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PROYECTO FIN DE CARRERA

NITROGEN REMOVAL IN ANAMMOX BIOREACTORS

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PREFACE

AND

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This report constitutes the final project of Begoña García Lapeña, student of Chemical Engineering at the University of Valladolid, Spain. She has been a visiting scholar at the Department of Chemical and Environmental Engineering in the University of Arizona (Tucson, AZ, USA), from September 2011 to June 2012. It has been supervised by Dr. Sierra (Full Professor at the Department of Chemical and Environmental Engineering) and Dr Field (Full Professor at the Department of Chemical and Environmental Engineering)

I want to say thank you to my advisors Dr. Sierra and Dr. Field for giving the opportunity to carry out this work in the University of Arizona.

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AND

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ABSTRACT

In recent years, different biological processes have been developed to remove nitrogen from wastewaters. One of these processes is the Anaerobic Ammonium Oxidation process (Anammox) in which ammonium is oxidized to nitrogen using nitrite as oxidant. The anammox process was discovered in 1977 and presents may advantages compared to more conventional nitrogen removal processes as nitrification and dentitrification. Particularly important among these advantages are the lower energy consumption and no need for oxygen or external source of organic matter.

Knowledge of the main parameters that influence the activity of anammox bacteria is critical to optimize the process performance. The main objectives of this study are as follows: *1)* to investigate approaches to increase biomass retention in the anammox process in order to reduce the startup period; *2)* to study the kinetics of the process, and *3)* to investigate the toxicity of two important wastewater components (nitrite and H_2S). In this project, all these objectives have been study with the help of three different types of reactors.

Operation of a membrane bioreactor (MBR) for 300 days at a hydraulic retention time (HRT) of 1 d demonstrated that the system could reach a volumetric nitrogen removal of 26.4 mg N/L d with nitrite and ammonium removal efficiencies of 99 and 95%, respectively. Under these conditions the biomass reached a value of around 900 mg VSS/L. The biomass in the effluent cannot be measured because is practically zero, so the membrane bioreactor has a good biomass retention.

Batch tests were used to determine the kinetic of the process and the influence of pH and the toxicity of H_2S. There are different studies about the kinetics of the anammox process, but few have considered the simulatenous utilization ammonium and

nitrite. Process kinetics was studied using a mathematic model based on Monod microbial growth kinetics. The kinetic parameters determined for anammox granular sludge were: K_S NH_4^+ = 0.680 mM; K_S NO_2^- = 0.339 mM, and for anammox flocculent sludge (K_S NH_4^+ = 0.544 mM; K_S NO_2^- = 0.375 mM).

The pH value has a very significant impact on the activity of anammox bacteria. The anammox activity was almost constant in the pH range from 7.2 to 7.5 with a maximum activity at a pH of 7.3. Outside of this range, the anammox activity decrease considerably. Sulfide (H_2S), a wastewater contaminant commonly found in reducing environments, is highly inhibitory to anammox bacteria. A significant decrease in the anammox activity, up to 65%, in the presence of 0.5 mM of H_2S; however addition of stoichiometric levels of Fe^{2+} , a cation that forms very insoluble salts with sulfide, eliminated microbial inhibition.

Laboratory-scale upflow bioreactors were used to study the toxicity of the nitrite on the anammox process. Three continuous different reactors were operating for more than 120 and fed an influent containing 4 mM NH_4^+ and different concentrations of nitrite. Reactor 1 (R1) was fed with a stoichiometry nitrite-ammonium ratio (NO_2^-/NH_4^+ = 1.15), and considered the control to compare the results with the other two reactors. Reactor 2 (R2) had a 30% excess of nitrite (NO_2^-/NH_4^+ =1.64), and the reactor 3 (R3) had a 50% excess of nitrite (NO_2^-/NH_4^+ =2.64). During a period of 120 d the volumetric load was increased up to a value of 56.96 for R1, 38.87 for R2 and 34.52 for R3 (in mmol N-removed/Ld) The activity was measured to compare the evolution of the reactors. The reactors obtained a high efficient removal for NH_4^+ (88.3%, 98.5%, and 98%), nevertheless the reactors could not remove the excess of nitrite (the efficiencies for each reactor are: 97.5%, 68.8%, and 62.5%). Two activity tests demonstrated that the residual nitrite decreased the activity in the anammox process.

ABREVIATIONS

ANAMMOX	Anaerobic ammonium oxidation
CANON	Completely autotrophic nitrogen-removal over nitrite
COD	Chemical oxygen demand
DO	Dissolved oxygen content
GCE	Granular anammox sludge
HRT	Hydraulic retention time
MBR	Membrane bioreactor
MCL	Maximum contaminant level
SAA	Specific anammox activity
SBR	Sequencing batch reactor
SEC	Suspended enrichment culture
TKN	Total Kjeldahl nitrogen
VSS	Volatile suspended solids
TSS	Total suspended solids

INTRODUCTION

1. INTRODUCTION

During the last years the increase in the level of development and its consequent increase in the industrial human activities have generated an increase in the production of wastes and wastewater. This increase in the level of development has also generated an growth in the standard of living and awareness over the environmental problem generated and the need to look for environmental friendly solutions.

At this moment environmental management at the industry is oriented to the minimization of waste production and the application of every time more adequate treatment technologies to decrease the final disposal of the wastes that can no be minimized.

Water is an essential resource for the development of life and its conservation is one is the main objective of different national agencies all over the world. To improve the ecological and sanitary conditions of surface and ground waters is necessary to limit the pollution load sent to these systems. One of the main impacts on water flows are those related with the release of nutrients, basically nitrogen and phosphorous.

The increase in the basic knowledge of the processes implied in nutrients removal has allowed the development of new technological systems that can treat wastewater with nutrients in a more safe and economical way.

1.1 Nitrogen

Nitrogen (N) is a natural element in the environment that is essential for plant and animal growth, maintenance and reproduction. The main species of inorganic

nitrogen are ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), and elemental nitrogen gas (N_2). Organic compounds containing nitrogen (e.g. proteins, amino acids) are also common in wastewaters.

Nitrogen levels in surface- and groundwater vary naturally and some amount in a water body is not necessarily harmful. In fact, nitrogen is essential to maintain the health of the organisms that live there. However, when too much nitrogen enters surface waters, it can cause the ecosystem to become unbalanced.

Nitrogen is a part of a natural, healthy aquatic ecosystem. It supports the growth of underwater plants, which produce oxygen and habitat that supports growth and reproduction of aquatic organisms. Nitrogen also supports the growth of algae, a natural part of many aquatic ecosystems. Algae are found in shallow water or near the surface of the water body, where they can access sunlight to photosynthesize. As a result, algae are a food source for some species of fish and shellfish.

Contamination caused by an excessive concentration of nitrogen in water is a growing concern. High levels of nitrogen in lakes, rivers, streams, and drinking water sources cause the degradation of these water bodies and harm fish, wildlife, and human health. Nitrogen is often a result of human activities and they speed up the growth of algae in surface waters to an unhealthy level in a process called eutrophication. The algae grow out of control and form what is called an algal bloom. These algal blooms can cause many problems for underwater plants and animals, as well as humans. One effect that algal blooms have is the reduction (hypoxia) or elimination (anoxia) of oxygen in the water. When algal organisms die can block sunlight from reaching underwater plants growing at lower depths, which causes a loss of very important habitat. When algal organisms die, they sink to the bottom of the water body where

oxygen-consuming bacteria break them down (along with any other dead material that may be present). As a result of this decomposition process, oxygen levels are reduced in the water. In addition to hypoxia and/or anoxia, algal blooms can also produce toxins or other negative effects, such as bad smells, ecological problems, loss of property or aesthetic value, clogged drinking water filters, drinking water taste and odor problems, and increased drinking water treatment costs. Harmful algal blooms can cause human health problems through recreational contact or the consumption of contaminated fish and shellfish. The effects of excess nitrogen and phosphorus in surface waters can occur close to and downstream of their sources. Rivers and streams carry excess nitrogen and phosphorus downstream, where some effects can take place in the river/stream far from the source of pollution. In addition, rivers/streams carry nutrients downstream into lakes and estuaries.

The NH_4^+ is toxic to fish and it is also an oxygen demanding substance. Ammonium is biologically oxidized to produce nitrate (eq. 1). This oxidation consumes 4.57 mg O_2 per mg of ammonium nitrogen oxidized.

$$NH_4^+ + 2\,O_2 + microbial\ activity \rightarrow NO_3^- + H_2O + 2\,H^+ \qquad \text{[Eq 1]}$$

Nitrogen, along with co-contaminants such as pathogens, chemicals, and pharmaceuticals, is also found in excess in groundwater, which some homes use as their drinking water source. At levels above 10 mg $N\text{-}NO_3^-$/L, nitrates in groundwater can cause human health effects, such as "blue baby syndrome" (Manassaram et al. 2010). To protect human health, it is important that nitrate levels be below this 10 mg $N\text{-}NO_3^-$ /L maximum contaminant level (MCL) when the groundwater is used as drinking water.

1.2 Removal of Nitrogen from Wastewaters

Nitrogen is present in wastewater in several species, the important ones being organic nitrogen (both soluble and particulate), ammonium/ammonia and possibly some nitrate. In the activated sludge process several reactions may occur, that will change the speciation of the nitrogenous matter.

Nitrogenous matter in wastewaters is mainly composed of ammonium nitrogen (which can be present as gaseous (NH_3) and ionic form (NH_4^+), depending on pH and temperature and soluble or particulate organic nitrogen (urea, amino acids and other organic compounds with an amino group). Sometimes wastewaters contain traces of oxidized forms of nitrogen, mainly nitrite and nitrate (Eq. 1). Different to organic matter, nitrogenous matter can be defined quantitatively and unequivocally by one parameter: the nitrogen concentration in its different forms. In practice, spectrophotometric tests and specific ion electrodes are used to determine the concentrations of ammonium, nitrate and nitrite. Organic nitrogen can be determined after its conversion to ammonium nitrogen by chemical digestion. The sum of the organic nitrogen and ammonium concentrations is called Total Kjeldahl Nitrogen (TKN).

Nitrogen compounds can be removed from wastewater by a variety of physicochemical (eg. reverse osmosis, ion exchange, stripping) and biological processes In which several reactions may occur, that change the form of nitrogenous matter.

Conventionally, nitrogen removal from wastewater by biological processes involves well-know aerobic nitrification followed by anoxic denitrification. Biological nitrogen removal has become common because of its low cost and high efficiency as compared to physical and chemical treatment (van Donger et al., 2001a,b). However,

nitrogen removal by biological processes is seldom used in wastewater with high NH_4^+ concentration but low carbon content. In some cases the available carbon in these wastewaters is insufficient for the denitrification process and an external carbon source such as acetate, glucose, ethanol, methanol or methane gas must be added. All these external sources are expensive and substantially increase the cost of operation. In recent years, the development of novel treatment methods (*i.e.*, partial nitrification followed by anammox treatment) that are based on autotrophic microorganisms has expanded the application of biological processes to wastewaters containing high N/C ratios. These advanced treatment methods for the removal of nitrogen nutrient will be discussed in Section 1.3.

The main microbial conversions implicated in the biological nitrogen removal processes are discussed below. Figure 1 illustrates the biological nitrogen cycle.

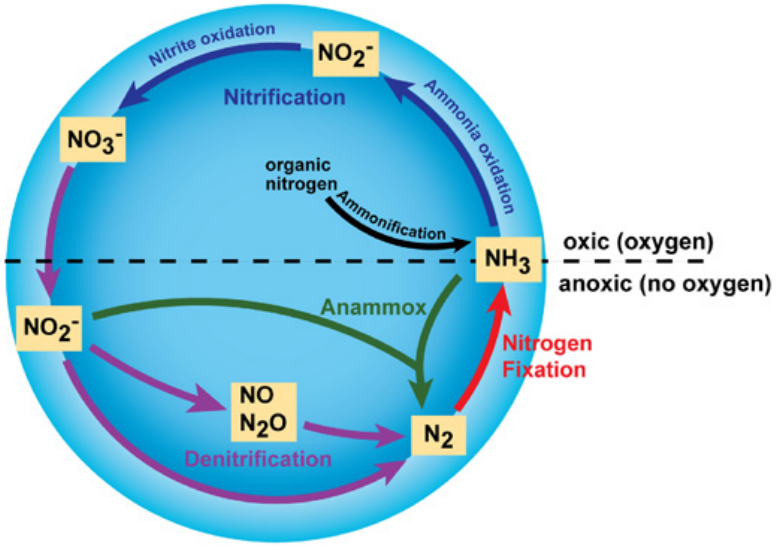

Figure 1: Nitrogen cycle (Bernhard, 2010)

1.2.1 Ammonification/assimilation

Ammonification is the conversion of organic nitrogen into ammonium, whereas the inverse process, the conversion of ammonium into organic nitrogen, is called bacterial anabolism or assimilation. Considering that the pH in mixed liquor is typically near the neutral point of pH = 7, ammonium will be present predominantly in its ionic form (NH_4^+) and the following reaction equation may be written:

$$RNH_2 + H_2O + H^+ \leftrightarrow ROH + NH_4^+ \qquad \text{[Eq 2]}$$

1.2.2 Nitrification

Nitrification is the biological oxidation of ammonium, with nitrate as the end product. The reaction is mediated by specific bacteria and is a two-step process: in the first step ammonium is oxidized to nitrite by autotrophic bacterial species such as *Nitrosomonas* spp. The complementary step, oxidation of nitrite to nitrate, is mediated by species such as *Nitrobacter* spp. Both *Nitrosomonas* and *Nitrobacter* can only develop biochemical activity in an environment containing dissolved oxygen (van Haandel, et al., 2006). The two steps can be written as:

$$\begin{aligned} NH_4^+ + {}^{3}\!/_{2}\, O_2 &\rightarrow NO_2^- + H_2O + 2H^+ \\ NO_2^- + {}^{1}\!/_{2}\, O_2 &\rightarrow NO_3^- \end{aligned} \qquad \text{[Eq 3]}$$

And the global equation as:

$$NH_4^+ + 2\,O_2 \rightarrow NO_3^- + H_2O + 2H^+$$ [Eq 4]

1.2.3 Denitrification

Denitrification is the biological reduction of nitrate to molecular nitrogen, with organic matter generally used as a reductor. It is also a two steps process and for organic matter with a general structural formula $C_xH_yO_z$, the half reactions of this redox process can be expressed as:

Oxidation reaction:

$$C_xH_yO_z + (2x - z)\,H_2O \rightarrow x\,CO_2 + (4x + y - 2z)\,H^+ + (4x + y - 2z)\,e^-$$

[Eq 5]

Reduction reaction:

$$\tfrac{1}{5}\,NO_3^- + \tfrac{6}{5}\,H^+ + e^- \rightarrow \tfrac{1}{10}\,N_2 + \tfrac{3}{5}\,H_2O$$

[Eq 6]

Overall redox reaction:

$$C_xH_yO_z + \tfrac{(4x + y - 2z)}{5}\,H^+ + \tfrac{(4x + y - 2z)}{5}\,NO_3^- \rightarrow x\,CO_2 + \tfrac{(2x + 3y - z)}{5}\,H_2O + \tfrac{(4x + y - 2z)}{10}\,N_2$$

[Eq 7]

The TKN concentration in municipal sewage typically is in the range of 40 to 60 mg N/L, *i. e.* a fraction in the range of 0.06 to 0.12 of the influent COD. About 75 percent of the total TKN concentration is ammonium nitrogen and the remaining 25 percent is predominantly organic nitrogen.

