Tutor/s

Dr. Isaac Fernández Rodríguez
Departament d'enginyeria química

Grau en Enginyeria Química

Treball Final de Grau

Autotrophic removal of nitrogen from wastewater: future trends.
Eliminació autòtrofa de nitrogen d'aigües residuals: Tendències futures.

Cristina Saladrich Català

June of 2015

Universitat de Barcelona

Dos campus d'excel·lència internacional
B:KC Barcelona Knowledge Campus
HUB^c Health Universitat de Barcelona Campus

Hi ha una força motriu més poderosa que el vapor, l'electricitat i l'energia atòmica: la voluntat.

Albert Einstein

Vull dedicar aquestes línies a totes les persones que m'han ajudat al llarg d'aquest treball.

Primer de tot als meus pares, per al suport constant que m'han donat durant tot el treball.

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REPORT

CONTENTS

1. SUMMARY

Anaerobic ammonium oxidation (Anammox) is considered one of the most promising alternative processes for the conventional nitrification and denitrification. It is an efficient and cost effective process; environmentally sustainable and provides many advantages compared with the conventional process such as lower sludge production, lower oxygen demand, no requirement of an external carbon source and the lower N_2O gas emission. It is performed by an autotrophic bacteria of the order of Planctomycetales called Anammox bacteria (characterized with low growth rate).

The process can be done either with a single stage, two stages or three stages. In the single stage process there is an interaction between the aerobic ammonium oxidizing bacteria and the anaerobic ammonium oxidizing bacteria in the same reactor. The most common systems used are the granular and the biofilm systems, where the ammonium oxidizing bacteria (AOB) grow in the outer layer. In this process the space is reduced and the maintenance and operational cost are also reduced. In the two-stage process there is firstly a partial nitrification where the nitrite is produced and afterwards there is an Anammox reactor where the nitrite and the remaining ammonium are converted to dinitrogen. This process has more control of the dissolved oxygen and it is ease to optimize. The three-stage process is composed of a pretreatment stage (where the biodegradable organic substances are removed), primary stage (where the partial nitrification occurs) and finally the Anammox process.

Compared with sidestream full-scale application, the mainstream is more challenging since the out-selection of nitrite oxidizing bacteria (NOB) and heterotrophic denitrifiers is more difficult. The application of the process in the sidestream of the wastewater treatment plants has been developed positively and is a mature technology, implemented in more than a hundred full-scale plants. Not being the same for the mainstream application, that still has some key factors that concern its performance.

There are three main challenges to be addressed in order to implement the Anammox process in a full-scale plant in the mainstream line. The most important one is the stabilization of

the operation performance since the Anammox bacteria are sensitive to the surroundings and have low growth rate. Another challenge is the ratio COD/N where if it is higher than 1 the denitrifiers may outcompete the Anammox bacteria because they are thermodynamically more favored. However if chemical oxygen demand (COD) is removed before the Anammox process and it is send to an Anaerobic digester, this enables carbon energy recovery. And lastly, the out selection of the NOB in front of the AOB.

There have been more than one hundred of articles about the research achievements on these issues. This work summarizes the most actual research achievements, in order to comprehend in which position we are as to implement this efficient process.

Keywords: Advanced biological treatment, Anammox, main line, nitrogen removal, partial nitritation, sustainability, wastewater.

2. RESUM

El procés Anammox (oxidació anaeròbica d'amoni) es considerat una de les alternatives més prometedores per a la substitució del tradicional tractament biològic de nitrificació i desnitrificació. És un procés eficient tant en termes d'energia com en termes econòmics. Té avantatges respecte el procés convencional i altres processos, ja que és ambientalment sostenible, requereix una demanda d'oxigen més baixa, redueix la producció de fangs, no necessita una aportació exterior de carboni i per últim, redueix l'emissió de N_2O a l'atmosfera, reduint tanmateix els gasos d'efecte hivernacle. Aquest procés es dut a terme per a una bactèria de l'ordre dels "Planctomycetales", anomenada Anammox bactèria (caracteritzada pel seu lent creixement).

El procés es pot dur a terme mitjançant una, dues o tres etapes. En el procés d'una etapa s'efectua una interacció, dins d'un mateix reactor, entre les bactèries aeròbiques oxidants i les anaeròbiques. Els sistemes de biofilm i granular són els més comuns i utilitzats: les bactèries aeròbiques creixen a la part externa de les capes i les anaeròbiques a la part interna. L'efectivitat d'aquest procés es troba en la reducció tant de l'espai com dels costos provinents de les operacions i del seu manteniment.

En el procés de dues etapes s'efectua una nitrificació parcial on el nitrit produït és enviat a una segona etapa on es realitza el procés Anammox (el nitrit i l'amoni restant són convertits a nitrogen). Aquest procés proporciona més control sobre l'oxigen dissolt i facilita l'optimització de les diferents etapes, però econòmicament és més car que el d'una etapa (donat que tot es troba per duplicat).

El procés de tres etapes està compost per un pretractament, on les substàncies biodegradables orgàniques són eliminades, un posterior tractament primari, on s'efectua una nitrificació parcial, i finalment el procés d' Anammox.

Si s'observa l'aplicació de l' Anammox a les línies secundàries és pot veure un creixement positiu, sent implementant en més de 100 plantes arreu del món. Tot el contrari passa en les línies principals, ja que hi han factors importants que preocupen el seu rendiment.

Centrant-nos en els reptes més importants per a poder implementar el procés a escala industrial s'ha observat que primerament s'ha d'estabilitzar el rendiment d'operació, donat a que les bactèries Anammox són sensibles al seu entorn i tenen un creixement molt lent, cosa que provoca una posada en marxa molt lenta. S'ha de tenir en compte, també, la relació COD/N donat a que si es major que 1 les bactèries desnitrificadores guanyen a les d' Anammox ja que són, termodinàmicament parlant, més favorables. Tot i que l'objectiu és disminuir aquesta relació, també és pot aconseguir una millora energètica si es condueix el carboni a un digestor Anaeròbic provocant un augment en el total energètic de la planta. L'últim repte a tenir present és la competitivitat que hi ha entre les bactèries d'amoni oxidants amb les del nitrit.

S'han publicat molts articles sobre aquest procés i sobre com solucionar aquests problemes, en aquest treball es pot veure resumit les actualitzacions dels reptes que encara existeixen i de les solucions proposades per als diferents investigadors d'arreu del món.

Paraules clau: tractament biològic avançat, Anammox, línies principals, eliminació del nitrogen, nitrificació parcial, sostenibilitat, aigües residuals.

3. INTRODUCTION

Nitrogen cycle is one of the most important biogeochemical cycles in the nature. The removal of the nitrogen compounds in the wastewater treatment plants are important due to the nitrogen compounds (their main form is ammonia) are pollutants for the water, toxic to the aquatic invertebrate and vertebrate species and have a depleting effect on the oxygen of the water; although at the same time they stimulate the growth of plants (Dapena-Mora et al. 2007; Paredes et al. 2007). According to EU directive 91/271/EEC the discharge of the nitrogen has to be regulated. It sets out the requirements for the discharge that depend on the localization. In the table (1) the parameters for a stringent treatment in an urban wastewater discharge are reflected:

Table 1. Effluent requirements for municipal wastewater discharge. Adapted from EU directive 91/271/EEC.

Parameter	**Concentration**		**reduction**
Total nitrogen	10.000-100.000 h-e.	>100.000 h.e.	
	15 mg N/L	10 mg N/L	70-80 %

Most of the wastewater has ammonium, a nutrient for the plants. However this component can be oxidized in a natural water because it is chemically reduced and at low concentration the non ionized form is toxic for the aquatic life (Paredes et al. 2007), so it has to be removed.

The traditional nitrification/denitrification process is being replaced by new technologies due to the improvement of different aspects.

The reaction (1) of the traditional biological process of ammonium removal is:

$$NH_4 + O_2 \longrightarrow NO_2^- + O_2 \longrightarrow NO_3^- \longrightarrow N_2 \quad (1)$$

During the nitrification process (aerobic conditions) the ammonia is oxidized to nitrate and during the denitrification process the nitrate is reduced to dinitrogen gas (Dapena-Mora et al. 2007) with consumption of organic matter as electron donor (reaction 1). The denitrification depend on the COD/N ratio and on the biodegradability of the electron donor. When the wastewater to be treated does not have enough biodegradable COD, an electron donor has to be added, increasing the costs.

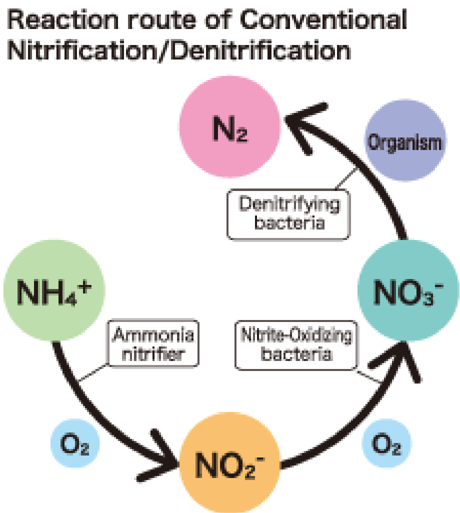

Figure 1. Route of nitrification and denitrification. Extracted from: http://nett21.gec.jp/GESAP/themes/themes4_8_2.html

3.1. Partial nitrification and denitrification process

In this new process (partial nitrification) the nitrogen oxidation (reaction 1) is stopped on nitrite. Usually the nitrite is accumulated in the reactor due to the control of the hydraulic retention time and the temperature. After that, the denitrification takes place from nitrite to nitrogen gas, and there is an addition of carbon source if needed.

This new process has two stages, firstly there is a reactor with aeration (normally it is a Single reactor system for High-rate Ammonium Removal Over Nitrite, SHARON; van Dongen et al. 2001) that produces the nitrite and after there is a heterotrophic denitrification reactor. Since the nitrogen oxidation is stopped in nitrite, the requirements of oxygen and carbon source are decreased (Paredes et al. 2007).

