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TESIS

Novel technologies for WWTP optimization in footprint, nutrients valorization, and energy consumption.

Doctor en Ingeniería Química y Ambiental por la Universidad de Santiago de Compostela.

NICOLÁS MORALES PEREIRA

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Nicolás Morales Pereira

Ph. D. Thesis

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Enxeñaría Química

Novel technologies for WWTP optimization in footprint, nutrients valorization, and energy consumption

Memoria presentada por:

Nicolás Morales Pereira

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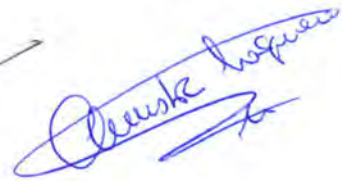
Ramón Méndez Pampín, Catedrático de Ingeniería Química, José Luis Campos Gómez, Profesor Contratado Doctor de Ingeniería Química y Anuska Mosquera Corral, Profesora Titular de Ingeniería Química de la Universidad de Santiago de Compostela,

Informan:

Que la memoria titulada “Novel technologies for WWTP optimization in footprint, nutrients valorization, and energy consumption”, que para optar al grado de Doctor de Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, presenta Don Nicolás Morales Pereira, ha sido realizada bajo nuestra inmediata dirección en el Departamento de Ingeniería Química de la Universidad de Santiago de Compostela.

Y para que así conste, firman el presente informe en Santiago de Compostela, el 10 de enero de 2014.

Ramón Méndez Pampín	Anuska Mosquera Corral	José Luis Campos Gómez
		

Esta tesis fue presentada el 6 de junio de 2014 en la Escola Técnica Superior de Enxeñaría (E.T.S.E.) de la Universidad de Santiago de Compostela ante el tribunal compuesto por:

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Obteniendo la calificación de *Sobresaliente Cum Laude* y la mención de *Doctorado Europeo*.

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Nico

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SUMMARY AND OBJECTIVES

The continuous development of the human society causes stress to natural systems: pollution increase, deforestation, global resource depletion, etc. Water resource contamination and water scarcity are among the major challenges to be confronted by humanity in the 21st century. Present wastewater treatment technologies were developed between the end of the 19th century and during 20th century. Technology evolved from simple aqueduct sewerages that dumped in waterways without treatment to a complete treatment; including physical, chemical and biological treatments, aimed to remove solids, organic matter and main nutrients. Nowadays, novel concepts are taken into account in order to face up to new environmental, economic and social limitations. At the same time, these new concepts have to deal with aspects like the population growth, the global climate change, the water scarcity, etc.

Up to now, wastewater treatment plants (WWTPs) can be defined as systems where organic matter, nitrogen and phosphorous are removed from wastewater using energy. However, this concept should be changed in the next years in order to improve these systems in terms of economic feasibility and environmental impact. In this way, wastewater must be seen as a renewable and recoverable source of energy, resources and water. This is feasible by means of the application of new treatment systems and technologies. In addition, the new wastewater treatments should require smaller implantation areas and produce lower amounts of sub-products, as greenhouse gases or excess sludge, compared to conventional ones.

According to this modern approach, the present thesis has been focused on the study of some alternatives to improve the WWTPs by the application of new technologies. In this way, the main improvements expected from this thesis are related to:

- The reduction in the sludge production in the biological processes used to remove organic matter and nitrogen from the wastewater.
- The improvement of the settleability of the biomass in the biological reactors.
- The treatment of wastewater in order to facilitate its application as fertilizer and the recovery of nutrients from the wastewater.

- The decrease of energy requirements for the wastewater treatment to achieve the energy self-sufficiency.

In this way, in Chapter 3, the aerobic granulation technology was applied to the treatment of swine slurry. This technology allows reducing the excess sludge production in the WWTPs and the requirements of area needed for its implantation by the formation of granular biomass with good settling properties. Aerobic granulation was mainly obtained in Sequencing Batch Reactors (SBRs) as it was the case of the pilot plant scale reactor used in Chapter 3. Nevertheless, in Chapter 4, the possibility of obtaining this kind of good settling biomass in a continuous reactor was researched. In Chapter 5, ammonia sulphate and struvite, nutrient rich minerals, were recovered from the sludge liquid fraction and separately collected urine. This combination allowed the recovery of nitrogen and phosphorus from wastewater streams. Finally, in Chapter 6, the applicability of the Anammox (ANaerobic AMMonium OXidation) process for the nitrogen removal from the water line of WWTPs was researched, with the aim of increasing the energy self-sufficiency of the wastewater treatment.

The main contents and the specific objectives corresponding to each chapter of the present thesis will be described in more detail in the following paragraphs.

In Chapter 1, an overview of the state of the art of the future challenges related to the wastewater treatment, and some of the novel technologies and new solutions which have emerged in the last years, are presented. Special attention is paid to the new technologies applied to nutrients removal and recovery, and aerobic granular biomass, which are studied in the present thesis. An actualized literature review about the performed studies up to date in these fields is presented.

In Chapter 2, a description of the analytical methodologies applied to determine the conventional parameters used in the thesis for the wastewater and biomass characterization is provided. Some usual parameters like the pH, dissolved oxygen (DO), chemical oxygen demand (COD) or solids concentration were measured following the instructions of the Standard Methods. The Fluorescent in situ Hybridization (FISH) technique, applied to the identification of the microbial populations involved in the biological processes, is briefly described.

Other analyses were performed after being adapted to the research of this thesis, like the characterization of aerobic granular biomass by Sludge Volume Index (SVI), the determination of the biomass density of the aerobic granules, the application of the digital image analysis to determine the morphology and the average diameter of the granules or the measurement of their Poly-Hydroxy-Alkanoates (PHA) concentration. The calculations made to determine parameters which allowed the analysis of the obtained results used through the thesis are also presented in this chapter.

The specific analytical methods and calculations used in a single part of the work, and the different experimental setups are described in the corresponding chapter.

With respect to the aerobic granulation, most of the studies concerning the physical properties and performance of aerobic granular biomass were carried out in laboratory scale SBRs. These reactors are operated in sequential cycles that comprise: filling, reaction, settling and withdrawal phases. The cycles are characterized by the short length of the filling and settling phases, aiming to the development of biomass in the shape of granules in aerobic conditions. However, a relevant number of studies performed in pilot or full scale systems are not available yet. Furthermore, when working with industrial wastewater, the fluctuations of inlet feeding characteristics can difficult the achievement of stable operation conditions. Thus, in Chapter 3, the startup and performance of a granular aerobic pilot plant scale SBR reactor, with a working volume of 100 L and treating the wastewater from a pig farm was studied. Since the availability to withstand shock loadings is stated as one of the characteristics of the aerobic granular biomass, the objective of this work was to test the effect of this high variability in the feeding composition on the performance of a pilot-scale granular SBR treating swine slurry. This information is indispensable in order to carry out the scale up and application of these systems to industrial level, since this is the last purpose of the design of new technologies.

The pig slurry used in this research was characterized by its highly variable composition, in terms of organic matter and nitrogen content, and C/N ratio. This variability was further increased by the dilution with tap water. In a first stage, the performance of the pilot plant during the starting up and the process of granular biomass formation were studied. In a later stage, the reactor and granular biomass capacities of withstanding the variable fed conditions were researched.

Granulation process was achieved in the reactor, and the physical properties of the biomass remained rather stable during the operational period of more than 300 days. The granular biomass obtained in the reactor showed excellent settling properties, with the sludge volume index values varying between 27 and 60 mL/g TSS. The good settling properties facilitate the granular biomass separation from the effluent and its retaining inside the reactor. The first aerobic granules were obtained after 9 days of operation, and their average size during the operation time varied between 2.0 and 2.8 mm. The reactor showed a good biomass retention capacity to select for granular biomass. In this way, solids concentrations from 5 to 12 g VSS/L were achieved. However, its efficiency to retain the solids contained in the fed pig slurry was low and as a consequence, the solids concentration in the effluent of the reactor was similar to that measured in the influent.

Organic matter removal efficiencies were not affected by the fluctuations of the applied organic loading rates (OLR) but by the non-biodegradable fraction of the swine slurry. For this reason, the average organic matter removal efficiencies never reached values higher than

80%. Ammonia load was mainly oxidized to nitrite and, in this way, the ammonia removal efficiency was around 76%, even with the high variability of the wastewater fed to the reactor. However, denitrification was practically not observed during the experimental period.

In order to evaluate the performance of the reactor, cycle measurements were carried out on different days of operation. The biodegradable organic matter was easily removed in the first minutes of the aeration phase in all cycles, during the so called feast period. During this time, a reduction in the DO concentration due to the quick organic matter oxidation was measured. Once the biodegradable organic matter was consumed, the DO concentration in the bulk liquid started to increase (famine period), while the fraction of non biodegradable COD remained unaltered. When the organic matter was depleted, nitrate and nitrite concentrations increased in the bulk liquid, but total nitrogen concentration remained almost constant. This observation suggests that simultaneous nitrification-denitrification processes did not occur during aeration phase. Concentrations of PHAs measured in the biomass confirmed that bacteria stored organic matter in the form of these compounds during feast period and consumed them during first minutes of the famine period.

FISH technique was applied for the characterization of the obtained biomass in order to follow the evolution of the microbial populations during the granulation process, and during the operation of the reactor once the granules were formed. The detected bacterial populations indicated an evolution from those present in the inoculated sludge to those composing the aerobic granules. Filamentous organisms were present primarily in the inoculums, while they were washed out from the system as aerobic granules developed. The nitrifying microbial population was mainly composed by members of *Nitrosomonas* spp. as ammonia oxidizing bacteria, and a smaller amount of nitrite oxidizing bacteria belonging to the phylum *Nitrospirae*, in correspondence with the nitrite accumulation observed in the reactor.

The application of the concepts of the granular SBR technology for the upgrading of existing WWTPs could be limited by the different required operational conditions and the geometry of both column-type SBR and conventional activated sludge reactors. Transforming a continuous system into an SBR suitable to obtain aerobic granules is sometimes difficult. Thus, the objective of the Chapter 4 was to define the appropriate operational conditions to develop granular biomass in continuous stirred tank reactors (CSTR) with geometry similar to the conventional activated sludge reactors used in the WWTPs, which height to diameter ratio is usually around 1. The production of aerobic granules in a continuous reactor would open a new perspective to the application of this technology for the improvement of already existing WWTPs.

In order to achieve the aerobic granular biomass development, a biomass selection system, based on the reactor hydrodynamics and progressive shortening of the hydraulic retention time (HRT), was utilized in the reactor. This device consisted of a tube semi-submerged in the liquid media through which the effluent of the reactor was discharged. In this

tube, an upflow velocity of around 10 m/h was fixed, and consequently, particles with a settling velocity smaller than this fixed upflow velocity were washed out from the reactor, while the solids with good settling properties were retained. Hydraulic retention times of 6, 3 and 1 hour were tested in reactors of 3 and 6 L.

Aerobic granular-like biomass was formed in the reactor when an HRT of 1 hour and a hydraulic pressure of 10 m/h of settling velocity in the effluent discharge tube were applied. On the other hand, floccular biomass accumulated in the reactor when an HRT of 3 and 6 hours were used. The granules presented an average diameter as high as 7 mm and a large settling velocity of around 36-48 m/h. These values were remarkably higher than the typical values of settleability of activated sludge and were comparable to those from aerobic granules formed in SBRs.

However, the SVI and density values were worse compared to those corresponding to the aerobic granules formed in SBRs. During the formation of the aggregates, the SVI_{10} value decreased gradually from 700 mL/g TSS to 127 mL/g TSS, while SVI corresponding to aerobic granules cultivated in SBR reactors can vary around 30-40 mL/g TSS. Biomass density of the aggregates in the continuous reactor varied between 7 and 11 g $VSS/L_{granule}$. These values are relatively low compared to that of 43.5 g $VSS/L_{granule}$ corresponding to granules formed in SBRs. The biomass produced in this continuous system will be more easily separated from the liquid phase, reducing the need of big settlers, and facilitating the management and disposal of the sludge produced during wastewater treatment.

Regarding organic matter and nitrogen removals, OLRs as high as 4.8-6.0 g COD/L·d were treated with removal percentages of around 60%, due to the presence of a non-biodegradable COD fraction. Nitrogen removal varied between 10 and 15%, and it can be attributed completely to biomass growth, while nitrification and denitrification processes did not occur in the reactor.

To our knowledge, this aerobic granular-like biomass was the first reported to have been formed in a continuous CSTR reactor with geometry similar to the activated sludge reactors, with a height to diameter ratio around 1.

The objectives of Chapter 5 were the evaluation of the pretreatment of the separately collected urine and its further co-treatment in an air stripping system. In this air stripping reactor, the urine was mixed with the sludge liquid from an anaerobic digester. The experiments performed in this work provided preliminary results, and more experiments are needed in order to test the viability of the system, but first results were promising.

In this chapter, a novel ammonia stripping system was used to treat the supernatant from an anaerobic digester. This liquid stream in the WWTPs is characterized by a relatively low flow and a high concentration of nitrogen. This system was operated at full scale in a municipal WWTP, combined to a previous CO_2 pre-stripper and to a subsequent ammonia

sorber unit. With this combination, the ammonia was recovered as an ammonia sulphate solution at the end of the treatment. This nitrogen rich solution was sold to local farmers who used it as fertilizer. In this way, a waste product was turned into a valuable product. With respect to the system operation, the presence of the CO_2 pre-stripper, which reduced the consumption of chemicals in the overall system, increased the pH of the liquid and facilitated the deprotonation of the ammonia. Consequently, the CO_2 pre-stripper increased the nitrogen recovery in the sorber unit, while allowed reducing 50% of the NaOH consumption.

The efficiency of this system was further increased when pre-treated urine was added to the supernatant liquid. Urine was collected separately by means of No-mix toilets and dry urinals. Then, it was pre-treated in a reactor in order to remove and recover the phosphorus which was present in high concentrations in the urine. Struvite, a phosphorus rich mineral used as fertilizer, precipitated in the reactor after magnesium oxide was added to the collected urine. Struvite crystals with an average size of 42 to 80 μm were formed in the reactor.

The first experiments showed the feasibility of the combined treatment system of supernatant liquid and urine in the ammonia stripping reactor including a CO_2 pre-stripper. In this way, an increase of 10% in the liquid flux by the addition of the urine represented a 40% increase of the ammonia concentration in the inlet of the stripping unit. Even if the efficiency of the nitrogen removal was lower than the values reached during the optimization and simulations performed during the operation without urine addition, the achievement of these percentages generated a proportional increase in the fertilizer production. The fertilizer production rise partially covered the chemical and operational costs of the ammonia stripping system. In addition, operational problems due to the treated urine addition were not reported by the WWTP operators; however, the length of the experiments was too short to discard this possibility.

In Chapter 6, the autotrophic nitrogen removal at low temperatures (15-20 ºC) was performed using a Completely Autotrophic Nitrogen Removal Over Nitrite (CANON) system with granular biomass. This system was operated to research its application to remove nitrogen from the water line of the WWTPs.

The CANON system is the combination of the partial autotrophic nitritation, oxidation of half of the present ammonium to nitrite by the Ammonia Oxidizing Bacteria (AOB), and the Anammox process, where ammonia and nitrite are combined to produce nitrogen gas under oxygen limiting conditions. The Anammox processes have successfully been applied to rich ammonia streams at temperatures above 25 ºC, as it is the case of the supernatant from anaerobic sludge digesters. However, little information about the application of Anammox process at low temperature and nitrogen concentration, like it is the case of the main stream of the WWTP, is available. As advantages, the CANON system presents the fact that it requires less aeration energy and no organic carbon source for the conversion to nitrogen gas, compared to the conventional nitrification/denitrification processes. Furthermore, larger

amounts of organic matter are available for methane production, which increase the energy recovery in the WWTPs, as the organic matter is not needed for heterotrophic denitrification.

The use of slow growing autotrophic microorganisms, as it is the case of the Anammox bacteria, implies the use of reactor systems characterized by high biomass retention capacities. In this case, the biomass retention was enhanced through the use of biomass in the form of granules grown in an SBR system. AOB bacteria developed in the outer layers of the granules where oxygen and ammonia were present. On the other hand, Anammox bacteria grown in deeper layers, where oxygen was depleted and ammonia and nitrite were available.

The CANON system was initially operated at 20 ºC and fed with moderate concentrations of ammonia (150-250 mg NH_4^+-N/L). Later the temperature of operation was decreased to 15 ºC. Finally, an influent containing low ammonia concentrations (50-80 mg NH_4^+-N/L) was applied in order to simulate the conditions of the water line of a municipal WWTP where the organic matter was previously removed.

At 20 ºC, the biomass retention in the SBR was satisfactorily achieved, and solids concentrations of more than 12 g VSS/L were obtained. Nitrogen removal rate values of 0.45 g N/L·d were achieved, reaching a 70% of nitrogen removal efficiency. However, when the process was operated at 15 ºC the low biomass growth and the difficulties to control the low dissolved oxygen concentrations in the bulk liquid implied the need of testing different reactor configurations. In addition, the low oxygen concentration, temperature and nitrogen load facilitated the development of a third microbial group, the nitrite oxidizing bacteria (NOB). The NOB compete with Anammox for the nitrite and with the AOB for the oxygen. The overcoming of these undesired microorganisms was a challenge in the last part of this research. In order to solve these drawbacks, the inclusion of mechanical stirring, the use of a reactor with a different height to diameter ratio, and variations in the settling time were tested. Two laboratory scale SBRs were used during the experimentation with a working volume of 1.5 L and 4.0 L, respectively.

Three main factors should be pointed out as crucial to control the performance of the AOB and Anammox bacteria in a CANON system operated at low temperature and nitrogen load: 1) to achieve a high biomass retention; 2) to achieve an equilibrium between the AOB and Anammox activities and 3) to avoid the NOB development in the biomass.

In a WWTP, the conventional activated sludge reactor could be substituted by a continuous aerobic granular reactor, where the organic matter would be removed, while the nitrogen would be removed in the Anammox based process.

The different alternatives of treatment research in this thesis are expected to lead to more efficient and sustainable wastewater treatment systems.

Chapter 1

INTRODUCTION

Summary

In this chapter, some of the challenges that the water and wastewater treatment processes have to face up nowadays are briefly described. New processes, new technologies and new paradigm shifts in view of water are under development. The solutions that were feasible and adequate for the 20th century are not valid anymore for the 21st century. Global population growth, climate change, energetic crisis, new pollutants, more and more stringent effluent quality requirements… seriously challenge the efficiency of the classical wastewater treatment plants.

The present thesis was focused on some of the new technologies under development in recent years which cover some of these challenges.

Aerobic granulation was used in some of the research experiments. It is a new biological process which produces less sludge, allows for high sludge retention times and has a small plant footprint, among other advantages, compared to the conventional activated sludge process. In addition, aerobic granules can combine different biological processes in the same reactor.

Wastewater carries a high amount of valuable nutrients; their recovery is one of the challenges of water research. The combination of urine separate treatment, production of struvite, and ammonia stripping can increase the nutrients recovery from wastewater through the production of fertilizers.

Anammox based processes have proved their efficiency in the nitrogen removal of the reject water. Nowadays, the scientific challenge is to apply these processes to the main stream of wastewater treatment plants, at low temperatures and nitrogen concentrations, with the multiples advantages that can be achieved.

1

OUTLINE

1.1 WATER AND WASTEWATER IN THE 21ST CENTURY

Modern wastewater treatment technology was developed during the 19th and 20th centuries. It evolved from simple aqueduct sewerages that dumped in waterways without treatment to a complete treatment. Modern wastewater treatments technologies include physical, chemical and biological treatments, aimed to remove solids, organic matter and nutrients (Figure 1.1 and Figure 1.2). Development of sanitation was considered the greatest medical advance since 1840, according to a reader survey run by the British Medical Journal (Ferriman, 2007).

However, to ensure an adequate supply and quality of water was one of the most serious challenges faced by human groups from the beginnings of civilization (Cassardo and Jones, 2011). Channels for conveying wastewater to fields for fertilization purposes have been used in Syria and Palestine since the earliest Neolithic societies (De Feo *et al.*, 2010). Sewers were already used in some households in the Mesopotamian Empire, 3500 to 2500 BC, as well as latrines leading to cesspits (Lofrano and Brown, 2010). The world's first urban sanitation systems were constructed in Harappa and Mohenjo-Daro in the Indus Valley, in the present Pakistan (Webster, 1962). Initially, wastewater was passed through tapered terra-cotta pipes into a small sump. Then solids settled and accumulated in the sump, while the liquids overflowed into drainage channels in the street. In Europe, the Minoan (3200-1100 BC) and Etruscan (800-100 BC) civilizations developed basic water and wastewater technologies: water harvesting and distribution systems, cisterns, groundwater and wells, drainage and sewerage systems (Koutsoyiannis *et al.*, 2008, Angelakis *et al.*, 2013). All the Minoan palaces applied strategies to dispose of water and wastewater with open terracotta or stone masonry conduits (Angelakis *et al.*, 2013). These systems were found in the sewage system of Knossos palace (Angelakis *et al.*, 2005). Furthermore, the Romans developed and improved these water and wastewater infrastructures. In this way, the largest known ancient sewer, the *Cloaca Maxima* in Rome, was built in the 6th century BC (Lofrano and Brown, 2010).

Nevertheless, after the Roman Empire collapse, the infrastructures, culture and knowledge of water were forgotten and abandoned. In the western countries the sanitary “dark ages” began (Lofrano and Brown, 2010). In an unprecedented historical regression, water came to be drawn from rivers and wells and to be discharged without treatment.

Unless some exception, sewage treatment did not reemerge until second half of 19th century, with the development of some new technologies (Figure 1.1). Primary treatment was developed: the Mouras automatic scavenger (1860), the septic tanks patented by Donald Cameron in 1895, the Imhoff Tank designed by Karl Imhoff in 1906, among others. Secondary treatment consisted of: trickling filter (1893), first patent related to attached growth processes for a moving cylinder with wooden slats (1900) or the activated sludge (Ardern and Lockett, 1914). Advanced treatment came later by means of: biological denitrification in an activated

sludge process (Ludzack and Ettinger, 1962), biological removal of nitrogen and phosphorous in a single sludge system (Barnard, 1975), membrane systems (1980s) and so on.

Nowadays, new concepts are being taken into account in order to face up to new environmental, economic and social limitations, coping with the population growth, the global climate change, water scarcity, etc. (Cassardo and Jones, 2011). Completely new approaches, or the recovery of some old forgotten solutions, are necessary in order to design the modern water and wastewater treatment systems.

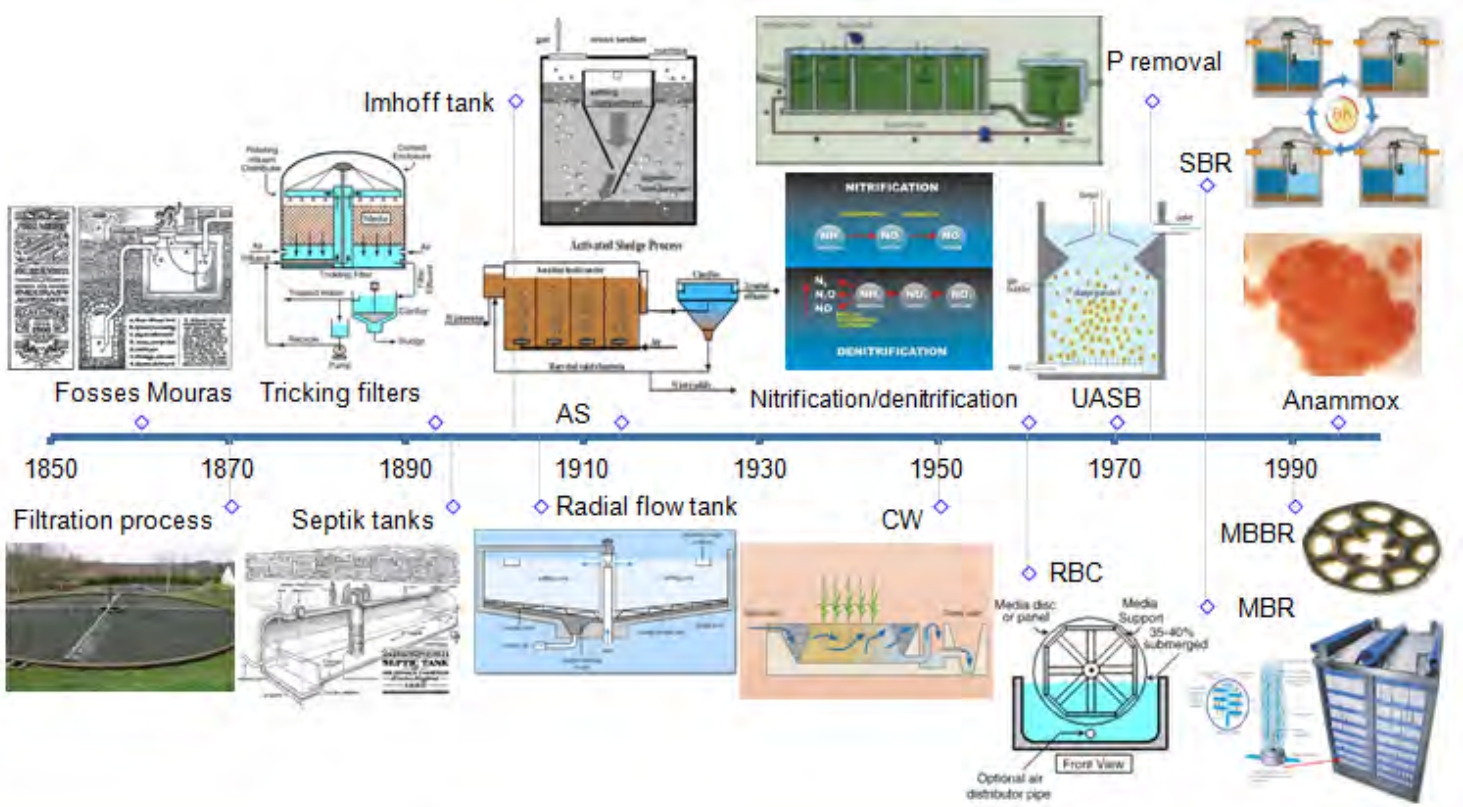
The new designs should be done with sustainability in mind, as the energy-intensive and chemical-dependent systems in current use are completely unsustainable. The present water cycle in most urban areas includes:

- a water collecting and transport system, usually several kilometers away from urban population centers;
- a treatment of all the volume to make it drinkable;
- a simple water usage, as only a small fraction is used for drinking or cooking;
- and then its treatment in huge energy and chemical consuming wastewater treatment plants, which treat a mix of black, grey, yellow, industrial and rain waters.

Consequently, the present water cycle is a totally unsustainable cycle.

This situation will be further aggravated by global climate change, which is altering water supply and storage patterns in ways that make existing water management infrastructures less effective.

Figure 1.1. Evolution of wastewater treatment: AS - Activated sludge; CW - constructed wetlands; RBC - Rotating biological reactors; UASB - Upflow anaerobic sludge blanket; MBRs - Membrane biological reactors, SBR - Sequencing Batch Reactors; MBBR - Moving Bed Biofilm Reactors. Adapted from: Lofrano and Brown (2010).



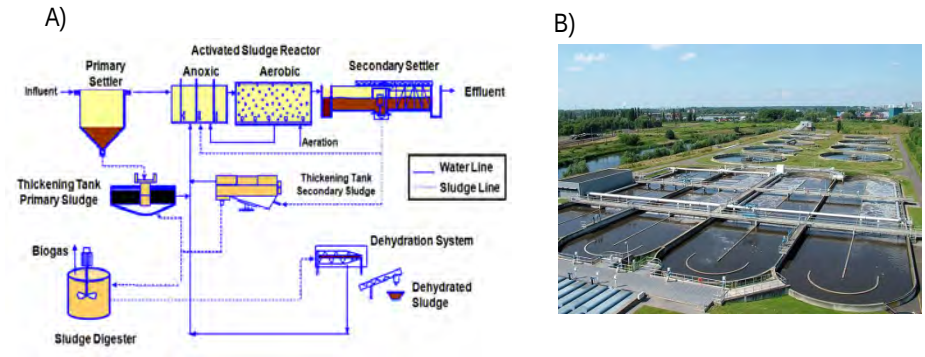


Figure 1.2. A) Simplified configuration of a conventional wastewater treatment plant. B) Image of a wastewater treatment plant (Annabel, 2009).

In a step forward (Figure 1.3), wastewater has to be seen as a renewable and recoverable source of energy, resources and water by means of the use of advanced treatment systems, which allow the obtaining of the following improvements:

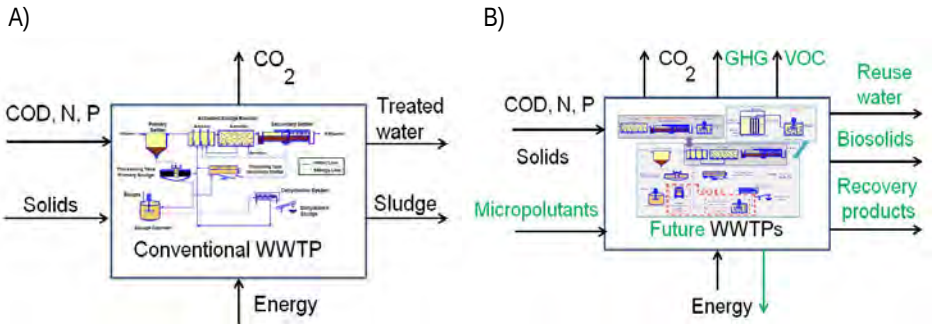


Figure 1.3. From the present-day conventional wastewater treatment plant (A) to the wastewater treatment plant of the future (B).

- to achieve a better water quality, for a full water reuse (Molinos-Senante *et al.*, 2011);
- to be more energy efficient, minimizing energy requirements and even producing energy (Siegrist *et al.*, 2008, Garrido *et al.*, 2013);
- to produce fewer by-products, as sludge or gaseous emissions, and to optimize their management (Préndez and Lara-González, 2008, Fine and Hadas, 2012, Yan *et al.*, 2013);
- to have a small plant footprint in urban areas. Decentralized systems can be seen as an option (Libralato *et al.*, 2012, Suriyachan *et al.*, 2012);
- to recover nutrients (Etter *et al.*, 2011, Hülsen *et al.*, 2014) and other compounds, as the biopolymers (Moralejo-Gárate *et al.*, 2011), cellulose as a biomass resource (Honda *et al.*, 2002) or different kind of metals (Petrov and Nenov, 2004, Manipura and Burgess, 2008, Fabbricino *et al.*, 2013);

- to minimize impacts on environment (emissions, greenhouse gases) and to treat emerging contaminants, as pharmaceutical and personal-care substances (Fernandez-Fontaina *et al.*, 2012, Rodríguez-García *et al.*, 2012).

According to this new approach the present thesis has been focused on the study of some alternatives to improve the WWTPs by the application of new technologies. This is the case of the systems based on aerobic granular biomass, systems which use separated collecting units for nutrients recovery or those based on new processes, as the Anammox processes. All these different alternatives are expected to lead to more efficient and sustainable wastewater treatment systems. These alternatives act over one or more aspects that are considered as susceptible of improvement. In this way, the main improvements expected from this thesis are related to:

- the reduction in the sludge production in the biological processes used to remove organic matter and nitrogen from the wastewater;
- the improvement of the settleability of the biomass in the biological reactors;
- the treatment of wastewater in order to facilitate its application as fertilizer and the recovery of nutrients from the wastewater;
- the decrease of energy requirements for the wastewater treatment to achieve the energy self-sufficiency.

However, this is a small part of the water cycle where humans can act upon. Furthermore, in order to understand the global situation of the water in the world several aspects have to be considered.

1.2 WATER DEMAND AND SANITATION AS POPULATION GROWS

The world population reached 7 thousand million in 2011 and is projected to reach 9 thousand million in 2050 (U.N., 2009). However, its growth has slowed down since the 1970s and it is expected to continue its downward trend. On the contrary, water demand is continually increasing at higher rates than population growth. This water demand growth comes mainly from countries with high rates of economic growth and large current populations. In these countries, people, agriculture and industry demand more water and with higher quality as stated by the United Nations World Water Assessment Programme (2009).

Although water covers 70% of the Earth surface, less than 3% of this water is fresh (Figure 1.4). As less than 1% of the freshwater is readily accessible and is unevenly distributed throughout the planet, water scarcity (Rijsberman, 2006) (Figure 1.5) can be the origin of local and international conflicts (Figure 1.6). Some authors talk about *"water war"* or conflicts due to water disputes (Barnaby, 2009, Rahaman, 2012). By 2025, nearly 2 thousand million people will be living in countries or regions with absolute water scarcity, and two-thirds of the world population could be living under stressed water conditions (World Economic

Forum, 2009). This situation will be further exacerbated by global climate change, which is altering water supply and storage patterns in ways that make existing water management infrastructure less effective.

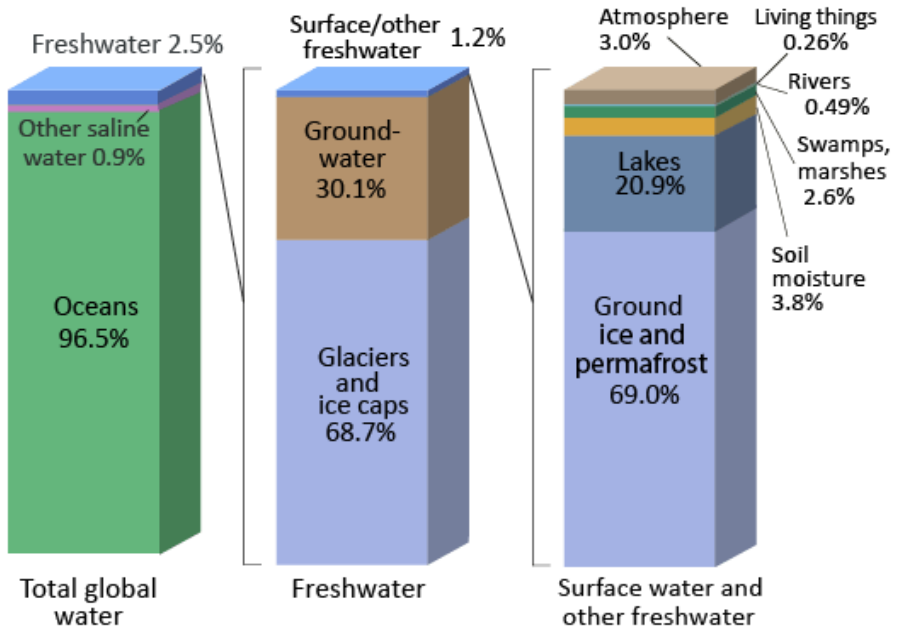


Figure 1.4. Global distribution of water in the world. (Shiklomanov, 1993, The USGS Water Science School, 2013).

Even with those water conflicts and water scarcity, the world is on track to meet the Millennium Development Goal (MDG) target on drinking water. By 2015, more than 90% of the human population will have access to drinking water sources. On the contrary, the world will fail to meet the MDG sanitation target. Population without improved sanitation, around 2.5 thousand million in 2015, is not decreasing notably and water pollution is on the rise globally (United Nations World Water Assessment Programme, 2009). In Spain, the situation has evolved from only 18% of the population connected to wastewater collection and treatment systems in 1980, to more than 90% connected in 2008 (Figure 1.7). Around 50% of these systems installed in Spain use tertiary treatment.

Water contamination agents related to human activities are: microbial pathogens, nutrients, oxygen-consuming substances, heavy metals and persistent organic matter, as well as suspended sediments, nutrients and pesticides. More than 80% of sewage in developing countries is discharged untreated, polluting rivers, lakes and coastal areas. Globally, the most prevalent water quality problem is eutrophication. Eutrophication is a result of high-nutrient loads, mainly phosphorus and nitrogen, dumped to natural water sources. That problem will grow due to the increasing food production caused by the increasing population. The river

input of nitrogen loads into coastal ecosystems is expected to increase around 10-15% in the future, caused by the increasing food production (M.E.A., 2005).

1.3 WATER AND ENERGY

The overlap between energy and water is known as the energy-water nexus. Water resources rely on energy, and vice versa. Water and energy cannot be seen separately when the objective is the sustainability of the water cycle (Hofman *et al.*, 2011). Energy can account for 60-80% of water transportation and treatment costs and up to 14% of total water utility costs. According to the United Nations World Water Assessment Programme (2009), the world will need almost 60% more energy in 2030 than in 2020 and renewable-energy resources alone are not sufficient to meet that demand. Electricity is required for potable water production and also for wastewater treatment. Energy consumption is a major contributor to the operation cost of wastewater systems. The costs for energy usually amount up to 10–30% of the total operation costs. Nowadays, electricity is the largest non staff operating cost item for companies involved in water management. In addition, water and wastewater treatment can account for more than half of the electricity bills of many municipalities (Elliott, 2005). Aeration is the main contributor and can account for approximately 60% of the energy used for wastewater treatment (Figure 1.8).

However, the potential energy contained in wastewater and biomass exceeds the energy needed for their treatment by several folds (Heidrich *et al.*, 2010). This value can be 2-4 times more in some cases based exclusively on chemical energy (Tchobanoglous and Leverenz, 2009), not including thermal or hydraulic energy, which varies considerably from facility to facility. If thermal or hydraulic energy is taken into account, the energy content can be as high as ten times the energy for treatment. Energy embedded in water is the sum of energy input into water along the various points of the water use cycle (Figure 1.9). Clearly not all the energy embedded in wastewater (Table 1.1) (Tchobanoglous, 2012) can be extracted in a useful form.

Nevertheless, nowadays, wastewater can be viewed as a resource instead of an energy consumer due to the concept of biorefinery (Olguín, 2012) and the application of new technologies that improve the energy recovery. One of these alternatives is the microbial fuel cell (MFC) which could directly convert the chemical energy contained in the wastewater into electrical energy (Oh *et al.*, 2010).

1

Table 1.1. Energy content of wastewater (Tchobanoglous and Leverenz, 2009).

Constituent	Value	Unit
Average heat in wastewater	41900	MJ/10°C·10^3 m^3
Chemical oxygen demand (COD) in wastewater	250-800	mg/L
Chemical energy in wastewater, COD basis	12-15	MJ/kg COD
Chemical energy in primary sludge, dry	15.0-15.9	MJ/kg TSS
Chemical energy in secondary biomass, dry	12.4-13.5	MJ/kg TSS

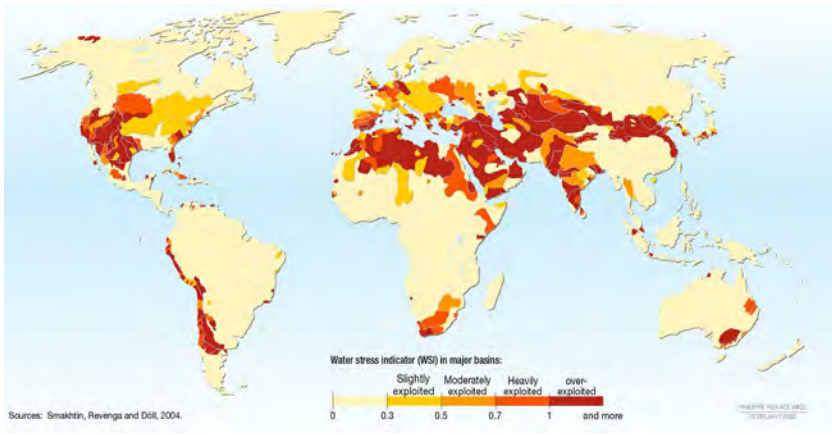


Figure 1.5. The Water Stress Indicator (WSI) in major basins (Rekacewicz, 2006).

Figure 1.6. Map showing geographic location where conflicts over water have occurred from year 2000 to 2009. Data from: Gleick (2009). The types of conflict include: control of water resources, military tool (water used as a weapon), political tool, terrorism, military target and development disputes.

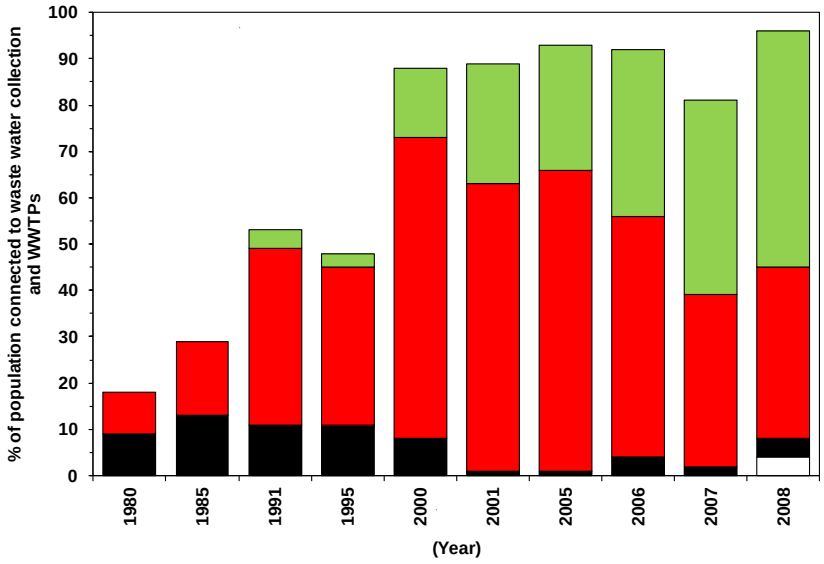


Figure 1.7. Percentage of population in Spain connected to wastewater collection and treatment systems (Urban WWTPs) and type of treatment over the period 1980 to 2008. Collected without treatment (□) and primary (■), secondary (■) and tertiary (■) treatment. Data from: European Environment Agency (EEA) and Eurostat, 2012.

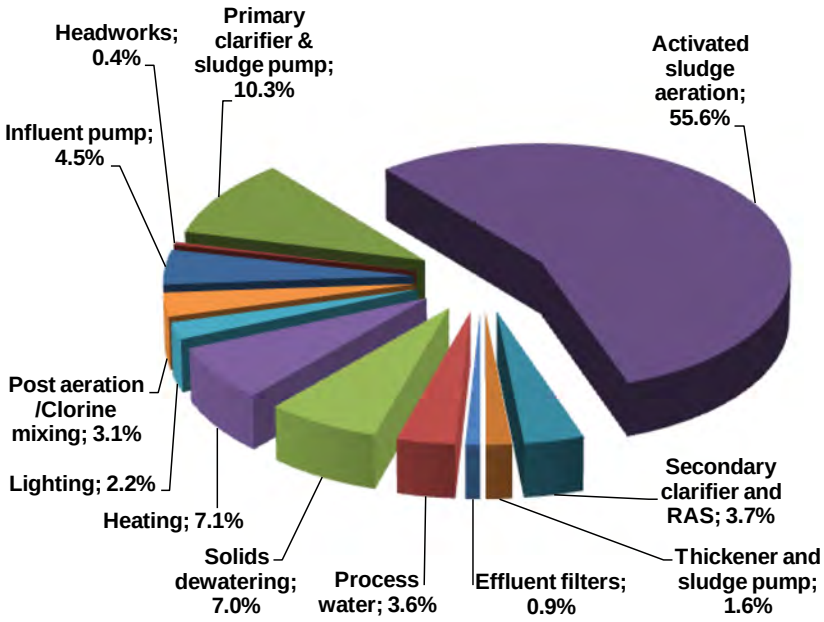


Figure 1.8. Energy usage in biological treatment systems in wastewater treatment plants. Data from: Tchobanoglous (2012).

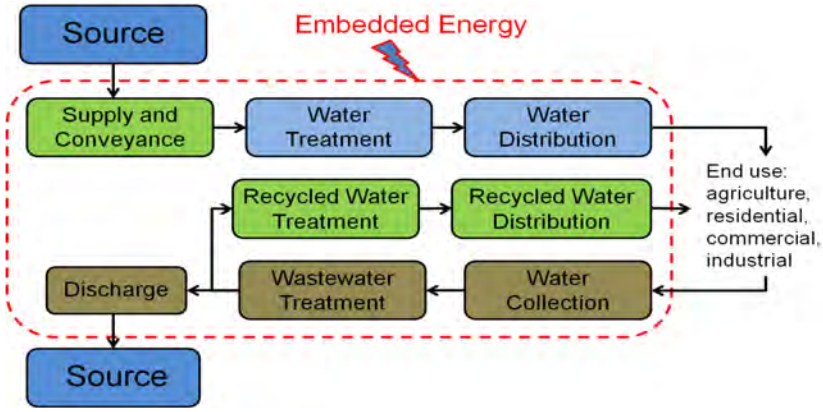


Figure 1.9. Embedded energy in the water use cycle
Adapted from: California Energy Commission (2005).

New practices, technologies and information are being used in order to achieve a self-sufficient energy operation of the WWTP. Then, even a net energy production from wastewater can be achieved (Table 1.2), in order to convert the actual concept of wastewater treatment from an "energy sink" to an "energy source" (Garrido *et al.*, 2013). In fact, some of the world's best performing WWTPs produce over 100 percent of the energy needed to operate (Wett *et al.*, 2007, Crawford, 2010). The implantation of some innovations in the biological treatment processes, mainly changes from aerobic to anaerobic or anoxic processes, has the potential to significantly reduce the energy demand at a treatment facility by acting over different stages of the process:

- Improved Screening. The use of fine screens on collection mains or trunks at the pump stations in satellite treatment facilities are innovative steps that can recover particulate matter before deposition. Improved screening can reduce the particle size, protecting further mechanical equipment. This prevents the loss of chemical energy, reduces the need for new facilities, and improves process and infrastructure sustainability (Tchobanoglous and Leverenz, 2009).
- Sidestream Treatment. Liquid sidestreams are extremely rich in N and P loads. The treatments used to remove these nutrients increase considerably the energy demand in conventional systems. Reductions in the load from these sidestreams have the potential to decrease the energy demand and the required size of the secondary treatment system. Treatment processes as DEMON, Anammox, and phosphorus removal processes can be applied to these sidestreams (Ahn and Choi, 2006, Wett, 2007, Vázquez-Padín et al., 2009a).
- Low Energy Secondary Treatment. The application of the Anammox process to the main stream of the WWTP would allow the development of new treatment concepts that use the advantageous metabolic pathways unique to these organisms (van Loosdrecht et al., 2004, Winkler et al., 2012).

Table 1.2. Summary of energy recovery potential using established technologies (WERF, 2011).

Biomass Technology	% of Net Energy "Gap" Reduction Possible	Other Technology	% of Net Energy "Gap" Reduction Possible
Anaerobic Digester (AD) biogas with boilers	13-57%	Enhanced solids removal	10-71%
AD biogas with cogeneration engines	11-61%	Anaerobic primary treatment	25-139%
AD biogas with micro turbines	5-38%	Heat recovery (per °C recovered)	13- 49%
AD biogas with turbines	7-46%	Hydrokinetic (per meter of head)	0%
AD biogas with fuel cell	6-42%	Ammonia as fuel	-6-12%
AD biogas after waste activated sludge pretreatment	-2-60%	Heat from centrate	13-49%
AD biogas with Co-digestion	2-128%	Microbial fuel cells	8-110%
Incineration	2-69%	Biofuel from algae	-39-208%
Gasification	-9-82%		

Many types of technologies and opportunities exist to convert wastewater chemical energy into different forms of energy. In this way, the wastewater treatment plant can be viewed as a net energy producer. Some of these technologies are well established; others are innovative technologies that will require additional research and development.

The most typical wastewater to energy process is based on the use of sludge produced during both primary settling and biological wastewater treatment, thought anaerobic digestion. In the anaerobic digestion, the readily biodegradable portion of the volatile solids in sludge is converted to biogas (mainly methane, 60-65% and carbon dioxide, 35-40%). Biogas is collected and converted into electricity, and heat can be recovered from the power generation units to heat the digesters, or to generate steam power. Research is focused on the use of co-digestion, in order to increase the energy recovery with the addition of some high-strength organic wastes (food-processing operation wastes, fats, oil, grease, animal manure...) (Li *et al.*, 2011, Iacovidou *et al.*, 2012, Marañón *et al.*, 2012, Regueiro *et al.*, 2012). Moreover, different pretreatments, as thermal hydrolysis, mechanical disintegration, electrical pulse treatment, etc. are applied in order to improve the methane production and its generation rate (Val del Río *et al.*, 2011, Carlsson *et al.*, 2012, Appels *et al.*, 2013).

The other widely used alternative is the thermal conversion through incineration, gasification, pyrolysis, supercritical water oxidation or steam reformation. In thermal conversion the entire volatile fraction of the biomass is either completely or partially oxidized. Energy can be recovered from the heat liberated during the oxidation, or in some technologies, from gaseous or carbon-based solid residues.

Other emerging alternatives convert solids to gases under aerobic conditions to produce synthetic gas or biofuel:

- Bio-hydrogen can be produced from wastewater using microbial electrolysis cells (Escapa *et al.*, 2012). MFCs generate electricity from the organics present in wastewater and are a promising innovative approach of renewable energy production from wastewater (Oh *et al.*, 2010).
- In the algae bioreactors wastewater and nutrients are used to stimulate algae growth (Pruvost et al., 2011). Then, the residual algae are digested with wastewater solids to produce biogas, which is purified and used as fuel for vehicles (Figure 1.10). To reach the enhanced algal yield, additional CO_2 can be obtained by the thermal transformation of external biomass (i.e. sludge from a wastewater treatment plant), together with internal biomass as the digestate from residual algae and wastewater solids.
- Cellulosic biofuel can be generated in constructed wetlands where energy output is used for biofuel production (Liu *et al.*, 2012a).

Energy can also be recovered from heat from warm water, as hot water is discharged into the sewer system. The average temperature of wastewater leaving the house is 27 ºC (SenterNovem, 2006, Lazarova *et al.*, 2012), while that temperature was 10 ºC when it entered the house (Hofman *et al.*, 2011). The total heat loss through sewage water in the Netherlands is about 110 W/cap (Watt per capita) and accounts for approximately 40% of the total heat loss of a modern house (Hofman *et al.*, 2011). This means that the heat loss via sewage is nearly ten times higher than the energy demand for drinking water supply and wastewater treatment together (Hofman *et al.*, 2011). Energy consumption of drinking water supply is 2.6 W/pe, (Watt population equivalent) and wastewater treatment around 7 W/pe (Hofman *et al.*, 2011). Temperature of grey water is higher, about 38-40 ºC because of warm showers, baths and hot laundry (Roest *et al.*, 2010).

Several options to recover this heat embedded in water are under research. The heat content of water from households can be recovered within buildings combined with Aquifer Thermal Energy Storage (ATES) systems (Lee, 2013) (Figure 1.11), within houses, i.e. shower heat exchangers (Figure 1.12) (Wong *et al.*, 2010), from the sewer, or at wastewater treatment plants (Frijns *et al.*, 2013). Similar to a geo-exchange system, electric heat pumps transfer thermal energy from warm sewage (12–25 ºC) to a higher temperature useful for residential space heating and domestic hot water (Figure 1.13). Compared to geoexchange, sewer heat

recovery is more efficient due to higher heat source temperature and lower installation costs (Roger Bayley Inc., 2009).

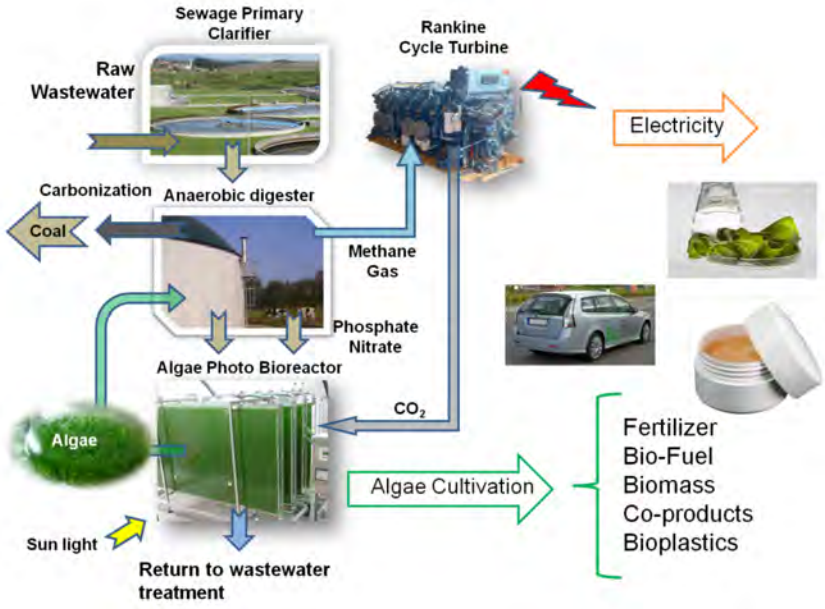


Figure 1.10. Micro algae water treatment processes.

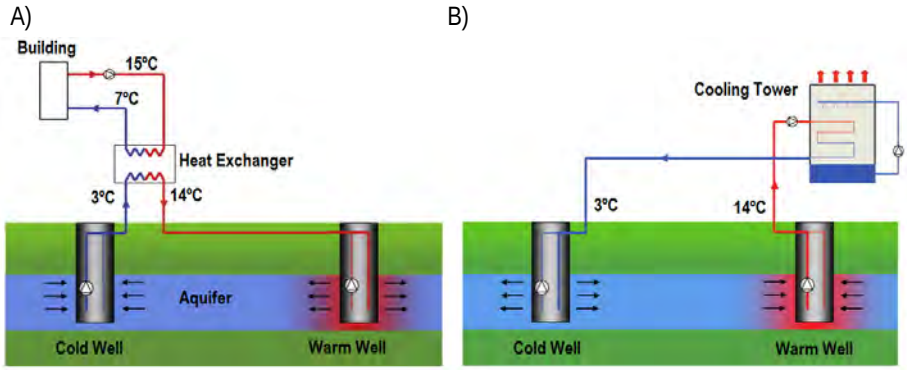


Figure 1.11. Aquifer Thermal Energy Storage: A) summer operation - cooling; B) winter operation - heating. Reprinted from: Ghaebi *et al.* (2014).

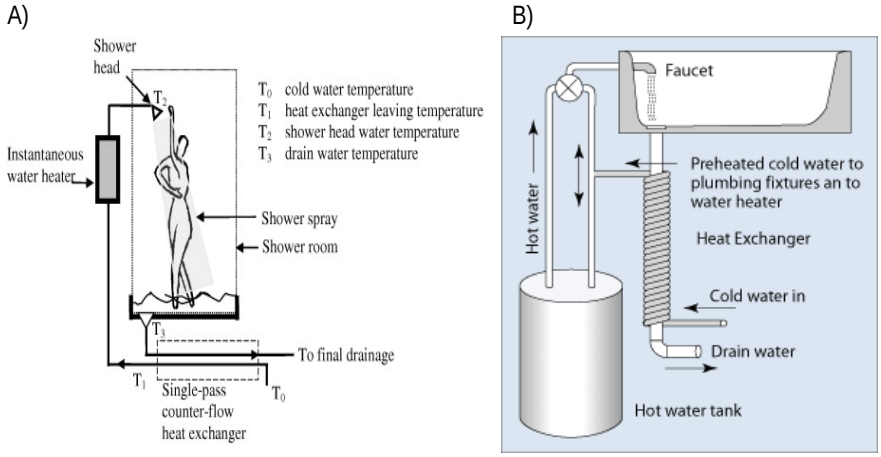


Figure 1.12. A) Scheme of a shower installation with a single-pass counter-flow heat exchanger. Reprinted from: Wong *et al.* (2010). B) Diagram of a drain water heat recovery system. Reprinted from: U.S. Department of Energy (2013).

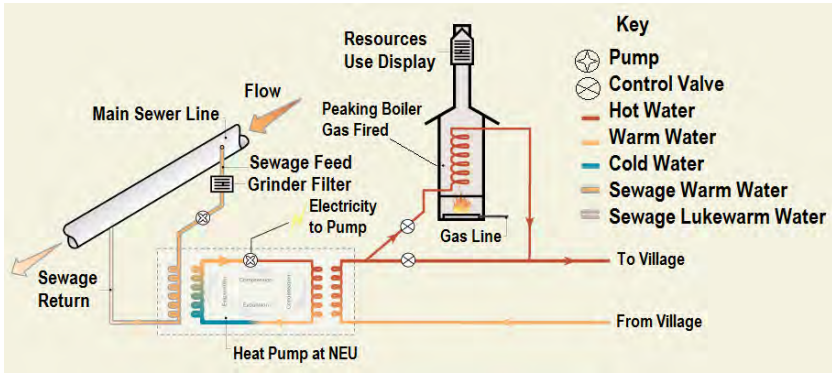


Figure 1.13. Heat recovery from the wastewater: the Neighborhood Energy Utility (NEU) provides heat and hot water in the City of Vancouver.
Adapted from: Roger Bayley Inc. (2009).

WWTPs can also recover hydraulic energy by installing large turbines to capture this energy and produce electricity, or installing micro-hydro water turbines or hydrokinetic devices in channels and conduits prior to discharge (NACWA, 2009).

However, these emerging and promising alternatives are still under development. Consequently, until now, most of the WWTPs are energy consumers. As an example, the cost of lipid production for diesel production from algae, to achieve a 10% return was determined to be around US$ 32/L for open ponds and US$ 68/L for photo bioreactors (Davis *et al.*, 2011). And by the year 2009, there were only three sewer heat recovery systems worldwide that recovered heat from untreated sewage, two in Oslo, Norway and one in Tokyo, Japan (Roger Bayley Inc., 2009).

The main objective of the wastewater industry has been to meet water quality requirements without major energy considerations. In consequence, WWTPs are hardly ever designed with energy efficiency in mind. Hernández-Sancho *et al.* (2011) found that only 10% of the WWTPs analyzed in their research were energetically efficient. Until these promising new technologies for energy recovery are developed and widely applied, the main objective of WWTPs has to be the optimization of the energy.

A global analysis of the wastewater treatment train in terms of the current water quality requirements and the optimal integration of the energy issues has been performed: to improve energy efficiency, to maximize the use of sludge for energy production and to recover energy from internal or external sources. The National Renewable Energy Laboratory of the U.S. Department of Energy (Daw *et al.*, 2012) and the United States Environmental Protection Agency (E.P.A., 2013) suggested some strategies to improve the energy efficiency in the municipal wastewater treatment facilities:

- Operational control. System controls that use supervisory control and data acquisition (SCADA) feedback and variable frequency drives (VFD) can be used to optimize effluent quality and energy consumption.
- Installation of variable-frequency drives which adjust the speed of an electric motor by modulating the power being delivered.
- Upgrade to energy-efficient motors and motor systems, doing that energy can be saved, maintenance costs reduced and the environment protected.
- Heating, cooling and ventilation system upgrades and use of bright lights.
- Managing electrical load using energy-efficient strategies such as reducing peak demand, shifting to off-peak hours, and improving the power factors of motors.
- Repair and replacement. A best practice for facilities is to regularly evaluate the condition, performance and remaining useful life of process equipment.
- Biomass. There are significant environmental and financial tradeoffs between providing higher quality biomass and the subsequent energy demands. Sustainable biomass treatment, transport, and end-use can reduce economic and environmental costs.
- Infiltration, inflow and leaks. A considerable amount of energy is wasted treating groundwater that infiltrates systems through pipes that are broken or out of alignment. Similarly, breaks and leaks in the collection system increase the energy required to pump sewage to the treatment plant.
- On-site renewable energy. A best practice for all communities is to look for opportunities to incorporate on-site renewable energy at their facilities: solar, wind, biodiesel…
- Use of co-generation or combined heat and power energy and enhance the production of biogas. Wastewater treatment facilities that have anaerobic digesters create methane gas as a by-product of digestion of biomass.

- Conservation. Effective outreach within the community can yield substantial reductions in water use and wastewater generation.

Organic matter content of wastewater has a chemical energy by oxidation of 18 W/pe. In contrast, the total energy input of the water cycle is approximately 10 W/pe. Consequently, new technologies in wastewater treatment plants that improve the energy recovery from this organic matter can create an energy self-sufficient water cycle. The best options for energy production from wastewater are production of biogas and using dried sludge for power generation (Hofman *et al.*, 2011). In addition, energy content of nutrients present in water is about 45 MJ/kg N for N-fertilizer and 29 MJ/kg P for P-fertilizer production. The energy recovery potential of these nutrients is 6.1 W/cap for nitrogen and 0.7 W/cap for phosphorus (Hofman *et al.*, 2011).

1.4 NUTRIENTS IN WASTEWATER

The production of artificial fertilizers has been increasing continually after the Second World War (Figure 1.14), with a rate of 600% between 1950 and 2000 (IFA, 2006), reaching a rate of 100 million tonnes of nitrogen used per year (Glass, 2003). However, this high rate of fertilizer production for modern agriculture is mainly dependent on phosphorus derived from phosphate rock, which is a non-renewable resource.

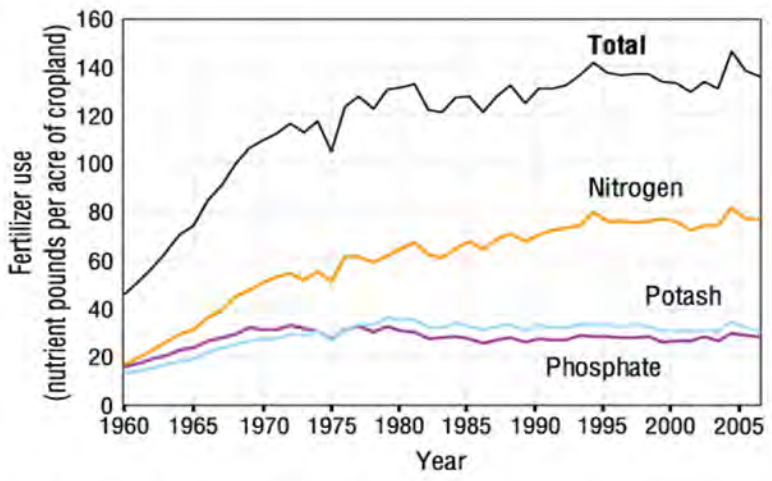


Figure 1.14. Commercial fertilizer use in the U.S.A. in the period 1960-2005 based on sales data. Reprinted from: U.S. Environmental Protection Agency, (2008).

On the other hand, nitrogen and phosphorus are the primary causes of environmental eutrophication in surface waters (E.P.A., 2007). Presence of excess of these nutrients (Figure 1.15 and Figure 1.16) produces different alterations in the natural cycles of rivers, lakes and seas: it promotes excessive plant growth and decay (Figure 1.17), severely reduces the general water quality, provokes undesirable impacts on biodiversity, water quality, fish stocks

and reduces the recreational use value of the environment. The annual cost of eutrophication in the U.S.A. is estimated in US $ 2200 million (Dodds *et al.*, 2009).