In the activated sludge process, organic nitrogen is converted rapidly and almost quantitatively to ammonium nitrogen (ammonification). If nitrification occurs and the oxygenation capacity is sufficient, the oxidation of ammonium nitrogen will be almost complete. If after nitrification the formed nitrate is removed by denitrification, the total nitrogen concentration in the effluent is in general smaller than 5 to 10 mg N/L, with a nitrogen removal efficiency of 90 percent or more (van Haandel, et al., 2006).

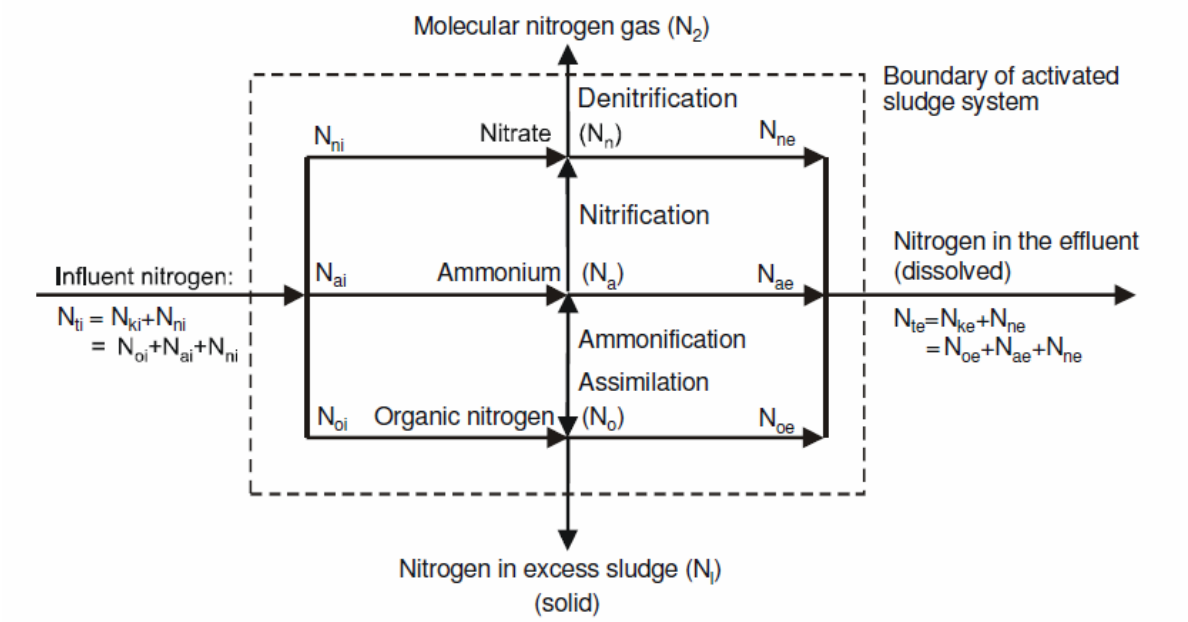

Figure 2. Schematic representation of the forms of nitrogenous material and their reactions in the activated sludge process (van Haandel, et al., 2006).

In the past few years, several novel and low cost-effective biological nitrogen removal processes have been developed, including partial nitrification, nitrifier

denitrification, anaerobic ammonium oxidation (the Anammox process), and its combined system, CANON (Completely Autotrophic Nitrogen-removal Over Nitrite).

1.3 Anammox process

The anaerobic ammonium oxidation (Anammox) is a chemo-lithoautotrophic biological conversion process, mediated by a group of Planctomycete bacteria as shown Figure 3.

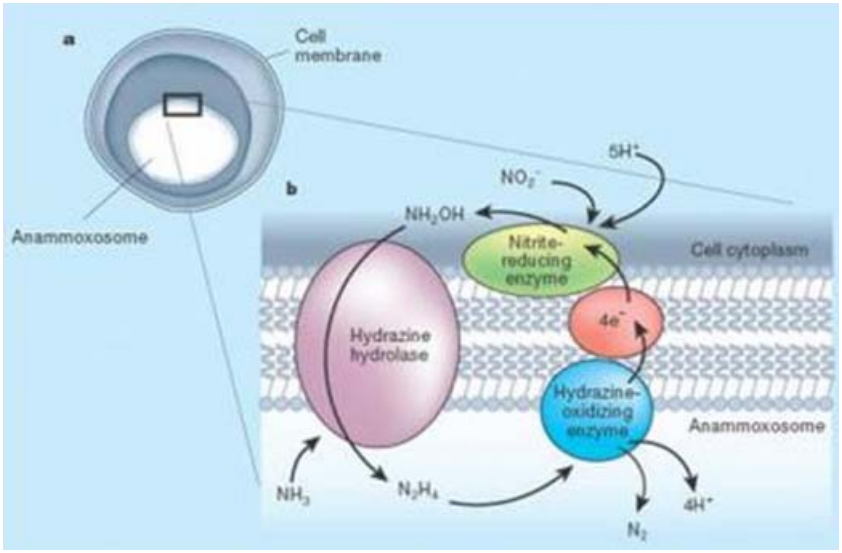

Figure 3. Biochemical pathway of the Anammox reaction (Young-Ho Ahn, 2006)

The remarkable advance in the molecular biological techniques has disclosed a great variety of information on biodiversity of the Anammox bacteria. These bacteria have a highly unusual physiology, in that they live by consuming ammonia in the absence of oxygen. Anammox is highly exergonic and linked to the energy metabolism of the organisms involved, this process involves the oxidation of ammonia with nitrite as the electron acceptor to yield gaseous nitrogen.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad [\Delta G^{o\prime} \text{ -357 kJ/mol}] \qquad \text{[Eq. 8]}$$

The stoichiometry of the Anammox reaction based on mass balance over Anammox enrichment cultures is represented by the next equation [Eq. 9].

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 0.066\ CH_2O_{0.5}N_{0.15} + 1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O$$
[Eq 9]

The anammox process uses ammonium as an elector donor and nitrite as an electron acceptor under anaerobic conditions. Anammox organisms grow with CO_2 as the sole carbon source. There is no need for addition of external carbon sources, leading avoidance of an incomplete conversion of organic matters. The power consumption can be reduce by 60% in this process over those of the nitrification-denitrification process, resulting in significant savings in operational costs (Abma et al., 2007) The low biomass yields in this autotrophic process could also reduce the burden of the sludge treatment (Jiachun, 2010).

Table 1 compares different processes orf nitrogen removal. Table 2 compares the anammox process with other nitrificacion-denitrification processes

Table 1. Comparison of various nitrogen removal processes (Young-Ho, 2006).

Reaction	First phase		Second phase	
	Oxygen (g O_2/g N)	Alkalinity (g $CaCO_3$/g N)	Alkalinity (g $CaCO_3$/g N)	Organic (g COD/g N)
Nitrification-Denitrification	4.57 [4.18]	7.14 [7.07]	(3.57)	3.7
Nitritation-Denitritation	3.46 [3.16]	7.14 [7.07]	(3.57)	2.3
Partial nitritation-Anammox	1.71-2.06	3.57	0.24	-
CANON	1.94	3.68	-	-

[] calculated by combined dissimilation-synthesis equations; () alkalinity production in heterotrophic denitrification/denitration.

Table 2: Physiological parameters for anaerobic and aerobic ammonium oxidation (Young-Ho 2006)

Parameters	Unit	Anammox	Nitrification
pH range		6.7-8.3	Variable
Temperature range	ºC	20-43	≤42
Free energy	kJ/mol	-367	-275
Biomass yield	mol/mol C	0.07	0.08
	g protein/g NH_4-N	0.07	0.1
Aerobic rate	nmol/min/mg protein	0	200-600
Anaerobic rate	nmol/min/mg protein	60	2
Growth rate	1/h	0.003	0.04
Doubling time	days	10.6	0.73
K_s NH_4^+	μM	5	5-2,600
K_s NO_2^-	μM	<5	n.a
K_s O_2	μM	n.a	10-50
NO_2^- inhibition of NH_4^+ consumption	g NO_2^--N/L	Ki =0.8, α=0.8	Usually
NO_2^- inhibition of NO_2^- consumption	g NO_2^--N/L	Ki =0.1, α=0.7	n.a
Protein content of biomass	g protein/g SS	0.6	Variable

As anammox bacteria, which are strictly anaerobes and autotrophs, are difficult to enrich, applications of this process is partly limited by the availability of anammox biomass. The dissolved oxygen, temperature, pH and influent nitrite concentration are considered as the primary factors for the anammox reaction. Additionally, the presence of organic matter is not suitable for the growth of anammox bacteria. The inorganic carbon source was shown to be a limiting factor in continuous nitrification experiments. It is widely accepted that carbon limitation may be the main reason for the dramatic decrease in growth and activity of nitrifying bacteria below neutral pH. It has been reported that the anammox activity increased as the influent bicarbonate concentration increased from 1.0 to 1.5 g/L in a sequenced batch reactor (SBR) (Yang, et al., 2010).

Therefore, it is very likely that the concentration of inorganic carbon may play an important role in the enrichment of anammox microorganisms.

This process reports considerable benefits, such as reduced energy cost, biomass production, and carbon requirement. The primary limitation of this process is the long start-up period for anaerobic ammonium oxidation, typically a few months after inoculation with ammonium oxidizing bacteria (Pongsak, et al.. 2008). However, after a suitable startup period, the system is inexpensive to maintain and simple to operate. Also, the extremely slow growth rate of Anammox bacteria, with an approximate doubling time of 11 days, is the major obstacle for implementation of Anammox process. A long start-up period is thus expected in Anammox process. Faster growth of Anammox bacteria was achieved in a membrane bioreactor (MBR) (the doubling time was less than 10 days), resulting in an unprecedented purity of the enrichment of 97.6% (van der Star et al., 2008).

Some factors have been found to be able to influence the start-up of anammox process including hydraulic retention time (HRT), dissolved oxygen (DO), inoculum, temperature, wastewater composition and nitrogen compound concentration (Tang et al., 2010a; Tsushima et al., 2007). Recently, reactor configuration was also demonstrated to have a significant impact on cultivation of anammox bacteria (Hu et al., 2010).

A reactor configuration with high biomass retention is essential for anammox process due to their slow growth rate. Membrane bioreactor (MBR) and sequencing batch reactor (SBR) are widely-applied bioreactors for wastewater treatment (Shannon et al., 2008; Gao et al., 2010). MBR can prevent product inhibition and the outflow of suspended cells (Jagersma et al., 2009). Several studies have proved that a submerged

MBR was an excellent tool for enriching slow-growing microorganisms such as methanotrophic archaea (Meulepas et al., 2009) and anammox bacteria (van der Star et al., 2008). However, the anaerobic condition of a submerged MBR could be interrupted if membrane modules are frequently replaced due to biofouling (Gao et al., 2009), which could harm anammox bacteria (Meulepas et al., 2009). An external MBR could avoid such interruption. To date, studies that investigated the start-up of anammox process by using an external MBR are very limited and the comparison of SBR versus MBR in anammox start-up is also rare.

One of the most critical aspects in the anammox process stability is nitrite, since it is the electron acceptor in the process and converted by anammox bacteria, but also a potential inhibiting compound. Nitrite concentrations as low as 5 and 40 mg N/L have been reported as strongly inhibitory (Wett, 2007 and Fux, 2003). Strous et al. (1999), who first reported the adverse effect of nitrite, found a complete but reversible, inhibition of the process at 100 mg N/L. Other authors reported similar concentrations as detrimental for the anammox process, but indicated that the nitrite inhibition was either as reversible or irreversible (e.g. Fux et al., 2004; Jetten et al., 2005; Lopez et al., 2008; Van Dongen et al., 2001). A few reports indicate even higher nitrite tolerance (Cho et al., 2010; Dapena-Mora et al., 2007; Egli et al., 2001; Fernandez et al., 2012) with the highest reported non-inhibitory value reported by Kimura et al. (2010) (toxicity threshold higher than 300 mg N/L).

OBJECTIVES

2 OBJECTIVES

Anammox is an innovative biological process for the removal of nitrogen that offers significant advantages compared to conventional treatment processes such as nitrification - denitrification. The process was recently discovered (1977) and it is not well understood so that it is necessary to study the behavior of the process.

The main objectives of this project are:

- Characterize the metabolic activity of anammox bacteria and the kinetics of the anammox process using two different inocula, a granular sludge and a flocculent enrichment culture. Batch experiments were used in this study.

- Study the effect of different parameters (pH, H_2S) on the metabolic activity of anammox bacteria in a flocculent enrichment culture using batch experiments.