The tables below summarized the pros and cons of this new process (Table 2) and also the main effects of the important parameters (Table 3):

Table 2: Advantages and disadvantages of PN and DN process (adapted from Paredes et al. 2007)

Pros	**Cons**
- low oxygen requirement in the first stage (25% less). - lower consumption of organic carbon (save 40%). - reaches just 30% of the traditional sludge production. - saves resources.	- if there is a low content of alkalinity, its depletion can reduce the pH and stop the reaction (at pH< 6,5 the reaction does not take place). - HNO_2^- is an inhibiting component and concentrations higher than 0.2 mg HNO_2/L inhibit the process.

Table 3: Effects of different parameters

Control:	**Effects**
-Temperature	-T>25ºC: ammonium oxidizers can outcompete the nitrite oxidizers (Paredes et al. 2007).
- pH	-pH: if the pH is low the carbon dioxide can be removed by stripping. And if the pH is high the mineral carbon is present in carbonate species. -An optimum pH is required for the nitrification by autotrophic microorganisms (Paredes et al. 2007). -It can also cause inhibition through formation of free ammonia and nitrous acid.
-Free ammonia in the reactor	-Free ammonia and free nitrous acid inhibits oxidizers of ammonium and nitrite.
-Salts	- High salinity has negative effects in the wastewater treatment plants (Paredes et al. 2007). - Ammonia oxidizers are more sensitive to salt stress (at short and long term) (Mousaa et al. 2006).
-Dissolved oxygen in the reactor	-Dissolved oxygen is a controlling factor. If the oxygen is limited it can generate a stable conversion of ammonium to nitrite, but if it is too low, conversion is reduced too. If its concentration is too high nitrite can be oxidized to nitrate (Paredes et al. 2007)

To solve some of the problems, different types of reactors have been implemented:

- **Stirred biofilm reactor:** The reactor works in a way where in the outer part of the biofilm the ammonium oxidizers are densely packed and in the deeper part, small clusters of nitrite oxidizers are created. In consequence the ammonia oxidizers are much more exposed to oxygen than nitrite oxidizers. As it is said before (Table 2) with low concentrations of oxygen the nitrite oxidizers can be outcompeted.

- **SHARON**: is a Single reactor with High activity Ammonia Removal Over Nitrite. (Hellinga et al. 1998). It has a high efficiency converting the ammonium into nitrite. The dilution rate is higher than the growth of the nitrite oxidizing bacteria (NOB), but lower than the growth rate of the ammonium oxidizing bacteria (AOB) and as a consequence there is a high production of nitrite. After the SHARON reactor, an Anammox process (reaction 2) (Section 5) is usually employed to convert the nitrite into nitrogen gas. Commonly the reactor operates within a temperature range of 25-40 ºC (Dapena-Mora et al. 2007). In the SHARON there is no biomass retention, consequently the sludge retention time is the same as the hydraulic retention time, and the pH has to be relatively high, because when the ammonium is converted the alkalinity goes down and it can inhibit the process (Dapena-Mora et al. 2007).

The equation (2) of this process is:

$$NH_4^+ + HCO_3^- + 0,75O_2 \rightarrow CO_2 + 0,5\ NH_4^+ + 0,5NO_2^- + 5H_2O \qquad (2)$$

3.2. Surface flow wetlands (SSF):

Surface flow wetlands has similar mechanisms as the biofilm reactor (Paredes et al. 2007) and depends on the vegetation and on the hydraulic flow. The biofilm is growing on soil particles and on the root surface of plants located in wetlands fed with wastewater. The biofilm allows the aerobic and anaerobic process to occur simultaneously inside the system. These two zones (the aerobic and anaerobic zone) are formed with the oxygen released by the plants in the wetlands. Then the ammonia oxidizers uses this oxygen to oxidize the ammonium to nitrate and at the same time in the anaerobic zone the nitrate is reduced by organic compounds present in the wastewater and also released from the plants.

The vegetation, the hydraulic flow and the microorganisms have a main role in the nitrogen removal (figure 2). The main issue of this process is that it strongly depends on the season, with far higher performance in the summer than in the winter (because of the temperature) (Paredes et al. 2007).

However the organic carbon source is from the plants and also is the dissolved oxygen, so it is less expensive than the other processes. On the contrary, this process is usually slow and not suitable for high flow rates of wastewater or very concentrated residual streams.

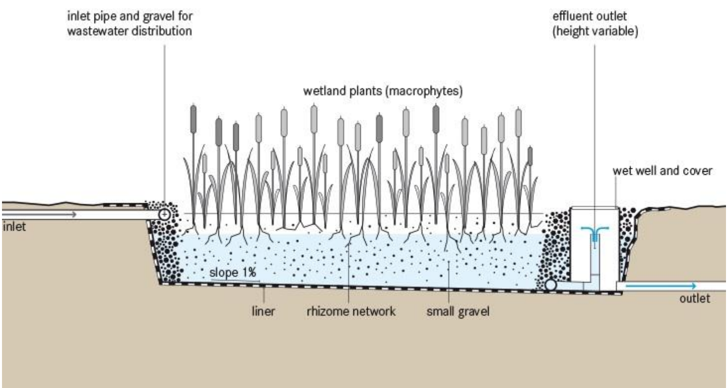

Figure 2.Constructed wetland. Source: Tilley et al. (2014)

3.3. Partial nitrification + ANAerobicOXidation of AMMonium (Anammox):

Partial nitrification (PN)/ Anammox process is addressed for a high ammonium nitrogen load and low organic matter concentration. The first in discovering this process were Mulder et al. (1995). It is a cost effective and low energy alternative to the conventional process, because the consumption of the organic carbon is decreased and also is the aeration (Okabe et al. 2011). In the Anammox process autotrophic bacteria are used and the nitrite is the electron acceptor (figure 3). The reaction of PN/ Anammox is:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O \quad (3)$$

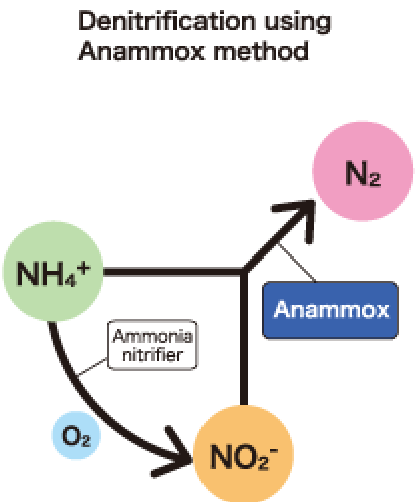

Figure 3.Anammox process. Extracted from http://nett21.gec.jp/GESAP/themes/themes4_8_2.html

The Anammox bacteria are autotrophic and have a variety of characteristics depending on their location (They are found in several different types of environments such as the marine (Schimd et al. 2007)). They are sensitive to some organic sources and to alcohols due to an enzyme called hydroxylamine oxidoreductase (Yang et al. 1992).

In the table 4 below the pros and cons of this process are summarized:

Table 4. Advantages and disadvantages of the Anammox process (Paredes et al 2007):

Pros	**Cons**
- Reactors havean efficient biomass retention. - Lower cost in aeration. - In order to work just half of the ammonium has to be converted to nitrite in a previous step. - No need for external carbon source. - Low surplus sludge production.	- Low growth rate. -The activity of the Anammox bacteria decreases when the temperature does too. -Sensitive to oxygen and nitrite.

The process of partial nitrification and Anammox is performed with one or two different reactors, depending on if it is a single or two stage system. If the process is in two stages, the first one is usually a SHARON reactor with an Anammox reactor after (Hellinga et al. 1998; van Dongen et al. 2001). If is a one stage process, the most well-known is the CANON (Completely Autotrophic Nitrogen Removal Over Nitrite) (Third et al. 2001).

Some reactors has been studied in order to reach high removal rates:

- Sequencing batch reactor (SBR) (Lettinga et al. 1980): SBR is one of the most widely used anaerobic reactors for wastewater, because it can handle short Hydraulic Retention Times (HRT). (Zhao et al. 2008).

- Air lift reactor (Sliekers et al. 2003).

- Fixed bed reactor (FBR) (Strous et al. 1997).

- Fluidised-bed reactor (FIBR) (Mulder et al. 1995; Strous et al. 1997).

- Membrane sequencing batch reactors (Trigo et al. 2006).

- Up- flow anaerobic sludge blanket reactors (Ahn et al. 2004).

- Rotating biological contactor (RBC) (Patwardhan, 2003) is a biofilm reactor used for the process Oxygen-limited autotrophic nitrification denitrification (OLAND) (Kuai and Verstraere, 1998). Liu et al. 2008 reported a start up within 100 days by reducing the hydraulic retention time and increasing the nitrogen loading rate in the influent.

- Membrane bioreactor (MBR) is one of the best alternatives for a quick and stable start up, with a total retention of biomass, but with some limitations of the membrane bioreactors (Trigo et al. 2006).

This process will be discussed more in detail in Section 5.

3.4. Denitrifying ammonium oxidation (Deamox):

Deamox is a combination of Anammox reactor with autotrophic denitrifying conditions (to generate nitrite from nitrate while converting sulfide to sulfate) (Mulder et al 2006). It was created as to overcome the nitrite production limitations, because it does not require a separated production of nitrite (Kalyuzhnyi et al. 2006). The reaction of the Deamox is (4):

$$4NO_3^- + HS^- \rightarrow 4NO_2^- + SO4^{2-} \quad (4)$$

And the flow diagram for this process is (figure4):

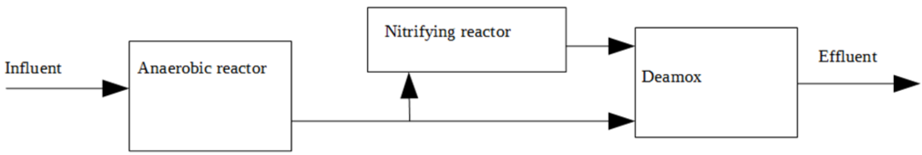

Figure 4. Flow diagram of the Deamox process. Adapted from Kalyuzhnyi et al. (2006).