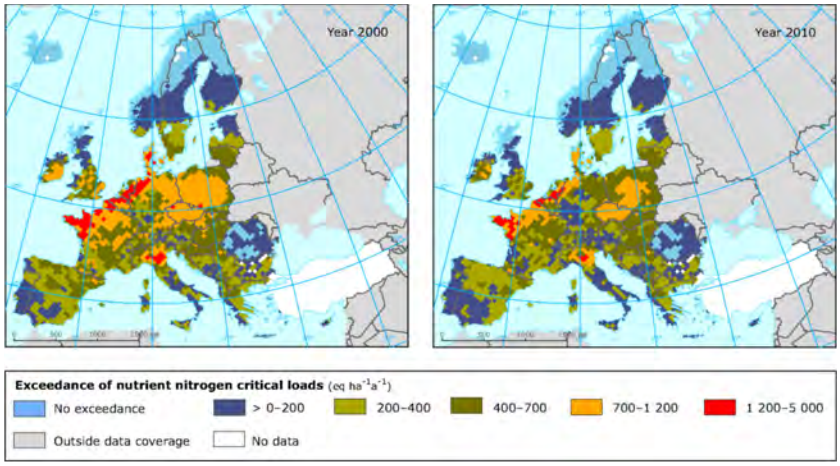


Figure 1.15. Exceedance of critical loads for eutrophication due to the deposition of nitrogen in 2000 and 2010. Reprinted from: European Environment Agency (2012a).

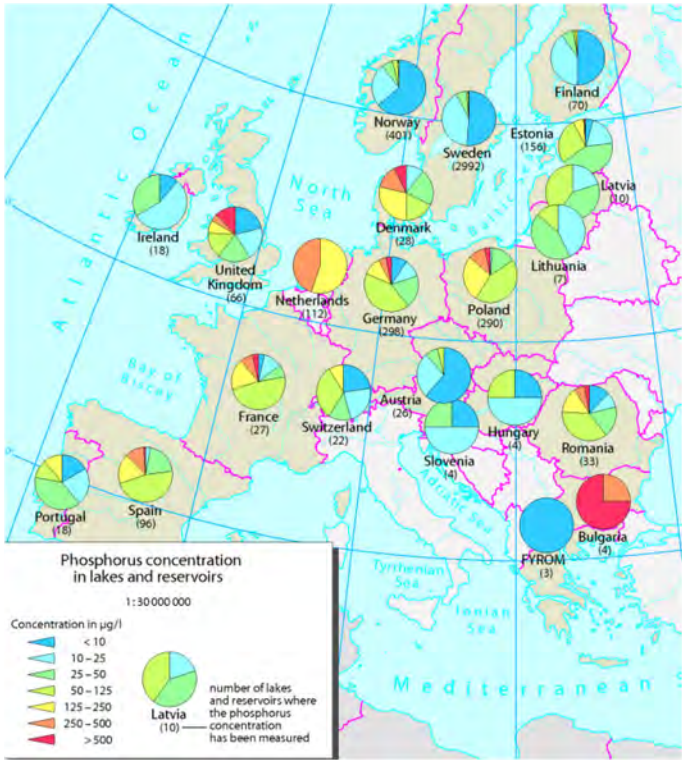


Figure 1.16. Phosphorus concentration in European lakes and reservoirs Reprinted from: European Environment Agency (2012b).

Major sources of nutrients discharges include wastewater, agriculture and atmospheric deposition of nitrogen from burning fossil fuels. Agricultural sources, as fertilizer leaching, runoff from cultivated fields and manure from concentrated livestock operations, and aquaculture are the principal sources of nutrient impairment in waterways in the United States and the European Union (Figure 1.18). On the other hand, urban wastewater is the primary source in Asia and Africa (Selman and Greenhalgh, 2009).

In Table 1.3 the main phosphorus sources in each EU member state are indicated. The data are based partly on phosphate input information (detergents) and partly on calculations. The calculation for human phosphate production assumes that each person in the EU generates 0.7 kg P each year and that 50% of this is available. Release of nutrients to the environment is expected to increase in the future (Selman and Greenhalgh, 2009) as a result of the enlarging global trends in population growth, energy use and agricultural production. However, eutrophication can be reversed by controlling the nutrient inputs to waterways (Smith and Schindler, 2009).

Table 1.3. Phosphate sources in Europe in 1992 (percentage from each source and total) (Morse *et al.*, 1993)

Member State	Human	Detergents	Livestock	Fertilizers	Industry	Background	Total (1000 t P/year)
Austria	20	10	36	16	6	12	13
Belgium	26	11	43	7	8	5	13
Denmark	12	11	55	11	5	6	15
Finland	18	9	17	15	3	38	9
France	18	15	31	19	6	11	106
Germany	28	3	44	12	6	7	97
Greece	21	7	18	34	5	15	17
Ireland	9	7	49	24	2	9	15
Italy	35	2	26	18	8	11	56
Netherlands	23	3	57	9	5	3	24
Portugal	24	14	27	16	7	12	15
Spain	19	16	18	26	7	14	72
Sweden	21	10	15	14	7	33	14
UK	24	19	29	14	8	6	82

Key assumptions and calculations: Human sources: 1.6 g P/cap·d (human waste) + 0.3 g P/cap·d (household waste) = 0.7 kg P/cap·year * population * 50% (availability). Detergent use: kg P/cap·year * population * 50% (availability). Animal sources: 9.5 kg P/ce·year * ce (cattle equivalent) * 10% (availability). Fertilizer/Manure: 0.6 kg P/ha·year loss from 30 kg P/ha·year application * agric area. Industry inputs: 20 % of domestic sources. Background: 0.2 kg P/ha·year (0.1 in Finland, Norway and Sweden) * whole area.

A) B)

Figure 1.17. A) Eutrophication at a waste water outlet in the Potomac River, Washington, D.C. Retrieved from: Trubetskoy (2012). B) Algal bloom warning in Loch Melvin, Ireland. Retrieved from: Webb (2012).

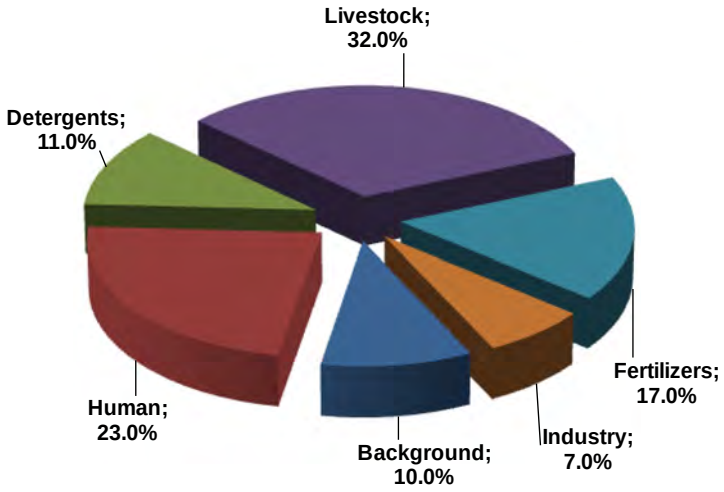


Figure 1.18. Phosphate sources in Europe in 1992. Data from Morse *et al.* (1993).

In order to reverse eutrophication trends and to mitigate nutrient losses, different legislation and normative were approved throughout the world to minimize the amounts of nutrients discharged (Table 1.4), as European Union directives:

- The urban wastewater treatment directive aims to protect the environment from the adverse effects of urban wastewater discharges and discharges from certain industrial sectors (EEC, 1991b).

- The nitrates directive aims to protect water quality across Europe by preventing nitrates from agricultural sources polluting ground and surface waters and by promoting the use of good farming practices (EEC, 1991a).
- The Water Framework Directive (WFD) lays down a strategy to fight against the pollution of water, including adopting specific measures against pollution by individual pollutants or groups of pollutants presenting a significant risk to the aquatic environment (EEC, 2000).

Both problems, phosphorus run out and eutrophication can be solved at the same time with technologies for nutrients recovery from wastewater streams (Figure 1.19). The fertilizer value of the nutrients discharged to the sewer systems in Norway per year is US$ 30 million. In addition, 15-20% of the current mineral fertilizer used could be substituted by fertilizer derived from wastewater. In the case of developing countries these percentages can achieve values up to 40-50% (Vråle and Jenssen, 2005).

The fertilizer value of human excreta was already recognized from the beginning of civilization. In this way, the Ancient Greeks used public latrines and later conducted the wastewater outside the city to agricultural fields, where wastewater was used for irrigation and to fertilize crops and orchards (Henze *et al.*, 2008). At the beginning of 20th century, a plan for separate collection of toilet water through a vacuum sewer and grey and storm water was developed by Mr. Liermur. The collected sewage was not treated, but directly spread out over land as fertilizer. However, water logging became a problem, and the continuous expansion of the cities made it more difficult to find sufficient land nearby (Henze *et al.*, 2008).

Table 1.4. Requirements for discharges from urban wastewater treatment plants to sensitive areas which are subject to eutrophication. One or both parameters may be applied depending on the local situation. The values for concentration or for the percentage of reduction shall apply (EEC, 1991b).

Parameters	Concentration	Minimum percentage of reduction (1)
Total phosphorus	2 mg/L P (10000-100000 pe) 1 mg/L P (more than 100000 pe)	80
Total nitrogen (2)	15 mg/L N (10000-100000 pe) 10 mg/L N (more than 100000 pe) (3)	70-80

(1) Reduction in relation to the load of the influent.
(2) Total nitrogen means: the sum of total Kjeldahl-Nitrogen (organic N+NH_3), nitrate (NO_3^-)-nitrogen and nitrite (NO_2^-)-nitrogen.
(3) Alternatively, the daily average must not exceed 20 mg N/L N. This requirement refers to a water temperature of 12 °C or more during the operation of the biological reactor of the wastewater treatment plant. As a substitute for the condition concerning the temperature, it is possible to apply a limited time of operation, which takes into account the regional climatic conditions.

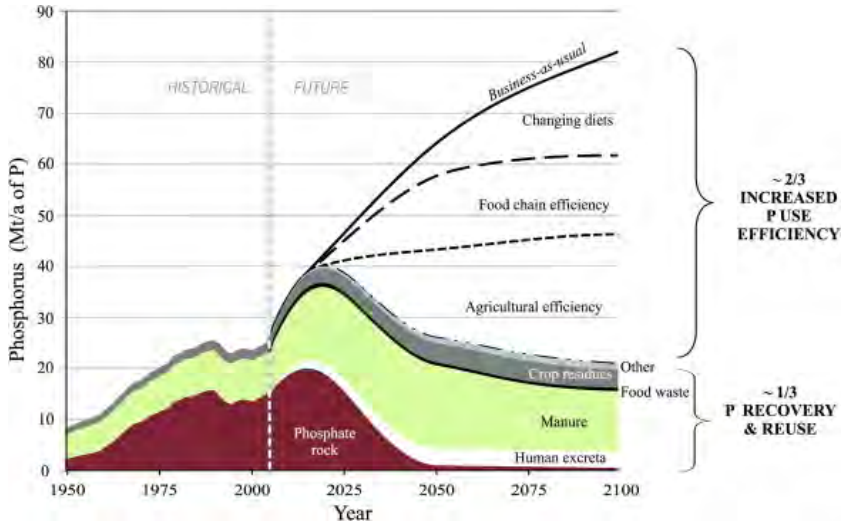


Figure 1.19. Sustainable scenario for meeting long-term future phosphorus demand through phosphorus use efficiency and recovery. Reprinted from: Cordell *et al.* (2011).

1.4.1 Nutrients recovery

Phosphorus source for artificial fertilizer is a phosphate rock, which is a non-renewable resource. Worldwide phosphate production in 2010 was around 176 million tonnes and current global reserve life of phosphate ore is not clear and might be depleted in 50-160 years (Steen and Agro, 1998, Roberts and Stewart, 2002, Cordell *et al.*, 2009, Heffer and Prud'homme, 2010). While there are commercially exploitable amounts of phosphate rock in several countries, almost 90% of phosphate reserves are located in four countries: Morocco/Western Sahara, China, South Africa and the United States (Figure 1.20 and Figure 1.21). Those countries with no domestic reserves could be particularly vulnerable in the case of global shortfalls (UNEP, 2011).

For these reasons, phosphorus reserves are gaining importance in the geopolitical strategies (Wellstead, 2012, Cordell and White, 2013), especially taking into account the sovereignty conflict over Western Sahara (Zoubir and Benabdallah-Gambier, 2005). Global reserves can be concentrated in just 7 countries by 2100 and therefore, these will be the only countries able to produce phosphate rock (Cooper *et al.*, 2011). Consequently, phosphate prices are expected to increase in the long-term as lower ore grades are mined and more expensive technology has to be employed (Figure 1.22) (Cordell and White, 2013).

Virtually, all the phosphorus that humans consume in food is excreted in urine and feces, with an estimated of 3 million tonnes produced globally each year (Cordell *et al.*, 2009). Different technologies are applied in wastewater treatment plants in order to remove the

phosphorus. The cost of this removal depends on the configuration of the system (Jiang *et al.*, 2004): filtration for particulate phosphorus, membrane technologies, precipitation, physical-chemical adsorption, biological assimilation, enhanced biological phosphorus removal (EBPR)... Most of these processes produce wastes, which need to be landfilled or incinerated (Bhuiyan *et al.*, 2008).

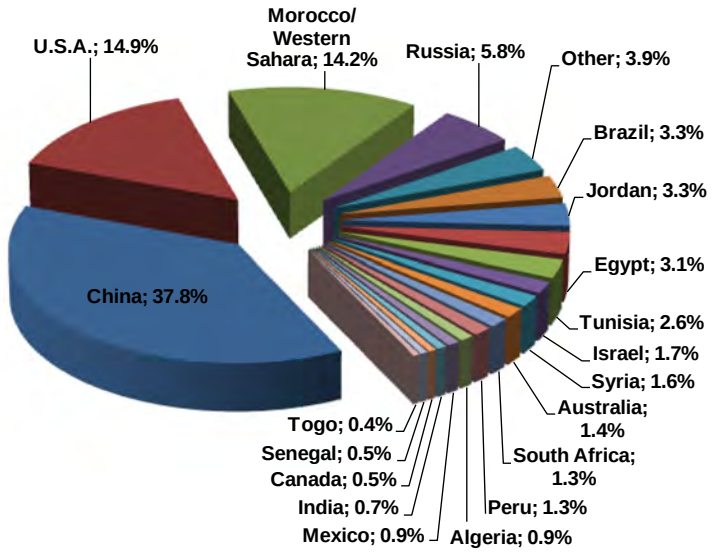


Figure 1.20. Global phosphate rock concentrate production percentage (based in 191 million tonnes/year). Data from: U.S. Geological Survey (2012).

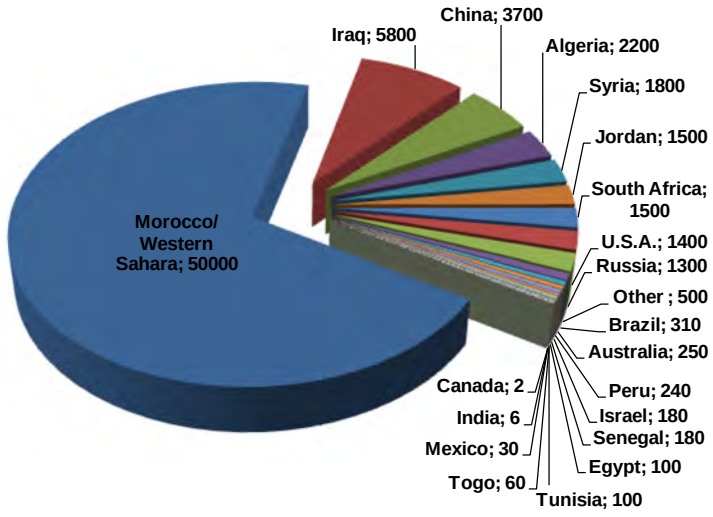


Figure 1.21. Worldwide reserves in millions of tonnes. Data from: U.S. Geological Survey (2012).

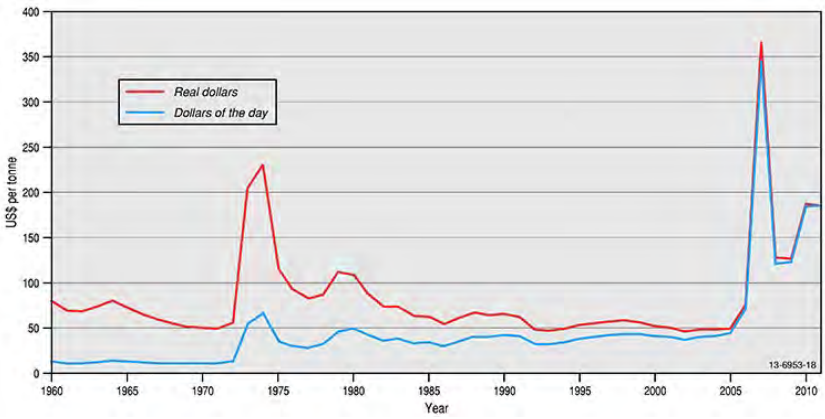


Figure 1.22. Phosphate prices for phosphate rock (Morocco). Reprinted from: Geoscience Australia (2013).

Another option is the recovery of phosphorus as a by-product by means of crystallization processes where minerals are obtained. Main minerals recovered are magnesium ammonium phosphate hexa-hydrate ($NH_4MgPO_4 \cdot 6H_2O$, MAP or struvite) and hydroxyapatite ($Ca_5(PO_4)_3OH$). These compounds can be used in agriculture as fertilizers (Figure 1.23). Struvite formation, as any other crystal build up, occurs by nucleation followed by crystal growth.

In Figure 1.24 the struvite crystal formation process from urine is shown. When a source of magnesium is added to the urine, the reaction of formation of struvite takes place (first nucleation or crystal birth). Then crystals continue to grow until equilibrium is achieved.

A) B)

Figure 1.23. A) and B) Fertilizer based on struvite, sold in the Japanese market (Ueno and Fujii, 2001).

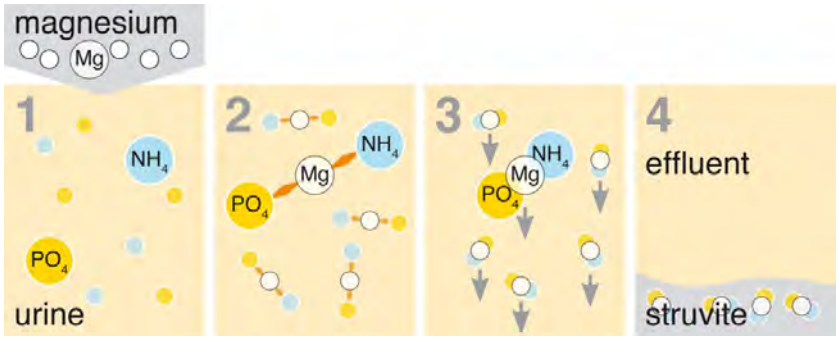
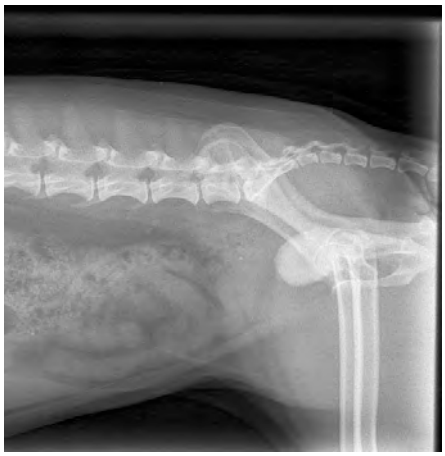


Figure 1.24. Struvite formation in urine (EAWAG, 2009).

Struvite urinary stones and crystals are also formed readily in the urine of animals (Figure 1.25 A) and humans that are infected with ammonia-producing organisms and can produce urinary tract calculi (Wang *et al.*, 1997). They are potentiated by alkaline urine and high magnesium excretion (high magnesium/plant-based diets). They are also potentiated by a specific urinary protein in domestic cats, which cause them health problems, as kidney stones (Matsumoto and Funaba, 2008). Uncontrolled struvite precipitation in the pipes, pumps, centrifuges and aerators of wastewater treatment plants (Figure 1.25 B) is also a problem (Stratful *et al.*, 2004, Barat *et al.*, 2009). According to Benisch *et al.* (2000), the struvite precipitation causes a economic annual cost that ranges around $0.53 and $2.65 per cubic meter and day of secondary dry weather capacity for the majority of treatment plants with anaerobic digesters.

A)



B)

Figure 1.25. A) Lateral radiographs of a canine abdomen showing a large bladder stone (urolith). Retrieved from: (Edudent, 2012). B) Submerged pump cover of struvite in a WWTP. Retrieved from: Vázquez-Padín (2013).

After a survey among Swiss farmers, Lienert *et al.* (2003) found a positive acceptance of a urine-based fertilizer product. A percentage of 57% of farmers considered the use of these fertilizers as a good or a very good idea, and 42% would purchase such a product. Of those that would not buy the fertilizer, 76% indicated that they had no need for it. Around 30% of all farmers had concerns regarding micropollutants. That was one of the main reasons to refute the purchase of urine-base fertilizers.

A research by Ueno and Fuji, (2001) showed the viability of recover struvite as a fertilizer in Japan. The recovered struvite contains almost no hazardous materials and exhibits equivalent or better fertilizer effectiveness than conventional chemical fertilizers. Doyle and Parsons, (2002) summarized distinct costs of producing and selling struvite in different countries. Costs for struvite production vary from $140/tonne in Australia to $460/tonne in Japan ($1075-3540/tonne of P, assuming phosphorus content of 13% in struvite) and contrasts with the $40-50/tonne paid for phosphate rock ($200-250/tonne of P, assuming phosphorus content of 20% in phosphate rock). The market value of struvite proposed by these authors varies highly between $9 and $1885/tonne (Doyle and Parsons, 2002).

1.4.1.1 Urine separation

Urine is a liquid by-product of the human body that is secreted by the kidneys through a process called urination. Urine represents only 1% of the volume of wastewater produced. However, it is estimated to contribute to around 80% to the total nitrogen, 70% of the potassium and up to 50% to the total phosphate loads in municipal wastewaters (Wilsenach *et al.*, 2007) (Figure 1.26). The separation of this high loaded strength from domestic wastewater presents several advantages in the operation of WWTPs: the reduction of the presence of many problematic micropollutants, the removal of one of the main nitrogen contributors, the reduction of the volume necessary for the nitrification/denitrification process and so on. Moreover, the treatment of separated urine allows recovering nutrients in the form of mineral precipitates. Wilsenach and van Loosdrecht (2006) suggested that by separating only 50% of the produced urine, compact and energy-efficient treatment technologies without nitrification, denitrification, and phosphorus removal are possible, because the remaining nutrients are removed through sludge activity.

The easy way to have separated urine will be to segregate the urine at the source, in the moment of the excretion with the use of special toilets and urinals (Figure 1.27 A and B). The urine separating toilets were invented in Sweden in the 1980s (Jönsson, 2002) and made the construction of urine separating systems possible.

Obviously, urine separation has also some disadvantages. Neither the toilets nor the other components of the system have been on the market for a long time, and the system has had some initial troubles. The main problems have been the toilets themselves and the toilet seals, but the studies and research done in this field have now solved or reduced these problems. The implication of the users is also necessary (Figure 1.27 C). Lienert and Larsen

(2006) have studied the acceptance of this technology in Switzerland, where this kind of toilets have in general a good level of acceptance by the users.

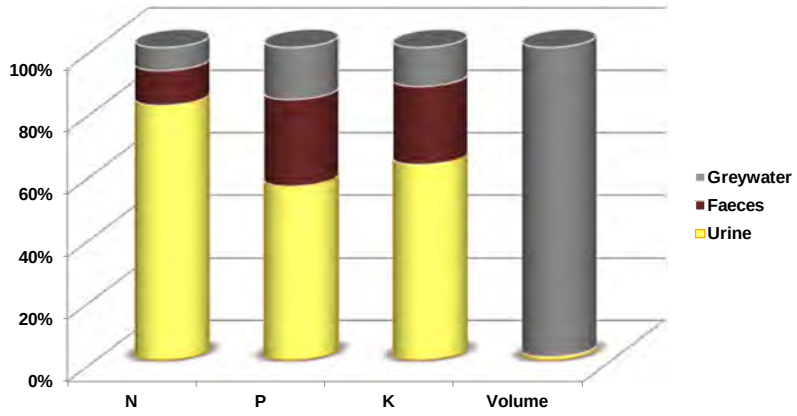


Figure 1.26. Nutrients content and percentage of greywater, feces and urine in wastewater, in Swedish domestic wastewater. The mean production per person and day is: 13.5 g N, 1.8 g P and 4.0 g K in a volume of 150-200 L. Data from: Höglund (2001).

Urine separation and struvite precipitation present also other advantages. The footprint of a struvite crystallization reactor is considerably smaller than that of biological P removal infrastructure; and the process has fewer problematic operational concerns (Wang *et al.*, 2005). Struvite crystallization may have the potential to cheaply remove phosphate and ammonium from wastewater relative to biological P removal and standard nitrification/denitrification techniques or caustic stripping (Webb and Ho, 1992). Moreover, struvite sales could cover part of the operational cost of the treatment plant.

A) B) C)

Figure 1.27. A) Separating urine toilet. B) No water urinal. C) Separating urine toilet user's instructions, retrieved from: Sustainable Sanitation (2013).

Urine from healthy persons is quite stable and hardly contains any microorganisms (Udert *et al.*, 2006). The average composition of fresh and stored urine is summarized in Table 1.5.

Table 1.5. Reference values for fresh urine and stored urine per person and day (Udert *et al.*, 2006).

		Fresh urine			Stored urine
		Average	*coefficient of variation* %	Data range	
Total nitrogen	g N/m³	9200	20	-	9200
Total ammonia	g N/m³	480	29	-	8100
Ammonia NH_3	g N/m³	0.3	-	-	2700
Urea	g N/m³	7700	20	-	0
Total Phosphate	g P/m³	740	14	-	540
Calcium	g/m³	190	22	-	0
Magnesium	g/m³	100	21	-	0
Potassium	g/m³	2200	-	1300-3100	2200
Total carbonate	g C/m³	0	-	-	3200
Sulphate	g SO_4/m³	1500	29	-	1500
Chloride	g/m³	3800	-	2300-7700	3800
Sodium	g/m³	2600	-	1800-5800	2600
pH		6.2	8	-	9.1
Alkalinity	mM	22	-	-	490
COD	g O_2/m³	10000	4000	-	10000
Volume	L	1.25	0.61	-	1.25

In fresh urine, about 85% of nitrogen is fixed as urea and about 5% as total ammonia. Ubiquitous urea-hydrolyzing bacteria catalyze the hydrolysis of urea to ammonia and bicarbonate (Udert *et al.*, 2006), causing a strong pH increase, as it is shown in equation (1.1). Only few days are necessary for complete urea depletion in the collection tank (Udert *et al.*, 2003).

$$NH_2(CO)NH_2 + 2\,H_2O \rightarrow NH_3 + NH_4^+ + HCO_3^- \qquad (1.1)$$

After urine is collected separately, it is available for its processing. The main options are:

- Urine used directly as a liquid fertilizer (Kirchmann and Pettersson, 1995). However, direct reuse of urine is not widely applied in industrialized countries. This is mainly due to issues related to possible effect of micropollutants contained in urine, a perceived hygiene risk, and an expected soil and plant sensitivity to the high salt concentrations (Maurer *et al.*, 2002).
- The existing sewer network could be used for transporting urine to a treatment facility at night when the sewers are empty (Larsen and Gujer, 1996), or it can be transported

by trucks. Another option is to improve the existing collection system for source separated resource streams (Figure 1.28 A and B).

- A wide variety of processes can be performed to treat the collected urine. Maurer *et al.* (2006) defined seven main purposes of a treatment unit: volume reduction, P-recovery, N-recovery, stabilization, hygienization, removal of micropollutants and biological nutrient removal.

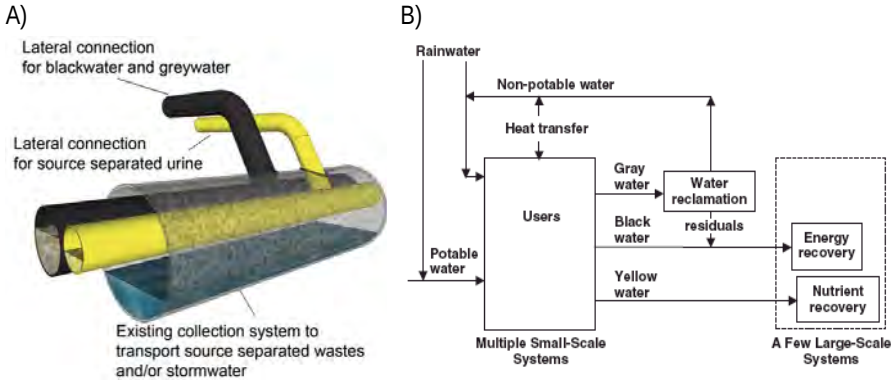


Figure 1.28. A) Source separated wastewater streams with the modification of existing collection system (Tchobanoglous, 2012). B) Schematic drawing of an integrated urban-water and resource-management system (Daigger, 2008).

1.4.1.2 Fertilizer production by ammonia stripping from sludge liquid fraction

Ammonia stripping by means of air supply is a unit process which brings air and water into intimate contact to transfer volatile substances from the water into the air. In a waste stream, ammonia nitrogen exists in aqueous solution as either ammonium ion (NH_4^+) or ammonia (NH_3) (Idelovitch and Michail, 1981). The degree to which ammonia forms the ammonium ion depends on the pH of the solution, following equation (1.2). This dependence is shown in Figure 1.29 at 0, 20, and 40 °C (Huang and Shang, 2004). This equilibrium is the basis of the ammonia-stripping process, a simple desorption process used to lower the ammonia content of a wastewater stream. In this process, the pH of the water is raised in such a way that the equilibrium of equation (1.2) displaces to NH_3 (aq) formation. Then, this gas is removed from the solution by water/gas exchange.

$$NH_4^+ \leftrightarrow NH_3(aq) + H^+$$
$$NH_4^+ + OH^- \leftrightarrow NH_3(aq) + H_2O \qquad (1.2)$$

1. Below pH 7 and at 20 ºC, virtually all the ammonia will be soluble in the form of ammonium (Idelovitch and Michail, 1981).

2. Above pH 11.5, virtually all the ammonia will be present as a dissolved gas. At pH 10.5, most of the ammonia (about 95%) is found in the gaseous form (Idelovitch and Michail, 1981).

3. In the range between 7 and 11, both ammonium ions and dissolved gas coexist.

4. Percentage of dissolved gas increases with temperature and pH and as a consequence, temperature and pH favor the removal of ammonia from solution.

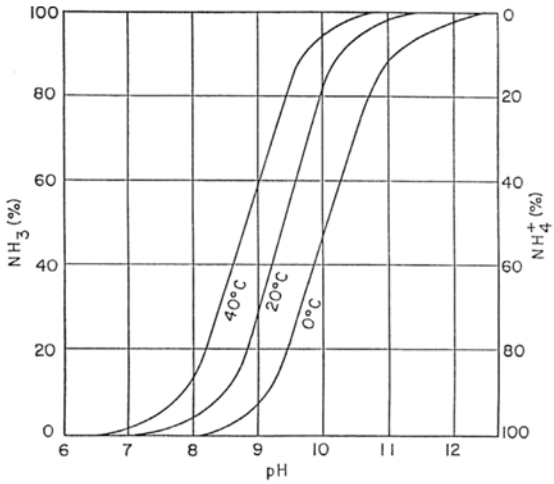


Figure 1.29. Effects of pH and temperature on the distribution of ammonia and ammonium ion in water. Reprinted from: Huang and Shang (2004).

After the ammonia desorption from the wastewater, this compound can be recovered as ammonium sulfate or other salt, using a closed-loop reactor. In the closed-loop system, ammonia is absorbed in sulphuric acid, to produce ammonium sulphate, which may have potential for being used as an agricultural fertilizer. The clean gas coming from this unit is then recycled back to the stripper.

One of the most ammonium concentrated streams in a WWTP is the sludge liquid fraction. It results out of the anaerobic sludge digestion and subsequent sludge treatment, like dewatering and drying. Sludge liquid contains between 600 and 1200 mg NH_4^+-N/L, as well as presents a higher temperature, typically 30 ºC, compared to influent wastewater (Wilsenach, 2005). In anaerobic conditions, nitrogen remains in the form of ammonium and its concentrations are not reduced during this process (Rousseau *et al.*, 2008). Consequently, the anaerobic effluent needs to be additionally treated to remove the nitrogen load. Ammonia stripping of the anaerobically digested effluent in a biogas plant is feasible due to the available heat from biogas utilization, low investment and basic pH of an anaerobically digested effluent (Bonmatí and Flotats, 2003). These authors showed that with pH above 11.5, temperature of

80 ºC and 3 hours of retention time in the batch stripping column, up to 99% of ammonium nitrogen can be stripped from the wastewater. Thus, an important pH increase is necessary to obtain a good ammonia removal from wastewater. That increase can be produced by the addition of chemicals as NaOH or $Ca(OH)_2$ for example. However, this can significantly increase the cost of the process.

An alternative to reduce the costs is the use of a CO_2-pre-stripper column, previously to the stripping reactor. The pH of the anaerobic digestion effluent can be increased from about 7 to about 9 using this alternative (Lei *et al.*, 2007). After heating the ammonia rich liquid, ammonium and bicarbonate are partly transformed to the gaseous components free ammonia and carbon dioxide. The stripping rate for CO_2 is higher, by two orders of magnitude, than that for ammonia. This happens since the dimensionless Henry's law constant is 0.011 and 0.95 for ammonia and CO_2, respectively (Fattah *et al.*, 2008). Carbon dioxide could be stripped in a first stripper column without losing too much of free ammonia in the off-gas and with significantly lower air flow than in the ammonia stripper. With the inclusion of the additional CO_2 stripping column (Figure 1.30), most of the nitrogen is in the form of NH_3 prior to enter the ammonia stripper because of the pH increase, reducing the amount of base solution that is needed for the removal of ammonia in the overall system (Figure 1.31).

As a by-product of the stripping process, the wastewater treatment plant will produce ammonium sulphate, a marketable fertilizer. This product can be applied to soils by means of the Controlled Uptake Long Term Ammonium Nutrition fertilization (CULTAN), a type of injection of fertilizer (Figure 1.32). Using this technique, the entire amount of nitrogen needed for a plant to grow is injected at one time, with a more precise application and a more uniform distribution of the fertilizer. Injecting nitrogen into the soil leads to a higher dry matter content of the plant. In addition, it allows the spread of nitrogen regardless the conditions of the field, reduces soil compaction caused by tractors moving across the field and produces lower nitrate and N_2O losses than conventional fertilization (Kozlovský *et al.*, 2009).

1.4.2 Nutrients removal by Anammox based processes

1.4.2.1 Conventional Nitrogen removal processes

Nitrogen is usually present in the wastewaters as ammonium or organic nitrogen. The conventional technologies for nitrogen removal in current wastewater treatment plants are based on the Nitrification/Denitrification processes. Initially, ammonia is oxidized to nitrite in the nitritation process, equation (1.3) by Ammonia Oxidizing Bacteria (AOB). Then, the nitrite is oxidized to nitrate by Nitrite Oxidizing Bacteria (NOB), following equation (1.4), in the nitratation process. These processes imply an oxygen consumption of 4.57 kg O_2/kg $N_{oxidized}$. In the denitrification process, the nitrate and/or nitrite presents in the wastewaters is reduced to molecular nitrogen in anoxic conditions by the action of heterotrophic bacteria, following

equation (1.5). Organic carbon is needed as the electron donor, while nitrate acts as the last electron acceptor in the respiratory chain substituting the O_2 molecule.

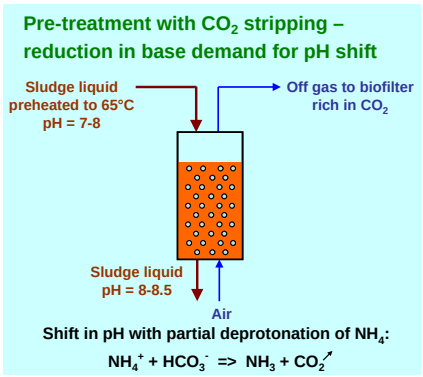


Figure 1.30. CO_2 stripping pre-treatment scheme.

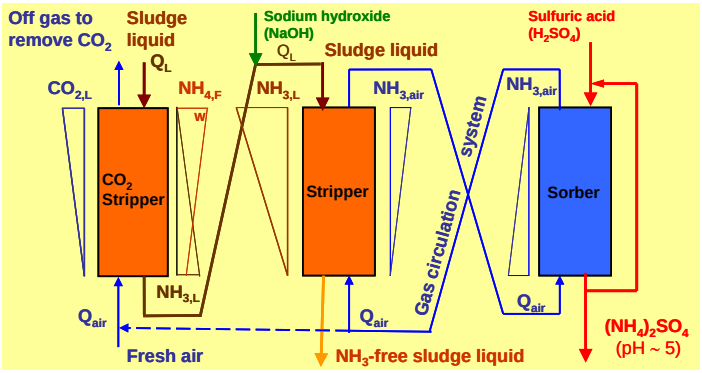


Figure 1.31. Functional principle of free ammonia stripping and sorption with pre-treatment of the supernatant by CO_2 stripping.

A)

B)

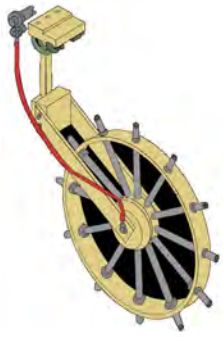


Figure 1.32. A) Fluid fertilizers injection. Retrieved from: (Werktuigendagen, 2009) B) Block diagram of a spur wheel. Retrieved from: (Sommer *et al.*, 2010).

The most common and widely distributed denitrifying bacteria are *Pseudomonas* species, which can use hydrogen, methanol, carbohydrates, organic acids, alcohols, benzoates, and other aromatic compounds for denitrification (Metcalf & Eddy *et al.*, 2002). For wastewaters with lower chemical oxygen demand to nitrogen ratios (COD/N), external addition of a carbon source such as methanol is needed to avoid the limitation of the denitrification process.

$$NH_4^+ + 1.5\,O_2 + 2\,HCO_3^- \rightarrow NO_2^- + 3\,H_2O + 2\,CO_2 \quad (1.3)$$

$$NO_2^- + 0.5\,O_2 \rightarrow NO_3^- \quad (1.4)$$

$$NO_3^- + 0.625\,CH_3COOH \rightarrow HCO_3^- + 0.25\,CO_2 + 0.5\,N_2 + 0.75\,H_2O \quad (1.5)$$

The AOB and NOB are two phylogenetically unrelated groups with different growth rates. The way their growth rates are affected by parameters like temperature, pH, free ammonia, dissolved oxygen (DO) concentration, etc. is different. These differences can be used to outcompete NOB and to uncouple both reaction rates (Sinha and Annachhatre, 2007). NOB inhibition conduces to an accumulation of nitrite, which can be exploited by the partial nitrification/denitrification process (Figure 1.33).

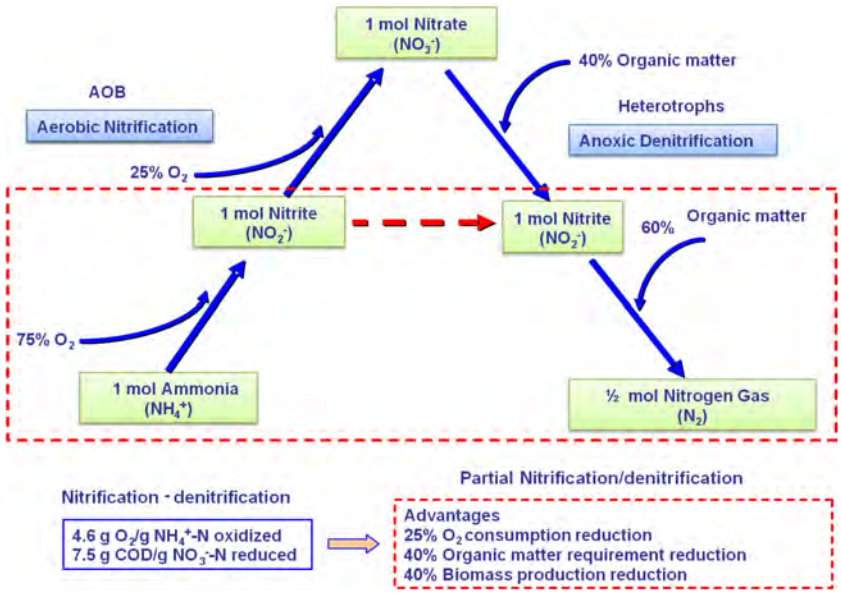


Figure 1.33. Comparison of nitrification/denitrification and partial nitrification/denitrification processes.

With the application of partial nitrification/denitrification process, a saving of 25% of oxygen and 40% of organic carbon source can be obtained. An additional advantage of this technology is that the sludge production is 40% of that corresponding to nitrification/denitrification processes (van Kempen *et al.*, 2001). The partial nitrification/denitrification strategy has been implemented in different reactor configurations (Sinha and Annachhatre, 2007), as the SHARON reactor (Single reactor for High-activity Ammonia Removal Over Nitrite) developed by Hellinga *et al.* (1998).

1.4.2.2 The Anammox processes

The ANaerobic AMMonium OXidation (Anammox) process, involving a new route in the nitrogen cycle (Figure 1.34), was predicted thermodynamically by Broda, (1977) and experimentally observed in the 1990's decade (Mulder *et al.*, 1995). Soon after its discovery, the potentiality of this unknown process was applied to the environmental field (Strous *et al.*, 1997). Furthermore, Anammox bacteria have been found to be essential components of the global nitrogen cycle and account for 50% of the world nitrogen turnover (Pennisi, 2012).

Anammox are chemolitoautotrophic bacteria capable of converting ammonium together with nitrite directly to dinitrogen gas, in the absence of any organic carbon source, following equation (1.6) (Strous *et al.*, 1998). Nitrite acts as electron acceptor in the reaction. The highly toxic "rocket fuel" hydrazine (N_2H_4) and nitric oxide (NO) are the two intermediates of this process (Kartal *et al.*, 2011). A small amount of nitrate is also produced in the anabolism of Anammox bacteria.

$$\begin{aligned} NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow \\ 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O \end{aligned} \quad (1.6)$$

Ammonium and nitrite are consumed on an almost equimolar basis, thus, half of ammonia in the wastewater treatment plant must be oxidized to nitrite by means of a partial nitritation process.

Anammox bacteria share features with all three domains of life, *Bacteria*, *Archaea* and *Eukarya*, making them extremely interesting from an evolutionary perspective. Anammox bacteria belong to the genus *Plantomycetes (Strous et al., 1999a)*, a phylum of the domain *Bacteri.* Different species of Anammox bacteria have been identified in the last years: Candidatus *Brocadia anammoxidans*, Candidatus *Kuenenia stuttgartiensis,* Candidatus *Scalindua brodae,* Candidatus *Anammoxoglobus propionicus,* Candidatus *Brocadia fulgida… (Schmid et al., 2000, Schmid et al., 2003, Kartal et al., 2007, Kartal et al., 2008)*. They are structurally distinct from other bacteria in that they have intracytoplasmic membranes that compartmentalize the cell in organelles. These membranes are called anammoxosomes (Jetten *et al.*, 2001). These bacteria also contain unique membrane lipids named ladderanes (Damste *et al.*, 2005, van Niftrik and Jetten, 2012).

The optimal temperature and pH of operation of Anammox bacteria have been found to be 35 ºC and 8, respectively. These bacteria are characterized by a low productivity; 0.038 g VSS/g $N_{removed}$, and a slow growth rate with large doubling times as long as 11 d (Strous *et al.*, 2002). Although Anammox bacteria were recently identified, these microorganisms are truly ubiquitous. They appear to be present in virtually any N-containing ecosystems with a pronounced suboxic zone or chemocline (a layer caused by a strong, vertical chemistry gradient within a body of water), marine sediments, wastewater treatment plants, etc. (Thamdrup and Dalsgaard, 2002, Dalsgaard *et al.*, 2003, Kuypers *et al.*, 2003, Francis *et al.*, 2007).

The application of Anammox processes to the removal of nitrogen in the wastewater treatment plants can produce an important reduction of energy consumption and resources needs (Figure 1.35).

The cost of the nitrogen removal using Anammox process has been estimated as 1 €/kg $N_{removed}$, while other conventional nitrogen removing techniques cost around 2–4 €/kg $N_{removed}$ (van Dongen *et al.*, 2001). The combination of the partial nitritation and Anammox processes requires less than half of the aeration energy, and no carbon source presence, compared to conventional nitrification and heterotrophic denitrification processes (Siegrist *et al.*, 2008). Furthermore, less sludge is produced as a by-product in comparison to the application of the nitrification/denitrification or the partial nitrification/denitrification strategies.

1.4.2.3 CANON process

Two different configurations are used to combine ammonia oxidation and Anammox processes: a two reactors configuration (Hellinga *et al.*, 1998) with each process carried out in different units, and a single stage system under oxygen limiting conditions.

Different acronyms have been used to define the single stage alternative: OLAND (Oxygen-Limited Aerobic Nitrification and Denitrification) (Windey *et al.*, 2005) and aerobic Deammonification DEMON (Wett, 2006). These names were based on the idea that the ammonia oxidizing bacteria carried out the denitrification process. However, nowadays, it is known that Anammox bacteria are the responsible bacteria for the denitrification process, becoming the CANON acronym (Completely Autotrophic Nitrogen removal Over Nitrite) (Sliekers *et al.*, 2002) the most suitable one to define the process.

The low biomass yield of Anammox and ammonia oxidizing bacteria (Strous *et al.*, 1999b) is at the same time an advantage, due to its low sludge production, and a disadvantage, due to the need of good biomass retention capacity of the reactor. In consequence, systems with a high sludge retention time are needed, such as systems based in a biofilm-based setup: rotating contactors (Pynaert *et al.*, 2003), moving bed reactors (Cema *et al.*, 2006), fixed bed reactors (Furukawa *et al.*, 2006) and granular biomass reactors

(Fernandez *et al.*, 2008); or based in membrane bioreactors (Trigo *et al.*, 2006, van der Star *et al.*, 2008).

Figure 1.34. The updated nitrogen cycle with autotrophic nitrogen removal (Anammox) Adapted from: Campos *et al.* (2010).

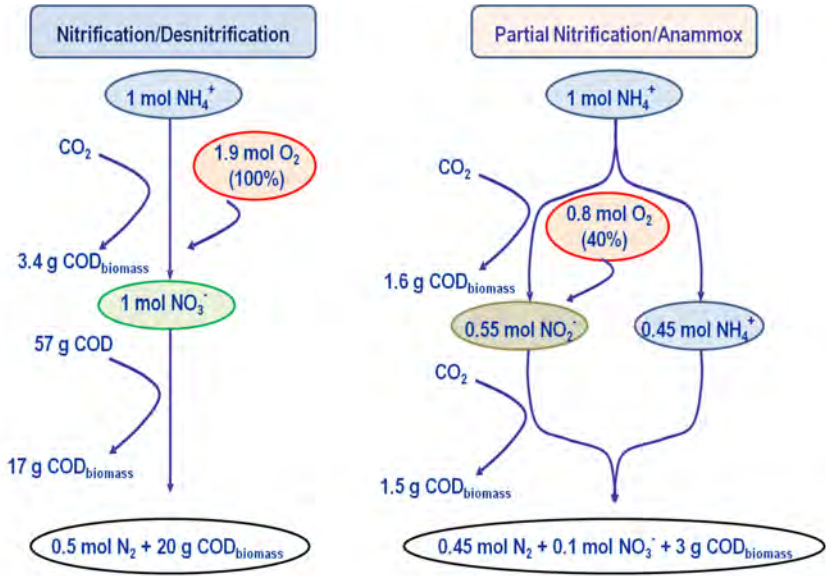


Figure 1.35. Comparison between the combination of nitrification/denitrification processes and the partial nitrification/Anammox processes for the treatment of wastewaters with low COD/N ratio.

Reactors based on granular biomass are suitable to develop the CANON process according to the “magic bead concept” (Santos *et al.*, 1996). The system is based on the

establishment of aerobic and anoxic zones within the granule and on the physical separation of the nitrifying and Anammox populations. In this way, AOB can grow in the outer part of the aggregates, where they produce nitrite and consume oxygen to provide anoxic conditions in the inner part of the granule. In this anoxic zone, ammonium, left from the incomplete AOB activity, and nitrite, produced during partial nitrification, have to be present in order to allow the growth of Anammox bacteria (Vázquez-Padín *et al.*, 2009a). A schematic overview of these reactions in a biofilm system is shown in Figure 1.36. This stratification of microorganisms was demonstrated through fluorescence in situ hybridization (FISH) and microsensors measurements (Nielsen *et al.*, 2005, Vázquez-Padín *et al.*, 2010b).

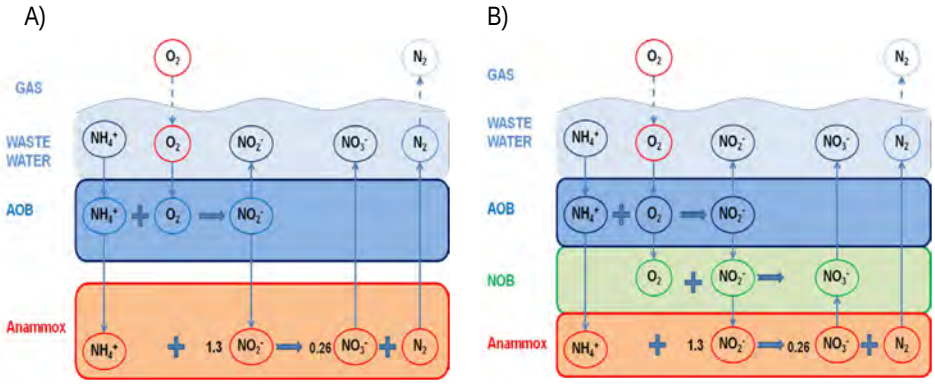


Figure 1.36. Schematic overview of the aerobic and anoxic reactions in a CANON biofilm system A) without and B) with the inclusion of NOB.

In order to maintain the stability of the process some operational considerations must be taken into account:

a) The nitrifying activity should be high enough to deplete oxygen in the outer part of the biofilm and to avoid its presence in the inner layers. Thus, to obtain anoxic conditions in the inner part of the biofilm, the thickness of the biofilm must be larger than the oxygen penetration depth.
b) Enough ammonia should be present in the bulk liquid in order to allow the total consumption of oxygen by AOB and to remove in the anoxic zone all the nitrite generated during partial nitrification. To avoid a complete penetration of the biofilm, ammonia should be in excess in such a way that the penetration distance of O_2 was shorter than that of ammonia. This condition is fulfilled when $C_{NH_4^+}$>0.48 C_{O_2} in the bulk liquid (Campos *et al.*, 2010).
c) The activity of AOB in the external layer should be high enough to protect Anammox bacteria from the penetration of dissolved oxygen, but their activity must be controlled to avoid inhibition of Anammox bacteria by high nitrite concentrations. This inhibition can occur when the diffusion rate of nitrite to the anoxic zone is higher than the nitrite consumption by Anammox bacteria.

Based on these considerations, a control strategy was defined by Vázquez-Padín *et al.* (2010b) to regulate the concentration of ammonia and nitrite by using on-line ammonia and nitrite analyzers. The variables to control were the dissolved oxygen (DO) concentration and the hydraulic retention time value (HRT), using the air flow rate and the inlet flow rate as actuation elements.

In Figure 1.37 the different scenarios for the different ranges of ammonium and nitrite concentrations are represented. Low NH_4^+ and NO_2^- concentrations would limit the activities of AOB and/or Anammox bacteria. Assuming that the diffusion coefficients of NH_4^+ and O_2 are barely similar, AOB would be limited by ammonia when the ratio DO/NH_4^+ in the bulk liquid is higher than 3.4 g O_2/g N. The limitation of AOB by ammonia would expose the reactor to a risk of failure since the granules would be fully penetrated by oxygen, which would temporally inhibit Anammox bacteria and enhance the undesired growth of NOB. On the other hand, high NH_4^+ and/or NO_2^- concentrations have the following disadvantages: both substrates can inhibit AOB and Anammox bacteria with a consequent detriment of the produced effluent quality. Moreover, maintaining concentrations of NO_2^- higher than the optimal ones involves higher costs of aeration (Vázquez-Padín *et al.*, 2010b).

Fluctuations of the influent temperature can also affect the process stability since it has effects on both AOB and Anammox activities. As the ammonia oxidation and Anammox processes have a comparable activation energy value, a decrease of temperature would affect to the intrinsic kinetics of both processes in a similar way. Then, in order to maintain a balance between ammonia oxidation and Anammox rates, the oxygen penetration depth should be kept constant by decreasing the oxygen concentration in the bulk liquid (Vázquez-Padín *et al.*, 2011).

CANON processes have been successfully applied to treat nitrogen-rich wastewaters short of carbon such as the case of the supernatant from municipal anaerobic sludge digesters (van Dongen *et al.*, 2001, Vázquez-Padín *et al.*, 2009b, Vázquez-Padín *et al.*, 2009c) or industrial wastewaters (Windey *et al.*, 2005, Figueroa *et al.*, 2012). These processes have been successfully applied at full scale (Wett, 2006, van der Star *et al.*, 2007).

1.4.2.4 Application of Anammox processes to the main stream of WWTP

Until now, the application of the Anammox process has been mainly focused on wastewaters with high temperatures (>30 ºC) and high ammonia concentration (>500 mg N/L). Nevertheless, when low nitrogen concentrations (30-45 mg NH_4^+-N/L) were used, the temperature of operation was always higher than 20 ºC (De Clippeleir *et al.*, 2011, Ma *et al.*, 2011). However, previous results showed the possibility to operate this kind of process at low/moderate temperatures with different reactor configurations (Dosta *et al.*, 2008, Qiao *et al.*, 2010, Vázquez-Padín *et al.*, 2010a, Hendrickx *et al.*, 2012). These new findings can expand the range of application of such technology, to remove nitrogen from landfill leachates,

effluents from psychrophilic anaerobic digesters and even from the main stream of municipal WWTPs, in order to improve the overall nitrogen removal efficiency (Vázquez-Padín *et al.*, 2011, Morales *et al.*, 2012).

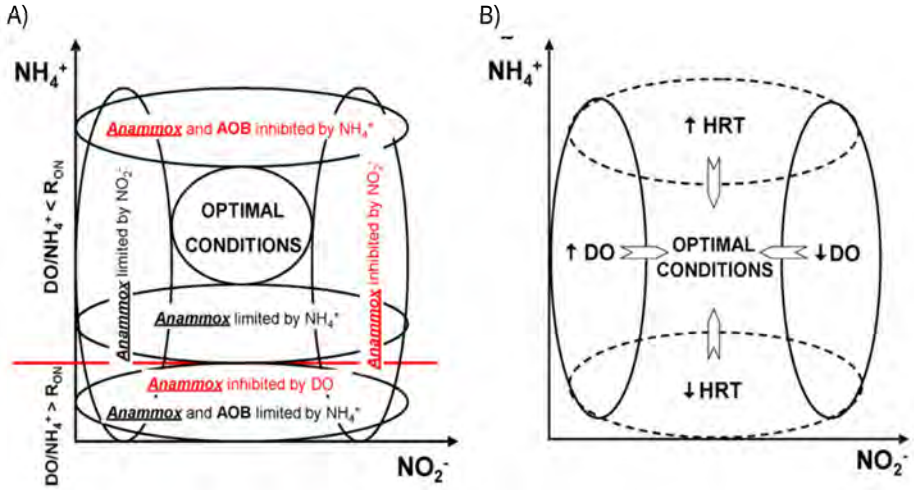


Figure 1.37. A) Zones defining the different operational conditions in a CANON reactor depending on different NH_4^+ and NO_2^- concentrations in the bulk liquid. B) Control strategy to return the CANON reactor to optimal conditions depending on NH_4^+ and NO_2^- concentrations in the bulk liquid. Figures retrieved from: Vázquez-Padín *et al.* (2010b).

The recovery of energy from the raw wastewater could be improved by the application of Anammox based processes to the main stream in the WWTPs (van Loosdrecht *et al.*, 2004), which offer a way to recover self-sufficiency in the WWTPs (Garrido *et al.*, 2013). In this case, the Anammox based process could be combined with either an anaerobic psychrophilic digestion (Álvarez *et al.*, 2008) or an aerobic stage operated at a low solids retention time (Wett *et al.*, 2007) to remove the organic matter, followed both by an anaerobic digestion of the generated sludge. No organic matter is then partially wasted during nitrogen removal via denitrification. With such strategy, the recovery of energy from the organic matter contained in the wastewater as methane production is maximized. At the same time, the nitrogen disposal requirements are fulfilled since both organic matter and nitrogen are removed in separated processes. Anammox based processes can be applied in both sludge and water lines of a conventional WWTP at several configurations in order to improve its energetic efficiency (Morales *et al.*, 2012). Garrido *et al.* (2013) determined an energy self-sufficient potential of 111% in a scenario with a high-rate aeration conventional activated sludge and an Anammox process as tertiary treatment for nitrogen removal.

The application of Anammox based processes systems directly to the water line in the WWTP presents two main disadvantages: 1) low biomass growth rate due to the temperature of operation under the optimum range; 2) low net biomass production due to the low nitrogen

content of the stream and to the biomass wash-out provoked by the high flow. Reactor systems with good biomass retention are required to solve these problems, like those based on biofilm or granular biomass (Vázquez-Padín *et al.*, 2009a, De Clippeleir *et al.*, 2011, Winkler *et al.*, 2012). Both bacterial populations Anammox and AOB need a minimum solid retention time at 15 ºC of 85 and 2.4 d respectively (Wiesmann, 1994, Strous *et al.*, 1999b). Since AOB are located in the outer layers of biofilms or granules, Anammox are protected from erosion and, therefore, washed-out biomass due to detachment is mainly composed by AOB. This implies that the minimum SRT needed is that imposed by AOB.

The start-up of the reactor at a low temperature, until the needed biomass concentration for steady-state operation is achieved, will take several months, even when a system with high biomass retention capacity is used. In order to avoid this constraint, the required amount of biomass can be produced in a separated unit, where the Anammox based systems are operated at optimum temperature values and high ammonia concentrations. Taking into account the maximum growth rate and activation energy values of Anammox biomass (Strous *et al.,* 1999b), a period of 50 d is needed to produce the required biomass amount at 30 ºC. This value would be approximately 4 times higher in the case that the biomass was produced at 15 ºC (Dosta *et al.*, 2008). A sudden change of temperature has no effects on the stability of the biological process if the operational conditions are properly controlled (Winkler *et al.*, 2012). In other case, a progressive adaptation of biomass to lower operating temperatures is suggested (Dosta *et al.*, 2008).

1.5 BIOLOGICAL COMPACT TREATMENT SYSTEMS

One of the main concerns of wastewater design is the space required for the plant construction, especially in big cities where the available area is limited. The biological treatment of wastewater in the WWTPs is often accomplished by means of the application of conventional activated sludge systems. These systems generally require large surface areas for implantation. They also require biomass separation units due to the usually poor settling properties of the sludge. In addition, low sludge concentrations can be achieved with the activated sludge, which limits the volumetric load of the system. These requirements will increase in the future, as urban population is constantly growing worldwide. The World Resources Institute (1996) projected that urban population will double in size to near 5000 million between 1995 and 2025. Many WWTPs initially built outside the urban area had become engulfed by residential areas, and industry started to treat its own wastewater (Henze *et al.*, 2008). Consequently, more compact treatment processes have been developed during recent years to be applied in regions with little area available: high population density regions, big cities, industrial areas, coastal areas…

One option to reduce the volume of the required units is the development of systems based on the improvement of biomass retention (Campos *et al.*, 2009); for example, by performing actions to enhance the settleability properties of the activated sludge as biofilms or

aerobic granules. Another alternative can be the use of membranes to obtain a better biomass separation in a lower area.

These compact treatment systems also contribute to the reduction in the sludge production. Sludge produced in WWTPs amounts to a small percentage (around 1%) of the volume of treated wastewater in a conventional activated sludge treatment. However, the disposal costs of the sludge can represent between 20 and 60% of the total operation costs of a urban WWTP, considering manpower, energy and sludge disposal (Foladori *et al.*, 2010). The cost for treatment and disposal of sludge has been estimated to vary from 250 to 1000 € per tonne of dry mass, with an average of around 470 €, according to the type of treatment and disposal (Paul and Liu, 2012). A further increase in these costs is expected in the near future (Foladori *et al.*, 2010).

1.5.1 Membrane systems

Membranes biological reactors (MBRs) are systems involving the combination of permselective or semi-permeable membrane filtration for solid/liquid separation, instead of secondary clarifiers, combined with biological treatment (Figure 1.38). Submerged MBRs need only half the land area of a conventional activated sludge process. Clarification is achieved in a single filtration stage in place of the conventional multi-stage process (Gander *et al.*, 2000). MBRs have been applied at full scale (Krzeminski *et al.*, 2012). However, the relatively high costs for membrane separation (Fenu *et al.*, 2010) and membrane life have hindered the spread of this technology. In most cases, MBR systems remain more expensive than conventional activated sludge treatments for equivalent capacities, even with the last decades reduction of capital and operational costs (De Wilde *et al.*, 2008). These high costs are mainly due to membrane installation and replacement costs, as well as higher energy demand, compared to conventional activated sludge systems (Verrecht *et al.*, 2010). In this way, the energy demand of MBRs in municipal WWTPs is around 2–4 times higher (Cornel *et al.*, 2003), with aeration being 50% of this energy demand (Gil *et al.*, 2010). Their tendency to foul is another drawback of MBR systems. Fouling is the general term given to those phenomena responsible for increasing membrane hydraulic resistance and the decrease in the permeate flux (Gander *et al.*, 2000).

1.5.2 Biofilm systems

Biofilms (Figure 1.39) form when bacteria adhere to surfaces in moist environments by excreting a slimy, glue-like substance. In nature, biofilms almost always consist of rich mixtures of many species of bacteria, as well as fungi, algae, yeasts, protozoa, other microorganisms, debris and corrosion products. Biofilms are held together by sugary molecular strands, collectively termed extracellular polymeric substances (EPS).

Biofilm growth is used in wastewater treatment processes, due to the high biomass concentrations that can be obtained in these systems. Another advantage is the lower sludge production compared with the activated sludge process. In aerobic systems, the growth of biomass adhering to support materials has been widely studied in airlift or fluidized bed reactors (Heijnen *et al.*, 1990, Tijhuis *et al.*, 1996).