- Investigate the treatment capacity of anammox membrane bioreactors.

- Investigate the inhibitory impact of nitrite on anammox bacteria using continuous upflow bioreactors inoculated with granular sludge.

MATERIALS

3. MATERIALS

3.1 Microorganisms

Two anammox cultures (granular and suspended) enriched in the specie Candidatus Brocadia were inoculated to evaluate the inhibitory effects of various environmental conditions and toxic compounds on the anammox activity. The suspended enrichment culture (SEC) (Sun et al., 2011) was washed with sterilized NaCl (1%) solution, centrifuged and re-suspended with sterile MilliQ water before it was transferred into batch assays. This anammox enrichment culture was originally developed from a returned activated sludge (RAS-Ina) from a local wastewater treatment plant (Ina Road, Tucson, Arizona) [Sun et al., 2011]. The granular anammox sludge (GEC) was obtained from a full-scale anammox bioreactor operated by Paques BV (Balk, The Netherlands). The volatile suspended solids (VSS) content of the anammox granular sludge was 4.8±0.2% of the wet weight. The granular samples were washed with basal medium and sieved immediately before use to remove fines.

3.2 Basal Medium

The basal mineral medium was prepared using ultrapure water (Milli-Q system; Millipore) and contained the following compounds (mg/L): NH_4HCO_3 (214); $NaNO_2$ (246); $NaH_2PO_4 \cdot H_2O$ (58); $CaCl_2 \cdot 2H_2O$ (100); $MgSO_4 \cdot 7H_2O$ (200); $NaHCO_3$ (2,500); and 1.0 mL/L of two trace element solutions. Trace element solution 1 contained (in mg/L) $FeSO_4$ (5,000) and ethylenediamine-tetraacetic acid (EDTA) (5,000). Trace element solution 2 contained (in mg/L) EDTA (1,500); $ZnSO_4 \cdot 7H_2O$ (430);

$CoCl_2$•$6H_2O$ (240); $MnCl_2$ (629); $CuSO_4$•$5H_2O$ (250); Na_2MoO_4•$2H_2O$ (220); $NiCl_2$•$6H_20$ (190); Na_2SeO_4•$10H_2O$ (210); H_3BO_3 (14); $NaWO_4$•$2H_2O$ (50).

3.3 Batch Assays

To evaluate the effects of various factors on anammox activity, batch assays were performed in shaken flasks (160 mL), which were incubated in a shaker at 115 rpm in a dark climate-controlled room at 30±2˚C. The re-suspended anammox biomass was added to the assays at 5% (v:v) for SEC and 0.6 g VSS/L for GEC. Serum flasks were supplied with 100 mL basal mineral medium (pH 7.4-7.5), containing bicarbonate (2.5 g/L) as the only added carbon source as described above, except specific conditions mentioned. The flasks for the anaerobic assays were sealed with rubber stoppers, and then the medium and headspace were purged with He/CO_2 (80/20, v/v) for 20 min to exclude elemental oxygen (O_2) from the assay. All assays were conducted in duplicate. Headspace samples were analyzed periodically for molecular nitrogen (N2) with a pressure lock gas tight syringe (1710RN, 100 μL (22s/2″/2), Hamilton Company, Reno, Nevada USA) to monitor the anammox activity. Flushed headspace controls incubated with just water were monitored to ensure that background levels of N_2 were low. Liquid samples were extracted at the beginning and at the end of the test for analysis.

Figure 4 shows the bottles where the batch experiments were carried out.

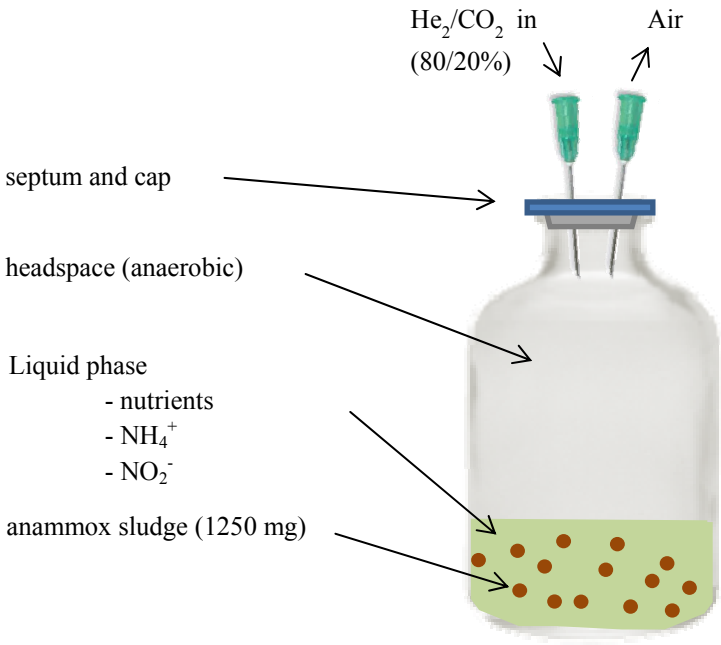

Figure 4: Set-up used in the batch bioassays.

3.3.1 Activity Tests

The medium used for the activity tests was also supplemented with a stoichiometric mixture (1.32:1 mol NO_2^-: mol NH_4^+) of NO_2^- (3.57 mM) as the electron acceptor and NH_4^+ (2.71 mM) as electron donor, except specific concentrations described.

The activity is calculated with the maximum slope of nitrogen removal divided by the time and the gVSS (N_2-N- mmol/h g VSS).

3.3.2 Kinetic Tests

These batch experiments were made with the granular sludge (3.75 g in each bottle) and also with the flocculent sludge (50 mL in each bottle). The sludge samples

were obtained from the different anammox reactors. The bottles were incubated all the time at 30ºC with a mix rate of 200 r.p.m.

3.4 Membrane Bioreactor Operation

The MBR is a cylindrical reactor with a total capacity of 7 L (16 cm inner diameter and 24 cm height). The reactor is in a room at a controlled temperature of 30ºC. The reactor was covered to protect anammox bacteria from light and was sealed to maintain the anaerobic conditions. The liquid volume was 5 L and the headspace was flushing continuously with He/CO_2 (80/20, v/v) to maintain the anaerobic conditions. The MBR had a submerged hollow fiber membrane module of curtain shape. The hollow fiber membrane was made of PVDF, had a pore size of 80 nm and a total area of 0.1 m^2. The membrane was arranged in the center of the reactor to ensure the complete biomass retention. The length of the membrane was 21 cm and it is from the company PALL (Port Washington, NY, USA). The membrane was cleaned two times at month. The steps are:

- Membrane replacement

  Some of the sludge inside the membrane bioreactor is adhered to the membrane. Once a week the membrane of the reactor has to be replaced for cleaning. Always the membrane must be in a liquid because is hydrophobic. If the membrane gets dry, the fibers can break.

  To remove the membrane:

  - Stop the agitation.
  - Put down the agitator and remove the agitator head (the out tube can be tense).

- Remove the top of the reactor.
- Remove the membrane and replace with a clean one. The dirty membrane is place in a volumetric flask with water.
- In basal medium the dirty membrane is flushed with He/CO^2 for 20-30 min. The gas helps to take off the sludge from the fibers.
- After flushing the membrane, very carefully with a finger remove as much sludge as you can.
- The sludge is returned to the reactor.
- Prepare dissolution of 2% NaOH with sodium hypochlorite (3 mL/L). The membrane has to be submerged in the dissolution for 3 h.
- After the 3 h, aerate the membrane for 5 min.
- Clean the membrane with MilliQ water.
- Introduce the membrane during 1.5 h in citric acid dissolution (1.5%).
- Clean the membrane with demineralized water.
- Store the membrane in a dissolution containing 1g KCl/L until its use.

An scheme of the reactor is shown in Figure 5 and a picture of the membrane module utilized is shown on Figure 6.

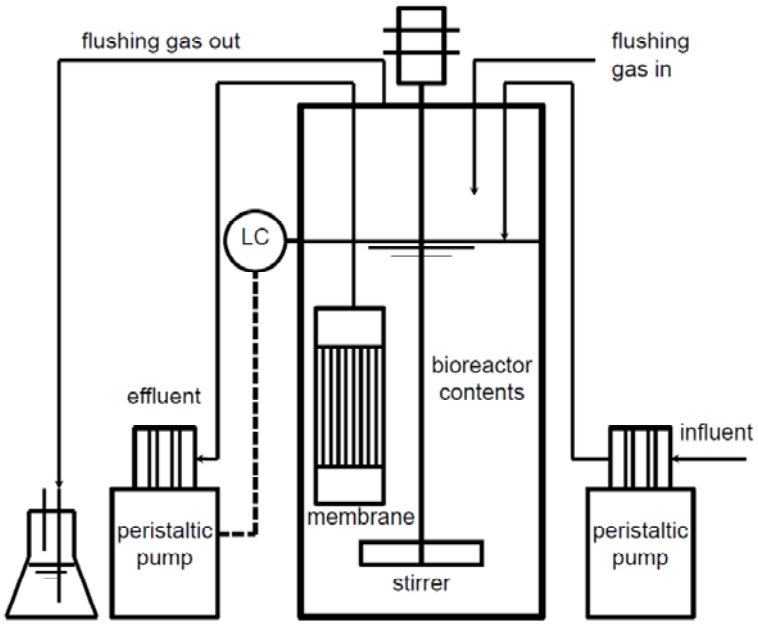

Figure 5: Diagram of the membrane bioreactor used in this study.

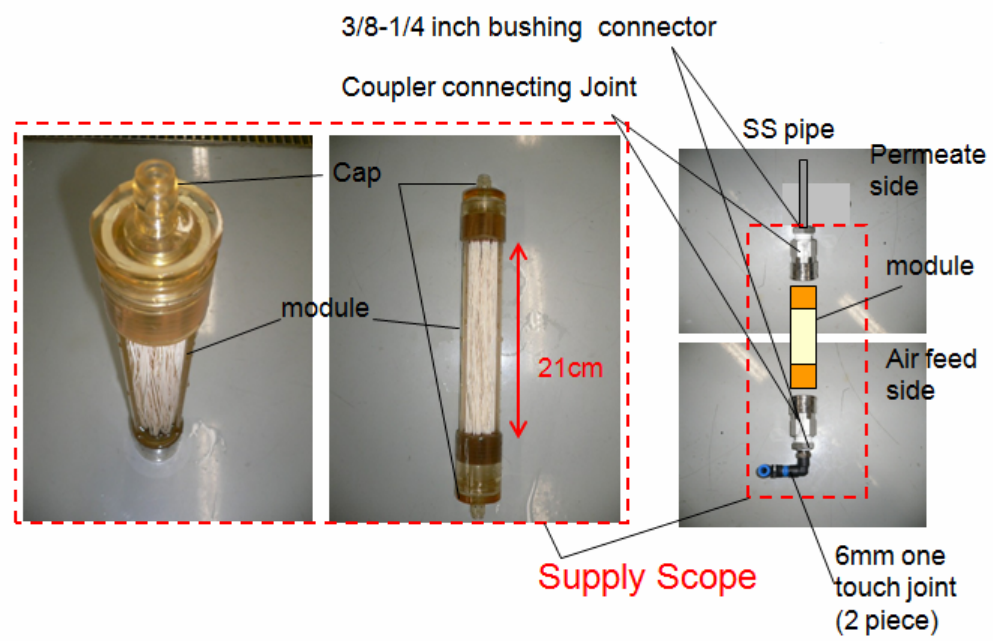

Figure 6: Characteristics of the membrane modules used in the membrane bioreactor.

The MBR was continuously fed with the synthetic wastewater using a peristaltic pump and, in the same way, the permeate was sucked up via the hollow fiber membrane module. The MBR was operated in the mode of constant flux. The MBR has a stirrer that worked at a speed of 100 rpm to keep biomass suspended as free cells. The MBR was operated with the following conditions: hydraulic retention time (HRT) of 1.5 d, temperature of 30ºC, and the pH value was controlled at around 7.2 to 7.5. Initially, the medium concentrations of NH_4HCO_3 and $NaNO_2$ were both set to around 50 mg N/L and the N-loading rate was increased by increasing the concentrations of NH_4HCO_3 and $NaNO_2$ in the feed vessel. The synthetic wastewater was prepared two times at week to avoid the changes in feed composition due to biological activity or other influencing factors. Samples from the influent and effluent (inside the reactor) were taken daily. The samples were centrifuged for 10 min to remove the possible biomass.

The reactor was inoculated with the dispersed anammox biomass enriched at very low concentrations (< 10 mg VSS/L). For 300 days the reactor worked with different concentrations of NH_4^+ and NO_2^- in the influent and also different loading rates. The influent and effluent pumps were checked every month. The pH, NH_4^+, NO_2^- and the flow were analysed every day. The membrane and the pressure were checked every two weeks. Samples of the reactor suspension were taken for activity assays, DNA isolation and quantification (this is not analize yet), and volatile suspended solids (VSS) and total suspended solids (TSS) analysis.

3.5 Operation of Up Flow Granular Reactors

The SBR are cylindrical reactors with a total capacity of 450 mL (16 cm inner diameter and 24 cm height). The reactors were located in an incubator at 30ºC. The

incubator was all the time without light except when taking the samples to prevent the growth of photosynthetic organisms. The reactors were sealed to maintain the anaerobic conditions. The liquid volume was 400 mL. Anammox granular sludge was used as inoculum (15 g wet sludge).

