, pH reduction did not compromised the reactor performance but AOB was possibly affected due to the reduction of nitrite and increase of ammonium present in the effluent. Such results can be explained due to the granular sludge structure. AOB are primarily affected by pH decrease due to its external presence on the granules. Reinforcing this argument, in the period B, between 15^{th} and 18^{th} day, under presence of sulfide, the AMX activity increased more than the AOB (Figure 3.3).

Sears *et al.* (2004) observed complete inhibition of AOB in nitrifying cultures on the presence of 0.50 mg S L^{-1} of soluble sulfide. Same authors suggested that sulfide reacts with other elements present in the reactor, affecting the availability of micronutrients such as iron, zinc, manganese and copper and consequentially inactivating the AMO enzyme, responsible for one of the intermediate steps of nitrification. Beccari *et al.* (1980) concluded that nitrification activity was reduced 67% at sulfide concentration of 5.0 mg S L^{-1} in an experiment with typical compounds of a coke plant wastewater. Beristain-Cardoso *et al.* (2010) had indication of 30% inhibition at 5.1 ± 0.5 mg S L^{-1} after a stabilization period in the presence of sodium thiosulfate. Jiang *et al.* (2009) showed that the use of nitrifying biofilm previously acclimatized with H_2S, makes it possible to keep ammonium removal efficiencies higher than 95%, even with high loading of H_2S (164 g m^{-3} h^{-1}). Such result suggest that the level of inhibition sulfide could be affected by several factors. In this way, the acclimatization of biomass would be an alternative to improve reactor performance.

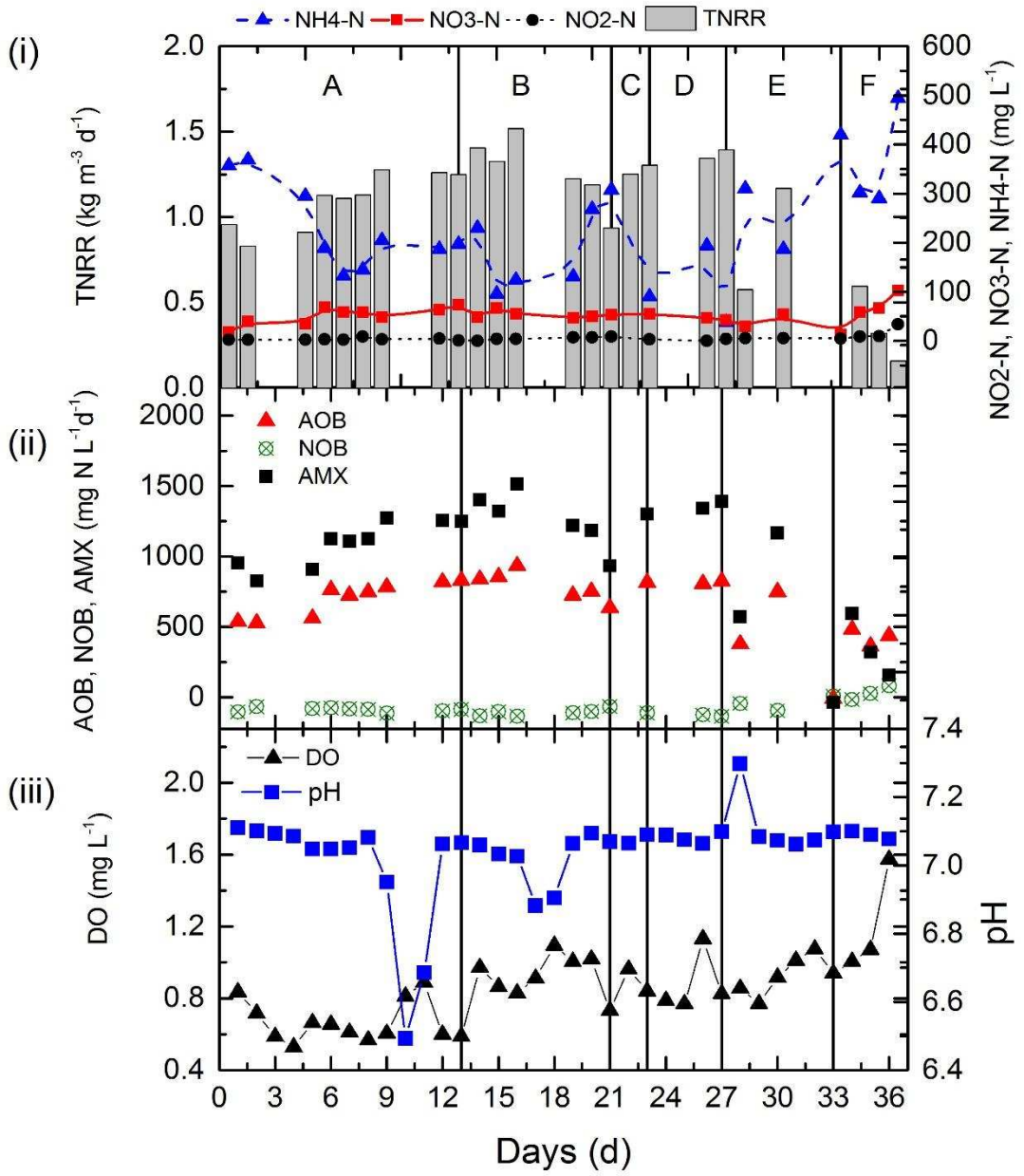

Figure 3.3 - (i) Performance of reactor during the experiment: (A) reactor stabilization; (B) 0.10 mg S L^{-1}; (C) without sulfide; (D) 0.10 mg S L^{-1}; (E) 0.25 mg S L^{-1}; (F) without sulfide; NO_2-N and NO_3-N concentrations are relative to output (ii) Activities of NOB, AOB, AMX. (iii) Average concentrations Oxygen (mg L^{-1}) and pH the reactor

After the third day during the B period in the presence of sulfide (16th day), the reactor was stabilized to its new condition with TNRR values higher than the period of sulfide absence. However, operating problems occurred and the pH decreased to 6.88 ± 0.40 and 6.90 ± 0.15 during the 17th and 18th day of test, respectively. Throughout the experiment, a decrease in the pH was observed on days 10, 11, 17 and 18. In all cases, the pH decrease was observed after a high increase in the TNRR. According to Jetten *et al.* (1999), anammox process consume alkalinity. During the low pH period, what possibly happened was a combination of operational problems in the control of pH and low alkalinity provided by wastewater. The pH values tend to below 7.00, when TNRR was higher than 1.26 kg m^{-3} d^{-1}. AOB and anammox (AMX) activity were above 784.3 mg L^{-1} d^{-1} and 1272.4 mg L^{-1} d^{-1}, respectively, which demonstrates the importance of providing carbonate to a satisfactory performance of the reactor.

TNRR was maintained at 1.23 to 1.19 kg m^{-3} d^{-1} followed by a reduction of 0.93 kg m^{-3} d^{-1} on day 21, fact that led to deciding in stopping the addition of sulfide in order to evaluate the reactor's performance recovery. Even with the reduction of TNRR the reactor did not accumulate NO_2-N and NO_3-N (Figure 3.3). Such fact probably explains why the reactor recovered quickly, reaching TNRR of 1.30 kg m^{-3} d^{-1}. Another 0.10 mg S L^{-1} were again added and after four days of recovery, TNRR achieved 1.39 kg m^{-3} d^{-1}, value of removals that was similar to those in the first addition of 0.10 mg S L^{-1}. Therefore, the concentration of sulfide added was increased to 0.25 mg S L^{-1} on the 28th day. Unfortunately, there was loss control in pH increases on the first day with 0.25 mg S L^{-1}. The reactor had high activity; however, pH was 7.30 ± 0.19 during two days and TNRR was 0.57 kg m^{-3} d^{-1}. The reactor became unstable with an average TNRR of 0.36 kg m^{-3} d^{-1} and accumulation of NO_2-N and NO_3-N was observed. Thus, it was decided to interrupt the test. Summary of the results found in each phase are shown in Table 3.2.

Table 3.2 -Summary of the ANAMMOX® reactor performance throughout the experiment.

	Phases	Period (days)	Substrate concentration (mg NH_4-N L^{-1})	Sulfide concentration (mg S L^{-1})	Average TNRR (mg L^{-1})	Removal of ammonium (%)
A	**Reactor Stabilization**	13	731.8[a]	0.00	1.26±0.01[a]	73.20±0.01
B	**0.10 mg S L^{-1}**	8	711.9	0.10	1.27±0.20	73.60±0.09
C	**Without sulfide**	2	625.0	0.00	1.28±0.03	80.94±0.07
E	**0.10 mg S L^{-1}**	4	672.5	0.10	1.37±0.03	83.96±0.14
F	**0.25 mg S L^{-1}**	6	549.8	0.25	0.57±0.60	38.17±0.38
G	**Without sulfide**	3	588.3	0.00	0.20±0.22	39.06±0.11

[a]: Average of the last three days which the reactor was stabilized.

Due to the addition of sulfide at concentrations near to the method detection limit and the possible interferences in solubility, it was not possible to accurately determine concentration of soluble sulfide that caused inhibitory effect to the reactor. Part of the sulfide could be lost by precipitation, oxidation or stripping. According to Sears *et al.* (2004), the sulfide fluctuation values are inevitable in experiments with biological reactors due to the presence of aerators, metallic ion or the dependence of pH. In acidic solutions (pH less than 7.00), hydrogen sulfide (H_2S) is the dominant form, but in basic solutions (pH greater than 7.00), the disulfide is dominant (Lens *et al.*, 1998). Sulfide as S^{2-} predominantly occurs only at strong alkaline conditions, such as when pH is above 9.5 (Morse *et al.* 2002). Sulfide always was kept 98% of the amount added in H_2S form (equation 7) throughout the experiment, even with variations in pH. Sulfide's most toxic form is unionized H_2S since it is easily able to permeate the cell membrane (Tursman *et al.* 1988). Even with interfering factors in solubility of sulfide, the sludge's always in contact with such toxic form.

The reactor became more susceptible to the presence of inhibitory secondary factors after addition of sulfide. At pH (7.30 ± 0.19) and sulfide (0.25 mg S L^{-1}) on day 28 was inhibitory and the reactor was unable to regain performance while for days 17 and 18, the pH at 6.88 ± 0.4 and 0.1 mg S L^{-1}, activity was recovered at the reactor, after interrupting the addition of sulfide. According to Dapena-Mora *et al.* (2007), anammox activity is 60% reduced with 64 mg S L^{-1} and completely inhibited with 160 mg S L^{-1}. While Van de Graaf *et al.* (1996) found results that the activity is increased. Jung *et al.* (2007) concluded that hydrogen sulfide provides an ideal reducing environment for the anammox bacteria. Through

their experiments, the authors detected increased activity after addition of hydrogen sulfide to keep oxidation reduction potential (ORP) between -150 and -200mV.

These contradictory results could be explained by the interference in the solubility of sulfide, the differences in the methodology employed, and/or different operating modes and reactors with small volumes. Some studies only evaluated the inhibition of anammox activity with specific species of bacteria such as *Candidatus Brocadiasinica* and *Candidatus Kueneniastuttgartiensis* (VAN DE GRAAF *et al.*, 1996; DAPENA-MORA *et al.*, 2007; OSHIKI *et al.*, 2011). More research should be conducted at more realistic conditions with a broader diversity of species.

3.5. Conclusions

The highest TNRR under sulfide's presence was 1.52 kg m^{-3} d^{-1} on 16^{th} day. It was not possible to determinate the sulfide inhibitory concentration to ANAMMOX® reactor. However, the presence of sulfide became the reactor sensitive to pH variations. Due to the granular structure, AOB are primarily affected. The combination pH (7.30 ± 0.19) and sulfide (0.25 mg S L^{-1}) reduced irreversibly the TNRR. While the pH was 6.88 ± 0.4 and 0.1 mg S L^{-1}, the reactor had loss of activity but it was able to recover after interrupting the addition of sulfide.

We recommend, in future research, to establish sulfide's effect in long-terms and improve the method to reduce interference in the solubility of sulfide.

4. CHAPTER III - ANAMMOX® PERFORMANCE UNDER ACIDIC CONDITIONS

4.1. Abstract

The partial nitrification/anammox process with one-step system has been implemented with satisfactory performance in full-scale. Inhibition causes are widely studied; however, operation windows are not well defined. This study evaluated the ANAMMOX® system under acidic short-term conditions. The reactor did not present considerable reduction on removal efficiencies at pH ranges between 7.10 ± 0.02 and 6.80 ± 0.01. Performance was inhibited and recovered at pH 6.61 ± 0.01 and 6.71 ± 0.01, respectively. The inhibitory effect of the pH causes accumulation of nitrite and consequently increases free nitrous acid (FNA) concentration, which is toxic to anammox bacteria. In addition, sludge granules reduced size when exposed to pH 6.61 ± 0.01.