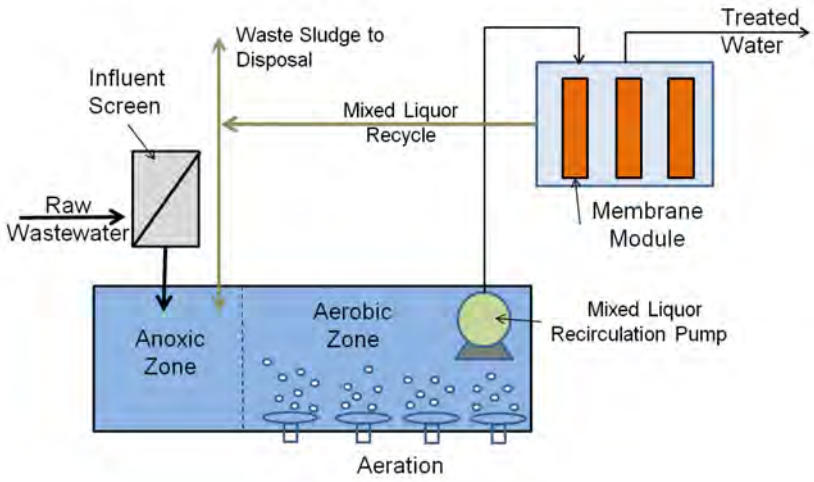


Figure 1.38. Simplified membrane bioreactors schematic.

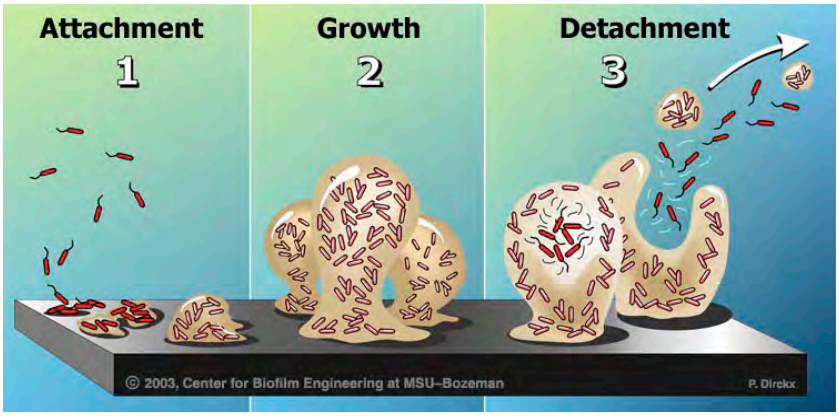


Figure 1.39. The biofilm life cycle: 1) initial attachment events, 2) the growth of complex biofilms, and 3) detachment events by clumps of bacteria or by a 'swarming' phenomenon within the interior of bacterial clusters. Retrieved from: Montana State University Center for Biofilm Engineering (2003).

There are already many different biofilm systems in use, such as moving bed biofilm reactor (MBBR), trickling filters, rotating biological contactors (RBC), fixed media submerged biofilters, fluidized bed reactors, etc. (Odegaard, 2006).

In the MBBR reactor, plastic pieces with a high specific surface area act as carrier material for the attachment of active bacteria (Figure 1.40). In this way, a high concentration of biomass can be maintained in the biological reactor, increasing the treatment capacity, but occupying the same volume.

An RBC unit (Figure 1.41) typically consists of a series of closely spaced large flat or corrugated discs that are mounted on a common horizontal shaft and are partially or completely submerged in wastewater (Cortez *et al.*, 2008). Wastewater flows through partially submerged rotating shafts where biomass is carried. The shaft continually rotates at 1 to 10 rpm (Patwardhan, 2003) while a layer of biofilm grows on the wetted surface of each disk. During the rotation, the disk leaves the wastewater and moves through the air, when oxygen is transferred to the slime. As the slime reenters the wastewater, excess solids and waste products are stripped off the media.

The trickling or percolating filter (TF) was introduced in 1890 and is one of the earliest systems for biological wastewater treatment. A TF consists of a permeable medium made of a bed of rock, slag or plastic over which wastewater is distributed to trickle through. The organic load is absorbed and degraded by the biomass, while the liquid drains to the bottom where it is collected (Figure 1.42). As the biofilm layer thickens, it eventually sloughs off into the treated effluent. The treated wastewater and solids are piped to a settling tank where the solids are separated. Usually, part of the liquid from the settling chamber is recycled to improve wetting and flushing of the filter medium, optimizing the process and increasing the removal rate.

New systems are continuously developing based on improvements, combinations or new designs based on biologic biofilm growth: the ultra-compact biofilm reactor (UCBR) (Koh *et al.*, 2012), the combination of MBBR with ballasted floc solids separation (Haegh *et al.*, 2010), integrated fixed film activated sludge (IFAS) (McQuarrie *et al.*, 2004), etc.

Biological aerated filters (BAFs) are submerged three-phase fixed-media reactors for wastewater treatment, which combine filtration with biological carbon reduction, nitrification or denitrification. A major characteristic of BAF reactors is the use of filter media. The media is either in suspension or supported by a gravel layer at the base of the filter. The dual purpose of this media is to support highly active biomass that is attached to it and to filter suspended solids in one unit. BAF is operated either in upflow or downflow configuration, depending on design specified by manufacturer. Trapped solids and growing biomass gradually block the filter pathways. These obstructions are cleared from time to time by air-scouring or back-washing with treated effluent. The BAFs have been applied to nitrogen removal using external carbon sources, as well to simultaneous biological phosphorus and nitrogen removal using a wastewater carbon source (Sammut *et al.*, 1994).

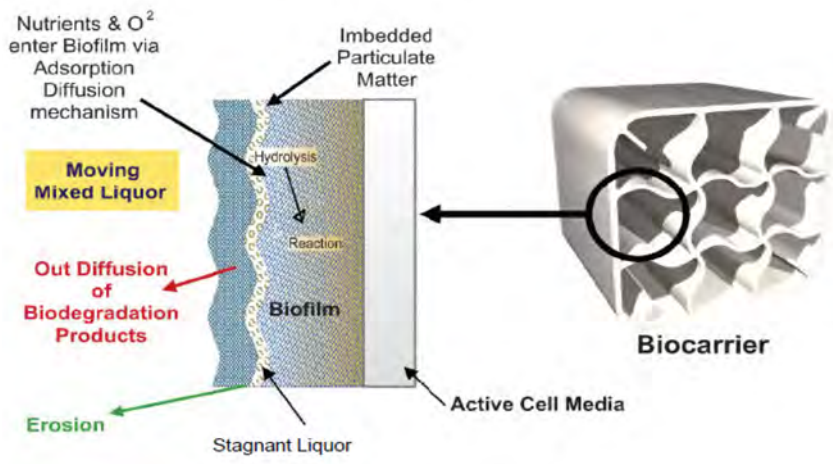


Figure 1.40. Scheme of a biocarrier in a MBBR (HeadworksBIO).

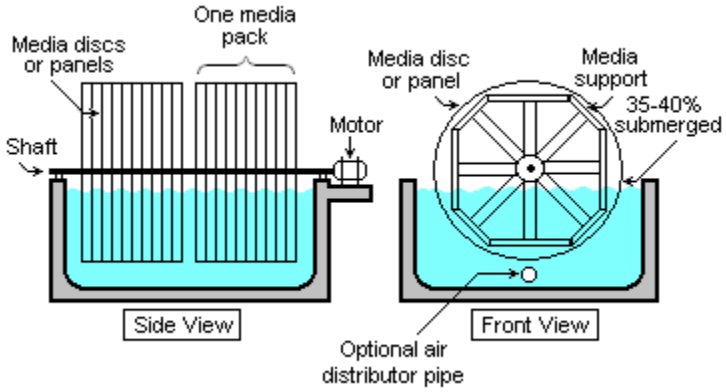


Figure 1.41. Rotating biological contactor. Retrieved from: Mbeychok (2007)

1.5.3 Aerobic granular systems

Granular growth is a particular case of biofilm growth, in which no carrier material is required. Bacteria are self-immobilized forming microbial aggregates. Initial applications of granular biomass were associated to anaerobic process: Upflow Anaerobic Sludge Blanket (UASB), Expanded Granular Sludge Blanket (EGSB) or Internal Circulating (IC) Reactors. The first studies about granular biomass developed in aerobic conditions were published in the early 1990's. Mishima and Nakamura (1991) developed aerobic granules in an Aerobic Upflow Sludge Blanket (AUSB) reactor. However, until the late 1990's the aerobic granular technology did not emerge (Campos *et al.*, 2009) with the use of Sequencing Batch Reactors (SBRs).

A)

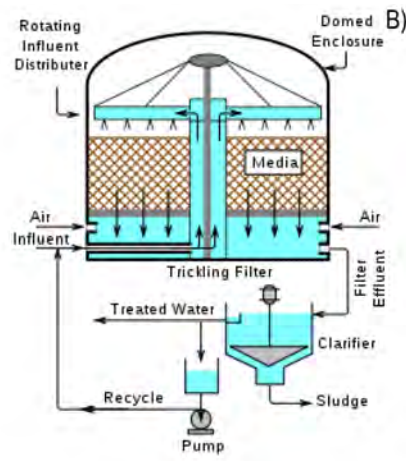


Figure 1.42. A) Scheme of a typical trickling filter (Mbeychok, 2011).
B) Sewage treatment trickling filter bed using plastic media in a small rural treatment plant at Beddgelert sewage treatment, Gwynedd, Wales, U.K. (Velela, 2005).

The definition of "aerobic granule" emerged from the discussions which took place at the 1st IWA-Workshop Aerobic Granular Sludge, in Munich (2004) and literally stated that:

"Granules making up aerobic granular activated sludge are to be understood as aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear and which settle significantly faster than activated sludge flocs" (de Kreuk *et al.*, 2005a).

According to this definition, aerobic granules are biomass structures, which fulfill the following requirements:

- The values of the sludge volume index after 10 and 30 minutes of settling (SVI_{10} and SVI_{30}, respectively) do not differ more than a 10% (Schwarzenbeck *et al.*, 2004) meaning that no thickening of the biomass occurs after settling.
- Granules do not coagulate and settle as separate units.
- The position of microorganisms is fixed and it does not change quickly as in an activated sludge floc due to the existence of a matrix of biomass and EPS.
- No carrier material is intentionally involved or added.
- The minimum size of the granules is considered to be around 0.2 mm (de Kreuk *et al.*, 2005a), in order to be able to separate them from a sludge sample by sieving.
- Bacterial aggregates with an SVI_{10} value equal or lower than 60-70 mL/g TSS are conventionally considered as aerobic granules.

1.5.3.1 Advantages and drawbacks of aerobic granulation

Aerobic granular technology has several advantages compared to activated sludge processes, such as good biomass retention capacity, ability to withstand shock and toxic

loadings (Tay *et al.*, 2005b), and presence of aerobic and anoxic zones inside the granules (Tay *et al.*, 2002) to perform simultaneously different biological processes. The reduction in the sludge production compared with activated sludge is another great advantage.

Initially, the aerobic granules were developed in SBR systems to oxidize the organic matter from the wastewaters (Beun *et al.*, 1999). Later, the operational conditions allowed the development of aerobic granules, which are capable to remove at the same time nutrients: nitrogen and phosphorus (de Kreuk *et al.*, 2005b). When organic matter oxidation and nitrogen removal occur inside a granule, the microorganisms which require aerobic conditions are situated on the granule surface, being in the outermost layer the aerobic heterotrophs which oxidize the organic matter. While the nitrifying organisms, which oxidize the ammonia to nitrite/nitrate, are located a little in depth (Figure 1.43). Inside the granules, where the dissolved oxygen is absent, the denitrifiers are located to perform the reduction of nitrite/nitrate to nitrogen gas. If the granule is big enough, an anaerobic zone in the core of the granule can exist. In those cases where phosphorus is simultaneously removed, the distribution of the different processes is as indicated in Figure 1.44. This stratification also happens during the formation of CANON granules, where ammonia oxidizing bacteria occupy an external nitrification zone while Anammox locate in a more internal zone (Vázquez-Padín *et al.*, 2010b) where oxygen cannot penetrate.

The biomass grows as compact and dense microbial granules, enabling better biomass retention in the reactor, which is important for substrate conversion capabilities and for implantation. The high biomass concentration that can be achieved with aerobic granular reactors allows to treat high organic loading rates (OLRs), up to 15 g COD/L·d (Moy *et al.*, 2002).

A comparison between the activated sludge systems and the aerobic granular technology indicates that the latter presents several improvements regarding costs and quality of the produced effluent. Some of these advantages are indicated in Table 1.6.

Table 1.6. Comparison between activated sludge systems and aerobic granular systems (de Bruin *et al.*, 2004).

Parameter	Activated sludge to remove nitrogen	Aerobic granular system
Effluent quality	Good	Similar or better
Process stability	Good	Similar or better
Implantation surface	100%	25%
Energy consumption	100%	< 65-75%
Sludge production	100%	Similar or better
Building costs	100%	Significantly lower
Operational costs	100%	Significantly lower

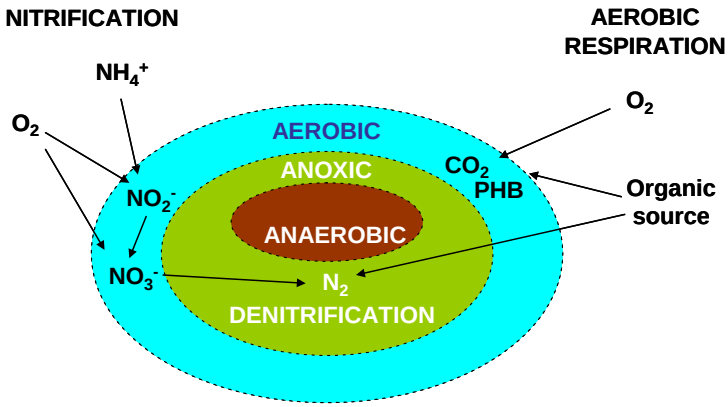


Figure 1.43. Distribution of the organic matter and nitrogen removal processes inside a granule. Adapted from: Mosquera-Corral *et al.* (2012).

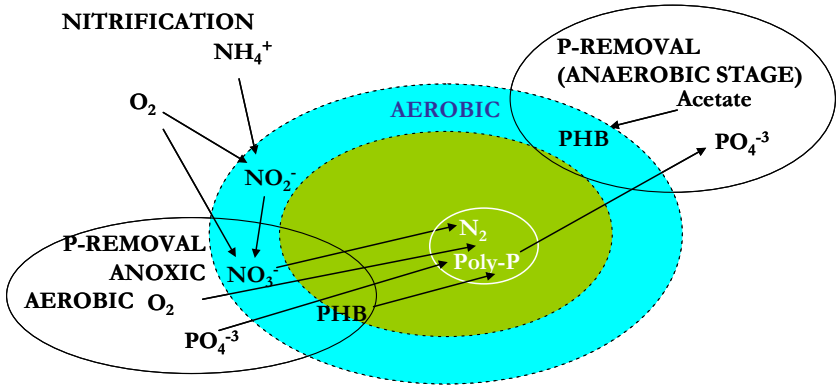


Figure 1.44. Distribution of the organic matter, nitrogen and phosphorous removal processes inside a granule. Adapted from: Mosquera-Corral *et al.* (2012).

However, there are still some drawbacks, which need further research like the fact that the produced effluents contain solids concentrations over 75 mg TSS/L. Consequently, these effluents need additional treatment to accomplish the discharge requirements. This solids content can be reduced by means of filtration systems, as membrane systems, settlers, sand filters, etc. Furthermore, the aeration cost is relatively high due to the need of large air flows to keep the required DO concentration and the appropriated mixture. In contrast, when slow-growing microorganisms, such as the nitrifiers or phosphorous removal bacteria, are used, the DO requirements decreased (de Kreuk and van Loosdrecht, 2004). Additionally, during the start-up, large quantities of the added inoculum are washed out from the reactor, decreasing temporarily the quality of the produced effluent. This can be avoided by inoculating the reactor with previously developed granular biomass (Liu *et al.*, 2005).

1.5.3.2 Aerobic granules formation

Aerobic granulation is a gradual process from seed sludge to compact aggregates, further to granular sludge and finally to mature granules (Tay *et al.*, 2001a). Verawaty *et al.* (2013) proposed a conceptual model describing how granules grow up to a certain critical size. Then, granules that have managed to grow larger than the critical size tend to break/attrite and in this way, they reduce in size down to the critical one, achieving a steady-state distribution of granule sizes (Figure 1.45). Critical size and size distribution depend on the operating conditions of the reactor, wastewater characteristics, aeration, reactor geometry, mixing, and solids concentration.

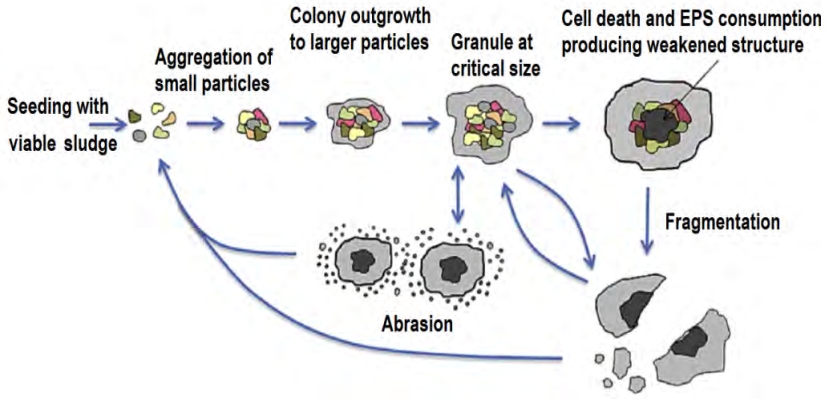


Figure 1.45. Conceptual model for granule formation and breakage/attrition. Reprinted from: Verawaty *et al.* (2013).

The key operational factors that promote aerobic granulation in SBRs have already been established:

- Feast-famine regime. The heterotrophic biomass must be cyclically subjected to periods of availability (feast) and lack (famine) of organic substrate in the liquid phase. During the feast period, the organic matter is oxidized and stored inside the bacteria cells as glycogen, lipids or Poly-β-Hidroxyalkanoates (PHA), like the Polyhydroxybutyrate (PHB). On the contrary, during the famine period, the bacteria grow on the stored compounds (Beun *et al.*, 2002).
- Short settling time. Biomass with good settling properties is retained inside the reactor, while the flocculent biomass is washed out. In aerobic granulation research, a short settling time has been commonly used to enhance aerobic granulation in SBRs (McSwain *et al.*, 2004, Qin *et al.*, 2004). Settling time is considered as a key factor for granulation. In fact, when long settling times are applied, poorly settling sludge flocs cannot be effectively withdrawn; and they may overtake granule-forming microorganisms (Campos *et al.*, 2009).

- Hydrodynamic shear force. Formation of aerobic granules and their physical granule integrity is stimulated by high shear forces (Chen *et al.*, 2007). The shear force in aerobic granular systems is achieved by means of mechanical stirring and/or aeration. The collisions between the granules provoke the detachment of weakly attached materials on the surface of the aggregates, helping to maintain their high densities and smooth surfaces. In fact, some authors reported that aerobic granules could be formed only above a threshold shear force value, in terms of superficial upflow air velocity, of 1.2 cm/s in a column SBR (Tay *et al.*, 2001b). More regular, rounder and more compact aerobic granules were developed at high hydrodynamic shear forces (Liu and Tay, 2002).
- Other factors. Several parameters have been reported as important for aerobic granulation, such as: substrate composition, fed organic load, exopolymeric substances formation, presence of divalent cations, and DO concentration.

However, once these important parameters are controlled, aerobic granule formation shows an important flexibility regarding operation conditions. This technology has been applied to treat different kinds of wastewaters (Arrojo *et al.*, 2004, Inizan *et al.*, 2005, Figueroa *et al.*, 2008) and under different operational conditions of oxygen limitation or low temperature (de Kreuk *et al.*, 2005c).

1.5.3.3 Aerobic granular reactor configuration. Continuous or discontinuous?

Nowadays, the development of aerobic granules is mostly focused on the application of column-type Sequencing Batch Reactors (SBRs) with a large height to diameter ratio (H/D), since these systems fulfill most of the needed requirements for this aim: short settling periods, alternative feast-famine periods and high shear forces. These SBRs operate in sequential cycles distributed in different operational phases: filling, reaction, settling, effluent withdrawal and idle time (Figure 1.46).

Nevertheless, the application of the concepts of the granular SBR technology for the upgrading of existing WWTPs could be limited by the different operational conditions and geometry of both column-type SBR and conventional activated sludge reactors. Transforming a continuous system into a SBR suitable to obtain aerobic granules is difficult.

Curiously, first applications of granular biomass grown in aerobic systems were developed on continuous systems, on the Aerobic Upflow Sludge Blanket (AUSB) reactor in a research performed by Mishima and Nakamura (1991) (Figure 1.47). This system consisted of two different units: 1) an AUSB reactor where high upflow velocities were maintained in order to achieve a high hydraulic selection pressure to retain only granular biomass and to wash out suspended biomass, 2) an external oxygenation chamber where pure oxygen was added to the recycled effluent. This kind of operational strategy promoted sludge aggregation and also allowed most of the produced biomass to be retained in the form of a well-settled sludge

blanket (Sharma and Huang, 2004). However, this system required high recirculation rates in order to obtain suitable oxygen transfer rates to remove pollutants and used pure oxygen, which increased the operational costs. Thus, this work was not really appreciated at that moment (Mosquera-Corral *et al.*, 2012). In consequence, there are very few reported works on AUSB and this kind of reactor was no further developed.

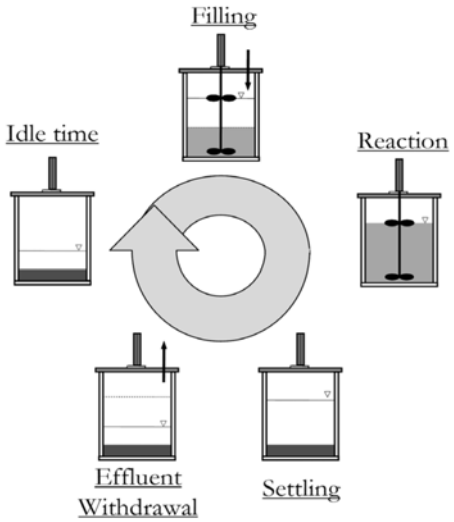


Figure 1.46. Operational phases of a cycle from a SBR. Retrieved from: Mosquera-Corral *et al.* (2012).

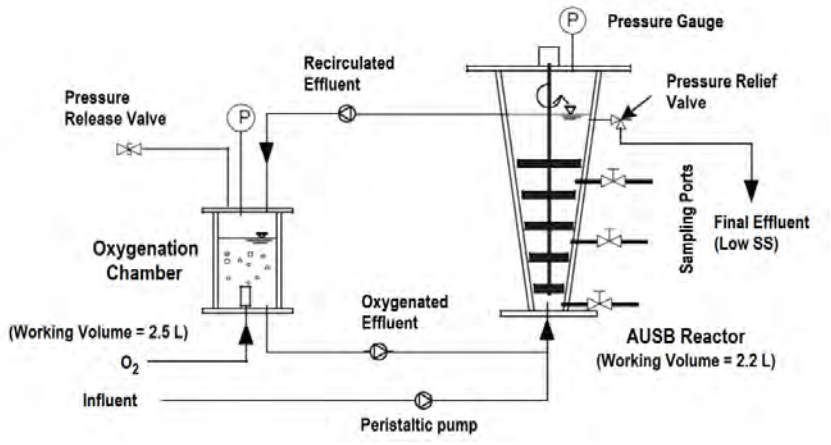


Figure 1.47. Schematic of the AUSB reactor set-up.
Reprinted from: Sharma and Huang (2004).

Biofilm Airlift Suspension reactors (BAS) (Figure 1.48) were also used for the development of aerobic biofilm and granular biomass (van Loosdrecht *et al.*, 1995). In the top

of the reactor, a phase separator allowed separating the biomass from the treated liquid (Figure 1.49 A). Campos *et al.* (2000) used a similar system for the development of nitrifying granules in the so called Nitrifying Activated Sludge Airlift reactor (NASA) (Figure 1.49 B).

Tsuneda *et al.* (2003) used an Aerobic Upflow Fluidized Bed reactor (AUFB) for the development of nitrifying granular biomass. From the bottom of the reactor, wastewater was continuously fed. Aeration was carried out via a porous air diffuser ball at a rate of 1.0 L/min. A solid–liquid separator was placed on the top of the reactor to prevent the outflow of suspended sludge from the reactor (Figure 1.50).

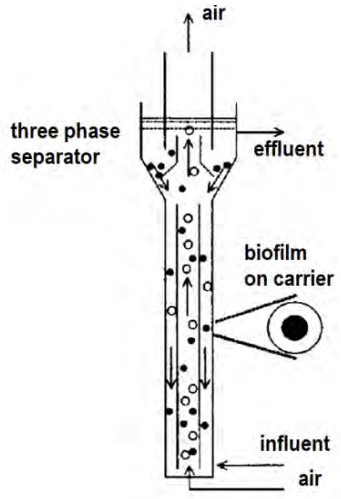


Figure 1.48. Schematic representation of the BAS reactor.
Reprinted from: van Loosdrecht *et al.* (1995).

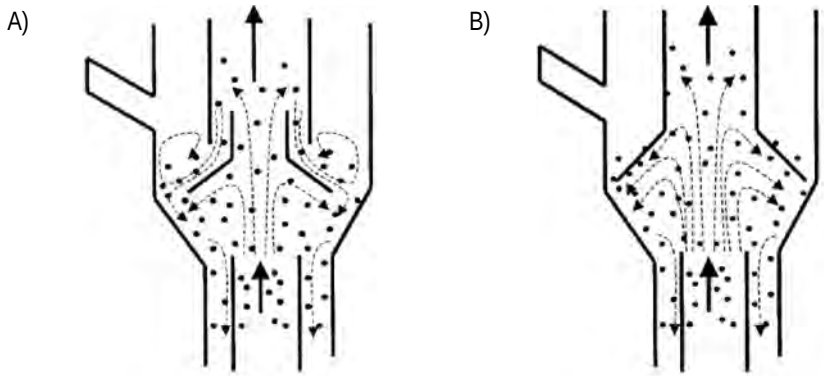


Figure 1.49. Design of the tree separator phase: A) BAS design.
B) NASA design. Reprinted from: Campos *et al.* (2000).

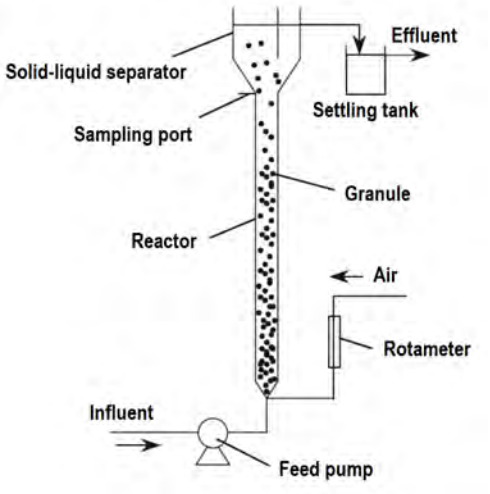


Figure 1.50. Schematic illustration of the AUFB reactor.
Reprinted from: Tsuneda *et al.* (2003).

More recently, Liu *et al.* (2012b) developed a new reactor for the growth of aerobic granules: the Continuous-flow bioreactor with aerobic Granular Sludge and Self-Forming Dynamic MemBRane (CGSFDMBR). This system includes four principal reaction tanks: the sequencing batch airlift reactor tank, the settling tank, the dynamic membrane bioreactor tank and the sludge selection tank (Figure 1.51). However, the formation of aerobic granules in that reactor was not completely demonstrated, as the reactor was completely inoculated with already formed granules, the characteristics of the granules worsened after a few weeks of operation, and no biomass concentration data was published.

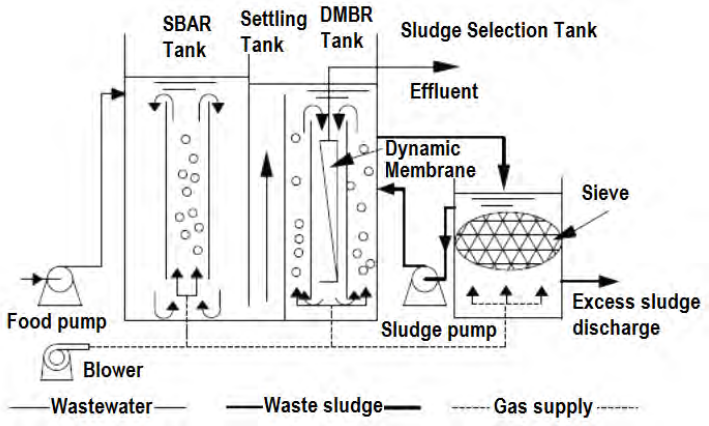


Figure 1.51. Diagram of the CGSFDMBR wastewater treatment process. Reprinted from: Liu *et al.* (2012b).

1.5.3.4 Scale-up applications

The research on aerobic granulation was initially focused on the use of lab-scale reactors fed with synthetic media. Nowadays, the technology has been developed and demonstration plants have been implemented.

Tay *et al.* (2005a) operated a pilot plant for the development of aerobic granular biomass treating a synthetic effluent. The pilot plant reactor had a height of 1.6 m and a diameter of 0.19 m, being the working volume 34 L. Inizan *et al.*, (2005) performed two experiments at pilot scale using a synthetic medium and the effluent of a pharmaceutical company. The reactors had 1.8 m of height and 0.2 m of diameter, with a working volume of 40 L. More recently, a 1 m^3 pilot plant was operated by Ni *et al.* (2009) to treat urban wastewater reaching COD and nitrogen removal percentages of 90-95% (Figure 1.52 A).

Scale-up of granular systems leads to modification of the hydrodynamic conditions, which are very important for the formation and maintenance of stability of aerobic granules. Jungles *et al.* (2011) studied the start-up and performance at different OLRs of a pilot-scale aerobic granular SBR reactor (Figure 1.52 B) fed with synthetic effluent. During the last phase of the experiment, the stability of the system was tested by rapid changes in the applied OLR. The obtained results showed that this technology is suitable to obtain high efficiencies in terms of COD and nutrient removal. These authors also stated that the selection of an adequate settling time related to the minimum settling velocity of the particles is an important parameter for the formation of aerobic granules.

A) B)

Figure 1.52. A) View of the aerobic granular pilot plant installed in the WWTP Zhuzhuanjing (China). Reprinted from: Ni *et al.* (2009).
B) View of the aerobic granular pilot plant operated in the University of Santiago de Compostela.

A pilot plant with a capacity of 5 m^3/h has been operated in The Netherlands from October 2003 to treat urban wastewater (de Bruin *et al.*, 2005). From the results of this pilot plant, two municipal units were constructed in The Netherlands and South Africa under the

trade mark "Nereda® technology", and one municipal facility was adapted to this technology in Portugal. The first full-scale Nereda plant for the treatment of municipal wastewater was located at Epe, the Netherlands, in summer 2011, and treats a peak flow of 1500 m^3/h (59000 pe).

To summarize, the aerobic granulation technology is extending its application to different kinds of domestic and industrial wastewaters, in order to remove organic matter and nutrients. In addition, its excellent settling properties can be used for the development of biomass with slow growing rate, as nitrifiers and the Anammox bacteria.

1.6 SCOPE OF THIS THESIS

As it was described in this introduction, several new technologies and improvements of conventional ones are under development in the field of wastewater treatment. Some of them are under study at laboratory scale, while others are already being applied at pilot or demonstration scale. Each specific case needs a specific solution chosen among all the available alternatives relying on the huge variability of the wastewater characteristics, the environmental and economical requirements, the advantages and disadvantages of all these technologies, the situation of the facilities, etc.

Some of the new technologies, previously cited in this introduction, can be applied to remove organic matter and/or nutrients from industrial and/or urban wastewaters and are studied in this thesis.

In the present thesis, a technology based on aerobic granular biomass will be initially applied to treat industrial wastewaters. This aerobic granular biomass is selected because it requires less area for its implantation and can be a substitute for the conventional activated sludge treatment. Furthermore, the good settling characteristics of the sludge can also permit the reduction in size or the elimination of the subsequent settling units. In addition, the reduction in the sludge production would facilitate and reduce the cost of its post-treatment. This system will be used to treat the effluent from a pig farm. The operation of this unit will be evaluated in terms of the reduction in the sludge production, the improvement of settleability, the capacity to treat high and fluctuating loads of organic matter and nitrogen, etc.

The aforementioned advantages of the aerobic granulation technology could be used to improve already constructed urban WWTPs. If the development of such granular biomass is feasible in continuous operated reactors, this technology could be applied to remove the organic matter from the urban wastewater in a more efficient way, producing lower amounts of sludge, with better settling properties. As the aerobic granular biomass allows for the accumulation of high biomass concentrations, the volume of the already constructed reactors could accomplish with the treatment of higher loads of contaminated water. Old facilities can be expanded with simple modifications, using the same area of implantation.

Regarding the nutrients removal, the source separation of human urine can reduce the needs of biological treatment to remove nitrogen and phosphorus in the WWTPs. In this way space requirements, energy consumption and sludge production can be diminished. In addition, separate urine treatment facilitates the nutrients recovery by struvite production. This mineral can be used as fertilizer due to its phosphorus rich composition. The inclusion of an air stripping system in the sludge line of the WWTPs also facilitates the nitrogen recovery and its reuse as fertilizer.

Finally, the use of Anammox based process to autotrophically remove the nitrogen from the sludge line, and in a step forward, directly from the water line of the WWTPs can contribute to important energy savings in the wastewater treatment. These processes use less oxygen, produce less sludge and do not need organic matter. In this way, more organic matter is available for its transformation in methane trough anaerobic digestion. The use of a combined two-stage biological treatment in the water line, using a high loaded first stage for carbon removal and a second stage combining partial nitrification and Anammox process for nitrogen removal, offers a way to achieve energy self-sufficiency in the WWTPs.

With all this in mind a possible strategy could be to substitute the conventional activated sludge reactor by a continuous aerobic granular reactor, where the organic matter would be removed, while the nitrogen would be removed in the subsequent partial nitrification-Anammox based system.

However, these are only some alternatives. In each particular case the most adequate treatment should be selected, taking into account the different advantages and disadvantages of the different available alternatives and the restrictions of the different regulations regarding discharge limits and so on. In conclusion, a perfect system, valid in all areas, does not yet exist, however this should be taken as an advantage because… *a perfect system does not stimulate new ideas (Foladori et al., 2010)*.

1.7 REFERENCES

Ahn, Y. H. and Choi, H. C. (2006). Autotrophic nitrogen removal from sludge digester liquids in upflow sludge bed reactor with external aeration. *Process Biochemistry* 41(9): 1945-1950.

Álvarez, J. A., Armstrong, E., Gómez, M. and Soto, M. (2008). Anaerobic treatment of low-strength municipal wastewater by a two-stage pilot plant under psychrophilic conditions. *Bioresource Technology* 99(15): 7051-7062.

Angelakis, A., De Feo, G., Laureano, P. and Zourou, A. (2013). Minoan and Etruscan Hydro-Technologies. *Water* 5(3): 972-987.

Angelakis, A. N., Koutsoyiannis, D. and Tchobanoglous, G. (2005). Urban wastewater and stormwater technologies in ancient Greece. *Water Research* 39(1): 210-220.

Annabel (2009). Overview of the Aquafin waste water treatment plant of Antwerpen-Zuid, located in the south of the agglomeration of Antwerp.

Appels, L., Houtmeyers, S., Degrève, J., Van Impe, J. and Dewil, R. (2013). Influence of microwave pre-treatment on sludge solubilization and pilot scale semi-continuous anaerobic digestion. *Bioresource Technology* 128(0): 598-603.

Ardern, E. and Lockett, W. T. (1914). Experiments on the oxidation of sewage without the aid of filters. *Journal of the Society of Chemical Industry* 33(10): 523-539.

Arrojo, B., Mosquera-Corral, A., Garrido, J. M. and Méndez, R. (2004). Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research* 38(14-15): 3389-3399.

Barat, R., Bouzas, A., Martí, N., Ferrer, J. and Seco, A. (2009). Precipitation assessment in wastewater treatment plants operated for biological nutrient removal: A case study in Murcia, Spain. *Journal of Environmental Management* 90(2): 850-857.

Barnaby, W. (2009). Do nations go to war over water? *Nature* 458(7236): 282-283.

Barnard, J. L. (1975). Biological nutrient removal without the addition of chemicals. *Water Research* 9(5–6): 485-490.

Benisch, M., Clark, C., Sprick, R. G. and R., B. (2000). Struvite deposits: a common and costly nuisance. *WEF Operation Forum*.

Beun, J. J., Hendriks, A., Van Loosdrecht, M. C. M., Morgenroth, E., Wilderer, P. A. and Heijnen, J. J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research* 33(10): 2283-2290.

Beun, J. J., Dircks, K., Van Loosdrecht, M. C. M. and Heijnen, J. J. (2002). Poly-β-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures. *Water Research* 36(5): 1167-1180.

Bhuiyan, M. I. H., Mavinic, D. S. and Koch, F. A. (2008). Phosphorus recovery from wastewater through struvite formation in fluidized bed reactors: A sustainable approach. *Water Science and Technology* 57: 175-181.

Bonmatí, A. and Flotats, X. (2003). Air stripping of ammonia from pig slurry: Characterisation and feasibility as a pre- or post-treatment to mesophilic anaerobic digestion. *Waste Management* 23(3): 261-272.

Broda, E. (1977). Two kinds of lithotrophs missing in nature. *Zeitschrift für allgemeine Mikrobiologie* 17(6): 491-493.

California Energy Commission (2005). California's Water – Energy Relationship.

Campos, J. L., Mendez, R. and Lema, J. M. (2000). Operation of a nitrifying activated sludge airlift (NASA) reactor without biomass carrier. *Water Science and Technology* 41(4-5): 113-120.

Campos, J. L., Figueroa, M., Mosquera-Corral, A. and Mendez, R. (2009). Aerobic sludge granulation: state-of-the-art. *International Journal of Environmental Engineering* 1(2): 136-151.

Campos, J. L., Vázquez-Padín, J. R., Fajardo, C., Fernández, I., Mosquera-Corral, A. and Méndez, R. (2010). Anammox based processes for nitrogen removal. In: *Innovative Technologies for Urban Wastewater Treatment Plants.* Omil, F. (ed), Lápices 4. Santiago de Compostela, Spain: 59-86.

Carlsson, M., Lagerkvist, A. and Morgan-Sagastume, F. (2012). The effects of substrate pre-treatment on anaerobic digestion systems: A review. *Waste Management* 32(9): 1634-1650.

Cassardo, C. and Jones, J. A. A. (2011). Managing Water in a Changing World. *Water* 3(2): 618-628.

Cema, G., Szatkowska, B., Plaza, E., Trela, J. and Surmacz-Gorska, J. (2006). Nitrogen removal rates at a technical-scale pilot plant with the one-stage partial nitritation/Anammox process. *Water Science and Technology* 54(8): 209-217.

Cooper, J., Lombardi, R., Boardman, D. and Carliell-Marquet, C. (2011). The future distribution and production of global phosphate rock reserves. *Resources, Conservation and Recycling* 57(0): 78-86.

Cordell, D., Drangert, J.-O. and White, S. (2009). The story of phosphorus: Global food security and food for thought. *Global Environmental Change* 19(2): 292-305.

Cordell, D., Rosemarin, A., Schröder, J. J. and Smit, A. L. (2011). Towards global phosphorus security: A systems framework for phosphorus recovery and reuse options. *Chemosphere* 84(6): 747-758 Reprinted with permission from Elsevier

Cordell, D. and White, S. (2013). Sustainable Phosphorus Measures: Strategies and Technologies for Achieving Phosphorus Security. *Agronomy* 3(1): 86-116.

Cornel, P., Wagner, M. and Krause, S. (2003). *Investigation of oxygen transfer rates in full scale membrane bioreactors*. 3rd World Water Congress of the International Water Association: Biological treatment processes. Selected proceedings, Melbourne, Australia, 7-12 April 2002., IWA Publishing House.

Cortez, S., Teixeira, P., Oliveira, R. and Mota, M. (2008). Rotating biological contactors: a review on main factors affecting performance. *Reviews in Environmental Science and Bio/Technology* 7(2): 155-172.

Crawford, G. W. (2010). *Best practices for sustainable wastewater treatment: Initial case study incorporating European experience and Evaluation Tool Concept*, WERF. Alexandria, VA, USA.

Chen, Y., Jiang, W., Liang, D. T. and Tay, J. H. (2007). Structure and stability of aerobic granules cultivated under different shear force in sequencing batch reactors. *Applied Microbiology and Biotechnology* 76(5): 1199-1208.

Daigger, G. T. (2008). New Approaches and Technologies for Wastewater Management. *The Bridge. Technologies for Clean Water* 38(3): 38-45.

Dalsgaard, T., Canfield, D. E., Petersen, J., Thamdrup, B. and Acuna-Gonzalez, J. (2003). N-2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. *Nature* 422(6932): 606-608.

Damste, J. S. S., Rijpstra, W. I. C., Geenevasen, J. A. J., Strous, M. and Jetten, M. S. M. (2005). Structural identification of ladderane and other membrane lipids of planctomycetes capable of anaerobic ammonium oxidation (anammox). *Febs Journal* 272(16): 4270-4283.

Davis, R., Aden, A. and Pienkos, P. T. (2011). Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy* 88(10): 3524-3531.

Daw, J., Hallett, K., DeWolfe, J. and Venner, I. (2012). Energy efficiency strategies for municipal wastewater treatment facilities. Golden, Colorado., National Renewable Energy Laboratory.

de Bruin, L. M. M., de Kreuk, M. K., van der Roest, H. F. R., Uijterlinde, C. and van Loosdrecht, M. C. M. (2004). Aerobic granular sludge technology: an alternative to activated sludge? *Water Science and Technology* 49(11-12): 1-7.

de Bruin, L. M. M., van der Roest, H. F. R., de Kreuk, M., van Loosdrecht, M. C. M., Bathe, S., McSwain, B. S. and Schwarzenbeck, N. (2005). Promising results pilot research aerobic granular sludge technology at WWTP Ede. In: *Aerobic Granular Sludge*. (ed), IWA Publishing. London: 135-142.

De Clippeleir, H., Yan, X., Verstraete, W. and Vlaeminck, S. (2011). OLAND is feasible to treat sewage-like nitrogen concentrations at low hydraulic residence times. *Applied Microbiology and Biotechnology* 90(4): 1537-1545.

De Feo, G., Laureano, P., Drusiani, R. and Angelakis, A. (2010). Water and wastewater management technologies through the centuries.

de Kreuk, M. K. and van Loosdrecht, M. C. M. (2004). Selection of slow growing organisms as a means for improving aerobic granular sludge stability. *Water Science and Technology* 49(11-12): 9-17.

de Kreuk, M. K., McSwain, B. S., Bathe, S., Tay, S. T. L., Schwarzenbeck, N. and Wilderer, P. A. (2005a). Discussion outcomes. In: *Aerobic Granular Sludge*. Bathe, S. *et al.*. (ed), IWA. London UK: 153–169.

de Kreuk, M. K., Heijnen, J. J. and van Loosdrecht, M. C. M. (2005b). Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. *Biotechnology and Bioengineering* 90(6): 761-769.

de Kreuk, M. K., Pronk, M. and van Loosdrecht, M. C. M. (2005c). Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. *Water Research* 39(18): 4476-4484.

De Wilde, W., Richard, M., Lesjean, B. and Tazi-Pain, A. (2008). Towards standardisation of the MBR technology? *Desalination* 231(1–3): 156-165.

Dodds, W. K., Bouska, W. W., Eitzmann, J. L., Pilger, T. J., Pitts, K. L., Riley, A. J., Schloesser, J. T. and Thornbrugh, D. J. (2009). Eutrophication of US Freshwaters: Analysis of Potential Economic Damages. *Environmental Science & Technology* 43(1): 12-19.

Dosta, J., Fernandez, I., Vazquez-Padin, J. R., Mosquera-Corral, A., Campos, J. L., Mata-Alvarez, J. and Mendez, R. (2008). Short- and long-term effects of temperature on the Anammox process. *Journal of Hazardous Materials* 154(1-3): 688-693.

Doyle, J. D. and Parsons, S. A. (2002). Struvite formation, control and recovery. *Water Research* 36(16): 3925-3940.

E.P.A., Ed. (2007). *Biological Nutrient Removal Processes and Costs*. Washington, DC, USA, United States Environmental Protection Agency.

E.P.A. (2013). "Water & Energy Efficiency in Water and Wastewater Facilities." Retrieved January 2013, from http://www.epa.gov/region9/waterinfrastructure/technology.html#area.

EAWAG (2009). How to produce fertilizer from urine: Struvite.

Edudent (2012). A large bladder stone (urolith) in a small dog, as shown in an x-ray. http://en.wikipedia.org/wiki/File:A_large_bladder_stone_(urolith)_in_a_small_dog,_as_shown_in_an_x-ray.JPEG Creative Commons Attribution-Share Alike 3.0 Unported

EEC (1991a). Council Directive 91/676/EEC, of 12 December 1991, Concerning the Protection of Waters Against Pollution Caused by Nitrates from Agricultural Sources. European Economic Community. Brussels, Belgium.

EEC (1991b). Council Directive 91/271/EEC of 21 May 1991 concerning urban waste-water treatment. European Economic Community. Brussels, Belgium.

EEC (2000). Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy.

Elliott, R. N. (2005). Roadmap to Energy in the Water and Wastewater Industry. Washington, D.C., American Council for an Energy-Efficient Economy.

Escapa, A., Gómez, X., Tartakovsky, B. and Morán, A. (2012). Estimating microbial electrolysis cell (MEC) investment costs in wastewater treatment plants: Case study. *International Journal of Hydrogen Energy* 37(24): 18641-18653.

Etter, B., Tilley, E., Khadka, R. and Udert, K. M. (2011). Low-cost struvite production using source-separated urine in Nepal. *Water Research* 45(2): 852-862.

European Environment Agency. (2012a). "Exceedance of critical loads for eutrophication due to the deposition of nutrient nitrogen in 2010" Retrieved February, 2013, from http://www.eea.europa.eu/data-and-maps/figures/exceedance-of-critical-loads-for-eutrophication-due-to-the-deposition-of-nutrient-nitrogen-in-2010.

European Environment Agency. (2012b). "Phosphorus concentration in European lakes and reservoirs." Retrieved February, 2013, from http://www.eea.europa.eu/data-and-maps/figures/phosphorus-concentration-in-european-lakes-and-reservoirs.

Fabbricino, M., Naviglio, B., Tortora, G. and d'Antonio, L. (2013). An environmental friendly cycle for Cr(III) removal and recovery from tannery wastewater. *Journal of Environmental Management* 117(0): 1-6.

Fattah, K. P., Sabrina, N., Mavinic, D. S. and Koch, F. A. (2008). Reducing operating costs for struvite formation with a carbon dioxide stripper. *Water Science and Technology* 58: 957-962.

Fenu, A., Roels, J., Wambecq, T., De Gussem, K., Thoeye, C., De Gueldre, G. and Van De Steene, B. (2010). Energy audit of a full scale MBR system. *Desalination* 262(1–3): 121-128.

Fernandez-Fontaina, E., Omil, F., Lema, J. M. and Carballa, M. (2012). Influence of nitrifying conditions on the biodegradation and sorption of emerging micropollutants. *Water Research* 46(16): 5434-5444.

Fernandez, I., Vazquez-Padin, J. R., Mosquera-Corral, A., Campos, J. L. and Mendez, R. (2008). Biofilm and granular systems to improve Anammox biomass retention. *Biochemical Engineering Journal* 42(3): 308-313.

Ferriman, A. (2007). BMJ readers choose the "sanitary revolution" as greatest medical advance since 1840. *BMJ* 334(7585): 111-111.

Figueroa, M., Mosquera-Corral, A., Campos, J. L. and Mendez, R. (2008). Treatment of saline wastewater in SBR aerobic granular reactors. *Water Science and Technology* 58(2): 479-485.

Figueroa, M., Vázquez-Padín, J. R., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2012). Is the CANON reactor an alternative for nitrogen removal from pre-treated swine slurry? *Biochemical Engineering Journal* 65(0): 23-29.

Fine, P. and Hadas, E. (2012). Options to reduce greenhouse gas emissions during wastewater treatment for agricultural use. *Science of The Total Environment* 416(0): 289-299.

Foladori, P., Andreottola, G. and Ziglio, G. (2010). *Sludge Reduction Technologies in Wastewater Treatment Plants*, IWA Publishing.

Francis, C. A., Beman, J. M. and Kuypers, M. M. M. (2007). New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. *ISME J* 1(1): 19-27.

Frijns, J., Hofman, J. and Nederlof, M. (2013). The potential of (waste)water as energy carrier. *Energy Conversion and Management* 65(0): 357-363.

Furukawa, K., Lieu, P. K., Tokitoh, H. and Fujii, T. (2006). Development of single-stage nitrogen removal using anammox and partial nitritation (SNAP) and its treatment performances. *Water Science and Technology* 53(6): 83-90.

Gander, M., Jefferson, B. and Judd, S. (2000). Aerobic MBRs for domestic wastewater treatment: a review with cost considerations. *Separation and Purification Technology* 18(2): 119-130.

Garrido, J. M., Fdz-Polanco, M. and Fdz-Polanco, F. (2013). Working with energy and mass balances: a conceptual framework to understand the limits of municipal wastewater treatment. *Water science and technology* 67(10): 2294-2301.

Geoscience Australia (2013). Long-term phosphate prices since 1960 for phosphate rock (Morocco), 70% BPL, contract, f.a.s. Casablanca. http://www.australianminesatlas.gov.au/aimr/images/phosphate-fig2.jpg Creative Commons Attribution 3.0 Australia

Ghaebi, H., Bahadori, M. N. and Saidi, M. H. (2014). Performance analysis and parametric study of thermal energy storage in an aquifer coupled with a heat pump and solar collectors, for a residential complex in Tehran, Iran. *Applied Thermal Engineering* 62(1): 156-170. Reprinted with permission from Elsevier.

Gil, J. A., Túa, L., Rueda, A., Montaño, B., Rodríguez, M. and Prats, D. (2010). Monitoring and analysis of the energy cost of an MBR. *Desalination* 250(3): 997-1001.

Glass, A. D. M. (2003). Nitrogen use efficiency of crop plants: Physiological constraints upon nitrogen absorption. *Critical Reviews in Plant Sciences* 22(5): 453-470.

Gleick, P. H. (2009). "Water Conflict Chronology." Retrieved November 2012, from http://www.worldwater.org/conflict/index.html.

Haegh, M., Sj, lin, C., Dahl, C. P. and Hansen, A. T. (2010). Small Footprint Wastewater Treatment Plants. The Combination of Moving Bed Biofilm Reactors with Ballasted Floc Solids Separation. *Proceedings of the Water Environment Federation* 2010(7): 270-283.

HeadworksBIO Advantages & Applications of MBBR Technologies.

Heffer, P. and Prud'homme, M. (2010). *Fertilizer Outlook 2010 - 2014*. 78th IFA Annual Conference, Paris, May - June 2010.

Heidrich, E. S., Curtis, T. P. and Dolfing, J. (2010). Determination of the Internal Chemical Energy of Wastewater. *Environmental Science and Technology* 45(2): 827-832.

Heijnen, S. J., Mulder, A., Weltevrede, R., Hols, P. H. and van Leeuwen, H. L. J. M. (1990). Large-scale anaerobic/aerobic treatment of complex industrial wastewater using immobilized biomass in fluidized bed and air-lift suspension reactors. *Chemical Engineering & Technology* 13(1): 202-208.

Hellinga, C., Schellen, A. A. J. C., Mulder, J. W., van Loosdrecht, M. C. M. and Heijnen, J. J. (1998). The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water. *Water Science and Technology* 37(9): 135-142.

Hendrickx, T. L. G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H. and Buisman, C. J. N. (2012). Autotrophic nitrogen removal from low strength waste water at low temperature. *Water Research* 46(7): 2187-2193.

Henze, M., Van Loosdrecht, M. C. M. and Ekama, G. A. (2008). *Biological Wastewater Treatment: Principles, Modelling and Design*, IWA Pub.

Hernández-Sancho, F., Molinos-Senante, M. and Sala-Garrido, R. (2011). Energy efficiency in Spanish wastewater treatment plants: A non-radial DEA approach. *Science of the Total Environment* 409(14): 2693-2699.

Hofman, J., Hofman-Caris, R., Nederlof, M., Frijns, J. and van Loosdrecht, M. C. (2011). Water and energy as inseparable twins for sustainable solutions. *Water Science and Technology* 63(1): 88-92.

Höglund, C. (2001). Evaluation of microbial health risks associated with the reuse of source separated human urine. PhD thesis, Royal Institute of Technology.

Honda, S. i., Miyata, N. and Iwahori, K. (2002). Recovery of biomass cellulose from waste sewage sludge. *Journal of Material Cycles and Waste Management* 4(1): 46-50.

Huang, J. C. and Shang, C. (2004). Air Stripping. In: *Handbook of Environmental Engineering.* Wang, L. K. *et al..* (ed), The Humana Press Inc., Totowa, NJ. New Jersey Volume 4: Advanced Physicochemical Treatment Processes: Disclaimer: "Springer and the original publisher is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media".

Hülsen, T., Batstone, D. J. and Keller, J. (2014). Phototrophic bacteria for nutrient recovery from domestic wastewater. *Water Research* 50(0): 18-26.

Iacovidou, E., Ohandja, D.-G. and Voulvoulis, N. (2012). Food waste co-digestion with sewage sludge – Realising its potential in the UK. *Journal of Environmental Management* 112(0): 267-274.

Idelovitch, E. and Michail, M. (1981). Nitrogen Removal by Free Ammonia Stripping from High pH Ponds. *Journal (Water Pollution Control Federation)* 53(9): 1391-1401.

IFA. (2006). "Production and International Trade Statistics Available online: http://www.fertilizer.org/ifa/HomePage/STATISTICS/Production-and-trade." International Fertilizer Industry Association Retrieved 21 August 2013.

Inizan, M., Freval, A., Cigana, J. and Meinhold, J. (2005). Aerobic granulation in a sequencing batch reactor (SBR) for industrial wastewater treatment. *Water Science and Technology* 52(10-11): 335-343.

Jetten, M. S. M., Wagner, M., Fuerst, J., van Loosdrecht, M., Kuenen, G. and Strous, M. (2001). Microbiology and application of the anaerobic ammonium oxidation ('anammox') process. *Current Opinion in Biotechnology* 12(3): 283-288.

Jiang, F., Beck, M. B., Cummings, R. G., Rowles, K. and Russell, D. (2004). Estimation of costs of phosphorus removal in wastewater treatment facilities. *Water Policy Working Paper*.

Jönsson, H. (2002). Urine separation – Swedish experiences. In: *Urban areas – rural areas and recycling – the organic way forward,* . Magid, J. *et al..* (ed), NJF (Nordic Association of Agricultural Scientists). Tjele.

Jungles, M. K., Figueroa, M., Morales, N., Val del Río, A., da Costa, R. H. R., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Start up of a pilot scale aerobic granular reactor for organic matter and nitrogen removal. *Journal of Chemical Technology and Biotechnology* 86(5): 763-768.

Kartal, B., Rattray, J., van Niftrik, L. A., van de Vossenberg, J., Schmid, M. C., Webb, R. I., Schouten, S., Fuerst, J. A., Damsté, J. S., Jetten, M. S. M. and Strous, M. (2007). Candidatus "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Systematic and Applied Microbiology* 30(1): 39-49.

Kartal, B., van Niftrik, L., Rattray, J., de Vossenberg, J. L. C. M. V., Schmid, M. C., Damste, J. S. S., Jetten, M. S. M. and Strous, M. (2008). Candidatus 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. *Fems Microbiology Ecology* 63(1): 46-55.

Kartal, B., Maalcke, W. J., de Almeida, N. M., Cirpus, I., Gloerich, J., Geerts, W., Op den Camp, H. J. M., Harhangi, H. R., Janssen-Megens, E. M., Francoijs, K.-J., Stunnenberg, H. G., Keltjens, J. T., Jetten, M. S. M. and Strous, M. (2011). Molecular mechanism of anaerobic ammonium oxidation. *Nature* 479(7371): 127-130.

Kirchmann, H. and Pettersson, S. (1995). Human urine - Chemical composition and fertilizer use efficiency. *Fertilizer Research* 40(2): 149-154.

Koh, L. C., Zhang, H., Nguyen, T. T. and Ong, S. (2012). Performance of an ultra-compact biofilm reactor treating domestic and synthetic wastewaters. *Water Science and Technology* 65(3): 6.

Koutsoyiannis, D., Zarkadoulas, N., Angelakis, A. and Tchobanoglous, G. (2008). Urban water management in ancient Greece: legacies and lessons. *Journal of Water resources planning and management* 134(1): 45-54.

Kozlovský, O., Balík, J., Černý, J., Kulhánek, M., Kos, M. and Prášilová, M. (2009). Influence of nitrogen fertilizer injection (CULTAN) on yield, yield components formation and quality of winter wheat grain. *Plant, Soil and Environment* 55(12): 536-543.

Krzeminski, P., Langhorst, W., Schyns, P., de Vente, D., Van den Broeck, R., Smets, I. Y., Van Impe, J. F. M., van der Graaf, J. H. J. M. and van Lier, J. B. (2012). The optimal MBR configuration: Hybrid versus stand-alone — Comparison between three full-scale MBRs treating municipal wastewater. *Desalination* 284(0): 341-348.

Kuypers, M. M. M., Sliekers, A. O., Lavik, G., Schmid, M., Jorgensen, B. B., Kuenen, J. G., Damste, J. S. S., Strous, M. and Jetten, M. S. M. (2003). Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* 422(6932): 608-611.

Larsen, T. A. and Gujer, W. (1996). Separate management of anthropogenic nutrient solutions (human urine). *Water Science and Technology*. Singapore, Singapore. 34: 87-94.

Lazarova, V., Choo, K. H. and Cornel, P. (2012). *Water-Energy Interactions in Water Reuse*, IWA Publishing.

Lee, K. (2013). Aquifer Thermal Energy Storage. In: *Underground Thermal Energy Storage*. (ed), Springer London. 59-93.

Lei, X., Sugiura, N., Feng, C. and Maekawa, T. (2007). Pretreatment of anaerobic digestion effluent with ammonia stripping and biogas purification. *Journal of Hazardous Materials* 145(3): 391-397.

Li, C., Champagne, P. and Anderson, B. C. (2011). Evaluating and modeling biogas production from municipal fat, oil, and grease and synthetic kitchen waste in anaerobic co-digestions. *Bioresource Technology* 102(20): 9471-9480.

Libralato, G., Volpi Ghirardini, A. and Avezzù, F. (2012). To centralise or to decentralise: An overview of the most recent trends in wastewater treatment management. *Journal of Environmental Management* 94(1): 61-68.

Lienert, J., Haller, M., Berner, A., Stauffacher, M. and Larsen, T. A. (2003). How farmers in Switzerland perceive fertilizers from recycled anthropogenic nutrients (urine). *Water Science and Technology*. 48: 47-56.

Lienert, J. and Larsen, T. A. (2006). Considering user attitude in early development of environmentally friendly technology: A case study of NoMix toilets. *Environmental Science and Technology* 40(16): 4838-4844.

Liu, D., Wu, X., Chang, J., Gu, B., Min, Y., Ge, Y., Shi, Y., Xue, H., Peng, C. and Wu, J. (2012a). Constructed wetlands as biofuel production systems. *Nature Clim. Change* 2(3): 190-194.

Liu, H., Li, Y., Yang, C., Pu, W., He, L. and Bo, F. (2012b). Stable aerobic granules in continuous-flow bioreactor with self-forming dynamic membrane. *Bioresource Technology* 121(0): 111-118. Image reprinted with permission from Elsevier

Liu, Q. S., Liu, Y., Tay, S. T. L., Show, K. Y., Ivanov, V., Benjamin, M. and Tay, J. H. (2005). Startup of pilot-scale aerobic granular sludge reactor by stored granules. *Environmental Technology* 26(12): 1363-1369.

Liu, Y. and Tay, J. H. (2002). The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. *Water Research* 36(7): 1653-1665.

Lofrano, G. and Brown, J. (2010). Wastewater management through the ages: A history of mankind. *Science of the Total Environment* 408(22): 5254-5264.

Ludzack, F. J. and Ettinger, M. B. (1962). Controlling Operation to Minimize Activated Sludge Effluent Nitrogen. *Journal Water Pollution Control Federation* 34(9): 920-931.

M.E.A. (2005). *(Millennium Ecosystem Assessment) Ecosystems and human well-being: wetlands and water synthesis*, World Resources Institute. Washington, DC

Ma, B., Zhang, S., Zhang, L., Yi, P., Wang, J., Wang, S. and Peng, Y. (2011). The feasibility of using a two-stage autotrophic nitrogen removal process to treat sewage. *Bioresource Technology* 102(17): 8331-8334.

Manipura, A. and Burgess, J. E. (2008). Biological nitrogenous compound removal and subsequent rhodium recovery from simulated metal refinery wastewater. *Minerals Engineering* 21(9): 652-659.

Marañón, E., Castrillón, L., Quiroga, G., Fernández-Nava, Y., Gómez, L. and García, M. M. (2012). Co-digestion of cattle manure with food waste and sludge to increase biogas production. *Waste Management* 32(10): 1821-1825.

Matsumoto, K. and Funaba, M. (2008). Factors affecting struvite ($MgNH_4PO_4 \cdot 6H_2O$) crystallization in feline urine. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1780(2): 233-239.

Maurer, M., Muncke, J. and Larsen, T. (2002). Techniques for nitrogen recovery and reuse. . In: *Water and Resource Recovery in Industry*. Lens, P. *et al.*. (ed), IWA Publishing. London, UK.

Maurer, M., Pronk, W. and Larsen, T. A. (2006). Treatment processes for source-separated urine. *Water Research* 40(17): 3151-3166.

Mbeychok (2007). Rotating_Biological_Contactor. http://commons.wikimedia.org/wiki/File:Rotating_Biological_Contactor.png, Public Domain

Mbeychok (2011). Trickle_Filter. http://en.wikipedia.org/wiki/File:Trickle_Filter.svg, Public Domain

McQuarrie, J., Rutt, K., Seda, J. and Haegh, M. (2004). Observations from the First Year of Full-scale Operation - The IFAS/BNR Process at the Broomfield Wastewater Reclamation Facility, Broomfield, CO. *Proceedings of the Water Environment Federation* 2004(7): 274-285.

McSwain, B. S., Irvine, R. L. and Wilderer, P. A. (2004). The influence of settling time on the formation of aerobic granules. *Water Science and Technology* 50 (10): 195-202.