In the previous figure (Figure 4) it is shown the flow diagram of the Deamox process. Firstly there is a pre-treatment step (anaerobic reactor) where the most common reactor is the Up-flow Anaerobic Sludge Blanket (UASB). After, the Deamox reactor is partially fed with the effluents from the anaerobic reactor and from the nitrifying reactor (that is used to generate the nitrate) (Kalyuzhnyi et al. 2006).

There are two configurations of the Deamox that has been studied, the sulphide-driven Deamox that was the first one to be discovered and is the one described above, and the second type is the organic-drive Deamox (heterotrophic) (Kalyuzhnyi and Gladchenko et al 2009).

This process has some advantages, foremost there is no complex control for the production of the nitrite. Secondly there are no high levels of nitrite, and the last one is that because of the denitrifying conditions in the Deamox reactor, the growth of granules is increased (Mulder et al. 1995).

4. OBJECTIVES

Partial nitrification and Anammox processes have given a step forward in the removal of nitrogen from wastewater, even though there are still issues about the implementation of them in the main line of the municipal wastewater plants. The main goals of this study are:

- Having knowledge about these new processes and their feasibility and reliability (basic concepts).

- Review the issues and limitations of the processes.

- Raising information about the implementation in full-scale (both treating mainstream and sidestream effluents).

- Evaluate the advantages and disadvantages and contrasting with the conventional treatments.

5. ANAMMOX PROCESS

The anaerobic ammonium oxidation is a biologically catalyzed process where the nitrite and the ammonium are converted to dinitrogen gas by autotrophic Anammox organisms that belong to the order of the *Brocadiales* (He et al. 2015). The ammonium is pre-oxidized to nitrite and then, this nitrite together with the remaining ammonium is converted to dinitrogen gas (Okabe et al. 2011). The Anammox reaction was firstly discovered in Gist-Brocades, Delft (Netherlands) where an effluent from a methanogenic reactor was treated (Suneethi et al. 2014). It is a good sustainable bioprocess for wastewaters with low carbon/nitrogen ratio. And also, it is a cost saving alternative for the nitrification and denitrification (figure 5).

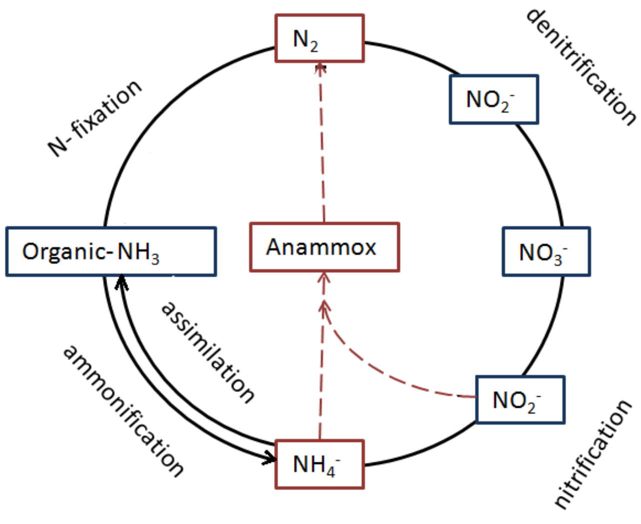

Figure 5. Nitrogen cycle with Anammox. Adapted from Trimmer et al. 2003.

The bacteria used for the process are called Anammox bacteria (Mulder, 1985; Strous et al, 1999), and with nitrite as an electron acceptor they do an autotrophic ammonium oxidation (Strous et al. 1999).

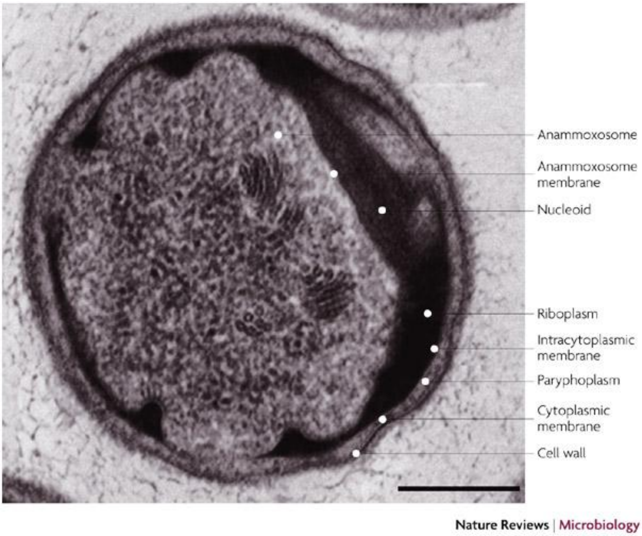

Figure 6. "*Candidatus Kuenenia*" adapted from Gijs Kuenen, J. (2008)(Scale bar corresponds to 200 nm and the bacteria was high pressure frozen).

In 2000 Strous studied the optimum characteristics of the process: the optimum temperature was found to be 40 +/- 3 ºC and the optimum pH was about 8.

These bacteria are sensitive to extreme temperatures, to extreme salinity and to the presence of organic matters. Nevertheless, it is reported that the Anammox sludge can slowly adapt to low temperature and high salinity (Xing et al. 2015).

In order to improve the performance in the full scale implementation of the Anammox granules, the settling velocity, the size distribution, the morphology of the bacteria and the extracellular polymeric substances have been studied so far (Mulder et al. 1995; Tang et al. 2011; Lu et al. 2012).

5.1. Stoichiometry

Strous et al. (1998) reported the first full stoichiometry of the Anammox metabolism. The modelization was achieved in a reactor SBR, and it was obtained by mass balancing with 90% of retention of growing biomass with stable conditions. Although the electron balance had a 15% of error, that error was related to the nitrogen source (ammonium or nitrate and nitrite) since it was not considered in the description.

A recent study (Lotti et al. 2014) characterized the Anammox bacteria for a more detailed stoichiometry with more stable conditions; the equation 4 is the last accurate stoichiometry for the Anammox process. Furthermore this stoichiometry had smaller error in electron balance and more accuracy in carbon balance than the Strous (1998) experiment. The main difference between both was that Strous et al. (1998) did not considerate nitrite and nitrate as a nitrogen source.

$$1NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071CH_{1,74}O_{0,31}N_{0,20} + 2.002H_2O \quad (4)$$

5.2. Kinetics

Another characteristic that has been studied recently are the kinetic characteristics (combination of dynamic modeling and continuous operation) of a suspended cell Anammox culture.

Lotti et al. (2014) studied the kinetic characteristics under unstable conditions.

The conditions of this experiment are listed below (Table 5):

Table 5. The conditions of the Anammox kinetic characterization (adapted from Lotti et al. 2014):

Wastewater	Dokhaven-sluisjexdijkWWTP (Rotterdam)
Bacteria	Brocadia (using Fluorescence in situ hybridization, FISH)
Volume (L)	1.8
HRT (days)	1.67
Argon(95%)+CO_2(5%) (ml/min)	50
Pressure (hPa)	12
pH	6.8-7.5
Temperature (ºC)	30
Solid retention time (SRT)	Controlled by a peristaltic pump

To determine the kinetic parameters a Monod equation (equation 1) was used:

$$qs = qs_{max} * (\frac{cs}{cs+ks}) \qquad \text{(eq.1)}$$

cs: concentration of the substance

ks: half saturation constant

qs: biomass specific uptake rate.

The nitrite half saturation constant (ks) was determined at pH=7: 0.035 mgN/l. The differences between the result of this experiment and earlier experiments (Puyol et al. 2014, Lotti et al. 2012, Strous et al. 1999) were the conditions and the methods of cultivating and investigating the biomass.

The maximum specific growth rate was **0.21 d^{-1}** was higher than earlier experiments or reported data (Cao et al. 2013, Isaka et al.2006, van der Star et al. 2008, Tsushima et al 2007) because there was a high growth rate in the suspension system and all the cells were highly active.

Free cell enrichment of Anammox bacteria was obtained in the study of van der Star et al. (2008) where there was an addition of yeast extract with absence of selectivity, high growth rate, low shear stress and complete anaerobiosis. The growth rate was low because some of the free cells were removed with the effluent and then in the calculations the high sludge retention was underestimated.

5.3. Microbiology

The Anammox reaction is done by Anammox bacteria from the phylum Planctomycetes, which uses the ammonia as an electron donor, and the nitrite as an electron acceptor. *Planctomycete*s have an specific gene sequence: 16S rRNA stablished. The Anammox bacteria belong to the *Brocadiales* related to the *Planctomycetales* (He et al. 2015).

The Anammox bacteria exist in many different environments, in natural ecosystems, in natural and artificial low oxygen zones, terrestrial ecosystems, freshwater, and marine sediments (Amano et al. 2007; Humbert et al. 2010; Jaeschke et al. 2010; Figure 7). They thrive both at high and low temperature.

Till now there are six Anammox genera (one is a marine genus and the other five are freshwater genera) that have been identified, the Candidatus “Brocadia”, Candidatus “Kuenenia”, Candidatus “Scalindua” (marine one), Candidatus “Jettenia”, Candidatus “Anammoxoglobus” and Candidatus “Anammoximicrobium” (Schimd et al 2005, He et al 2015).

Recently, it has been found that some species (while forming ammonium and nitrite as intermediate) have the capacity to use nitrate as an electron acceptor to oxidize volatile fatty acids (Güven et al 2005; Kartal et al. 2007a,2007b; Winkler et al. 2012).