4.2. Introduction

ANAMMOX® system is a technology developed over 20 years as a promising alternative to remove high loads of nitrogen in wastewater. The central feature of this system is the potential operating costs reduction due to lower demand of oxygen and biomass production (VAN DONGEN *et al.*, 2001). The first time this technology has been applied in full scale was in Rotterdam at 2002. It was used on wastewater from sludge reject water treatment at the municipal wastewater treatment plant of Waterboard Hollandse Delta (WHSD). Presently, there are already 24 plants of ANAMMOX® system operating on different types of wastewater such as distillery, tanning and food industries. ANAMMOX® system consists of simultaneous occurrence of partial nitrification and anammox process, which are performed in only one-step with granular sludge. Part of the NH_4^+-N is converted to NO_2^--N by ammonium-oxidizing bacteria (AOB) (equation 8). According to equation 9, Third *et al.*, (2001), remaining NH_4^+-N and the NO_2^--N produced is converted to dinitrogen (N_2) by anammox bacteria. The overall reaction (equation 10) generates H^+, which results in pH reduction on wastewater without sufficient alkalinity.

Partial nitrification:

$$1.32\ NH_4^+ + 1.98\ O_2 \rightarrow 1.32\ NO_2^- + 1.4\ H_2O + 2.64\ H^+ \quad (8)$$

Anammox process:

$$NH_4^+ + 1.32\ NO_2^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O \quad (9)$$

Overall reaction:

$$NH_4^+ + 0.85 O_2 \rightarrow 0.13\ NO_3^- + 0.435\ N_2 + 1.4\ H^+ + 1.43\ H_2O \quad (10)$$

The pH is a parameter of importance to ensure the anammox activity. Strous *et al.* (1999b) reported that pH interval for anammox is 6.7–8.3. Anammox's activity was maintained stable (TANG *et al.*, 2010b) even with pH 8.5-9.3 while using granular sludge. It incurs, under acidic conditions, alteration in pH gradient of cell. The energy generation by ATPases and active transport are the metabolic process affected, which uses NO_2^--N as transporters (DAPENA-MORA *et al.*, 2007; LU *et al.*, 2013).

Furthermore, the pH affects directly the concentration of ammonia in the free form (FA) and in free nitrous acid (FNA). Several authors (STROUS *et al.*, 1998; DAPENA-MORA *et al.*, 2007; JAROSZYNSKI *et al.*, 2011) report that these compounds as potentially toxic. The FA has an inhibitory effect since ammonia can easily penetrate and dissolve the cell membrane lipid compound (SPEECE 1996; GALLERT and WINTER, 1997). How FNA acts in the inhibition and its mechanisms is yet to be clarified. Low pH reduces the concentration of ammonia in the free form (FA) but increases the free nitrous acid (FNA) while high pH, increases FA concentration and decreases FNA concentration (MOSQUERA-CORRAL *et al.*, 2005; JIN *et al.*, 2012). The pH is adjusted to neutral conditions in order to avoid inhibition by FA or FNA (JIN *et al.*, 2012).

This study aimed to evaluate ANAMMOX® system performance under acidic short-term conditions. It was considered short-term period lower than 8 days. Test were conducted on lab-scale dimension using a CSTR reactor of 9 liters and pH was progressively reduced at ranges of 7.10, 7.00, 6.80, 6.70 and 6.60, according to the reactor's performance. Results were compared with literature data and practical implications for the application of technology.

4.3. Material and Methods

4.3.1. Synthetic wastewater

The description of the influent is mentioned in section 3.3.1.

4.3.2. Design, ANAMMOX® reactor operation and experiment procedures.

The pH inhibition test was conducted in a CSTR of 9 liters. Design and operation were described in detail on section 3.3.2. Start-up occurred adding 3 liters of tap water, 3 liters of influent and 3 liters of mixed sludge from two different origins. Half of the added sludge came from a municipal wastewater treatment plant in the UK and the other one from a combination of industrial (potato processing factory) and dewatering liquor from the digester at a municipal wastewater treatment plant.

The tests begun 9 days after the reactor's stabilization. Experiments were set to evaluate the reactor performance with progressive reduction in the pH of 7.00, 6.80 and 6.60. Each pH was planned to operate for 7 days, approximately 18 cycles of HRT. When a strong loss of activity was detected, we returned the reactor the initial condition of 7.10 in order to evaluate its recoverability. Due to the results found during the tests, the pH range of 6.70 was also evaluated at the end of the test.

4.3.3. Analytical procedure

Concentrations (mg L^{-1}) of chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrite (NO_2^--N) and nitrate (NO_3^--N) of the influent and effluent were determined daily. The analysis followed the same procedures of the section 3.3.3.

The size distribution of the granules were measured at the end of each pH range using image analysis. Digital images were provided by a single-lens reflex camera (Nikon D5100, 18-55 mm) and processed by *ImageJ* software. The method consists in taking approximately 50 ml of sludge samples spread over on a plain white surface. The images were converted to grayscale and then the number and individual size of each granule were identified. Ferret's diameter concept was applied to determine the granule's size. Feret's Diameter is the biggest distance between two internal points, also known as the caliper length.

4.3.4. Mathematical Models

Estimates of FNA concentration as HNO_2 was calculated based on Anthonisen *et al.* (1976). The equation (11) correlates the factors pH and temperature on the equilibrium constant of nitrous acid.

$$Free\ nitrous\ acid\ as\ HNO_2(\mu g/L) = \frac{46}{14} x\ \frac{NO_2 - N}{K_a \times 10^{pH}}; \quad (11)$$

$$K_a = e^{(-2{,}300/273+T)} \quad (12)$$

Where NO_2-N is the concentration of nitrite in effluent (mg N L^{-1}), T is the temperature in degrees Celsius (°C) and K_a is the ionization constant of the nitrous acid equilibrium.

The reactor had sludge loss during the experiment. Thus, such factor was considered on the evaluation of the reactor performance. The parameters settings were the total nitrogen removal rate (TNRR) and the volume of sludge (V_s) inside the reactor measuring every change in the operation of the pH (equation 13).

$$TNRR/_{V_s} = \frac{(NH_4^+ - N + NO_2^- - N + NO_3^- - N)_{inf} - (NH_4^+ - N + NO_2^- - N + NO_3^- - N)_{eff}}{HRT \times V_s} \quad (13)$$

Where NH_4^+-N_{inf}, $NO_2^-$$N_{inf}$, NO_3^--N_{inf} are ammonium, nitrite and nitrate concentrations in the influent (mg N L^{-1}), NH_4^+-N_{eff}, NO_2^--N_{eff}, NO_3^--N_{eff} are ammonium, nitrite and nitrate concentrations in the effluent (mg N L^{-1}), HRT is hydraulic retention time expressed in days and V_s is the volume of sludge inside the reactor.

4.4. Results and Discussion

As Figure 4.1 shows, along the 38 days of observation, reactor's performance was directly affected by pH. After the first day of startup, the reactor presented rapidly, satisfactory performance (500.3 kg m^{-3} d m_s^{-3}) and stable operating parameters. It operated for 8 days at optimal conditions with average pH of 7.10 ± 0.01 and average TNRR/Vs of 463.20 ± 40.5 kg m^{-3} d m_s^{-3} presenting no considerable reduction in removal efficiencies at pH ranges 7.10 and 6.80. Values observed between pH 7.10 and 6.80 are presented in Table 4.1. However, after 21 days of testing, the sludge volume in the reactor decreased 10%.

Table 4.1 - Summary of the reactor performance throughout the experiment.

pH	Graphic area	Period (days)	FNA (µg L^{-1})	Average TNRR (kg m^{-3} d^{-1})	V_s (l)	Average TNRR/V_s (kg m^{-3} d^{-1} m_s^{-3})
7.09 ± 0.01	A	8	1.65 ± 0.22	1.39 ± 0.23	3.0	463.90 ± 40.50
7.01 ± 0.03	B	6	2.60 ± 0.32	1.35 ± 0.11	3.0	451.40 ± 35.40
6.80 ± 0.01	C	7	6.56 ± 1.62	1.25 ± 0.09	2.7	461.30 ± 32.50
6.61 ± 0.01	D	2	134.97 ± 55.05	0.22 ± 0.13	2.7	82.10 ± 49.9
7.10 ± 0.02	E	5	4.83 ± 0.79	1.26 ± 0.19	2.4	523.50 ± 8.90
6.71 ± 0.01	F	7	60.99 ± 39.05	0.48 ± 0.28	2.3	207.20 ± 119.90
7.10 ± 0.01	G	3	4.36 ± 0.76	1.18[a]	2.3	512.90[a]

[a]: It was only considered the value of the third day, when the reactor had already recovered.

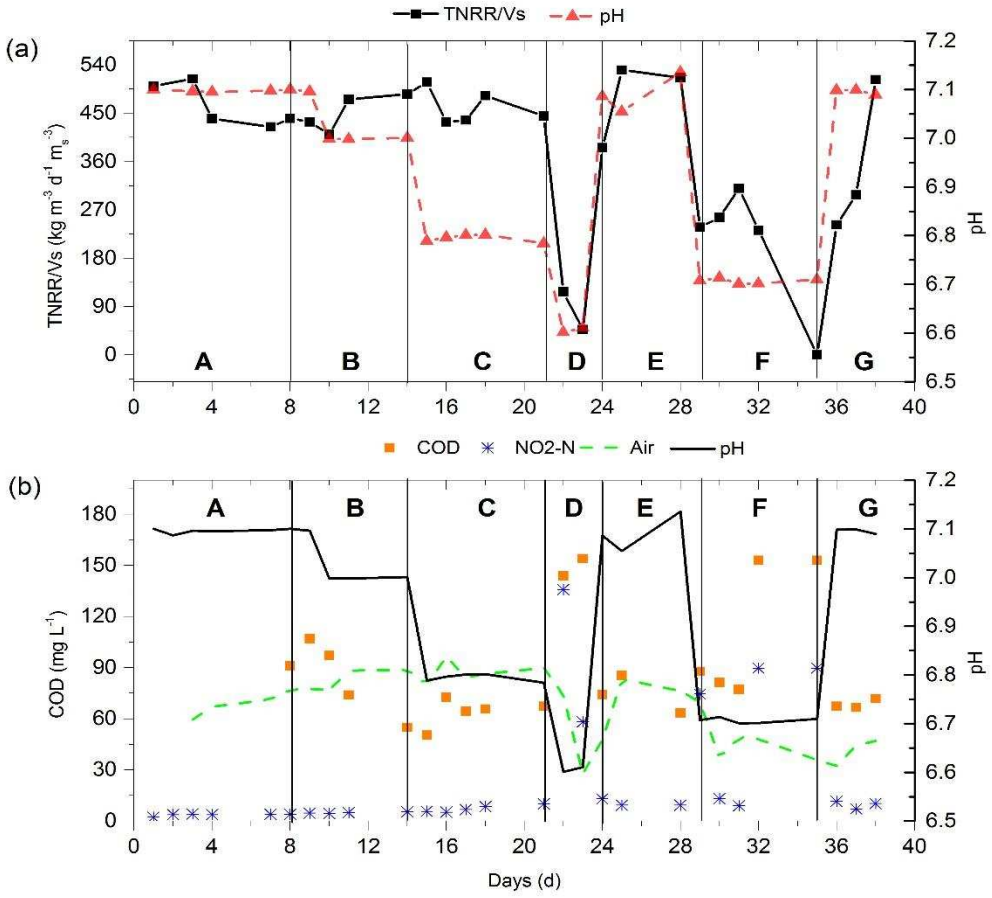

Figure 4.1 - (a) TNRR/Vs (kg m^{-3} d^{-1}) and pH values during the experiment. (b) COD and NO_2-N L^{-1} (mg L^{-1}), Airflow (L h^{-1}) for 40 days of test. Both graphics are divided by the period of each tested pH. These areas are as follows: A (7.09 ± 0.01), B (7.01 ± 0.03), C (6.80 ± 0.01), D (6.61 ± 0.01), E (7.10 ± 0.02), F (6.71 ± 0.01) and G (7.10 ± 0.01).

At pH values lower than 6.71 ± 0.01, it was observed substantial loss in nitrogen removal efficiencies (Figure 4.1). The reactor was exposed for two days (D) to pH 6.61 ± 0.01 with average TNRR/V_s of 82.10 ± 49.9 kg m^{-3} d m_s^{-3} and seven days (F) to pH 6.71 ± 0.01 with average TNRR/V_s of 207.20 ± 119.90 (Figure 4.1, Table 4.1). The observed concentration of NH_4-N on the effluent was lower than the increase in NO_2-N concentration. Hypothetically, the pH had more inhibitory effect on anammox bacteria than the aerobic ammonium-oxidizing bacteria (AOB). The optimum pH for anammox bacteria is between 7.80 and 8.00 (LANGONE *et al.,* 2014; STROUS *et al.,* 1999b). As for AOB, studies reported different optimum pH values for nitritation. According to Van Hulles *et al.*, (2010) optimal pH interval for AOB is 7.00-8.00. Thus, AOB group versatility, could explain the results obtained.