Metcalf & Eddy, I., Tchobanoglous, G., Burton, F. and Stensel, H. D. (2002). *Wastewater Engineering: Treatment and Reuse*, McGraw-Hill Companies, Incorporated.

Mishima, K. and Nakamura, K. (1991). Self-immobilization of aerobic activated sludge - a pilot study of the aerobic upflow sludge blanket process in municipal sewage treatment. *Water science and technology* 23(4-6): 981-990.

Molinos-Senante, M., Hernández-Sancho, F. and Sala-Garrido, R. (2011). Cost–benefit analysis of water-reuse projects for environmental purposes: A case study for Spanish wastewater treatment plants. *Journal of Environmental Management* 92(12): 3091-3097.

Montana State University Center for Biofilm Engineering. (2003). "Biofilm formation in 3 steps." Retrieved January 2013, 2013, from http://www.biofilm.montana.edu/node/2390 Reproduced with authorization from Center for Biofilm Engineering.

Moralejo-Gárate, H., Mar'atusalihat, E., Kleerebezem, R. and Loosdrecht, M. M. (2011). Microbial community engineering for biopolymer production from glycerol. *Applied Microbiology and Biotechnology* 92(3): 631-639.

Morales, N., Val del Río, A., Vázquez-Padín, J. R., Méndez, R., Mosquera-Corral, A. and Campos, J. L. (2012). Application of the Anammox process to the main stream of WWTPs. *9th Edition Int. Symposium of Sanitary and Environmental Engineering (Sidisa2012)*. Milano (Italy).

Morse, G. K., Lester, J. N. and Perry, R. (1993). The Economic and Environment Impact of Phosphorus Removal from Wasetwater in the European Community. Selper Publications. London.

Mosquera-Corral, A., Figueroa, M., Morales, N., Val del Río, Á., Campos, J. L. and Méndez, R. (2012). Aerobic granulation technology In: *Innovative Technologies for Urban Wastewater Treatment Plants (2nd Edition)* Omil, F. *et al.*. (ed), Lápices 4, . Santiago de Compostela.

Moy, B. Y. P., Tay, J. H., Toh, S. K., Liu, Y. and Tay, S. T. L. (2002). High organic loading influences the physical characteristics of aerobic sludge granules. *Letters in Applied Microbiology* 34(6): 407-412.

Mulder, A., Vandegraaf, A. A., Robertson, L. A. and Kuenen, J. G. (1995). Anaerobic Ammonium Oxidation Discovered in a Denitrifying Fluidized-Bed Reactor. *Fems Microbiology Ecology* 16(3): 177-183.

NACWA (2009). (National Association of Clean Water Agencies), Renewable Energy Recovery Opportunities from Domestic Wastewater.

Ni, B. J., Xie, W. M., Liu, S. G., Yu, H. Q., Wang, Y. Z., Wang, G. and Dai, X. L. (2009). Granulation of activated sludge in a pilot-scale sequencing batch reactor for the treatment of low-strength municipal wastewater. *Water Research* 43(3): 751-761.

Nielsen, M., Bollmann, A., Sliekers, O., Jetten, M., Schmid, M., Strous, M., Schmidt, I., Larsen, L. H., Nielsen, L. P. and Revsbech, N. P. (2005). Kinetics, diffusional limitation and microscale distribution of chemistry and organisms in a CANON reactor. *Fems Microbiology Ecology* 51(2): 247-256.

Odegaard, H. (2006). Innovations in wastewater treatment: the moving bed biofilm process. *Water Science and Technology* 53(9): 17-33.

Oh, S. T., Kim, J. R., Premier, G. C., Lee, T. H., Kim, C. and Sloan, W. T. (2010). Sustainable wastewater treatment: How might microbial fuel cells contribute. *Biotechnology Advances* 28(6): 871-881.

Olguín, E. J. (2012). Dual purpose microalgae–bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a Biorefinery. *Biotechnology Advances* 30(5): 1031-1046.

Patwardhan, A. W. (2003). Rotating Biological Contactors: A Review. *Industrial & Engineering Chemistry Research* 42(10): 2035-2051.

Paul, E. and Liu, Y. (2012). *Biological Sludge Minimization and Biomaterials/Bioenergy Recovery Technologies*, Wiley.

Pennisi, E. (2012). Water Reclamation Going Green. *Science* 337(6095): 674-676.

Petrov, S. and Nenov, V. (2004). Removal and recovery of copper from wastewater by a complexation-ultrafiltration process. *Desalination* 162(0): 201-209.

Préndez, M. and Lara-González, S. (2008). Application of strategies for sanitation management in wastewater treatment plants in order to control/reduce greenhouse gas emissions. *Journal of Environmental Management* 88(4): 658-664.

Pruvost, J., Van Vooren, G., Le Gouic, B., Couzinet-Mossion, A. and Legrand, J. (2011). Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. *Bioresource Technology* 102(1): 150-158.

Pynaert, K., Smets, B. F., Wyffels, S., Beheydt, D., Siciliano, S. D. and Verstraete, W. (2003). Characterization of an autotrophic nitrogen-removing biofilm from a highly loaded lab-scale rotating biological contactor. *Applied and Environmental Microbiology* 69(6): 3626-3635.

Qiao, S., Matsumoto, N., Shinohara, T., Nishiyama, T., Fujii, T., Bhatti, Z. and Furukawa, K. (2010). High-rate partial nitrification performance of high ammonium containing wastewater under low temperatures. *Bioresource Technology* 101(1): 111-117.

Qin, L., Liu, Y. and Tay, J. H. (2004). Effect of settling time on aerobic granulation in sequencing batch reactor. *Biochemical Engineering Journal* 21(1): 47-52.

Rahaman, M. M. (2012). Water wars in 21st century: speculation or reality? *International Journal of Sustainable Society* 4(1): 3-10.

Regueiro, L., Carballa, M., Álvarez, J. A. and Lema, J. M. (2012). Enhanced methane production from pig manure anaerobic digestion using fish and biodiesel wastes as co-substrates. *Bioresource Technology* 123(0): 507-513.

Rekacewicz, P. (2006). Water Scarcity Index

Rijsberman, F. R. (2006). Water scarcity: Fact or fiction? *Agricultural Water Management* 80(1–3): 5-22.

Roberts, T. L. and Stewart, W. M. (2002). Inorganic Phosphorus and Potassium Production and Reserves. *Better Crops* 86(2): 6-7.

Rodríguez-García, G., Hospido, A., Bagley, D. M., Moreira, M. T. and Feijoo, G. (2012). A methodology to estimate greenhouse gases emissions in Life Cycle Inventories of wastewater treatment plants. *Environmental Impact Assessment Review* 37(0): 37-46.

Roest, K., Hofman, J. and Loosdrecht, M. C. M. (2010). The Dutch water cycle can produce energy. *H2O* 43(25/26): 47-51.

Roger Bayley Inc. (2009). Chapter 5, Energy. In: *The Challenge Series. The 2010 Winter Olympics: The Southeast False Creek Olympic Village*. (ed). Vancouver, Canada: Licensed under a Creative Commons Attribution 2.5 Canada License.

Rousseau, P., Steyer, J. P., Volcke, E. I. P., Bernet, N. and Béline, F. (2008). Combined anaerobic digestion and biological nitrogen removal for piggery wastewater treatment: a modelling approach. *Water Science and Technology* 58 (1): 133-141.

Sammut, F., Rogalla, F., Goncalves, R. F. and Penillard, P. (1994). Practical experiences with removing nitrogen and phosphorus on aerated biofilters. In: *Nutrient Removal from Wastewaters.* Horan, N. J. *et al..* (ed). Basel, Switzerland: 335–345.

Santos, V. A. P. M., Bruijnse, M., Tramper, J. and Wijffels, R. H. (1996). The magic-bead concept: an integrated approach to nitrogen removal with co-immobilized micro-organisms. *Applied Microbiology and Biotechnology* 45(4): 447-453.

Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J. W., Schleifer, K. H. and Wagner, M. (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Systematic and Applied Microbiology* 23(1): 93-106.

Schmid, M., Walsh, K., Webb, R., Rijpstra, W. I. C., van de Pas-Schoonen, K., Verbruggen, M. J., Hill, T., Moffett, B., Fuerst, J., Schouten, S., Damste, J. S. S., Harris, J., Shaw, P., Jetten, M. and Strous, M. (2003). Candidatus "Scalindua brodae", sp nov., Candidatus "Scalindua wagneri", sp nov., two new species of anaerobic ammonium oxidizing bacteria. *Systematic and Applied Microbiology* 26(4): 529-538.

Schwarzenbeck, N., Erley, R. and Wilderer, P. A. (2004). Aerobic granular sludge in an SBR-system treating wastewater rich in particulate matter. *Water Science and Technology* 49(11-12): 41-46.

Selman, M. and Greenhalgh, S. (2009). Eutrophication: Sources and Drivers of Nutrient Pollution. *World Resources Institute Policy Note*

SenterNovem (2006). Watergebruik in Woningen en Warmteterugwinning uit Huishoudelijk Afvalwater (water use in houses and heat recovery from domestic wastewater). Utrecht, Report of TU Delft and Cauberg Huygen.

Sharma, K. and Huang, J. (2004). High rate treatment by aerobic upflow sludge blanket (AUSB) with external oxygenation. *Water Science & Technology* 50(8): 193-200.

Shiklomanov, I. (1993). World fresh water resources. In: *Water in Crisis: A Guide to the World's Fresh Water Resources*. Gleick, P. H. *et al..* (ed), Oxford University Press.

Siegrist, H., Salzgeber, D., Eugster, J. and Joss, A. (2008). Anammox brings WWTP closer to energy autarky due to increased biogas production and reduced aeration energy for N-removal. *Water Science and Technology* 57(3): 383-388.

Sinha, B. and Annachhatre, A. (2007). Partial nitrification: operational parameters and microorganisms involved. *Reviews in Environmental Science and Biotechnology* 6(4): 285-313.

Sliekers, A. O., Derwort, N., Campos, J. L., Strous, M., Kuenen, J. G. and Jetten, M. S. M. (2002). Completely autotrophic nitrogen removal over nitrite in one single reactor. *Water Research* 36(10): 2475-2482.

Smith, V. H. and Schindler, D. W. (2009). Eutrophication science: where do we go from here? *Trends in Ecology & Evolution* 24(4): 201-207.

Sommer, K., Derivative work: chris and derivative work: Fornax (2010). Funktionsskizze eines Spornrades für die CULTAN-Düngung nach Prof. Karl Sommer. http://commons.wikimedia.org/wiki/File:CULTAN-D%C3%BCngung_Spornrad.svg Creative Commons Attribution-Share Alike 3.0 Unported

Steen, I. and Agro, K. (1998). Phosphorus availability in the 21st century: Management of a non-renewable resource. *Phosphorus and Potassium*(217): 25–31.

Stratful, I., Scrimshaw, M. D. and Lester, J. N. (2004). Removal of struvite to prevent problems associated with its accumulation in wastewater treatment works. *Water Environment Research* 76(5): 437-443.

Strous, M., Van Gerven, E., Zheng, P., Kuenen, J. G. and Jetten, M. S. M. (1997). Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (Anammox) process in different reactor configurations. *Water Research* 31(8): 1955-1962.

Strous, M., Heijnen, J. J., Kuenen, J. G. and Jetten, M. S. M. (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Applied Microbiology and Biotechnology* 50(5): 589-596.

Strous, M., Fuerst, J. A., Kramer, E. H. M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K. T., Webb, R., Kuenen, J. G. and Jetten, M. S. M. (1999a). Missing lithotroph identified as new planctomycete. *Nature* 400(6743): 446-449.

Strous, M., Kuenen, J. G. and Jetten, M. S. M. (1999b). Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology* 65(7): 3248-3250.

Strous, M., Kuenen, J. G., Fuerst, J. A., Wagner, M. and Jetten, M. S. M. (2002). The anammox case - A new experimental manifesto for microbiological eco-physiology. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 81(1-4): 693-702.

Suriyachan, C., Nitivattananon, V. and Amin, A. T. M. N. (2012). Potential of decentralized wastewater management for urban development: Case of Bangkok. *Habitat International* 36(1): 85-92.

Sustainable Sanitation (2013). Instruction poster for urine-diverting dry toilet (UDDT) in low-income area Bulbul near Nairobi, Kenya. http://www.flickr.com/photos/gtzecosan/10543355195/sizes/o/in/set-72157637061451965/ Creative Commons Attribution 2.0 Generic

Tay, J. H., Liu, Q. S. and Liu, Y. (2001a). The role of cellular polysaccharides in the formation and stability of aerobic granules. *Letters in Applied Microbiology* 33: 222-226.

Tay, J. H., Liu, Q. S. and Liu, Y. (2001b). The effects of shear force on the formation, structure and metabolism of aerobic granules. *Applied Microbiology and Biotechnology* 57(1-2): 227-233.

Tay, J. H., Ivanov, V., Pan, S. and Tay, S. T. L. (2002). Specific layers in aerobically grown microbial granules. *Letters in Applied Microbiology* 34(4): 254-257.

Tay, J. H., Liu, Q. S., Liu, Y., Show, K., Ivanov, V. and Tay, S. T. L. (2005a). A comparative study of aerobic granulation in pilot- and laboratory-scale SBRs. In: *Aerobic Granular Sludge.* (ed), IWA Publishing. Munich: 125-133.

Tay, S. T. L., Moy, B. Y. P., Jiang, H. L. and Tay, J. H. (2005b). Rapid cultivation of stable aerobic phenol-degrading granules using acetate-fed granules as microbial seed. *Journal of Biotechnology* 115(4): 387-395.

Tchobanoglous, G. and Leverenz, H. (2009). Impacts of New Concepts and Technology on the Energy Sustainability of Wastewater Management. *Climate Change, Sustainable Development and Renewable Resources in Greece*. Thessaloniki, Greece.

Tchobanoglous, G. (2012). *Wastewater treatment trends in the 21st century*. The 3rd International Sede Boqer Conference on Water Technologies 2012 Advanced Technologies in Water Management, Ben-Gurion University of the Negev, Israel.

Thamdrup, B. and Dalsgaard, T. (2002). Production of N-2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Applied and Environmental Microbiology* 68(3): 1312-1318.

The USGS Water Science School (2013). The World's Water. http://ga.water.usgs.gov/edu/earthwherewater.html, Public Domain

Tijhuis, L., Hijman, B., Loosdrecht, M. C. M. and Heijnen, J. J. (1996). Influence of detachment, substrate loading and reactor scale on the formation of biofilms in airlift reactors. *Applied Microbiology and Biotechnology* 45(1-2): 7-17.

Trigo, C., Campos, J. L., Garrido, J. M. and Méndez, R. (2006). Start-up of the Anammox process in a membrane bioreactor. *Journal of Biotechnology* 126(4): 475-487.

Trubetskoy, S. (2012). Eutrophication at a waste water outlet in the Potomac River, Washington, D.C. http://commons.wikimedia.org/wiki/File:Potomac_green_water.JPG Creative Commons Attribution-Share Alike 3.0 Unported

Tsuneda, S., Nagano, T., Hoshino, T., Ejiri, Y., Noda, N. and Hirata, A. (2003). Characterization of nitrifying granules produced in an aerobic upflow fluidized bed reactor. *Water Research* 37(20): 4965-4973. Image reprinted with permission from Elsevier

U.N. (2009). World Population Prospects: The 2008 Revision. United Nations Department of Economic and Social Affairs, Population Division. *Population Newsletter* 87: 1-3.

U.S. Department of Energy (2013). Diagram of a drain water heat recovery system. http://energy.gov/sites/prod/files/styles/borealis_article_hero_respondlarge/public/drainwater_heat_recover.gif, Public Domain

U.S. EPA. (2008). Chapter 4. Land. In: *EPA's 2008 Report on the Environment (Final Report).* (ed), U.S. Environmental Protection Agency, Washington, D.C. USA. Public Domain.

U.S. Geological Survey. (2012). "USGS Minerals Information: Mineral Commodity Summaries." Retrieved February, 2013, from http://www.minerals.usgs.gov/minerals/pubs/mcs/.

Udert, K. M., Larsen, T. A., Biebow, M. and Gujer, W. (2003). Urea hydrolysis and precipitation dynamics in a urine-collecting system. *Water Research* 37(11): 2571-2582.

Udert, K. M., Larsen, T. A. and Gujer, W. (2006). Fate of major compounds in source-separated urine. *Water Science and Technology* 54: 413-420.

Ueno, Y. and Fujii, M. (2001). Three years experience of operating and selling recovered struvite from full-scale plant. *Environmental Technology* 22(11): 1373-1381.

UNEP (2011). *Year book 2011: Emerging issues in our global environment*, United Nations Environment Programme.

United Nations World Water Assessment Programme , UN-Water and UNESCO (2009). *Water in a changing world*, UNESCO Publishing. Paris

Val del Río, A., Morales, N., Isanta, E., Mosquera-Corral, A., Campos, J. L., Steyer, J. P. and Carrère, H. (2011). Thermal pre-treatment of aerobic granular sludge: Impact on anaerobic biodegradability. *Water Research* 45(18): 6011-6020.

van der Star, W., Miclea, A. I., van Dongen, U., Muyzer, G., Picioreanu, C. and van Loosdrecht, M. C. M. (2008). The membrane bioreactor: A novel tool to grow anammox bacteria as free cells. *Biotechnology and Bioengineering*.

van der Star, W. R. L., Abma, W. R., Blommers, D., Mulder, J.-W., Tokutomi, T., Strous, M., Picioreanu, C. and van Loosdrecht, M. C. M. (2007). Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. *Water Research* 41(18): 4149-4163.

van Dongen, U., Jetten, M. S. M. and van Loosdrecht, M. C. M. (2001). The SHARON-Anammox process for treatment of ammonium rich wastewater. *Water Science and Technology* 44(1): 153-160.

van Kempen, R., Mulder, J. W., Uijterlinde, C. A. and Loosdrecht, M. C. M. (2001). Overview: full scale experience of the SHARON process for treatment of rejection water of digested sludge dewatering. *Water Science and Technology* 44(1): 145-152.

van Loosdrecht, M. C. M., Eikelboom, D., Gjaltema, A., Mulder, A., Tijhuis, L. and Heijnen, J. J. (1995). Biofilm structures. *Water Science and Technology* 32(8): 35-43.

van Loosdrecht, M. C. M., Hao, X., Jetten, M. S. M. and Abma, W. (2004). Use of Anammox in urban wastewater treatment. *Water Science and Technology: Water Supply* 4(1): 8.

van Niftrik, L. and Jetten, M. S. M. (2012). Anaerobic Ammonium-Oxidizing Bacteria: Unique Microorganisms with Exceptional Properties. *Microbiology and Molecular Biology Reviews* 76(3): 585-596.

Vázquez-Padín, J. R., Fernández, I., Figueroa, M., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2009a). Applications of Anammox based processes to treat anaerobic digester supernatant at room temperature. *Bioresource Technology* 100(12): 2988-2994.

Vázquez-Padín, J. R., Figueroa, M., Fernández, I., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2009b). Post-treatment of effluents from anaerobic digesters by the Anammox process. *Water science and technology* 60(5): 1135-1143.

Vázquez-Padín, J. R., Pozo, M. J., Jarpa, M., Figueroa, M., Franco, A., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2009c). Treatment of anaerobic sludge digester effluents by the CANON process in an air pulsing SBR. *Journal of Hazardous Materials* 166(1): 336-341.

Vázquez-Padín, J. R., Figueroa, M., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2010a). Nitrifying granular systems: A suitable technology to obtain stable partial nitrification at room temperature. *Separation and Purification Technology* 74(2): 178-186.

Vázquez-Padín, J. R., Mosquera-Corral, A., Campos, J. L., Méndez, R. and Revsbech, N. P. (2010b). Microbial community distribution and activity dynamics of granular biomass in a CANON reactor. *Water Research* 44(15): 4359-4370.

Vázquez-Padín, J. R., Fernández, I., Morales, N., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Autotrophic nitrogen removal at low temperature. *Water science and technology* 63(6): 1282-1288.

Vázquez-Padín, J. R. (2013). Personal Communication.

Velela (2005). Trickling filter bed 2 w. http://commons.wikimedia.org/wiki/File:Trickling_filter_bed_2_w.JPG Creative Commons Attribution-Share Alike 3.0 Unported

Verawaty, M., Tait, S., Pijuan, M., Yuan, Z. and Bond, P. L. (2013). Breakage and growth towards a stable aerobic granule size during the treatment of wastewater. *Water Research* 47(14): 5338-5349. Image reprinted with permission from Elsevier.

Verrecht, B., Maere, T., Nopens, I., Brepols, C. and Judd, S. (2010). The cost of a large-scale hollow fibre MBR. *Water Research* 44(18): 5274-5283.

Vråle, L. and Jenssen, P. D. (2005). Source separated wastewater a new resource for producing mineral fertilizer. *3rd. International Conferenc on Ecological Sanitation*. Durban.

Wang, J., Burken, J. G., Zhang, X. and Surampalli, R. (2005). Engineered struvite precipitation: Impacts of component-ion molar ratios and pH. *Journal of Environmental Engineering* 131(10): 1433-1440.

Wang, L. P., Wong, H. Y. and Griffith, D. P. (1997). Treatment options in struvite stones. *Urologic Clinics of North America* 24(1): 149-162.

Webb, K. M. and Ho, G. E. (1992). Struvite ($MgNH_4PO_4 \cdot 6H2O$) solubility and its application to a piggery effluent problem. *Water Science and Technology* 26(9-11): 2229-2232.

Webb, R. (2012). Algal bloom warning, Loch Melvin. http://s0.geograph.org.uk/geophotos/03/05/98/3059829_3170e9c0.jpg Creative Commons Licence. Attribution-ShareAlike 2.0 Generic

Webster, C. (1962). The Sewers of Mohenjo-Daro. *Journal Water Pollution Control Federation* 34(2): 116-123.

Wellstead, J. (2012). "Political Risks in MENA Phosphate Markets." Retrieved March, 2013, from http://potashinvestingnews.com/4799-political-risks-mena-phosphate-markets-tunisia-morocco-jordan-syria-afric.html.

WERF (2011). Energy Management-Exploratory Team Report, Water Environment Research Foundation.

Werktuigendagen (2009). John Deere 7430 with Joskin liquid manure trailer at Werktuigendagen 2009. http://commons.wikimedia.org/wiki/File:John_Deere_7430.jpg Creative Commons Attribution-Share Alike 2.0 Generic

Wett, B. (2006). Solved upscaling problems for implementing deammonification of rejection water. *Water Science and Technology* 53(12): 121-128.

Wett, B. (2007). Development and implementation of a robust deammonification process. *Water Science and Technology* 56(7): 81-88.

Wett, B., Buchauer, K. and Fimmi, C. (2007). Energy self-sufficiency as a feasible concept for wastewater treatment systems. *Asian Water* September: 21-24.

Wiesmann, U. (1994). Biological nitrogen removal from wastewater. In: *Advances in Biochemical Engineering/Biotechnology*. Fletcher, A. (ed), Springer Berlin / Heidelberg. 51: 113-154.

Wilsenach, J. (2005). *DESAR: Options for Separate Treatment of Urine. STOWA report number 2005-11*, STOWA. Utrecht

Wilsenach, J. A. and van Loosdrecht, M. C. M. (2006). Integration of Processes to Treat Wastewater and Source-Separated Urine. *Journal of Environmental Engineering* 132(3): 331-341.

Wilsenach, J. A., Schuurbiers, C. A. H. and van Loosdrecht, M. C. M. (2007). Phosphate and potassium recovery from source separated urine through struvite precipitation. *Water Research* 41(2): 458-466.

Windey, K., De Bo, I. and Verstraete, W. (2005). Oxygen-limited autotrophic nitrification-denitrification (OLAND) in a rotating biological contactor treating high-salinity wastewater. *Water Research* 39(18): 4512-4520.

Winkler, M. K. H., Kleerebezem, R. and van Loosdrecht, M. C. M. (2012). Integration of anammox into the aerobic granular sludge process for main stream wastewater treatment at ambient temperatures. *Water Research* 46(1): 136-144.

Wong, L. T., Mui, K. W. and Guan, Y. (2010). Shower water heat recovery in high-rise residential buildings of Hong Kong. *Applied Energy* 87(2): 703-709.

World Economic Forum (2009). Thirsty Energy: Water and Energy in the 21st Century. *Energy Vision Update 2009*.

World Resources Institute (1996). *World Resources 1996-97: Guide to the Global Environment*, Basic Books.

Yan, P., Ji, F., Wang, J., Fan, J., Guan, W. and Chen, Q. (2013). Evaluation of sludge reduction and carbon source recovery from excess sludge by the advanced Sludge reduction, Inorganic solids separation, Phosphorus recovery, and Enhanced nutrient Removal (SIPER) wastewater treatment process. *Bioresource Technology* 150(0): 344-351.

Zoubir, Y. H. and Benabdallah-Gambier, K. (2005). The United States and the North African Imbroglio: Balancing Interests in Algeria, Morocco, and the Western Sahara. *Mediterranean Politics* 10(2): 181-202.

Chapter 2

MATERIALS AND METHODS

Summary

The analytical methods used during the experimental work performed in this thesis are described within this chapter. A brief description of the conventional parameters used for the wastewater (organic matter, nitrogen compounds, pH, dissolved oxygen, solids, etc.) and biomass characterization is presented. The biomass was characterized by means of parameters such as biomass density of the granules, sludge volume index and techniques such as digital image analysis, electronic microscopy and stereomicroscope.

Identification of the different bacterial populations presented in the biomass samples was researched by Fluorescent In Situ Hybridization (FISH). The procedure to determine the biomass activity is also presented in this chapter.

The specific analytical methods used in a single part of the work, and the various experimental setups are described in the corresponding chapter. The descriptions of the general calculations, like biomass production, performed in the different chapters of this thesis are also provided. Other more specific calculations used exclusively in one section of the thesis are described in the corresponding chapter.

2

OUTLINE

2.1 ANALYSIS OF THE LIQUID PHASE

In this section, the methods used for the determination of the conventional parameters measured in the wastewater are described. Samples were filtered through a pore size filter of 0.45 µm (MF-Millipore, Millipore) in order to remove suspended solids for soluble fraction analysis.

2.1.1 Chemical Oxygen Demand (COD)

The Chemical Oxygen Demand (COD) is the amount of oxygen required to oxidize the organic matter present in a liquid sample (wastewater) using a strong chemical oxidant (potassium dichromate) in an acid medium. A catalyst (silver sulphate) is used to improve the oxidation of some organic compounds. After digestion, the remaining amount of unreduced $K_2Cr_2O_7$ is titrated with ferrous ammonium sulphate to determine the amount of $K_2Cr_2O_7$ consumed, being the amount of oxidable matter calculated in terms of oxygen equivalent.

The total and soluble Chemical Oxygen Demand concentrations (COD_t and COD_s) were determined following the method described by Soto *et al.,* (1989), which is a modification from the method 5220C of the Standard Methods for the Examination of Water and Wastewater (APHA-AWWA-WPCF, 2005). COD_t was determined using the raw sample, while filtered samples were used to determine COD_s.

2.1.1.1 Reagents

- Standard potassium dichromate digestion solution: 10.216 g of $K_2Cr_2O_7$ and 33 g of $HgSO_4$ were dissolved in 500 mL of distilled water. Then, 167 mL of concentrated H_2SO_4 were added. Then, the solution was cooled to room temperature and, finally, diluted to 1000 mL.
- Sulphuric acid reagent: 10.7 g of Ag_2SO_4 were added to 1 L of concentrated H_2SO_4. The solution was used after 2 days of preparation.
- Ferroin indicator solution: 1.485 g of $C_{18}H_8N_2 \cdot H_2O$ (phenanthroline monohydrate) and 0.695 g of $SO_4Fe \cdot 7\ H_2O$ were dissolved in 100 mL of distilled water.
- Standard potassium dichromate solution 0.05 N. An amount of 1.226 g of $K_2Cr_2O_7$, previously dried at 105 ºC for 2 hours, was dissolved in 500 mL of distilled water.
- Standard ferrous ammonium sulphate titrant (FAS) 0.035 N: 13.72 g of $Fe(NH)_4(SO)_2 \cdot 6\ H_2O$ were dissolved in distilled water. Then, 20 mL of concentrated H_2SO_4 were added and, finally, the solution was cooled and diluted to 1000 mL

2.1.1.2 Determination procedure

This procedure is applicable to samples with COD concentrations between 90-900 mg/L. Place 2.5 mL of sample in 10-mL Pyrex tubes. Add 1.5 mL of digestion solution and 3.5 mL of sulphuric acid reagent slowly on the wall of the tube slightly inclined (to avoid mixing). A blank sample using distilled water is prepared in the same way. This blank acts as "reference", representing the COD of the distilled water. After being sealed with Teflon and tightly capped, the tubes are finally mixed completely and placed in the block digester (HACH 16500-100) preheated to 150 ºC. The duration of the digestion period is 2 h.

After digestion, the tubes are cooled to room temperature. Then, the content of the tubes is transferred into a beaker and, once added 1-2 drops of ferroin indicator, the solution is titrated under rapid stirring with standard FAS. The FAS solution is standardized daily as follows: Place 5 mL of distilled water into a small beaker. Add 3.5 mL of sulphuric acid reagent. Cool to room temperature and add 5 mL of standard potassium dichromate solution (0.05 N). Add 1-2 drops of ferroin indicator and titrate with FAS titrant. The end-point is a sharp color change from blue-green to reddish brown. Molarity of FAS solution is calculated using the equation (2.1).

$$M_{fas} = \frac{5 \cdot 0.05}{V_{fas}} \qquad (2.1)$$

where:

M_{fas}: molarity of FAS (mol/L), and

V_{fas}: volume of FAS consumed in the titration (mL).

The COD is calculated with the equation (2.2):

$$COD = \frac{(A - B) \cdot M_{fas} \cdot 8000}{V} \qquad (2.2)$$

where:

COD: Chemical Oxygen Demand (mg O_2/L); A: mL of FAS solution consumed by the blank; B: mL of FAS solution consumed by the sample; 8000: milliequivalent weight of oxygen x 1000 mL/L.

2.1.2 Total Organic Carbon (TOC)

Organic carbon in liquid samples may include a variety of organic compounds in different oxidation states. Total Organic Carbon (TOC) is a more convenient and direct expression of total organic content than Chemical Oxygen Demand (COD), but does not provide the same information. Unlike COD, TOC is independent of the oxidation state of the organic matter and does not measure other organically bound elements, such as nitrogen and hydrogen, and inorganic compounds that can contribute to the oxygen demand measured by COD (APHA-AWWA-WPCF, 2005). To determine the quantity of organically bound carbon, the organic molecules must be broken down and converted to a single carbon molecular form that can be measured quantitatively. The TOC concentration was determined by a Shimadzu analyzer (TOC-5000) as the difference between the Total Carbon (TC) and the Inorganic Carbon (IC) concentrations. The instrument was connected to an automated sampler (Shimadzu, ASI-5000-S). The TC concentrations were determined from the amount of CO_2 produced during the combustion of the sample at 680 °C, using platinum immobilized over alumina spheres as catalyst. The IC concentrations were obtained from the CO_2 produced during the chemical decomposition of the sample with H_3PO_4 (25%) at room temperature. The CO_2 produced was optically measured with a non dispersive infrared analyzer (NDIR) after being cooled and dried. High-purity air was used as carrier gas at a flow of 150 mL/min. A curve comprising 4 calibration points in the range of 0 to 1 g C /L, using potassium phthalate as standard for TC and a mixture of sodium carbonate and bicarbonate (Na_2CO_3/$NaHCO_3$, 3:4 w/w) for IC, was used for the quantification.

2.1.3 Nitrogen

In waters and wastewaters, the forms of nitrogen of greater interest are, in order of decreasing oxidation state, nitrate, nitrite, ammonia and organic nitrogen. All these forms, as well as nitrogen gas (N_2), are biochemically inter-convertible following the processes of the nitrogen cycle. Organic nitrogen is defined functionally as the organically bound nitrogen in the trinegative oxidation state, but it does not include all organic nitrogen compounds. Analytically, organic nitrogen and ammonia can be determined together and have been referred to as “Total Kjeldahl Nitrogen” (TKN), a term that reflects the technique used in their determination. Total inorganic nitrogen (IN) is the sum of the nitrate and nitrite forms.

2.1.3.1 Total (TN), Inorganic (IN) and Total Kjeldhal Nitrogen (TKN)

TKN was determined in a total organic nitrogen analyzer (Rosemount-Dohrmann DN-1900) equipped with a quimio-luminiscence detector with two channels. One channel determined the Total Nitrogen (TN), by oxidation at high temperature, and the other determined the Inorganic Nitrogen (IN), by a chemical reduction. TKN was determined as the difference between TN and IN. All the nitrogen presented in the sample was catalytically oxidized to nitrous oxide (NO).

The process for TN determination occurred in two steps. The first step was a catalytic (Cu as catalyst) oxidation in the combustion tube at 850 °C and with pure oxygen (1 atm) as carrier gas. The second one was the chemical reduction of residual NO_2 with H_2SO_4 at 80 ºC and catalyzed by $VaCl_3$. For the IN determination, only the second step (chemical reduction) was used. The NO obtained in the two steps was dried and forced to react with O_3 producing an unstable excited state NO_2*. The change back of this oxide to its fundamental state released a proton, from which the determination of TN and IN was carried out by quimio-luminiscence, using a multiplicator tube. The instrument was calibrated with a certified standard solution (KNO_3, 20 mg N/L) using a response factor method.

2.1.3.2 Ammonia, method Bower, Holm-Hansen

Total ammonia-nitrogen (NH_4^+-N) was determined spectrophotometrically by a method in which indophenol blue was produced by the reaction of ammonia with salicylate and hypochlorite, in the presence of sodium nitroprusside (Bower and Holm-Hansen, 1980). This method is safer than the phenol–hypochlorite method, 4500-NH_3 F of (APHA-AWWA-WPCF, 2005), because phenol is not used, but it may not be suitable for field determinations because of photosensitivity. The characteristic colors produced by increasing concentrations of ammonia make the assay useful for the direct, visual estimation of ammonia in culture systems.

Reagents:

- Reagent A: Solution of 0.28 g/L of sodium nitroprusside and 440 g/L of sodium salicylate.
- Reagent B: Solution of 18.5 g/L of NaOH and 120 g/L of sodium citrate.
- Reagent C: Standard commercial solution of sodium hypochlorite.
- Reagent D: Solution prepared mixing 7 parts of reagent B and 1 part of reagent C. Reagent D was stable for 1 hour after preparation.

Determination Procedure:

- Add 120 µL of reagent A and 200 µL of reagent D to 1 mL of sample (diluted if necessary).
- Store, protected from light, for more than 2 hours but less than 3 hours.
- Measure it at 640 nm and compare the obtained absorbance to a calibration curve.

2.1.3.3 Ammonia, phenate method

Ammonia concentration was determined following the method 4500-NH_3 F. (Phenate method) (APHA-AWWA-WPCF, 2005). The method is based on the reaction of NH_3 with HClO

and phenol, forming a strong-blue compound (indophenol) which can be colorimetrically determined using a spectrophotometer (Shimadzu UV- 1603, UV-Visible) at 635 nm.

Reagents preparation:

- Solution 1 (Phenol-nitroprusiate): 15 g of phenol and 0.05 g of sodium nitroprusiate were added to 250 mL of buffer solution. The buffer solution was prepared adding 30 g of $Na_3PO_4 \cdot 12\ H_2O$, 30 g $Na_3C_6H_5O_7 \cdot 2\ H_2O$ and 3 g EDTA per liter, adjusted to pH 12.
- Solution 2 (Hipochloride): 15 mL of commercial bleach were mixed with 200 mL of NaOH 1 N and filled up to 500 mL with distilled water.

Determination procedure:

2.5 mL of sample (diluted if necessary to get a maximum concentration of 1 mg NH_4^+-N/L) were placed in a 10 mL tube. 1.0 and 1.5 mL of solution 1 and 2, respectively were added to the sample. After waiting 45 minutes at room temperature, the concentration of NH_4^+-N was measured in a spectrophotometer (CECIL- 7200) at 635 nm. The quantification was done with a 5-7 points calibration curve in the range of 0-1 mg NH_4^+-N/L, using NH_4Cl as standard (Figure 2.1).

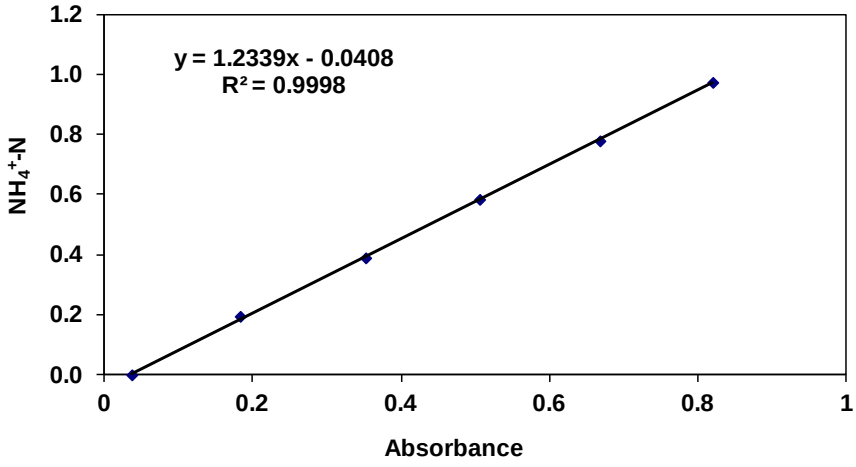


Figure 2.1. Calibration curve for ammonia determination by the phenate method.

2.1.3.4 Nitrite

Nitrite concentration was determined following the method 4500-NO_2^--B (Colorimetric Method) described in the Standard Methods for the Examination of Water and Wastewater (2005). Nitrite is determined through the formation of a reddish purple azo dye produced at pH

2.0-2.5 by coupling diazotized sulphanilamide with N-(1-napththyl)- ethylenediamine dihydrochloride (NED dihydrochloride).

Reagents preparation:

- Sulphanilamide: 10 g of sulphanilamide were dissolved in 100 mL of concentrated HCl and 600 mL of distilled water. After cooling, the volume was filled up to 1 L with distilled water.
- NED: 0.5 g of NED were dissolved in 500 mL of distilled water.

Determination procedure:

A volume of 0.1 mL of each reagent was added to 5 mL of sample, diluted if necessary to fit the concentration range of the method. After waiting 20 minutes for color stabilization, the sample was measured in a spectrophotometer (CECIL-7200) at 543 nm. The quantification was done with 6-8 points calibration curve in the range of 0-0.25 mg NO_2^--N/L, using $NaNO_2$ as standard (Figure 2.2).

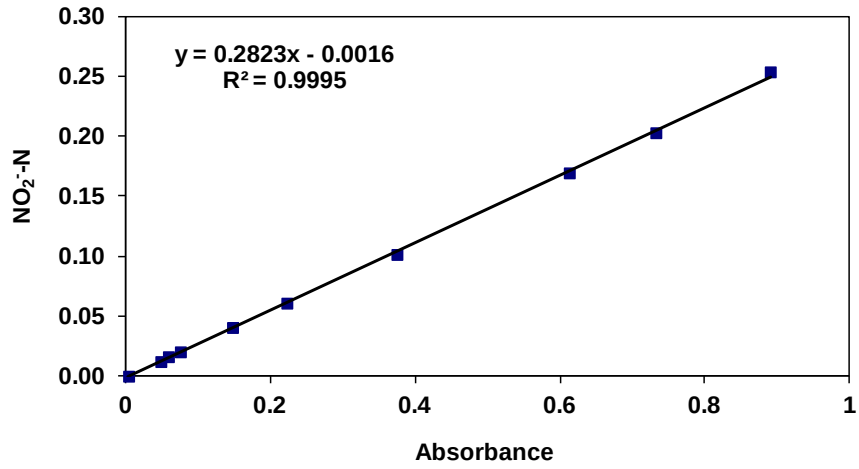


Figure 2.2. Calibration curve for nitrite.

2.1.3.5 Nitrate

Nitrate concentration was determined following the method 4500-NO_3^--B (Ultraviolet Spectrophotometric Screening Method) described in the Standard Methods for the Examination of Water and Wastewater (2005). Measurement of UV absorption at 220 nm enables rapid determination of NO_3^- ions. Because dissolved organic matter also may absorb at 220 nm and NO_3^- does not absorb at 275 nm, a second measurement at 275 nm is used to correct the NO_3^- value.

Determination procedure:

A volume of 0.1 mL of HCl 1 N was added to 5 mL of sample (diluted if necessary to get a maximum concentration of NO_3^--N of 2.5 mg/L). Afterwards, the absorbance at 220 and 275 nm was measured in a spectrophotometer (CECIL-7200). The absorbance related to nitrate was obtained by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm. The quantification was done with a 6-8 points calibration curve within the range of 0-3 mg NO_3^--N/L, using KNO_3 as standard (Figure 2.3).

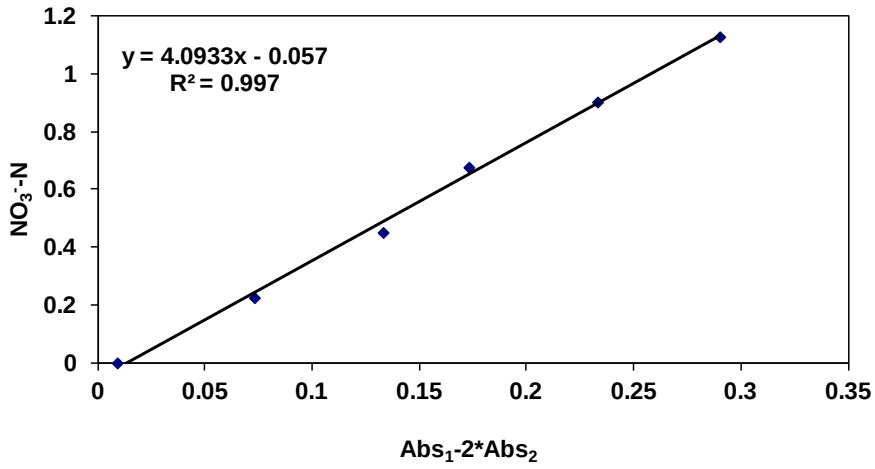


Figure 2.3. Calibration curve for nitrate.

2.1.4 Inorganic ions

The anions nitrite (NO_2^-), nitrate (NO_3^-), chloride (Cl^-), bromide (Br^-), phosphate (PO_4^-), sulphate (SO_4^-), thiosulphate ($S_2O_3^{-2}$) and the cations lithium (Li^+), sodium (Na^+), ammonium (NH_4^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) were determined by ion chromatography (IC) with an Advanced Compact IC system (861, Metrohm) equipped with a CO_2 suppressor (MCS 853, Metrohm) and a sample processor (AG 838, Metrohm). Anions were determined with a "Metrosep A column" (250 x 4.0 mm) and a mobile phase (buffer) with 3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$ at a flow rate of 0.7 mL/min. Cations were determined with a column (250 x 4.0 mm) (Metrosep C3, Metrohm) and nitric acid 3.5 mM was used as mobile phase. The injection volume of the sample was 20 µL and data collection was done by using the Processor software IC Net 2.3.

Reagents:

- Mobile phase for anions: Na_2CO_3 3.2 mM (339.2 mg Na_2CO_3 in 1000 mL of deionised water) and $NaHCO_3$ 1.0 mM (84 mg $NaHCO_3$ in 1000 mL of distillated water).
- Mobile phase for cations: Nitric acid 3.5 mM (0.243 mL of nitric acid 65% in 1000 mL of distillated water).
- Standard commercial solutions for anions and cations (Fluka).

Determination Procedure:

Table 2.1 shows the calibration range for the different inorganic ions' concentrations. Therefore, in some samples, a dilution with distilled water was performed in order to fit to these ranges.

Table 2.1. Calibration ranges for the different inorganic ions (mg/L).

Anion	Low value	High value	Cation	Low value	High value
Cl^-	1.0	100	Li^+	0.05	5
NO_2^-	0.05	5	Na^+	1.5	150
NO_3^-	0.5	50	NH_4^+	0.1	10
Br^-	0.2	20	K^+	0.5	50
PO_4^{3-}	0.5	50	Mg^{2+}	0.5	50
SO_4^{2-}	1.5	150	Ca^{2+}	0.5	50
$S_2O_3^{2-}$	1.5	150			

2.1.5 Other control parameters

2.1.5.1 pH

The pH value was measured using different instruments in the laboratory and pilot plant scale reactors. In each experimental setup was specified the on-line device. The pH measurements of the liquid samples were performed with an electrode (52-03, Crison Instruments) equipped with an automatic compensatory temperature device (21-910-01, Crison Instruments) and connected to a measurement instrument (pH). The sensibility of the instrument was ±1 mV, corresponding to 0.01 pH units. The electrode was calibrated at room temperature with two standard buffer solutions of pH 7.02 and 4.00.

2.1.5.2 Dissolved oxygen (DO)

Dissolved oxygen was measured using different instruments in the laboratory and pilot plant scale reactors. The specific used device is specified in the corresponding chapter.

- Dissolved oxygen pocket meter (Oxi 330i, WTW) equipped with a membrane covered galvanic dissolved oxygen sensor (CellOx® 325, WTW).
- Digital multimeter (HQ40D, Hach Lange) equipped with a Luminescence-based DO probe (Intellical LDO, Hach Lange).
- A Luminescence-based DO probe (LDO Process sensor, Hach Lange) connected to a controller (SC-100, Hach Lange).

2.2 BIOMASS CHARACTERIZATION

2.2.1 Solids concentrations (TSS and VSS)

Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) concentrations were determined according to the methods 2540 D (Total Suspended Solids Dried at 103-105 °C) and 2540 E (Fixed and Volatile Solids Ignited at 550 °C) reported in Standard Methods for the Examination of Water and Wastewater (APHA-AWWA-WPCF, 2005).

2.2.2 Sludge Volume Index

The Sludge Volume Index (SVI) determination is defined in the Standard Methods for the Treatment of Water and Wastewater (APHA-AWWA-WPCF, 2005) as the volume in milliliters occupied by 1 g of a suspension after 30 minutes of settling. However, as suggested at the "1st IWA-Workshop Aerobic Granular Sludge" (Munich, 2004) and by Schwarzenbeck *et al.,* (2004) another parameter, the SVI_{10} (SVI after 10 minutes of settling) was used in all the chapters of this work instead of SVI_{30} (SVI after 30 minutes of settling) since it is more representative for granular biomass (de Kreuk *et al.*, 2007). A low SVI_{30} value does not necessarily imply sludge granulation and vice versa. Nevertheless, a granular sludge bed does consolidate much faster, i.e., the terminal SVI_{30} is already reached after 10 minutes of settling.

2.2.3 Density of the granules

The biomass density (as mass of granules per volume of granules) was determined using the method described by Beun *et al., (2002)* and modified in the laboratory of Environmental Engineering and Bioprocesses. First, a known amount of a homogeneous biomass sample was taken from the reactor and weighed (W_1) in a tare weighed graduated cylinder (W_2). Then, a known amount of liquid was removed from the sample (W_3). A known volume of a dextran blue solution (1 g/L) was added to a known volume of sample of granular sludge, in a volume ratio of about 1:1. The mixture was gently mixed, and subsequently, the

granules were allowed to settle (W_4). A known amount of the liquid above the settled granules was removed, and a sample was taken from it. This fraction (Abs_1) and the original dextran blue solution (Abs_0) were analyzed by a spectrophotometer at 620 nm. Subsequently the volume occupied by the biomass in the reactor sample was calculated, since dextran blue only binds to water and not to biomass. By measuring also the dry weight of the reactor sample (APHA-AWWA-WPCF, 2005) the density of the granules can be calculated as gram of biomass per liter of granule. The density was calculated from equation (2.3):

$$\rho_{granule} = VSS \frac{W_1 - W_2}{W_4 - W_2 - \left(\frac{Abs_0}{Abs_1} \cdot (W_4 - W_3) \right)} \quad (2.3)$$

Being:

$\rho_{granule}$: The density of the granules (g VSS/$L_{granule}$)

VSS: Volatile Suspended Solids concentration (g/L)

W_1: weight of the graduated cylinder with sample (g)

W_2: graduated cylinder weight (g)

W_3: weight of the graduated cylinder with sample after removal of liquid (g)

W_4: weight of the graduated cylinder after dextran blue addition (g)

Abs_0: Absorbance of the dextran blue solution (1 g/L)

Abs_1: Absorbance of the sample

2.2.4 Average diameter of the granules

Morphology of the granules was followed by image analysis. Images of the granular sludge were taken with a digital camera (Coolsnap, Roper Scientific Photometrics) combined to a stereomicroscope (Stemi 2000-C, Zeiss). For digital image analysis the software Image ProPlus® was used (Figure 2.4). The procedure of average diameter determination is as follows:

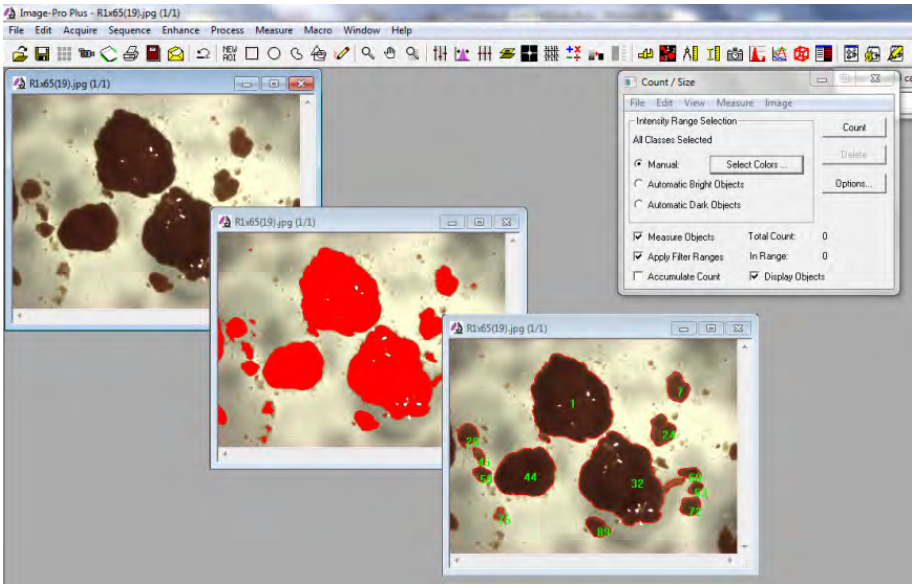


Figure 2.4. Example of the use of Image ProPlus® software.

- Definition of the range of colors corresponding to the area of interest in the image, i.e. the granules (Manual or Automatic).
- Selection of the measurements of interest.
- Exporting of the data of interest selected with the software (e.g., area, aspect, roundness, average diameter, etc.) to a worksheet.
- Utilization of the histogram tool (data analysis tool pack) to calculate the frequency and construction of the histogram. The average diameter can be calculated from a frequency, surface or volumetric distribution.

The average diameter obtained from the software corresponded to the mean feret diameter of the granules. The feret diameter was calculated as an average value from the shortest and the longest measured segments of each granule.

2.2.5 Elemental analysis

The elemental analysis of the biomass was performed in order to know the composition as $C_nH_aO_bN_cS_d$. First, the sample was dried at 105 ºC for 24 hours, and then it was crushed to obtain a homogeneous powder. The quantity of sample necessary was between 1-3 mg. The elemental analysis technique is based on the complete and instantaneous oxidation (combustion) of the sample and the determination of the gases from the combustion through a thermal conductivity detector model CHNS FISONS EA 1108 (for C, H, N and S) and model CARLO ERBA EA 1108 (for oxygen). The results were expressed as percentage of compound in the sample.

2.2.6 Poly-Hydroxy-Alkanoates (PHA)

The Poly-Hydroxy-Alkanoates (PHA) content in the biomass was measured according to a modification of the method of Pijuan *et al.,* (2005).

2.2.6.1 Reagents preparation

Acidified methanol (10% H_2SO_4) with internal standard: A volume of 20 mL of H_2SO_4 (98%) was added drop to drop to 140 mL of pure methanol (99.8%) and then, a volume of 200 mL was completed with pure methanol. 20 mg of benzoic acid (internal standard) was dissolved in this solution.

Standards: The quantity of standard necessary was weighted in Pyrex® tubes by triplicate. The calibration of the method was performed using as standards 3-hydroxybutyric acid and 3-hydroxyvaleric acid copolymer (88:12) (Aldrich) to quantify Poly-Hydroxy-Butyrate (PHB) and Poly-Hydroxy-Valerate (PHV), respectively. Poly-Hydroxy-2-MethylValerate (PH2MV) was quantified using 2-hydroxycaproic acid (98%) (Aldrich).

2.2.6.2 Determination procedure

The biomass sample collected from the reactor was centrifuged at 4700 rpm, during 6 minutes, and then, the supernatant was removed in order to stop the biological activity of the biomass. The samples were stored in ice until their freeze-drying.

To freeze-dry the tubes containing the samples were covered with Parafilm®, perforated with some small holes in order to avoid that the biomass burst. The samples were inside the freeze-drier for around 24 hours (depending on the volume of the sample) with the following operational conditions: -40 ºC and 0.1 atm.

Then, an amount around 30 mg of freeze-dried sludge was weighted and placed it in Pyrex® tubes by triplicate (taking note about the exact quantity of sludge weighed). A volume of 4 mL of acidified methanol (10% H_2SO_4) and 4 mL of chloroform were added to the tubes which are finally closed. Samples were digested during 20 h at 100 ºC. The same procedure was required for the standards.

After cooling, free acids must be extracted from the organic phase. In order to extract them, 1 mL of milliQ water was added and the tubes were shaked vigorously using the vortex. Then, the tubes were stood until the two phases (organic and aqueous) were separated. Then, 1 mL of the organic phase was extracted, filtrated with glass wool and dried with free sodium sulphate before putting it into the Gas Chromatography (GC) vial.

The analyses were performed in a GC system (Agilent 6850). A volume of 1 µL of the organic phase was injected in 7:1 split mode in a column HP-INNOWAX (30 m × 0.25 mm × 0.25 µm). Results were expressed as weight percentage of PHA in the total biomass.

2.2.7 Specific Anammox Activity assays

The batch assays used to estimate the Anammox activity were performed according to the methodology described by Dapena-Mora *et al., (2007)* based on the measurement along time of the overpressure generated by the nitrogen gas produced in completely closed vials with a total volume of 38 mL and 25 mL of liquid volume. The overpressure in the headspace was measured using a differential pressure transducer 0-5 psi, linearity 0.5% of full-scale manufactured by Centerpoint Electronics. Biomass concentration at the beginning of the experiment was fixed around 1.0 g VSS/ L (this value can be changed if the expected activity is too low). Before the beginning of the batch test, the biomass was washed almost three times with phosphate buffer (0.143 g KH_2PO_4/L and 0.747 g K_2HPO_4/L). The pH value was fixed at 7.8, and the temperature was fixed at a value depending on the conditions to be analyzed. Gas and liquid phases were purged with inert gas (Helium or Argon) to remove the oxygen from the bulk liquid and headspace. The vials were placed in a thermostatic shaker, fixing the agitation speed at 150 rpm and the temperature T at the desired value until stable conditions were reached. Initial concentrations of substrates were 70 mg NH_4^+-N/L and 70 mg NO_2^--N/L. The production of N_2 was determined in the gas phase as the increment of pressure in the headspace of the vials, measured by means of the pressure transducer device.

Maximum Specific Anammox Activity (SAA) was estimated from the maximum slope of the curve described by the cumulative N_2 production along the time and related to the biomass concentration in the vials. The N_2 gas production rate (moles N_2/min) was calculated from the maximum slope of the curve describing the pressure increase in the vial along time (α) (atm/min) using the equation (2.4).

$$\frac{dN_2}{dt} = \alpha \frac{V_G}{R \cdot T} \qquad (2.4)$$

being V_G the volume of the gaseous phase (L), R the ideal gas coefficient (atm·L/mol·K) and T the temperature (K). The SAA (g N_2-N g VSS/ d) is calculated from the N_2 gas production rate and the biomass concentration in the vial (g VSS/L) using equation (2.5):

$$SAA = \frac{dN_2/dt}{X \cdot V_L} \frac{28 \text{ g N}}{\text{mol } N_2} \frac{1440 \text{ min}}{\text{d}} \qquad (2.5)$$

being V_L the volume of the liquid phase (L).

Since the values of the affinity constant of the Anammox bacteria for ammonium and nitrite are lower than 10 μM and 5 μM, respectively (Strous *et al.*, 1999), it can be considered that the activity measured is the maximum activity for the range of nitrite and ammonium concentrations used.

2.2.8 Respirometric assays

Activity tests were performed in order to measure the nitrifying capability of the biomass by means of a respirometric method (López-Fiuza *et al.*, 2002, Mosquera-Corral *et al.*, 2005a) in a BOM 5300 (YSI Inc) device equipped with two oxygen selective probes (YSI 5331). Respirometric batch experiments were performed in hermetically closed vials of 15 mL with a useful volume of 10 mL. Between 100 and 200 mg VSS/L were suspended in a buffer solution (1.25 mL/L traces; NaCl, 0.2 g/L; K_2HPO_4, 1.0 g/L; KH_2PO_4, 0.8 g/L; $MgSO_4$, 0.2 g/L; $MgCl_2 \cdot H_2O$, 0.3 g/L) of pH 7.0. The liquid and biomass mixture inside the vessel were bubbled with air for 15 minutes to reach the oxygen saturation. Meanwhile, the software for the data acquisition (Labtech) was initiated, and the two electrodes for oxygen measurement were calibrated to 100% oxygen saturation. To begin the experiment, aeration was removed and the oxygen probes were connected with the vessels and tightly closed avoiding the presence of bubbles.

The oxygen depletion was monitored during the time by means of the connection of the oxygen electrode to the data acquisition system (Figure 2.5). The endogenous respiration was measured during enough time to obtain the slope of the consumed oxygen (g O_2/L·d). The substrate (initial concentrations of NH_4^+ or NO_2^- of 60 mg N/L) was injected into the vial (10 µL) and then the slope of the oxygen consumption due to biomass activity was determined (g O_2/L·d).

After the experiment, the solids content in each of the vials was determined according to the Standard Methods (APHA-AWWA-WPCF, 2005). Finally, the specific activity of the biomass is determined by dividing the oxygen consumption rate by the solids content, which can be referred to the specific substrate by the use of the stoichiometric coefficient.

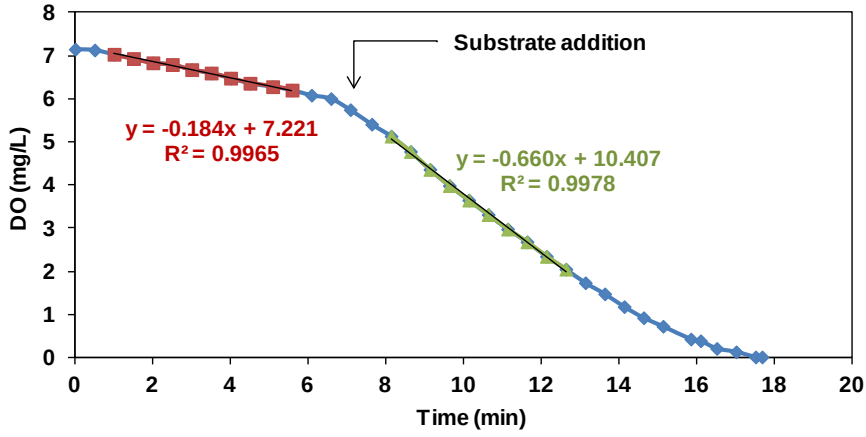


Figure 2.5. Oxygen depletion during endogenous respiration (red) and substrate consumption (green).

2.3 MICROBIOLOGICAL DETERMINATIONS

Molecular techniques based on the rRNA of *Bacteria* and *Prokaryotes* are presented in the next section. The FISH technique makes the identification of microorganisms at any desired taxonomical level possible, depending on the specificity of the used probe. It is the only quantitative molecular biology technique, although quantification is either complex or tedious and subjective.

2.3.1 Identification of bacteria populations by FISH

The abundance of the different populations of microorganisms presented in the sludge samples of the reactors was researched by Fluorescent In Situ Hybridization (FISH). With this technique, specific regions in the 23S or 16S rRNA are detected with fluorescently labeled probes. If the corresponding domain, phylum, genus or species are present, the probe hybridizes to the targeted sequence and can later be detected microscopically. According to Amann *et al., (1995)* a typical FISH protocol includes four steps: the fixation and permeabilization of the sample; hybridization of the targeted sequence to the probe; washing steps to remove the unbound probe; and finally, the detection of labeled cells by microscopy or flow cytometry. This protocol must be applied to disrupted biomass; therefore, the granules must be disintegrated before starting the procedure. To achieve the granular biomass breakage, biomass was sonicated for 1 minute at 65% of amplitude using a probe sonicator (UP200s, Dr. Hielscher). The time of sonication was selected in order to achieve the breakage of the granules but not of the cells.

During hybridization, the cells were exposed to high temperatures, detergents and osmotic gradients. Thus, fixation of the cells was essential in order to maintain the morphological integrity of the cells. Fixation of cells with glutaraldehide resulted in considerable auto fluorescence of the specimen. Auto fluorescence was minimized by fixation in freshly prepared (not older than 24 h) 4% paraformaldehyde solution in phosphate buffer solution (PBS).

After fixation, the cells were immobilized on a microscopic slide and used for hybridization with 16S rDNA probes. In order to avoid non-specific binding of the rDNA probes, the hybridization was done at stringent conditions (46 ºC, 0-65% formamide; Table 2.2) and specimens were washed with wash buffer (48 ºC). The targeted organisms can be detected by the characteristic fluorescence of the dye contained in the probe.

The fluorochromes used to detect the hybridized rRNA were FLUOS (5(6)-carboxyfluorescein-Nhydroxysuccinimide ester) and Cy3 (indocarbocyanine). To visualize all cells in a sample the stain 4,6-diamidino-2-phenylindole (DAPI) was used. Its application can provide insight into the existence of archaeobacteria and eukaryotes, like e.g. protozoa. For analysis of the slides, an epifluorescence microscope (Axioskop 2 plus, Zeiss) in combination

with a digital camera (Coolsnap, Roper Scientific Photometrics) was used. The phylogenetic tree reflecting the different probes applied in this study indicating the bacteria detected by each probe are shown in Figure 2.6. The probes applied in this study are listed and detailed in Table 2.3.

The three probes for the domain of eubacteria (EUB338, EUB338 II and EUB338 III) were applied together in all samples to get an impression of the relative abundance of the microorganisms detected by more specific probes. In comparison with DAPI, they provided evidence of non-eubacteria present in the sample. For further discussion, it has to be kept in mind that samples can never be 100% representative. Thus the fact that no bacteria of a certain kind were present in the sample can always be attributed to unrepresentative sampling as well. Still this error it was tried to be kept small.