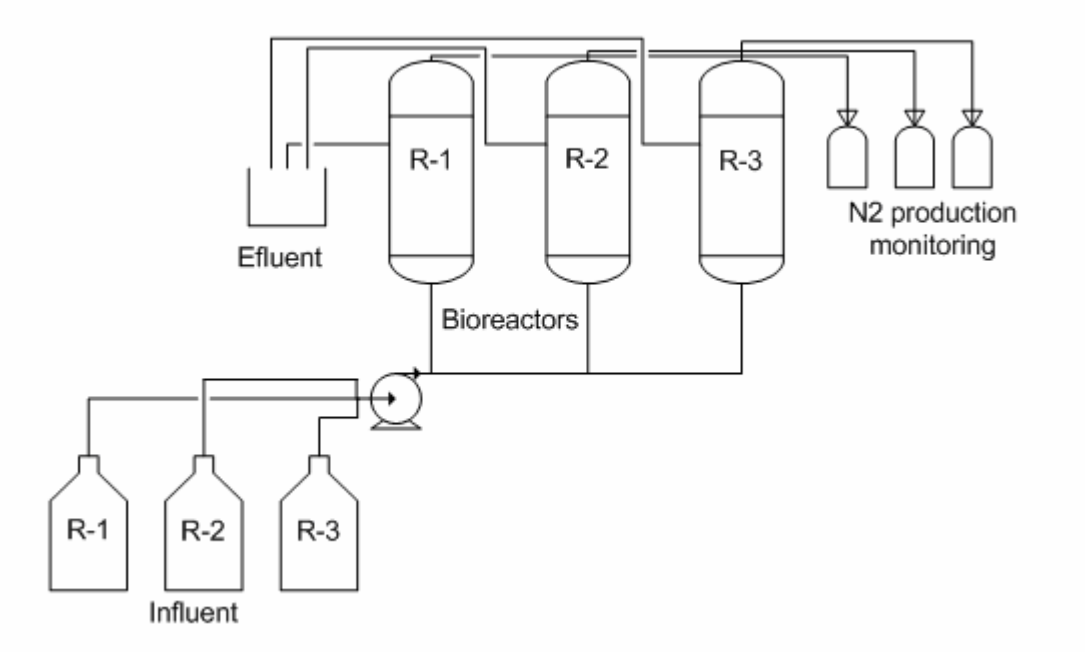

Figure 7: Diagram of the upflow anaerobic bioreactors utilized in this study.

Three different granular upflow reactors were used to study nitrite inhibition. The up flow reactors were continuously fed with the synthetic wastewater by means of a peristaltic pump, and operated under a constant flux. The reactors were not stirred. The upflow reactorswere operated at the following conditions: hydraulic retention time (HRT) of 0.23 d, temperature of 30ºC, pH was controlled at around 7.2 to 7.5. Initially, the medium concentrations of NH_4HCO_3 and $NaNO_2$ were both set to around 50 mg N/L and the N-loading rate was increased by increasing the concentrations of NH_4HCO_3

and $NaNO_2$ in the feed vessel. The synthetic wastewater was replaced two times at week to avoid the changes in feed composition due to biological activity or other influencing factors.

For 2 months the three reactors were working under the same conditions. Then, in the first reactor (R1) the fed was maintained and the reactor operated as the control one, the second reactor (R2) was fed with an excess of nitrite (30%) and the third reactor (R3) with a higher nitrite excess (50%).

The influent pump was checked every month. The pH, NH_4^+, NO_2^-, N_2, and the flow were analysed every day. Samples of the reactor suspension were taken for activity assays, DNA isolation and quantification, and VSS and TSS analysis.

3.6 Analytical Methods

NO_3^- and NO_2^- were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS18 analytical column (4 x 250 mm) and an AG18 guard column (4 x 50 mm). NH_4^+ was determined using Mettler Toledo SevenMulti ion selective meter with selective NH_4^+ electrode (Mettler Toledo, Columbus, OH, USA). N_2 were analyzed using a Hewlett Packard 5890 Series II gas chromatograph fitted with a Carboxen 1010 Plot column (30 m x 0.32 mm) and a thermal conductivity detector. Other analytical determinations (e.g., pH, TSS, VSS, etc.) were conducted according to Standard Methods (APHA, 1999).

3.7 Chemicals

The chemical used in the experimental work are shown in the Table 3

Table 3: Chemicals used

FORMULA	PURITY	COMPANY
NH_4HCO_3	98%	ACROS
$NaNO_2$	99%	ACROS
$NaH_2PO_4•H_2O$	99%	Fisher Scientific
$CaCl_2•2H_2O$	70%	Fisher Scientific
$MgSO_4•7H_2O$	100%	Fisher Scientific
$NaHCO_3$	99%	Fisher Scientific
$FeSO_4$	99%	Sigma
EDTA	99%	Spectrum
$ZnSO_4•7H_2O$	99%	EM Science
$CoCl_2•6H_2O$	98%	Mallinckrodt
$MnCl_2$	98%	Sigma-Aldrich
$CuSO_4•5H_2O$	98%	Sigma-Aldrich
$Na_2MoO_4•2H_2O$	99%	Sigma-Aldrich
$NiCl_2•6H_20$	99%	Sigma-Aldrich
$Na_2SeO_4•10H_2O$	98%	Sigma-Aldrich
H_3BO_3	98%	Sigma-Aldrich
$NaWO_4•2H_2O$	99%	Sigma-Aldrich

RESULTS

AND

DISCUSSION

4. RESULTS AND DISCUSSION

Three different experiments were performed. The results are shown below.

4.1 Batch experiments

Batch experiments were carried out for two main reasons. The first is to characterize the metabolic activity and kinetics of the anammox process. The second is to evaluate the effects of varying pH and sulphide levels on anammox sludge activity.

4.1.1 Characterization of anammox sludge

4.1.1.1 Activity test

In the activity test ammonium and nitrite were in the stoichiometric ratio. The production of N_2 gas was measured. Figure x represents the nitrogen production during the experiment with granular sludge. The maximum specific anammox activity is 4.65 N_2-N- mmol/h g VSS.

Figure 7 represents the nitrogen production and the nitrite and ammonium consumption in the flocculent sludge. The maximum specific anammox activity is 4.15 N_2-N- mmol/h g VSS.

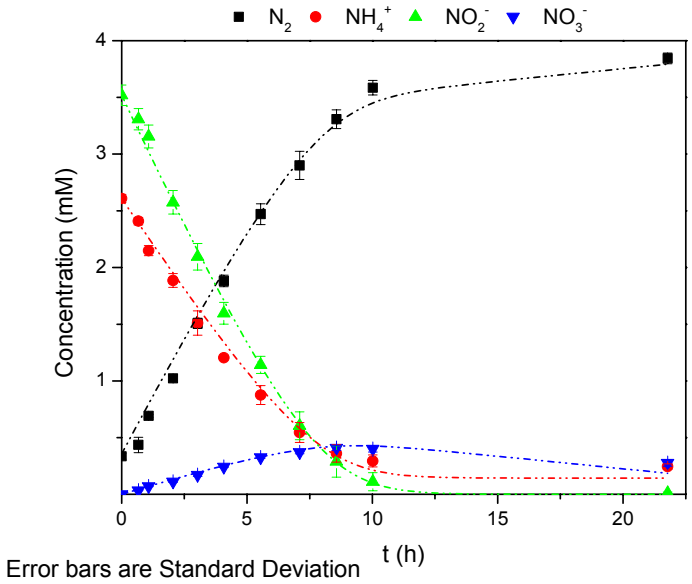

Figure 7: N_2 production in anammox activity test of granular sludge

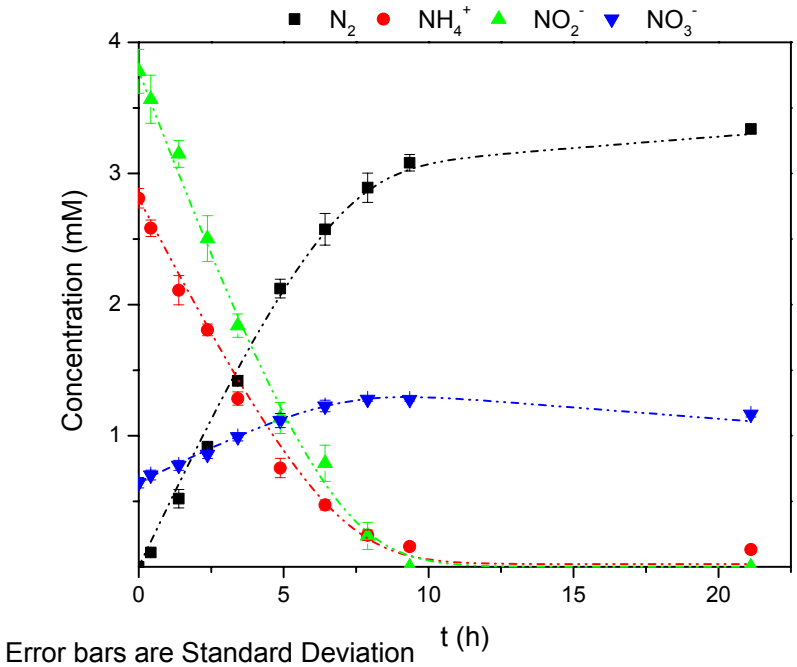

Figure 8: N_2 production in Anammox activity test of flocculent sludge

4.1.1.2 Kinetics

Monod model (Eq. (10)) is the most popular model to describe the kinetics of pollutant biodegradation (Block, et al,. 1996; Goudar, et al., 2001). The maximum substrate conversion rate (q_{max}) and half saturation constant (K_s) can be obtained according to Monod model. The model is represented as follows:

$$q = \frac{\left(dS/dt\right)_u}{X} = \frac{Q(S_0 - S_e)}{VX} = \frac{q_{\max} S}{K_s + S} \qquad \text{[Eq. 10]}$$

where:

q, q_{max} – substrate conversion rate and the maximal substrate conversion rate, respectively (1/d);

X – the concentration of biomass in reactor (mg/L);

K_s – half saturation constant (mg/L); and

S – substrate concentration (mg/L).

The anammox process uses nitrite and ammonium to produce nitrogen gas and nitrate (Eq. 9). To know the kinetic of the process the results of this experiment were analyzed with the computer program Scientist. The Monod equation for each compound is:

$$\frac{d[NH_4^+]}{dt} = -V_{NH_4^+ \max} \frac{[NH_4^+]}{K_{S\,NH_4^+} + [NH_4^+]} \cdot \frac{[NO_2^-]}{K_{S\,NO_2^-} + [NO_2^-]} \quad \text{[Eq 11]}$$

$$\frac{d[NO_2^-]}{dt} = -V_{NO_2^- \max} \frac{[NO_2^-]}{K_{S\,NO_2^-} + [NO_2^-]} \cdot \frac{[NH_4^+]}{K_{S\,NH_4^+} + [NH_4^+]} \quad \text{[Eq 12]}$$

$$\frac{d[N_2]}{dt} = V_{N_2 \max} \frac{[NH_4^+] \cdot [NO_2^-]}{\left(K_{S\,NH_4^+} + [NH_4^+]\right) \cdot \left(K_{S\,NO_2^-} + [NO_2^-]\right)} + V_{desn} \quad \text{[Eq 13]}$$

$$\frac{d[NO_3^-]}{dt} = V_{NO_3^- \max} \frac{[NH_4^+] \cdot [NO_2^-]}{\left(K_{S\,NH_4^+} + [NH_4^+]\right) \cdot \left(K_{S\,NO_2^-} + [NO_2^-]\right)} - V_{desn} \quad \text{[Eq 14]}$$

A first run of assays, performed in triplicate, was carried out to assess the accuracy of the method to estimate the specific anammox activity (SAA) with an excess of nitrite (2.7 mM NH_4^+, 3.95 mM NO_2^-). In this run, consumption and production of nitrogenous compounds in the liquid and gas phases, respectively, was evaluated (Experiments 11-13; Figure 7).

A second run of assays, also ran in triplicated, was carried out to study the consumption and the production of the elements in the reaction of the anammox with the granular sludge. This second run had an excess of ammonium tested (3.0 mM NH_4^+, 3.57 mM NO_2^-). In this run, consumption and production of nitrogenous compounds in the liquid and gas phases, respectively, was evaluated (Experiments 21-23; Figure 8).

The runs were repeated with the flocculent sludge, the batch experiment procedure was similar to that previously described in the first and second run. The results are shown in Figure 8. The reason to use an excess of ammonium or nitrite was to obtain the saturation constant that is easy to get if the final concentration is not zero. In the runs with ammonium excess, not all the ammonium was consumed, and in the runs with nitrite excesses not all the nitrite is consumed.