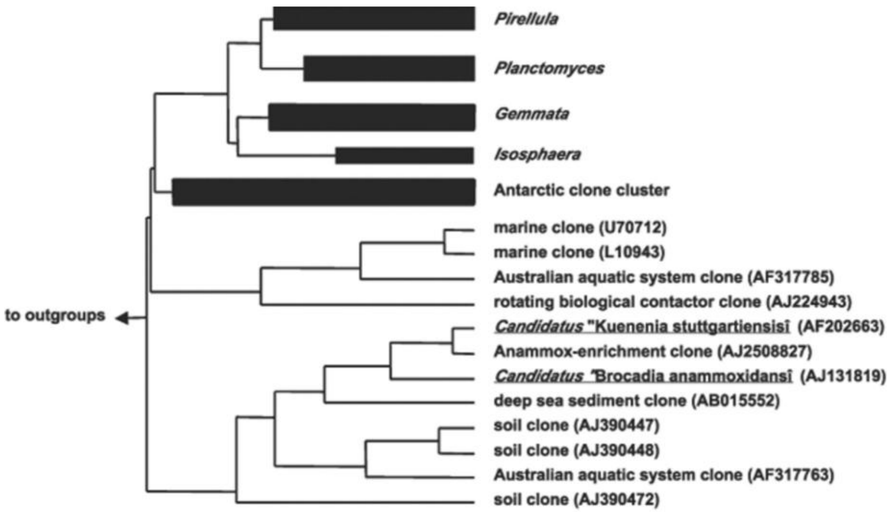

Figure 7. Different branches of Anammox bacteria. The tree is based on results of maximum likelihood analyses from different data sets (Schmidt et al. 2002).

For the single stage process an interaction of an anaerobic Ammonium Oxidizing Bacteria (An. AOB) and the Aerobic Ammonium Oxidizing Bacteria (Aer. AOB) is required. The first one includes *Candidatus Brocadia, Candidatus Jettenia, Candidatus Anammoxoglobus, Candidatus Scalindua and Candidatus Kuenenia*, and the last includes *Genus I Nitrosomonas*, *Genus II Nitrosococcus, and Genus III Nitrosospira* (Abbas et al. 2014).

A new genus in the Anammox line of the planctomycetes has been recently found (with a 91% of similarity of the 16 S rRNA gene sequence Anammox bacteria) (Kartal et al. 2008) establishing a new line in the Planctomycetes. The lastly specie to be discovered was the Candidatus “Anammoxoglobuspropionicus” (in the figure 8).

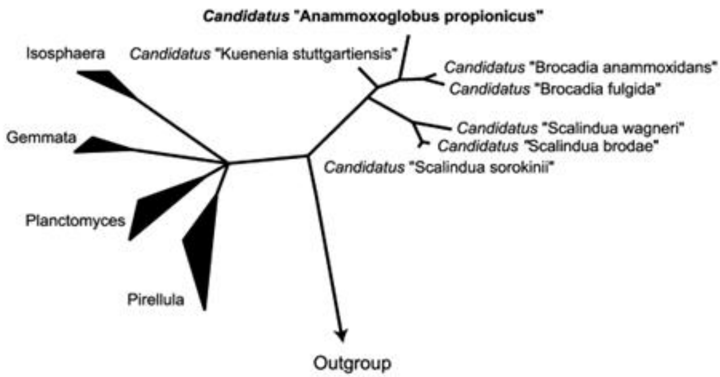

Figure 8. Anammox genera (Kartal et al. 2008).

In the figure before (figure 8) it can be seen the different Anammox genera or species. The planctomycetales have six branches and these branches are splitted in different species.

The dominant Anammox type in an environment where there is acetate or propionate is the *"Candidatus Brocadia fulgida"* (Kartal et al. 2008). And when the substrate concentration is low, the dominant is "*Candidatus Kuenenia"* (Ding et al. 2013). With the Fluorescence In Situ Hybridization (FISH) measurements (tool to verify which strains are present in the sludge) it has been discovered that this specie (*"Candidatus Brocadiafulgida")* is the dominant Anammox strain, and has the ability to oxidize the acetate, and outcompete other types of Anammox bacteria in presence of acetate. Also, it can compete with heterotrophic bacteria in presence of many types of organic dissolved compounds (as long as they are not toxic), and has more advantages at low temperatures. Hendrickx et al. (2014) reconfirmed that the "Candidatus

Brocadia Fulgida" has high specific Anammox activity and in low temperatures had highly enriched biomass (prerequisite for full-scale application).

5.4. ANAMMOX TECHNOLOGIES:

For both PN/Anammox technologies (i.e. one or two steps) there are several common advantages over the conventional nitrification and denitrification: there is a reduction in the energy consumption for aeration, there is no requirement of an organic donor of electrons, the required resources are reduced, there is a high volumetric nitrogen removal rate, and there is a reduction of the greenhouse gases emission, so the costs associated with the external carbon source and the aeration for the nitrification are decreased (Abbas et al 2014). As mentioned, the PN/Anammox process can be carried out with a single stage, two stages or three stages.

5.4.1 Single- stage

The single stage (the direct application was done by Xu et al. 2007) is based on two microbial populations living together. In the same reactor there is an interaction of aerobic ammonium oxidizing bacteria and anaerobic ammonium oxidizers (Anammox), under limiting oxygen conditions. In this process granular or biofilm systems are used. The granular sludge is set up by a self-aggregation in the granules and with large surface area (Abbas et al. 2014). In the granules, Anammox and AOB grow together, but the last grows in the outer layer (Winkler et al. 2012) (Figure 9). The single stage has some advantages and disadvantages. Firstly, there is a reduction of the space since it only requires a single reactor and, as a consequence, there is an investment saving. Also the maintenance cost is reduced and the operating expenses too (Abbas et al. 2014). However, the conversion of the ammonia to nitrite is determined by the concentrations of ammonium and oxygen. If the environment is with low temperatures then it has to be a thick biofilm and high dissolved oxygen concentration is required (the thickness of the biofilm depends on the temperature and on the oxygen). The required oxygen has to be sufficient to oxidize the ammonium but not as much to oxidize the nitrite to nitrate. To reach the sufficient ammonium surface load, the required time is estimated from 5 to 10 years, because Anammox is a very low growth bacterial population (Abbas et al. 2014).

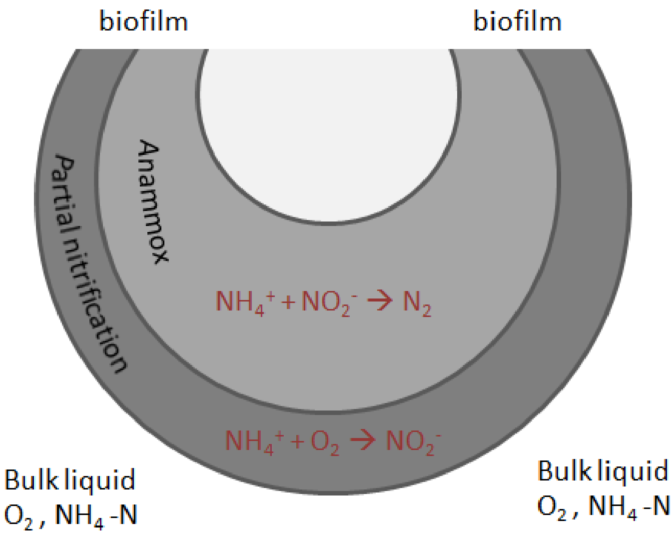

Figure 9. PN/Anammox in the biofilm.

The reaction (5) of the single stage process is (Sliekers et al. 2003):

$$NH_4^+ + 0.85\ O_2 \rightarrow 0.44\ N_2 + 0.11\ NO_3^- + 1.43\ H_2O + 0.14\ H^+ \qquad (5)$$

There are different names for the single stage nitrogen removal depending on who developed it:

- <u>Oxygen-Limited Autotrophic Nitrification–Denitrification (OLAND)</u> developed at Ghent University (Belgium) (Hippen et al. 1997).

- Completely Autotrophic Nitrogen removal Over Nitrite (CANON), developed at Delft University (Sliekers et al. 2002).

- Aerobic /Anoxic Deammonification (DEMON), Hanover University (Germany) (Kuai et al. 1997).

- Single-stage Nitrogen removal using Anammox and Partial nitritation (SNAP), Kumamoto University (Japan) (Lieu et al. 2005).

- Simultaneous partial Nitrification, Anammox and Denitrification (SNAD), Dalian University of Technology (China) (Chen et al. 2009).

The advantages of the single stage over the two stage process is that it reaches higher nitrogen removal rate, has lower hydraulic retention time, and it does not require control of the nitrite (Abbas et al. 2014).

5.4.2 Two stage system

The two stage system (Liang and Liu 2008) has two reactors in series: first of all there is a partial nitrification and then an Anammox process. The affluent to the Anammox step has to contain about 50% of ammonium and 50% of nitrite (Paredes et al. 2007). The nitrite is produced in a separated tank and is fed into the Anammox reactor (Winkler et al. 2012). The main disadvantage is the initial cost because as it has two reaction tanks, many devices are duplicated and entails a higher cost for the implementation. Also, it is required more space, which can be a problem if the available area is limited or expensive.

The two stages system has several advantages, chiefly in the control of the process. Since the partial nitrification is done in one reactor and the Anammox process in another, the control of the dissolved oxygen is easier (inhibition protection) and easier to optimize each process independently.

For the two-stage system there is a new method called the **reverse two-stage partial nitrification-Anammox.** The typical two stage system is reversed where the Anammox reactor is located at the upstream of the bioreactor of partial nitrification and the effluent is partially recycled. It is a promising new method because it solves the problem with the nitrite that the "non reverse" system has and also reaches an efficient nitrogen removal in a wastewater with high ammonia concentration and low C/N ratio (Xu et al. 2014). The figure below (Figure 10) shows a two stage system and the reverse system.

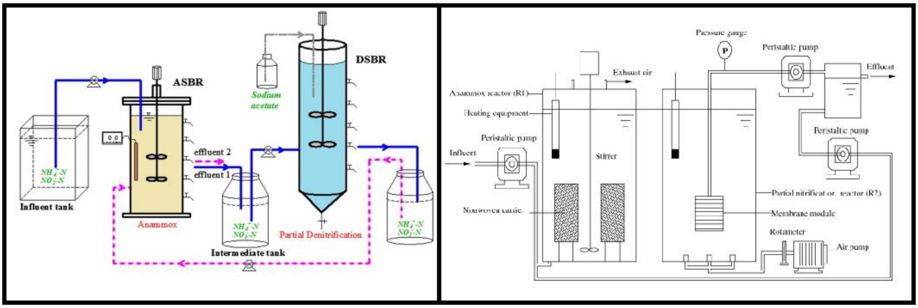

Figure 10. The right figure is a two stage system (PN-Anammox), and in the left is the reverse two stage PN- Anammox (Adapted from Du et al. 2014; Xu et al. 2014).