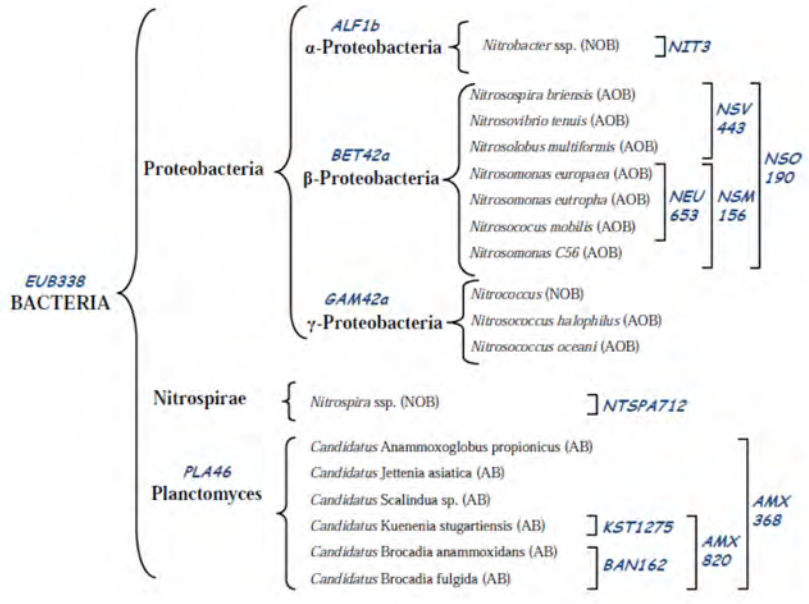


Figure 2.6. Different probes applied and the main bacteria detected by each probe (AOB: ammonium-oxidizing bacteria, NOB: nitrite-oxidizing bacteria and AB: Anammox bacteria).

2.3.2 Reagents preparation

- PBS (3x): An amount of 0.49 g KH_2PO_4 was dissolved in 80 mL of milliQ water, then 2.3 g of NaCl were added and the pH value was adjusted to 7.2. Finally, the volume was adjusted to 100 mL.
- PBS (1x) was prepared by a 1:3 dilution of PBS (3x) in milliQ water.
- Fixative solution: First, 6.5 mL milliQ were heated to 60 °C and 0.4 g of paraformaldehyde were added to them. One drop of 1 M NaOH was added

and the solution was shaken vigorously until it had nearly clarified (1-2 min). Then, 3.3 mL of PBS (3x) were added and the pH was adjusted to 7.2 with HCl (one drop 1 M HCl). Finally, the solution was filtered through 0.2 µm membrane filter.

- Hybridization buffer: The buffer was prepared into a 2 mL eppendorf by mixing: 360 µL of NaCl 5 M and 40 µL of Tris/HCl (1 M) (pH 8.0). The percentage of formamide of the hybridization buffer was selected according to the used probe (% FA in Table 2.2). Finally, 4 µL of sodiumdodecylsulfate 10% (w/v) were added to the mixture.

Table 2.2. Formamide and water added to the hybridization buffer.

% Formamide (v/v)	**Formamide (µL)**	**MilliQ (µL)**
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
55	1100	500
60	1200	400

Table 2.3. Probes used for fluorescent in situ hybridization and the formamide (FA) concentration used during hybridization.

Probe	Target site 16S	Probe sequence (5'→3')	% FA	Target organisms	Ref.[a]
EUB 338	338-355	GCT GCC TCC CGT AGG AGT	0-50	Bacteria domain	[1]
EUB 338 II	338-355	GCA GCC ACC CGT AGG TGT	0-50	*Planctomycetales*	[2]
EUB 338 III	338-355	GCT GCC ACC CGT AGG TGT	0-50	*Verrucomicrobiales*	[2]
ALF1B	19-35	CGT TCG YTC TGA GCC AG	20	*α-proteobacteria, some δ-proteobacteria, Spirochaetes*	[3]
BET42a	1027-1043	GCC TTC CCA CTT CGT TT	35	*β-proteobacteria*	[3]
GAM42a	1027-1043	GCC TTC CCA CAT CGT TT	35	*γ-proteobacteria*	[3]

Probe	Target site 16S	Probe sequence (5'→3')	% FA	Target organisms	Ref.[a]
NEU653	653-670	CCC CTC TGC TGC ACT CTA	40	Most *halophilic* and halotolerant *Nitrosomonas* spp.	[4]
Competitor		TTC CAT CCC CCT CTG CCG			
Nso190	189-207	CGA TCC CCT GCT TTT CTC C	55	Ammonio-oxidizing-β-Proteobacteria	[5]
Nsm156	156-174	TAT TAG CAC ATC TTT CGA T	5	*Nitrosomonas* spp., *Nitrosococcus* mobilis	[5]
NIT3	1035-1052	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter* spp.	[6]
Competitor		CCT GTG CTC CAG GCT CCG			
Ntspa712	712-732	CGC CTT CGC CAC CGG CCT TCC	35	Most members of phylum Nitrospira	[7]
Competitor		CGC CTT CGC CAC CGG GTT CC			
PLA46	46-63	GAC TTG CAT GCC TAA TCC	20	Planctomycetes	[8]
Amx820	820-841	AAA ACC CCT CTA CTT AGT GCC C	35	*Candidatus* "Brocadia anammoxidans"	[9]
Kst157	157-174	GTT CCG ATT GCT CGA AAC	25	*Candidatus* Kuenenia stuttgartiensis	[9]
Ban162	162-179	CGG TAG CCC CAA TTG CTT	40	*Candidatus* Brocadia anammoxidans	[9]
Pae997	997-1014	TCT GGA AAG TTC TCA GCA	0	*Pseudomonas* spp.	[10]
PAR1244	1244-1262	GGA TTA ACC CAC TGT CAC C	20	*Paracoccus*	[11]

[a] References: [1] Amann *et al.,* (1990); [2] Daims *et al.,* (1999); [3] Manz *et al.,* (1992); [4] Wagner *et al.,* (1995); [5] Mobarry *et al.,* (1996); [6] Wagner *et al.,* (1996); [7] Daims *et al.,* (2001); [8] Neef *et al.,* (1998); [9] Schmid *et al.,* (2001); [10] Amann *et al.,* (1996); [11] Neef *et al.,* (1996).

2.4 CALCULATIONS

2.4.1 SBR operational cycle analysis

The activity of the different microbial populations presented in the SBR was calculated using the concentration profiles during a whole cycle of operation, based on the procedure described by Mosquera-Corral *et al.,* (2005b) and according to the representation of Figure 2.7.

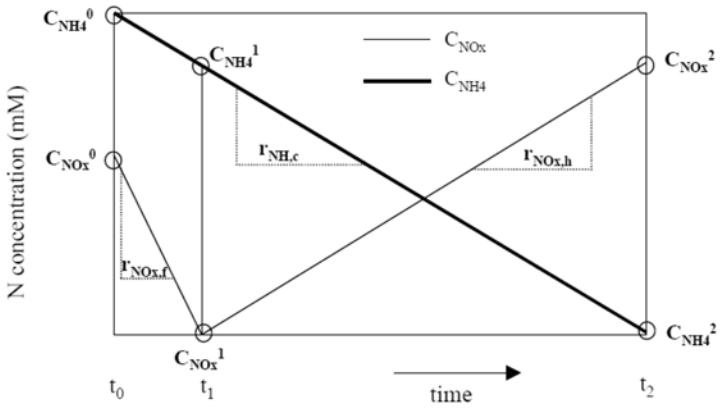


Figure 2.7. Schematic representation of the nitrogen compounds concentrations during the cycle and definition of the calculated parameters.

The consumption rates for ammonia (rNH_4^+) and for nitrogen oxides (nitrite or nitrate) ($rNO_{x\,f}$) in the feast phase and the production rate of nitrogen oxides (nitrite or nitrate) ($rNO_{x\,h}$) during famine phase were calculated using the equations (2.6), (2.7) and (2.8).

$$r_{NH_{4\,c}^+} = \frac{C_{NH_4^+}^0 - C_{NH_4^+}^2}{(t_2 - t_0)/60} \tag{2.6}$$

$$r_{NO_{x\,f}^-} = \frac{C_{NO_x^-}^0 - C_{NO_x^-}^1}{(t_1 - t_0)/60} \tag{2.7}$$

$$r_{NO_{x\,h}^-} = \frac{C_{NO_x^-}^1 - C_{NO_x^-}^2}{(t_1 - t_2)/60} \tag{2.8}$$

being t_0 the time at the beginning of the cycle, t_1 the time at the end of the feast phase, t_2 the time at the end of the famine phase and C_c^t the concentration of each compound (c) in a certain time (t), expressed in mg N/L. Specific rates were calculated by dividing the consumption rates by the solids concentration.

2.4.2 Estimation of the nitrogen assimilated and denitrified.

The main processes for nitrogen removal in the aerobic granular SBR were nitrogen assimilation for biomass growth and nitrification-denitrification. The sum of the amount of nitrogen removed from the liquid phase by both ways provides the value of nitrogen removed (NR). In order to discern between the percentages of nitrogen removal achieved by each of these mechanisms a nitrogen balance was performed to the reactor to determine the amount

of nitrogen used for growth. For each selected period, the amount of biomass produced was estimated from the biomass increase in the reactor and the amount of biomass washed out in the effluent using equation (2.9)

$$\Delta W_p = \Delta X_r \cdot V_r + \overline{X}_{Eff} \cdot Q \cdot \Delta t \quad (2.9)$$

Where ΔW_P is the amount of produced biomass (g VSS), ΔX_r the change of biomass concentration during each period (g VSS/L), V_r the reactor volume (L), $\overline{X}_{Eff}$ the average biomass concentration washed out in the effluent (g VSS/L), Q the flow rate (L/d) and Δt the length of each period (d).

Considering a general composition of the biomass as $C_5H_7NO_2$, the averaged amount of nitrogen assimilated for biomass growth ($N_{assimilated}$, g N) was calculated using equation (2.10) as:

$$N_{assimilated} = \Delta W_p \frac{14 \text{ g-mol N}}{113 \text{ g-mol biomass}} \quad (2.10)$$

$N_{removed}$ (*g N*) during such period of operation was calculated using equations and, being ΔN the nitrogen balance, NH_4^+-N_{Inf}, NO_2^--N_{Inf}, and NO_3^--N_{Inf} the ammonium, nitrite, nitrate (mg N/L) in the influent, and NH_4^+-N_{Eff}, NO_2^--N_{Eff}, and NO_3^--N_{Eff} the ammonium, nitrite and nitrate concentrations in the effluent respectively.

$$\Delta N = \left(NH_4^+\text{-}N_{Inf} + NO_2^-\text{-}N_{Inf} + NO_3^-\text{-}N_{Inf}\right) - \left(NH_4^+\text{-}N_{Eff} + NO_2^-\text{-}N_{Eff} + NO_3^-\text{-}N_{Eff}\right) \quad (2.11)$$

$$N_{removed} = \Delta N \cdot Q \cdot \Delta t \quad (2.12)$$

Nitrogen removed by denitrification ($N_{denitrified}$) was calculated by the difference between $N_{removed}$ and $N_{assimilated}$.

2.4.3 Biomass production

The growth yield of microorganisms (Y_{Obs}) in aerobic granules expressed in terms of gram of biomass produced per gram of organic matter removed (biomass production) was calculated for selected operational periods. The amount of biomass produced was calculated according to equation (2.9), and the amount of organic matter removed was calculated from the experimental data obtained from the performance of the reactor in the selected period of time, as the difference between the average COD concentrations in the influent and effluent. Finally, the obtained amount of biomass is divided by the amount of COD removed according to equation (2.13).

$$Y_{obs} = \frac{\Delta W_p}{\left(\overline{COD_{Inf}} - \overline{COD_{Eff}}\right)\cdot Q\cdot \Delta t} \quad (2.13)$$

Where Y_{obs} is the growth yield of aerobic granules (g VSS /g COD), ΔW_P the amount of produced biomass (g VSS) COD_{inf} and COD_{eff} the average COD concentration in the influent and effluent (g COD/L), Q: flow rate (L/d) and Δt: length of each period (d).

2.4.4 Nitrogen removal rates in the Anammox Processes

Ammonia and nitrite oxidation rates (AOR and NOR, respectively) and nitrogen removal rate by Anammox bacteria (ANR) in g N/L·d, and Ammonia removal efficiency (AR) and Nitrogen removal efficiency (NR) in %, were estimated based on nitrogen balances and the Anammox process stoichiometry, equation (2.14), according to equations: (2.15), (2.16), (2.17), (2.18), (2.19) and (2.20).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O \quad (2.14)$$

$$\Delta N = \left(NH_4^+\text{-}N_{Inf} + NO_2^-\text{-}N_{Inf} + NO_3^-\text{-}N_{Inf}\right) - \left(NH_4^+\text{-}N_{Eff} + NO_2^-\text{-}N_{Eff} + NO_3^-\text{-}N_{Eff}\right) \quad (2.15)$$

$$AOR = \frac{\left(NH_4^+\text{-}N_{Inf}\right) - \left(NH_4^+\text{-}N_{Eff}\right) - \left(\Delta N / 2.04\right)}{HRT} \quad (2.16)$$

$$NOR = \frac{NO_3^-\text{-}N_{Eff} - NO_3^-\text{-}N_{Inf} - \left(0.26\cdot \Delta N / 2.04\right)}{HRT} \quad (2.17)$$

$$ANR = \frac{\Delta N}{HRT} \quad (2.18)$$

$$AR = \frac{NH_4^+\text{-}N_{Inf} - NH_4^+\text{-}N_{Eff}}{NH_4^+\text{-}N_{Inf}}\cdot 100 \quad (2.19)$$

$$NR = \frac{\Delta N}{NH_4^+\text{-}N_{Inf} + NO_2^-\text{-}N_{Inf} + NO_3^-\text{-}N_{Inf}}\cdot 100 \quad (2.20)$$

Being HRT the hydraulic retention time (d).

2.5 REFERENCES

Amann, R., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. and Stahl, D. A. (1990). Combination of 16S ribosomal-RNA-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial-populations. *Applied and Environmental Microbiology* 56(6): 1919-1925.

Amann, R., Ludwig, W. and Schleifer, K. H. (1995). Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiological Reviews* 59(1): 143-169.

Amann, R., Ludwig, W., Schulze, R., Spring, S., Moore, E. and Schleifer, K. H. (1996). rRNA-targeted oligonucleotide probes for the identification of genuine and former pseudomonads. *Systematic and Applied Microbiology* 19(4): 501-509.

APHA-AWWA-WPCF (2005). *Standard methods for the examination of water and wastewater. American Public Health Association/American Water Works Association/Water Environment Federation*. Washington DC, USA

Beun, J. J., van Loosdrecht, M. C. M. and Heijnen, J. J. (2002). Aerobic granulation in a sequencing batch airlift reactor. *Water Research* 36(3): 702-712.

Bower, C. E. and Holm-Hansen, T. (1980). A Salicylate–Hypochlorite Method for Determining Ammonia in Seawater. *Canadian Journal of Fisheries and Aquatic Sciences* 37(5): 794-798.

Daims, H., Bruhl, A., Amann, R., Schleifer, K. H. and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology* 22(3): 434-444.

Daims, H., Nielsen, J. L., Nielsen, P. H., Schleifer, K. H. and Wagner, M. (2001). In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology* 67(11): 5273-5284.

Dapena-Mora, A., Fernandez, I., Campos, J. L., Mosquera-Corral, A., Mendez, R. and Jetten, M. S. M. (2007). Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. *Enzyme and Microbial Technology* 40(4): 859-865.

de Kreuk, M., Kishida, N. and van Loosdrecht, M. C. M. (2007). Aerobic granular sludge: state of the art. *Water Science and Technology* 55 (8-9): 75-81.

López-Fiuza, J., Buys, B., Mosquera-Corral, A., Omil, F. and Méndez, R. (2002). Toxic effects exerted on methanogenic, nitrifying and denitrifying bacteria by chemicals used in a milk analysis laboratory. *Enzyme and Microbial Technology* 31(7): 976-985.

Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K. H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria - problems and solutions. *Systematic and Applied Microbiology* 15(4): 593-600.

Mobarry, B. K., Wagner, M., Urbain, V., Rittmann, B. E. and Stahl, D. A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology* 62(6): 2156-2162.

Mosquera-Corral, A., Gonzalez, F., Campos, J. L. and Mendez, R. (2005a). Partial nitrification in a SHARON reactor in the presence of salts and organic carbon compounds. *Process Biochemistry* 40(9): 3109-3118.

Mosquera-Corral, A., de Kreuk, M. K., Heijnen, J. J. and van Loosdrecht, M. C. (2005b). Effects of oxygen concentration on N-removal in an aerobic granular sludge reactor. *Water Res* 39(12): 2676-2686.

Neef, A., Zaglauer, A., Meier, H., Amann, R., Lemmer, H. and Schleifer, K. H. (1996). Population analysis in a denitrifying sand filter: conventional and in situ identification of Paracoccus spp. in methanol-fed biofilms. *Applied and Environmental Microbiology* 62(12): 4329-4339.

Neef, A., Amann, R., Schlesner, H. and Schleifer, K. H. (1998). Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology-Uk* 144: 3257-3266.

Pijuan, M., Guisasola, A., Baeza, J. A., Carrera, J., Casas, C. and Lafuente, J. (2005). Aerobic phosphorus release linked to acetate uptake: Influence of PAO intracellular storage compounds. *Biochemical Engineering Journal* 26(2-3): 184-190.

Schmid, M., Schmitz-Esser, S., Jetten, M. and Wagner, M. (2001). 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. *Environmental Microbiology* 3(7): 450-459.

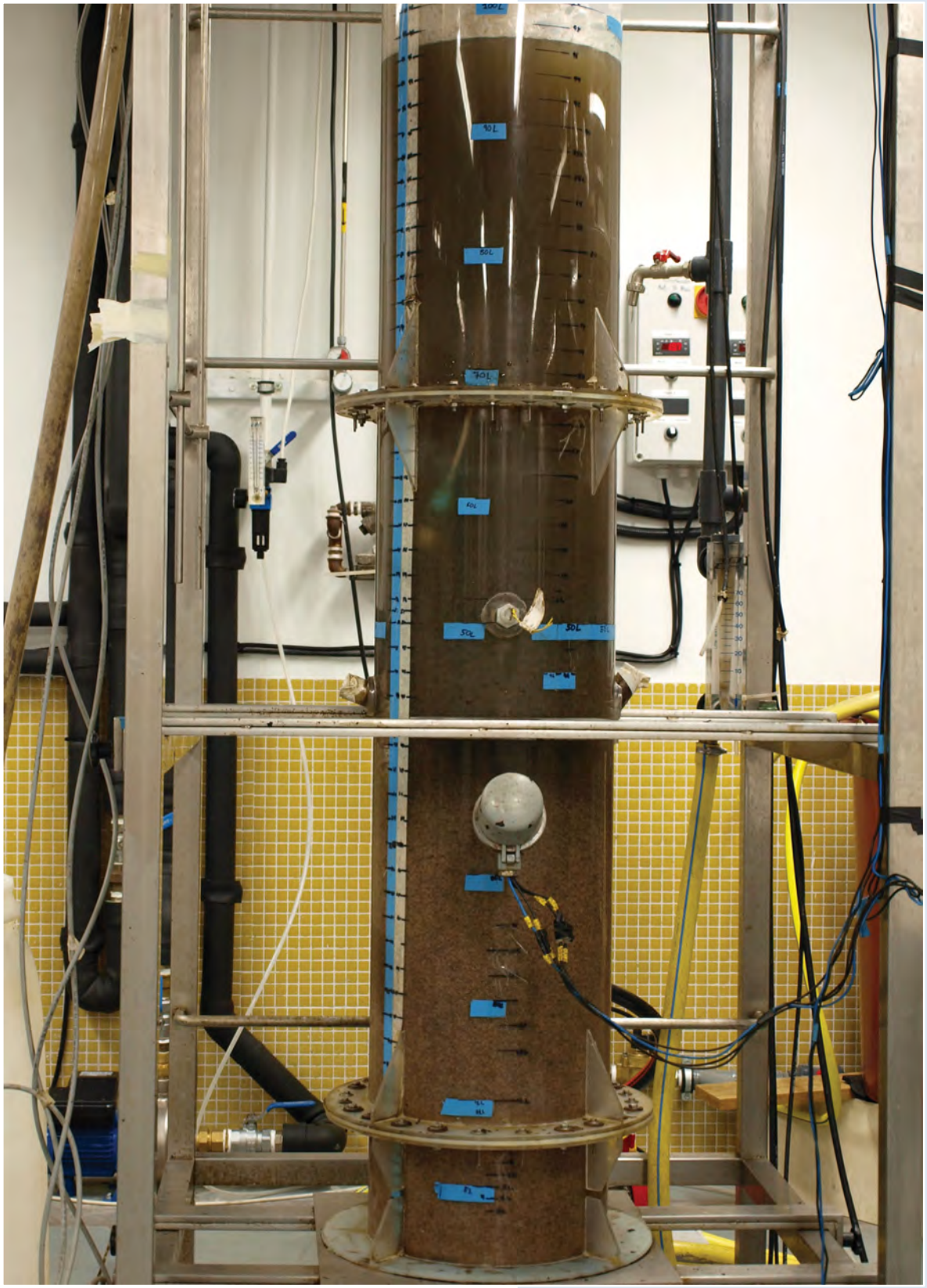
Schwarzenbeck, N., Erley, R. and Wilderer, P. A. (2004). Aerobic granular sludge in an SBR-system treating wastewater rich in particulate matter. *Water Science and Technology* 49(11-12): 41-46.

Soto, M., Veiga, M. C., Mendez, R. and Lema, J. M. (1989). Semi-micro C.O.D. determination method for high-salinity wastewater. *Environmental Technology Letters* 10(5): 541-548.

Strous, M., Kuenen, J. G. and Jetten, M. S. M. (1999). Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology* 65(7): 3248-3250.

Wagner, M., Rath, G., Amann, R., Koops, H. P. and Schleifer, K. H. (1995). In-situ identification of ammonia-oxidizing bacteria. *Systematic and Applied Microbiology* 18(2): 251-264.

Wagner, M., Rath, G., Koops, H. P., Flood, J. and Amann, R. (1996). In situ analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology* 34(1-2): 237-244.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Chapter 3

OPERATION OF AN AEROBIC GRANULAR PILOT SCALE SBR PLANT TO TREAT SWINE SLURRY [1]

Summary

A pilot scale Sequencing Batch Reactor (SBR) was operated in order to remove organic matter and nitrogen from swine slurry characterized by its high variable composition. Aerobic granules successfully developed in the reactor during the first weeks of operation. The physical properties of the biomass remained rather stable during the long-term operation of the reactor (307 days), despite the high variation in the organic and nitrogen applied loading rates (OLR and NLR), which varied from 1.4 to 6.3 kg $COD_s/m^3 \cdot d$ and from 0.5 to 2.5 kg $N/m^3 \cdot d$, respectively. Furthermore, the C/N ratio of the feeding also varied in a wide range (1.9-9.4 g COD_s/g N). The reactor had a good biomass retention capacity to select for granular biomass. However, its efficiency to retain the solids present in the feeding was low. Consequently, the VSS concentration in the effluent was similar to that in the influent. Aerobic granulation in SBR systems appears as an interesting alternative to treat slurry in small livestock facilities, where the implementation of anaerobic digestion systems is not a feasible option or the removal of nitrogenous compounds is required.

[1] Morales N., Figueroa M., Fra-Vázquez A., Val del Río A., Campos J. L., Mosquera-Corral A. and Méndez R. (2013). Operation of an aerobic granular pilot scale SBR plant to treat swine slurry. Process Biochemistry 48(8), 1216-1221.

OUTLINE

3.1 INTRODUCTION

Spain is the second European swine livestock producer, after Germany, and the fourth in the world (FAOSTAT, 2010). Pig population in 2011 in Spain was of around 25.6 million heads, which represented the 17% of the total production in the Europe-27 (Figure 3.1), (Eurostat, 2011). These animals produced around 25 million tones of swine slurry per year (Yagüe and Quílez, 2010). Factors such as type of livestock, diet, housing system or waste handling system affect the concentration of nutrients in pig slurry and the daily manure production values, which vary widely from herd to herd. Traditional waste treatment in farms consisted in the use of manure and slurry as nutrients for agriculture. However, the amount of nutrients that can be applied to soils is limited and depends on the soil characteristics and the current nutrient levels. For instance, the maximum load of total nitrogen to be spread by year on lands in Europe should be less than 170 kg of nitrogen per hectare, while nitrate levels in groundwater are set to a maximum of 50 mg NO_3^-/L or 11.3 mg $N\text{-}NO_3^-$/L (EEC, 1991).

The production of biogas thought an anaerobic digestion of carbonaceous compounds for waste treatment and energy recovery has been encouraged by the European Union through energy policies (European Parliament, 2008). It can reduce volatile organic compounds emissions, control odors, mineralize nutrients, and improve its fertilizing properties and energy recovering. Nevertheless, the anaerobic digestion has the disadvantage of its low efficiency regarding the nitrogen removal, high capital investment, and requirement of specialist technical input and control. In this way, an anaerobic digestion plant with a capacity of 100000 t/year costs around 3 million euro, while it can generate 1.5 million of Nm^3/year of biogas with a 65% of methane (Angulo, 2004). Centralized collective treatment facilities for small plants can solve part of the limitations, when the transport costs are affordable (Flotats *et al.*, 2009).

Nevertheless, the ammonia removal is still not solved. A valid solution when the extension of available land to absorb the nutrients is limited has to be developed. There is not a unique solution and numerous technologies have been used in order to treat the livestock waste as main treatment or pre/post-treatment, mainly with the objective of nitrogen removal based on nitrification and denitrification processes. Nowadays, Anammox treatment is also an option due to its potential of removing nitrogen from wastewater characterized by a low C/N ratio as anaerobic effluents (Vázquez-Padín *et al.*, 2009) with a reduction of costs compared to other technologies (STOWA, 1996). Despite the promising results of this novel technology (Figueroa *et al.*, 2012), until now, there are not so many applications at full scale. In small and isolated farms, swine waste treatment needs the application of robust, but flexible in operation, systems which can remove the organic matter content and the nutrients. Anaerobic lagoons are another option widely used to treat swine slurry, however, there are environmental and health concerns related to this technology (Vanotti *et al.*, 2009).

As the objective is to adapt the nitrogen level to the available land, this treatment technology can be no completely effective or can be applied only to a fraction of the produced slurry. In this way, high nutrient loaded slurry can be applied to the soil, involving low transport costs, while another fraction of slurry is treated.

A nitrification/denitrification system based on the aerobic granular technology can fulfill these requirements (de Kreuk *et al.*, 2005). This technology has been applied for the simultaneous removal of organic matter, nitrogen and phosphorus to different kinds of wastewaters (Arrojo *et al.*, 2004, Adav *et al.*, 2007, Figueroa *et al.*, 2011). It has several advantages compared to activated sludge processes, such as good biomass retention, the presence of aerobic and anoxic zones inside the granules to perform different biological processes simultaneously, and the ability to withstand shock loadings (Adav *et al.*, 2008). In order to achieve the successful granulation of the biomass some factors must be fulfilled, such as the existence of a feast-famine regime, short settling time, or high shear stress conditions (Campos *et al.*, 2009).

Most of the studies concerning the physical properties and performance of aerobic granular biomass were carried out in laboratory scale sequencing batch reactors (SBRs). However, not many studies are available performed in pilot or full scale systems (Tay *et al.*, 2005, Ni *et al.*, 2009, Jungles *et al.*, 2011). In this sense, to test the behavior of this granular biomass, full scale evaluation is important. At full scale, other factors such the hydrodynamics of the bulk liquid, oxygen and substrate transfer e.g., will change due to the scale-up effect.

In addition, when working with industrial wastewater, the fluctuations of inlet feeding characteristics can difficult the achievement of a stable operation and the nutrients removal efficiency requirements. Since the availability to withstand shock loadings (Thanh *et al.*, 2009) is stated as one of the characteristics of the aerobic granular biomass (Adav *et al.*, 2008), the objective of this work was to test the effect of this high variability in the feeding compositions of a pilot scale granular SBR treating swine slurry to remove organic matter and nitrogen.

3.2 MATERIALS AND METHODS

3.2.1 Reactor set-up and operational conditions

Experimental work was performed in a bubble column reactor (Figure 3.2). The reactor had total and working volumes of 125 L and 100 L, respectively. The total reactor height was 177 cm, the working height was 150 cm and the diameter was 30 cm. Then, the effective height to diameter ratio (H/D) was 5. The reactor was operated in cycles of 3 hours distributed in: 3 min of feeding, 165-171 min of aeration, 4-10 min of settling and 2 min of

effluent withdrawal. A ceramic dome fine bubble diffuser placed at the bottom of the reactor was used to supply 65-100 L/min of air.

Temperature (18-22 ºC), pH and Dissolved Oxygen (DO) concentration were measured and registered on-line during the entire experiment. Peristaltic pumps were used to feed the reactor through the top of the reactor, and to discharge the effluent through a port placed at middle height of the reactor. The volumetric exchange ratio was fixed at 50%, while the Hydraulic Retention Time (HRT) was of 6 h.

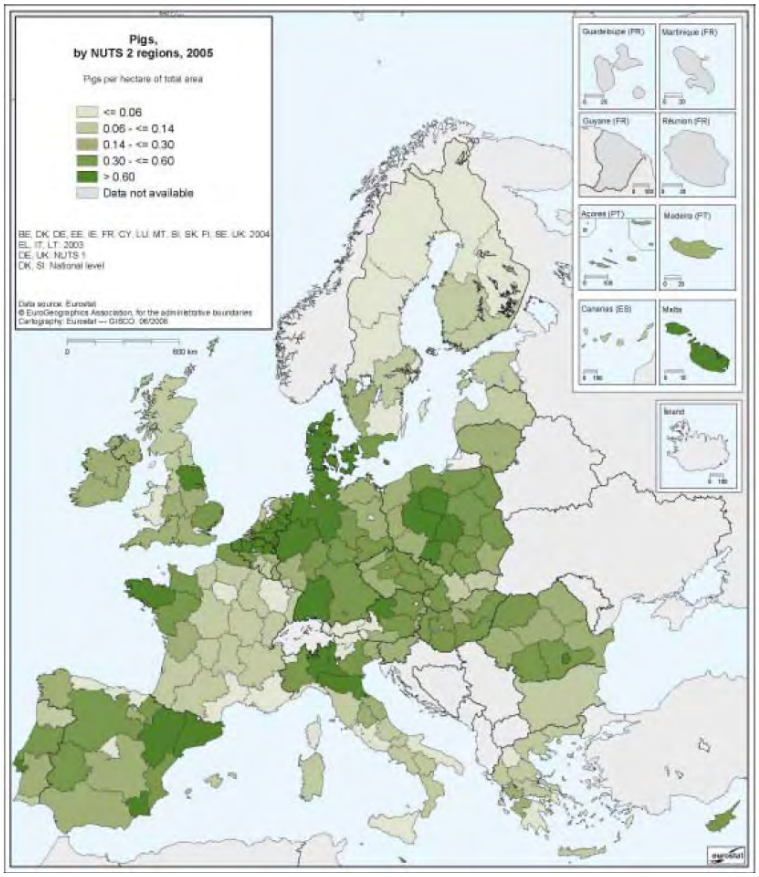


Figure 3.1. Pigs per hectare of total area in Europe in 2005 (Eurostat, 2011).

The inoculum was flocculent activated sludge collected from a municipal wastewater treatment plant (WWTP). The reactor was fed with slurry from a pig farm open basin. The different applied dilutions with tap water (10 to 50%), and the variable composition of each collected stock amount, originated the high variations in the Organic and Nitrogen Loading Rates (OLR and NLR). The reactor was operated during 307 days with two different approaches (Table 3.1). During Stage I the applied OLR was maintained around 1.9 kg COD/m^3·d. Then, on day 76, the share of swine slurry in the feeding was increased and the

operation with high variability in terms of feeding characteristics was tested. The OLR ranged from 1.75 to 6.26 kg COD/m^3·d during Stage II.

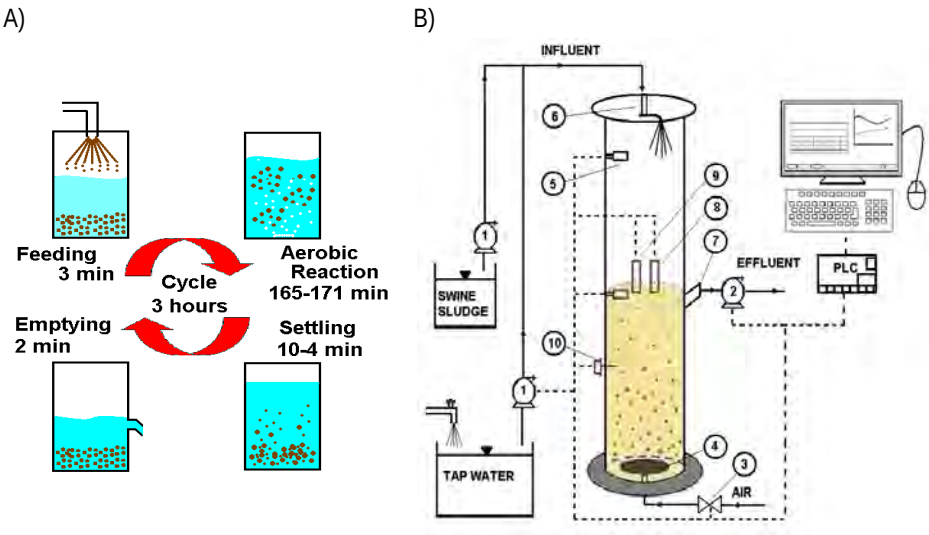


Figure 3.2. a) Distribution of the operational cycle and b) scheme of the pilot granular SBR: (1) Feeding pumps. (2) Effluent pump. (3) Air valve. (4) Ceramic dome fine bubble diffuser. (5) Maximum level sensor. (6) Influent port. (7) Effluent port. (8) Dissolved oxygen sensor. (9) pH sensor. (10) Temperature sensor.

3.2.2 Analytical methods

The pH value, nitrate, nitrite and ammonium concentrations, Sludge Volume Index at 30 and 10 minutes (SVI_{30} and SVI_{10} respectively) and Total and Volatile Suspended Solids (TSS, VSS) concentrations were determined according to the Standard Methods (APHA, 1998). A luminescence-based DO probe (LDO Process sensor, Hach Lange) connected to a controller (SC-100, Hach Lange) was used to measure the DO concentration. The poly-hydroxyalkanoates (PHA) concentration was measured using a modification of the method developed by Pijuan *et al.* (2005), where an amount around 30 mg of lyophilized sludge samples was digested and methylated with 4 mL of acidulated methanol (10% H_2SO_4) and 4 mL of chloroform during 20 h at 100 ºC. Benzoic acid was used as internal standard and the analyses were performed in a Gas Chromatography system (Agilent 6850). The morphology and size distribution of granules were regularly measured by means of an image analysis procedure using a stereomicroscope (ZEISS 2000-C).

Microbial populations were detected by the Fluorescence in Situ Hybridization (FISH) technique. Biomass samples from the reactor were collected, disrupted and fixed according to the procedure described by Amann *et al.* (1995) with freshly prepared 4% paraformaldehyde solution. Hybridization was performed at 46 °C for 2.5 h followed by a washing step at 48 °C for 15 min. General probes used for *in situ* detection of bacterial

cells: EUB338mix, for all *Bacteria*, and ALF1b and BET42a probes for α- and β-subclass of the *Proteobacteria*. More specific probes were: NSO190 for *β-Proteobacteria* ammonia-oxidizing, Nsm156 for *Nitrosomonas* spp., Nitspa712, for *Nitrospira* spp., CFX1223+GNSB941, specific for phylum *Chloroflexi*, and SNA probe that targets *Sphaerotilus natans*. The probes were 5' labeled with fluorochromes FITC and Cy3 and all bacteria stained by DAPI. Fluorescence signals were recorded with an acquisition system coupled to an Axioskop 2 epifluorescence microscope (Zeiss, Germany).

Table 3.1. Feeding composition.

Parameter	Stage I	Stage II
Days	1-76	77-307
COD_t (mg/L)	660 ± 95	1890 ± 552
COD_s (mg/L)	487 ± 80	1003 ± 261
OLR (kg COD_s /(m^3·d))	1.91 ± 0.34	4.00 ± 1.02
NH_4^+-N (mg/L)	148 ± 70	249 ± 68
NLR (kg N /(m^3·d))	0.31 ± 0.06	1.00 ± 0.27
COD_s:N ratio (g/g)	3.89 ± 1.44	4.27 ± 2.33
TSS (g/L)	0.11 ± 0.06	0.47 ± 0.17
VSS/TSS (g/g)	0.92 ± 0.04	0.87 ± 0.08

3.2.3 Calculations

The Sludge Retention Time (SRT) was calculated as proposed by Liu and Tay (2007). The amount of biomass produced in a period of operation was estimated from the biomass increase in the reactor and the amount of biomass washed out in the effluent using equation (3.1).

$$\Delta W_P = \Delta X_r \cdot V_r + X_{eff} \cdot Q \cdot \Delta t \quad (3.1)$$

Where: ΔW_P: amount of produced biomass (g VSS), ΔX_r: change of biomass concentration in the reactor during each period (g VSS/L), V_r: reactor volume (L), X_{eff}: average biomass concentration washed out in the effluent (g VSS/L), Q: flow rate (L/d), Δt: length of each period (d).

Considering a general composition of the biomass as $C_5H_7NO_2$ the averaged amount of nitrogen assimilated for biomass growth ($N_{assimilated}$ in g N) was calculated using equation (3.2).

$$N_{assimilated} = \Delta W_P \cdot \frac{14 \text{ g-mol N}}{113 \text{ g -mol biomass}} \quad (3.2)$$

$N_{removed}$ (g N) during such period of operation was calculated using equations (3.3) and (3.4), being ΔN the nitrogen balance, NH_4^+-N_{inf}, NO_2^--N_{inf}, and NO_3^--N_{inf} the ammonium, nitrite, nitrate concentrations (mg N/L) in the influent, and NH_4^+-N_{eff}, NO_2^--N_{eff}, and NO_3^--N_{eff} the ammonium, nitrite and nitrate concentrations (mg N/L) in the effluent respectively.

$$\Delta N = (NH_4^+\text{-}N_{inf} + NO_2^-\text{-}N_{inf} + NO_3^-\text{-}N_{inf}) - (NH_4^+\text{-}N_{eff} + NO_2^-\text{-}N_{eff} + NO_3^-\text{-}N_{eff}) \quad (3.3)$$

$$N_{removed} = \Delta N \cdot Q \cdot \Delta t \quad (3.4)$$

Nitrogen removed by denitrification ($N_{denitrified}$) was calculated by the difference between $N_{removed}$ and $N_{assimilated}$.

3.3 RESULTS AND DISCUSSION

3.3.1 Granule formation and physical properties

The pilot reactor was operated during 307 days. During the first 15 days of operation the settling time was progressively shortened from 10 to 5 min, which provoked the almost complete wash-out of the inoculated activated sludge and the selection of biomass with better settling properties. The minimum settling velocity ($V_{s\ min}$) for the biomass to be retained in the reactor was 9.0 m/h. In this way, on day 9 of operation, the first granules with an average diameter of 1.1 mm and filamentous structures in their surface were observed (Figure 3.3 A).

The size of the granules fluctuated during Stage I from 1.1 to 2.3 mm, presumably due to the episodes of breakage and new granules formation (Figure 3.3 B, C and D and Figure 3.5), which are part of the granular biomass evolution (Tay *et al.*, 2005). In Stage II the average diameter of the granules increased due to the increase in the applied OLR (Figure 3.4). This value reached a maximum value of 3.2 mm on day 99 and varied most of the time between 2.0 and 2.8 mm (Figure 3.5). Granules with smooth surfaces dominated in the reactor after start-up period. Moy *et al.* (2002) also observed an increase in the average size of the granules and a change in the appearance of the granules when they increased the applied OLR, from a fluffy to a smooth outer appearance. The size of the granules formed during the experimental period, which ranged from 1.1 to 3.2 mm, was among the optimal values suggested by Toh *et al.* (2003) for and optimal performance and economically effective aerobic granular SBR reactor, which ranged from 1.0 to 3.0 mm.

The time needed to achieve granular biomass is highly dependent on the operational conditions, wastewater composition and reactor type. The size of the reactor is not a handicap for the granulation process while the key parameters are controlled. Several examples are available regarding aerobic granules formation in aerobic pilot reactors. In

this way, Jungles *et al.* (2011) operated the same pilot scale SBR reactor of this study, but fed with a synthetic solution and using a $V_{s\ min}$ of 7.5 m/h. In that case, small granules were obtained after 6 days of operation; however, the biomass was a mixture of small granules with a high amount of suspended biomass. These authors later increased the $V_{s\ min}$ to 11 m/h, and finally to 15 m/h, in order to obtain good aerobic granules. In the present work, with swine slurry wastewater instead of a synthetic solution, a $V_{s\ min}$ of 9.0 m/h was enough to achieve a complete granulation. Figueroa *et al.* (2011), working also with pig slurry, obtained granules with a diameter of 1.9 mm after 10 days of operation of a laboratory scale SBR reactor treating an OLR of 2.2 kg COD/m^3·d and applying a $V_{s\ min}$ of about 8 m/h. Ni *et al.* (2009) obtained the first granules, which had a relatively small size of about 0.2-0.8 mm, after 80 days of operation in a pilot scale SBR reactor which treated a low-strength municipal wastewater (OLR being 1.0 kg COD/m^3·d). Moreover, Isanta *et al.* (2012) obtained complete granulation after 51 days treating a low-strength municipal wastewater at pilot scale.

On the other hand, when the reactor is seeded with stored granules, the time needed for the formation of new granules is clearly shorter. In this way, Liu et al. (2005) obtained new granules after only 5 days of operation, when they operated a pilot scale SBR reactor, using a synthetic wastewater with an OLR of 1.2-2.4 kg COD/m^3·d, as the initial selection of biomass with good settling properties during the start-up period is skipped.

In the present work, the settling properties of biomass rapidly improved along the operation period (Figure 3.5). The SVI_{30} of the inoculum was about 380 mL/g TSS, while the biomass present in the reactor during the first week already had a SVI_{10} below 80 mL/g TSS. This parameter varied between 27 and 60 mL/g TSS throughout the remaining experimental period. The improvement of the settling properties of the biomass is one of the main characteristics of aerobic granular technology (Arrojo *et al.*, 2004). SVI for aerobic granules is usually below 80 mL/g TSS, while the SVI for floccular biomass is above 120 mL/g TSS (Toh *et al.*, 2003). Previously, the aerobic granules obtained by Figueroa *et al.* (2011), in a reactor fed with swine slurry, showed SVI_{10} values between 20 and 75 mL/g TSS, whereas the values of the biomass from the pilot plant SBR operated by Jungles *et al.* (2011) decreased from 190 to 26 mL/g TSS during the operation period. Also Isanta *et al.* (2012) reduced the SVI from 200 to 13-16 mL/g TSS in their pilot scale SBR reactor. In fact, Toh *et al.* (2003) found a relation between the granule size and the SVI values. The smallest granule size had a smallest SVI, and that value increased gradually as the granules grew bigger in size. A similar relation was found in the aerobic granules grown in this study (Figure 3.5 and Figure 3.6). In this way, during Stage II, average diameter and SVI decreased continuously. Later, until around day 225, the average diameter increased, and an increase in the SVI was measured. However, the SVI gradually recovered the previous values (around 30 mL/g TSS) despite the size of the granules did not decrease in the same way.

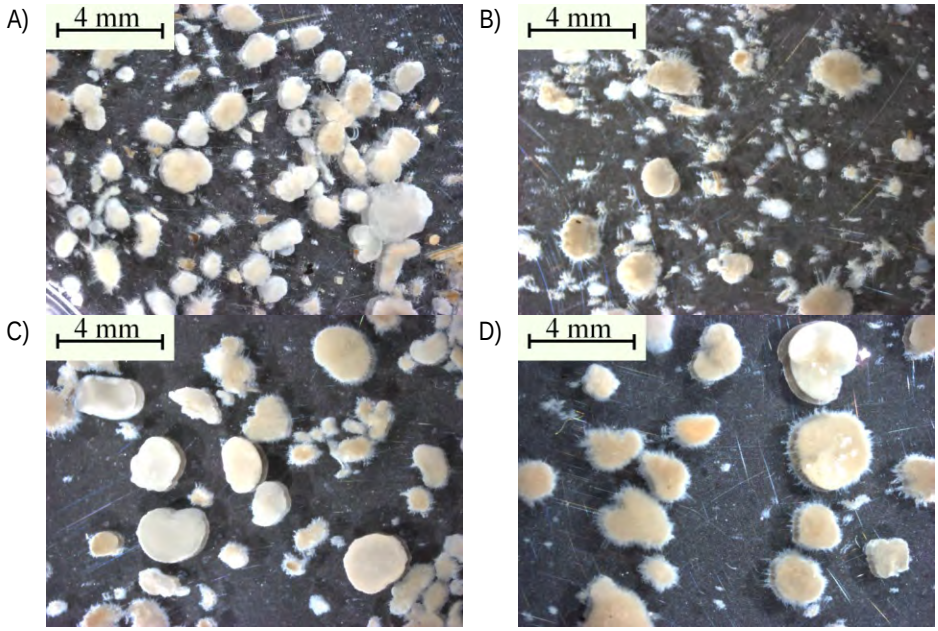


Figure 3.3. Images showing the aerobic granules evolution in the SBR reactor during Stage I. A) day 9, B) day 15, C) day 19 and D) day 26.

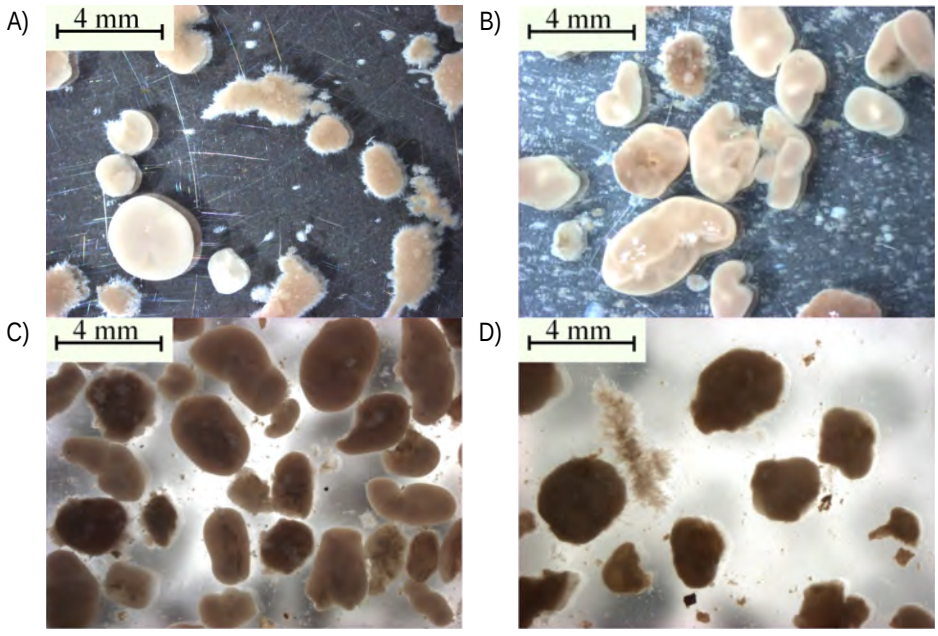


Figure 3.4. Images showing the aerobic granules evolution in the SBR reactor during Stage II. A) day 41, B) day 99, C) day 120 and D) day 286.

The high biomass retention is one of the main advantages of aerobic granulation. Solids concentration inside the reactor reached a value up to 2-3 g VSS/L during the start-up period (Figure 3.5).

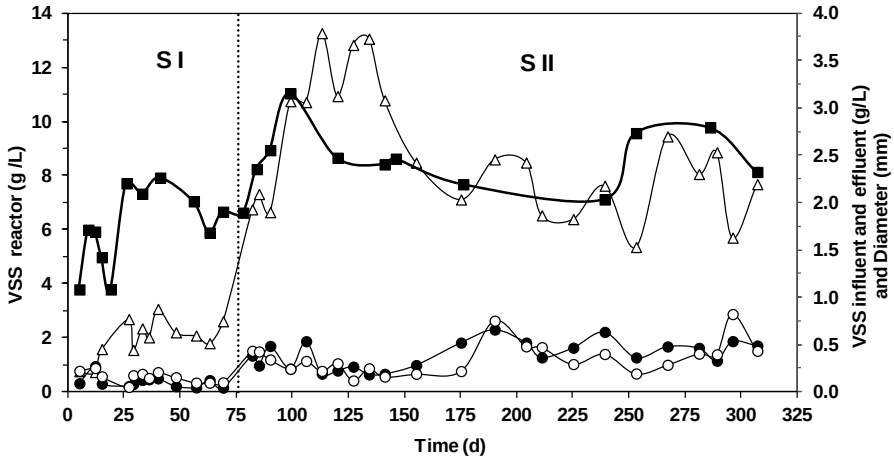


Figure 3.5. Solids concentration in the influent (●), in the reactor (Δ) and in the effluent (○) in g VSS/L and average diameter of the granules in mm (■).

The biodegradability of the aerobic granular biomass produced in the reactor was studied by Val del Río *et al.* (2011), with and without thermal treatment. These authors concluded that anaerobic biodegradability of aerobic granules, which was around 33%, was similar to that obtained for an activated sludge (30-50%) and demonstrated the feasibility of their anaerobic digestion. The thermal pre-treatment before the anaerobic digestion was proposed as a good option to enhance the biodegradability: between 20% at 60 ºC and 88% at 170 ºC with respect to the untreated sludge (Val del Río *et al.*, 2011).

The biomass concentration in the effluent during Stage I was always below 0.25 g VSS/L, indicating a good biomass retention capacity of the reactor, as no granules were observed in the effluent. Jungles *et al.* (2011) reported a maximum solids concentration in the effluent below 0.20 g VSS/L during the start up of the pilot scale SBR reactor. With the increase of the applied OLR in Stage II, the biomass concentration in the reactor reached values as high as 11-13 g VSS/L.

During both stages, the solids concentration in the effluent was dependent on its concentration in the influent, since the system was able to retain the biomass that grew in form of aerobic granules, but those solids that enter with the feeding were washed out. In average, the solids concentration in the effluent was around 0.05 and 0.01 g VSS/L higher than in the influent during Stages I and II, respectively. A pre- or post- treatment of these solids should be integrated in the overall treatment in order to achieve the limit values required for the discharge effluent quality (de Bruin *et al.*, 2004). The effect of particulate or

colloidal suspended solids in aerobic granules was studied by Schwarzenbeck *et al.* (2004) showing that the COD removal efficiency is strongly dependent on the particle size and that it increases with decreasing the particle size. These authors showed that particles bigger than 50 μm were removed at 40% efficiency from a SBR reactor with a $V_{s\ min}$ of 3.0-7.5 m/h. Inizan *et al.* (2005) also observed that a relatively high concentration of suspended solids in the inlet were not removed from the reactor and this fact deteriorated its performance with regard to the total COD removal. However, these authors obtained good removal efficiencies when industrial wastewaters containing biodegradable COD_s were treated.

The average SRT value calculated in the pilot scale reactor was around 3.8 days in Stage I (Figure 3.6). This value increased in Stage II, matching up with the higher biomass concentration, and reached an average value of 11.7 d. Once the biomass stabilized around 8 g VSS/L (Figure 3.5), from day 175 on, the SRT stabilized to around 5.1 d (Figure 3.6).

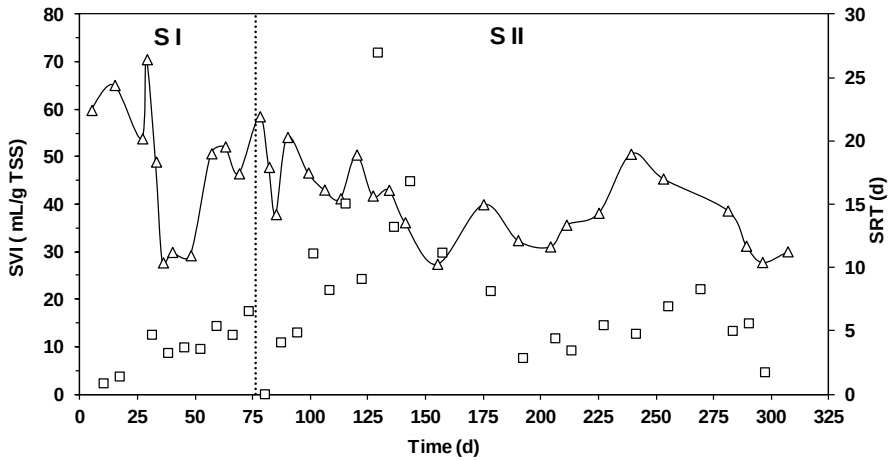


Figure 3.6. SVI_{10} of the biomass in mL/g TSS (Δ) and SRT of the biomass in days (□).

3.3.2 Organic matter and Nitrogen removal

In Stage I, the applied OLR was of 1.91 ± 0.34 kg $COD_s/m^3 \cdot d$ (Figure 3.7) to promote the formation and development of aerobic granules as this value was previously found appropriated when the reactor was fed with a synthetic feeding (Jungles *et al.*, 2011). Then, in Stage II, the OLR was increased and varied in a wide range (minimum 1.75 and maximum 6.26 kg $COD_s/m^3 \cdot d$) in order to test the stability performance of the reactor. These modifications in the OLRs applied did not alter the physical properties of the granules, and the performance of the reactor in terms of removal efficiency was in average of about 73 ± 12 % in stage I and 61 ± 18 % in stage II. Average pH in the influent was 7.6, while that parameter in the effluent was 7.2 probably caused by the nitrification process and the low amount of denitrification. Ammonia stripping due to high ammonia concentration of

swine slurry and the extended aeration time applied was considered negligible at these pH values.

At the end of the experiment, the removal efficiency dropped dramatically due to the increase of the non-biodegradable fraction of COD present in the pig slurry fed to the reactor. A variation of the biodegradable/non-biodegradable COD ratio in the feeding was observed during the operational time, caused by the degradation of the biodegradable COD in the farm and the subsequent increase of the non- biodegradable fraction. The presence of a high fraction of non-biodegradable organic matter provoked elevated COD_s concentrations in the effluent with the consequent decrease of removal efficiency. The non-biodegradable fraction widely varied, corresponding to up to 80% of the inlet COD_s in some periods, higher than those values of 10% reported for anaerobic biodegradability (González-Fernández *et al.*, 2008) and for aerobic granular treatment (Figueroa *et al.*, 2011). These authors also found that the removal efficiency and granules properties remained stable when OLRs from 2 to 8 kg $COD_s/m^3 \cdot d$ were applied.

Thus, highly variable characteristics of the influent and particularly the presence of a non-biodegradable fraction provoked the maintenance of a good effluent quality in swine wastewater treatments difficult (Shin *et al.*, 2005).

As a consequence of the high variability of the applied OLR and the feeding COD/N ratio, the NLR ranged between 0.3 and 1.4 kg $N/m^3 \cdot d$ (Figure 3.8). The average ammonia removal efficiency was around 56.4 ± 13.9% in Stage I and 76.6 ± 8.7% in Stage II. Ammonia was mainly oxidized to nitrite, although some amounts of nitrate were also measured in the effluent (Figure 3.9).

Isanta *et al.* (2012) also observed a partial nitrification (80% of nitrification to nitrite) when they operated a pilot scale granular SBR reactor. These authors suggested that granules size can limit the oxygen flux towards the inner part of the granules. Having in mind that the higher the granule size, the lower the specific granular surface where nitrite oxidizing bacteria (NOB) can grow due to the stratification between heterotrophs, ammonia oxidizing bacteria (AOB) and NOB usually observed in biofilms (Terada *et al.*, 2003). As a consequence AOB outcompete NOB due to low oxygen concentrations as suggested by Hanaki *et al.* (1990) in a suspended-growth reactor.

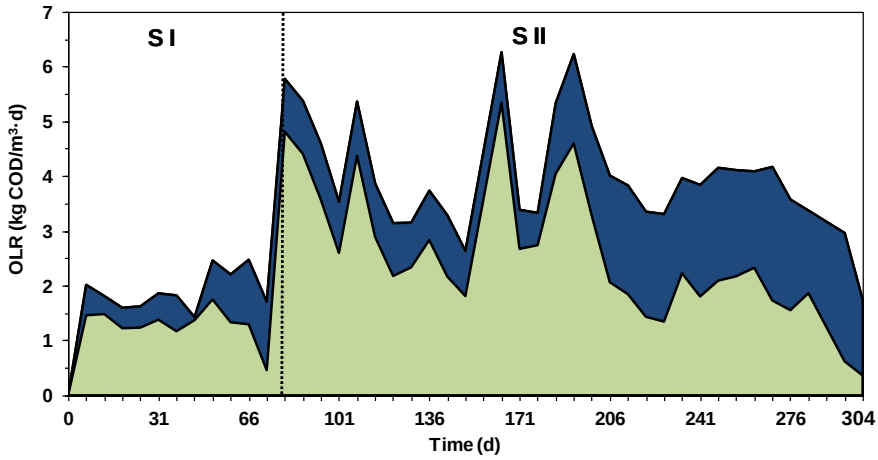


Figure 3.7. OLR fed to (■) and removed from (■) the SBR in kg $COD_S/m^3 \cdot d$.

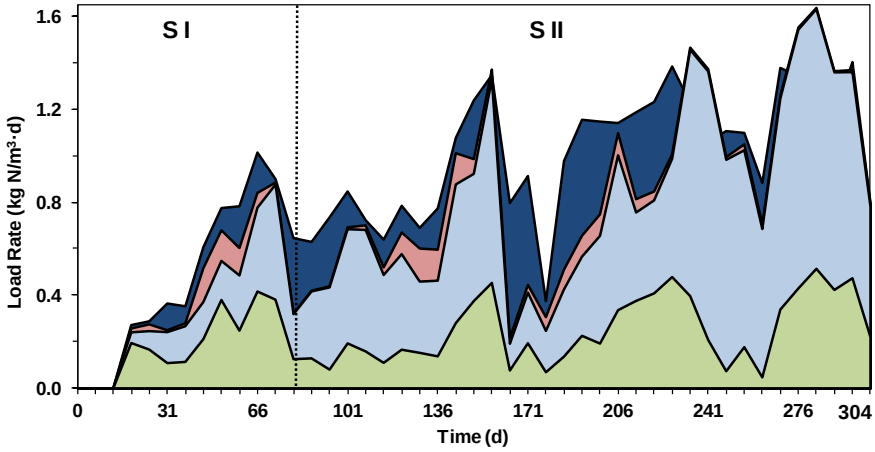


Figure 3.8. NLR in the influent (■) in kg $N/m^3 \cdot d$, and Ammonia (■), Nitrite (■) and Nitrate (■) loads in the effluent (accumulated values) in kg $N/m^3 \cdot d$.

Overall nitrogen removal efficiencies reached values up to 30% in Stage I (Figure 3.8), but the average nitrogen removal during that stage was about 16.4 ± 8.3%. In Stage II, the high variability of the feeding composition provoked the variability of the nitrogen removal efficiency that reached values up to 73%; however, it was highly unstable, especially after day 230 of operation. Until day 230 the average nitrogen removal efficiency was 27.4 ± 18.5%. Then, the overall nitrogen removal was almost completely lost, and nitrite accumulated in the reactor.

During periods of high biomass growth, the $N_{removed}$ was used for assimilation as well as denitrified. In this way, at the beginning of Stage I from days 17 to 45, when the overall N

removal was around 19%, slightly higher than the average in Stage I, the solids concentration increased from 1.15 to 3.06 g VSS/L and the $N_{assimilated}$ accounted for 42% of the $N_{removed}$. In stage II, from days 80 to 115, with an overall N removal about 27.3% (the average of Stage II), the $N_{assimilated}$ added up to 16% of the $N_{removed}$.

In consequence, the system was not achieving a good nitrogen removal with the operation strategy tested via denitrification or simultaneous nitrification-denitrification. These results contradict previous ones obtained from laboratory scale experiments (Figueroa *et al.*, 2011), where overall nitrogen removal reached values of 70% with denitrification and simultaneous nitrification-denitrification observed in the operational cycles.

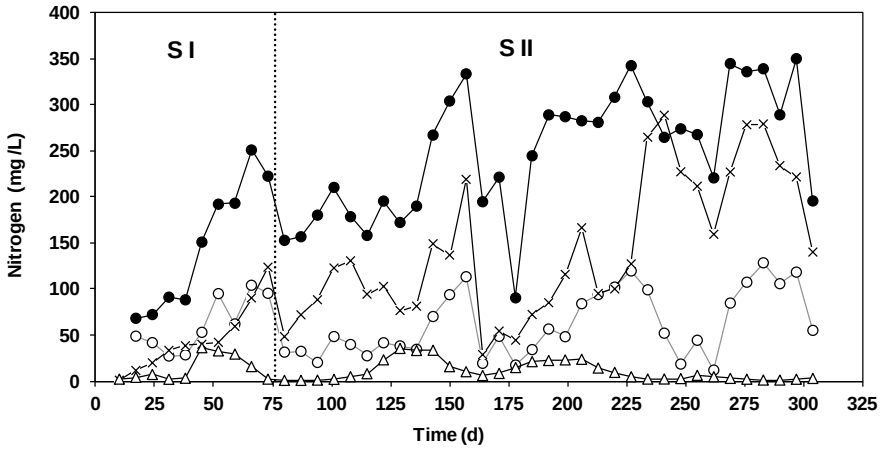


Figure 3.9. Concentrations of NH_4^+-N in the influent (●), and NH_4^+-N (○), NO_2^--N (×) and NO_3^--N (△) in the effluent.

3.3.3 Operational cycles

In order to evaluate the performance of the reactor, cycle measurements were carried out. COD_s, PHA and nitrogenous compounds concentrations, pH, and DO values were measured along operational cycles, on different days of operation. The data from day 56 are shown in Figure 3.10 and Figure 3.11.

The biodegradable organic matter was easily removed in the first minutes of the aeration phase in all cycles, in no more than 20-25 minutes, during the so called feast period as it can be observed from the obtained concentration profiles. During this time, a reduction in the DO concentration due to the quick organic matter oxidation was measured. Once the biodegradable organic matter was consumed, the DO concentration in the bulk liquid started to increase during the famine period, although it did not reach the saturation level due to the ammonia oxidation and endogenous respiration, while the fraction of non-

biodegradable COD remained unaltered. An increase in COD_s was measured at the end of each cycle, which can be due to hydrolysis processes of the colloidal particles of the swine slurry.