In both cases, in pH 6.71 ± 0.01 (D) and pH 6.61 ± 0.01 (F), the accumulation of NO_2-N was detected (Figure 4.1). The airflow was reduced to decrease the accumulation of nitrite. Nevertheless, its concentration sustained higher than those observed on pH above 6.80 ± 0.01. On 22th day, it was verified the highest concentration with 135.75 mg NO_2-N L^{-1} when pH was set at 6.61 ± 0.01. The NO_2^--N accumulation is not directly toxic, however, low pH increases the concentration of NO_2^- in the form of FNA (JIN *et al.,* 2012; MOSQUERA-CORRAL *et al.,* 2005). Several authors (DAPENA-MORA *et al.*, 2007; JAROSZYNSKI *et al.*, 2011; STROUS *et al.*, 1998) reported FNA as potentially toxic.

Studies results with anammox granular sludge and similar operation are presented in comparison with this test in Table 4.2 As it can be seen, different results were observed regarding FNA's concentration. Tang *et al.* (2010a, 2010b) observed inhibition with FNA above 19.9 ± 5.0 µg L^{-1}, while Chen *et al.* (2011) reported loss in efficiency to values above 134.4 µg L^{-1} at pH 7.0. However, FNA concentration levels must be under control, because of the anammox process's sensibility under acidic conditions. The anammox activity is significantly influenced by small pH variations. The combination of low pH and high concentrations of nitrite proves to be the worst operating condition to the efficiency of reactor. In this study, due the low concentration of NO_2^--N, it was possible to maintain the reactor with a good performance even at pH 6.80.

Table 4.2 - Comparison of results with other research with similar parameters of operations.

Reactor designer	**T (°C)**	**pH**	**HRT (h)**	**NO_2^--N (mg L^{-1})**	**FNA (µg L^{-1})**	**Inhibition**	**References**
CSTR	36	6.6	9.0	97± 54[a]	134.1 ± 55.5	82%[d]	This study
CSTR	36	6.7	9.0	55± 41[b]	60.9 ± 39.1	60%[d]	This study
CSTR	36	6.8	9.0	7 ± 2[b]	6.6 ± 1.5	no inhibition	This study
Upflow anaerobic sludge blanket (UASB)	35	6.8	14.2	280	77.7	12%[e]	Tang *et al*., 2010a
Upflow anaerobic bed-filter (UBF)	35	6.8	9.1	380	19.9 ± 5.0[c]	31%[f]	Tang *et al*., 2010b
Expanded granular sludge bed (EGSB)	35	6.8 – 7.0	1.5 – 8.0	768	213.1 - 134.4[c]	24%[f]	Chen *et al.*, 2011

[a]: Average NO_2^- concentration exposure for 2 days; [b]: Average NO_2^- concentration exposure for 7 days; [c]: Calculated based on experimental data and NO_2^- concentration of influent; [d]: TNRR/Vs efficiency; [e]: SAA; [f]: TNRR efficiency.

Although the pH below 6.71 ± 0.01 has inhibitory effect on short-term, activity was recovered after increasing the pH to 7.10. Table 4.1 summarizes the reactor performance throughout the experiment.

Low pH was also responsible for reducing the size of the granules. The reactor started up with an average ferret diameter of 2.9 mm and significant difference was not found ($p < 0.01$) of the mean of the ferrets for pH above 6.71 ± 0.01. Figure 4.2 represents the distribution of granules at each pH range. The curve of pH 6.60 differs from the others due to an increase of 1537.5% in the granules with diameter less than 0.5 mm. Furthermore, in the same period, COD increased 53.2% (Figure 4.1). It is possible that a process of deterioration of the granule at pH 6.61 ± 0.01 have started. The reactor also had an increase in the COD concentration to pH 6.71 ± 0.01. However, it was not enough to affect the average ferret diameter of the granules. Sludge loss observed in the reactor throughout experiment cannot be attributed only to reduction of pH (Figure 4.2). The three-phase separator used in the test may have been inappropriate for biomass retention.

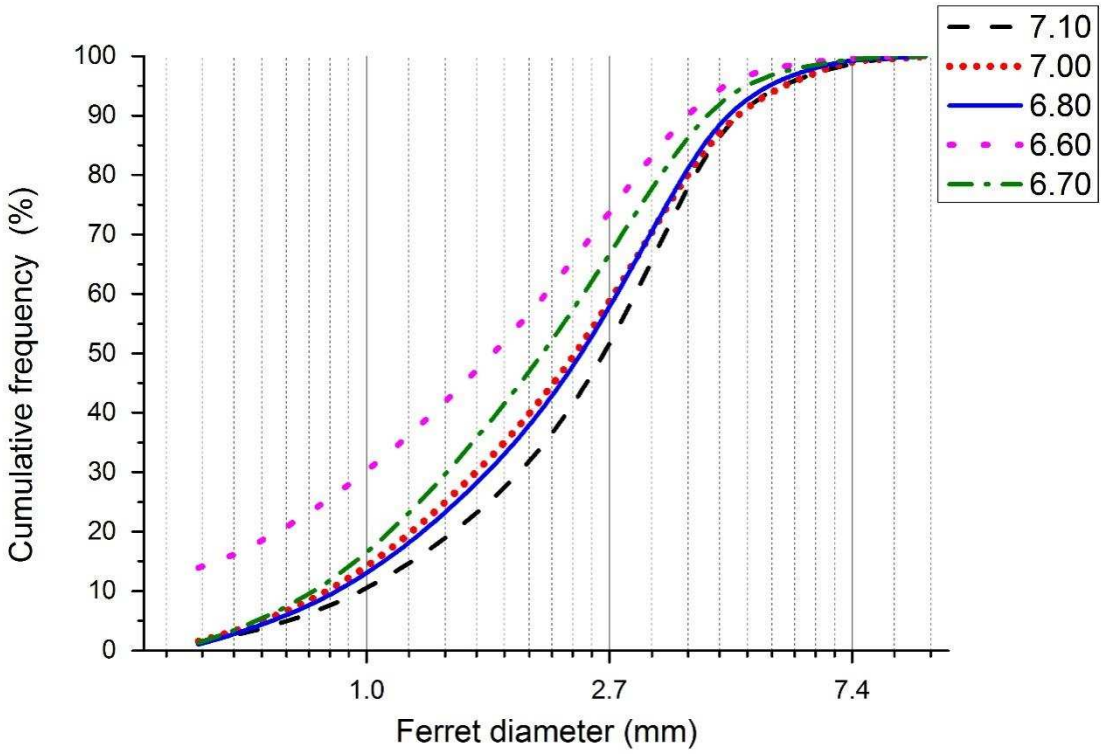

Figure 4.2 - Accumulated granular distribution per pH range.

4.5. Conclusions

The conclusions that could be drawn from the results are summarized as follows:

1) The inhibitory effect on pH values lower than 6.8 ± 0.01 decreases the anammox activity, causing accumulation of nitrite. Part of the nitrite under acidic conditions is in the form of FNA, which is toxic for the anammox bacteria. Therefore, nitrite is a second inhibition factor in low pH. It is essential the control of FNA concentration.
2) ANAMMOX® system could operate at pH 6.80 ± 0.01 with TNRR/Vs higher than 461.30 ± 32.50, when FNA concentration is maintained below 6.56 µg L^{-1}.
3) A strong loss of activity was detected when the reactor operated at pH 6.61 ± 0.01 for 2 days and pH 6.71 ± 0.01 during 7 days. In this period, TNRR/Vs was lower than 207.20 ± 119.90kg m^{-3} d^{-1} m_s^{-3}. Nevertheless, in both cases the reactor was able to recover performance with TNRR/Vs higher than it was observed in the beginning of the test, at pH 7.09 ± 0.01. After such recovery, the reactor operated with TNRR/Vs above 512.90 kg m^{-3} d^{-1} m_s^{-3}.
4) The sludge granule reduced its size when exposed to pH 6.61 ± 0.01 for two days

5. CHAPTER IV – TEMPERATURE'S SHORT-TERM EFFECTS ON THE ANAMMOX® SYSTEM

5.1. Abstract

Experiences of Anammox process in full-scale has been usually applied in the wastewater treatment at temperatures above 30 °C in order to operate under optimum conditions. The maintenance of anammox and anaerobic ammonia-oxidizing bacteria (AOB) and suppression of nitrite-oxidizing bacteria (NOB) at low temperature are key issues to a stable operation of the reactor and satisfactory nitrogen removal. Application at lower temperatures would allow extending the employment at different scenarios with significant reduction of its operating costs. This study aimed to evaluate ANAMMOX® system at temperature between 36 and 25°C in short-term. Tested temperatures had no effect on reactor performance. Stepwise, lowering the temperature (36 – 25 °C) did not result in nitrate accumulation. Apparent activation energy (Ea) was 36 kJ mol^{-1} to temperatures between 36 - 29°C.

5.2. Introduction

Anammox process has explored part of the biological nitrogen cycle, which has not been completely understood (MOSTAFA, 2012). However, anammox reactors have already been widely installed around the world at full-scale. Wastewaters have been treated successfully and are characterized as having low carbon/nitrogen ratios, very high ammonium concentrations and temperature higher than 30°C (VANHULL *et al.*, 2010). The reactor operation at lower temperatures would allow extending the potential applications of anammox-related processes to different scenarios with significant reduction of operating costs (JETTEN *et al.,* 1999; KARTAL *et al*., 2007; SIEGRIST *et al*., 2008).

Studies have shown that anammox bacteria exists in different natural systems with depleted oxygen zones (BURGIN and HAMILTON, 2007) and available NO_x (NO_3^- and NO_2^-), including marine ecosystems (KUYPERS *et al.,* 2003; RYSGAARD *et al.,* 2004), freshwater ecosystems (ZHANG *et al.,* 2007), terrestrial ecosystems (HUMBERT *et al.,* 2010) and even sea ice (RYSGAARD *et al.,* 2008). Anammox activity can be found in natural systems at temperatures ranging from -2 to 85°C (RYSGAARD *et al.,* 2004; BYRNE *et al.,* 2009; JETTEN *et al.,* 2009; GAO and TAO, 2011) although several authors observed that the optimum temperature for the operation in wastewater treatment of the anammox process is between 30 and 40°C (STROUS *et al.,* 1999b; EGLI *et al.,* 2001; JIN *et al.,* 2013b).

Isaka *et al.* (2007) reported that a total nitrogen removal rate (TNRR) of 11.5 kg N m^{-3} d^{-1} was achieved at 37°C while the value of TNRR was 8.1 kg N m^{-3} d^{-1} when temperature was 20 – 22 °C. At low temperatures, the growth rate slows down and low activities of anammox bacteria and ammonium-oxidizing bacteria (AOB) decreases significantly the removal capacity (ISAKA *et al.*, 2007; VAZQUEZ-PADÍN *et al.*, 2009b). Furthermore, partial nitritation is harder to achieve at low temperatures since aerobic nitrite-oxidizers (NOB) are expected to have higher growth rate than AOB at 10 – 20°C (HELLINGA *et al.*, 1998).Cui (2012). It was verified that at temperature of 15°C, the TNRR decreased from 83% to 63%, anammox activity decreased by 27% and the same AOB remained active while NOB increased activity by 43%.

Despite such difficulty, several laboratory studies reported one-stage partial nitritation/anammox at temperatures lower than 25°C and reasonable removal of nitrogen (SZATKOWSKA *et al.*, 2007; VAZQUEZ-PADÍN *et al.*, 2011; WINKLER *et al.*, 2011; DE CLIPPELEIR *et al.*, 2013; HU *et al.*, 2013; LOTTI *et al.*, 2014). Dosta *et al.* (2008) reported that the anammox process could be successfully operated at 18°C. Hu *et al.* (2013) kept activity stable in long-term lab-scale SBR at 12°C and low concentration of ammonium. However, the state of the art is not developed enough to operate a full-scale reactor at low temperatures. In particular, little attention has been directed to the detailed response of the microbial community to temperature changes (JIN *et al.*, 2013a; PERSSON *et al.*, 2014).

This study aimed to evaluate short-term effects that temperatures between 25 - 36°C have on the stability of the ANAMMOX® reactor.

5.3. Material and Methods

5.3.1. Synthetic wastewater

The description of the influent is mentioned in section 3.3.1.

5.3.2. Experiment procedures, design and ANAMMOX®'s operation

Design, operation and start-up are more detailed at section 4.3.2. The reactor was monitored for 61 days in which the temperature was progressively reduced as shown in Table 5.1. The experiment was set to change the temperature every week. Due to operational problems, it was necessary to raise the temperature to 36°C in order to stabilize the reactor between 28 °C and 27 °C.