Table 4: Conditions in the kinetic experiment for the granular sludge

	RUN 1					
	11	12	13	21	22	23
SLUDGE	Granular	Granular	Granular	Granular	Granular	Granular
$[NO_2^-]$	3.95	3.95	3.95	3.57	3.57	3.57
$[NH_4^+]$	2.7	2.7	2.7	3.0	3.0	3.0

Table 5: Conditions in the kinetic experiment for the flocculent sludge

	RUN 2					
	11	12	13	21	22	23
SLUDGE	Flocculent	Flocculent	Flocculent	Flocculent	Flocculent	Flocculent
$[NO_2^-]$	3.95	3.95	3.95	3.57	3.57	3.57
$[NH_4^+]$	2.7	2.7	2.7	3.0	3.0	3.0

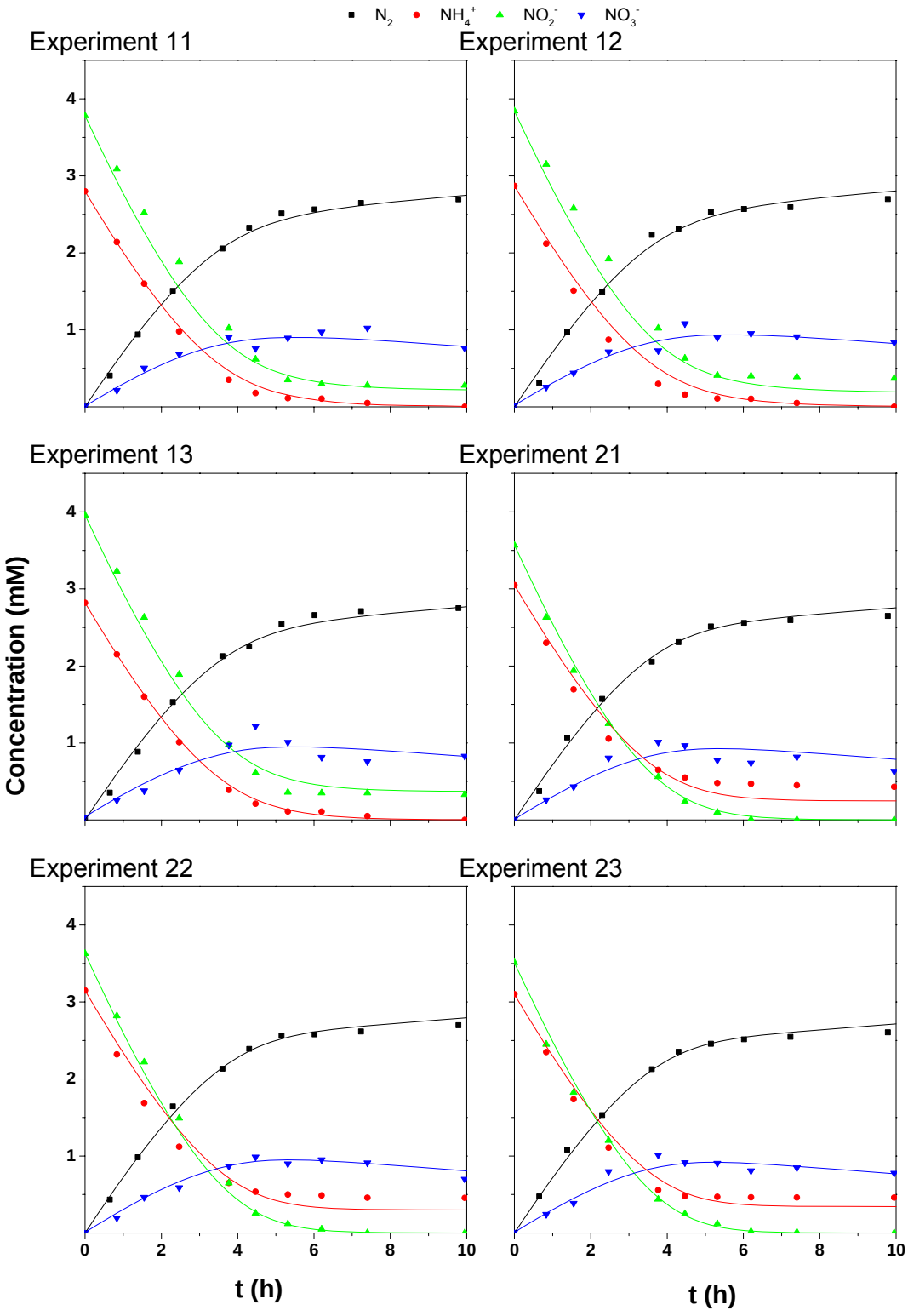

Figure 7: Kinetics of anammox process using granular sludge. ■ N2, ♦ NH_4^+, ▲NO_2^- , ▼ NO_3^-

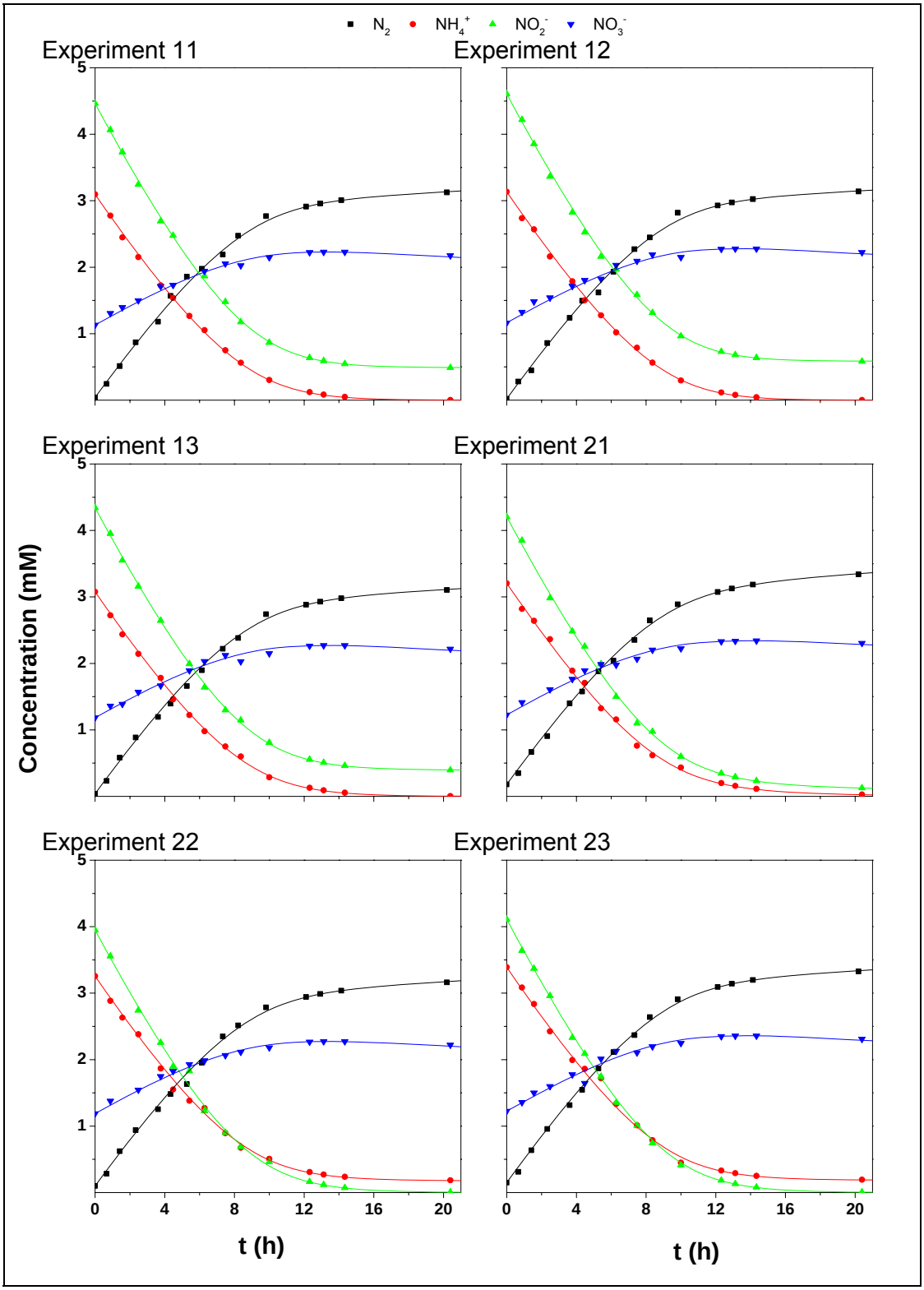

Figure 8: Kinetics of anammox process using flocculent sludge. ■ N_2, ♦ NH_4^+, ▲NO_2^- , ▼ NO_3^-

The values obtained for the Monod parameters are:

Table 6: Fitting parameters of experimental data to the model represented in Eq 10

Parameter	Granular sludge	Flocculent sludge
VNH_4^+max (mmol/ g VSS h)	0.7018 ± 0.0005	0.6756 ± 0.0072
VNO_2^-max (mmol/g VSS h)	0.8939 ± 0.0006	0.8666 ± 0.0096
VN_2max (mmol/g VSS h)	0.5945 ± 0.0004	0.6108 ± 0.0078
VNO_3^-max (mmol/g VSS h)	0.2901 ± 0.0002	0.2883 ± 0.0054
Vdesn (mmol/ g VSS h)	0.0238 ± 0.0000	0.0204 ± 0.0019
K_S NH_4^+ (mM)	0.680 ± 0.151	0.544 ± 0.043
K_S NO_2^- (mM)	0.339 ± 0.100	0.375 ± 0.041
R^2	0.996	1.00

With this batch experiment can be observed that the flocculent sludge has high speeds with low ammonium concentrations. However, the granular sludge works better with low nitrite concentrations.

In this experiment both the ammonium and the nitrate were evaluated. Up to our knowledge there is not any similar experiment in the literature because usually only one of the parameters is evaluated. Trimmer, et al., (2004) have reported *Ks* NO_2^- values for anammox microorganisms as low as 2.5 μM. However, in their experiment very low concentrations of nitrite and ammonium were used (between 1 and 20 μM). For a good application of the Monod model, higher concentrations are needed. Other authors have reported *Ks* NO_2^- values of 0.54 mM (Jaroszynki, et al., 2012) or 0.20 mM (Shou-Qing, et al., 2012), that are similar to the one determined in this study (0.34 mM). For the analysis of anammox kinetics in continuous bioreactors, models as Monod, Contois,

Stover Kincannon or Grau have been used (Shou-Qing, et al., 2010). The best is the Stover Kincanno (Trimmer, et al., 2004).

4.1.2 Effects of pH and sulphide on Anammox activity

4.1.2.1 pH

The pH of the medium was modified by the addition of different amounts of sodium bicarbonate. As shown in Figure 9, the anammox activity was almost constant in the pH range from 7.2 to 7.5 with a maximum activity at a pH of 7.3. Out of this pH range a serious decrease in activity occurred. The activity was almost completely inhibited at pH 7.9 and below 7.0 there was also a dramatic activity decrease.

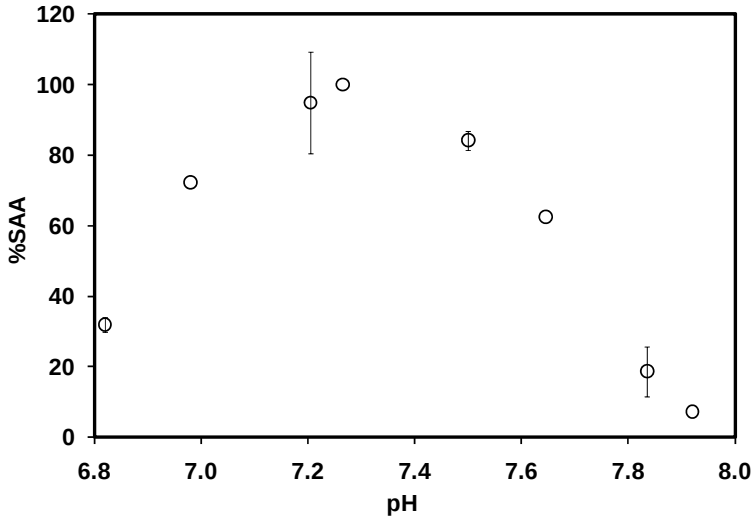

Figure 9: Effect of pH on anammox activity

A batch experiment was conducted to evaluate the impact of maintaining a pH value of 6.6 for 12 h on the activity of the anammox biomass. The experiment consisted in four bottles (two were the control and the other two were the run 1 and 2). The control worked at pH 7.2 during all the experiment. The other two bottles were incubated at pH

6.6 for 12 h, and after this time, some sodium bicarbonate was added to adjust the pH to 7.3. The nitrogen production was measured at different times. In Figure 10 it can be observed that after 12 h the anammox process produced some nitrogen gas, so little by little the process recovered the activity (the activity after 27 h was 1.25 N_2-N- mmol/h g VSS), but this was much lower than that in the control (the activity in the control after 27 h was 6.29 N_2-N- mmol/h g VSS). So, only 23.15% of the activity was recovered.

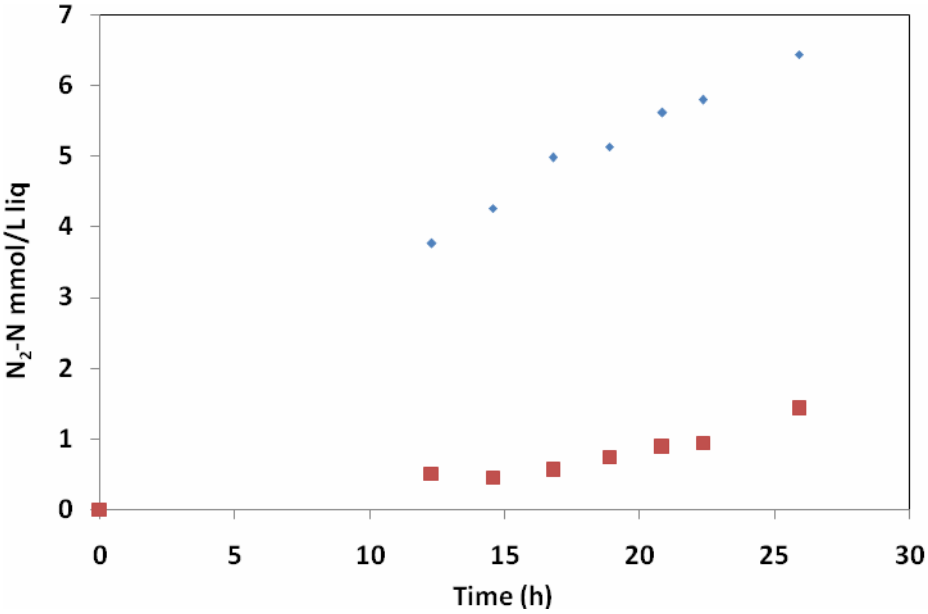

Figure 10: nitrogen production. The diamonds represent the control and the squares represent the experiment.

If in the anammox process the pH value is lower than the optimum during some hours the activity decrease, but increasing the pH to the optimum the process can partially recover the activity.

4.1.2.2 Inhibitory effect of H_2S

From an engineering point of view, the importance of the anammox process is related to its application for nitrogen removal from wastewaters. It is known that the use of anammox process combined with partial nitrification would lead to an important reduction of operational costs compared to conventional nitrification–denitrification processes. This process is applicable to wastewaters characterized by low carbon to nitrogen ratio content and high ammonia concentrations. Furthermore, it is common that these streams also contain compounds that may inhibit the Anammox process such as chloride, sulphide, sulphate. Therefore, to study the possible toxicity of the wastewater is necessary to know the feasibility of this treatment.