Concentrations of PHA measured in the biomass (Figure 3.10) confirmed that bacteria stored organic matter in the form of these compounds during feast period and consumed them during first minutes of famine period.

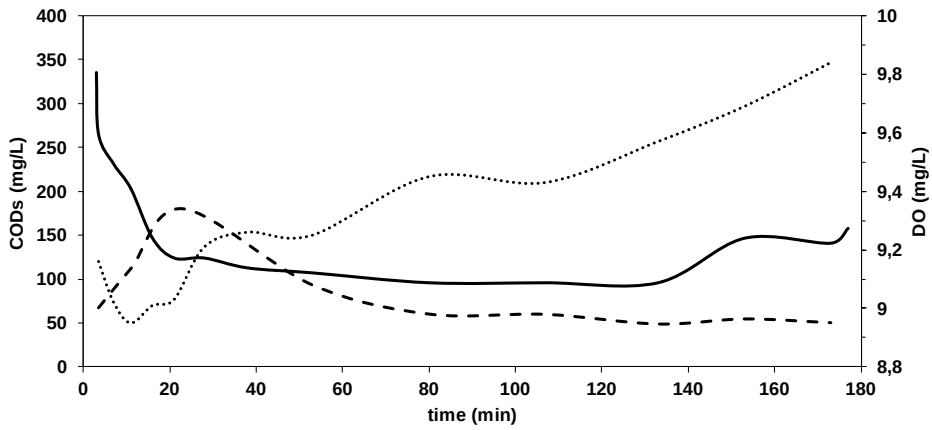


Figure 3.10. A) Evolution of COD_s (——), DO (····) and PHA (— —) concentrations (mg/L) during an operational cycle in the SBR at day 56 (Stage I).

The consumption rate for ammonia measured during the cycle in Stage I on day 56 was 212 mg NH_4^+-N/g VSS·d. In Stage II that value ranged from 44.3 to 213 mg NH_4^+-N/g VSS·d on days 133 and 295, respectively. Once the organic matter was depleted, nitrate and nitrite concentrations increased in the bulk liquid, but total nitrogen concentration remained almost constant. This observation suggests that simultaneous nitrification-denitrification processes did not occur during aeration phase, contrary to that observed in other experiments (Figueroa *et al.*, 2011, Jungles *et al.*, 2011). In the experiments performed by these authors the denitrification process using storage compounds can justify the removal of nitrogen during famine phase as suggested by Qin *et al.* (2005).

After the PHA consumption in the approximately first 60 minutes of famine period, PHA values continued rather constant most of the remained time of the cycle, indicating that organic matter was no longer available for denitrification.

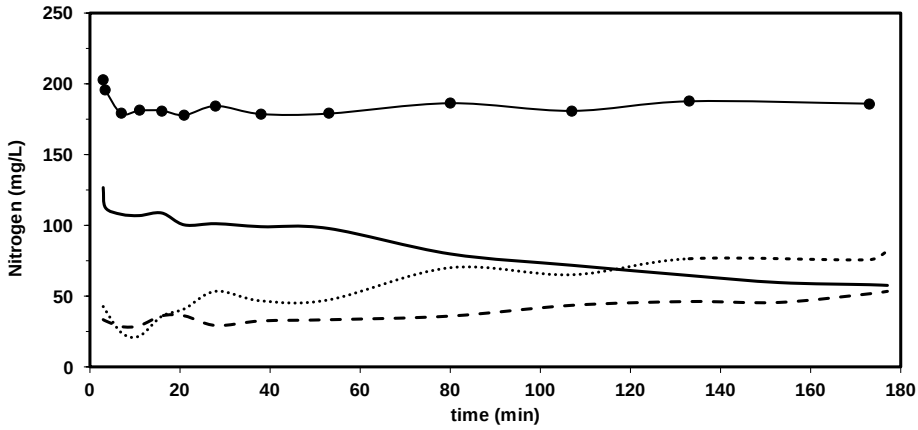


Figure 3.11. Evolution of NH_4^+-N (——), NO_2^--N (····), NO_3^--N (— —) and TN: NH_4^+-N + NO_2^--N + NO_3^--N (—●—) concentration in mg N/L during an operational cycle in the SBR on day 56 (Stage I).

Figueroa *et al.* (2011) observed simultaneous nitrification-denitrification in their operational cycles, in spite of aeration in the 15 first minutes of the cycle, and once the organic matter was depleted it occurred using pre-accumulated poly-b-hydroxybutyric acid (PHB). Isanta *et al.* (2012) also observed nitrite accumulation in their SBR cycle studies, as 56% of total nitrogen at the end of the cycle was converted to nitrite and 19% to nitrate. However, these authors observed the complete nitrite and nitrate consumption through denitrification after the static feeding period, which lasted one hour, when readily degradable COD was available and without aeration.

The short feeding period used in our study hindered a higher denitrification using the COD added during the feeding in a similar way to that observed by Isanta *et al.* (2012). On the other hand, the consumption of readily biodegradable organic matter at the beginning of the cycle and the small amount of PHA available in the granule, made simultaneous nitrification-denitrification not possible, as it happened in the reactor operated by Figueroa *et al.* (2011).

The accumulation of non-readily biodegradable CODs can conduct to low available COD/N ratios, which are not suitable for denitrification, even via nitrite, which has a requirement of 2-4 g/g for the COD/N ratio (Mulder, 2003). In this case, a different alternative must be used. The autotrophic ammonia removal by means of Anammox based processes (Vázquez-Padín *et al.*, 2011) can be suitable as partial nitrification was already obtained in the granular SBR reactor.

3.3.4 Microbial populations

General probes were applied to samples collected during the reactor operation to detect the main classes of bacteria involved in the process (EUB338mix, ALF1b and BET42a probes). The hybridized bacteria belonged mainly to *β-Proteobacteria* subclass, and represented an important fraction in comparison with all the positives from EUB338mix probe. On the other hand, no positive results were obtained for *α-Proteobacteria*.

Positive results were obtained from CFX1223+GNSB941 probes, specific for phylum *Chloroflexi* (Figure 3.12 A), and SNA probe, which targets *Sphaerotilus natans* within *β-Proteobacteria* (Figure 3.12 B), on samples collected during the first days of operation. Days after, a reduction of the fraction of these filamentous-shape bacteria was observed. Probably these bacteria came with the flocculent activated sludge used as inoculums, and then were washed from the system due to their poor settling properties.

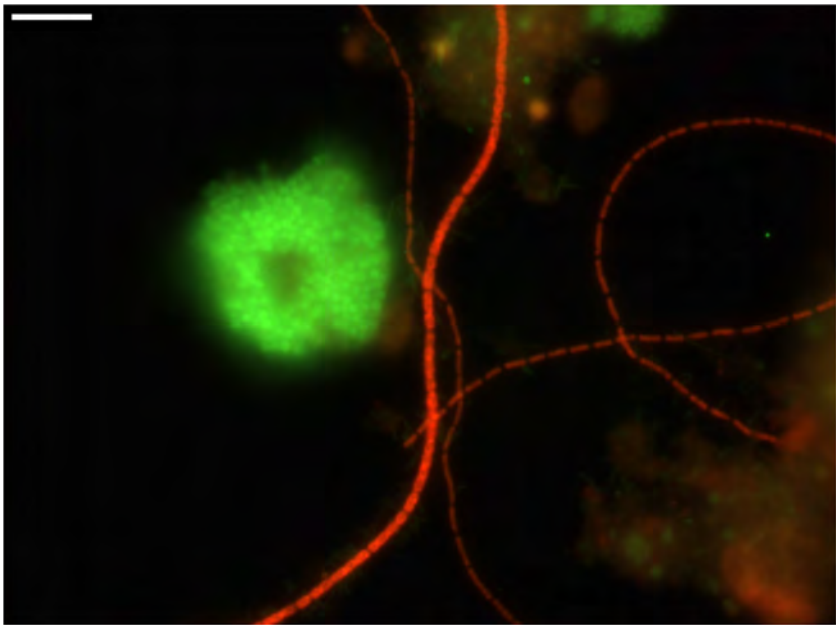
Positive results were obtained with NSO190 probe, designed for *β-Proteobacteria* (AOB), in correspondence with the detection of ammonia oxidation activity in the reactor. Individual cells of AOB were detected when the ammonia oxidation activity started (around day 15, Figure 3.13 A) while AOB cells appeared grouped into clusters when this activity increased (Figure 3.13 B). The Nsm156 probe, specific for *Nitrosomonas* spp., was applied and positive results can indicate the presence of *Nitrosomonas communis* and *Nitrosomonas oligotropha* (Figure 3.14), which are among the most commonly AOB species found in nitrifying WWTPs (Nielsen *et al.*, 2009).

The Ntspa712 probe was applied to test the presence of nitrite oxidizing bacteria (NOB) belonging to phylum *Nitrospirae*. These are the dominant NOB in the majority of nitrifying WWTPs (Daims *et al.*, 2001). However, only a small fraction of bacteria hybridized with this probe (Figure 3.15). These results are in correspondence with the results obtained in the reactor, as ammonia was mainly oxidized to nitrite, and only a small fraction of nitrate was produced.

The differences in the relative abundance of each group of bacteria (heterotrophic, AOB and NOB) can be due to the competition for the same subtract, in this case the dissolved oxygen, where NOB were the less favored. Isanta *et al.* (2012) observed a ratio of the AOB and NOB fractions in the range 5-8 using FISH analysis when obtained partial nitrification in their reactor.

From the obtained results an evolution of the bacterial populations present in the inoculum to those observed in the granular biomass occurs. Further work is needed to establish a correlation between the microbial populations identified and the granulation process.

A)



B)

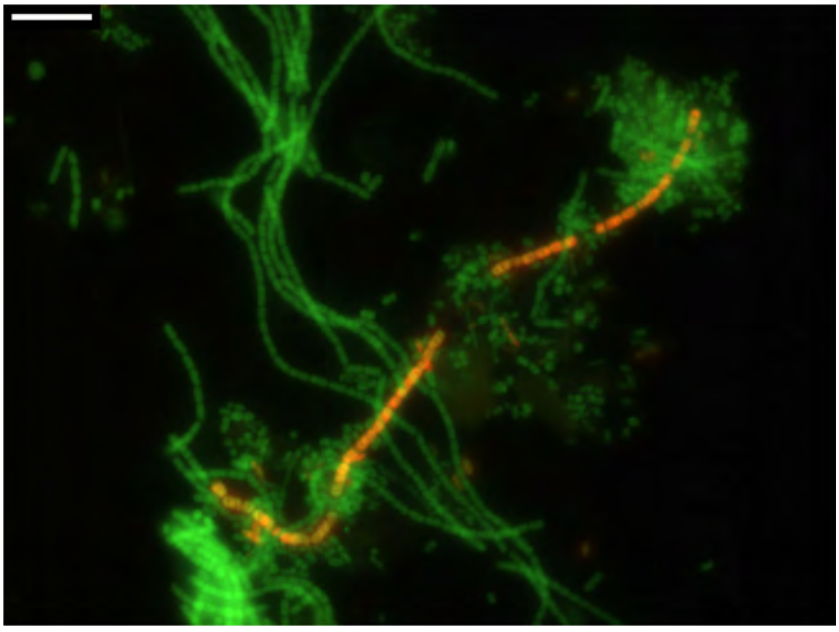
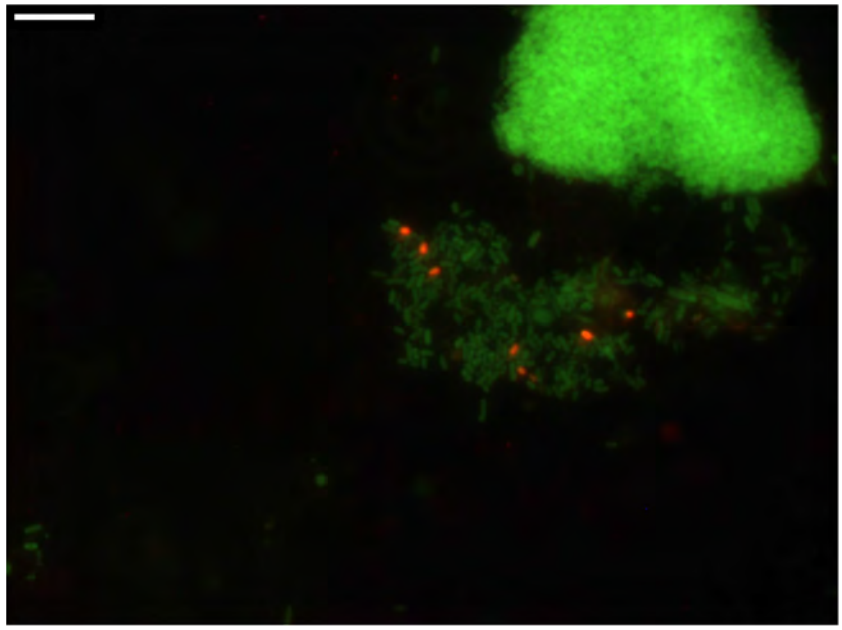


Figure 3.12. FISH analysis of a biomass sample from day 2 of operation. The bar represents 10 µm.
A) Filamentous shape bacteria of phylum *Chloroflexi* (CFX1223+GNSB941 probes: Cy3, red) and all bacteria (EUB338mix: FITC, green).
B) Filamentous bacteria *Sphaerotilus natans* (SNA probe: Cy3, red) and all bacteria (EUB338mix: FITC, green).

A)



B)

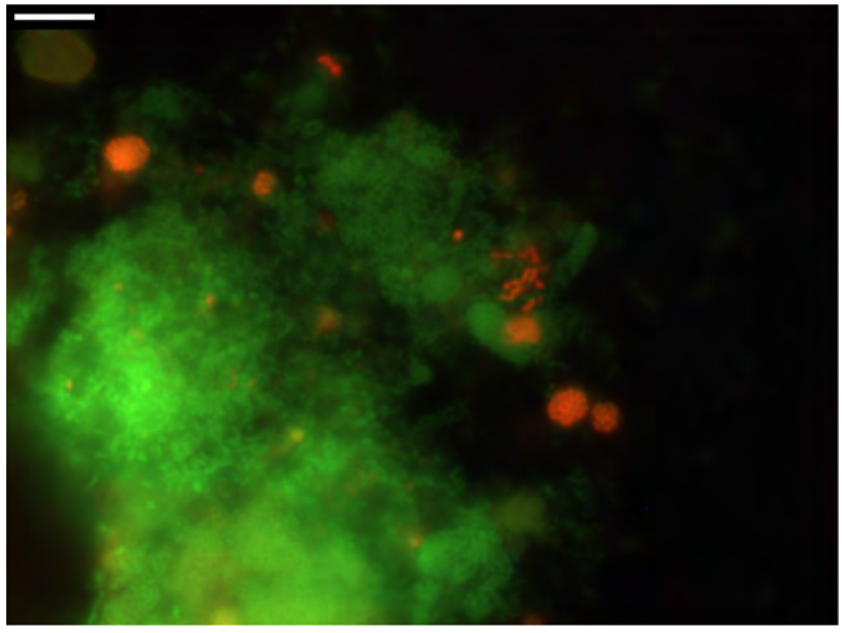


Figure 3.13. FISH analysis for AOB stained with Nso190 probe (Cy3, red) and all bacteria with EUB338mix (FITC, green). A) Biomass sample from day 16. B) Biomass sample from day 35. The bar represents 10 µm.

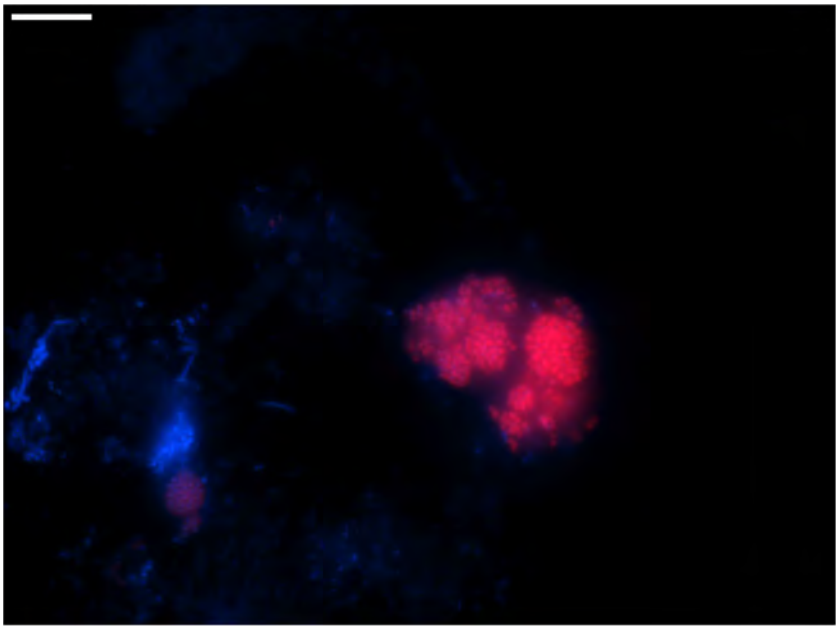


Figure 3.14. FISH analysis of a biomass sample from day 56 of operation. The bar represents 10 μm. Bacteria of *Nitrosomonas* spp. (Nsm156 probe; Cy3, red) and DAPI (blue).

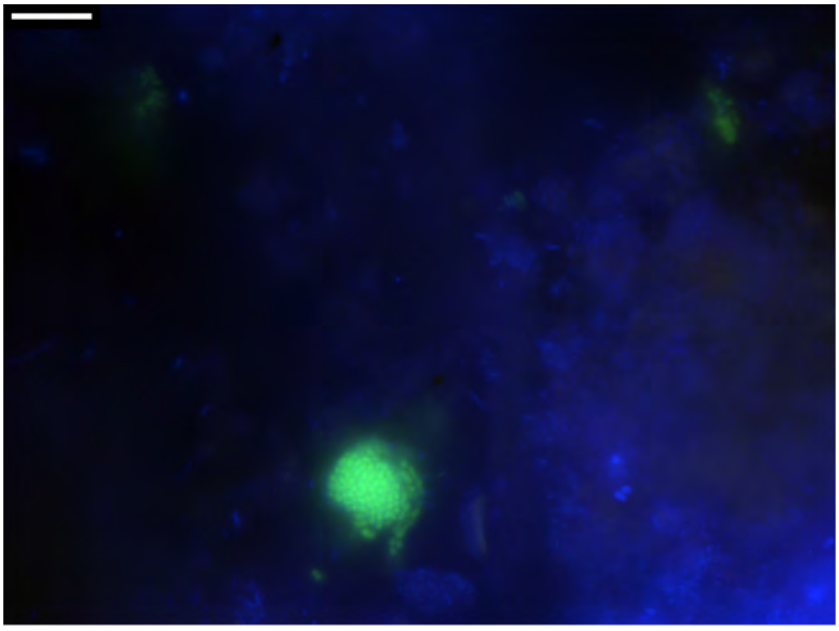


Figure 3.15. FISH analysis of a biomass sample from day 56 of operation. The bar represents 10 μm. Bacteria of phylum *Nitrospirae* (Ntspa712 probe; FITC, green) and DAPI (blue).

3.4 CONCLUSIONS

The aerobic granular technology was tested for its implementation as nitrogen and organic matter removal system for small swine farms. Granules were formed in a pilot scale reactor after 9 days of operation when the reactor was fed with an OLR of 1.9 kg $COD_s/m^3 \cdot d$ and the minimum settling velocity imposed to the biomass to be retained in the reactor was of 9 m/h. The average granule diameter stabilized around 3 mm, with a SVI_{10} lower than 50 mL/g TSS, and the solids concentration in the reactor ranged between 5 and 12 g VSS/L.

Even though the reactor had a good biomass selection capacity, it was not able to retain the solids present in the pig slurry. Therefore, a pre- or post- treatment of these solids should be integrated in the overall treatment in order to achieve the required values for the effluent.

Aerobic granules could withstand the variations in the OLR (1.74-6.26 kg $COD_s/m^3 \cdot d$), NLR (0.3-1.7 kg $N/m^3 \cdot d$), and COD/N ratio (1.9-9.4 g COD_s/g N) that can be usually observed in this kind of effluents. Organic matter removal efficiencies were not affected by OLRs fluctuations, but by the non-biodegradable fraction of the swine slurry.

Ammonia load was mainly oxidized to nitrite; the ammonia removal efficiency was around 76% even with the high variability of the wastewater fed to the reactor in Stage II. However, denitrification was practically not observed during the experiment as it was revealed by the cycle analysis.

The detected bacterial populations indicated an evolution from the inoculated sludge to those composing the aerobic granules. Filamentous organisms were present mainly in the inoculums while they were washed out from the system as granules developed.

The nitrifying microbial population was mainly composed by members of *Nitrosomonas* spp. as ammonia oxidizing bacteria, and a fewer amount of nitrite oxidizing bacteria belonging to phylum *Nitrospirae*, in correspondence with the nitrite accumulation observed in the reactor.

3.5 REFERENCES

Adav, S. S., Lee, D. J. and Ren, N. Q. (2007). Biodegradation of pyridine using aerobic granules in the presence of phenol. *Water Research* 41(13): 2903-2910.

Adav, S. S., Lee, D. J., Show, K. Y. and Tay, J. H. (2008). Aerobic granular sludge: Recent advances. *Biotechnology Advances* 26(5): 411-423.

Amann, R., Ludwig, W. and Schleifer, K. H. (1995). Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiological Reviews* 59(1): 143-169.

Angulo, J. (2004). La digestión anaerobia en el tratamiento de efluentes de granjas de porcino. (Spanish) (The anaerobic digestion in the treatment of swine farm effluents.) *Ingeniería Química* 409(Enero): 102-108.

APHA (1998). *Standard methods for the examination of water and wastewater*, American Public Health Association/American Water Works Association/Water Environment Federation. Washington DC, USA

Arrojo, B., Mosquera-Corral, A., Garrido, J. M. and Méndez, R. (2004). Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research* 38(14-15): 3389-3399.

Campos, J. L., Figueroa, M., Mosquera-Corral, A. and Mendez, R. (2009). Aerobic sludge granulation: state-of-the-art. *International Journal of Environmental Engineering* 1(2): 136-151.

Daims, H., Nielsen, J. L., Nielsen, P. H., Schleifer, K. H. and Wagner, M. (2001). In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology* 67(11): 5273-5284.

de Bruin, L. M. M., de Kreuk, M. K., van der Roest, H. F. R., Uijterlinde, C. and van Loosdrecht, M. C. M. (2004). Aerobic granular sludge technology: an alternative to activated sludge? *Water Science and Technology* 49(11-12): 1-7.

de Kreuk, M., Heijnen, J. J. and van Loosdrecht, M. C. M. (2005). Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. *Biotechnology and Bioengineering* 90(6): 761-769.

EEC (1991). Council Directive 91/676/EEC, of 12 December 1991, Concerning the Protection of Waters Against Pollution Caused by Nitrates from Agricultural Sources. European Economic Community. Brussels, Belgium.

European Parliament (2008). Report on Sustainable Agriculture and Biogas: a need for review of EU Legislation (2007/2107(INI)), European Parliament, Committee on Agricultural and Rural Development.

Eurostat. (2011). "Food: From farm to fork statistics. Livestock. Pig population (WEB)." 2012.

FAOSTAT. (2010). "Agricultural production data (Live animals)." 2012.

Figueroa, M., Val Del Río, A., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Treatment of high loaded swine slurry in an aerobic granular reactor. *Water science and technology* 63(9): 1808-1814.

Figueroa, M., Vázquez-Padín, J. R., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2012). Is the CANON reactor an alternative for nitrogen removal from pre-treated swine slurry? *Biochemical Engineering Journal* 65(0): 23-29.

Flotats, X., Bonmatí, A., Fernández, B. and Magrí, A. (2009). Manure treatment technologies: On-farm versus centralized strategies. NE Spain as case study. *Bioresource Technology* 100(22): 5519-5526.

González-Fernández, C., Nieto-Diez, P. P., León-Cofreces, C. and García-Encina, P. A. (2008). Solids and nutrients removals from the liquid fraction of swine slurry through screening and flocculation treatment and influence of these processes on anaerobic biodegradability. *Bioresource Technology* 99(14): 6233-6239.

Hanaki, K., Wantawin, C. and Ohgaki, S. (1990). Nitrification at low levels of dissolved oxygen with and without organic loading in a suspended-growth reactor. *Water Research* 24(3): 297-302.

Inizan, M., Freval, A., Cigana, J. and Meinhold, J. (2005). Aerobic granulation in a sequencing batch reactor (SBR) for industrial wastewater treatment. *Water Science and Technology* 52(10-11): 335-343.

Isanta, E., Suárez-Ojeda, M. E., Val del Río, Á., Morales, N., Pérez, J. and Carrera, J. (2012). Long term operation of a granular sequencing batch reactor at pilot scale treating a low-strength wastewater. *Chemical Engineering Journal (Lausanne)* 198–199(0): 163-170.

Jungles, M. K., Figueroa, M., Morales, N., Val del Río, A., da Costa, R. H. R., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Start up of a pilot scale aerobic granular reactor for organic matter and nitrogen removal. *Journal of Chemical Technology and Biotechnology* 86(5): 763-768.

Liu, Q. S., Liu, Y., Tay, S. T. L., Show, K. Y., Ivanov, V., Benjamin, M. and Tay, J. H. (2005). Startup of pilot-scale aerobic granular sludge reactor by stored granules. *Environmental Technology* 26(12): 1363-1369.

Liu, Y. Q. and Tay, J. H. (2007). Influence of cycle time on kinetic behaviors of steady-state aerobic granules in sequencing batch reactors. *Enzyme and Microbial Technology* 41(4): 516-522.

Moy, B. Y. P., Tay, J. H., Toh, S. K., Liu, Y. and Tay, S. T. L. (2002). High organic loading influences the physical characteristics of aerobic sludge granules. *Letters in Applied Microbiology* 34(6): 407-412.

Mulder, A. (2003). The quest for sustainable nitrogen removal technologies. *Water Science and Technology* 48(1): 67-75.

Ni, B. J., Xie, W. M., Liu, S. G., Yu, H. Q., Wang, Y. Z., Wang, G. and Dai, X. L. (2009). Granulation of activated sludge in a pilot-scale sequencing batch reactor for the treatment of low-strength municipal wastewater. *Water Research* 43(3): 751-761.

Nielsen, H., Daims, H. and Lemmer, H. (2009). *FISH handbook for biological wastewater treatment*, IWA Publishing. London, UK

Pijuan, M., Guisasola, A., Baeza, J. A., Carrera, J., Casas, C. and Lafuente, J. (2005). Aerobic phosphorus release linked to acetate uptake: Influence of PAO intracellular storage compounds. *Biochemical Engineering Journal* 26(2-3): 184-190.

Qin, L., Liu, Y. and Tay, J. H. (2005). Denitrification on poly-beta-hydroxybutyrate in microbial granular sludge sequencing batch reactor. *Water Research* 39(8): 1503-1510.

Schwarzenbeck, N., Erley, R. and Wilderer, P. A. (2004). Aerobic granular sludge in an SBR-system treating wastewater rich in particulate matter. *Water Science and Technology* 49(11-12): 41-46.

Shin, J. H., Lee, S. M., Jung, J. Y., Chung, Y. C. and Noh, S. H. (2005). Enhanced COD and nitrogen removals for the treatment of swine wastewater by combining submerged membrane bioreactor (MBR) and anaerobic upflow bed filter (AUBF) reactor. *Process Biochemistry* 40(12): 3769-3776.

STOWA (1996). One reactor system for ammonia removal via nitrite (In dutch). Report no 96-01. STOWA. Utrecht, The Netherlands.

Tay, J. H., Liu, Q. S., Liu, Y., Show, K., Ivanov, V. and Tay, S. T. L. (2005). A comparative study of aerobic granulation in pilot- and laboratory-scale SBRs. In: *Aerobic Granular Sludge.* (ed), IWA Publishing. Munich: 125-133.

Terada, A., Hibiya, K., Nagai, J., Tsuneda, S. and Hirata, A. (2003). Nitrogen removal characteristics and biofilm analysis of a membrane-aerated biofilm reactor applicable to high-strength nitrogenous wastewater treatment. *Journal of Bioscience and Bioengineering* 95(2): 170-178.

Thanh, B. X., Visvanathan, C. and Aim, R. B. (2009). Characterization of aerobic granular sludge at various organic loading rates. *Process Biochemistry* 44(2): 242-245.

Toh, S. K., Tay, J. H., Moy, B. Y. P., Ivanov, V. and Tay, S. T. L. (2003). Size-effect on the physical characteristics of the aerobic granule in a SBR. *Applied Microbiology and Biotechnology* 60(6): 687-695.

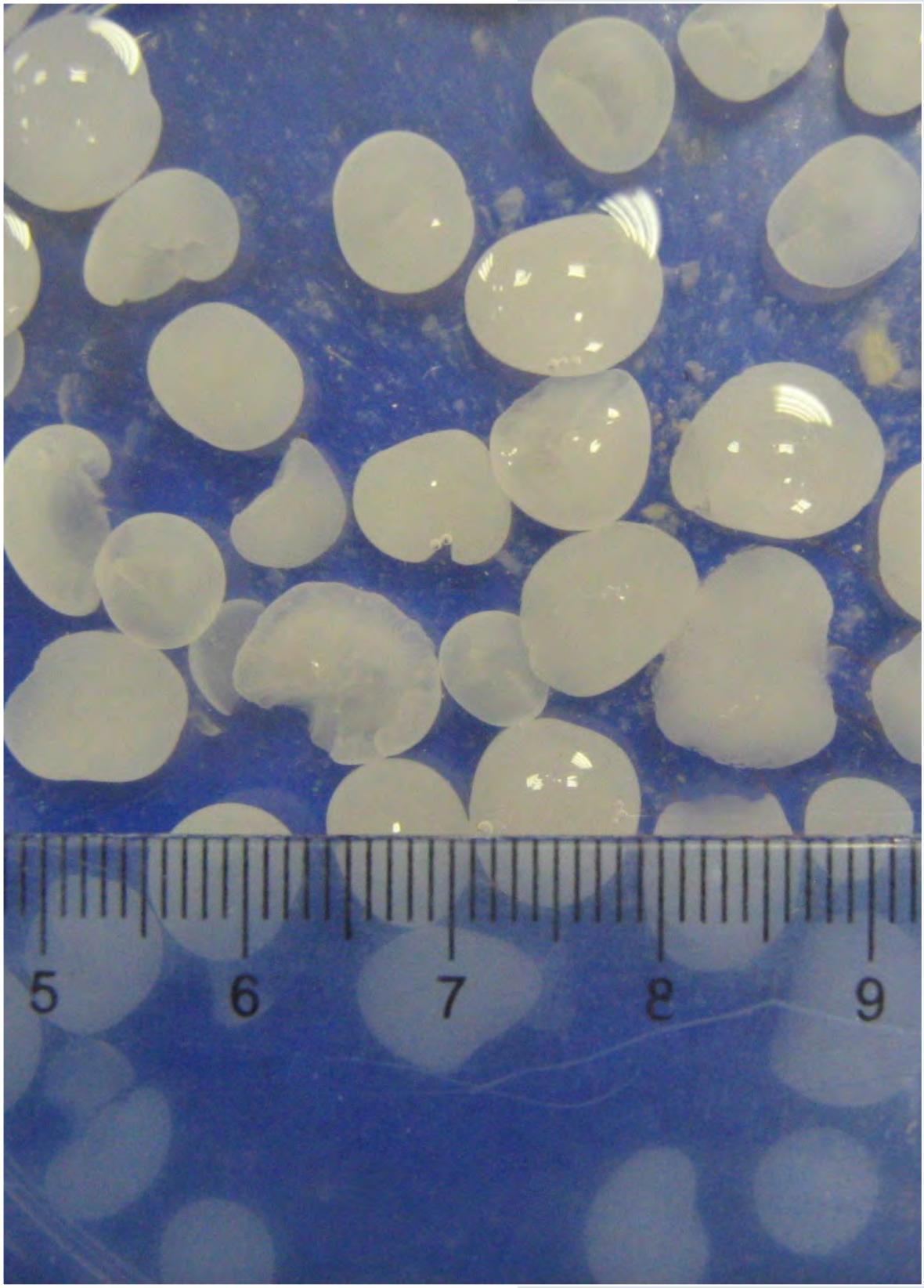
Val del Río, A., Morales, N., Isanta, E., Mosquera-Corral, A., Campos, J. L., Steyer, J. P. and Carrère, H. (2011). Thermal pre-treatment of aerobic granular sludge: Impact on anaerobic biodegradability. *Water Research* 45(18): 6011-6020.

Vanotti, M. B., Szogi, A. A., Millner, P. D. and Loughrin, J. H. (2009). Development of a second-generation environmentally superior technology for treatment of swine manure in the USA. *Bioresource Technology* 100(22): 5406-5416.

Vázquez-Padín, J. R., Figueroa, M., Fernández, I., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2009). Post-treatment of effluents from anaerobic digesters by the Anammox process. *Water science and technology* 60(5): 1135-1143.

Vázquez-Padín, J. R., Fernández, I., Morales, N., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Autotrophic nitrogen removal at low temperature. *Water science and technology* 63(6): 1282-1288.

Yagüe, M. and Quílez, D. (2010). Direct and residual response of wheat to swine slurry application method. *Nutrient Cycling in Agroecosystems* 86(1): 161-174.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Chapter 4

AEROBIC GRANULAR-TYPE BIOMASS DEVELOPMENT IN A CONTINUOUS STIRRED TANK REACTOR [1]

Summary

A continuous stirred tank reactor (CSTR) was operated in order to define the appropriate operational conditions to obtain aerobic biomass grown in the form of granules. A selection pressure of 10 m/h of minimum settling velocity for the biomass to be retained in the reactor ($V_{s\ min}$) was imposed while the hydraulic retention time (HRT) was gradually decreased from 6 to 1 h to promote the wash-out of the suspended biomass. At HRT values of 6 and 3 h, filamentous bacteria were dominant in the biomass. The formation of aerobic granular-type biomass was only achieved when the HRT was fixed at 1 h. The physical properties of these granules were: sludge volume index of 127 mL/g TSS, density of 11 g VSS/$L_{granule}$, settling velocity of 36-48 m/h and average diameter of 6.8 mm. Abundance of filamentous species was different at the tested HRTs. When the HRT was fixed at 6 hours filamentous shape bacteria appeared in large amounts, while at the HRT of 1 hour the bacterial shape was mainly bacillus, while filamentous shape bacteria were absent. Microbial populations were mainly composed by members of *Comamonadaceae* and *Rhodocyclaceae* families within β-*Proteobacteria*, and members of the subclass γ-*Proteobacteria*.

[1] Morales, N., Figueroa, M., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2012). Aerobic granular-type biomass development in a continuous stirred tank reactor. *Separation and Purification Technology* 89(0), 199-205.

OUTLINE

4.1 INTRODUCTION

The biological treatment of effluents in wastewater treatment plants (WWTPs) is often accomplished by means of the application of conventional activated sludge systems. In these systems, the aerobic organic matter oxidation occurs in a continuous stirred tank reactor. These treatment technologies generally require large surface areas for their implantation and the presence of biomass separation systems, due to the usually poor settling properties of the activated sludge. The low settling velocity of biomass flocs also limits the maximum biomass concentration inside the reactors to about 3 g VSS/L. Taking into account a typical design value of food to microorganism ratio (F/M) of 1 g COD/g COD, used in activated sludge processes, the maximum achievable reactor capacity is about 3 kg COD/m^3·d.

One possible action to reduce the volume of the required units is the development of systems based on the improvement of biomass retention, for example, by modifying the operational conditions in order to obtain granular sludge. Granular growth is a particular case of biofilm growth, in which no carrier material is required. Bacteria are self immobilized forming microbial aggregates. Initial applications of granular biomass were associated to anaerobic process: Upflow Anaerobic Sludge Blanket (UASB), Expanded Granular Sludge Blanket (EGSB) or Internal Circulating (IC) Reactors.

Despite first studies of aerobic granulation were performed using continuous systems using the AUSB (Aerobic Upflow Sludge Blanket) reactor (Mishima and Nakamura, 1991, Shin *et al.*, 1992), only a few studies were developed with aerobic granules in continuous reactors. In addition, most of these studies used column-type reactors, as Biofilm Airlift Suspension (BAS) reactor (van Benthum *et al.*, 1996), Nitrifying Activated Sludge Airlift (NASA) reactor (Campos *et al.*, 2000), Aerobic Upflow Fluidized Bed (AUFB) reactor (Tsuneda *et al.*, 2003), or more recently with the complex combination of reactors Continuous-Flow Granular Self-Forming Dynamic Membrane Bioreactor (CGSFDMBR) (Liu *et al.*, 2012).

Nowadays, the development of aerobic granules is mostly focused on the application of column-type SBR reactors with a large height to diameter ratio (H/D). The use of this kind of reactors allows maintaining not only a high hydraulic selection pressure on the biomass by applying short settling periods (Liu and Tay, 2004) but also other environmental conditions, such as alternative feast-famine periods and high shear forces, which promote the aggregation of the biomass (Beun *et al.*, 1999).

Granular SBRs were successfully used to treat various kinds of effluents at lab scale (Arrojo *et al.*, 2004, Schwarzenbeck *et al.*, 2005, Figueroa *et al.*, 2008). Nowadays, there are several pilot scale reactors in operation (de Bruin *et al.*, 2005, Inizan *et al.*, 2005, Ni *et al.*, 2009, Jungles *et al.*, 2011).

Nevertheless, the application of the concepts of the granular SBR technology for the upgrading of existing WWTPs could be limited by the different operational conditions and different geometry of both column-type SBR and conventional activated sludge reactors. Then, transforming a continuous system into a SBR suitable to obtain aerobic granules is difficult. Consequently, the research on the feasibility of the production of aerobic granules in a continuous reactor would open a new perspective to the application of the aerobic granular technology for the improvement of already existing WWTPs.

Therefore, the objective of the present work was to define the suitable operational conditions to develop granular biomass in continuous stirred tank reactors (H/D<1). A biomass selection system based on the reactor hydrodynamics and progressive shorting of the HRT applied to the reactor was used. The performance of the aerobic granular system was evaluated in terms of carbon and nitrogen removal efficiencies, and special attention was paid to the physical properties and bacterial populations of the granular biomass.

4.2 MATERIALS AND METHODS

4.2.1 Reactor description

Two cylindrical continuous stirred tank reactors (CSTRs) with a working volume of 6 L and 3 L (R1 and R2, respectively) were operated in a continuous mode (Figure 4.1). Their H/D ratios were 0.7 and 0.5 m/m, respectively (Figure 4.1). Dissolved oxygen (DO) was supplied continuously by means of air flow (2-6 L/min) through air spargers to promote the formation of small bubbles. A stirrer provided with flat impellers was used in some experimental stages (Table 4.1).

A set of two peristaltic pumps was used to introduce in a continuous mode the concentrated feeding solution (flow 1-6 mL/min) and the dilution water (flow 10-100 mL/min), while a third peristaltic pump simultaneously discharged the effluent (flow 10-100 mL/min). To avoid unbalances between influent and effluent flows which were difficult to adjust in exact values, a level controller turned off/on the effluent pump. It can be estimated that pumps were in operation simultaneously 99% of the time, so the flow per minute was essentially identical. The reactor was operated at room temperature (15-25 ºC), and without pH control, which ranged between 7.1 and 8.3.

In order to select the biomass with a high settling velocity, the effluent was discharged through a tube semi-submerged in the liquid media which fixed the upflow velocity at 10 m/h (v_{up}). With this design, particles with a settling velocity (v_{set}) smaller than this fixed upflow velocity will be washed out from the reactor. Biomass with good settling properties will be retained in the system, and eventually it will form granules, or at least it will become a biomass with better settling properties than a conventional activated sludge. Since the influent flow rate changed during the different operational stages, the diameter of the

discharge tube was changed to maintain a constant upflow velocity. Tubes with 1.2, 1.6, 2.0 and 2.8 cm of diameter were used.

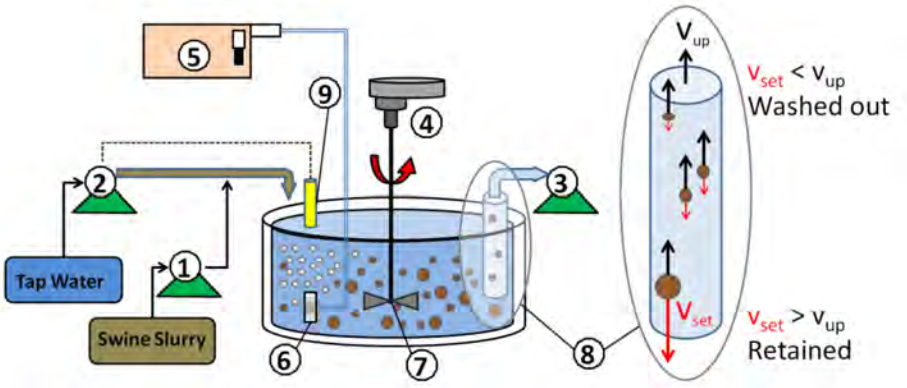

Figure 4.1. Reactor layout: Feeding pumps (1 and 2), effluent discharging pump (3), stirrer (4), air pump (5), spargers (6), flat impeller (7), effluent discharge tube (8) and liquid level controller (9), solids settling velocity (v_{set}) and upflow liquid velocity (v_{up}).

A) B)

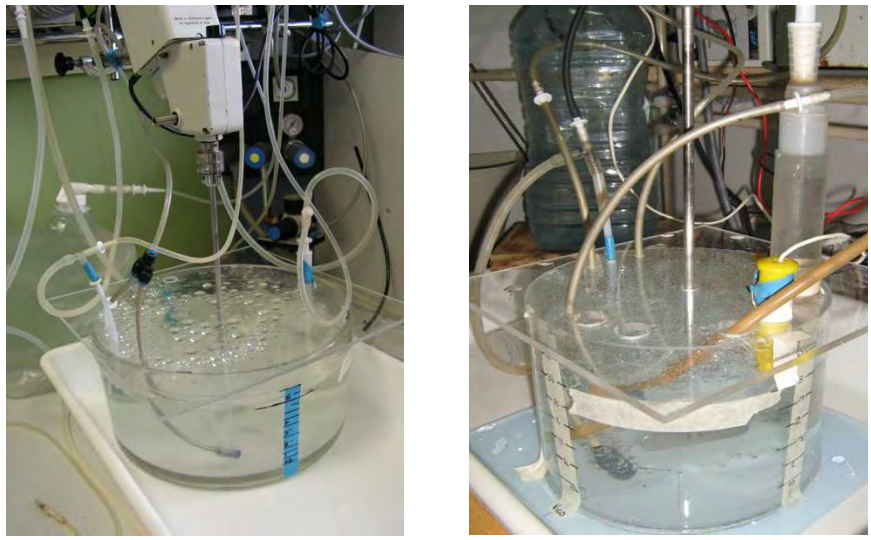

Figure 4.2. Images of used reactors: A) R1 (6 L) and b) R2 (3 L).

Table 4.1. Operational conditions and feeding composition of R1 and R2.

Reactor	R1					R2
Stages	S I		S II		S III	S I
Days (d)	0-78	79-108	109-131	132-184	185-260	1-120
HRT (h)	6		3		1	1
Mechanical stirring	Yes	Yes	No	Yes	Yes	Yes
Effluent tube diameter (cm)	1.2	1.6	1.6	1.6	2.8	2.0
COD (mg COD/L)	600	600	600	600	200-500	200-250
OLR (g COD/L·d)	2.4	4.8	4.8	4.8	4.8-12.0	4.8-6.0
NLR (g N/L·d)	0.5	0.8	1.2	1.2	0.9-2.2	1.4-1.7
DO (mg/L)	5.0	3.8	3.0	2.5	6.1	8.6
VSS influent (g/L)	0.06-0.19	0.14-0.26	0.07-0.11	0.06-0.10	0.02-0.10	0.02-0.06
pH influent	7.1-8.1	7.1-8.1	7.9-8.1	7.5-8.1	6.9-7.3	7.3-8.3

4.2.2 Feeding composition

The feeding supplied to the reactors was the liquid fraction of pig slurry, which was diluted with tap water to fix the influent COD concentration at the required values (Table 4.1). The slurry was collected monthly from a pig farm located in San Marcos (Santiago de Compostela) and it was sieved through 1 mm diameter mesh and settled overnight. It was kept refrigerated at 4 °C to avoid degradation before entering the reactor.

4.2.3 Inoculum

Reactor R1 was initially inoculated with flocculent activated sludge collected from a municipal WWTP. A sample of 500 mL of settled sludge was added to the reactor. On day 205 of operation the reactor was re-started up using pig slurry as inoculum. The initial biomass concentration was of 0.75 g VSS/L. Pig slurry itself was used as inoculum for reactor R2.

4.2.4 Operational conditions

Reactors R1 and R2 were operated in a continuous mode under aerobic conditions during 260 and 120 days, respectively. Reactor R1 was operated in three stages differenced by the decreasing of HRT with the consequent increase of the Organic Loading Rate (OLR). In the case of R2, the optimum conditions to form aerobic granules obtained during the operation of R1 were applied for its start-up (Table 4.1).

4.2.5 Analytical methods

Solids concentration (TSS, VSS), sludge volume index (SVI), settling velocity, pH value, nitrate, nitrite and ammonia concentrations were determined according to the Standard Methods (APHA, 1998). DO concentration was measured with a dissolved oxygen electrode (DurOx 325) connected to a meter (WTW Oxi 340i). Density of the granules as g $VSS/L_{granule}$ was measured using the dextran blue method described by Beun *et al.* (2001). Chemical Oxygen Demand (COD) was determined by a semi-micro method (Soto *et al.*, 1989). Concentrations of total organic carbon (TOC) and inorganic carbon (IC) were measured with a Shimadzu analyzer (TOC-5000).

The morphology and size distribution of the granules were measured regularly by using an image analysis procedure (Tijhuis *et al.*, 1994) with a stereomicroscope (Stemi 2000-C, Zeiss).

The elemental analysis of the biomass was carried out with a dried biomass sample by means of the Elemental Thermo Finnigan model Flash 1112.

Microbial populations were followed by the Fluorescence in Situ Hybridization (FISH) technique. Biomass samples from the reactor were collected, disrupted and fixed, according to the procedure described by Amann *et al.* (1995) with 4% paraformaldehyde solution. Hybridization was performed at 46 ºC for 90 minutes. Bacterial cells were hybridized with the following FISH probes: EUB338mix, for the domain Bacteria, ALF1b, BET42a and GAM42a probes for the *α-, β-* and *γ-* subclasses of the *Proteobacteria*. More specific probes were: Cte probe, for *Comamonas* spp., *Acidovorax* spp., *Hydrogenophaga* spp. and *Aquaspirillum* spp., Rhoc-1425 for some member of family *Rhodocyclaceae*, Pae997 probe, specific for *Pseudomonas* spp., and NEU653 probe, that targets most halophilic and halotolerant *Nitrosomonas* spp. Details on oligonucleotide probes are available at probeBase (Loy *et al.*, 2007). The probes were 5' labeled with the fluorochromes FITC and Cy3. Fluorescence signals were recorded with an acquisition system coupled to an Axioskop 2 epifluorescence microscope (Zeiss, Germany).

In order to quantify the bacterial population, for each hybridization experiment at least 20 randomly chosen images were recorded, and the ratio of the area of those cells labeled by the specific probe to the area of all bacteria stained by DAPI was determined by digital image analysis using Image ProPlus (Media Cybernetics) (Crocetti *et al.*, 2002).

Morphological studies of the biomass were performed with Scanning Electron Microscopy (SEM) (Digital SEM 440, Leica) controlled by a computer system and provided with a magnification capacity ranging from 15 to 290000 folds. For SEM analysis the sludge samples were washed three times for 10 minutes with phosphate buffer 0.05 N at a pH value of 7.4. Subsequently they were fixed with a solution of glutaraldehyde 3% in phosphate buffer for 3 hours. After fixation, the sample was dehydrated using acetone

solutions with increasing acetone concentrations (30, 50, 70 and 100%). Later, the sample was shaded with gold and observed under the scan electron microscope.

4.3 RESULTS AND DISCUSSION

4.3.1 Reactor performance

The organic matter removal efficiency, expressed in terms of soluble COD, ranged between 50-80% and 30-60% for R1 and R2, respectively (Figure 4.3 and Figure 4.4). A similar maximum removal efficiency of soluble COD close to 80% was also obtained by Vanotti *et al.* (2009) and Karakashev *et al.* (2008) during aerobic and anaerobic treatment of pig slurry, respectively. From previous works it can be considered that the fraction of the slowly biodegradable or non-biodegradable organic matter present in the pig slurry (Shin *et al.*, 2005) can achieve values from 10 to 55% (Magrí *et al.*, 2009, Figueroa *et al.*, 2011).

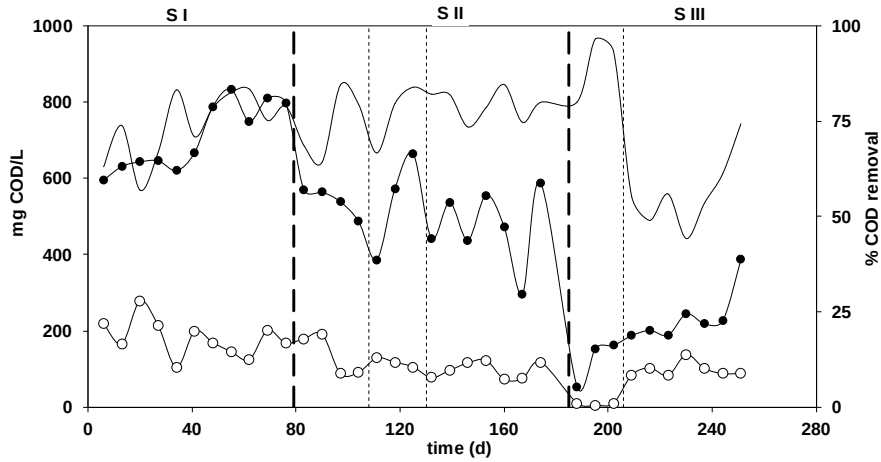

Figure 4.3. COD concentration in the influent (●) and effluent (○) and percentage of removed COD (—) in reactor R1.

Nitrogen removal efficiency in both reactors varied between 10 and 15% during the whole operational period. Small amounts of nitrite and nitrate (under 10 mg N/L) were measured in the effluent during all the experimental operation. Nitrogen removal can be attributed only to biomass assimilation, i.e. during the period with granule formation around 80% of the removed nitrogen was used for biomass growth.

Since the nitrogen removal efficiency was very low, a post-treatment of the effluent would be necessary to fulfill the disposal requirements. The absence of nitrifying activity could be due to the low solids retention time (SRT) values obtained. These values were always lower than 2 days, even when granules with good settling properties were developed in the reactor.

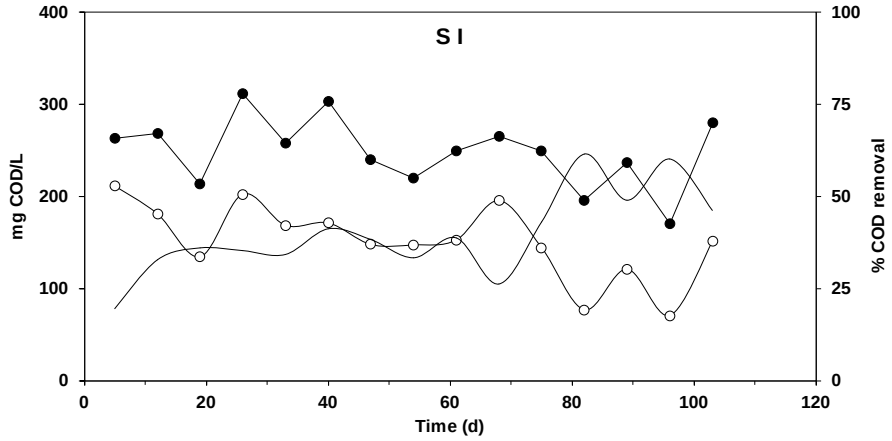

Figure 4.4. COD concentration in the influent (●) and effluent (○) and percentage of removed COD (—) in reactor R2.

No improvement in the biomass retention was observed during Stage I of operation R1 (Figure 4.5) since the biomass concentration in the effluent was very similar to that present inside the reactor (0.4-0.6 g VSS/L). Tijhuis *et al.,* (1994) stated that, in order to develop biofilms on carrier materials in an airlift suspension reactor, the hydraulic retention time must be shorter than the inverse of the maximum growth rate of the suspended bacteria.

Following this concept, in the present work the HRT was reduced to 3 h during Stage II. However, no positive effect on the biomass retention was observed. Then, mechanical stirring was switched off to decrease the applied shear stress on the biomass and try to promote the development of biomass aggregates. Under these conditions, biomass concentration rapidly increased inside the reactor, reaching values of 6 g VSS/L, while biomass concentration in the effluent ranged between 0.2 and 0.4 g VSS/L. The biomass concentration in the reactor increased up to 10 g VSS/L in spite of the restoration of the mechanical stirring from day 132. As the physical characteristics of the biomass were unsuitable due to the presence of filamentous bacteria, it was sieved and the liquid fraction obtained was used as inoculum on Stage III.

However, the growth of filamentous bacteria took place again and, in only 5 days, the biomass concentration reached again values of 10 g VSS/L. In addition to this, the dissolved oxygen concentration during this period was below 2 mg O_2/L. In previous research works (Liu and Liu, 2006) it was identified the low dissolved oxygen concentration as a possible cause of filamentous growth in aerobic granules. On day 205, this biomass was discarded and the pig slurry was directly used as inoculum to re-start the reactor in the operational conditions of Stage III. The pig slurry was directly used as inoculum due to the fact that in the initial operational days the inoculated sludge was observed to be fully

removed from the system making it similar to seed or not the reactor. Having in mind that the pig slurry contained a certain amount of organic solids it was assumed that these solids would act as an inoculum.

Under these operational conditions the appearance of aerobic granular-type biomass took place on day 227. A biomass concentration in the reactor of around 1.0-1.5 g VSS/L was reached at the end of this stage, while the solids concentration in the effluent was of 0.15 g VSS/L.

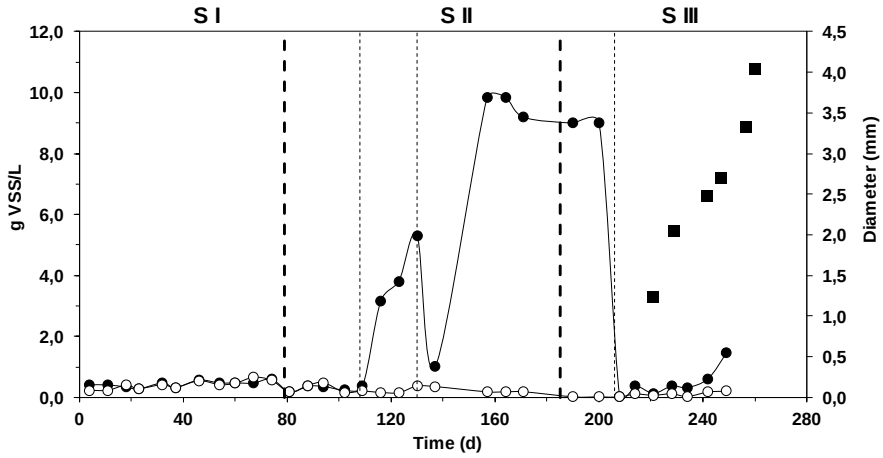

Figure 4.5. Solids concentration inside the reactor (●) and effluent (○) and average granule diameter (■) in millimeters in R1.

Similar results were obtained during the operation of R2 (Figure 4.6), where pig slurry was used as inoculum, and biomass concentration reached values around 1.0-1.2 g VSS/L, while solids concentration in the effluent did not exceed 0.15 g VSS/L.

First granules were observed in this reactor after 24 days of operation. Variations in the VSS concentration and in the average size of the granules are usual in aerobic granular reactors, especially during the start up period (Beun *et al.*, 1999). The application of low HRTs, lower than the inverse of the maximum heterotrophic bacterial growth rate, which ranges for heterotrophic bacteria around 3.0-13.2 d^{-1} (Henze *et al.*, 1987), allowed the washed out of microorganisms that did not form aggregates or biofilms (Tijhuis *et al.*, 1994).

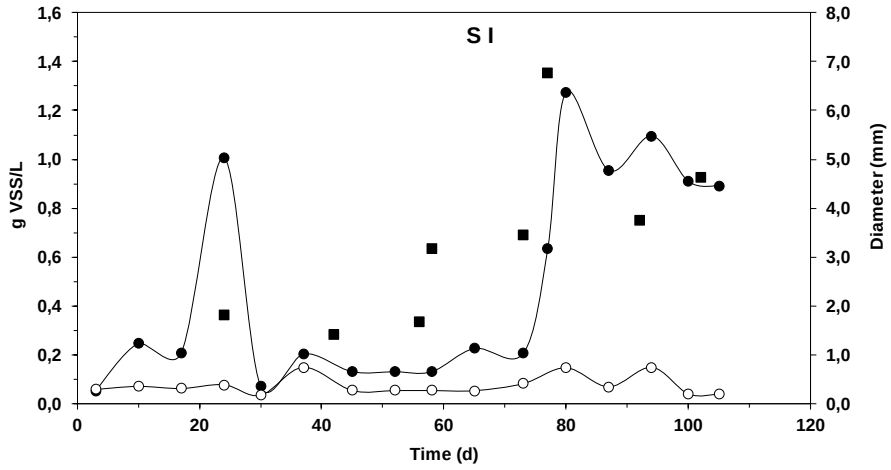

Figure 4.6. Solids concentration inside the reactor (●) and effluent (○) and average granule diameter (■) in millimeters in R2.

The average biomass yield coefficient estimated during Stages I and II of R1 was about 0.55 g VSS/g $COD_{removed}$. This value agrees with that of 0.4-0.6 g VSS/g $COD_{removed}$ observed in continuous activated sludge systems (Droste, 1997) and SBR systems with the feeding supplied during the reaction period (Klimiuk and Kulikowska, 2006). In those stages when aerobic granular type biomass was present (Stage III of R1 and Stage I of R2), the average biomass yield coefficient calculated decreased down to 0.44 and 0.37 g VSS/g $COD_{removed}$, respectively, which is higher than that of 0.2 g VSS/g $COD_{removed}$ obtained for aerobic granules cultivated in SBRs (Liu *et al.*, 2005a, Liu *et al.*, 2005b, Liu and Tay, 2007, Figueroa *et al.*, 2011). The difference between both values could be explained by the low SRT achieved in this work due to the deficient biomass retention.

Tay *et al.* (2001) observed that the sludge production in aerobic granular systems was 30% less than in the conventional activated sludge technology. Those authors operated SBR reactors, and when granules were produced, the average biomass yield was around 0.34 g VSS/g COD, while in reactors with flocculent biomass that parameter was around 0.48 g VSS/g COD.

4.3.2 Biomass characteristics

The biomass present in reactor R1 during Stage I and the first days of Stage II was mainly filamentous biomass, with poor settling properties (Figure 4.7 A and B). Once the mechanical stirring was switched off, the biomass formed a gelatinous structure that occupied most of the reactor (Figure 4.7 C). This kind of biomass was again developed during first days of Stage III until it was removed, and pig slurry was used as inoculum. In Stage III (Figure 4.7 D-H) and during the operation of R2 (Figure 4.8 A-F) a biomass

aggregation process occurred, and aerobic granules dominated the reactor. Aggregates had a white color, a smooth surface, and a regular round shape.

A deeper analysis of the surface of the granules can be observed in Figure 4.9, where SEM pictures of an aerobic granule are shown. Aggregation of bacteria can be inferred from the surface detail pictures (Figure 4.10) where individual bacteria are linked together with extra polymer substances (EPS) (Liu *et al.*, 2004, Adav *et al.*, 2008a).

The average volumetric diameter of the aggregates continuously increased during Stage III (Figure 4.5) and during the operation of R2 (Figure 4.6). The average diameter reached a value of 4 mm in Stage III and of 6.8 mm during the operation of R2. The granular size distribution was similar to that found previously in granular SBRs fed with industrial wastewater (Arrojo *et al.*, 2004). Granules with a diameter larger than 6.0 mm in the final days of operation of R2 represented about 96% of the biomass volume (Figure 4.11 F) while on day 42 represented only 15% of it (Figure 4.11 B). The size of the granules formed in R1 and R2 was larger than those corresponding to aerobic granules formed in a SBR fed also with diluted pig slurry (Figueroa *et al.*, 2011).

During the formation of the aggregates, the SVI_{10} value decreased gradually from 700 mL/g TSS to 127 mL/g TSS. This value was larger than the SVI_{10} value corresponding to aerobic granules cultivated in SBR reactors, where values around 30-40 mL/g TSS were reached (Schwarzenbeck *et al.*, 2004, Figueroa *et al.*, 2011).

Biomass density of the aggregates in R2 varied between 7 and 11 g $VSS/L_{granule}$. These values are relatively low compared to that of 43.5 g $VSS/L_{granule}$ corresponding to granules formed in SBR reactors treating pig slurry (Figueroa *et al.*, 2011).