Table 5.1. Reactor operation strategy

Temperature	36°C	33°C	30°C	29°C	28°C	36°C	27°C	26°C	25°C
Period (days)	7	7	8	7	7	5	6	9	5

5.3.3. Analytical procedures

Concentrations (mg L^{-1}) of ammonium (NH_4^+-N), nitrite (NO_2^--N) and nitrate (NO_3^--N) of the influent and effluent were determined daily. The chemical oxygen demand (COD), the total suspended solids (TSS) and volatile suspended solids (VSS) were set for each change of temperature. The analyses follow the same procedures described in section 3.3.3.

The data analysis was performed by generalized linear model (GLM) with logarithmic link and gamma distribution to point the effects of different temperatures in the reactor efficiency in order to take into account the skewed distribution of these variables. For better interpretation, the coefficients obtained from the GLM were exponentiated, which represent the arithmetic mean ratios (AMR) in continuous outcome variables between the explanatory variables being compared.

5.3.4. Mathematical Models

The reactor was unable to effectively retain biomass during the test. Thereby the efficiency was evaluated on basis of total nitrogen removal rate (TNRR) and the volume of sludge (Vs) inside the reactor, measured daily. The Equation (7) describing TNRR/Vs is mentioned in section 4.3.4.

According to Hao *et al.* (2002). The dependence of a biological reaction on temperature was evaluate using the modified Arrhenius equation (equation 14)

$$Ln(TNRR) = \frac{-E_a}{RT} + LnA \tag{14}$$

Where TNRR is the average of total nitrogen removal rate per range evaluated kg m^{-3} d^{-1}; Ea is apparent activation energy J mol^{-1}; R is ideal gas constant, 8.314 J kg^{-1} K^{-1}; T is thermodynamic temperature K; and A refers to Arrhenius constant.

5.4. Results and discussion

Figure 5.1 describes TNRR/Vs' values found throughout the experiment. The mean was 515.2 kg m^{-3} d^{-1} m_s^{-3} and the interquartile range was of 441.1 - 595.9 kg m^{-3} d^{-1} m_s^{-3}. The maximum observed value was 720.2 kg m^{-3} d^{-1} m_s^{-3} and the minimum was 341.6 kg m^{-3} d^{-1} m_s^{-3}. The distribution of variable has asymmetric features. The curve represents the log transformation of the variable TNRR/Vs to correct the asymmetry and transform variables in normal distribution.

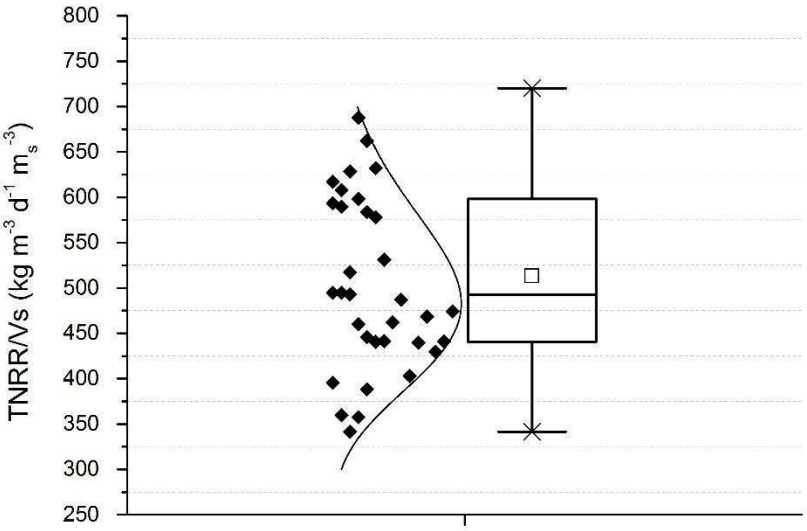

Figure 5.1- Boxplot of TNRR throughout the test and log non-normal distribution of data (n= 36).

The temperature reduction had no effect on the results of the TNRR/Vs observed (β_1= -0.0058, 95%CI: 5.906-6.931). The Spearman's correlation was -0.1451, i.e. inverse and weak. The ANAMMOX® reactor remained stable on all operating parameter. The nitrite and nitrate concentrations did not change significantly, leading us to affirm that the reduction on temperature of 36 °C to 25°C over 61 days had no inhibitory effect (Figure 5.2). Jin *et al.* (2013a) found a decrease of 33.5% on the anammox activity, working short-term on the maximum biomass specific activity when the temperature has changed from 30°C to 25°C. As for Hu *et al.* (2013), after reducing 5°C, reported no adverse effects as his study shows. Lackner *et al.* (2014) applied a survey on experience in full-scale partial nitration/anammox process. They reported that in 13 systems, temperature variations had small effect on process

performance. Only on one case did the high variations in temperature within a short-term (8°C within one week) resulted in a significant influence on reactor performance.

Overcapacity at this range of temperature could be explained by the fact that the sludge granular structure produces a temperature gradient that protects the anammox bacteria. Heylen *et al.* (2012) observed that with the protective role of the polymeric matrix present in aggregated cultures, anammox bacteria tolerance to cold was enhanced. Moreover, AOB have an optimal temperature of around 28°C (ALAWI *et al.*, 2007) and at 10°C to 15°C, NOB had a higher activity than AOB (YANG *et al.*, 2007) which explains the low accumulation of nitrite and nitrate in the present study.

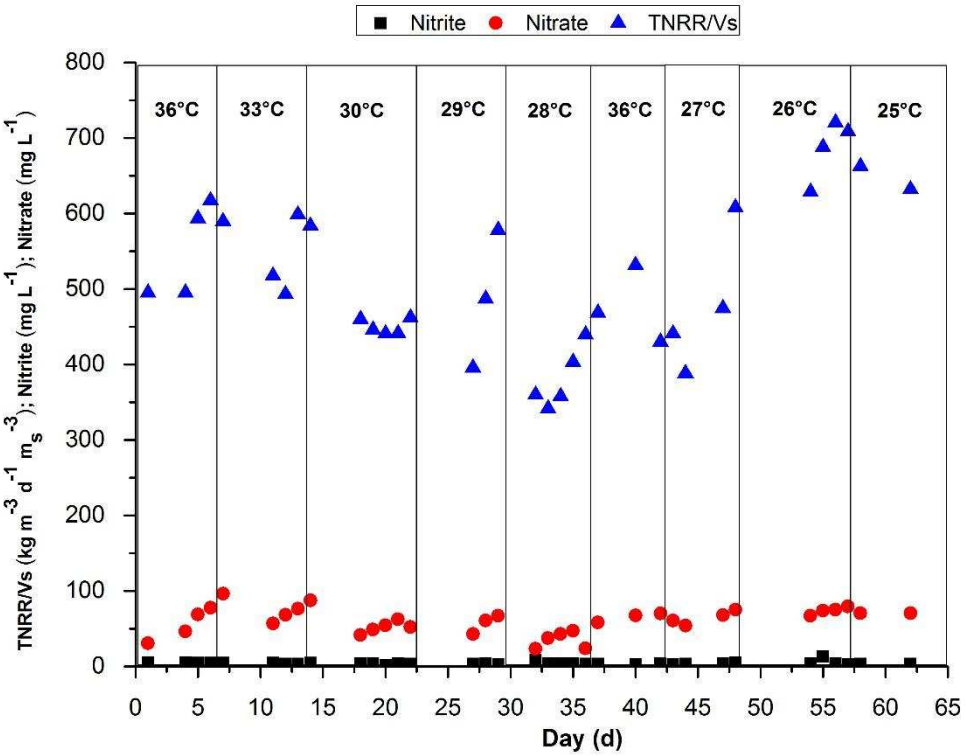

Figure 5.2- The performance of ANAMMOX® reactor during the test.

Despite the slow growth rates and low activities of anammox bacteria and AOB at temperature lower than 25°C, several laboratory studies have reported one-stage partial nitritation/anammox with reasonable removal of nitrogen (SZATKOWSKA *et al.*, 2007; DOSTA *et al.*, 2008; VAZQUEZ-PADÍN *et al.*, 2011; WINKLER *et al.*, 2011; DE

CLIPPELEIR *et al.*, 2013; HU *et al.*, 2013; LOTTI *et al.*, 2014). Dosta *et al.* (2008) reported that anammox process could be successfully operated at 18°C. Hu *et al.* (2013) kept stable activity in a lab-scale SBR in long-term at 12°C and low concentration of ammonium. Cema *et al*. (2007) also showed that satisfactory anammox activity was possible at the temperature around 20°C.

Unfortunately, this study lacked time to evaluate at long-term. However, the strategy of acclimatization seemed promising by some authors (VAZQUEZ-PADIN *et al.*, 2011; WINKLER *et al.*, 2011;HU *et al.* 2013; JIN *et al.*, 2013a; LOTTI *et al.*, 2014). Hu *et al.* (2013), after long-term cultivation at 12°C, were able to decrease the optimum temperature for anammox bacteria from 35 to 25°C. Nevertheless, most studies are limited to the use of low ammonium concentrations (<100 mg NH_4^+ - N loading rate) (VAZQUEZ-PADIN *et al.*, 2011; WINKLER *et al.*, 2011), rarely applied for wastewater with a NH_4^+−N content exceeding 500 mg N L^{-1} (HU *et al.*, 2013; LOTTI *et al.*, 2014).

The Ea was plot and calculated by modified Arrhenius equation (HAO *et al.*, 2002). Analysis was divided in two temperature ranges (36°C – 29°C and 28°C - 25°C), so it could have better linear fit (Figure 5.3). The range of 36°C – 29°C has a small slope, which means that kinetic reaction only varies slightly with the temperature, confirming previous analyses. While for the range of 28°C – 25°C, we observe an increase in efficiency when reducing temperature, fact characterized as a non-Arrhenius behavior. Therefore, it was not possible to establish the Ea. In addition, we cannot affirm that anammox process has a non-Arrhenius kinetic reaction. Due to the short period of analysis and the reduction only at one unit of temperature, the ANAMMOX® reactor could still be in the process of stabilization, being possible to observe different results of TNRR.

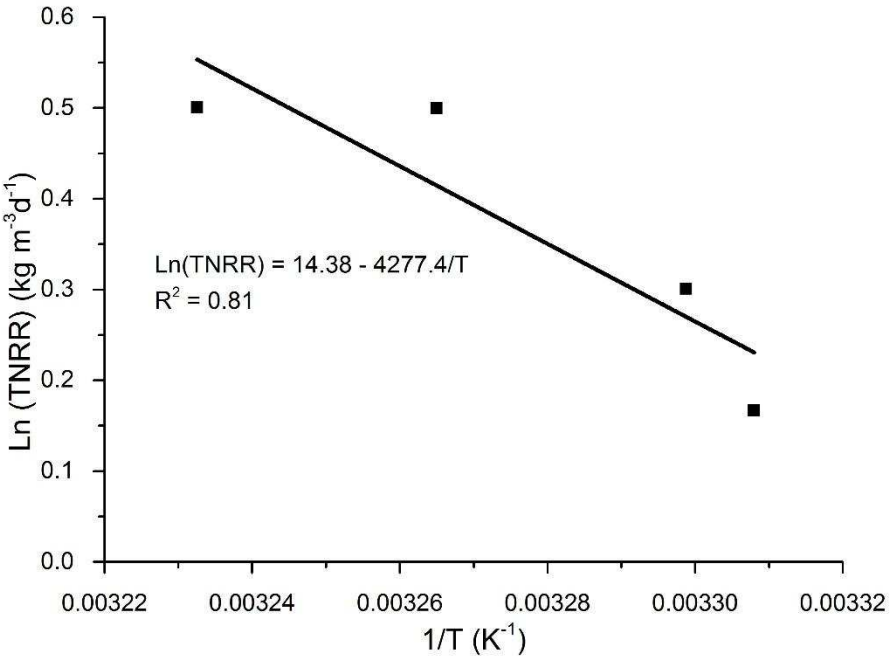

Figure 5.3- Arrhenius plot that provides relationship between TNRR and temperature (36 °C – 29°C).

Table 5.2 presents an overview of the values obtained in different studies and presented also in this study. The estimated Ea in this study was 36 kJ mol^{-1}, which is much lower than studies using granular biomass reported. Similar results were obtained by JETTEN, *et al.* (1999) and ISAKA *et al.* (2007). They reported a value of 43 and 33 kJ mol^{-1}, respectively. Both experiments of Dalsgaard and Thamdrup (2002) and Rysgaard *et al.*, (2004) evaluated biomass in marine sediments, reporting values of Ea closer to other studies assessed in mesophilic temperature. This is an indication that long periods of acclimatization and the oxygen limitation could be alternatives for reactor's operation at lower temperatures.

Table 5.2 - Overview of apparent activation energy of Anammox reaction (Ea).