The effect of sulphide on SAA was tested because SO_4^{2-} reduction takes place quite often in anaerobic digesters, mainly transforming it into H_2S. Concentrations of sulphide between 1 and 2 mM have been reported to caused a decrease of 60% of SAA, and SAA was absent at concentrations of sulphide above 5 mM (Dapena Mora, et al., 2006). These results disagree with those of van de Graaf et al. (1996) who found stimulation of the activity in both batch and continuous assays. These authors used nitrate as electron donor for the Anammox biomass and explained their results considering that sulphide could reduce nitrate to nitrite, which is the authentic electron donor of the process. These results suggest that an effective operation of the previous treatment stage is necessary to cause the oxidation of organic matter and sulphide, preventing their entry into the anammox reactor.

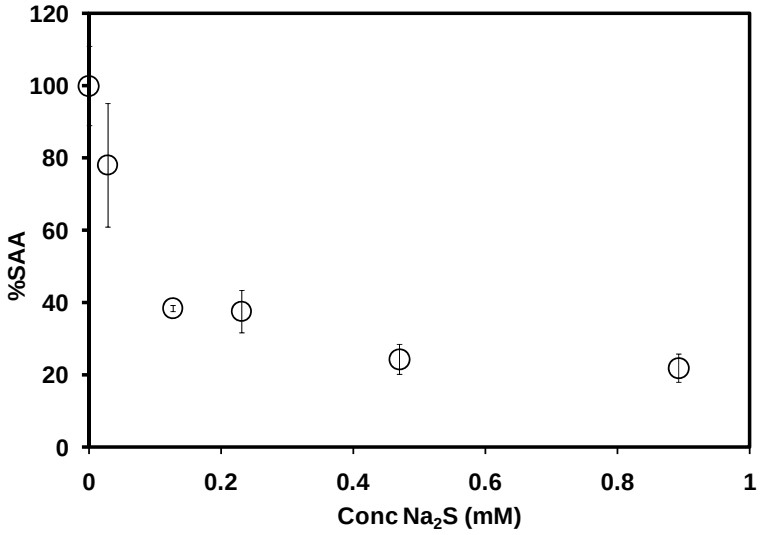

Figure 11: Effect of sulfide on Anammox activity (Dapena-Mora, et al,. 2006)

As the H_2S can be precipitated with ferrous iron (Fe^{2+}), in this study was added iron with H_2S to observe the influence on the activity of the anammox process. The experiment consisted of seven different runs (each run were duplicated). The control run is sludge with the NH_4^+/NO_2^- in stoichiometric ratio. The abiotic run did not have sludge. Operational conditions of the work are shown on Table 7.

Table 7: Conditions used in the batch experiment inhibitory effect of H_2S

Bottle	Treatment Name	Sludge (g wet sludge)	Sludge Type	NO_2^- (mM)	NH_4^+ (mM)	Fe^{2+} (mM)	H_2S (mM)
Control	C1	1.25	Granular	1.07	2.37	0.0	0.0
	C2	1.25	Granular	1.07	2.37		
Abiotic $H_2S+NO_2^-$	A1	-	-	1.07	2.37	0.0	0.5
	A2	-	-	1.07	2.37		
FeTox	Fe1	1.25	Granular	1.07	2.37	0.79	0.0
	Fe2	1.25	Granular	1.07	2.37		
H_2S	1	1.25	Granular	1.07	2.37	0.0	0.5
	2	1.25	Granular	1.07	2.37		
H_2S+Fe 50	3	1.25	Granular	1.07	2.37	0.33	0.5
	4	1.25	Granular	1.07	2.37		
H_2S+Fe 100	5	1.25	Granular	1.07	2.37	0.66	0.5
	6	1.25	Granular	1.07	2.37		
H_2S+Fe 120	7	1.25	Granular	1.07	2.37	0.79	0.5
	8	1.25	Granular	1.07	2.37		

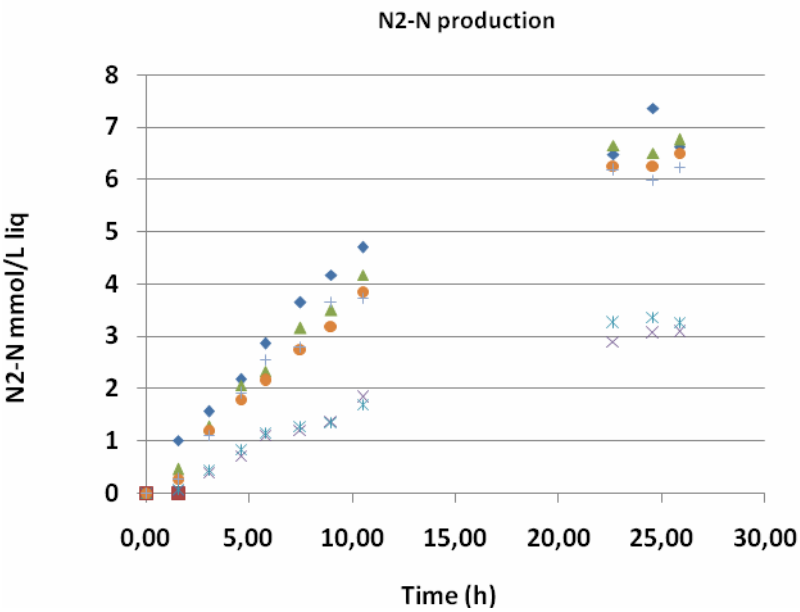

Figure 12: Nitrogen production with different concentrations of Fe and H_2S. Control (♦), abiotic (■), Fe tox (▲), H_2S (x), H_2S+Fe 50 (*), H_2S+Fe 100 (●), and H_2S+Fe 150 (+)

As the Figure 11 shows, the H_2S decrease the activity in the anammox process (65%), so it can inhibit the process. Figure 12 shows that the activity of the anammox decreased when low concentrations of iron were added. This is because not all the H_2S is precipitate and cause inhibition in the process. However, when the concentration of iron is enough to precipitate all the H_2S the anammox activity is the same as the control (the one operated without H_2S and Fe). Fe^{2+} can be used to precipitate the H_2S and removed the toxicity in the process.

4.2 Anammox Process in a Membrane Bioreactor (MBR)

The MBR was inoculated with a low concentration (< 10 mg VSS/L) of an Anammox enrichment culture obtained from an activated sludge (Figure 12A). The reactor was fed continuously with synthetic wastewater whose characteristics are described in chapter 3. The reactor was operated for more than 250 d. Figure 12B shows the external aspect of the bioreactor after 230 d of operation; the orange color is typical from Anammox bacteria.

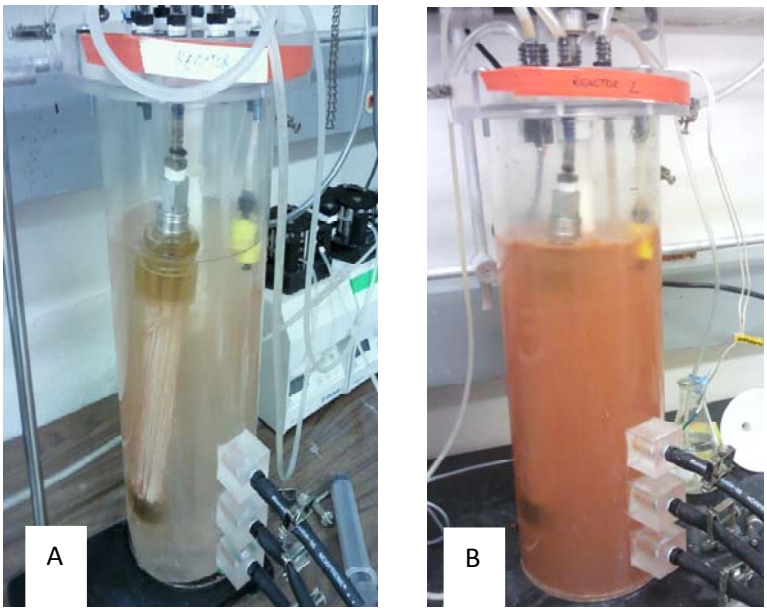

Figure 12: Membrane bioreactor: (A) 3 days after inoculation and after 230 days of operation (B).

During the operation of the reactor seven different periods, named A to G, can be distinguished according to feed characteristics and HRT applied. Initially the HRT was

set at 1.5 d (periods A-C), but later it was decreased to 0.9 d (period D) and finally to 1 d (periods F and G) (Figure 13).

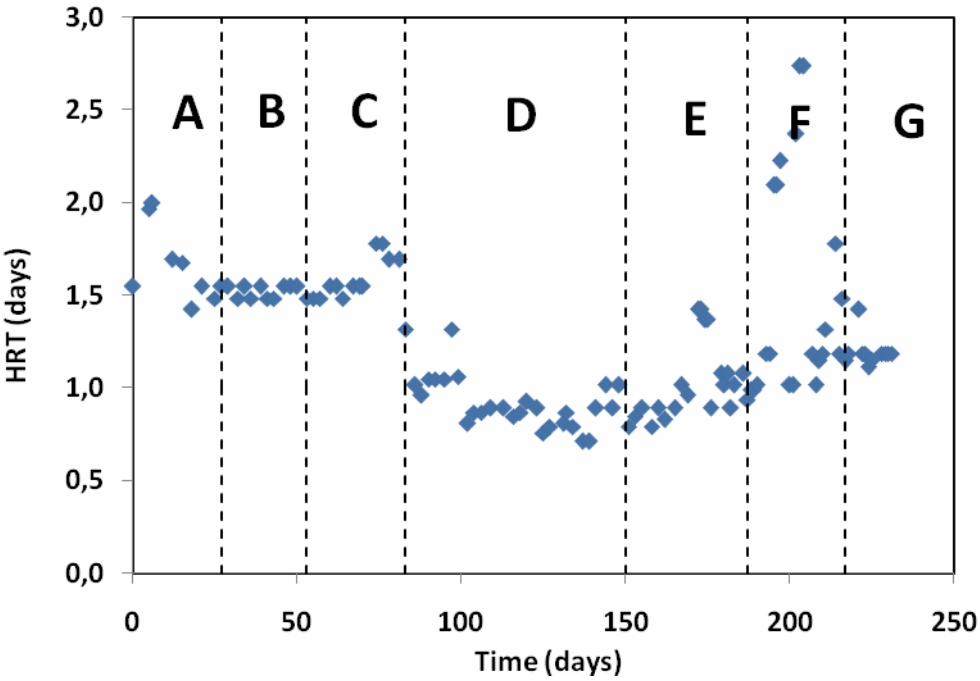

Figure 13: Evolution of the hydraulic retention time in the MBR.

A summary of the feed characteristics for each period and removal efficiencies are presented in Table 8.

Table 8: Conditions of operation of the MBR during the various experimental periods.

PERIOD	NO_2^- in	NO_2^- out	NH_4^+ in	NH_4^+ out	HRT	Volumetric load	NO_2^- removed	NH_4^+ removed
	mM				Days	mmole N-removed/(L d)	%	%
A (27 d)	7	0.06	7.0	1.0	1.5	8.6	99.1	85.7
B (26 d)	11	4.0	10.5	3.9	1.5	9.1	63.6	62.9
C (30 d)	8	4.0-0.06	8.0	1.0-3.9	1.5		50.0-99.3	51.3-87.5
D (104 d)	8	0.06	8.0	1.5	0.9	16.04	99.3	81.3
E (30 d)	9	0.06	9.0	1.5	0.9	18.27	99.4	83.3
F (30 d)	12	0.06	12.0	1.5	1.0	22.44	99.5	87.5
G (40d)	14	0.06	14.0	1.5	1.0	26.44	99.6	90.0

Note: the volumetric load (in mmole N-removed/(L d)) was calculated based on the removal of ammonium and nitrite.

The pH in the reactor was controlled at 7.2–7.5, by adding 3.75 g/L of sodium bicarbonate in the influent, in order to satisfy the strict requirement of Anammox growth and metabolism. The pH evolution in the continuous experiment can be observed in Figure 14.

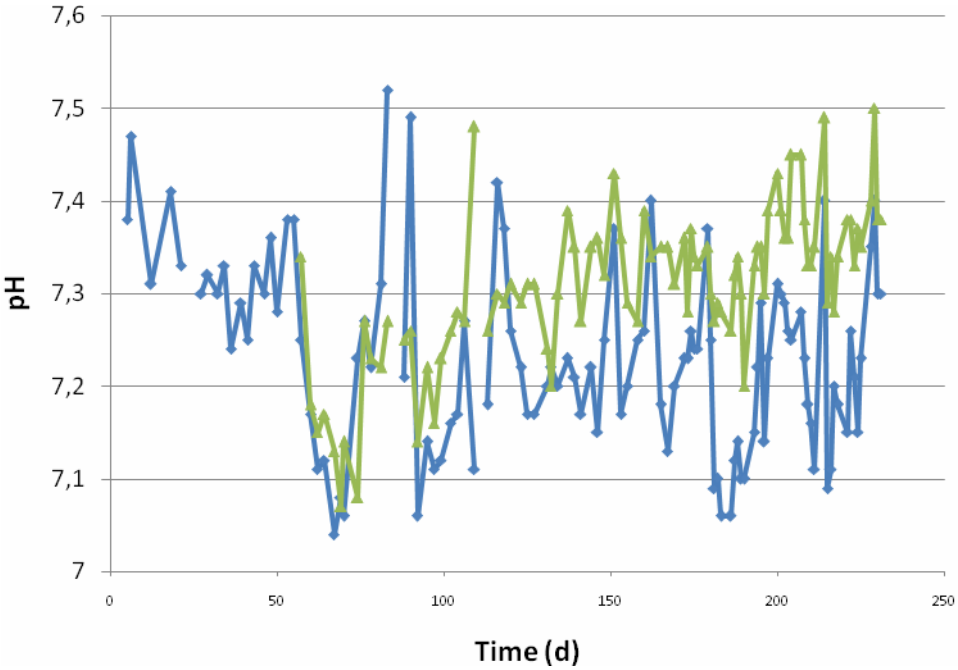

Figure 14: pH evolution. Influent (■) and effluent (▲).