5.4.3 Three stage system

The three stage system consists in three sequencing batch reactors, where the first one is a pretreatment SBR, then the partial nitritation SBR and the last is the Anammox SBR. The SBR has much more advantages than other reactors, for example, has a flexible operational mode, is more stable during the operation, and is a space saving reactor. In the pretreatment stage the biodegradable organic substances are removed, and as a consequence the negative effect in the Anammox process is reduced (figure 11). In the second stage the ammonia is oxidized to nitrite and the effluents of the first two stages are mixed together to be sent to the last stage where the Anammox reaction comes. The system is implemented for the mature landfill leachate treatment (Miao et al. 2014).

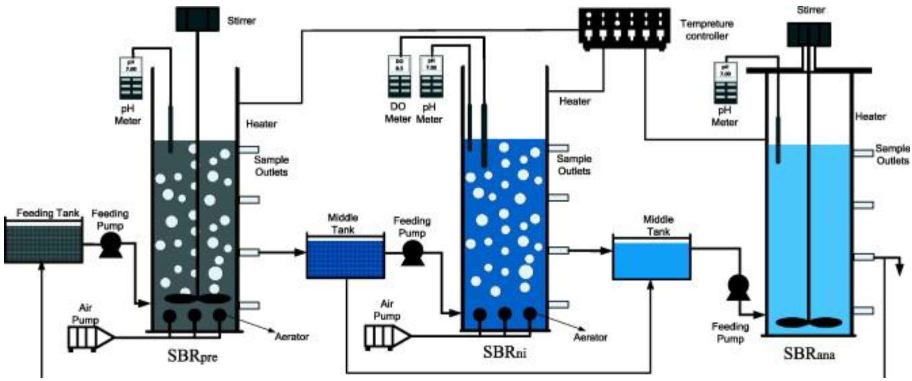

Figure 11. Three stage system (Miao et al. 2014).

5.5 MAIN FACTORS AFFECTING THE ANAMMOX PROCESS

5.5.1 Inhibition:

The Annamox process suffers inhibition by the substrates. Their presence improves the activity if the concentrations are low or moderate (ammonia, nitrite, HCO_3^-), however if there is an overload they can inhibit the process (He et al. 2015). Apart from the substrates, many other compounds are inhibitors of the Anammox process like the biodegradable organic matter, some heavy metals, phosphate and sulfide (Jin et al. 2012).

5.5.1.1 Free ammonia

Free ammonia is a competitive inhibitor for some of the bacteria (Jin et al. 2014). On one hand free ammonia vies to inhibit the enzyme *"nitrite oxidoreductase" (*found on the cell membrane of the NOB) and as a consequence the NOB metabolism too. On the other hand, it is used as a substrate to enrich the Anammox bacteria (Abbas et al. 2014). The studies showed that high level of free ammonia suppress the Anammox process (Quiao et al, 2010; Cho et al. 2011). Fernández et al. (2012) studied the long and short term effects of the ammonium concluding that at 35-40 mg NH_3-N/L the operation was extremely unstable and the nitrogen removal was near to negligible.

The different types of Anammox bacteria can endure somewhat different high ammonium concentrations (Candidatus *brocadia fugida* was reported as the most resistant; Jenni et al. 2013), and the biofilm reactors provided a better tolerance for the Anammox bacteria to free ammonia. Free ammonia is still an actual issue with variety of conclusions from different authors. Jubany et al. (2009) and Tora et al. (2010) shows that free ammonia could help the PN/Anammox process because it could be used to enrich AOB and wash-out the nitrite oxidizers. Otherwise, authors like Quiao et al. (2010) and Cho et al. (2011) showed that free ammonia is prejudicial for the process and can inhibit it. Also, there are some discrepancies about the maximum concentration of free ammonia due to the different operational conditions, sludge and microbial populations

One factor that affects free ammonia is the pH: at low pH the concentration of the free ammonia is decreased, and at high pH the concentration of ammonia is increased, according to the corresponding chemical equilibrium (Anthonisen et al 1976). So, to prevent too high free ammonia concentrations, the pH is adjusted close to pH neutral (Okabe et al. 2011). The relations of the pH with the free ammonia and free nitrous acid (FNA) are (eq.2 and eq.3):

$$FA = \frac{\left(\frac{17}{14}\right) * \left(TAN * 10^{pH}\right)}{\left(\exp\left(\frac{6334}{273 + ℃}\right) + 10^{pH}\right.} \qquad \text{(eq.2)}$$

$$FAN = \frac{\left(\frac{47}{14}\right) * (TNN)}{\exp\left(\frac{-2300}{273 + ℃}\right) * 10^{pH} + 1} \qquad \text{(eq.3)}$$

5.5.1.2 Nitrite

Various studies show that free nitrous acid has suppressing effects due to its biotoxicity and destabilizes the Anammox process (Quiao et al. 2010; Yamamoto et al. 2011; Okabe et al. 2011). Additionality, some studies reported that the inhibition also depends on the biomass characteristics and the operational conditions. Wett et al. (2007) showed that at 5 mg/L the process was inhibited, however, Strous et al. (1999) found that inhibition at 100 mg/L.

The pH has a large effect on this inhibition because it affects the equilibrium of the reaction 6 (Jin et al. 2012; eq.3):

$$HNO_2 \longleftrightarrow NO_2^- + H^+ \qquad (6)$$

Egli et al. 2001 reported that the responsible for the inhibition effect of the nitrite was the free nitrous acid. At low pH (lower than 7.1) the FNA had the maximum effect of inhibition and at high pH, the inhibition was caused by ionic nitrite(Puyol et al. 2014).

5.5.1. 3 Nitrous oxide

Nitrous oxide has a great potential to cause global warming effects. It composes the 10% of the total greenhouse gas emissions (Okabe et al. 2011). In the lab scale experiences, the PN/Anammox process still emits this compound. Although in the single stage system the emission is lower than the two stage system. In the two stage process the nitrous oxide is higher due to the DO (dissolved oxygen) limitation conditions and also because of the accumulation of the nitrite (Okabe et al. 2011).

Most of the produced nitrous oxide is released in the aeration phase. In the study of Zheng et al. (1994), during the partial nitrification 5.4% of the transformed nitrogen was emitted as nitrous oxide and in the study of Okabe et al. (2011) in the Anammox process 0.1 +/- 0.07% of the transformed nitrogen was also released as nitrous oxide.

In this last study, it was reported that the biological process responsible of producing this undesirable gas was not the Anammox. Instead, it was produced due to the incomplete heterotrophic denitrification caused by the low COD/N ratio (Okabe et al. 2011) and the production of it was located in the internal part of the Anammox granules, and the active consumption in above.

Nitrous oxide depends on the pH due to the inhibition of the nitrous oxide reductase at low pH (Knowles 1982). So in the Okabe et al. (2011) study, the authors reported that when the pH was decreased from 8.5 to 6.5 the emission of the gas was increased.

5.5.1.4 Organic matter

The Anammox bacteria are autotrophic so they do not require organic matter. But in the environment there are found different carbon sources that negatively affect the Anammox bacteria (Winkler et al. 2012). In wastewaters there are usually both nitrogen and organic carbon compounds (Kumar and Lin 2010). The inhibition by the organic carbon can happen in two different ways: firstly because the Anammox bacteria have a diversity of substrate and different metabolic pathways and the dominant species in the Anammox systems can change. Secondly because the heterotrophic bacteria grow faster than the autotrophic one in high concentration of organic matter conditions (He et al. 2015). The heterotrophic bacteria suppresses the Anammox and there is a decrease of nitrogen removal. To maintain a high autotrophic nitrogen removal the avoidance of the negative effect of the organic matter is required (Tang et al. 2014). For a combined removal of nitrogen, ammonia, sulfate and organic carbon a combined Anammox, sulfidogenesis and denitrification in one phase can be helpful (He et al. 2015).

Alcohols, aldehydes, phenols and antibiotics are some examples of biodegradable organic matters that inhibit the process (Güven et al. 2005). Some reports show that alcohols inhibit the Anammox activity (Jensen et al. 2007; Güven et al. 2005; Jin et al. 2012). It has been researched that methanol (an alcohol who was usually used as a supplementary carbon source) may inhibit the Anammox bacteria when is converted to formaldehyde because it destroys the protein and the enzyme activity by cross-linking the peptide chain (Jin et al. 2012; Isaka et al. 2008). The formaldehyde is formed because of the conversion of the methanol by the action of the enzyme hydroxylamine oxidoreductase, which is found in the Anammox bacteria (Kindaichi et al. 2004). The phenols are frequently found in some industrial wastewaters, and some studies showed that they also have an adverse effect in the metabolism of the Anammox (because of their biotoxicity) although it can be partially overcome by acclimation (Jin et al. 2013b). The last group, the antibiotics are found in a large number of environments. Although the dose-response has not been studied yet, a few researches have been done and arrived at the conclusion that some types of antibiotics (tetracycline, chloramphenicol) have bad effects in the process (Fernández et al. 2009; Yang and Jin 2013).

5.5.1.5 Salts

Salt is an important factor in Anammox process because high salinity results in high osmotic pressure that can inhibit the bacteria. And there are large volumes of salts in some industrial wastewaters. The Anammox bacteria from wastewater do not have a very high resistance to salts but it can be adapted by increasing salinity step by step (gradually) until 30 g NaCl/L (Jin et al. 2011; Dapena-Mora et al. 2010). Also, Kartal et al. (2006) demonstrated that the Anammox bacteria can be adapted to high concentrations of salts, if they are acclimated. Dapena-Mora et al. (2007) showed that sodium chloride does not affect the Anammox activity when the concentration is moderate.

The non-marine Anammox specie that was better adapted in systems with high concentrations of salts is the "*Ca. Kueneniastuttgartiensis*" (Yang et al. 2011).

A recent study (Xing et al. 2015) has been done in order to know if it is possible to operate at high salts concentrations and low temperature.

The results were that a shock of the temperature weakened the Anammox bacteria, however, the decrease of the temperature weakened the tolerance to the salt. Although these two parameters weakened the bacteria, it were acclimated to high salt conditions in a long term acclimation.