Temperature Range (°C)	Biomass	Ea (kJ mol^{-1})	References
10 – 20	Granule	72	Hendrickx *et al.*, 2012
20 – 43	Aggregated	70	Strous *et al.*, 1999b
22 – 28	Gel carrier with entrapped anammox bacteria	93	Isaka *et al.*, 2007
28 – 37	Gel carrier with entrapped anammox bacteria	33	Isaka *et al.*, 2007
-2 – 13	Marine sediment	51	Rysgaard *et al.*, 2004
6.5 – 37	Marine sediment	61	Dalsgaard and Thamdrup, 2002
15 – 35	Granule	43	Jin *et al.*, 2013[a]
20 – 30	Granule	70	Jetten, *et al.*, 1999
15 – 30	Granule	66	Lotti *et al.*, 2014
36 – 29	Granule	36	This study

The Table 5.3 summarizes the ANAMMOX® reactor performance throughout the experiment.

Table 5.3 -Summary of the ANAMMOX® reactor performance throughout the experiment

Temperature	Period (days)	NH_4-N effluent (mg L^{-1})	NO_2-N effluent (mgL^{-1})	NO_3-N effluent (mgL^{-1})	V_s (l)	Average TNRR/V_s (kg m^{-3} d^{-1} m_s^{-3})
36.2 ± 0.1	7	95.7 ± 81.5	5.0 ± 0.5	55.8 ± 21.3	3.0	550.1 ± 64.5
33.1 ± 0.4	7	57.8 ± 38.4	3.9 ± 0.8	74.3 ± 16.7	3.0	549.5 ± 52.40
30.0 ± 0.1	8	207.0 ± 89.6	3.6 ± 1.2	59.0 ± 17.6	2.8	482.3 ± 68.0
29.2 ± 0.5	7	255.0 ± 100.8	3.1 ± 0.5	55.7 ± 10.5	2.5	472.7 ± 67.8
27.8 ± 0.3	7	363.6 ± 45.5	5.2 ± 2.5	37.7 ± 10.3	1.9	367.4 ± 31.8
35.6 ± 0.7	5	156.2 ± 54.4	3.2 ± 0.3	49.9 ± 23.0	3.0	433.3 ± 1.2
26.7 ± 0.5	6	231.0 ± 73.2	3.3 ± 0.5	63.0 ± 0.5	2.7	479.6 ± 0.2
26.1 ± 0.1	9	114.2 ± 55.0	5.7 ± 4.2	74.2 ± 4.4	2.4	686.2 ± 40.9
25.3 ± 0.2	5	115.1 ± 48.2	3.3 ± 0.2	70.5	2.3	647.8 ± 21.4

5.5. Conclusions

The evaluated temperature ranges in this study between 36 °C and 25 °C had no effect on the results on the TNRR/Vs of the reactor. Moreover, the stepwise lowering of the temperature (36 – 25°C) did not result in nitrate accumulation on the reactor which represents the control of NOB activity in acceptable levels of operation. The maximum value of TNRR/Vs was 720.2 kg m^{-3} d^{-1} m_s^{-3} and the Ea was 36 kJ mol^{-1} for temperatures of 36 - 29°C. Due to the period of less than 8 days for each temperature tested, it can be inferred that operational problems in reactors with temperature reduction up until 25 °C does not cause losses in removal efficiency.

6. Final Considerations

Throughout the experiments performed in this thesis and full-scale experiences reported in the literature, the ANAMMOX® technology with partial nitritation/anammox process was shown to be an efficient system with a series of advantages in terms of operating costs when compared to conventional treatment systems (nitrification/denitrification).

In this study, it was observed that the ANAMMOX® reactor is sensitive to small variations in pH. At pH values lower than 6.71 ± 0.01, it was observed substantial loss in nitrogen removal efficiencies. Moreover, the sludge reduced its size when exposed to pH 6.61 ± 0.01. When pH variations occurred simultaneously in the presence of sulfide, the reactor lost ammonium removal efficiency. After interruption of the sulfide addition and control of pH to 7.10 (optimum conditions), when it was the case that the pH was at 7.30 ± 0.19 and the sulfide concentration was at 0.25 mg L^{-1}, the loss of activity was irreversible. While the pH was at 6.88 ± 0.4 and 0.1 mg S L^{-1}, the reactor was able to recover, which demonstrates sensibility of the ANAMMOX® reactor for theses parameters but on the other hand showed ability to recover from toxic shock.

In terms of temperature reduction, anammox process could be operated at temperatures lower than 36 °C without losses in removal efficiency and maintenance of anammox and anaerobic ammonia-oxidizing bacteria (AOB) with suppression of nitrite-oxidizing bacteria (NOB). The experimental work performed during the temperature test had no effect on the results of TNRR/Vs at temperatures between 36 and 25 °C. The results found in this research are summarized in Table 6.1.

Table 6.1- Operational window of Anammox process for the parameters tested.

Parameter		Effect	Recovery
pH range	7.10 – 6.80	No inhibition	-
	6.70	Inhibition	Yes
	6.60	Inhibition	Yes
Temperature range	36 – 25 °C	No inhibition	-
Sulfide concentration	0.1 mg S L^{-1}	Inhibition	Yes
	0.25 mg S L^{-1}	Inhibition	No

[a]: When the pH was 6.88 ± 0.4, [b]: Under pH at 7.30 ± 0.2.

For future research, it is recommended the evaluation of parameters, sulfide, pH and temperature in long-term. Therefore, it is the improvement in methodologies of solubility control of sulfide and experiments to verify the reactor performance at low temperature and under acidic conditions simultaneously. The application of anammox technology at low temperatures and low pH have major impact in the treatment of cold wastewater and low alkalinity.

REFERENCES

ABELING, U., SEYFRIED, C. F. **Anaerobic–aerobic treatment of high strength ammonium wastewater nitrogen removal via nitrite.** *Water Science and Technology*, v. 26, p. 1007–1015, 1992.

ABELIOVICH, A. **Nitrifying bacteria in wastewater reservoirs.** *Applied & Environmental Microbiology*, v. 53, p. 754-760, 1987.

ABDMA, W. R., SCHULTZ, C. E., WOUTERS, J. W., MULDER, J. W., VAN LOOSDRECHT, M. C. M., VAN DER STAR, W., STOUS, M., TOKUTOMI, T. **Full scale granular sludge ANAMMOX® process.** In: 4th Charted Institution of Water and Environmental Management Annual Conference**,** 2006. New Castle. Proceedings. 2006.

AHN, Y. H. **Sustainable nitrogen elimination biotechnologies: A review.** *Process Biochemistry*, v. 41, p. 1709-1721, 2006.

ALAWI, M., LIPSKI A., SANDERS, T., PFEIFFER, E. M., SPIECK, E. **Cultivation of a novel cold-adapted nitrite oxidizing betaproteo bacterium from the Siberian Arctic.** *ISME J.* v.1, p.256–264, 2007

ANTHONISEN, A. C. **Inhibition of nitrification by ammonia and nitrous acid.** *J.Water Pollut. Control Fed.*, v. 48, n. 5, p. 835–852, 1976.

ARAÚJO, J. C., CAMPOS, A. P., CORREA, M. M. S, SILVA, E. C., VON SPERLING, M., CHERNICHARRO, C. A. L. **Enriquecimento de bactérias anaeróbias oxidadoras de amônia - Anammox.** *Engenharia Sanitária e Ambiental*, 15, p. 205-212, 2010.

AKTAN, C. K., YAPSAKLI, K., MERTOGLU, B. **Inhibitory effects of free ammonia on Anammox bacteria.** *Biodegradation*, v.3, p.751–762, 2012.

BECCARI, M., PASSINO, R., RAMADORI, R., TANDOI, V. **Inhibitory effect on nitrification by typical compounds in coke plant wastewaters.** *Environmental Techonology Letters*, v.1, n. 5, p. 245-252, 1980.

BARNES, D., BLISS, P. J. *Biological control of nitrogen in wastewater treatment.* London: E. & F.N., 1983.

BERTINO, A. **Study on One-Stage partial Nitritation-Anammox process in Moving Bed Biofilm Reactors: a Sustainable Nitrogen Removal.** Stockholm/Torino, 2010, 193p. Dissertação (Mestrado). Politecnico di Torino in cooperation with Department of Sustainable Development, Environmental science and Engineering, Royal Institute of Technology (KTH), Stockholm/Torino, 2010.

BERISTAIN-CARDOSO, R., GÓMEZ, J., MÉNDEZ-PAMPÍN, R. **The behavior of nitrifying sludge in presence of sulfur compounds using a floating biofilm reactor.** *Bioresource Technology*, v.101, n.22, p.8593–8598, 2010.

BRODA, E. **Two kinds of lithotrophs missing in nature.** *Z AllgMikrobiol*, v. 17, p. 491-493, 1977.

BURGIN, A. J., HAMILTON, S. K. **Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways.** *Front. Ecol. Environ.* v.5, p.89–96, 2007.

BYRNE, N., STROUS, M., CREPEAU, V., KARTAL, B., BIRRIEN, J. L., SCHMID, M., LESONGEUR, F., SCHOUTEN, S., JAESCHKE, A., JETTEN, M., PRIEUR, D., GODFROY, A. **Presence and activity of anaerobic ammonium oxidizing bacteria at deep-sea hydrothermal vents.** *ISME J*.v.3, p.117–123, 2009.

CASAGRANDE, C. G., KUNZ, A., DE PRÁ, M. C., BRESSAN, C. R., SOARES, H. M. **High nitrogen removal rate using ANAMMOX process at short hydraulic retention time**. *Water Science & Technology*, v. 67, n. 5, p. 968-975, 2013.

CEMA, G., WISZNIOWSKI, J., ZABCZYNSKI, S., ZABLOCKA-GODLEWSKA, E., RASZKA, A., SURMACZ-GÓRSKA, J. **Biological nitrogen removal from landfill leachate by deammonification assisted by heterotrophic denitrification in a rotating biological contactor (RBC).** *Water Sci. Technol.*v.55, p.35–42, 2007.

CHEIS, D. Remoção de nutrientes, como fósforo e nitrogênio, no tratamento de esgotos. Disponível em: < http://www.revistatae.com.br/noticiaInt.asp?id=7454>. Acesso em 18 março 2015.

CHEN, J.W., JI, Q. X., ZHENG, P., CHEN, T. T., WANG, C. H., MAHMOOD, Q. **Floatation and control of granular sludge in a high-rate anammox reactor.** *Water Research*, v.44, p.3321-3328, 2010.

CHEN, T. T., ZHENG, P., SHEN, L. D., DING, S., MAHMOOD, Q. **Kinetic characteristics and microbial community of Anammox-EGSB reactor.** *J. Hazard. Mater*.v.190, p.28–35, 2011.

CUI, F. **Cold CANON: Anammox at low temperatures**. Delft, 2012, 118 p. Dissertação (Mestrado). Sanitary Engineering Section, Department of Water Management. Faculty of Civil Engineering and Geosciences. Delft University of Technology, Delft, 2012.

DALSGAARD, T., THAMDRUP, B. **Factors controlling anaerobic ammonium oxidation with nitrite in marine sediments.** *Appl. Environ. Microbiol.*v.68, n.8, p.3802–3808, 2002.

DAPENA-MORA, A., CAMPOS, J. L., MOSQUERA-CORRAL, A., MÉNDEZ, R. **Anammox process for nitrogen removal from anaerobically digested fish canning effluents.** *Water Science & Technology*, v. 53, n.12, p. 265–274, 2006.

DAPENA-MORA, A.; FERNANDEZ, I.; CAMPOS, J. L.; MOSQUERA-CORRAL, A.; MÉNDEZ, R.; JETTEN, M. S. M. **Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production.** *Enzyme Microb. Technol.* v. 40, n.4, p.859–865, 2007.

DE CLIPPELEIR, H., VLAEMINCK, S.E., DE WILDE, F., DAENINCK, K., MOSQUERA, M., BOECKX, P., VERSTRAETE, W., BOON, N. **One-stage partial nitritation/anammox at 15°C on pretreated sewage: feasibility demonstration at lab-scale.** *Appl. Microbiol. Biotechnol.* v. 97, n. 23, p. 10199-10210, 2013.

DOSTA, J., FERNÁNDEZ, I., VÁZQUEZ-PADÍN, J. R., MOSQUERA-CORRAL, A., CAMPOS, J.L., MATA-ÁLVAREZ, J., MÉNDEZ, R. **Short- and long-term effects of temperature on the Anammox process.** *J. Hazard. Mater.* v.154, p.688–693, 2008.

EGLI, K., FANGER, U., ALVAREZ, P. J. J. **Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate.** *Arch. Microbiol.* v.175, p.198–207, 2001.

FERNÁNDEZ, I., DOSTA, J., FAJARDO, C., CAMPOS, J. L., MOSQUERA-CORRAL, A., MÉNDEZ R. S**hort- and long-term effects of ammonium and nitrite on the Anammox process.** *Journal of Environmental Management*, v. 95, p. 170–174, 2012.