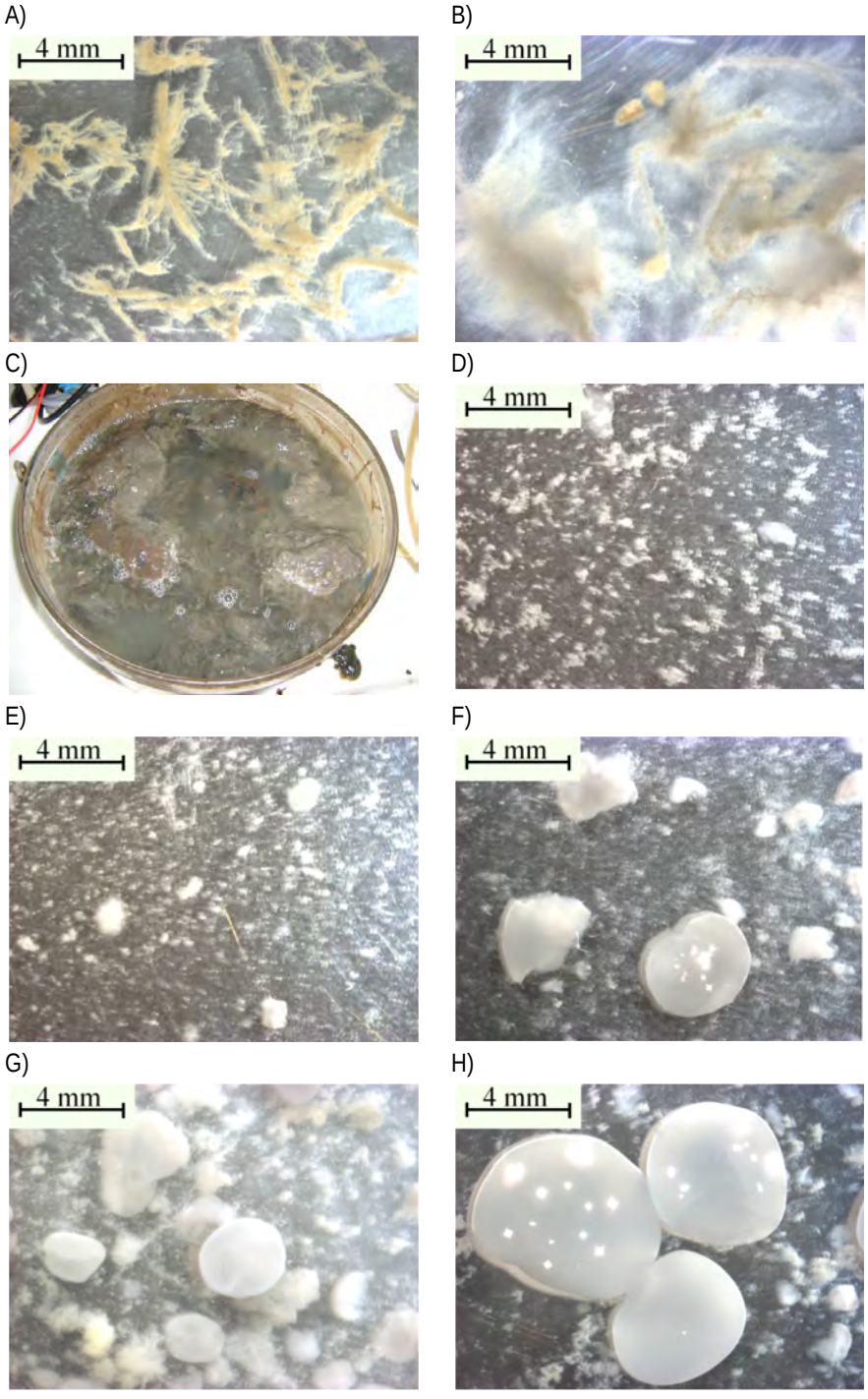

Figure 4.7. Biomass images from reactor R1 on days: A) 44 (Stage I), B) 105 (Stage II), C) 185 (Stage II), and on Stage III on days: D) 214, E) 221, F) 229, G) 242 and H) 247.

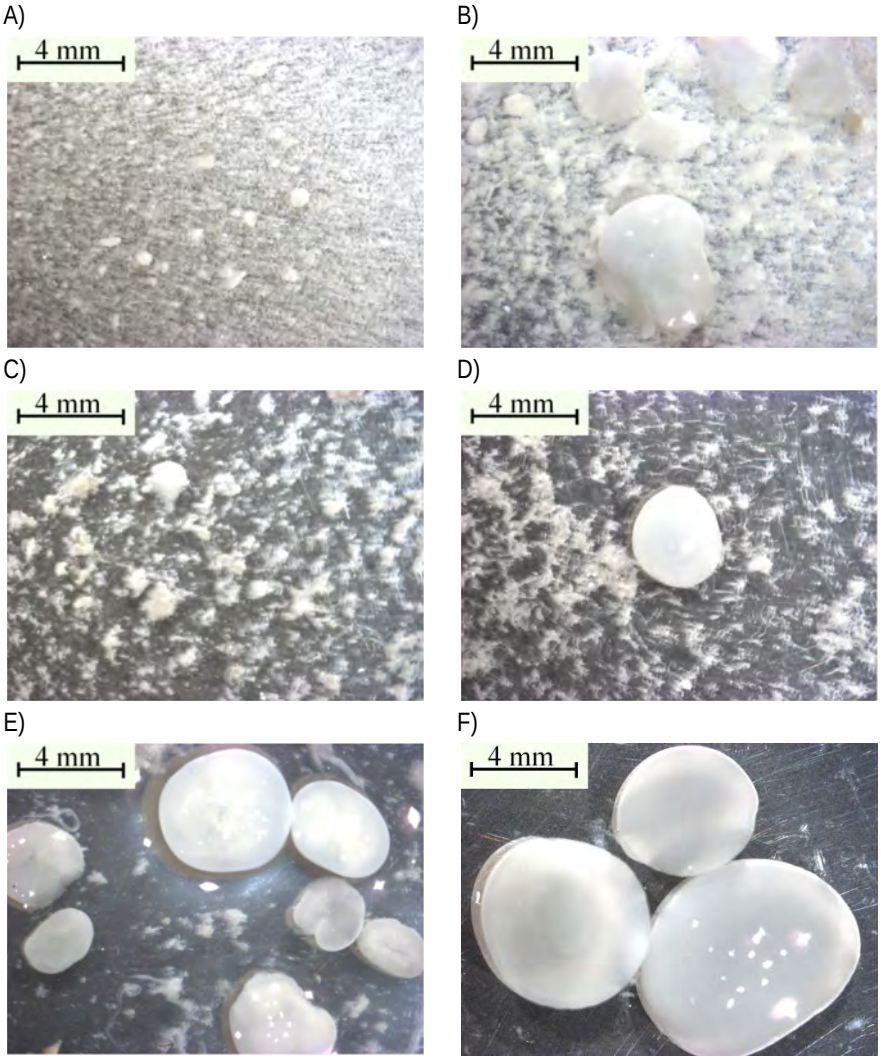

Figure 4.8. Biomass images from reactor R2 on days: A: 16, B) 24, C) 42, D) 56, E) 73 and F) 77.

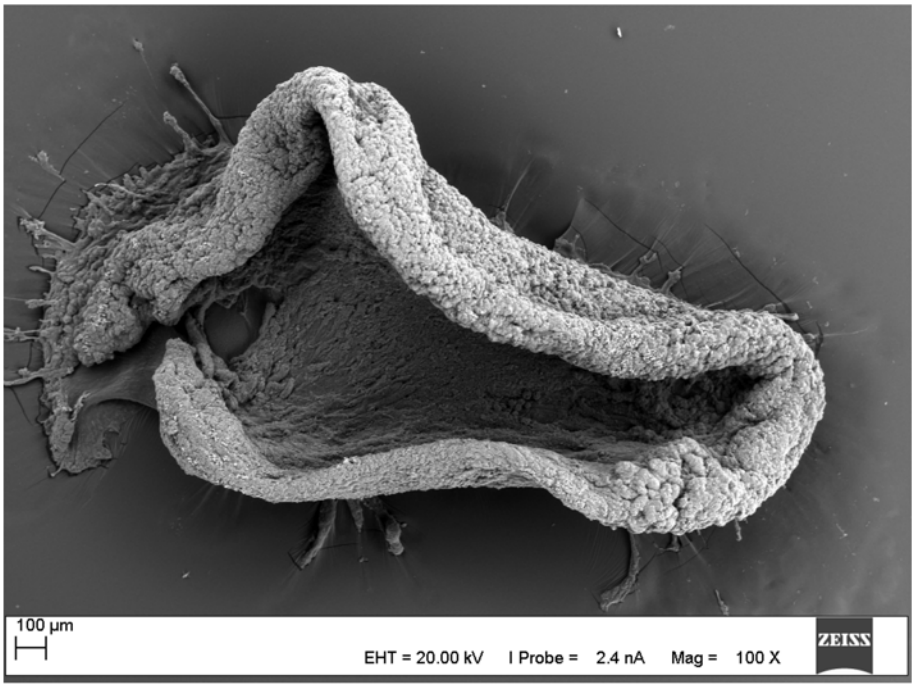

Figure 4.9. SEM image of an entire aerobic granule from reactor R2.

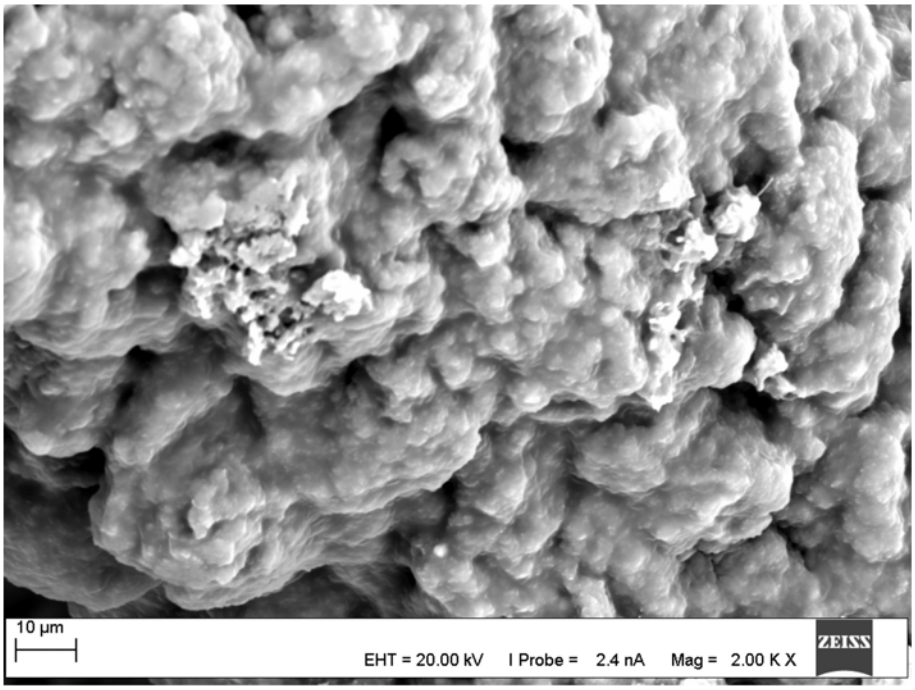

Figure 4.10. SEM image of an aerobic granule from reactor R2 (surface detail).

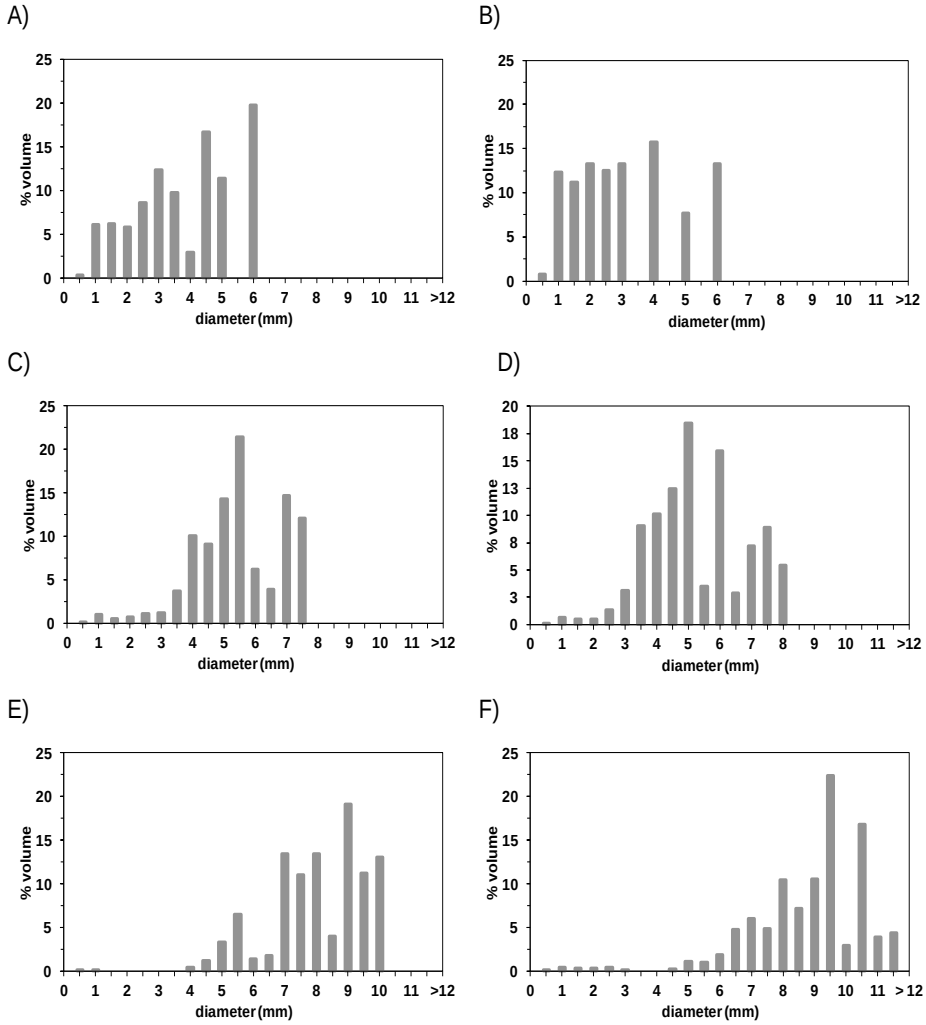

Figure 4.11. Volumetric distribution of the granules in R2 on days: A) 24, B) 42, C) 58, D) 73, E) 77 and F) 102.

However, the settling velocity values of the granules formed in both reactors varied between 36 and 48 m/h, which are similar to those of aerobic granules cultivated in SBR reactors (Jang *et al.*, 2003, Adav *et al.*, 2008b) and are remarkably higher than the typical values of settleability of activated sludge (Jin *et al.*, 2003). The biomass produced in this continuous system will be more easily separated from the liquid phase, reducing the need of big settlers. In addition it will be more easily dewatered due to the high cell hydrophobicity of aerobic granules (Liu *et al.*, 2003), facilitating the management and disposal of the sludge produced during wastewater treatment.

Elemental composition of aerobic granules observed in R1 was $CH_{1.68}N_{0.18}O_{0.55}S_{0.003}$, while in R2 was $CH_{2.10}N_{0.20}O_{0.64}S_{0.004}$. Both results were similar to those obtained in aerobic granular reactors with feeding media characterized by a C/N ratio of 5 g/g by Liu *et al.* (2003) and Yang *et al.* (2004).

The surface organic loading rates applied and treated by the aerobic granules ranged between 7-12 and 24-40 g COD/m^2·d for R1 (average diameter 4.0 mm) and R2 (average diameter 6.8 mm), respectively. Figueroa *et al.* (2011), using pig slurry as wastewater in a SBR, obtained granules with an average diameter of 5 mm. The surface organic loading rates of their granules reached values about 17 and 20 g COD/m^2·d. Westerman *et al.* (2000), using upflow aerated biofilms with an specific surface area of 140 m^2/m^3 for the treatment of flushed swine manure, achieved surface organic loading rates about 34 and 47 g COD/m^2·d.

4.3.3 Microbial Populations

Samples of sludge from R1 (day 64, Stage I) and R2 (day 120) were collected for a microbial population analysis with FISH. These samples were chosen because they represent the two different kinds of biomass formed in the continuous operated systems: fluffy biomass in Stage I and granular biomass from R2. A summary of the FISH probes applied and the obtained results is shown in Table 4.2.

A set of general probes was applied to the sample from R1 to detect the main classes of bacteria involved in the process (EUB338mix, ALF1b, BET42a and GAM42a probes). In Stage I (Figure 4.12), the hybridized bacteria belonged mainly to β- and γ-*Proteobacteria* subclasses and represented an important fraction in comparison with all the positives from EUB338I probe (30% in biovolume in both cases) while no α-Proteobacteria were detected. An important fraction of the β-*Proteobacteria* detected were filamentous shape bacteria, and also hybridized with Cte probe, specific for *Comamonas* spp., *Acidovorax* spp., *Hydrogenophaga* spp. and *Aquaspirillum* spp..

According to the phylogenetic affiliation of identified filamentous β-*Proteobacteria*, these bacteria can be classified within the genus *Curvibacter*, *Sphaerotilus natans* or *Leptothrix* (Nielsen *et al.*, 2009). However, considering the probe coverage and the morphology, these filamentous bacteria are probably organisms of genus *Curvibacter*. These bacteria are responsible of filamentous bulking episodes in activated sludge plants and decrease of sludge settleability. This observation corresponds to the settling properties of the biomass in Stage I when mainly activated sludge flocs with poor settling properties (SVI_{30} above 250 mL/g TSS) were produced.

Positive results were also obtained with Rhoc-1425 probe, designed for family *Rhodocyclaceae* within β-*Proteobacteria* (Figure 4.12, D). Positive results of these probe corresponded with the bacillus-shape bacteria that hybridized with probe Bet42a. This

family comprises bacteria belonging to the genera *Azoarcus*, *Thauera*, and *Zoogloea* that are abundant and believed to be the main denitrifiers in wastewater treatment plants (Wagner and Loy, 2002) although in this study the denitrifying activity was negligible.

The Pae997 probe, specific for *Pseudomonas* spp., was applied in order to test if these bacteria were the dominant γ-Proteobacteria in the sample; however, no positive results were obtained.

Table 4.2. FISH probes applied and results.

FISH Probes		R1 (day 64, Stage I)	R2 (day 120)
		fluffy biomass	granular biomass
General Probes	ALF1b (no α-Proteobacteria)	- ND	- ND
	BET42a (β-Proteobacteria)	+ (30%) Filamentous shape	+ (20%) Bacillus shape
	GAM42a (γ-Proteobacteria)	+ (30%) Filamentous shape	+ (15%) Bacillus shape
Specific Probes	Cte (Comamonas spp., Acidovorax spp., Hydrogenophaga spp. and Aquaspirillum spp.)	+	+
	Rhoc-1425 (Rhodocyclaceae within β-Proteobacteria)	+ Bacillus-shape	+
	Pae997 (Pseudomonas spp)	ND	+ (<1%)
	Nitrosomonas (AOB) (NEU653)	ND	ND

ND= Not detected

Results from the FISH analysis of the sample from R2 (biomass mainly in form of aerobic granules) were different from previous detailed ones (Figure 4.13). Although the hybridized bacteria belonged mainly to β- and γ -*Proteobacteria* classes the bacterial shape was mainly bacillus, and no filamentous shape bacteria were observed. An estimated biovolume percentage calculated for this sample was 20% for β-*Proteobacteria* and 15% for γ-*Proteobacteria*. Positive results for Cte and Rhoc-1425 probes were obtained, covering the major part of observed β-*Proteobacteria*. In this case, only a small fraction of bacteria hybridized with Pae997 probe (around 1%).

No positive results were obtained with specific probes for ammonia oxidizing bacteria *Nitrosomonas* (AOB) (NEU653). These results agree with the fact that no ammonia oxidation activity was detected in the reactor.

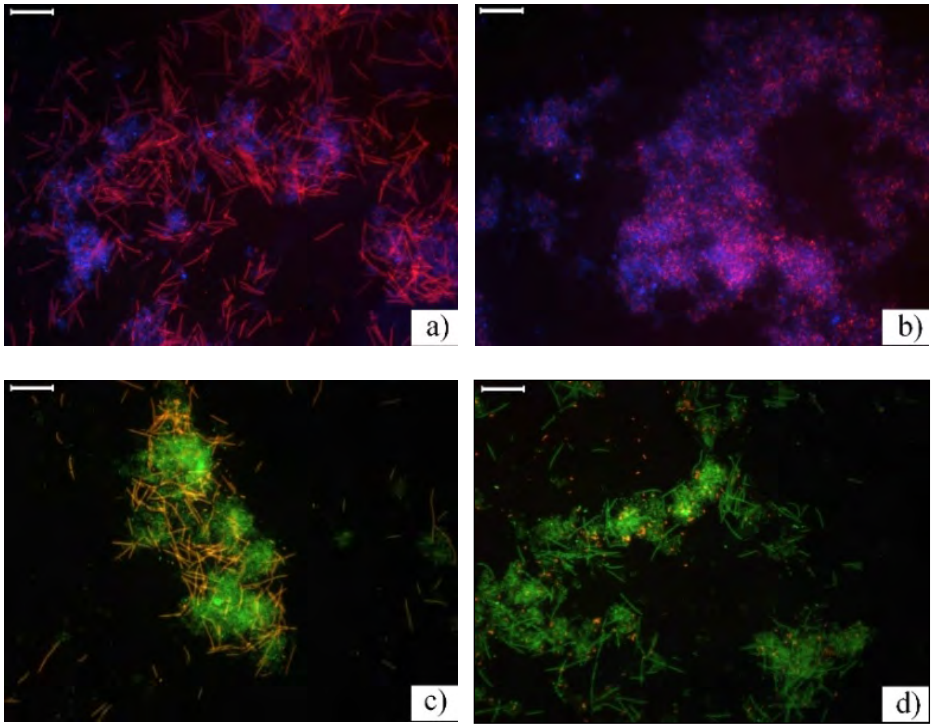

Figure 4.12. Images of biomass samples from R1 analyzed with FISH, Stage I. In all cases, the bar represents 25 µm.
a) Filamentous and bacillus shape bacteria of class β-*Proteobacteria* (Bet42a probe; Cy3, red) and DAPI (blue).
b) Bacteria of class γ-*Proteobacteria* (GAM42a probe; Cy3, red) and DAPI (blue).
c) Filamentous bacteria of the family *Comamonadaceae* (Cte probe; Cy3, red) and all bacteria (EUBmix; FITC, green).
d) Bacteria of family *Rhodocyclaceae* (Rhoc-1425 probe; Cy3, red) and all bacteria (EUBmix; FITC, green).

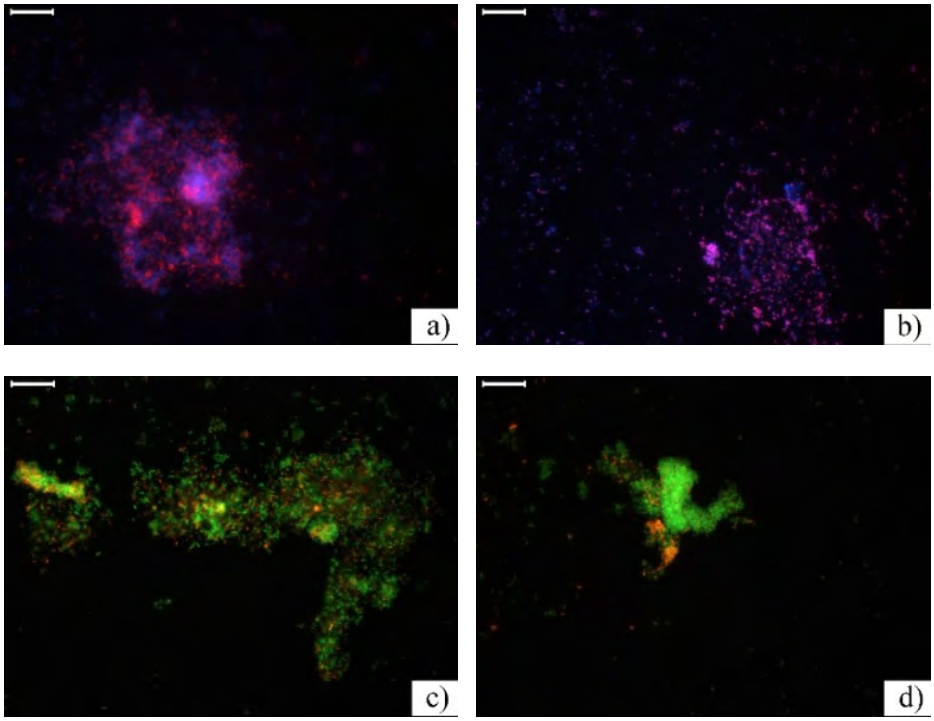

Figure 4.13. Images of biomass samples from R2 analyzed with FISH.
In all cases, the bar represents 25 μm.
a) Bacillus shape bacteria of class β-*Proteobacteria*
(Bet42a probe; Cy3, red) and DAPI (blue).
b) Bacteria of class γ-*Proteobacteria* (GAM42a probe; Cy3, red) and DAPI (blue).
c) Bacteria of family *Comamonadaceae* (Cte probe; Cy3, red) and
all bacteria (EUBmix; FITC, green).
d) Bacteria of family *Rhodocyclaceae* (Rhoc-1425 probe; Cy3, red) and
all bacteria (EUBmix; FITC, green).

4.4 CONCLUSIONS

Microbial aggregates were formed in a continuous stirred tank reactor with similar geometry to the activated sludge reactors used in WWTPs. The formation of these aggregates was achieved when a HRT of 1 hour and a hydraulic pressure of 10 m/h of settling velocity in the effluent discharge tube were applied during start up and operation of the system. At this point, OLRs as high as 4.8-6.0 g COD/L·d were treated with removal percentages around 60%.

Nitrogen removal varied between 10 and 15%, and can be attributed completely to biomass growth, while nitrification and denitrification processes did not occur in the reactor. In case nitrogen disposal requirements had to be fulfilled, a post-treatment would be necessary.

The aggregates formed in the system had a SVI_{10} of 127 mL/g TSS and a density of 11 g $VSS/L_{aggregate}$, worse values compared to aerobic granules formed in SBR reactors. However, these aggregates showed an average diameter as high as 7 mm, and a large settling velocity (36-48 m/h). These high values are comparable to those from aerobic granules formed in SBR reactors and allowed the biomass to remain inside the reactor. The average biomass yield coefficient of these microbial aggregates was of 0.37-0.44 g VSS/g COD. Besides the effect of hydrodynamic conditions, the origin of the inoculum should be taken into account to achieve microbial aggregation.

The presence of filamentous microorganisms could cause the failure in the microbial aggregation in R1 (Stages I and II), since no filaments were observed in R2. In both cases, the microbial population was mainly composed by members of *Comamonadaceae* and *Rhodocyclaceae* families within β-*Proteobacteria*, and members of class γ-*Proteobacteria*.

To our knowledge, these aerobic granules were the first reported to have been formed in a continuous CSTR reactor with geometry similar to the activated sludge reactors with a height to diameter ratio around 1.

More research is needed to establish the operational conditions which allow improving both physical properties of the biomass and the nitrogen removal efficiency.

4.5 REFERENCES

Adav, S. S., Lee, D. J. and Tay, J. H. (2008a). Extracellular polymeric substances and structural stability of aerobic granule. *Water Research* 42(6-7): 1644-1650.

Adav, S. S., Lee, D. J., Show, K. Y. and Tay, J. H. (2008b). Aerobic granular sludge: Recent advances. *Biotechnology Advances* 26(5): 411-423.

Amann, R., Ludwig, W. and Schleifer, K. H. (1995). Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiological Reviews* 59(1): 143-169.

APHA (1998). *Standard methods for the examination of water and wastewater*, American Public Health Association/American Water Works Association/Water Environment Federation. Washington DC, USA

Arrojo, B., Mosquera-Corral, A., Garrido, J. M. and Méndez, R. (2004). Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research* 38(14-15): 3389-3399.

Beun, J. J., Hendriks, A., Van Loosdrecht, M. C. M., Morgenroth, E., Wilderer, P. A. and Heijnen, J. J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research* 33(10): 2283-2290.

Beun, J. J., Heijnen, J. J. and van Loosdrecht, M. C. M. (2001). N-removal in a granular sludge sequencing batch airlift reactor. *Biotechnology and Bioengineering* 75(1): 82-92.

Campos, J. L., Mendez, R. and Lema, J. M. (2000). Operation of a nitrifying activated sludge airlift (NASA) reactor without biomass carrier. *Water Science and Technology* 41(4-5): 113-120.

Crocetti, G. R., Banfield, J. F., Keller, J., Bond, P. L. and Blackall, L. L. (2002). Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. *Microbiology* 148(11): 3353-3364.

de Bruin, L. M. M., van der Roest, H. F. R., de Kreuk, M., van Loosdrecht, M. C. M., Bathe, S., McSwain, B. S. and Schwarzenbeck, N. (2005). Promising results pilot research aerobic

granular sludge technology at WWTP Ede. In: *Aerobic Granular Sludge*. (ed), IWA Publishing. London: 135-142.

Droste, R. L. (1997). *Theory and practice of water and wastewater treatment.*, Wiley. New York

Figueroa, M., Mosquera-Corral, A., Campos, J. L. and Mendez, R. (2008). Treatment of saline wastewater in SBR aerobic granular reactors. *Water Science and Technology* 58(2): 479-485.

Figueroa, M., Val Del Río, A., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Treatment of high loaded swine slurry in an aerobic granular reactor. *Water science and technology* 63(9): 1808-1814.

Henze, M., Grady Jr, C. P. L. and Gujer, W. (1987). A general model for single-sludge wastewater treatment systems. *Water Research* 21(5): 505-515.

Inizan, M., Freval, A., Cigana, J. and Meinhold, J. (2005). Aerobic granulation in a sequencing batch reactor (SBR) for industrial wastewater treatment. *Water Science and Technology* 52(10-11): 335-343.

Jang, A., Yoon, Y.-H., Kim, I. S., Kim, K.-S. and Bishop, P. L. (2003). Characterization and evaluation of aerobic granules in sequencing batch reactor. *Journal of Biotechnology* 105(1-2): 71-82.

Jin, B., Wilén, B.-M. and Lant, P. (2003). A comprehensive insight into floc characteristics and their impact on compressibility and settleability of activated sludge. *Chemical Engineering Journal* 95(1-3): 221-234.

Jungles, M. K., Figueroa, M., Morales, N., Val del Río, A., da Costa, R. H. R., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Start up of a pilot scale aerobic granular reactor for organic matter and nitrogen removal. *Journal of Chemical Technology and Biotechnology* 86(5): 763-768.

Karakashev, D., Schmidt, J. E. and Angelidaki, I. (2008). Innovative process scheme for removal of organic matter, phosphorus and nitrogen from pig manure. *Water Research* 42(15): 4083-4090.

Klimiuk, E. and Kulikowska, D. (2006). Organics removal from landfill leachate and activated sludge production in SBR reactors. *Waste Management* 26(10): 1140-1147.

Liu, H., Li, Y., Yang, C., Pu, W., He, L. and Bo, F. (2012). Stable aerobic granules in continuous-flow bioreactor with self-forming dynamic membrane. *Bioresource Technology* 121(0): 111-118. Image reprinted with permission from Elsevier

Liu, Y., Yang, S. F. and Tay, J. H. (2003). Elemental compositions and characteristics of aerobic granules cultivated at different substrate N/C ratios. *Applied Microbiology and Biotechnology* 61(5): 556-561.

Liu, Y. and Tay, J. H. (2004). State of the art of biogranulation technology for wastewater treatment. *Biotechnology Advances* 22(7): 533-563.

Liu, Y., Liu, Y. Q., Wang, Z. W., Yang, S. F. and Tay, J. H. (2005a). Influence of substrate surface loading on the kinetic behaviour of aerobic granules. *Applied Microbiology and Biotechnology* 67(4): 484-488.

Liu, Y., Wang, Z. W. and Tay, J. H. (2005b). A unified theory for upscaling aerobic granular sludge sequencing batch reactors. *Biotechnology Advances* 23(5): 335-344.

Liu, Y. and Liu, Q. S. (2006). Causes and control of filamentous growth in aerobic granular sludge sequencing batch reactors. *Biotechnology Advances* 24(1): 115-127.

Liu, Y. Q., Liu, Y. and Tay, J. H. (2004). The effects of extracellular polymeric substances on the formation and stability of biogranules. *Applied Microbiology and Biotechnology* 65(2): 143-148.

Liu, Y. Q. and Tay, J. H. (2007). Influence of cycle time on kinetic behaviors of steady-state aerobic granules in sequencing batch reactors. *Enzyme and Microbial Technology* 41(4): 516-522.

Loy, A., Maixner, F., Wagner, M. and Horn, M. (2007). probeBase - an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Research* 35(suppl_1): D800-D804.

Magrí, A., Givernau, M., Baquerizo, G., Viñas, M., Prenafeta-Boldú, F. X. and Flotats, X. (2009). Batch treatment of liquid fraction of pig slurry by intermittent aeration: Process simulation and microbial community analysis. *Journal of Chemical Technology and Biotechnology* 84(8): 1202-1210.

Mishima, K. and Nakamura, K. (1991). Self-immobilization of aerobic activated sludge - a pilot study of the aerobic upflow sludge blanket process in municipal sewage treatment. *Water science and technology* 23(4-6): 981-990.

Ni, B. J., Xie, W. M., Liu, S. G., Yu, H. Q., Wang, Y. Z., Wang, G. and Dai, X. L. (2009). Granulation of activated sludge in a pilot-scale sequencing batch reactor for the treatment of low-strength municipal wastewater. *Water Research* 43(3): 751-761.

Nielsen, H., Daims, H. and Lemmer, H. (2009). *FISH handbook for biological wastewater treatment*, IWA Publishing. London, UK

Schwarzenbeck, N., Erley, R., Mc Swain, B. S., Wilderer, P. A. and Irvine, R. L. (2004). Treatment of malting wastewater in a granular sludge sequencing batch reactor (SBR). *Acta Hydrochimica Et Hydrobiologica* 32(1): 16-24.

Schwarzenbeck, N., Borges, J. M. and Wilderer, P. A. (2005). Treatment of dairy effluents in an aerobic granular sludge sequencing batch reactor. *Applied Microbiology and Biotechnology* 66(6): 711-718.

Shin, H. S., Lim, K. H. and Park, H. S. (1992). Effect of shear stress on granulation in oxygen aerobic upflow sludge bed reactors. *Water science and technology* 26(3-4): 601-605.

Shin, J. H., Lee, S. M., Jung, J. Y., Chung, Y. C. and Noh, S. H. (2005). Enhanced COD and nitrogen removals for the treatment of swine wastewater by combining submerged membrane bioreactor (MBR) and anaerobic upflow bed filter (AUBF) reactor. *Process Biochemistry* 40(12): 3769-3776.

Soto, M., Veiga, M. C., Mendez, R. and Lema, J. M. (1989). Semi-micro C.O.D. determination method for high-salinity wastewater. *Environmental Technology Letters* 10(5): 541-548.

Tay, J. H., Liu, Q. S. and Liu, Y. (2001). The role of cellular polysaccharides in the formation and stability of aerobic granules. *Letters in Applied Microbiology* 33: 222-226.

Tijhuis, L., van Loosdrecht, M. C. M. and Heijnen, J. J. (1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. *Biotechnology Bioengineering* 44: 595-608.

Tsuneda, S., Nagano, T., Hoshino, T., Ejiri, Y., Noda, N. and Hirata, A. (2003). Characterization of nitrifying granules produced in an aerobic upflow fluidized bed reactor. *Water Research* 37(20): 4965-4973. Image reprinted with permission from Elsevier

van Benthum, W. A. J., Garrido, J. M., Tijhuis, L., van Loosdrecht, M. C. M. and Heijnen, J. J. (1996). Formation and detachment of biofilms and granules in a nitrifying biofilm airlift suspension reactor. *Biotechnology Progress* 12(6): 764-772.

Vanotti, M. B., Szogi, A. A., Millner, P. D. and Loughrin, J. H. (2009). Development of a second-generation environmentally superior technology for treatment of swine manure in the USA. *Bioresource Technology* 100(22): 5406-5416.

Wagner, M. and Loy, A. (2002). Bacterial community composition and function in sewage treatment systems. *Current Opinion in Biotechnology* 13(3): 218-227.

Westerman, P. W., Bicudo, J. R. and Kantardjieff, A. (2000). Upflow biological aerated filters for the treatment of flushed swine manure. *Bioresource Technology* 74(3): 181-190.

Yang, S.-F., Tay, J.-H. and Liu, Y. (2004). Respirometric Activities of Heterotrophic and Nitrifying Populations in Aerobic Granules Developed at Different Substrate N/COD Ratios. *Current Microbiology* 49(1): 42-46.

Chapter 5

RECOVERY OF N AND P FROM URINE BY STRUVITE PRECIPITATION FOLLOWED BY COMBINED STRIPPING WITH DIGESTER SLUDGE LIQUID AT FULL SCALE [1]

Summary

A novel ammonia stripping method, including a CO_2 pre-stripper was used to treat a mix of supernatant liquor from an anaerobic digester and urine in order to recycle nitrogen as ammonium sulfate at full-scale in the WWTP Kloten/Opfikon (Switzerland). Waste streams were not generated, since the ammonia was recovered as a marketable nitrogen fertilizer, turning a waste product into a valuable product. The efficiency of this system was increased by the addition of pre-treated urine collected separately at EAWAG building. The separation step was performed by the use of water free urinals and urine diversion flush toilets. An increase of 10% in the liquid flux with the addition of the urine translated into a 40% increase of the ammonia concentration in the inlet of the stripping unit. The achievement of these percentages generated a proportional increase in the fertilizer production. The urine pre-treatment was carried out by adding magnesium to produce a precipitate of struvite. The first experiments with the combined treatment showed the feasibility of the combination of the separation and pre-treatment steps.

[1] Morales N., Boehler M., Buettner S., Liebi C. and Siegrist H. (2013). Recovery of N and P from urine by struvite precipitation followed by combined stripping with digester sludge liquid at full scale. *Water* 5(3), 1262-1278.

OUTLINE

5.1 INTRODUCTION

The industrial production of fertilizers containing nitrogen and phosphorous increased by 600% between 1950 and 2000 (IFA, 2006), reaching a rate of 100 million tons of nitrogen per year (Glass, 2003). Modern agriculture is highly dependent on phosphorus, which is derived from phosphate rock, a non-renewable resource. The phosphate rock global reserves might be depleted in 50–160 years (Steen and Agro, 1998, Roberts and Stewart, 2002, Cordell *et al.*, 2009, Heffer and Prud'homme, 2010).

On the other hand, nitrogen and phosphorus contained in wastewaters are the primary causes of environmental eutrophication in surface waters (E.P.A., 2007). Dodds *et al.* (2009) have estimated the annual cost of eutrophication in the United States in $2200 million. There are many efforts to break this trend, as for example the EU directive 91/676/EWG of the European Community (EEC, 1991). Both problems, nutrients depletion and eutrophication, can be reduced at the same time by the application of technologies for recovering nutrients out of wastewater streams.

Recycled sludge liquid from anaerobic digesters and separated collected urine are two wastewater streams of particular interest for nutrients recovery due to the high concentrations of nitrogen and phosphorus in relatively low flows. Percentages of nutrients present in chemical fertilizers that can be substituted by those recovered from wastewater vary and depend on several factors, one being whether a country has a net import or export of food (Jenssen *et al.*, 2007). In this way, Norway can substitute 15–20% of its mineral fertilizer (Jenssen and Vatn, 1991) while, according to Etnier and Jenssen (1997), more than 40% of the nutrients present in chemical fertilizers could be substituted by nutrients recovered from wastewater in developing countries.

Even if nitrogen can be recovered from wastewater using different techniques, until now, most of the WWTPs have implemented some kind of denitrification processes. The denitrification process implies the loss of nitrogen by returning it to the atmosphere as N_2 gas (Metcalf & Eddy *et al.*, 2002).

Nowadays, only around 10 treatment plants in Europe are using the air stripping process and produce fertilizer in form of ammonium sulphate. As nitrogen is not a finite resource, the economic and energetic balance has to be taken into account when the stripping alternative is evaluated. In this way, the combination of industrial ammonia production (around 30 MJ/kg $N_{produced}$ and 0.20 €/kg $N_{produced}$) (Wilsenach, 2002) plus nitrogen removal via Sharon/Anammox process (around 14 MJ/kg $N_{removed}$ and 3 €/kg $N_{removed}$, including amortization and energy costs) (Böhler and Liebi, 2012) is still economically more favorable than ammonia recovery, as free ammonia air stripping processes require 40–50 MJ/kg $N_{recovered}$ (including primary energy for chemical production and excess heat of WWTP) and costs are about 6 €/kg $N_{recovered}$ (Böhler and Liebi, 2012).

Energy consumption and costs for the base are a significant part of the overall operational costs in the stripping process. Consequently, an improvement of the stripping process efficiency and the economic exploitation of the by-products (fertilizers costs around 1.2 €/kg N) (Wilsenach, 2002) can reverse the situation. An alternative to reduce costs is the use of a CO_2-pre-stripper column, prior to the stripping reactor. Ammonium and bicarbonate are partly transformed to the gaseous components free ammonia and carbon dioxide. The latter can be stripped in a first stripper column with significantly lower air flow requirements than those of the ammonia stripper, and therefore, no more than 2–3% of the free ammonia will be lost in the off-gas. That is due to the fact that Henry's law constant for free ammonia (H_{NH_3}) is much lower than that for CO_2 (H_{CO_2}), 0.0006 and 1.1 at 20 °C respectively (Crittenden *et al.*, 2012). After the additional CO_2 stripping column, 50% of the nitrogen is in the form of NH_3, reducing the total amount of alkali needed for the ammonia removal by 50% (Siegrist *et al.*, 2013) as the pH of the anaerobic digestion effluent is increased from about 7 to 9 by CO_2 stripping (Lei *et al.*, 2007). Extra investment costs for the CO_2-stripper can be compensated by the decrease of alkali demand. In this way, if the specific benefit for the recovered N could be increased to 1 €/kg $N_{recovered}$, the specific treatment costs would decrease by about 20% (Böhler and Liebi, 2012).

Similar to the case of nitrogen, different treatment technologies are commonly applied in wastewater treatment plants in order to remove the phosphorus (Morse *et al.*, 1998). Most of these processes produce wastes, which need to be land filled or incinerated (Bhuiyan *et al.*, 2008). Another option is the recovery of phosphorus as a by-product, by means of crystallization processes where minerals with application in agriculture as fertilizers are produced: struvite ($NH_4MgPO_4 \cdot 6H_2O$) or hydroxyapatite [$Ca_5(PO_4)_3OH$].

Until now, the recovering costs as precipitates are higher than the current production costs derived from mineral extraction. Nevertheless, the phosphorous recovery seems to be favorable in order to meet the aims of sustainability. Shu *et al.* (2006) indicated that struvite production is technically feasible and economically beneficial. Up to 50% of the total phosphate load in municipal wastewaters comes from urine, which represents only 1% of the total volume of wastewater (Wilsenach *et al.*, 2007). The benefit of the selective collection and separated treatment of this high loaded stream from domestic wastewater is not only the economic profit of the struvite, being the potential income around 1.5 € per m^3 of urine (Wilsenach, 2002), but also the advantages in the operation of wastewater treatment plant: the reduction of many problematic micropollutants, the removal of one of the main nitrogen contributions, and the reduction of the volume necessary for the nitrification/denitrification process. By separating only 50% of the urine, the use of compact and energy-efficient treatment technologies without nitrification, denitrification, and phosphorus removal, is possible. In this way, the remaining nutrients are removed through the produced sludge (Wilsenach and van Loosdrecht, 2006).

The combination of urine and sludge liquid from the anaerobic digester in an ammonia stripping reactor, including a CO_2 pre-stripper can increase the overall nitrogen removal efficiency and the fertilizer production of the wastewater treatment plant.

Thus, the objectives of the present work were the evaluation of the pretreatment of the separately collected urine produced in EAWAG (Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland) and its further co-treatment in a stripping system where the sludge liquid from an anaerobic digester was treated. The urine pretreatment consisted of a precipitation step by reagents addition (magnesium) at pilot-scale. Reaction time and magnesium addition was evaluated in a pilot-scale reactor, and the precipitates produced were characterized in terms of composition and crystal size. The stripping process was performed in a full-scale plant comprising a CO_2 pre-stripper unit followed by an ammonia stripping system and a subsequent ammonia sulphate recovery unit.

5.2 MATERIALS AND METHODS

5.2.1 Urine separation, storage and treatment

Separated urine from the No-Mix toilets (manufactured by Roediger, Figure 5.1 A and B) (male and female) and water free urinals (male) (by HellBrok, Figure 5.1 C and D) was collected during several weeks.

Urine was stored in the basement of the EAWAG office building, in a 600 L water- and odor-tight storage tank (Figure 5.2) equipped with an overflow outlet, stirring mechanism, level sensor, sampling points and a pump to remove the effluent located at the bottom of the tank. The storage tank was also used as reactor for the urine treatment (struvite precipitation). After reaching a level of approximately 80% of the capacity of the storage tank a pre-defined dose of magnesium was added at the top of the tank.

Seven tanks of urine with the characteristics showed in Table 5.1 were collected and treated during the experimental work. Each tank in Table 5.1 represented the urine collected approximately in one week. The reaction time and optimal dose of magnesium were determined based on laboratory scale batch experiments performed with stored urine samples from the storage tank. The reactor mixing started at least 10 min before the addition of magnesium and it was working during the whole process (reaction and emptying).

The magnesium was added in different ways: MgO in powder, MgO pre-solved in 5 L of water and 15, 30 and 50% of the stoichiometric amount of HCl and in 100% of the stoichiometric amount of H_2SO_4, as it is indicated in Table 5.1. The minimum mixing period applied was of 30 min after the addition of the magnesium.

Figure 5.1. A) No-Mix toilet. B) Bottom side of a Roediger NoMix toilet. When somebody sits down on the toilet the valve is opened and the urine can flow out through the grey pipe into the urine collection tank. C) Section view of a water free urinal. D) Siphon and membranes of a water free urinal.

Samples were taken from the sample port located at the middle height of the reactor tank and filtered immediately (0.45 μm pore size) in order to follow the phosphorus removal from the urine. Then, the treated urine was transferred using the effluent pump and transfer to a 1 cubic meter plastic container. Filter bags of 81x18 cm and 0.5 m^2, made of Nylon Monofilament (BNM-050 and BNM-100, Infiltec GmbH, Speyer am Rhein, Germany, Figure 5.3 A), with a pore size of 50 μm and 100 μm, respectively were used for the separation of the solids from the treated urine. The filter bags were placed over the input port of the plastic container at the moment of the removal of the treated urine from the reactor in such a way that the urine passed through them while charging the plastic container (Figure 5.3 B). In some experiments, a pre filtration with the 100 μm filter bag was applied before the

filtration with the 50 μm filter bags. Filtered urine was transported from the EAWAG to the WWTP in the plastic containers and then mixed with the sludge liquid before entering the stripping treatment by a flow controlled pump station in the stripping experiments I and II.

Figure 5.2. Urine storage tank, at the basement of EAWAG office building.
1) Sample ports. 2) Effluent port. 3) Mixer. 4) Level sensor. 5) Women's toilet influent. 6) Men's toilet influent. 7) Hermetic tight. 8) Ventilation.

A)

B)

Figure 5.3. A) Nylon filter bag. B) Nylon filter bag in the input of the plastic storage tank.

Table 5.1. Reagents addition in the urine treatment experiments, concentrations of N and P in the stored urine and volume treated in each experiment.

Parameter	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7
Mg Source	MgO+ (15%) HCl	MgO + (30%) HCl	MgO + H_2SO_4	MgO + (50%) HCl	MgO	MgO	MgO
PO_4^{-3}-P (mg/L)	166.8 ± 0.4	156.0 ± 0.3	187.2 ± 0.4	194.1 ± 0.3	167.8 ± 0.2	174.4 ± 1.0	174.4 ± 1.0
NH_4^+-N (mg/L)	3200 ± 50	3900 ± 290	4990 ± 237	4930 ± 199	3780 ± 133	–	–
Volume (L)	600 ± 10	480 ± 10	510 ± 10	510 ± 10	430 ± 10	430 ± 10	510 ± 10

5.2.2 Ammonia air stripping process including a CO_2 pre-stripper

The full-scale stripping reactor including a CO_2 pre-stripper was located in the Kloten/Opfikon Wastewater Treatment Plant (60000 population equivalent, Glattbrugg, Switzerland). It was the first of these reactors installed in Switzerland (Böhler and Liebi,

2012). The ammonia stripping process was set in operation for the first time in fall 2010. Some of the characteristics of the stripping reactor are summarized in Table 5.2.

Table 5.2. Dimensions and operational conditions of the stripping system.

Parameter	Value	Units
Column height (CO_2 and Ammonia)	10	m
Packing bed height	6	m
Diameter	0.98	m
Liquid inflow	5–12	m^3/h
Air flow to the ammonia stripper	1000–6250	Nm^3/h
Gas flow to the CO_2 stripper	50–200	Nm^3/h
Average NH_4^+-N concentration inlet	1000	mg/L
Average NH_4^+-N concentration effluent	30	mg/L
Maximum removal rate	99	%

A scheme of the overall process is shown in Figure 5.4, including the stripping columns, and the urine co-treatment (Böhler *et al.*, 2012). Both stripping columns use special UV-stabilized Fluorpolymere packing with a honeycomb shape (CF 12F, Hewitech GmbH & Co, Ochtrup, Germany). This material has 12 mm of channel opening, a specific surface of 300 m^2/m^3 and a cross-flow channel structure. The ammonium rich stream is fed by the top of the CO_2-pre stripper where air is fluxed in counter flow.

The CO_2 stripping increases the pH of the liquid due to the displacement of carbonic acid system equilibrium (Cohen and Kirchmann, 2004). In order to avoid a CO_2 accumulation in the column, CO_2 rich gas is discharged after this unit, and fresh air is provided. Sodium hydroxide (NaOH solution at 50%, 1.524 kg/L, Thommen-Furler AG, Büren, Switzerland) is used to increase the pH of the liquid in the ammonia stripper up to a fixed value to favor the equilibrium displacement towards the NH_3, as showed in equation (5.1):

$$NH_4^+ + OH^- \rightleftharpoons NH_3(aq) + H_2O \quad (5.1)$$

This liquid is fed by the top of the stripper reactor, where the ammonia solved in the liquid phase is stripped to the gas phase. Then, the ammonia rich gas is washed in the sorber unit using a sulfuric acid solution (H_2SO_4 at 32% solution, 1.16 kg/L, Thommen-Furler AG, Büren, Switzerland). Ammonia sulphate is recovered as the end product, following equation (5.2). The gas washing is done until a solution with a density of 1.2 kg/L is obtained.

$$2\ NH_3 + H_2SO_4 \longrightarrow (NH_4)_2SO_4 \qquad (5.2)$$

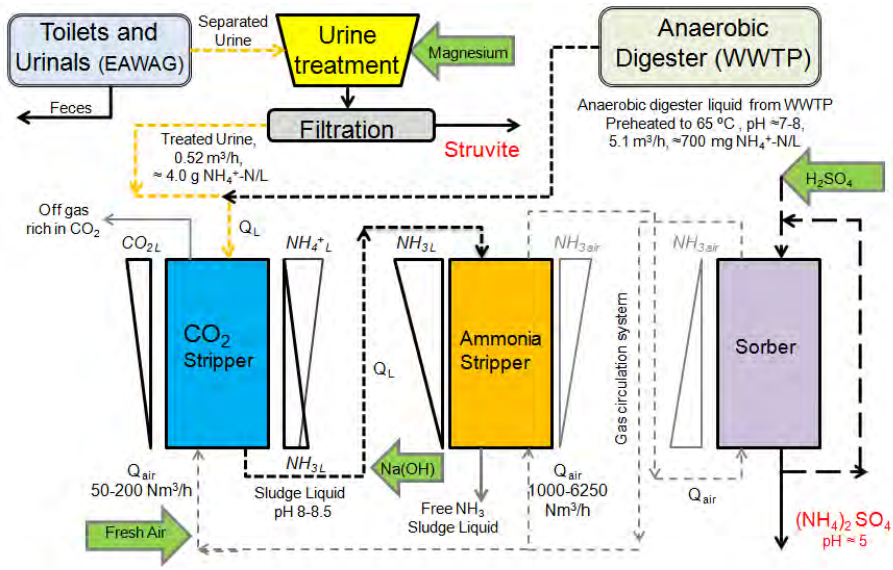

Figure 5.4. Flow-chart of the co-treatment of sludge liquid and treated urine.

The stripping factor (S) describes the balance between gas and water at the upper end of the stripping column. The minimum required amount of air (Q_{Air}) in relation to the supernatant (Q_{Liquid}) is in accordance with equation (5.3) (Siegrist *et al.*, 2013) to be at least $1/H_{NH_3}$ where H_{NH_3} is the NH_3 Henry's constant.

$$S = H_{NH_3} \cdot \frac{Q_{Air}}{Q_{Liquid}} \geq 1 \qquad (5.3)$$

The operation Q_{Air}/Q_{liquid} ratio used in the air stripping unit was in the range of 700–750 Nm^3/m^3 at 60 °C, while higher air to liquid ratios resulted in higher electricity consumption (Böhler *et al.*, 2012). Values for Henry's coefficients at different temperatures were determined following Crittenden *et al.,* (2012). Acid cleaning of the stripping reactor is executed daily using hydrochloric acid at pH 0–2 (HCl at 35%, Thommen-Furler AG, Büren, Switzerland) with an automated pH control.

Treated urine was dosed to the stripping treatment system in a percentage of 10% of the liquid sludge flux, which accounted for approximately 525 L/h in experiment I and II. The sludge liquid flux was maintained around 5.1 m^3/h. In experiment I the air flow in the stripping reactor was adjusted to 3600 Nm^3/h, the pH to 9.3 and the temperature to 60 °C, which were the operation conditions used by the WWTP operators at the time of the experimental work. Experiment II was divided in stages with different conditions of pH and air flow as it is shown in Table 5.3, while temperature set point remained at 60 °C.

Table 5.3. Sample conditions and times during experiment II.

Parameter	Stage I				Stage II				
Time (min)	0	25	43	75	89	95	125	162	178
Sampling	X	X	X	X	–	X	X	X	X
Urine addition	X	X	X	X	X	X	X	X	X
Modification in the operational conditions	–	–	–	–	X	–	–	–	–
pH set point	9.3				9.5				
Air flux (Nm^3/h)	3900				3900				

Parameter	Stage III			Stage IV	
Time (min)	179	195	210	211	235
Sampling	–	X	X	–	X
Urine addition	X	X	X	–	–
Modification in the operational conditions	X	–	–	X	–
pH set point	9.0			9.3	
Air flux (Nm^3/h)	3900			3900	

Note: Sampling, urine addition or modification in the operational conditions were performed at times indicated by "X".

5.2.3 Chemical and crystals analysis

Filtered urine (0.45 μm PET/GF) was used to measure the concentration of (ortho-) phosphate and ammonium with Lange cuvette tests (Hach Lange, GmbH, Düsseldorf, Germany). Magnesium and calcium concentrations were measured with ion chromatography. The pH of the samples was measured using a pH meter (WTW 340i, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany).

The composition of the solids (NH_4^+-N, PO_4^{3-}-P, Mg^{2+} and Ca^{2+}) was measured from solid samples recovered with the filter bags. These samples were previously dried (40 °C in a heater) and powdered. Then the analysis was performed with an amount of 0.05 g, which was dissolved in 1 mL of HCl (32%) and 25 mL of H_2O. Taking into account the magnesium added and the stoichiometric composition of struvite, the theoretical amount of struvite formed during the experiment was calculated. Then the PO_4^{3-} and Ca^{2+} in excess per mol of struvite formed and the calcium phosphate [$Ca_3(PO_4)_2$] that could be produced with such excess was calculated.

Microscopy images of the formed crystals were taken with a Scanning Electron Microscopy (Nova NanoSEM 230, FEI Company, Hillsboro, OR, USA) equipped with a high resolution field emission-SEM column, operated in low vacuum mode and equipped with a gaseous analytical detector (TV-rate low vacuum Solid-State BSED). Previously, the samples of the precipitate were filtered thought nuclepore filters and rinsed with nanopure water, in order to remove organics and salts. Size of the crystals measurements were

performed with a “Mastersizer 2000” (Malvern Instruments Ltd., Worcestershire, UK) with a measuring range from 0.02 μm to 2000 μm.

5.2.4 Chemical cost, electricity and fertilizer price

Price of chemicals and electricity cost for year 2011 (Table 5.4) were obtained from the WWTP data in Swiss Francs (CHF) (Büttner, 2011) The fertilizer price was calculated in relation with the average price of the ammonium nitrate in the last three years and the price of ammonia sulphate in the last year, using data from the Swiss Farmers Association (SBV, 2011), following equation (5.4):

$$\text{price}\ (NH_4)_2SO_{4\ 2011} = \\ = \frac{\text{price}\ (NH_4)_2SO_{4\ 2010}}{\text{Average price}\ NH_4NO_{3\ (2007\text{-}2009)}} \text{Average price}\ NH_4NO_{3\ (2008\text{-}2010)} \quad (5.4)$$

Table 5.4. Chemical, electricity and fertilizer price.

Year	Electricity (CHF/kWh)	NaOH (CHF/kg)	H_2SO_4 (CHF/kg)	HCl (CHF/kg)	NH_4NO_3 (CHF/100 kg)	$(NH_4)_2SO_4$ (CHF/1000 kg)
2011	0.1	0.36	0.23	0.52	–	50.6
2010	–	–	–	–	169.63	50.0
2009	–	–	–	–	199.95	–
2008	–	–	–	–	231.83	–
2007	–	–	–	–	162.49	–

5.3 RESULTS AND DISCUSSION

5.3.1 Urine pre-treatment

An important part of the phosphorus concentration present in the urine was already precipitated in the storage tank, and the measured values for phosphorus concentration before the magnesium addition were around 150–200 mg P/L (Table 5.1). Literature values for phosphorus concentration in fresh human urine samples are about 740 mg P/L (Udert *et al.*, 2003b). However, in the storage tank, that value can be reduced to 76 mg P/L due to the ureolysis (urea is hydrolyzed to ammonia by naturally-occurring bacteria) and subsequent struvite precipitation (Udert *et al.*, 2003a), while total phosphate precipitation is limited by the lack of magnesium.

A minimum magnesium to phosphorus ratio of 1.2 was determined after laboratory scale precipitation experiments: similar results to that obtained by Abegglen (2009) and Tettenborn *et al.* (2007) and higher than that obtained by Wilsenach *et al.* (2007). However,

these authors used synthetic urine. Finally, a ratio of 1.4 was used in order to guarantee a complete removal in the stored urine tank.

Ronteltap *et al.* (2007) obtained precipitation after 5–10 min of stirring when using $MgCl_2$ as a magnesium source. However, Abegglen (2009) needed 60 min to complete the reaction and suggested that the reason was the slow dissolution rate of MgO used in his experiment. MgO is poorly soluble in water (0.086 g/L) but sufficiently soluble in high-strength solutions (digester supernatant, urine...). In the present study, magnesium was added in different ways (in powder and solved in acid) to the tank scale experiments, in order to check the effect of the magnesium solubility in the stored urine (Table 5.1).

The phosphorus concentration in the tank scale experiments after the magnesium addition was quite similar (Figure 5.5). After 30 min of reaction, more than 95% of P was removed from the stored urine in all the experiments. There were no significant differences between the experiments where the MgO was added pre-dissolved in acid solution, and the experiments where the MgO was added directly as powder. Differences in the reaction rates of the tested magnesium precipitants might be observed with more frequent and faster measurements. However, for practical purposes, the simpler and more economical approach seems to be the use of magnesium oxide in powder form, as no extra chemicals are required and obtained results were equivalent after a reaction time of 30 min.

The pH of fresh urine samples collected in the inflow of the urine storage tank was around 7.2, the same value observed by Udert *et al.* (2003a). That value rose in the storage tank to values around 9.1–9.3 due to the ureolysis. Udert *et al.* (2003a) obtained a pH of 9.0 in stored urine. The precipitation experiments showed that the pH rose by 0.04 units after MgO addition in powder and dropped 0.04 units if MgO was pre-solved in acid, in consequence, no significant pH variations were produced by the reagent addition.

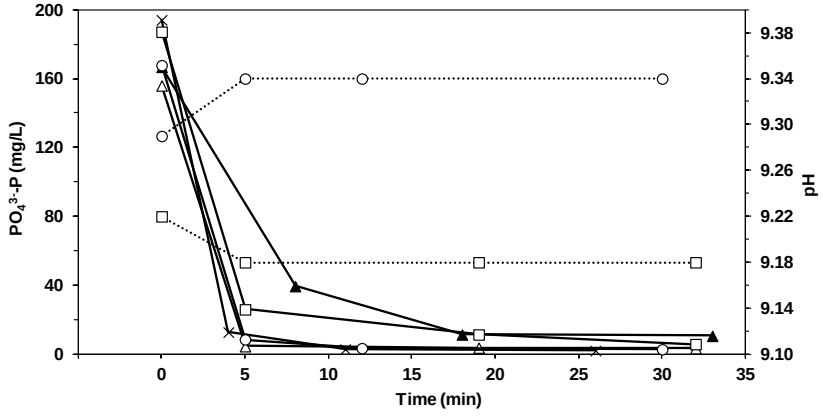

Figure 5.5. Evolution of phosphorus concentration (—) and pH (···) values in the reactor in the experiments: Tank 1 (▲), Tank 2 (△), Tank 3 (□), Tank 4 (×) and Tank 5 (○).

Regarding the formed precipitates, the size of the crystals in samples from Tank 1 was measured, before and after magnesium addition. In Figure 5.6, the particle size distribution in terms of volume percentage of both samples is compared. The average size of the crystals in the raw urine was about 75.3 μm (with a standard deviation of 123.9 μm). After the reaction, the average size increased slightly and reached a value about 79.3 μm (standard deviation 137.2 μm), with almost the same size distribution as in the initial sample. The average size of the crystals in the experiments Tanks 2–6 varied between 42.5 and 79.3 μm with a similar crystal size distribution to that obtained in Tank 1. The size of the crystals formed is in the range obtained by other authors. In this way, Ronteltap *et al.* (2007) observed that under the conditions typically found in hydrolyzed urine, larger crystals cannot be easily formed, leading to rather limited crystal sizes typical for struvite precipitation in urine (36–136 μm). These authors observed that there was no significant time dependent shift recognizable in the particle size, once the magnesium was added to the urine.

It is necessary to take into account that solids were presented in the storage tank even before the addition of magnesium; the elemental composition of these crystals determined with the gaseous analytical detector corresponded with magnesium ammonium phosphate (struvite) and with calcium phosphates. Some of the struvite crystals showed the typical coffin shape (Wierzbicki *et al.*, 1997), but there were no x-shaped branched crystals (Figure 5.7). It is possible that the mixing speed in the reactor avoided the formation of big crystals, and some crystals seem to be fragments of bigger crystals. In terms of further application as fertilizers, different struvite morphologies have been shown to have distinct dissolution kinetics while chemical differences may also affect solubility and phosphorus availability over time as the materials dissolve (Massey *et al.*, 2010).

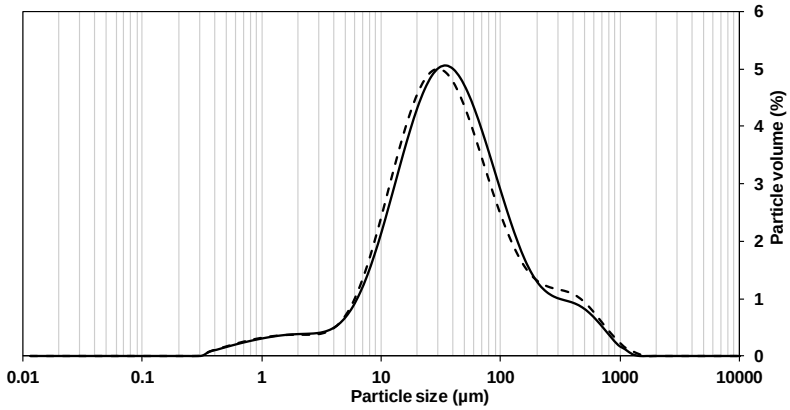

Figure 5.6. Crystal size distribution of particles in Tank1, before the addition of magnesium (—) and at the end of the experiment (- - -).