It is possible to operate in a low temperature and high salt conditions in two steps. It must be a production of biomass in an optimum temperature and then it has to be a slow adaptation of the biomass increasing the salt and decreasing the temperature in the same reactor.

Is possible the adaptation to high salt levels in the fresh water, where the calcium might act as a protection of the bacteria against salts stress (Lotti et al. (2014)). The velocity of the flocks calculated in the experiment of Lotti et al. 2014 is higher than the velocity calculated with the Stokes' law due to the porous fractal structure that permits an intra aggregate flow. Nakajima et al. (2008), showed that it is possible to enrich the AOB from marine environment (3.4% salt), using a continuous cultivation.

5.5.2. Low growth rate

The Anammox bacteria have a low growth rate which is susceptible to changing environments. The low growth rate is a problem that has been widely studied in order to increase the growth and in consequence the nitrogen removal rate. In the chapter 7 there are provided specific solutions for this factor.

The biomass retention is the key to achieve high nitrogen removal. The nitrogen removal is proportional to the population of Anammox bacteria, in consequence if there is a high population of Anammox bacteria it will be a high nitrogen removal.

A solution of having more population is the addition of fresh Anammox sludge in the reactor, which will increase the sludge concentration.

5.5.3. Temperature, DO and pH.

The temperature and the dissolved oxygen are key factors for the control of the process. The optimum temperature for the Anammox process is relatively high (between 30-40 ºC, because 37 ºC is the maximum temperature where there is activity of the Anammox bacteria and at 45 ºC the activity is already lost (Dosta et al. 2008; He et al. 2015)) but in many of the wastewaters that are not in a warm climate their temperature are low. It is studied that at low temperature the Anammox activity is reduced, but if there is an acclimatization of the bacteria at low temperatures, the problem can be solved (at least partially) (Xing et al. 2015). Studies showed that there is less negative effect if the temperature is decreased stepwise than with a sudden drop (Isaka et al. 2008; Persson et al. 2014). Isaka et al. (2008) showed that with a reduction of temperature (from 20 to 12 ºC) a high nitrogen removal can still be achieved. In a cold adaptation process the responsible for a good performance of the AOB is the shift to the optimum temperature (Hendrickx et al. 2014).

The dissolved oxygen is also a critical parameter (Okabe et al 2011) because at low concentrations there is not enough oxygen to complete the partial nitrification and the process is limited by substrate (oxygen) and at high concentration an irreversible inhibition of the Anammox biomass can also become, so it has to be controlled in order to not exceed these concentrations. At concentrations below 1 mg O_2 L^{-1} (Park and Noguera, 2004; Okabe et al 2011; Tokutomi, 2004) the AOB grows faster than the NOB.

The pH is an important control parameter for the Anammox process. When the nitrogen loading rate (NLR) increases also the pH does (Niu et al., 2008) because of the H^+ is consumed when the AOB uses nitrite (electronic acceptor) to oxidize ammonia. The optimum pH is 7.5 -8. (Egli et al. 2001), over 8.45 the Anammox reactor enters to a zone where a slight variation of the pH can conduce to an inhibition of the Anammox activity (Puyol et al. 2014).

Along this section 5.5 there are mentioned the main factors that affect the Anammox process. Many of these factors are related with the competition among the different microorganisms. In the figure below (figure 12) there is a scheme of the relation of the competitions between the different microorganisms.

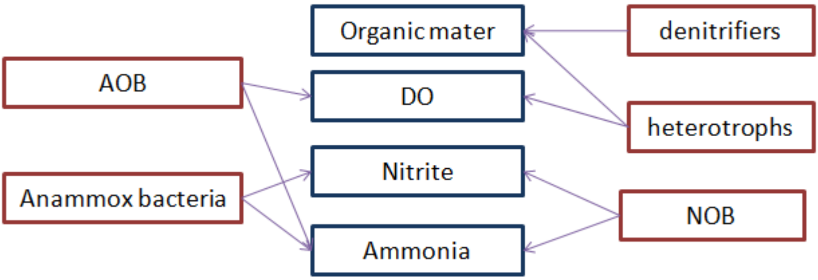

Figure 12.Competition among the different microorganisms.

6. FULL SCALE APPLICATIONS.

There have been at least one hundred applications of the new processes of partial nitritation and Anammox at full scale (Lackner et al. 2014) in the sidestream line, although the conditions of each plant are different due to the different wastewaters to be treated.

Almost all the plants have several conditions that are similar: the temperature of the wastewater is relatively high, there is a high strength ammonium wastewater, and a low ratio of carbon to nitrogen.

It has been studied the plants that were in operation in 2014, and more than 50% of them used sequencing batch reactors; 88% were single stage systems and 75% were applied to the sidestream treatment of municipal wastewater (Lackner et al. 2014).

The plants can be classified in three different ways to operate:

- Aeration supply: the feeding of the air can be intermittent or continuous.
- Biomass: the biomass can be suspended or attached.
- One stage process or two stage process.

Table 6.Few examples of full- scale implementations

SBR reactor	Continuous feeding	Intermittent feeding	Two stage process	Biofilm reactor
Zürich, Switzerland: the SBR is controlled via an ammonia sensor. (Joss et al. 2011). **Ingolstadt, Germany** **Rotterdam, Netherlands** **Gütersloh,Germany:** they implemented the SBR reactor after an storage tank (Schröder et al. 2009). **Strass, Austria:** Uses SBR Demon with a control of pH (under patent). (Wett et al, 2006).	**Gütersloh,Germany:** when the ammonia is over the limit the aeration is activated, and it is been stopped when the pH or the concentration decreases. **Hattingen, Germany:**has stirrers for the aeration. **Rhendawiedenbrück, Germany.**	**Zürich, Switzerland**: has two different feedings: at the beginning on each cycle and in the aeration phase (that controls the nitration and the ammonia). **Ingolstadt, Germany:** uses also the SBR reactor.	**Rotterdam, Netherlands:** has two stage process (SHARON/ ANAMMOX), but now they are trying to use the single stage. (Lackner et al. 2014). **RhendaWiedenbrück, Germany:** this plant uses a two stage suspended sludge.	**Mechernich, Germany:** (Hippen and Rosenwinkel, 1997). Uses a biofilm reactor with rotating biological contactors. **Kölliken, Switzerland.** (Siegrist et al 1998). **Pitsea, Great Britain.** (Schimd et al. 2003). **Hattingen, Germany:** the first biofilm full scale implemented. (Szatkowska et al. 2007). **Himmerfjärden, Sweden (2007):** they improved the plant using an integrated fixed film with activated sludge.

In the table above (table 6) the different full-scale implementations are divided according to different characteristics, depending on the type of reactor, the type of aeration used, or the number of stages.

The wastewater treatment plant in Zürich (Switzerland) had a successful start up but in winter the Anammox activity decreased, and it was thought that the problem was the season, but it did not happen again at the same season. So the reduction was because the DO was higher than 1 mg O_2/L due to an unidentified toxic substance (Joss et al. 2009). The wastewater treatment plant Niederglatt (started in April 2008) had a fast start up due to it had an inoculation of 100 m^3 of sludge from the WWTP of Zürich (Joss et al. 2009).

Some of the plants during the years had developed new improvements to achieve higher efficiency. In Ghent University it was developed a new process called OLAND (Vlaeminck et al 2009). It requires less money for the operation but it's control has high complexity. OLAND process has been implemented in DeSah, Bulgaria, and Sneek, Netherlands, but for black water treatment (Lackner et al. 2014). In 2015 a full-scale plant using an OLAND treatment is expected. In Germany they introduced a new material called: *terrana.* This is a material that has small splits of bentonitic splay that it is added to the suspended sludge. It helps to retain the Anammox bacteria and to stabilize the pH. The only problem of this material is that has high picks of loading Tss (Lackner et al. 2014).

Another improvement that has been done is the process Anitamox (Malmö, Sweden). This process improves the integrated fixed film with activated sludge. They have under patent the control based on ratio of ammonia/NO_3.

The main challenge nowadays is the implementation of the partial nitrification and Anammox in the mainstream line of municipal wastewater plants. Compared with the sidestream line, the mainstream is more challenging since the ammonium concentration is not high enough to produce FA to suppress NOB.

However, implanting it in the mainstream will improve the energetic efficiency of the plant. The strategy for the process will be the avoidance of the nitrite oxidation in order to obtain the target effluent quality and to maintain the stability. The best reactor that has been studied is the reactor with biofilm or granular sludge that maintains high sludge retention time. Nevertheless, it has problems because the dissolved oxygen will have to be maintained low in order to not destabilize the balance between Anammox and ammonia oxidation.

Just a few implementations has been done in a full-scale, two of them are in WWTP Glarnerland, Switzerland and in the WWTP Strass (Austria)(figure 13).

Figure 13. Plant of WWTP Strass, Austria (http://www.aaees.org/e3competition-winners-2013gp-research.php) .

6.1 Control of the plants:

The online monitoring is a reliable strategy to keep the stability of the process. The full-scale implementations determined that it is better if the process is as less manual as possible, due to human errors and also because the manual control much difficult. Online sensors are the sensors that have more demand, especially the ones for ammonium, nitrite and nitrate.

Almost 20-35% of the plants had problems with the process performance, which is normal because the plants are new and the process is still in progress of improvement (Lackner et al. 2014).

When in a plant the nitrate accumulation is problematic the best strategy is to control the air flow rate and the nitrogen species (Joss et al. 2011). The two main factors to be controlled are the pH and the DO. In different plants the time of the aeration varies (Lackner et al. 2014). The pH control in the reactor relies in the alkalinity and in the buffer capacity, and this will avoid the carbon dioxide limitation of the biomass. The control of DO is important, because several plants reported to have had an impact of DO sensor problems. A better control parameter is the air flow rate rather than the DO, because if there is a failure of the DO control systems it can lead to several consequences in the process (Joss et al. 2011).