FURUKAWA, K., INATOMI, Y., QIAO, S., QUAN, L., YAMAMOTO, T., ISAKA, K., SUMINO, T. **Innovative treatment system for digester liquor using anammox process.** *Bioresource Technology*, v. 100, p. 5437-5443, 2006.

GALLERT, C., WINTER, J. **Mesophilic and thermophilic anaerobic digestion of source sorted organic wastes: effect of ammonia on glucose degradation and methane production.** *Appl Microbiol Biot*, n.48, v.3, p.405–410, 1997.

GALLOWAY, J. N., **Nitrogen cycles: past, present, and future**; *Biogeochemistry*, n.70: p.153–226, 2004

GAO, D. W., TAO, Y. **Versatility and application of anaerobic ammonium oxidizing bacteria**. *Appl. Microbiol. Biotechnol.*v.91, n.4, p.887–894, 2011.

GRONTMIJ, N.V. **Sharon: Nitrogen Removal over Nitrite.** Disponível em: <http://www.grontmij.com/highlights/water-and-energy/Documents/SHARON-Nitrogen-Removal-over-Nitrite.pdf>. Acesso 23 de março 2015

GRUNDITZ, C., DALHAMMAR, G. **Development of nitrification inhibition assays using pure cultures of Nitrosomonas and Nitrobacter.** *Water Research*, v.35, p.433-440, 2001.

HAO, X., HEIJNEN, J. J., VAN LOOSDRECHT, M. C. M. **Model-based evaluation of temperature and inflow variations on a partial nitrification-ANAMMOX biofilm process.** *Water Res.*, v.36, p.4839–4849, 2002.

HANAKI, K., WANTAWIN, C., OHGAKI, S. **Nitrification at low-levels of dissolved-oxygen with and without organic loading in a suspended-growth reactor.** *Water Research*, v. 24, p. 297-302, 1990

HENDRICKX, T. L. G., WANG, Y., KAMPMAN, C.; ZEEMAN, G., TEMMINK, H., BUISMAN, C. J. N. **Autotrophic nitrogen removal from low strength waste water at low temperature.** *Water Res*. v.46, p. 2187–2193, 2012.

HELLINGA, C., SCHELLEN, A. A. J. C., MULDER, J. W.; VAN LOOSDRECHT, M. C. M., HEIJNEN, J. J. **The SHARON process: an innovative method for nitrogen removal from ammonium-rich wastewater.** *Wat. Sci. Tech.*v.37, n.9, p.135–142, 1998.

HEYLEN, K., ETTWIG, K., HU, Z.Y., JETTEN, M., KARTAL, B. **Rapid and simple cryopreservation of anaerobic ammonium-oxidizing bacteria**. *Appl. Environ. Microbiol.* n.78, p.3010–3013, 2012

HU, Z., LOTTI, T., DE KREUK, M., KLEEREBEZEM, R., VAN LOOSDRECHT, M. C. M., KRUIT, J., JETTEN, M., KARTAL, B. **Nitrogen removal by a nitritation-anammox bioreactor at low temperature.** *Appl. Environ. Microbiol.* v.79, n.8, p.2807-2812, 2013.

HUMBERT, S., TARNAWSKI, S., FROMIN, N., MALLET, M .P., ARAGNO, M., ZOPFI, J. **Molecular detection of Anammox bacteria in terrestrial ecosystems: distribution and diversity.** *ISME J.* v.4, p.450–454, 2010.

ISAKA, K., DATE, Y., SUMINO, T., YOSHIE, S., TSUNEDA, S. **Characteristic of anaerobic ammonium-oxidizing bacteria in an anaerobic biological filtrated reactor.** *Biotechnological Products and Process Engineering*, v. 70, p. 47-52, 2006.

ISAKA, K., SUMINO, T., TSUNEDA, S. **High nitrogen removal performance at moderately low temperature utilizing anaerobic ammonium oxidation reactions.** *J. Biosci. Bioeng.*v.103, n.5, p.486–490, 2007.

JAROSZYNSKI, L. W., CICEK, N., SPARLING, R., OLESZKIEWICZ, J. A. **Importance of the operating pH in maintaining the stability of anoxic ammonium oxidation (Anammox) activity in moving bed biofilm reactors.** *Bioresour. Technol.* v.102, n.14, p.7051–7056, 2011.

JETTEN, M., STROUS, M., VAN DE PAS-SCHOONEN, K. T., SCHALK, J., VAN DONGEN, L. G. J. M., VAN DE GRAAF, A. A., LOGEMAN N. S.; MUYZER, G.; VAN LOOSDRECHT, M. C. M., KUENEN, J. G. **The anaerobic oxidation of ammonium.** *FEMS Microbiol Rev*, n.22, p.421–437, 1999.

JETTEN, M., CIRPUS, I., KARTAL, B., VAN NIFTRIK, L., VAN DE PAS-SCHOONENK, T., SLIEKERS, O., HAAIJER, S., VAN DER STAR, W. R .L., SCHMID, M., VAN DE VOSSENBERG, J., SCHMID, T. I., HARHANGI, H., VAN LOOSDRECHT, M., GIJS KUENEN, J., OP DEN CAMP, H., STROUS, M. **1994–2004:10 years of research on the anaerobic oxidation of ammonium.** *Biochemical Society Transactions*, v. 33, n. 1, p. 119–123, 2005.

JETTEN, M., VAN NIFTRIK, L., STROUS, M., KARTAL, B., KELTJENS, J. T., DEN CAMP, H. J. M. O. **Biochemistry and molecular biology of anammox bacteria.** Critical Reviews in *Biochemistry and Molecular Biology*, v.44, p. 65-84, 2009.

JETTEN, M., WAGNER, M., FUERST, J., VAN LOOSDRECHT, M. C. M., KUENEN; J.G., STROUS; M. **Microbiology and application of the anaerobic ammonium oxidation (anammox) process.** *Curr. Opin. Biotechnol*.v.12, p.283-288, 2001.

JIANG, X., LUO, Y., YAN, R., TAY, J. H. **Impact of substrates acclimation strategy on simultaneous biodegradation of hydrogen sulfide and ammonia.** *Bioresour. Technol.,* v.100, p.5707–5713, 2009.

JIN, R. C., YANG, G. F., YU, J. J., ZHENG, P. **The inhibition of the Anammox process: A review.** *Chemical Engineering Journal*, v. 197, p. 67-79, 2012.

JIN, R. C., MA, C., YU, J. J. **Performance of an Anammox UASB reactor at high load and low ambient temperature.** *Chem. Eng. J*. v.232, p. 7-25, 2013a.

JIN, R.C., YANG, G. F., ZHANG, Q. Q., MA, C., YU, J. J., XING, B. S. **The effect of sulfide inhibition on the ANAMMOX process.** *Water Res.* v.47, p.1459–1469, 2013b.

JOSS, A., SALZGEBER, D., EUGSTER, J., KONIG, R., ROTTERMANN, K., BURGER, S., FABIJAN, P., LEUMANN, S., MOHN, J., SIEGRIST, H. **Full-scale nitrogen removal from digester liquid with partial nitritation and Anammox in one SBR.** *Environmental Science & Technology*, v. 43, n. 14,p. 5301–5306, 2009.

JUNG, J. Y., KANG, S. H., CHUNG, Y. C., AHN, D. H. **Factors affecting the activity of anammox bacteria during start up in the continuous culture reactor**. *Water Science &Technology*. v. 55, p.459-468, 2007.

KARTAL, B., RATTRAY, J., VAN NIFTRIK, L., VAN DE VOSSENBERG, J., SCHMID, M., WEBB, R., SCHOUTEN, S., FUERST, J., DAMSTE ´, J., JETTEN, M., STROUS, M. **Candidatus "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria.** *Syst. Appl. Microbiol.* v.30, p.39-49, 2007.

KIPERSTO, K A., NASCIMENTO, F. R. A., KIPERSTOK, A. C. **O tratamento em separado da urina e das fezes é uma solução viável ou uma utopia?** *Revista DAE*, v.183. 2010.

KUAI, L., VERSTRAETE, W. **Ammonium removal by Oxigen-Limited Autotrophic Nitrification-Denitrification system. Applied and Environmental**. *Microbiology*, v. 64, p. 4500- 4506, 1998.

KUENEN, J .G., JETTEN, M. **Extraordinary anaerobic ammonia-oxidizing bacteria.** *ASM News*, v.67, p.456-463, 2001

KUENEN, J. G. **Anammox bacteria: From discovery to application.** *Nature Reviews Microbiology*, v.6, n. 4, p. 320-326, 2008.

KUNZ, A., STEINMETZ, R., VANOTTI, A. S., SOARES, H. M. **Development of a New Generation Low Cost Treatment of Ammonia for Livestock Effluents Using Anammox and Nitritation.** In: *13th Ramiran International Conference*, 2008, Albena. Proceedings. Albena: FA. p.255-258, 2008.

KUNZ, A.,VANOTTI, M., SZOGI, A., GONZALES, M. C. G., NETO, G. F.S., SOARES H. M. **Development of Anammox process for animal waste treatment: Experiences in Brazil.** In: *International Symposium on Air Quality and Waste Management for Agriculture*, 2007, Broomfield. Proceedings. Broomfield: ASABE, 2007.

KUYPERS, M. M. M., SLIEKERS, A. O., LAVIK, G., SCHMID, M., JORGENSEN, B. B., KUENEN, J. G., DAMSTE, J. S. S., STROUS, M., JETTEN, M. **Anaerobic ammonium oxidation by Anammox bacteria in the Black Sea**, *Nature*, v.422,p.608–611, 2003.

LACKNER, S., GILBERT, E. M., VLAEMINCK, S. E., JOSS, A., HORN, H., VAN LOOSDRECHT, M. C. M. **Full-scale partial nitritation/anammox experiences – An application survey.** *Water Research*, v. 55, p. 292-303, 2014.

LANGONE, M., YAN, J., HAAIJER, S. C. M., OP DEN CAMP, H J. M., JETTEN, M S. M., ANDREOTTOLA, G. **Coexistence of nitrifying, anammox and denitrifying bacteria in a sequencing batch reactor.** *Frontiers in Microbiology*. v.5, n.28, p. 1-12, 2014.

LENS, P. N. L., VISSER, A., JANSSEN, A. J. H., POL, L. W. H., LETTINGA, G. **Biotechnological treatment of sulfate-rich wastewaters.** E*nvironmental Science and Technology*, v.28, p.41-88, 1998.

LOTTI, T., KLEEREBEZEM, R., VAN ERP TAALMAN KIP, C., HENDRICKX, T. L. G.; KRUIT, J.; VAN LOOSDRECHT, M. C. M. **Anammox growth on pretreated municipal wastewater.** *Environ. Sci. Technol.* v.112, n.1, p.98-103, 2014.

LU, W., DU, J., SCHWARZER, N. J., WACKER, T., ANDRADE, S. L. A., EINSLE, O. **The formate/nitrite transporter family of anion channels.** *Biol. Chem.* v.394, n.6, p.715–727, 2013.

MA, T., MA, H. R., DU, Z. P., JIA, W. **Sulfide distribution and toxicity on anaerobic treatment of tannery wastewater.** *Environmental Chemistry*, v.24, p.550-553, 2005.

MORSE, J.W., MILLERO, F.J., CORNWELL, J.C., RICKARD, D. **The chemistry of the hydrogen sulfide and iron sulfide systems in natural waters.** *Earth-Science Reviews*, v.24, p.1-42, 2002.

MOSTAFA, M.G. **Recent Advances in Anammox Bioreactors for Industrial Wastewater Reuse.** *J. Pet. Environ. Biotechnol*, v.3, p.108, 2012.

MOSQUERA-CORRAL, A., GONZALEZ, F,; CAMPOS, J.L., MÉNDEZ, R. **Partial nitrification in a SHARON reactor in the presence of salt and organic carbon compounds.** *Process Biochem.* v.40, n.9, p.3109–3118, 2005.

MULDER A., VAN DE GRAAF, A. A., ROBERTSON, L. A., KUENEN, J. G. **Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor.** *FEMS Microbiol*. Ecol., 16, p. 177–184, 1995.

OSHIKI, M., SHIMOKAWA, M., FUJII, N., SATOH, H., OKABE, S. **Physiological characteristics of the anaerobic ammonium-oxidizing bacterium 'CandidatusBrocadiasinica'**, *Microbiology* v.157,p.1706–1713, 2011.

PAQUES B.V. **Presença Global ANAMMOX**. Disponível em: <http://br.paques.nl/produtos/featured/anammox>. Acesso em 18 de março 2015.

PEREIRA, A. D. **Caracterização microbiana e avaliação do Efeito do fenol em um reator em batelada Sequencial com atividade Anammox**. Belo Horizonte, 2013, 128 p. Dissertação (Mestrado). Escola de Engenharia, Universidade Federal de Minas Gerais, Belo Horizonte, 2013.