As the anammox process may be inhibited when concentrations of nitrite-N are higher than 70 mg/L (1.52 mM) (Jetten et al., 1999), the ratio NH_4^+/NO_2^- in the influent of the MBR was higher than the stoichiometric ratio to prevent nitrite accumulation in the reactor and the possibility of inhibition by nitrite of the anammox process.

The evolution of nitrite and ammonium during the six phases in which the operational period has been divided is shown in Figures x and y. During Phase A, that can be considered the start-up period, the influent concentration of NO_2^- and NH_4^+ was 7 mM. In this period the concentrations of nitrite and ammonium in the effluent were stable and around 0.06 and 1.0 mM, respectively. The removal percentage was 99.1% for nitrite and 85.7% for ammonium.

After 27 days of operation, the concentration of NH_4^+ in the influent was increased to 11 mM, maintaining the influent nitrite concentration (Phase B). In this period (26 days), a continuous increase of ammonium and nitrite was observed in the effluent. This increase can be attributed to the fact that the process was not stable (the anammox need long start-up periods). At the end of the period the concentration of nitrite in the effluent had reached a value of 4 mM (56 mg/L) and it was decided to decrease the influent concentration to avoid possible inhibition by nitrite.

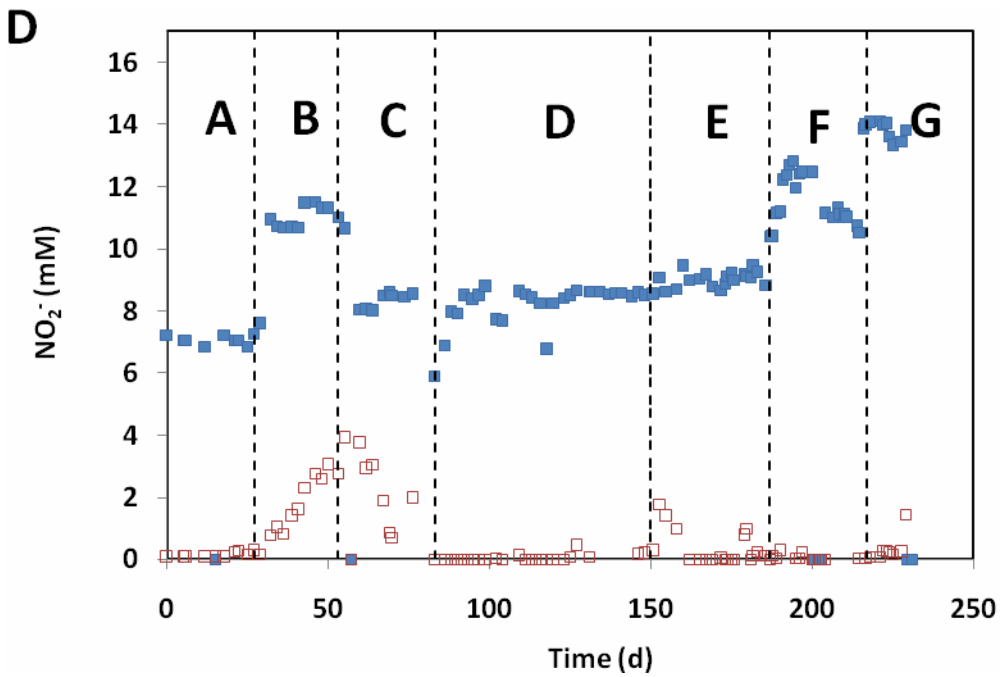

Figure 15: NO_2^- evolution in MBR. ■ Influent, □ effluent

During the phases C and D, the NH_4^+ concentration in the influent was decreased to 8 mM to prevent reactor overloading and facilitate recovery. After 30 days of operation (Phase C), the concentration of nitrite in the effluent was practically zero. Subsequently, in phase D the HRT was decreased to 0.9 days and the reactor operated stably for 104 d. The efficiency was 99.3% and 81.3% for NO_2^- and NH_4^+.

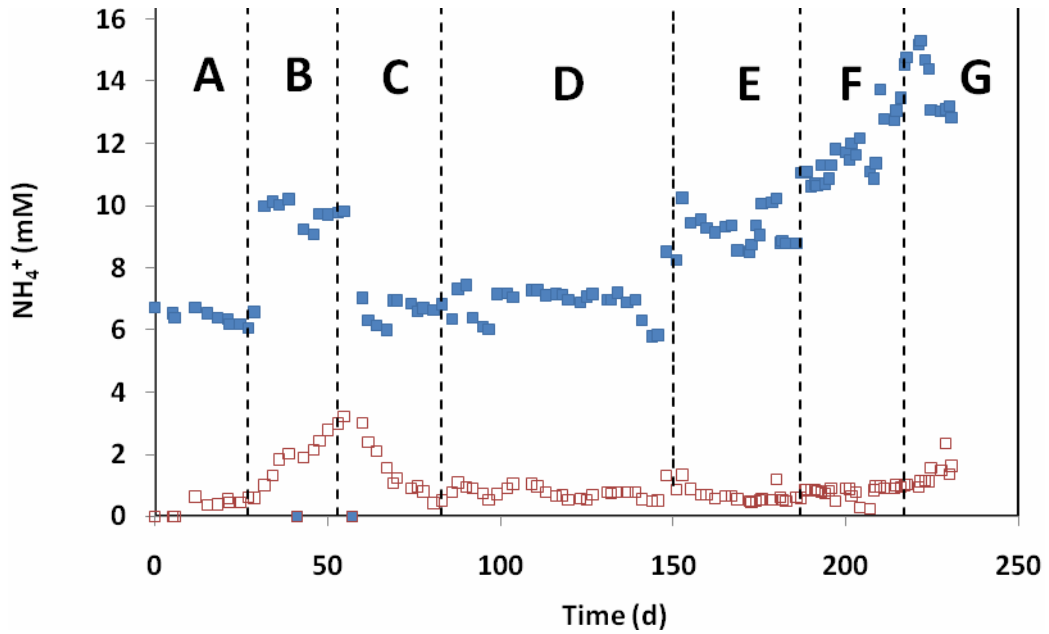

Figure 16: NH_4^+ evolution in MBR. ■ Influent, □ effluent

In phase E the concentration of ammonia and nitrite was increased to the same value as in the phase B to be sure that the start-up period was closed. The effluent concentration of nitrite and ammonium were stable and the removal percentage was the same as in the other phases, 99.3% for nitrite and 83.3% for ammonium. There were some days with a higher retention time. This was because one third of the biomass with liquid in the reactor was removed for some batch experiments. After one day, the biomass was returned to the reactor.

Once the anammox reactor was operating stably, with high removal efficiencies, the concentration of nitrite and ammonia was increased to 12 mM (Phase F). The HRT was

increased from 0.9 to 1.0 d. This phase lasted 30 d, operating stably. The efficiency was 99.5% for nitrite and 87.5% for ammonia.

During the last period of operation (Phase G), the reactor operated for more than 30 d with a concentration in the influent of 14 mM and with the same HRT than in phase F. Figure 15 shows that the NO_2^- concentration in the effluent (approx. 0.06 mM) was slightly lower than the concentration of NH_4^+ (approx. 1 mM) because the ammonium was feed in a stoichiometric excess (the ammonium/ nitrite ratio was 1.2).

The reactor started with a volumetric load of 8.6 mmol N-removed/(L d) (phase A) and actually the load is 26.44 mmol N-removed/(L d) (phase G). In all the periods (except in B and C) the removal efficiency was 99.6% for the nitrite and 89.3% for the ammonia.

During all the periods, the biomass in the effluent was practically zero, so the membrane had a good efficiency. These kinds of reactors are good for slow-growing microorganism because the membrane have a high efficient retention of biomass.

The VSS were analized in different periods of the continuous experiment (Table 9).

Table 9: Biomass concentration in the MBR

PERIOD	DAY	VSS (mg/L)
A	0	10
E	161	402
E	183	644
F	202	769
F	203	776
F	210	893

During the 250 days operating with the reactor, the VSS increased from less than 10 mg to 890 mg VSS/L during the period F.

4.3 Operation in flow upflow granular reactions

In these experiments the influence of the nitrite to ammonium ratio over the Anammox process was studied. Three reactors, whose characteristics were detailed in section 3.5, were operated in parallel and fed with different nitrite to ammonium ratio. The three reactors were inoculated with a concentration of 10 mg VSS/L of a granular anammox sludge (Figure 17) and they were fed continuously with synthetic wastewater whose characteristics are described in chapter 3.2. The stoichiometric ratio in the influent fed to reactor 1 (R1), reactor 2 (R2) had a 30% excess of nitrite and reactor 3 (R3) had a 50% excess of nitrite respectively. The reactors were operated continuously for more than 125 d.

Figure 17: Upflow reactors

During the operation of the reactor five different periods, named A to E, can be distinguished according to feed characteristics. The HRT was set at 0.23 d during all the periods. A summary of the feed characteristics for each period and removal efficiencies is presented in Table 10, 11, and 12.

Table 10: Conditions of operation of R1 during the various experimental periods.

PERIOD	NO_2^- in	NO_2^- out	NH_4^+ in	NH_4^+ out	HRT	N-volumetric removal load	NO_2^- removed	NH_4^+ removed
	mM				Days	mmol N-removed/(L d)	%	%
A (36 d)	4.0	0.2	3.0	0.3	0.23	28.26	95.0	90.0
B (60 d)	4.0	0.2	3.0	0.7	0.23	26.52	95.0	76.7
C (8 d)	4.5	0.2	3.4	0.7	0.23	37.4	95.6	82.5
D (12 d)	5.5	0.2	4.1	0.7	0.23	41.74	96.4	86.0
E (>5 d)	8.0	0.2	6.0	0.7	0.23	56.96	97.5	88.3

Table 11: Conditions of operation of R2 during the various experimental periods.

PERIOD	NO_2^- in	NO_2^- out	NH_4^+ in	NH_4^+ out	HRT	N-volumetric removal load	NO_2^- removed	NH_4^+ removed
	mM				Days	mmol N-removed/(L d)	%	%
A (36 d)	4.0	0.2	3.0	0.30	0.23	28.26	95.0	90.0
B (60 d)	4.0	1.0	2.0	0.06	0.23	24.96	75.0	97.0
C (8 d)	4.5	1.5	2.5	0.06	0.23	21.47	66.7	97.6
D (12 d)	5.5	2	3.0	0.06	0.23	25.83	63.6	98.0
E (5 d)	8.0	2.5	4.0	0.06	0.23	38.87	68.8	98.5

Table 12: Conditions of operation of R3 during the various experimental periods

PERIOD	NO_2^- in	NO_2^- out	NH4+ in	NH4+ out	HRT	N-volumetric removal load	NO_2^- removed	NH_4^+ removed
	mM				Days	mmole N-removed/(L d)	%	%
A (36 d)	4.0	0.2	3.0	0.30	0.23	28.26	95.0	90.0
B (60 d)	4.0	1.5	1.5	0.06	0.23	17.13	62.5	96.0
C (8 d)	4.5	2.0	2.0	0.06	0.23	19.30	55.6	97.0
D (12 d)	5.5	2.5	2.5	0.06	0.23	23.65	54.6	97.6
E (5 d)	8.0	3.0	3.0	0.06	0.23	34.52	62.5	98.0

Note: the volumetric load (in mmole N-removed/(L d)) was calculated based on the removal of ammonium and nitrite.

The pH in the reactors was controlled at 7.2–7.5, by adding 3.75 g/L of sodium bicarbonate in the influent, in order to satisfy the strict requirement of anammox growth and metabolism.

The three reactors were fed with the same concentration of NO_2^-. The evolution of nitrite and ammonium during the six phases in which the operational period has been divided is shown in Figures 18, 19, and 20. During Phase A, that can be considered the start-up period, each reactor was fed with the same influent concentration of NO_2^- and NH_4^+ of 4 and 3 mM, respectively. In this period the concentrations of nitrite and ammonium in the effluent were stable and around 0.2 and 0.3 mM, respectively. The removal percentage was 95.0% for nitrite and 90.0% for ammonium.

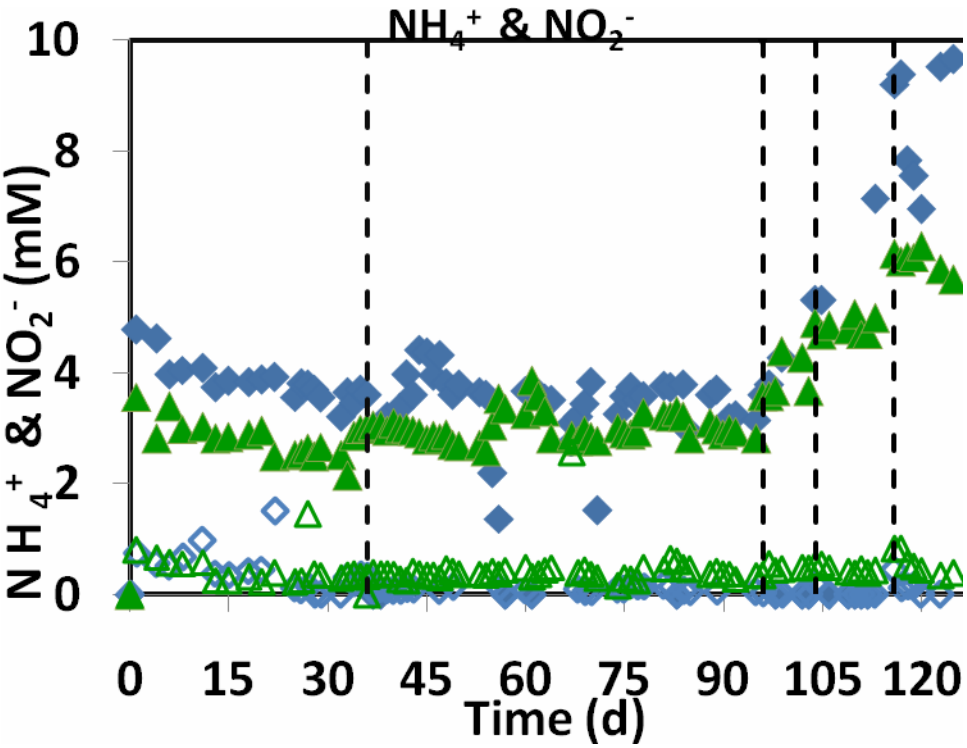

Figure 18: Amonium and nitrite in R1 that was fed an influent containing a 10% stoichiometric excess of NH_4^+. NH_4^+ influent (♦), NH_4^+ effluent (◊), NO_2^- influent (▲), NO_2^- effluent (Δ).