7. APPLICATION OF PN/ANAMMOX IN THE MAINSTREAM

As mentioned earlier, the problems of this new technology starts when it has to be implemented in the mainstream of the municipal wastewater plants. However, implementing it in the mainstream line will improve the energetic efficiency (Morales et al. 2015). During all these years the application in the sidestream has been widely studied and developed and it does not have significant problems, being a mature technology today.

In the mainstream line the application is still in progress. The main key factors that concerns the performance in the full scale implementation are the greenhouse gases emissions, the sludge production and the energy consumption (Mo and Zhang, 2013; Yerushalmi et al. 2013), but the main bottleneck is the stable operation of the PN process avoiding the nitrite oxidizing bacteria (Morales et al. 2015). Some of the issues can partially be solved with a two stage reactor. The effect of the dissolved oxygen on the Anammox biomass is suppressed, but as it is said in the previous chapter (section 5.4.2) using two stages the price for the implementation is higher than the single stage.

To operate in the mainstream a minimum amount of biomass is required, together with the effective control of the ammonia oxidation and the dissolved oxygen. There are three main challenges to be addressed in the process. Firstly there is a relatively high ratio of COD to nitrogen in the wastewater. If significant organic carbon reaches the Anammox process, it might lead the denitrifiers to out-compete the Anammox bacteria. Secondly the selective retention of AOB (ammonia oxidizing bacteria) over the NOB (nitrite oxidizing bacteria), and last the accumulation of the Anammox bacteria.

7.1 COD/N ratio:

As it is said in a previous section (5.5.1.3) the municipal wastewater contains nitrogen and organic carbon (Abbas et al. 2014), and if there is organic carbon in the water, the heterotrophic denitrifiers compete with the Anammox organisms for the nitrite. Ni et al. (2012) showed that if the ratio is low it does not affect to the Anammox reaction. But, if in the wastewater the ratio of COD/N is higher than 1 (Güven et al. 2005) the denitrifiers (heterotrophs) can outcompete the Anammox bacteria (autotrophs). From report to report the maximum admissible ratio of COD/N differs from 1 to 2 (Güven et al. 2005; Chamchoiet al.2008). In any case, the undesirable out competition by the denitrifiers occurs because according to Gibbs free energy the denitrification is thermodynamically more favorable than the Anammox process.

Regarding to this ratio, one of the main aims is the energy efficiency. In the Anammox process a fraction of the energy can be recovered as a biogas (from the sludge digestion the plant is able to produce electricity out of biogas). To recover the maximum energy and to have a major environmental sustainability firstly it must be implemented an anaerobic digester combined with biogas incineration (Corominas et al. 2013). Dereli et al. 2010 reported that co-digestion of the sewage sludge with organic fraction in the anaerobic degradation increases the methane and improves also the stability of the process. Besides with anaerobic digestion an energy recovery is done and contributes to the energy balance of the plant (Verstraete and Vlaeminck, 2011).

The solution for this problem is the removal of the biodegradable COD . The mechanism to improve this issue is (figure 14): at first stage there is a physic treatment by gravity that decreases the COD up to 30% and some solids are removed (removal of particulate COD). This would be an improved or upgraded of the primary settling step. In the second stage a reactor is used, normally a High Rate Activated Sludge (HRAS) reactor (Xu et al. 2015), in order to remove most of the soluble COD. As well, an anaerobic digester has to be operated to convert the organic carbon and produce the methane rich biogas (Kartal et al. 2010). To lead high energy recovery in this process a high load activated sludge is needed. The biomass with high growth yield is generated by the soluble organic matter (it will be later converted to biogas). The biomass generated is separated in the primary settler (mentioned above) with the colloidal and non degraded suspended material. Then, in the digestion process the organic matter concentrated is converted to biogas (methane).

In the HRAS reactor, there is an intracellular storage or biosynthesis and then the COD is separated as biomass. This reactor enables high C energy recovery and reduces the aeration demand. One of the problems that HRAS has is the unbalance sludge flow between the sludge returned to the stage CEPT/HRAS/AD and the sludge directed to AD (for biogas production). So if there is more sludge going to AD there is less sludge for the capture of COD. Additionally the anaerobic digester recovers high amount of the C energy however the nitrogen cannot be removed. It enables the recovery of the energy in form of methane. The reactors Upflow Anaerobic Sludge Blanket (USAB) and Expanded Granular Sludge Bed (EGSB) have been commonly implemented to remove the biodegradable organic matter with biogas generation (Xu et al. 2015). If the reactor UASB is not well controlled it may emit greenhouse gases (Liu et al. 2013). The reactor Chemically Enhanced Primary Treatment (CEPT) (Harleman et al. 1999) reaches higher COD/N ratios than the HRAS reactor and it does not has as many problems as the HRAS has (stability and quality of the sludge) (Xu et al. 2006). Many of the COD is removed in the stage one, and the excess sludge of the second stage (through PN/Anammox) is reduced due to the slow growth rates of the ammonium oxidizer bacteria and Anammox bacteria, and as a consequence the excess can be recycled for carbon capture in the stage one (Xu et al. 2015).

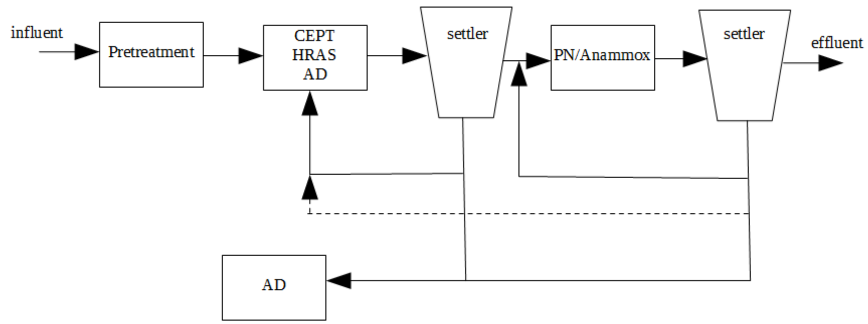

Figure 14. Proposal for the PN/Anammox process in the mainstream line. Adapted from Xu et al. (2015).

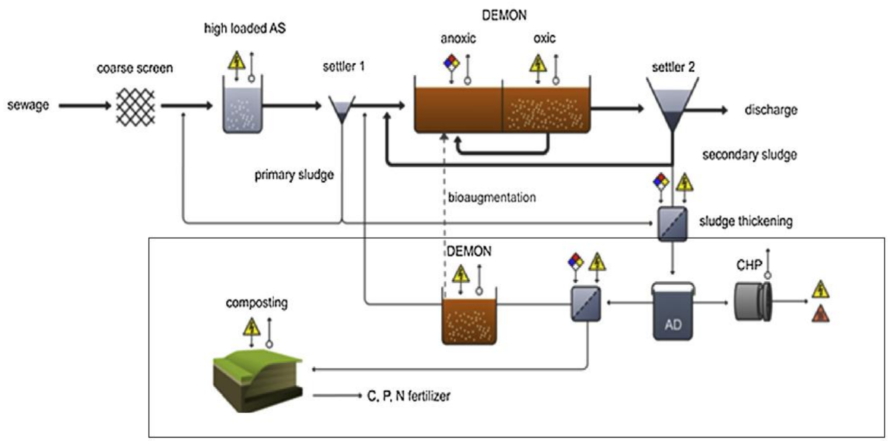

Figure 15. Schematic of the sewage treatment at the Strass plant.Adapted from Schaubroeck et al. 2015.

WWTP Strass, Autria has been put forward as an energy self-sufficient plant, making it a role model for most of the WWTP plants. In the figure above (figure 13) there is an schema of the plant where it can be seen in the mainstream line the two stage sludge system and in the sidestream line the DEMON reactor for the anaerobic digestion treatment and also there is upgraded the low load activated sludge to a DEMON. The reactor DEMON was implemented as to improve the nitrogen removal and the energy production. In Strass plant the **N_2O** is also a parameter that concerns since is a powerful harmful greenhouse gas (Joss et al. 2009). Schaubroeck et al. 2015 mentions that the N_2O is linked to the operational conditions, and the emissions are increased when there is a nitrite accumulation. The reactor DEMON has high risk for accumulation of the nitrite and as a consequence a high risk to emit more N_2O. Since there is a risk in the reactor it is crucial to control it as to reduce the environmental impact of the plant.

7.2 Retention of AOB in front of NOB:

The retention of AOB and the suppression of NOB at low temperature and low N load is one of the main limiting factors for the mainstream application that has to be highlightened (De Clippeleir et al. 2013; Wett et al. 2013). As mentioned in the previous chapter (5.3) there are two types of bacteria that interact in the process the Aer.AOB and An.AOB, the first one require ammonia and oxygen and the second require ammonia and nitrite. NOB compete for the oxygen with the Aer.AOB and for the nitrite with the An.AOB. Many attempts showed that most of the ammonia is oxidized into nitrate instead of dinitrogen gas due to the nitrite oxidizing bacteria (NOB). The objective in the mainstream is the out selection of the NOB in front of the AOB and the Anammox populations (Morales et al.2015). The conventional strategies for the NOB suppression are not usable because of the low temperature and the low ammonium concentration of the mainstream. At low temperature it is more difficult to prevent the growth of the NOB (because at low temperature NOB grows faster than AOB) (Hellinga et al. 1998), and as a consequence in the effluent there will be high nitrate concentrations (Winkler et al. 2012). Vazquez-Padin et al. 2009, reported that it will not be an inhibition if all the nitrite that is produced is consumed (equally).

A study in a lab-scale for a single stage reported that the optimum range of DO is 0.5-1 mg /L (Abbas et al. 2014). Sequencing fed batch of aerobic granular sludge reactor is the best option.

Below there are summarized the different methods to improve the retention of AOB in front of NOB:

- Increasing the temperature: it is a good strategy since the AOB growth faster than the nitrite oxidizers. The problem is that this strategy (that is one of the best) it is only possible in tropical climate due to the high cost of increasing the temperature.
- Combined control of the dissolved oxygen and the sludge retention time (Abbas et al. 2014; Szatkowska et al. 2007): Currently the control of DO is the best practical solution. The affinity to dissolved oxygen is larger for the AOB than the NOB when there is a system with DO limited conditions (Blackburne et al. 2008). The NOB requires more time to oxidize (it has to move from anoxic conditions to aerobic ones). The new approaches are focused on the development of the lag phase in the nitrate production.
    - The best option is to use an intermittent aeration (aerobic and anoxic conditions will be alternated). Although the continuous aeration has more advantages than the intermittent one, because of its simplicity (better monitoring) (Joss et al. 2009) and its higher performance for the control of the oxygen.