PERSSON, F., SULTANA, R., SUAREZ, M., HERMANSSON, M., PLAZA, E., WILÉN, B.M. **Structure and composition of biofilm communities in a moving bed biofilm reactor for nitritation–anammox at low temperatures.** *Bioresource Technology*, v.154, p.267-273, 2014.

REGINATTO, V., TEIXEIRA, R. M., PEREIRA, F., SCHMIDELL, W., FURIGO, J. R. A., MENES, R., ETCHEBEHERE, C., SOARES, H. M. **Anaerobic ammonium oxidation in a bioreactor treating slaughterhouse wastewater.** *Brazilian Journal of Chemical Engineering*, v. 22, p. 593-600, 2005.

_____.**Resolução nº 430, de 13 de maio de 2011**. Dispõe sobre as condições de lançamento de efluentes, complementa e altera a Resolução n° 357, de 17 de março de 2005, do Conselho Nacional do Meio Ambiente – CONAMA. 9 p. Disponível em: <http://www.legislacao.mutua.com.br/pdf/diversos_normativos/conama/2011_CONAMA_RES_430.pdf>. Acesso em: 18 fev. 2015.

RYSGAARD, S., GLUD, R. N., RISGAARD-PETERSEN, N., DALSGAARD, T. **Denitrification and anammox activity in Arctic marine sediments**. *Limnology and Oceanography*. v. 9, n.5, p. 493-1502, 2004.

RYSGAARD, S., GLUD, R.N., SEJR, M. K., BLICHER, M. E., STAHL, H. J. **Denitrification activity and oxygen dynamics in Arctic sea ice.** *Polar Biol.* v.31, n.5, p.527–537, 2008.

SCHEEREN, M. B., KUNZ, A., STEINMETZ, R. L. R., DRESSLER, V. L. **O processo ANAMMOX como alternativa para tratamento de águas residuárias, contendo alta concentração de nitrogênio.** *Revista Brasileira de Engenharia Agrícola e Ambiental*, v. 15, n. 12, p. 1289-1297, 2011.

SCHMID, M., WALSH, K., WEBB, R., IRENE, W., RIJPSTRA, C., VAN DE PAS-SCHOONEN, K., VERBRUG-GEN, M. J., HILL T., MOFFETT, B., FUERST, J., SCHOUTEN, S., DAMSTÉ, J. S. S., HARRIS, J., SHAW, P., JETTEN, M., STROUS, M. **Candidatus "Scalinduabrodae," sp. nov., Candidatus "Scalinduawagneri," sp. nov. Two New Species of Anaerobic Ammonium Oxidizing Bacteria.** *Syst Appl Microbiol,* v. 26, p. 529–538, 2003.

SCHMID, M. C., MAAS, B., DAPENA, A., DE, K.; VAN VOSSENBERG, J., VAN DE KARTAL, B., NIFTRIK, L., VAN SCHMIDT, I., CIRPUS, I., GIJS, J., WAGNER, M., DAMSTE, J. S. S., KUYPERS, M. M. M.; REVSBECH, N. P., MENDEZ, R.; JETTEN, M. S. M., STROUS, M., PAS-SCHOONEN, K. **MINIREVIEW: Biomarkers for In Situ Detection of Anaerobic Ammonium-Oxidizing (Anammox) Bacteria.** *Appl. Env. Microbiol*., v.71, n.4, p.1677-1684, 2005.

SIEGRIST, H., SALZGEBER, D., EUGSTER, J., JOSS, A. **Anammox brings WWTP closer to energy autarky due to increased biogas production and reduced aeration energy for N-removal.** *Water Sci. Technol.* v.57, n.3, p.383-388, 2008.

SEARS, K., ALLEMAN, J. E.; BARNARD, J. L., OLESZKIEWICZ, J.A. **Impacts of reduced sulfur components on active and resting ammonia oxidizers.** *J. Ind. Microbiol. Biotechnol.*, v.31, n.8, p.369–378, 2004.

SPEECE, R.E. **Anaerobic biotechnology for industrial wastewaters.** *Archae Press*, v. 22, p.25-68, 1996

STROUS, M., VAN GERVEN E., KUENEN J. G., JETTEN M. **Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (Anammox) sludge**. *Appl. Environ. Microbiol.,* v. 63, p. 2446–2448, 1997.

STROUS, M., HEIJNEN, J. J., KUENEN, J. G., JETTEN, M. **The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms.** *Appl. Microbiol. Biotechnol.*, v. 50,n. 5,p. 589–596, 1998.

STROUS, M., FUERST, J. A., KRAMER, E. H. M., LOGEMANN, S., MUYZER, G., VAN DE PAS-SCHOONEN, K. T., WEBB, R., KUENEN, J. G., JETTEN, M. **Missing lithotroph identified as new planctomycete.** *Nature*, v. 400, p. 446-449, 1999a.

STROUS, M., KUENEN, J. G, JETTEN, M. **Key physiology of anaerobic ammonium oxidation.** *Applied Environmental Microbiology*, v.65, n.7, p.3248-3250, 1999b.

STROUS, M., PELLETIER, E., MANGENOT, S., RATTEI, T., LEHNER, A., TAYLOR, M.W., HORN, M., DAIMS, H., BARTOL-MAVEL, D., WINCKER, P., BARBE, V., FONKNECHTEN, N., VALLENET, D., SEGURENS, B., SCHENOWITZ-TRUONG, C., MEDIGUE, C., COLLINGRO, A., SNEL, B., DUTILH, B.E., DEN CAMP, H.J.M.O.,VAR DER DRIFT, C., CIRPUS, I., VAN DE PAS-SCHOONEN, K.T., HARHANGI, H.R., van NIFTRIK, L., SCHMID, M., KELTJENS, J., VAN DE VOSSENBERG, J., KARTAL, B., MEIER, H., FRISHMAN, D., HUYNEN, M.A., MEWES, H., WEISSENBACH, J., JETTEN, M.S.M., WAGNER, M., LE PASLIER, D. **Deciphering the evolution and metabolism of an anammox bacterium from a community genome.** *Nature*,v.440, p. 790-794, 2006.

SUDARNO, U., WINTER, J., GALLERT, C. **Effect of varying salinity, temperature, ammonia and nitrous acid concentrations on nitrification of saline wastewater in fixed-bed reactors.** *Bioresour. Technol.* v.102, p.5665–5673, 2010.

SULTANA, R. **Partial nitritation/anammox process in a moving bed biofilm reactor operated at low temperatures.** Stockholm, 2014, 33p. Dissertação (Mestrado). Department of Sustainable Development, Environmental science and Engineering, Royal Institute of Technology (KTH), Stockholm, 2014.

SZATKOWSKA, B., CEMA, G., PLAZA, E., TRELA, J., HULTMAN, B. **A one-stage system with partial nitritation and Anammox processes in the moving-bed biofilm reactor.** *Water Sci. Technol.* v.55, p.19-26, 2007.

TANG, C. J., ZHENG, P., HU, B. L., CHEN, J. W., WANG, C. H. I**nfluence of substrates on nitrogen removal performance and microbiology of anaerobic ammonium oxidation by operating two UASB re actors fed with different substrate levels.** *J. Hazard. Mater*, v.181, p.19–26, 2010a

TANG, C. J., ZHENG, P., MAHMOOD, Q., CHEN, J. W. **Effect of substrate concentration on stability of Anammox biofilm reactors**. *J. Central South Univ. Technol*.v.17 p.79–84, 2010b

TANG, C. J., ZHEN, G P., WANG, C. H., MAHMOOD, Q., ZHANG, J.Q., CHEN, X. G., ZHANG L., CHEN J. W. **Performance of high loaded ANAMMOX UASB reactors containing granular sludge.** *Water Res*., v. 45, n. 1, p. 135–144, 2011.

THAMDRUP, B., DALSGAARD, T. **Production of N_2 through anaerobic ammonia oxidation coupled to nitrate reduction in marine sediments.** *Appl. and Environ. Microbiol*., v. 68, n. 3, p. 1312–1318, 2002.

THIRD, K. A., SLIEKERS, A. O., KUENEN, J. G., JETTEN, M. **The CANON system (Completely Autotrophic Nitrogen-Removal Over Nitrite) under ammonium limitation: interaction and competition between three groups of bacteria.** *Systematic and Applied Microbiology*, v. 2001, p. 588-596, 2001.

TURSMAN, J. F., CORK, D. J. **Influence of sulfate and sulfate-reducing bacteria on anaerobic digestion technology.** *Biological Waste Treatment.*, v.22, n.2, p.1-26, 1996.

VAN DONGEN, U., JETTEN, M., VAN LOOSDRECHT, M. C. M. **The SHARON–Anammox process for treatment of ammonium rich wastewater.** *Water Science & Technology*. v. 44, p. 153–160, 2001.

VAN DE GRAAF, A. A., MULDER, A., DE BRUIJN, P., JETTEN, M., ROBERTSON, L. A., KUENEN, J. G. **Anaerobic oxidation of ammonium is a biologically mediated process.** *Appl. Environ. Microbiol.* v. 61, p. 1246–1251, 1995.

VAN DE GRAAF, A. A., DE BRUIJN P., ROBERTSON, L. A., JETTEN, M., KUENEN, J. G. **Autotrophic growth of anaerobic ammonium-oxidizing microorganisms in a fluidized bed reactor.** *Microbiology*, v. 142, p. 2187-2196, 1996.

VAN DE GRAAF, A. A., BRUJIN, P., ROBERTSON, L. A., JETTEN M., KUENEN, J. G. **Metabolic pathway of anaerobic ammonium oxidation on the basis of 15N studies in a fluidized bed reactor.** *Microbiology*, v. 143, p.2415-2421, 1997.

VAN DE VOSSENBERG, J., WOEBKEN, D., MAALCKE, W. J., WESSELS, H. J. C. T., DUTILH, B. E., KARTAL, B., JANSSEN-MEGENS, E. M., ROESELERS, G., YAN, J., SPETH, D., GLOERICH, J., GEERTS, W.,VAN DER BIEZEN, E., PLUK, W., FRANCOIJS, K. J., RUSS, L., LAM, P., MALFATTI, S. A., TRINGE, S. G., HAAIJER, S. C. M.,OP DEN CAMP,H. J. M., STUNNENBERG, H.G., AMANN, R., KUYPERS, M. M. M., JETTEN, M. **The metagenome of the marine anammox bacterium 'Candidatus Scalindua profunda' illustrates the versatility of this globally important nitrogen cycle bacterium.** *Environ. Microbiol.*, v.15, n.5, p.1275–1289, 2012.

VAN DER STARW, R. L., ABMAW, R., BOLMMERS, D., MULDER, J. W., TOKUTOMI, T., STROUS, M., PICIOREANU, C., VAN LOOSDRECHT, M. C. M. **Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale Anammox reactor in Rotterdam.** *Water Res.*, v. 41, n. 18, p. 4149–4163, 2007.

VAN HULLES, W. H., VAN DE WEYER, H. J. P., MEESSCHAERT, B. D., VAN ROLLEGHEM, P. A., DEJANS, P., DUMOULIN, A.. **Engineering aspects and practical application of autotrophic nitrogen removal from nitrogen rich streams.** *Chemical Engineering Journal*, v. 162, p. 1–20, 2010.

VAZQUEZ-PADÍN, J., POZO, M. J., JARPA, M., FIGUEROA, M., FRANCO, A., MOSQUERA-CORRAL, A., CAMPOS, J. L., MÉNDEZ, R. **Treatment of anaerobic sludge digester effluents by the CANON process in an air pulsing SBR.** *Journal of Hazardous Materials*, v. 166, p. 336-341, 2009a.

VAZQUEZ-PADÍN, J. R., FERNANDEZ, I., FIGUEROA, M., MOSQUERA-CORRAL, A., CAMPOS, J. L., MENDEZ, R. **Applications of Anammox based processes to treat anaerobic digester supernatant at room temperature.** *Bioresour. Technol.*, v.100, p.2988-2994, 2009b.

VÁZQUEZ-PADÍN J. R, FERNÁNDEZ I., MORALES N., CAMPOS J. L., MOSQUERA-CORRAL A., MÉNDEZ R. **Autotrophic nitrogen removal at low temperature.** *Water Sci. Technol.* v.63, n.6, p.1282–1288, 2011.

YANG, Q., PENG, Y., LIU, X., ZENG, W., MINO, T., SATOH, H. **Nitrogen removal via nitrite from municipal wastewater at low temperatures using real-time control to optimize nitrifying communities.** *Environ. Sci. Technol.* v.41, p.8159–8164, 2007.

WIESMANN, U. **Biological nitrogen removal from wastewater.** *Adv. Biochem. Eng. Biotechnol.*, v. 51, p. 113-154, 1994.