After 36 days of operation, the characteristics of the feed were changed to operate with a different ratio for each reactor (phase B). R1 was operated with the same concentrations as in the phase A (10% over stoichiometric ratio). R2 was operated with a concentration of NH_4^+ in the influent of 2 mM and with the same concentration of NO_2^- as in the previous phase (stoichiometric). The third reactor was operated with the concentration of NH_4^+ in the influent as 1.5 mM. The ammonium removal was 95.0% for the three reactors, but the NO_2^- concentration in the effluent of R2 and R3 was higher than in R1 and depending of the excess that they had, 75.0% of nitrite removal

for R2 and 50.0% of nitrite removal for R3. This period lasted 60 d to stabilize the anammox process.

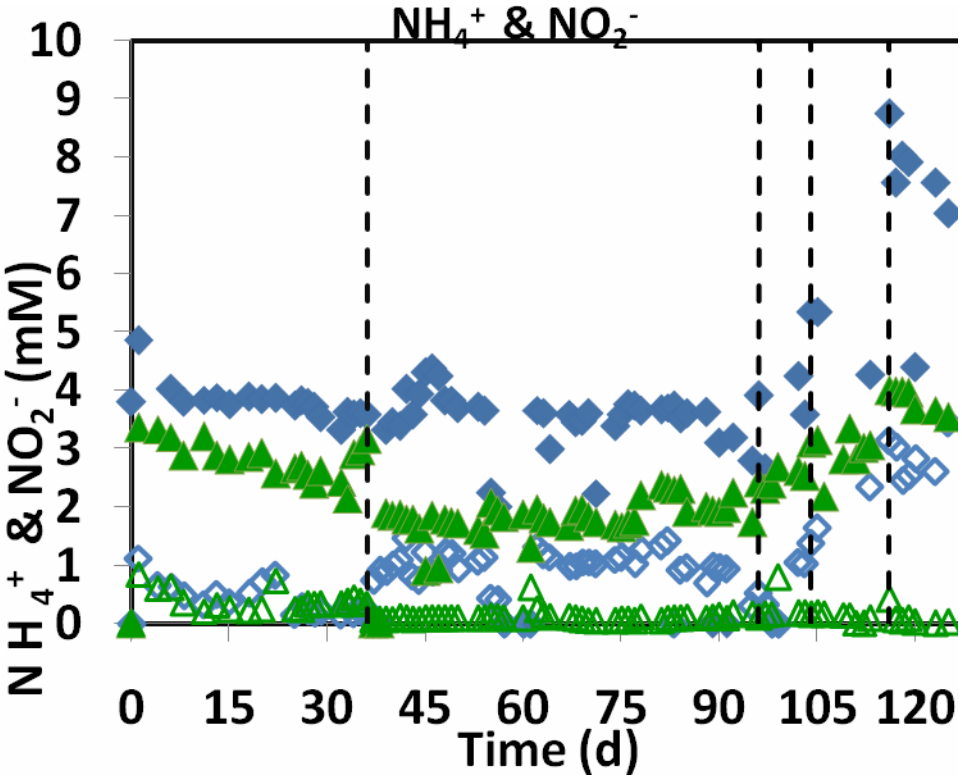

Figure 19: Amonium and nitrite in R2. NH_4^+ influent (♦), NH_4^+ effluent (◊), NO_2^- influent (▲), NO_2^- effluent (Δ)

The three next phases (C, D and E) lasted 8, 12 and 5 days and wanted to determine the influence of an increase in the volumetric load over the inhibition The removal efficiencies in these periods were similar to those obtained in period B. The reactors are still working.

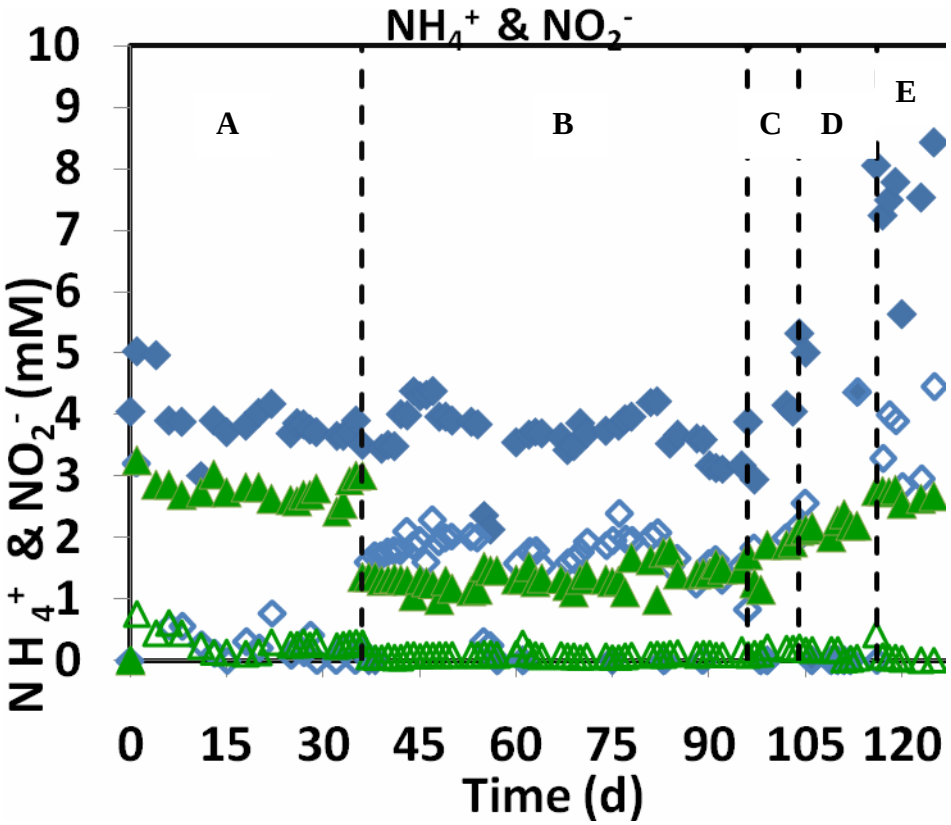

Figure 20: amonium and nitrite in R3. NH_4^+ influent (♦), NH_4^+ effluent (◊), NO_2^- influent (▲), NO_2^- effluent (Δ)

Batch activity tests were performed with biomass withdrawn from the three reactors after 91 d (Figure 21) and after 126 d of operation (Figure 22).

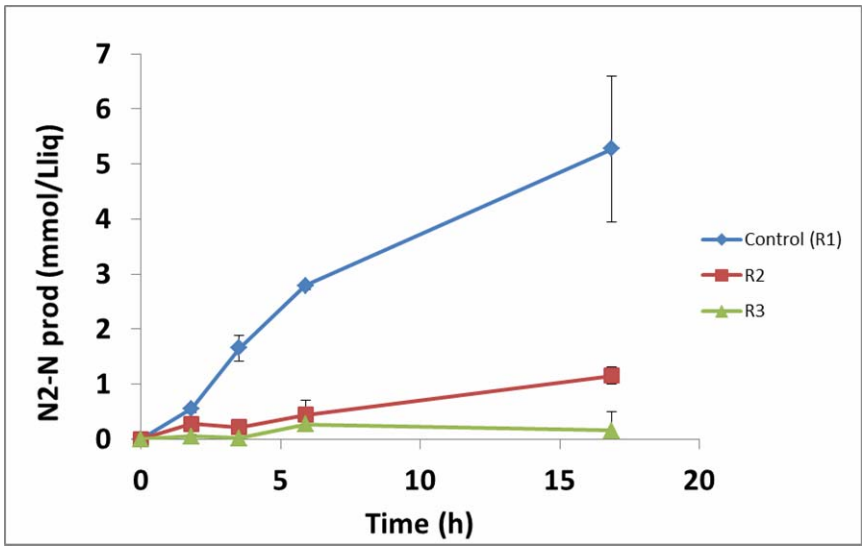

Figure 21: Activity test for the three reactors. Day 91

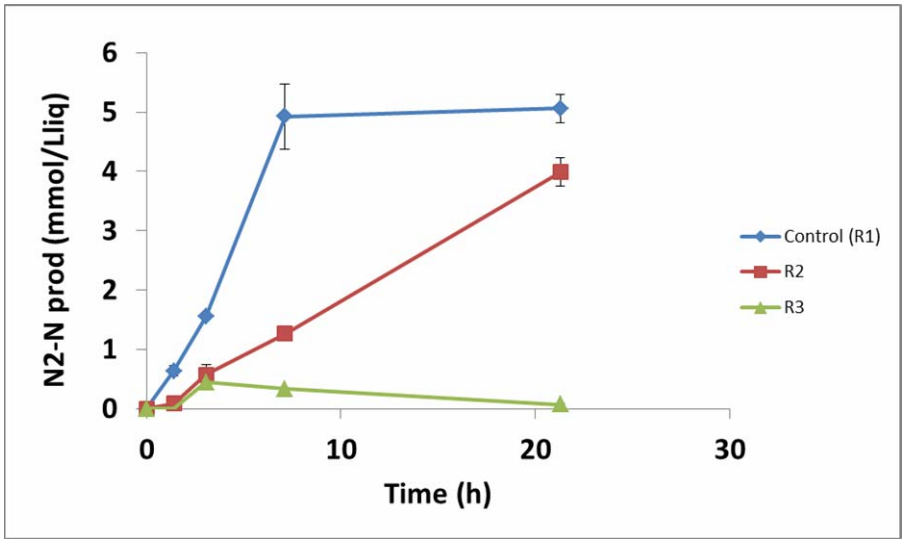

Figure 22: Activity test for the three reactors. Day 126

Table 13: Maximum specific anammox activity of the biomass in the three up flow reactors after 91d and 126 d of operation

	R1		R2		R3	
	91 days	**126 days**	**91 days**	**126 days**	**91 days**	**126 days**
Specific Activity (gN/gVSS d)	0.233	0.336	0.031	0.088	0.028	0.025
SAA (%)	100	100	13.38	26.31	12.25	7.42

The sludge from R3, that was fed an influent containing a 50% stochiometric excess of nitrite, only had a very low anammox activity, indicating that exposure to high residual concentrations of nitrite (up to 3 mM) inhibited the anammox process. The specific anammox activity of the sludge in R2, that was was fed with a 30% excess of nitrite, was also significantly lower compared to that of the control reactor (R1), albeit less than the sludge in R3. The residual concentration of nitrite in R2 was up to 2.5 mM compared to a maximum nitrite concentration of only 0.2 mM in the effluent of R1. These results indicate the importance of ensuring low residual nitrite concentrations to avoid deterioration of the anammox activity that could lead to reactor failure.

CONCLUSIONS

5. CONCLUSIONS

During nine months different reactors have been operated to study the behavior of anammox process and the influence of operational parameters over this process. The main conclusions obtained in the study are:

- Optimal conditions for the operation of anammox reactors include: pH values between 7.2 and 7.5, and a molar ratio NO_2^-/NH_4^+ = 1.3 in the influent.

- The optimum pH conditions for the anammox process are very restricteive, being the specific anammox activity almost constant in the pH range from 7.2 to 7.5 with a maximum activity at a pH value of 7.3. The activity was almost completely inhibited at a pH of 7.9 and below 7.0 there was also a dramatic activity decreased.

- Careful control of the pH value is required during the operation of anammox bioreactors to ensure process stability. Our results demonstrated that biomass exposure to sub-optimal pH values for a short period of time (a few hours) can lead to partial loss of the anammox activity *i.e.*, pH decrease from 7.3 to 6.5 followed by a return of the pH to the optimal value, lead to partial loss of the anammox activity.

- Hydrogen sulfide is highly inhibitory to anammox bacteria. Addition of ferrous iron (Fe^{2+}) can be a good solution to eliminate H_2S toxicity because the anammox process is not inhibited by Fe^{+2} or by the FeS precipitate formed.

- The kinetic study shows that the flocculent sludge works better with low concentrations of ammonium. However, the granular sludge works better with low concentrations of nitrite.

- The membrane bioreactor (MBR) system was shown to offer promise to reduce the lengthy periods generally required to startup anammox systems. The very small pore size of the reactor membrane (80 nm) precludes the washout of bacteria with the effluent and ensures high biomass retention. In this study, stable increase of the anammox biomass in the membrane bioreactor was observed (from <10 mg VSS/L to 900 mg VSS/L after more than 260 days).

- The three upflow sludge bed reactors were operated for more than 130 d with feed solutions containing different stoichiometric ammonium/nitrite ratios. Operation with residual effluent concentrations of nitrite ranging from 2.5 to 3.0 mM led to a sharp decrease in specific anammox activity (51%) compared to a control reactor operated with low residual nitrite levels (0.2 mM). These results indicate the importance of ensuring low residual nitrite concentrations to avoid deterioration of the anammox activity that could lead to reactor failure.

FUTURE WORK

6. FUTURE WORK

The results obtained in this work can help to increase the knowledge of the anammox process, however the continuation of work could allow to enhance this knowledge. Some interesting aspects that can be considered as future work are:

- Continuing the operation of the up flow reactors, increase the concentration of ammonium and nitrite in the feed to determine the maximum treatment capacity of the reactors operated with a stoichiometric excess of nitrite. This will confirm that exposure to high residual levels of nitrite can inhibit the anammox process and lead to reactor failure.
- Characterize the biomass present in the anammox reactors and its evolution. The sludge samples taken from the reactor for molecular ecology studies can be used for this purpose.
- To perform a complete mass balance of the different nitrogen species involved in the process to attain a better understanding of secondary reactions that take place.

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7. REFERENCES

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