    Therefore the intermittent aeration has to be optimized so as to know the exact quantity of aeration necessary. Okabe et al. (2011) reported that the optimum ratio of air flow rate to ammonium loading rate was below 0.1[(m^3-air d^{-1})(Kg-N$m^{-3}d^{-1}$)$^{-1}$] so as to have a stable partial nitrification. This new approach seems to be the solution, even though do not completely avoid the growth of the NOB and it must be joined with a short aerobic solids retention time (Regmi et al, 2014). Additionally, to maintain the alkalinity the DO has to be lower than 0.06 mg O/mg N/d (Bagchi et al. 2010).

- Another approach is using the biofilm reactor. It has been wide implemented and researched (there have been used several different reactors such as UASB, SBR, MBBR (moving bed biofilm reactor), DMBR (dynamic membrane reactor) and RBC (rotating biofilm contactor)). In this method the AOB grow in the outer layers producing nitrite and consuming oxygen. In the inner layers the ammonium left and the nitrite produced helps the Anammox growing bacteria. Some studies (Volcke et al. 2010; Winkler et al. 2011b) have shown that the nitrifiers grow more in the smaller granules because the granules have larger aerobic volume fraction. The only requirement needed is the control of the conditions for the Anammox bacteria and the AOB. To maintain the stability of the process there are some considerations to be addressed:

    - To obtain the anoxic conditions in the inner part of the thickness of the biofilm, it has to be larger than the oxygen penetration depth. (Gilbert et al. 2014a; Morales et al. 2015). The thickness depends on the temperature and on the dissolved oxygen concentration (Morales et al. 2015).
    - The concentration of the ammonia in the bulk liquid has to be about 0.48 times the concentration of the oxygen in the bulk liquid (Campos et al. 2010) so that there will be enough ammonia to remove the nitrite in the anoxic zone and to allow the total consumption of the oxygen.
    - The ammonium oxidizing bacteria in the outer layers have to be enough to not let the dissolved oxygen penetrate but not too high to produce high levels of nitrite (Vlaeminck et al. 2010). The temperature must be controlled in order to maintain the process stability (it affects both the Anammox activity and AOB) (Morales et al. 2015).

- Alternating the bioagumentation in the sidestream part of the ammonium oxidizing bacteria: This process reaches fasters start up (Xu et al. 2014) and improves the biodegradation of the phenolic compounds (Quan et al. 2003) if they are present. Although seems to be one of the best alternatives this process has some negative effects. Bartrolí et al. 2010 reported that the bioaugmentation improves the duration and the stability of the start-up in a biofilm airlift reactor.

The online monitoring is actively pursued nowadays in order to have a better control of the process. Most of the full-scale plants use online sensors for NH_4-N, NO_3-N, and less of NO_2-N because sometimes these sensors can be affected by some unreliability.

7.3 Anammox bacteria retention.

The Anammox bacteria have low growth rate which is also sensitive to some compounds and operation conditions. This is a drawback for the Anammox process (Strous et al. 1998). Due to the low growth rate the start up of the Anammox process is complicated and slow (specific growth rate: 0.065 day^{-1} (Strous et al. 1998)). Also, one of the failures in the process of start-up is the biomass washout due to the production of nitrogen gas bubbles (Chen et al. 2010). So, with the conventional microbiological methods the Anammox bacteria cannot be cultivated because of it slow growth (Chamchoi and Nitisoravut 2007). To have a successful start up a good biomass retention is needed in the reactor and also, sufficient amount of seeding (Ali et al. 2014b).

The temperature plays an important role due to its effects to the microbial activity. The Anammox bacteria as it is said in the previous chapter (Section 5) under low temperature grow even more slowly (duplication time equals 25 days or more (Hendrickx et al. 2012)), however with high temperature the growth of the bacteria is faster (duplication time 11 days) (Strous et al. 1998). If the temperature is lower than 20 ºC the Anammox bacteria require a long sludge residence time, which implies very good biomass retention (Lotti et al. 2014). Also the light leads the growth of phototrophic algae that can inhibit the bacteria (Uyanik et al. 2007). In the full scale plant is required a quick start up, and high biomass concentration to achieve high stability. The capability for the degradation of the pollutants at low temperature still lasts due to the cold adaptive capacity of the microorganisms (Guo et al. 2010).

The start up can be improved by having an appropriate seed of biomass and a good configuration of the reactor (Suneethi et al. 2014). To have good start up the key parameters are the seed, the operational strategy, the conditions, the type of reactor and the potential inhibitors. Various studies have been made as to know the best biological reactor for the cultivation of Anammox biomass (Dapena-Mora et al. 2004; Wang et al. 2009; Chamchoi and Nitisoravut 2007).

In order to develop the growth of Anammox bacteria one of the best alternatives was the Sequencing Batch Reactor (SBR) (that has efficient retention biomass, stable conditions and is simple). Chamchoi and Nitisoravut in 2007 used a SBR and the start up time was only 4 months.

So as to enrich the bacteria, the sludge retention time has to be increased. It has to be careful with the hydraulic retention time because if it is increased, the nitrogen loading rate is decreased. In order to solve the problem of the low growth rate, two solutions are shown: the influent flow rate to the partial nitrification must be increased (Winkler et al. 2012) or retaining efficiently the inoculated biomass in the reactor by immobilizing the biomass in gel beads (Isaka et al. 2006) (will allow high NLR and short HRT, and there won't be neither biomass washout or nitrite inhibition). The **gel beads** increase the effective diffusion in the biomass changing the matrix of it and improving the specific Anammox activity. Ali et al. (2014b) studied different types of gel beads[1], and reported that the mixture of polyvinyl alcohol with sodium alginate is the most efficient to improve the start up of the Anammox process.

[1]polyvinyl alcohol (PVA), Sodium alginate (SA), Polyethylene glycol (PEG), and PVA+ SA.

One solution is to reach the optimum speed (growth) by having a different unit where the biomass is produced with optimum temperature and high ammonia concentration (Morales et al. 2015) and accelerating the process by inoculating with enriched Anammox (Suneethi et al. 2014). Another solution is to adapt progressively the biomass to low operating temperatures. However showed that the sudden change of the temperature does not affect to the stability (Winkler et al, 2012a; Morales et al. 2015).

The **bioagumentation** is also a solution to speed up the start up of the bioreactor (Dabert et al. 2005). However it has some disadvantages for example the changing on-side operational conditions. This method has been applied in three different WWTP (Jiamusi, Mudanjiang, Taiping) (China). It is more cost-effective than the conventional method, and the discharge of the pollutants in the environment is reduced (Guo et al 2010). Guo et al. 2010 reported that bioaugmentation enables rapid and stable start up performance in WWTP with low temperatures if an specialized bacteria is added.

8. CONCLUSIONS

The Anammox process seems to be one of the most promising alternatives for the nitrogen removal in the wastewater treatment plants. This process combined with anaerobic digestion can turn the energy balance of the plant into positive. The conventional nitrification and denitrification is not a good choice to recover energy because it requires high energy input in order to remove the nitrogen and also it consumes biodegradable carbon. However the challenges for the application of the full-scale Anammox process in the mainstream line are still matter of research due to some issues and limitations.

- The prediction, the modeling and the design of the Anammox process is difficult because of the discrepancies of the different studies.
- The relatively high COD/N ratio can be solved by using a two stage system: Firstly having a carbon removal and then having the PN/Anammox, with an anaerobic digester, that recovers the energy as biogas.
- The intermittent aeration is one of the most promising solutions for the retention AOB in front of NOB. Many researchers are investigating the optimum ratio of the air flow rate.
- Speeding up the growth of the Anammox bacteria, produced in a different unit with high temperature and high levels of ammonia, is the best option to improve the Anammox bacteria retention. Another way is the step by step adaptation to low temperature.

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10. ACRONYMS

- Anammox: anaerobic ammonium oxidation
- AOB: ammonium oxidizing bacteria
- An. AOB: anaerobic ammonium oxidizing bacteria.
- Aer. AOB: aerobic ammonium oxidizing bacteria.
- AD: anaerobic digester.
- CEPT: chemically enhanced primary treatment.
- COD: chemical oxygen demand.
- C/N: carbon nitrogen ratio.
- Cs: concentration of the substrate.
- CANON: completely autotrophic nitrogen removal over nitrite.
- DN: denitrification.
- Deamox: denitryfing ammonium oxidation.
- DEMON: aerobic /anoxic deammonification.
- DO: dissolved oxygen.
- DMBR: dynamic membrane reactor.
- EGSB: expanded granular sludge bed.
- FA: free ammonia.
- FNA: free nitrous acid.
- FIBR: fluidized-bed reactor.
- FBR: fixed bed reactor.
- FISH: fluorescence in situ hybridization.
- HRT: hydraulic retention time.
- HRAS: high rate activate sludge.
- Ks: half saturation constant.
- MBR: membrane bioreactor.
- MBBR: moving bed biofilm reactor.

- NOB: nitrite oxidizing bacteria.
- NLR: nitrogen loading rate.
- OLAND: oxygen limited autotrophic nitrification denitrification.
- PN: partial nitrification.
- PVA: polyvinyl alcohol.
- PEG: polyethylene glycol.
- qs: biomass specific uptake rate.
- RBC: rotating biological contactor/rotating biofilm contactor.
- SA: sodium alginate.
- SHARON: single reactor system for high-rate ammonium removal over nitrite.
- SBR: sequencing batch reactor.
- SNAD: simultaneous partial nitrification, Anammox and denitrification.
- SNAP: single-stage nitrification removal using Anammox and partial nitrification.
- TNN: total nitrite nitrogen.
- TAN: total ammonium nitrogen.
- UASB: up-flow anaerobic sludge blanket.