WINKLER, M. K. H., KLEEREBEZE M. R., KUENEN, J. G., YANG, J., VAN LOOSDRECHT, M. C. M. **Segregation of biomass in cyclic anaerobic/aerobic granular sludge allows the enrichment of anaerobic ammonium oxidizing bacteria at low temperatures.** *Environ. Sci. Technol.* v.45, p.7330-7337, 2011.

WYFFELS, S., BOECKX, P., PYNAERT, K., VERSTRAETE, W., VAN CLEEMPUT, O. **Sustained nitrite accumulation in a membrane-assisted bioreactor (MBR) for the treatment of ammonium rich wastewater.** *Journal of Chemical Technology & Biotechnology*, v. 78, p. 412-419, 2003

ZHANG, Y., RUAN, X. H., OP DEN CAMP, H. J. M., SMITS, T. J. M., JETTEN, M., SCHMID, M. C. **Diversity and abundance of aerobic and anaerobic ammonium oxidizing bacteria in freshwater sediments of the Xinyi River (China)**.*Environ. Microbiol*.v.9, n.9, p.2375–2382, 2007.

ZHU, G., PENG, Y., LI, B., GUO, J., YANG, Q., WANG, S. **Biological removal of nitrogen from wastewater.** *Reviews Environmental Contamination & Toxicology*, v. 192, p.159-195, 2008.

ANNEX

Annex A

Annex A.1 presents raw data from the sulfide test, the measurement concentrations of NH_4^+-N,NO_2^--N and NO_3^--N in the inlet, inside the reactor, outlet as well as calculated total nitrogen removal rate (TNRR) and ammonium removal efficiency. The logged data from the mass flow controllers' gases and average DO are shown in Annex A.2.

Annex A.1 - Raw data of analyzes during sulfide test.

Time (day)	Influent (mg L^{-1})			Effluent (mg L^{-1})			TNRR (kg m^{-3} d^{-1})	Ammonium removal efficiency (%)
	NH_4^+-N	NO_2^--N	NO_3^--N	NH_4^+-N	NO_2^--N	NO_3^--N		
1	734	0.06	0.69	357	2.14	18	0.96	51.36
2	719	0.03	1.78	369	2.69	39	0.83	48.68
5	672.5	0.03	1.03	295	2.82	35	0.91	56.13
6	682.5	0.08	0.55	189.5	3.01	68.5	1.13	72.23
7	608	0.35	0.68	132.8	2.3	58.8	1.11	78.16
8	632.5	0.04	1.52	145	8.61	58.25	1.13	77.08
9	732.5	0.18	1	204.5	3.03	49	1.27	72.08
12	725.5	0.63	1.08	187	4.88	63.75	1.26	74.22
13	737.5	0.81	0.8	197	1.04	73.5	1.25	73.29
14	802	0	1.5	229.8	0.06	48	1.4	71.35
15	660	1.15	1.3	96	4.16	66.56	1.32	85.45
16	752.5	1.22	0.8	124	4.15	55.18	1.25	83.52
19	627.5	13.40	2.7	131.25	6.80	47.25	1.22	79.08
21	717.3	0.94	1.5	307.5	8.89	53.75	0.93	57.14
23	635	0.16	1.1	90.25	2.86	55.25	1.30	85.79
26	742.5	0	0.9	193.5	0.11	47	1.34	73.94
27	602.5	1.59	1.7	37.5	4.27	42.75	1.39	93.78
28	575	0.12	0.6	310	5.82	29.50	0.57	44.34
30	682	0.14	1.6	187	5.72	54.50	1.17	72.59
33	410	2.94	10.1	420	4.77	12.45	-0.04	-2.44
34	592.5	0	0.7	302.5	9.11	59.25	0.59	48.95
35	485	0.17	1.1	290.00	9.52	67	0.32	40.21
36	687.5	0.17	1.6	494.25	33.66	102.50	0.16	28.04

Annex A.2–Average DO and Flow of gases N_2, CO_2 and Air used throughout the experiment.

Time (day)	N_2 flow (L h^{-1})	N_2 accumulated (l)	CO_2 accumulated (l)	Airflow (L h^{-1})	Air acccumulated	DO (mg L^{-1})
1	34.1	20830.0	2506.4	20.6	17090.0	0.83 ± 0.06
2	-	-	-	-	-	0.72 ± 0.05
5	-	-	-	-	-	0.66 ± 0.06
6	35.9	24860.0	2820.1	26.9	19970.0	0.66 ± 0.05
7	-	-	-	-	-	0.61 ± 0.01
8	-	-	-	-	-	0.57 ± 0.03
9	-	-	-	-	-	0.60 ± 0.03
12	31.0	29950.0	3212.1	32.6	23970.0	0.60 ± 0.04
13	-	-	-	-	-	0.59 ± 0.03
14	30.3	30640.0	3220.0	46.4	24700.0	0.97 ± 0.18
21	45.4	34760.0	4351.5	27.0	31230.0	1.00 ± 0.24
22	52.8	37050.0	4610.3	29.2	3269.0	1.01 ± 0.19
26	65.1	42130.0	5069.5	27.8	35750.0	0.96 ± 0.18
27	56.4	43710.0	5359.7	42.1	36450.0	0.84 ± 0.03
28	54.6	44780.0	5619.8	32.4	37190.0	1.13 ± 0.15
30	54.5	46230.0	5758.0	37.8	38100.0	0.83 ± 0.11
33	64.9	50130.0	5948.5	34.9	40720.0	0.86 ± 0.14
34	66.1	51710.0	6071.3	38.4	41560.0	0.92 ± 0.02
36	63.5	54960.0	6370.5	31.4	43350.0	1.0 0.04

Annex B

Annex B contains the raw data from the reactor performance on pH test (Annex B.1), and results of the distribution of the granule size throughout the pH ranges evaluated (Annex B.2).

Annex B.1–Raw data of the reactor performance and operating conditions.

Time (day)	Influent (mg L^{-1})				Effluent (mg L^{-1})					Average pH	TNRR (kg m^{-3} d^{-1})
	NH_4^+-N	NO_2^--N	NO_3^--N	COD	NH_4^+-N	NO_2^--N	NO_3^--N	COD	FNA (µg L^{-1})		
1	726.00	0.00	1.81	-	157.50	2.26	6.31	-	1.01	7.10 ± 0.24	1.50
2	428.00	0.00	1.78	-	58.60	3.81	32.00	-	1.75	7.09 ± 0.17	0.90
3	835.00	0.00	1.60	-	195.00	4.13	60.00	-	1.86	7.10 ± 0.14	1.54
4	757.50	0.00	1.79	-	210.25	3.75	51.50	-	1.69	7.10 ± 0.06	1.32
7	772.50	0.00	1.69	-	245.00	4.06	48.50	-	1.82	7.10 ± 0.07	1.27
8	765.00	0.00	1.76	37.30	222.50	4.03	45.75	91.10	1.80	7.10 ± 0.09	1.32
9	757.50	0.00	1.75	38.20	210.25	4.63	57.25	107.00	2.08	7.10 ± 0.04	1.30
10	742.50	0.00	1.68	37.90	221.50	4.40	57.50	97.10	2.48	7.00 ± 0.06	1.23
11	750.00	0.00	1.70	34.30	155.00	4.95	57.50	74.00	2.79	7.00 ± 0.02	1.43
14	757.50	0.00	1.66	33.40	154.50	5.45	54.00	54.90	3.05	7.00 ± 0.09	1.46
15	665.00	0.00	1.72	31.60	93.00	5.69	55.00	50.40	5.20	6.79 ± 0.10	1.37
16	677.50	0.00	1.75	31.60	184.00	5.22	51.75	72.60	4.68	6.80 ± 0.05	1.17
17	690.00	0.00	1.69	33.50	191.50	6.77	51.50	64.40	6.01	6.80 ± 0.03	1.18

Annex B.1 – Continued from previous page.

18	775.00	0.03	1.74	36.80	226.00	8.56	54.25	65.70	7.59	6.80 ± 0.05	1.30
21	674.00	0.13	1.78	31.60	170.50	10.10	46.00	67.40	9.32	6.78 ± 0.04	1.20
22	688.00	0.00	1.82	31.20	385.00	135.75	50.50	144.00	190.92	6.60 ± 0.06	0.32
23	700.00	0.00	1.77	31.40	550.00	58.25	46.25	154.00	80.17	6.61 ± 0.06	0.13
24	772.00	0.00	1.70	34.10	376.00	13.05	38.25	74.20	6.01	7.09 ± 0.11	0.93
25	740.00	0.00	1.71	37.30	191.00	9.35	64.75	85.30	4.63	7.05 ± 0.13	1.27
28	764.00	0.00	1.70	32.20	235.00	9.35	57.50	63.40	3.84	7.14 ± 0.07	1.24
29	732.00	0.00	1.74	31.80	410.00	74.50	44.50	87.80	82.05	6.71 ± 0.06	0.55
30	840.00	0.03	1.65	39.20	575.00	13.15	33.00	81.30	14.28	6.71 ± 0.20	0.59
31	861.00	0.04	1.66	35.90	555.00	9.00	31.75	77.20	10.06	6.70 ± 0.03	0.71
32	901.00	0.22	1.59	45.80	580.00	89.75	33.25	153.00	100.21	6.70 ± 0.19	0.53
35	701.00	0.13	1.77	28.50	580.00	89.75	33.25	153.00	98.34	6.71 ± 0.06	0.00
36	736.00	0.00	1.71	29.60	491.00	11.55	26.75	67.30	5.17	7.10 ± 0.05	0.56
37	686.00	0.00	1.77	29.80	388.50	7.20	35.50	66.60	3.22	7.10 ± 0.06	0.69
38	799.00	0.00	1.78	31.00	309.50	10.30	39.75	71.90	4.71	7.09 ± 0.06	1.18

Annex B.2 - Granular distribution per pH value.

pH		0.05 mm	0.06 mm	0.10 mm	0.20 mm	0.30 mm	0.4 mm	0.5 mm	0.6 mm	0.7 mm	0.8 mm	0.9 mm	1.0 mm
7.10	No. of accumulated granules	91	155	527	1757	3477	5056	5589	5765	5882	5936	5966	5980
	Proportion of granules collected (%)	1.52	2.59	8.81	29.38	58.14	84.55	93.46	96.40	98.36	99.26	99.77	100.00
7.00	No. of accumulated granules	57	126	435	1402	2346	3081	3362	3484	3571	3597	3610	3615
	Proportion of granules collected (%)	1.58	3.49	12.03	38.78	64.90	85.23	93.00	96.38	98.78	99.50	99.86	100.00
6.80	No. of accumulated granules	40	120	435	1434	2556	3434	3742	3859	3925	3945	3953	3961
	Proportion of granules collected (%)	1.01	3.03	10.98	36.20	64.53	86.70	94.47	97.42	99.09	99.60	99.80	100.00
6.70	No. of accumulated granules	60	164	607	2067	3255	4009	4259	4336	4380	4407	4413	4416
	Proportion of granules collected (%)	1.36	3.71	13.75	46.81	73.71	90.78	96.44	98.19	99.18	99.80	99.93	100.00
6.60	No. of accumulated granules	615	721	1266	2489	3514	4140	4311	4363	4394	4409	4414	4417
	Proportion of granules collected (%)	0.14	0.16	0.29	0.56	0.8	0.94	0.98	0.99	0.99	1	1	1

Annex C

The raw data in this appendix refer to the temperature test (**Error! Reference source not found.**).

Annex C - Shows the concentrations of temperature, NO_2^--N, NO_3^--N and TNRR/Vs.

Temperature (°C)	NO_2^--N (mg L^{-1})	NO_3^--N (mg L^{-1})	TNRR/Vs
36	5.40	30.75	494.86
36	5.40	46.25	494.98
36	4.48	68.75	593.49
36	4.62	77.50	617.09
33	4.75	96.50	589.51
33	4.38	57.00	517.20
33	3.25	68.25	492.90
33	3.21	76.25	598.34
30	4.49	87.50	583.72
30	4.24	41.75	459.91
30	3.66	49.00	445.77
30	1.61	54.50	440.61
30	4.16	62.25	441.20
29	3.34	52.25	461.86
29	3.10	42.75	395.43
29	3.50	60.75	487.09
29	2.29	67.00	578.00
28	8.90	23.37	359.95
28	4.00	37.50	341.64
28	3.95	43.00	357.70
28	3.78	47.00	402.95
36	3.37	23.95	439.71
36	3.32	58.25	468.30
36	2.86	67.50	531.36
27	3.61	70.00	429.83
27	2.54	60.50	440.96
27	3.48	53.75	388.16
27	3.50	67.75	474.29
26	5.06	75.25	607.92
26	3.52	67.25	628.36
26	13.04	74.00	687.86
26	3.52	75.25	720.23
26	3.05	79.25	708.63
25	3.42	70.50	662.29
25	3.20	70.50	632.07