Struvite particle size affects the nitrogen release in the first weeks after planting; smaller particle sizes released more nitrogen (Nelson, 2000). However, the mentioned study was conducted with larger particle's sizes (>2 mm) than the obtained in the present experiments. It was also possible to distinguish the typical clusters of calcium phosphate in the samples from raw stored urine (Figure 5.7 B).

The composition of the solids, in terms of NH_4^+-N, PO_4^{3-}-P, Mg^{2+} and Ca^{2+}, recovered from the filters is shown in Table 5.5. It is important to emphasize the significant amount of Ca^{2+} found in the solid samples, which suggested that the solids recovered from the filter bags were not 100% struvite. Calcium is a major interfering ion affecting the deposit composition, decreasing struvite purity (Wang *et al.*, 2005). However, calcium increases the recovery and precipitation efficiency due to a phosphorus co-precipitation as struvite and calcium phosphates (Pastor *et al.*, 2008).

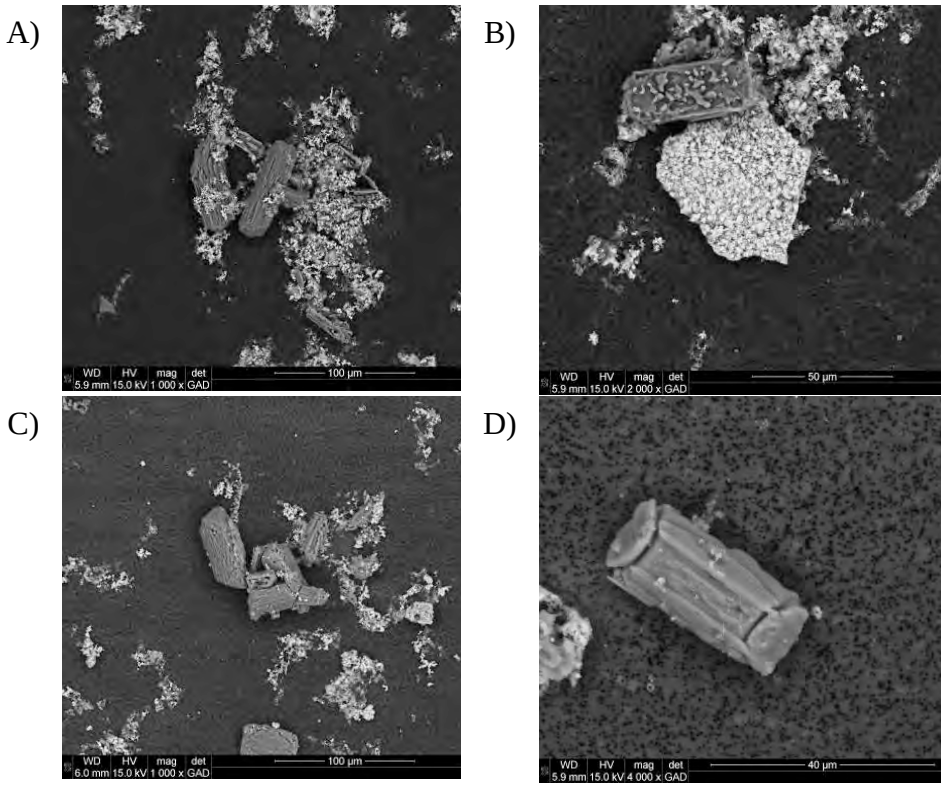

Figure 5.7. Scanning electron microscopy images of crystals in the reactor.

The theoretical production of struvite after the addition of magnesium in the urine collected in Tanks 1–6 was around 4.2 kg (2960 L with an average of 180 mg PO_4^{3-}-P/L).

Using the theoretical concentrations of phosphorus, magnesium and calcium in fresh urine (Tettenborn *et al.*, 2007) the theoretical maximum values of struvite (3.0 kg) and calcium phosphate (1.5 kg) that would be produced in the urine was determined. In the present work, the weight of solids actually recovered from the filter bags after grinding was about 3.2 kg, including solids formed before the magnesium addition, which is clearly lower than the calculated theoretical values. The solids recovery system needs to be improved in order to retrieve a higher percentage of nutrients.

Table 5.5. Composition of solids in mg of compound per gram of solid. Excess of PO_4^{3-} and Ca^{2+} consumed and $Ca_3(PO_4)_2$ produced per mol of struvite.

Sample	Composition mg/g solid				mol/mol$_{struvite}$		
	NH_4^+	PO_4^{3-}	Mg^{2+}	Ca^{2+}	PO_4^{3-}	Ca^{2+}	$Ca_3(PO_4)_2$
Sample 1 (Tanks 1 and 2)	52.1	367.7	67.0	55.0	0.40	0.50	0.17
Sample 2 (Tanks 2 and 3)	57.2	376.9	75.0	44.5	0.29	0.36	0.12
Sample 3 (Tanks 4 and 5)	55.3	370.8	71.0	45.5	0.34	0.39	0.13
Sample 4 (Tank 6)	63.6	380.0	80.0	27.0	0.22	0.21	0.07

From the previous results, it is inferred that the main objectives of the present work, which were the urine pre-treatment to remove the phosphate and to prevent precipitation in the stripping column together with the characterization of the formed crystals, were achieved. The maximization of the P rich solids recovery was not the objective because the experiments were accomplished in a reactor (storage tank) with a non optimized filtration system.

A full-scale reactor equipped with an automatic filter system should be designed for the continuous application of the urine pretreatment, as the recovery of phosphorous as struvite can have a high economic and environmental potential benefit, and probably can cover part of the investment costs for the urine separation and pre-treatment infrastructure. One option can be the scale up of the system used by Abegglen (2009) for decentralized wastewater treatment in a 4-person household. In addition, the magnesium source can be changed to other products with lower cost, as was remarked by Etter *et al.* (2011).

5.3.2 Ammonia stripping with sludge liquid and treated urine

Two experiments were performed in order to study the effect of the addition of a mix of urine and sludge liquid from the anaerobic digestion in the stripping unit. The temperature of the experiments was the value fixed by the WWTP operators, 60 °C, and the effect of its variation was not studied in this work. The change of the ammonia/ammonium ratio in favor

of ammonia is mainly affected by the pH, with less impact of the temperature (Guštin and Marinšek-Logar, 2011). In addition, temperature changes needed longer times to be applied in the full-scale stripping unit. With such temperature, the free ammonia/total ammonia ratio was 0.84 at pH 9.0, 0.91 at pH 9.3 and 0.94 at pH 9.5. The average pH of the pre treated urine measured at the moment of the addition to the stripping unit was 8.86 ± 0.02, while pH of the sludge liquid was 7.91 ± 0.06. That pH implied a deprotonation of 21% for the urine and 2.3–2.9% for the sludge liquid at 20 °C.

5.3.2.1 Experiment I

In first experiment with urine addition, no variations in the operational parameters of the stripping units were performed. The addition of urine was tested, in order to check the direct effect of the urine in the performance of the full-scale stripping reactor. With such conditions, the maximum nitrogen removal efficiency reached during the optimization phase of the reactor was 89% (Büttner, 2011), using a simulation in AQUASIM validated with experimental data from the full-scale WWTP.

The sludge liquid in the influent during experiment I had an average of 753 ± 19 mg N/L and was fed with a flow of 5.10 m^3/h. The pre-treated urine had a total nitrogen average concentration of around 3970 ± 93 mg N/L with a flow of 0.52 m^3/h, which provoked an increase on nitrogen concentration up to 1050 ± 120 mg N/L (around 40%) in the influent of the stripping units. Değermenci *et al.* (2012) determined that initial ammonia concentration has no significant effect on the ammonia removal rate. The treated liquid after the stripping processes had an average of 264 ± 4 mg N/L, which represented a TN removal efficiency of 76%. A representation of this experiment is shown in Figure 5.8.

The ammonia sulphate retrieved in the sorber column increased by a factor of 47% due to the extra ammonia addition. If the removal efficiency would have reached the maximum obtained with the simulation (89%) the recovery of fertilizer at the end of the stripping treatment should have increased by a factor of 54%. The air/liquid ratio before the addition of urine was 706 Nm^3/m^3, while that value was reduced to 643 Nm^3/m^3 during experiment I due to the addition of the urine flux, with the consequent decrease of the stripping factor by 9%.

5.3.2.2 Experiment II

With the assumption that the air flux was one of the limit parameters in experiment I, that parameter was changed in experiment II. The air ratio was fixed in 3900 Nm^3/h and the air/liquid ratio was increased up to 697 Nm^3/m^3.

Pre treated urine added in experiment II, had an average concentration of 3943 ± 224 mg NH_4^+-N/L and was fed with a flow of 0.52 m^3/h, while the sludge liquid had an average nitrogen concentration of 692 ± 17 mg NH_4^+-N/L with a flow of 5.10 m^3/h, resulting in an

increase of the ammonia content of about 41%. Samples were taken before the addition of treated urine (time 0), and at different times after the beginning of the experiment as shown in Table 5.3.

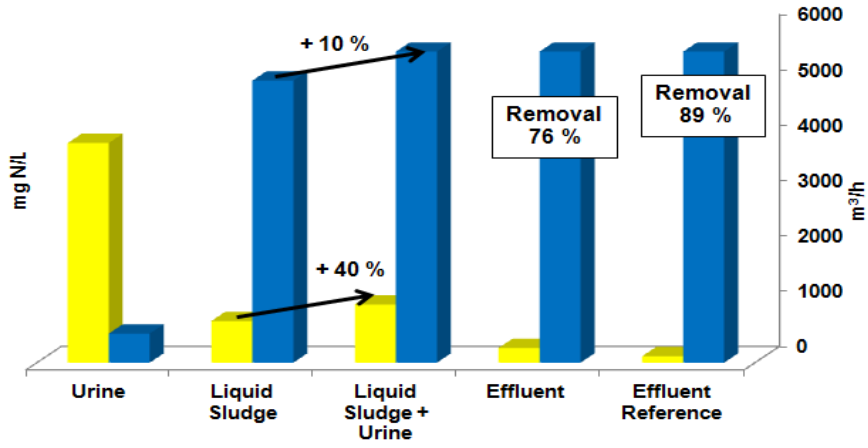

Figure 5.8. Total nitrogen concentration (■) in mg N/L and volumetric flow (■) in m³/h in the urine, liquid sludge, liquid sludge after the urine addition, effluent in the experiment I, and effluent for the removal efficiency reference (89%) (Büttner, 2011).

5.3.2.3 Removal efficiency and fertilizer recovery

The overall removal efficiency of the stripping processes without urine addition in experiment II (time 0) was around 68%, when the reactor treated 3300 g N/h, thus ammonia sulphate was produced at a rate of 2460 g N-$(NH_4)_2SO_4$/h. Once the urine was added to the influent, the overall removal efficiency slightly dropped to 65% (Stage I). However, the nitrogen treated was 5100 g N/h (41% more) and consequently, the ammonia sulphate recovered after the sorber column rose to 3350 g N-$(NH_4)_2SO_4$/h, which represents an increase of 36%. In stage II, the dose of NaOH was increased in order to reach a pH of 9.5, which facilitated the deprotonation of ammonium (94%). With such conditions, the nitrogen treated in the stripping reactors was 5250 g N/h and an improvement of the removal efficiency was obtained as it reached the 73%. The production of fertilizer was 3800 g N-$(NH_4)_2SO_4$/h, which represented an increase of 56% comparing with the observed at time 0. In Stage III, the pH set point was reduced to 9.0. The nitrogen in the influent of the stripping column was 5200 g N/h and a removal of 65% was reached (similar value to the obtained on Stage I), in consequence, also the increase in the ammonia sulphate produced reached a similar value to that obtained in Stage I.

After the urine addition, the operation conditions were reset (Stage IV), and all parameters recovered the stationary values observed before the urine addition experiment. Foam or clogging problems or other operational problems related to the addition of the urine

were not reported by the WWTP operators. However the length of the experiments was too short to discard completely this possibility.

Overall nitrogen removal in all stages was lower than expected values obtained from the simulation (Büttner, 2011), even with no urine addition (Table 5.6), which can suggest operational problems of the stripping unit no related to the urine addition.

5.3.2.4 Chemical and energy cost

Extra costs related to the urine addition in the stripping unit were caused by the necessary increase of some operational parameters as the air/liquid ratio or chemicals consumption.

The electric energy used variations were mainly related to the increase of air flux. In this way, the electricity consumed for aeration was around 4.5, 5.0 and 6.0 kWh/h when the air flow was fixed in 3600, 3900 and 4300 Nm^3/h respectively. The electricity cost was 0.1 CHF/kWh (around 0.08 €/kWh), in consequence, an increase of 11.1% in kWh consumed or 1.2 CHF/day was necessary to apply in experiment II, when the air flow was fixed in 3900 m^3/h.

The average amount of NaOH dosed in the stripping reactor before urine addition was around 132 L/day (average of the week). The measured values during experiment I and II were around 170 L/day. The NaOH consumption rose when urine was added, as the amount of NaOH dosed in the stripping reactor must be around 35 mol/m^3 (Büttner, 2011). Taking into account the NaOH price, the cost raised around 20.8 CHF/day. Siegrist (1996) reported a relation of 24 kg of NaOH (30%) per 1 kg of N removed in an air stripping system at 20 °C, while that rate in the present study was around 2.4 kg of NaOH (50%) in the stripping system with CO_2 pre-stripper at 60 °C. If the CO_2 stripping column was not included, the NaOH solution consumption should be around 55 mol/m^3 (Büttner, 2011), a 57% more.

The consumption of sulphuric acid is related with the amount of N removed; Siegrist (1996) reported 9.6 kg of H_2SO_4 per kg of N removed. The average daily use of sulphuric acid solution was around 205 L/day, while that value raised a 30–40% during the experiments with urine addition, which represented around 23 CHF/day.

Thermal energy costs also increased, as the urine had to be heated to 60 °C from ambient temperature. However, residual heat from the WWTP was used for that purpose, and no data to analyze were available. Costs related with the possible increase in chemical cleaning of the stripping unit were not possible to evaluate due to the short terms of experiments.

These extra costs associated with the urine addition can be directly compensated by the increase in fertilizer recover at the end of the stripping processes, which can vary around 5.3–7.8 CHF/day (+36–56%). Each kg of N removed need at least 3.5 kg of H_2SO_4 and will produce 4.7 kg of $(NH_4)_2SO_4$. In a cost study for a full-scale stripping plant with a CO_2 pre-stripper, the estimated costs for treatment were around 3–4.5 €/kg $N_{recovered}$ (depending on operation/ charge wise or continuously) (Zuleeg *et al.*, 2012). With the current price of the fertilizer, chemical cost was higher than fertilizer incomes. However, indirectly extra costs can be compensated by the reduction in the cost of aeration for nitrification, and by the reduction in the carbon source needed for denitrification in the biological reactors of the WWTP (Fux and Siegrist, 2004, Wett *et al.*, 2007).

A summary of the results of the two experiments with urine addition to the stripping columns is shown in Table 5.6. The experiments established that is possible to treat sludge liquid and pre-treated urine in the same stripping unit.

5.4 CONCLUSIONS

In this work, a set of experiments were performed in order to investigate the combination of urine treatment and ammonia stripping to treat a mixture of sludge liquid together with separated collected urine in a full-scale stripping reactor located in a municipal WWTP. The experiments performed in this work provided preliminary results, more experiments are needed in order to test the viability of the system, but first results are promising. The following conclusions could be drawn:

The addition of magnesium oxide in powder to the separated urine, combined with a filtration system is enough to produce and recover nutrients in form of struvite. A ratio of magnesium to phosphorus of 1.4 was used in order to remove more than 95% of phosphorus from the stored urine in a short time of less than 1 hour.

Struvite crystals with an average size of 42 to 80 μm were formed in the reactor (the urine storage tank). Nevertheless, the solids recovery system during urine pre-treatment needs to be improved in order to retrieve a higher percentage of nutrients. The separated urine collecting system should be connected to the stripping reactor in order to combine both processes and optimize the nutrients recovery system.

The addition of 10% volume of treated urine to the sludge liquid fed in the stripping treatment system produced an increase of 40% of the ammonia concentration.

The efficiency in the nitrogen removal was lower than the value reached during the optimization and simulations without urine addition. An increase in the NaOH dose, H_2SO_4 composition and air/liquid ratio was needed, due to the liquid flux increase. Nevertheless, an increase in the ammonia sulphate production rate (36%–56%) was observed during the

full-scale experiments with urine addition, which demonstrated the viability of the combined system.

Operational problems due to the treated urine addition were not reported by the WWTP operators; however, the length of the experiments was too short to discard this possibility.

Table 5.6. Summary of the main results of the different experiments.

Experiment (Stage)	I	II (S-I)	II (S-II)	II (S-III)
Temperature after the heat exchanger (°C)	60	60	60	60
Set point pH, after the NaOH dosage	9.3	9.3	9.5	9.0
Air flow in the stripping reactor (Nm^3/h)	3600	3900	3900	3900
Air flow in the CO_2 stripper (Nm^3/h)	100	100	100	100
Sludge liquid flow (m^3/h)	5.1	5.1	5.1	5.1
Urine flow added (L/h)	525	525	525	525
pH after the stripping	No data	7.9	8.1	8.0
Efficiency (%)	76	65	73	65
Efficiency % (Büttner, 2011)	89 (with 5.25 m^3/h)	89 (with 5.25 m^3/h and 4000 Nm^3/h)	97 (with 5.25 m^3/h and 4000 Nm^3/h)	80 (with 5.25 m^3/h and 4000 Nm^3/h)
Increase/decrease in the efficiency (%)	−13	−24	−24	−13

5.5 REFERENCES

Abegglen, C. (2009). Membrane bioreactor technology for decentralized wastewater treatment and reuse (Thesis). *Diss. ETH No. 17998.*

Bhuiyan, M. I. H., Mavinic, D. S. and Koch, F. A. (2008). Phosphorus recovery from wastewater through struvite formation in fluidized bed reactors: A sustainable approach. *Water Science and Technology* 57: 175-181.

Böhler, M. and Liebi, C. (2012). Reduzierter NaOH-Verbrauch bei der Ammoniak-Strippung durch den Einsatz einer vorgeschalteten CO2-Strippung – Betriebserfahrungen auf der ARA Klothen-Opfikon. (Reduction of base consumption in the free ammonia stripping process by means of a pre treatment by CO2-stripping - operational experiences at WWTP Kloten/Opfikon). *8th Conference on nitrogen rejection load - state of the art*. Heidelberg, Germany.

Böhler, M., Büttner, S., Morales, N., Liebi, C., Schachtler, M. and Siegrist, H. (2012). *Recovery of nutrients from ammonia rich sludge liquids and urine for the production of fertilizer by full scale air stripping or membrane stripping.* Proceedings of the IWA World Congress on Water, Climate and Energy, Dublin, Ireland.

Büttner, S. (2011). Gewinnung eines Düngers aus Abwasser - Optimierung des Verfahrens der Luftstrippung der Kläranlage Kloten/Opfikon. (Production of a fertilizer from sewage -

Optimization of the process of air stripping of the WWTP Kloten / Opfikon). *Diplomarbeit FH Weihenstephan-Triesdorf, angefertigt an der Eawag* Master Thesis.

Cohen, Y. and Kirchmann, H. (2004). Increasing the pH of wastewater to high levels with different gases - CO2 stripping. *Water, Air, and Soil Pollution* 159(1): 265-275.

Cordell, D., Drangert, J.-O. and White, S. (2009). The story of phosphorus: Global food security and food for thought. *Global Environmental Change* 19(2): 292-305.

Crittenden, J. C., Trussell, R. R., Hand, D. W., Howe, K. J. and Tchobanoglous, G. (2012). *MWH's Water Treatment: Principles and Design*, Wiley.

Değermenci, N., Ata, O. N. and Yildız, E. (2012). Ammonia removal by air stripping in a semi-batch jet loop reactor. *Journal of Industrial and Engineering Chemistry* 18(1): 399-404.

Dodds, W. K., Bouska, W. W., Eitzmann, J. L., Pilger, T. J., Pitts, K. L., Riley, A. J., Schloesser, J. T. and Thornbrugh, D. J. (2009). Eutrophication of US Freshwaters: Analysis of Potential Economic Damages. *Environmental Science & Technology* 43(1): 12-19.

E.P.A., Ed. (2007). *Biological Nutrient Removal Processes and Costs*. Washington, DC, USA, United States Environmental Protection Agency.

EEC (1991). Council Directive 91/676/EEC, of 12 December 1991, Concerning the Protection of Waters Against Pollution Caused by Nitrates from Agricultural Sources. European Economic Community. Brussels, Belgium.

Etnier, C. and Jenssen, P. D. (1997). The human waste resource in developing countries: Examples of options for reuse of nutrients in agriculture and aquaculture., Centre for International Environment and Development Studies; Noragric, report 35. Agr. Univ. Norway.

Etter, B., Tilley, E., Khadka, R. and Udert, K. M. (2011). Low-cost struvite production using source-separated urine in Nepal. *Water Research* 45(2): 852-862.

Fux, C. and Siegrist, H. (2004). Nitrogen removal from sludge digester liquids by nitrification/denitrification or partial nitritation/anammox: environmental and economical considerations. *Water Science and Technology* 50(10): 19-26.

Glass, A. D. M. (2003). Nitrogen use efficiency of crop plants: Physiological constraints upon nitrogen absorption. *Critical Reviews in Plant Sciences* 22(5): 453-470.

Guštin, S. and Marinšek-Logar, R. (2011). Effect of pH, temperature and air flow rate on the continuous ammonia stripping of the anaerobic digestion effluent. *Process Safety and Environmental Protection* 89(1): 61-66.

Heffer, P. and Prud'homme, M. (2010). *Fertilizer Outlook 2010 - 2014*. 78th IFA Annual Conference, Paris, May - June 2010.

IFA. (2006). "Production and International Trade Statistics Available online: http://www.fertilizer.org/ifa/HomePage/STATISTICS/Production-and-trade." International Fertilizer Industry Association Retrieved 21 August 2013.

Jenssen, P. D. and Vatn, A. (1991). *Ecologically sound wastewater treatment: Concepts and implementation*. Ecological engineering for wastewater treatment, Stensund, Bokskogen, Sweden.

Jenssen, P. D., Vråle, L. and Lindholm, O. (2007). *Sustainable wastewater treatment*. International conference on natural resources and environmental management and environmetal safety and health, Kuching, Malaysia.

Lei, X., Sugiura, N., Feng, C. and Maekawa, T. (2007). Pretreatment of anaerobic digestion effluent with ammonia stripping and biogas purification. *Journal of Hazardous Materials* 145(3): 391-397.

Massey, M. S., Ippolito, J. A., Davis, J. G. and Sheffield, R. E. (2010). Macroscopic and microscopic variation in recovered magnesium phosphate materials: Implications for phosphorus removal processes and product re-use. *Bioresource Technology* 101(3): 877-885.

Metcalf & Eddy, I., Tchobanoglous, G., Burton, F. and Stensel, H. D. (2002). *Wastewater Engineering: Treatment and Reuse*, McGraw-Hill Companies, Incorporated.

Morse, G. K., Brett, S. W., Guy, J. A. and Lester, J. N. (1998). Review: Phosphorus removal and recovery technologies. *Science of the Total Environment* 212(1): 69-81.

Nelson, N. O. (2000). *Phosphorus removal from anaerobic swine lagoon effluent as struvite and its use as a slow-release fertilizer. Masters Thesis, Soil Science Dept.,* North Carolina State University.

Pastor, L., Mangin, D., Barat, R. and Seco, A. (2008). A pilot-scale study of struvite precipitation in a stirred tank reactor: Conditions influencing the process. *Bioresource Technology* 99(14): 6285-6291.

Roberts, T. L. and Stewart, W. M. (2002). Inorganic Phosphorus and Potassium Production and Reserves. *Better Crops* 86(2): 6-7.

Ronteltap, M., Maurer, M. and Gujer, W. (2007). Struvite precipitation thermodynamics in source-separated urine. *Water Research* 41(5): 977-984.

SBV. (2011). "Landwirtschaftliche Monatszahlen." Retrieved March, 2011, from http://www.sbv-usp.ch/de/shop/landwirtschaftliche-monatszahlen/.

Shu, L., Schneider, P., Jegatheesan, V. and Johnson, J. (2006). An economic evaluation of phosphorus recovery as struvite from digester supernatant. *Bioresource Technology* 97(17): 2211-2216.

Siegrist, H. (1996). Nitrogen removal from digester supernatant - comparison of chemical and biological methods. *Water Science and Technology* 34(1–2): 399-406.

Siegrist, H., Laureni, M. and Udert, K. M. (2013). Transfer into the gas phase: ammonia stripping In: *Source Separation and Decentralization for Wastewater Management*. Larsen, T. A. *et al..* (ed), IWA publishing. London, UK.

Steen, I. and Agro, K. (1998). Phosphorus availability in the 21st century: Management of a non-renewable resource. *Phosphorus and Potassium*(217): 25–31.

Tettenborn, F., Behrendt, J. and Otterpohl, R. (2007). *Resource recovery and removal of pharmaceutical residues - Treatment of separate collected urine. Final report for Task 7 of the demonstration project "Sanitation Concepts for Separate Treatment of Urine, Faeces and Greywater" (SCST)*, Technical University of Hamburg. Harburg

Udert, K. M., Larsen, T. A., Biebow, M. and Gujer, W. (2003a). Urea hydrolysis and precipitation dynamics in a urine-collecting system. *Water Research* 37(11): 2571-2582.

Udert, K. M., Larsen, T. A. and Gujer, W. (2003b). Estimating the precipitation potential in urine-collecting systems. *Water Research* 37(11): 2667-2677.

Wang, J., Burken, J. G., Zhang, X. and Surampalli, R. (2005). Engineered struvite precipitation: Impacts of component-ion molar ratios and pH. *Journal of Environmental Engineering* 131(10): 1433-1440.

Wett, B., Buchauer, K. and Fimmi, C. (2007). Energy self-sufficiency as a feasible concept for wastewater treatment systems. *Asian Water* September: 21-24.

Wierzbicki, A., Sallis, J. D., Stevens, E. D., Smith, M. and Sikes, C. S. (1997). Crystal growth and molecular modeling studies of inhibition of struvite by phosphocitrate. *Calcified Tissue International* 61(3): 216-222.

Wilsenach, J. (2002). *Separate urine collection and treatment: options for sustainable wastewater systems and mineral recovery. STOWA report number 2002-39*, STOWA. Utrecht

Wilsenach, J. A. and van Loosdrecht, M. C. M. (2006). Integration of Processes to Treat Wastewater and Source-Separated Urine. *Journal of Environmental Engineering* 132(3): 331-341.

Wilsenach, J. A., Schuurbiers, C. A. H. and van Loosdrecht, M. C. M. (2007). Phosphate and potassium recovery from source separated urine through struvite precipitation. *Water Research* 41(2): 458-466.

Zuleeg, S., Wüthrich, F., Egli, C., Hürlimann, M., Grömping, M., Böhler, M. and Siegrist, H. (2012). ARA Altenrhein – Rücklaufbehandlung Variantenvergleich. (Cost comparison on different side stream treatment technologies for ammonia rich supernatant for WWTP Altenrhein). St. Gallen (Switzerland), ARA Altenrhein / Ingenieurbüro AG.


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Chapter 6

DYNAMICS OF THE CANON PROCESS TREATING LOW NITROGEN LOAD AT LOW TEMPERATURE [1]

Summary

Nowadays, the application of Anammox based processes to the main stream of WWTPs is under extensive research. The reason is to open the possibility of removing the nitrogenous compounds with less energy and chemical requirements. Furthermore, larger amounts of organic matter would be available for methane production. In this work, the long-term performance and stability of a CANON system operated at 20 and 15 ºC was researched. The system operated in stable conditions treating concentrations in the feeding of 150-250 mg NH_4^+-N/L, and achieved nitrogen removal rates of 0.45 and 0.33 g N/L·d at 20 and 15 ºC, respectively. However, when ammonia concentration was decreased to 50-80 mg NH_4^+-N/L and the reactor operated at 15 ºC, obtained nitrogen removal rate was of 0.06 g N/L·d. This rate was limited by the low dissolved oxygen concentration required in the bulk liquid to avoid the nitrite oxidation by nitrite oxidizing bacteria (NOB). Variations in the operational conditions affected the characteristics of the granular biomass: specific biomass activity, average size and density, and modified the oxygen depth penetration into the granule. One of the main challenges to be faced when low temperature and ammonia loads are applied is to avoid the development of NOB, which compete with Anammox bacteria, reducing the overall nitrogen removal capacity of the CANON system.

[1] Morales N., Val del Río, A., Vázquez Padín J.R., Mosquera-Corral A., Campos J. L. and Méndez R. (2014). Dynamics of the CANON process treating low nitrogen load at low temperature (In preparation)

OUTLINE

6.1 INTRODUCTION

The Anammox process (ANaerobic AMMonium OXidation), recently discovered, belonging to the nitrogen cycle, was predicted thermodynamically by Broda, (1977) and experimentally observed in the 90's (Mulder *et al.*, 1995). Soon after its discovery, the potentiality of this process in the environmental field was recognized (Strous *et al.*, 1997b).

The application of the Anammox process contributes to an important reduction in the energy and resources consumptions required for the removal of nitrogen in the wastewater treatment plants (WWTPs). This process is operated combined to a previous partial autotrophic nitritation process where the oxidation of half of the present ammonium to nitrite is performed by ammonia oxidizing bacteria (AOB). When this combined system is applied to wastewaters with low C/N ratio the process requires approximately half of the energy needed for aeration, and no carbon source addition for denitrification compared to conventional nitrification and heterotrophic denitrification processes (Siegrist *et al.*, 2008). Both autotrophic processes can take place simultaneously in a single reactor like in the Completely Autotrophic Nitrogen Removal Over Nitrite (CANON) system.

Although Anammox bacteria activity has been detected in Arctic marine sediments at temperatures as low as -1.3 ºC (Rysgaard *et al.*, 2004), hitherto, the application of the Anammox process to wastewater treatment has been mainly focused on the treatment of wastewaters at relatively high temperatures (>30 ºC) and elevated ammonia concentrations (>500 mg N/L). This is the case of the supernatant from anaerobic sludge digesters (van Dongen *et al.*, 2001). Successful full-scale applications of the Anammox process to the treatment of these wastewaters have been already developed (van der Star *et al.*, 2007, Wett, 2007).

Recently, experimental results proved the feasibility of the Anammox process to be operated at low/moderate temperatures with different reactor configurations (Dosta *et al.*, 2008, Qiao *et al.*, 2010, Vázquez-Padín *et al.*, 2010a, Hendrickx *et al.*, 2012). New developments in this line would expand the applicability of the process to remove nitrogen from landfill leachates, effluents from psychrophilic anaerobic digesters and even from the main stream of municipal WWTPs (Vázquez-Padín *et al.*, 2011, Morales *et al.*, 2012). However, until now, Anammox based process have been applied in cases where the temperature of the reactor was low (below 20 ºC), the applied nitrogen concentration was moderate or high (>200 mg/L) and vice versa (Table 6.1). Little information about the application of Anammox process at low temperature and nitrogen concentration, like it is the case of the main stream of the WWTPs, is available (De Clippeleir *et al.*, 2013, Hu *et al.*, 2013).

Calculations made based on mass balances indicate that the use of a combined two-stage biological treatment in the water line, comprising a high loaded first stage for carbon

removal and a second stage combining partial nitrification and Anammox process for nitrogen removal, offers a way to achieve energy self-sufficiency in the WWTPs (Garrido *et al.*, 2013). Wastewater treatment with the Anammox process in the main line would produce 24 watt hours per person equivalent per day (Wh/pe·d), compared to a consumption of 44 Wh/pe·d in conventional treatment (Kartal *et al.*, 2010). This indicates that the performance of the WWTPs will improve from an energetic and environmental point of view. Morales *et al.* (2012) checked several modifications of a conventional WWTP flowsheet in order to improve its sustainability (Figure 6.1) and found cost savings and N_2O emissions reductions can reach 68% and 83%, respectively, when Anammox process is applied to both sludge and main line.

Nevertheless, in order to apply the Anammox based processes to the main stream of WWTPs two crucial constraints have to be solved: a) the slow growth rate of the microorganisms at the low operational temperatures; b) the low biomass production due to the low substrate concentration in the wastewater. Due to these two situations, to guarantee the minimal biomass losses in the effluent will be of great importance. In addition, in these operational conditions nitrite oxidizing bacteria (NOB) will develop and compete with Anammox bacteria for the nitrite, producing nitrate instead of the desired N_2. If the conditions for its repression are not fulfilled the overall nitrogen removal efficiency of the system will decrease.

In general, the temperature of the wastewater is slightly higher than that of the water supply, because of the addition of warm water from the domestic use (Frijns *et al.*, 2013). Depending on the season and region the temperature varied in a wide range, but conventional values for most municipal WWTPs are around 10-20 ºC. In the case of operating single unit systems, where the ammonia oxidation to nitrite and the Anammox processes simultaneously occur in a biofilm, the temperature decrease would have a similar effect over the activity of AOB and Anammox bacteria. This is related to the fact that ammonia oxidation and Anammox processes have similar activation energy values, which are 68 and 70 kJ/mol, respectively (Strous *et al.*, 1999, Hao *et al.*, 2002a). To operate at lower temperatures increases the oxygen solubility in the bulk liquid and at the same time provokes the diminishing of the oxygen consumption by the AOB in the outer layers of the biofilm. The combination of both effects produces the increase of the oxygen penetration depth in the biofilm. Furthermore, if ammonium concentrations to be treated are low to maintain stable the operational conditions in terms of DO concentration is difficult. As the AOB are in charge of the production of the nitrite used by Anammox bacteria and of the consumption of the dissolved oxygen (both substances inhibitors of the Anammox bacteria), the fluctuations in their activity and, therefore, in the oxygen penetration depth can produce the unstable operation of the system. Among the biofilms, the granular biomass can achieve higher nitrogen removal performance due to their large surface area for mass transfer, compared to flat or cylindrical ones.

Table 6.1. Overview of one stage Anammox reactors operated at temperatures below 25ºC or low nitrogen loading rates. (SBR: Sequencing Batch Reactor, RBC: Rotating Biological Contactor, MBBR: Moving Bed Bioreactor, NLR: Nitrogen Loading Rate, NRR: Nitrogen Removal Rate, DO: Dissolved Oxygen).

Temperature (ºC)	Reactor	Reactor (volume)	DO (mg/L)	Influent (mg N/L)	NLR (g N/L·d)	NRR (g N/L·d)	HRT (h)	Ref.
20	SBR	1.5 L	3.1	225	0.9 ± 0.2	0.5	6	[1]
15			2.1	175	0.7 ± 0.2	0.2	6	
18 - 24	SBR	1.5 L	0.3 - 3.1	150 - 350	0.3 - 0.7	0.45	12	[2]
18 ± 3	SBR	2.9 L	1.5	190	1.0	0.9	7.7	[3]
25	RBC	3.6 L, 1.32 m^2	1.4	537	0.82	0.64	16	[4]
			1.2	278	0.84	0.56	8.3	
			1.4	146	0.83	0.47	4.2	
			1.2	66	0.85	0.44	2	
			1.4	29	0.85	0.30	1	
			1.2	31	0.84	0.38	1	
25	SBR	4.1 m^3	0.5 - 1.0	800	-	0.65	-	[5]
25	MBBR	2.1 m^3	1.9	568	0.85	0.39	16	[6]
22 - 36	SBR	500 m^3	0.3	1844	0.43	0.37	100	[7]
17 - 22	SBR	2.9 L	0.2 - 1.0	230	0.92	0.9	6	[8]
24	RBC	2.5 L, 1.32 m^2	1.3	60	1.44	0.75	1.8	[9]
22			1.4			0.71	1.7	
17			1.7			0.49	1.9	
16			2.8			0.52	1.1	
15			2.4			0.52	1.6	
14			3.1			0.60	1.1	

References: [1] Vázquez-Padín *et al.* (2011), [2] Vázquez-Padín *et al.* (2009), [3] Winkler *et al.* (2012), [4] De Clippeleir *et al.* (2011), [5] Vlaeminck *et al.* (2010), [6] Szatkowska *et al.* (2007), [7] Wett (2007), [8] Winkler *et al.* (2011), [9] De Clippeleir *et al.* (2013).

Table 6.1. (Continuation) Overview of one stage Anammox reactors operated at temperatures below 25ºC or low nitrogen load rates.

Temperature (ºC)	Reactor	Reactor (volume)	DO (mg/L)	Influent (mg N/L)	NLR (g N/L·d)	NRR (g N/L·d)	HRT (h)	Ref.
20	Bio Ceramic Filter	1.8 L	6.5 (in the effluent)	370 - 441	1.3	1.00	1.2 - 1.6	[10]
20				245 - 361	1.5	1.24	0.8 - 1.2	
24				165 - 218	1.4	1.04	0.6 - 0.8	
12	SBR	5 L	0.05	70	0.025	0.0225	67	[11]
17-36	SBR biocarriers	18 L	0.5	920- 3640	0.23 - 0.91	0.21 - 0.82	96	[12]
25	MBBR	200 L	1.5 - 3.5	800 - 1000	2.5 - 3.9 (g N/m²·d)	1.35-3.22 (g N/m²·d)	-	[13]
20	SBR	1.5 L	3.8	217	0.90	0.50	6	[14]
20			1.9	186	0.78	0.40		
15			2.8	192	0.79	0.13		
15			1.5	185	0.76	0.43		
15			1.3	74	0.34	0.03		
15			0.25	48	0.21	0.04		
15		4 L	0.17	53	0.11	0.06	12	

References: [10] Chang *et al.* (2013), [11] Hu *et al.* (2013), [12] Daverey *et al.* (2013), [13] Yang *et al.* (2011), [14]: This study.

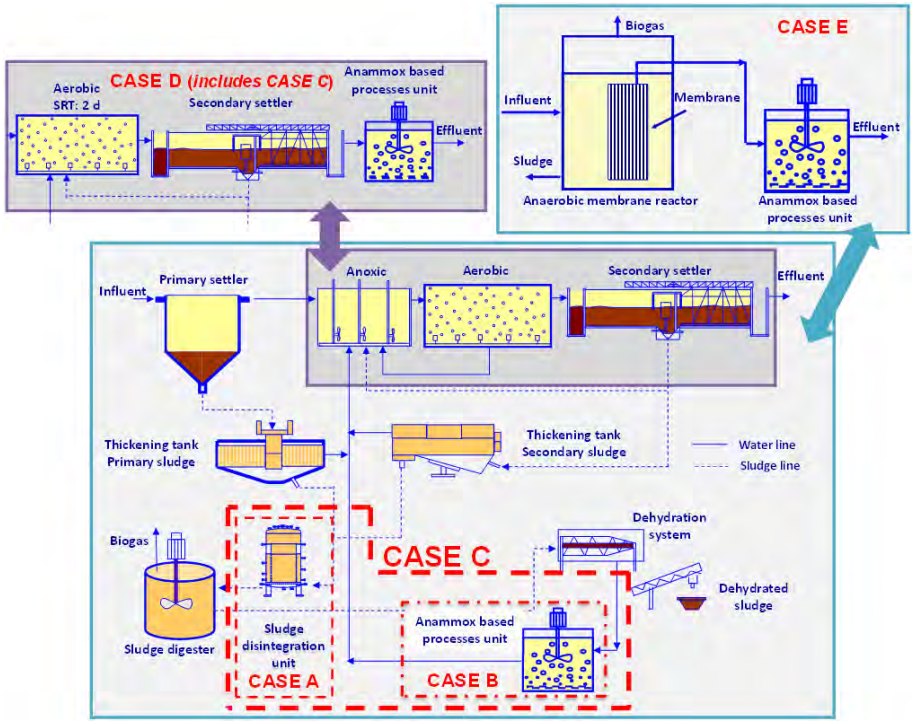

Figure 6.1. Possible modifications of a conventional WWTP to improve its energetic efficiency: sludge line (dotted lines); water line (boxes indicating the substituting units). Case A: the implementation of a sludge disintegration unit previously to the anaerobic sludge digester. Case B: the implementation of an Anammox based processes system to treat the supernatant of the anaerobic sludge digester. Case C: the implementation of both sludge disintegration unit and an Anammox based processes system in the sludge line. Case D: in addition to modification of Case C in the water line, an aerobic reactor operated at a sludge retention time of 2 d, in order to remove only organic matter and maximize the sludge production, followed by an Anammox based processes system, which replaced the nitrification-denitrification reactor. Case E: an anaerobic membrane reactor was used to remove the organic matter previously to the application of the Anammox based processes systems as single units of the WWTP (Morales *et al.*, 2012).

The activation energy value of nitrite oxidation (44 kJ/mol) (Hao *et al.*, 2002a) is lower to that of the Anammox process. Therefore, at low temperatures nitrite oxidation by NOB is promoted and a no desirable competition between NOB and Anammox biomass can be established. To avoid NOB activity at low temperature two possible strategies can be applied: 1) the NOB inhibition by free ammonia (FA) and/or free nitrite (FNA) (Anthonisen *et al.*, 1976), which is only feasible when the system is operated at high inlet ammonia concentrations; and 2) the operation at low DO concentrations which implies also a decrease of the AOB activity and, therefore, of the nitrogen removal capacity of the system.

In the case of the CANON granules, the oxygen penetration depth defines the existence of a borderline between AOB and Anammox, as oxygen inhibits the activity of the latter bacteria (Strous *et al.*, 1997a). Experimental research by Vlaeminck *et al.* (2010), Vázquez-Padín *et al.* (2010b) and mathematical simulations by Volcke *et al.* (2010) established that AOB are located at the outside layers of the granule, where oxygen and ammonia are present. The activity of AOB is directly related to the fraction of the granule which is under aerobic conditions (Vázquez-Padín *et al.*, 2011). In addition, oxygen limitation regulates the amount of nitrite produced and as a consequence also influences the Anammox activity. The oxygen penetration depth in biofilms varies typically in a range from 75 to 200 µm. Therefore, to guarantee the AOB access to DO and to maximize the reactor capacity, to maximize the surface area of the biofilm or granule is required (van Loosdrecht and Heijnen, 1993). Vázquez-Padín *et al.* (2010b), working with granular biomass from a CANON reactor, determined that the oxygen penetration depth enlarged from 100 to 350 µm, when the DO concentration in the bulk liquid was increased from 1.5 to 35.2 mg O_2/L. Due to the sizes of the granules, much larger than 700 µm and to the high oxygen demand in the surface layer because of the AOB activity, an anoxic zone is present in the core of the granule, where the Anammox process can take place. The NOB are located inside the granules, below the layer of AOB, where nitrite produced by the AOB is accessible and oxygen is still available (Figure 6.2). Anammox bacteria are situated in the anoxic part of the granule, but still close to the bulk liquid and AOB layer, as they need ammonium and nitrite for their activity.

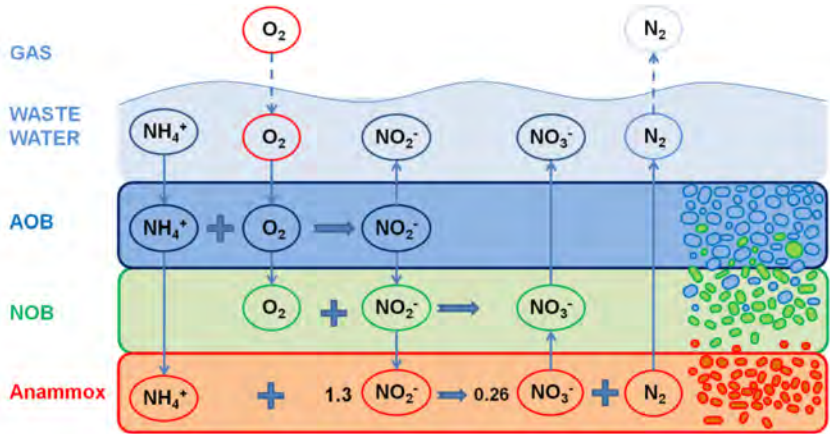

Figure 6.2. Schematic overview of the aerobic and anoxic reactions in a CANON biofilm system, consisting of three layers of bacteria: AOB (blue), NOB (green) and Anammox (red).

When operational conditions such as the temperature are changed, the dissolved oxygen (DO) concentration in the bulk liquid can be fitted in such a way that a constant oxygen penetration depth is maintained. In this way, although the ammonia oxidation and Anammox rates decrease, a dynamic behavior of the system is expected to avoid variations

in the ratio between both processes and keep the stable performance of the Anammox based process (Vázquez-Padín *et al.*, 2011).

The sizes of the granules also have an effect on the overall activity of the AOB. According to Volcke *et al.* (2010) Different granules have distinct surface/volume ratios and consequently, different oxygen depth penetrations. Vlaeminck *et al.* (2010) showed that the activity and abundance of AOB decrease and the activity and abundance of Anammox bacteria increase with the increase of the size of the granule creating a dynamic behavior of the systems in terms of the properties of the granules. The NOB out competition over Anammox bacteria can be produced in small granules, where most part of the biomass is under aerobic conditions (Volcke *et al.*, 2012).

It is clear that previous to the application of the Anammox based process to effluents characterized by low temperature and low ammonia concentrations several questions have to be faced.

The aim of this work was to study the performance of the autotrophic nitrogen removal at low temperatures, 15 and 20 ºC, in a CANON reactor with granular biomass. The system was initially fed with an influent containing moderate nitrogen concentrations (150-250 mg NH_4^+-N/L) in order to study its maximum capacity and long-term stability and, subsequently, with an influent containing nitrogen concentrations of 50-80 mg NH_4^+-N/L to mimic the conditions of the main stream of a WWTP pretreated for organic matter removal.

6.2 MATERIALS AND METHODS

Research was performed in two reactors: SBR 1 operated during 1676 days and SBR 2 operated during 186 days. The operation of SBR 1 was divided into six different stages, where the temperature, the applied nitrogen load rate and the DO concentration were modified. A summary of the operational stages and their main characteristics is compiled in Table 6.2.

6.2.1 Reactors description

A glass Sequencing Batch Reactor (SBR 1) with a working volume of 1.5 L (Figure 6.3 A and B) was used in stages A-I to A-VI. Dimensions of the unit were: total height of 465 mm, working height of 270 mm, and inner diameter of 85 mm. The height to diameter ratio (H/D) was 3.2. The volume exchange ratio was fixed at 50%. The hydraulic retention time was fixed at 0.25 d.

Table 6.2. Operational conditions and influent characteristics in the experimental stages.

Reactor	Stage	Days	DO (mg O_2/L)	Temperature (ºC)	$NH_4^+{}_{inf}$ (mg N/L)	NLR (g N/L·d)	pH
SBR 1	A-I	1-506 (506)	3.8±1.4	20	216±48	0.90±0.19	7.8±0.1
	A-II	507-623 (116)	2.8±1.3	15	193±26	0.79±0.10	7.8±0.1
	A-III	624-945 (321)	2.0±0.8	20	186±32	0.78±0.12	7.7±0.1
	A-IV	946-1120 (174)	1.5±0.3	15	185±24	0.76±0.10	7.7±0.2
	A-V	1250-1585 (335)	1.2±0.8	15	74±19	0.34±0.08	7.6±0.2
	A-VI	1600-1676 (76)	0.25±0.13	15	48±7	0.21±0.02	8.0±0.3
SBR 2	B-I	1-186 (186)	0.17±0.21	15	53±5	0.11±0.01	8.0±0.2

A mixture of N_2 gas and recycled off-gas from the reactor was supplied from the bottom of the reactor by using an air pump to promote the appropriated transfer of oxygen into the bulk liquid and to reach a suitable mixing (stages A-I to A-V). The DO concentration in the liquid phase was regulated by changing the ratio of fresh air to recycled air injected into the reactor. In stage A-VI a mechanical stirring system (100 rpm) was installed to provide the mixing of the biomass. During stage A-VI, the DO concentration was regulated changing the fresh air flow added to the reactor.

A glass Sequencing Batch Reactor (SBR 2) with a working volume of 4.0 L (Figure 6.3 C and D) was used in stage B-I. Dimensions of the unit were: total height of 340 mm, working height of 220 mm and inner diameter of 160 mm. The H/D ratio was 1.4. The volume exchange ratio was fixed at 25%, while the hydraulic retention time was fixed at 0.5 d. Fresh air was supplied from the bottom of the reactor by using an air pump. A mechanical stirring system (150 rpm) was installed to provide the mixing of the biomass.

In both cases, a set of two peristaltic pumps was used to introduce the feeding solution on the top of the reactor and to discharge the effluent at medium height in the SBR 1 and at 75% of the height in the SBR 2. A programmable logic controller Siemens model S7-224 CPU controlled the actuations of the pumps and valves, and regulated the different periods of the operational cycle. The pH was not controlled and ranged between 7.2 and 8.5. A thermostatic bath was installed to control the temperature inside the reactor at 15 and 20 ºC.

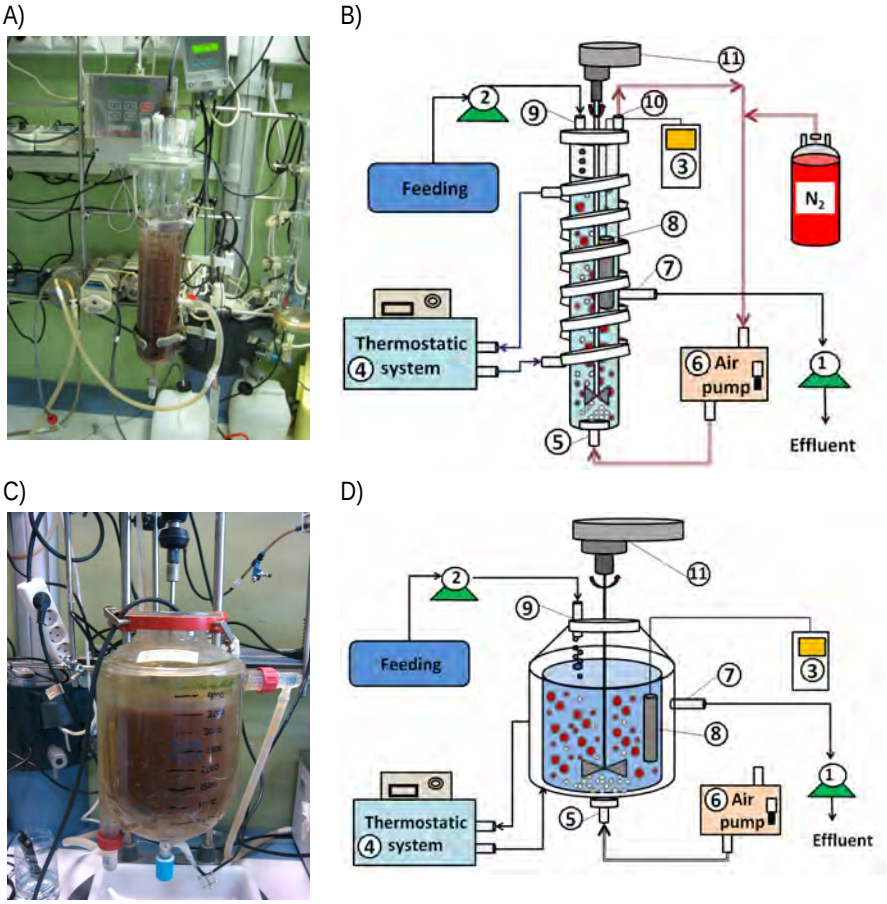

Figure 6.3. A) and C) images of the SBR 1 and SBR 2, respectively. B) and D): schemes of the experimental set ups for SBR 1 and SBR 2, respectively: 1) Effluent pump. 2) Influent pump. 3) Dissolved oxygen indicator. 4) Thermostatic bath. 5) Gas diffuser. 6) Air pump. 7) Effluent port. 8) Dissolved oxygen probe. 9) Influent port. 10) Recycled air port. 11) Mechanical stirring system (For SBR 1 only during stage VI).

6.2.2 Cycle distribution

The SBR reactors were operated in a semi continuous mode, as the feeding and aeration phases took place simultaneously during more than 90% of the cycle time. The duration of the operational cycles was of 3 hours distributed as follows:

- Stages A-I to A-V of SBR 1 according to the distribution described in Figure 6.4 A. The minimum settling velocity ($V_{s,min}$) for the biomass to be retained in the reactor was around 8.1 m/h.
- Stage A-VI of SBR 1 according to the distribution described in Figure 6.4 B. The $V_{s,min}$ varied from 8.1 to 0.3 m/h.

- SBR 2 according to the distribution described in Figure 6.4 C. The $V_{s,min}$ varied from 0.1 to 0.3 m/h.

6.2.3 Influent

Both CANON reactors were fed with the supernatant an anaerobic sludge digester in operation at a municipal WWTP, which was collected every month in 20 L containers and stored in a cold room at 4 ºC. The liquid supernatant was diluted with tap water (50-90%) in order to achieve the desired moderate (around 200 mg NH_4^+-N/L) and low (50-80 mg NH_4^+-N/L) ammonia concentrations of the media fed to the SBRs. Sodium bicarbonate was added to the influent to guarantee the presence of more than 50 mg IC/L in the effluent to avoid alkalinity limitations in the reactor.

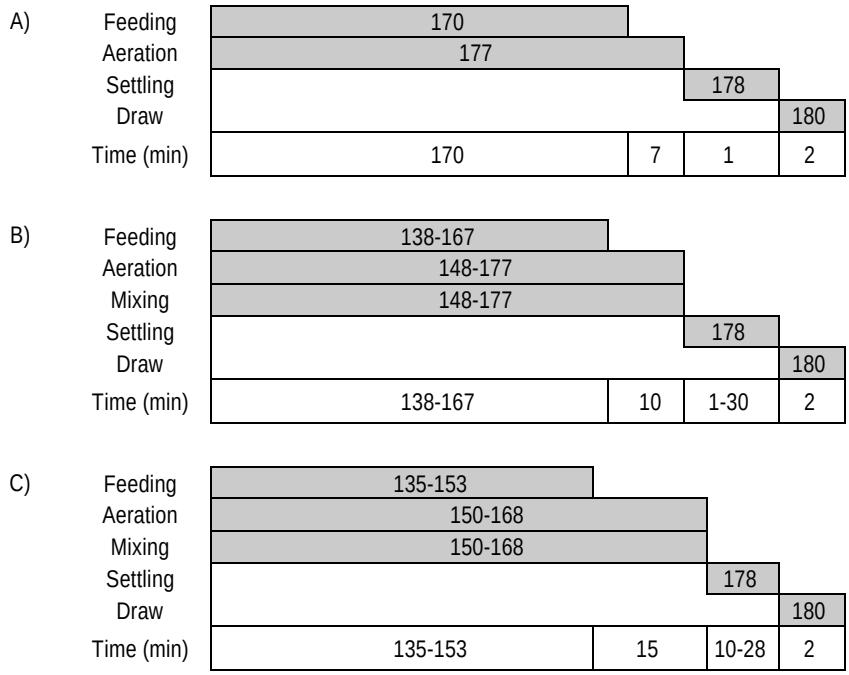

Figure 6.4. Distribution of the operational cycles of the granular SBRs in minutes. A) SBR 1 during stages A-I to A-V. B) SBR 1 during stage A-VI. C) SBR 2 during stage B-I.

6.2.4 Inoculums

CANON granules developed in a previous research (Vázquez-Padín *et al.*, 2011) were used as inoculum of SBR 1. These granules were produced in a granular nitrifying reactor operated under oxygen limiting conditions to promote the suitable conditions for the Anammox biomass development: 1) the presence of ammonia and nitrite in an equimolar

ratio; 2) high biomass retention capacity; and 3) the existence of an anoxic zone in the inner zones of the granules.

Later, granules from a 200 L CANON reactor treating the supernatant of an anaerobic sludge digester of a municipal WWTP at around 30 ºC were used in the performed re-inoculations on stages A-V (Vázquez-Padín *et al.*, 2012), A-VI and B-I (Vázquez-Padín *et al.*, 2013).

6.2.5 Analytical methods

The pH value and the concentrations of ammonia, nitrite, nitrate, biomass as volatile suspended solids (VSS) and total suspended solids (TSS) were determined according to the Standard Methods (APHA, 1998). Concentrations of total organic carbon (TOC) and total inorganic carbon (IC) were measured with a Shimadzu analyzer (TOC-5000). Dissolved Oxygen (DO) concentration was determined during stages A-I and A-II using a dissolved oxygen membrane meter (WTW CellOx 325) and with a luminescent dissolved oxygen probe (LDO, Hach Lange) from stage A-III on. The biomass density as mass of granules per volume of granules was determined using the dextran blue method described by Beun *et al.* (2002). Changes in morphology of the granules were followed by image analysis with a stereomicroscope (Stemi 2000-C, Zeiss) incorporating a digital camera (Coolsnap, Roper Scientific Photometrics). For the determination of the size distribution of the granules, the software Image Pro Plus was applied to the digital images of samples of granules. Almost 100 granules were analyzed in each sample. Average diameter of the granules and a size distribution in categories of 0.5 mm were determined supposing a spherical shape of the particles. Batch assays used to estimate the specific Anammox activity (SAA) were performed according to the methodology described by Dapena-Mora *et al.* (2007). This method was based on the measurement along time of the overpressure generated by the nitrogen gas produced in tightly closed vials with a total volume of 38 mL and 25 mL of liquid volume. The overpressure in the headspace was measured using a differential pressure transducer 0–5 psi (Centerpoint Electronics). Respirometric assays in order to determine the nitrifying capability of the biomass were performed by means of a respirometric method, using a Biological Oxygen Monitor (BOM, YSI model 5300) with two oxygen selective probes (YSI 5331) connected to a data acquisition system. Specific Anammox activity and respirometric assays were performed to the biomass from the reactor and to separated fractions of granules and flocculent biomass.

6.2.6 Calculations

6.2.6.1 Removal rates

Ammonia and nitrite oxidation rates (AOR and NOR, respectively) and nitrogen removal rate by Anammox bacteria (ANR) in g N/L·d, and Ammonia removal efficiency (AR)

and Nitrogen removal efficiency (NR) in %, were estimated based on the Anammox process stoichiometry showed in equation (6.1) and in nitrogen balances according to equations (6.2), (6.3), (6.4), (6.5), (6.6) and (6.7).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O \quad (6.1)$$

$$\Delta N = (NH_4^+\text{-}N_{Inf} + NO_2^-\text{-}N_{Inf} + NO_3^-\text{-}N_{Inf}) - (NH_4^+\text{-}N_{Eff} + NO_2^-\text{-}N_{Eff} + NO_3^-\text{-}N_{Eff}) \quad (6.2)$$

$$AOR = \frac{NH_4^+\text{-}N_{Inf} - NH_4^+\text{-}N_{Eff} - \Delta N/2.04}{HRT} \quad (6.3)$$

$$NOR = \frac{NO_3^-\text{-}N_{Eff} - NO_3^-\text{-}N_{Inf} - 0.26 \cdot \Delta N/2.04}{HRT} \quad (6.4)$$

$$ANR = \frac{\Delta N}{HRT} \quad (6.5)$$

$$AR = \frac{NH_4^+\text{-}N_{Inf} - NH_4^+\text{-}N_{Eff}}{NH_4^+\text{-}N_{Inf}} \cdot 100 \quad (6.6)$$

$$NR = \frac{\Delta N}{NH_4^+\text{-}N_{Inf} + NO_2^-\text{-}N_{Inf} + NO_3^-\text{-}N_{Inf}} \cdot 100 \quad (6.7)$$

Being NH_4^+-N_{Inf}, NO_2^--N_{Inf} and NO_3^--N_{Inf} the ammonium, nitrite and nitrate concentrations (g N/L) in the influent, and NH_4^+-N_{Eff}, NO_2^--N_{Eff} and NO_3^--N_{Eff} the ammonium, nitrite and nitrate concentrations in the effluent. HRT is the hydraulic retention time (d). Specific rates (in g N/g VSS·d) were determined taking into account the VSS concentration in the reactor.

The ratio AOR/ANR=0.637 indicates an ideal ratio between AOB and Anammox activities. The nitrite produced by AOB is completely consumed by the Anammox bacteria. The ratio AOR/ANR>0.637 indicates that a fraction of the nitrite is not consumed by the Anammox bacteria. In this case, if (AOR-NOR)/ANR=0.637, the NOB consume this nitrite. On the other hand, when (AOR-NOR)/ANR>0.637, nitrite will accumulate in the system.

Possible heterotrophic denitrification rate (HR) was considered with equation (6.8) and calculated using the TOC data, assuming a COD/TOC ratio of 3 (Mara and Horan, 2003).

$$HR = \frac{3 \cdot (TOC_{Inf} - TOC_{Eff})}{HRT} \quad (6.8)$$

being TOC_{Inf} and TOC_{Eff} the TOC concentrations (g C/L) in the influent and effluent. HR is expressed in g COD/L·d.

6.2.6.2 Biomass production

The biomass production corresponding to each population in the reactor was calculated based on the HR, AOR, NOR and ANR rates, calculated for each period according to equation (6.9) where the observed yield values (Y_{obs} in g VSS/g $Substrate_{removed}$) were calculated according to equation (6.10), being V_R, the volume of the reactor (L).

$$\text{biomass}\left(\frac{\text{g VSS}}{\text{d}}\right) = (AOR \cdot Y_{obs,\,AOB} + NOR \cdot Y_{obs,\,NOB} + ANR \cdot Y_{obs,\,Anammox} + HR \cdot Y_{obs,\,Het}) \cdot V_R \quad (6.9)$$

$$Y_{obs} = \frac{Y}{1 + f_b \cdot b \cdot SRT} \quad (6.10)$$

The biomass yield coefficients (Y in g VSS/g $Substrate_{removed}$) and decay rate constant (b in d^{-1}) of each bacterial group were taken from Hao *et al.* (2002b). The sludge retention time (SRT in days) of each period was calculated with equation (6.11), where VSS_r and VSS_{Eff} are the volatile suspended solids in the reactor and effluent respectively in g VSS/L, and HRT the hydraulic retention time expressed in days. The biodegradable fraction of the biomass (f_b) was calculated according to equation (6.12), assuming a value of 0.8 for the fresh biomass (f_b') taken from von Sperling (2007).

$$SRT = \frac{VSS_r \cdot HRT}{VSS_{Eff}} \quad (6.11)$$

$$f_b = \frac{f_b'}{1 + (1 - f_b') \cdot b \cdot SRT} \quad (6.12)$$

6.2.6.3 Oxygen depth penetration

The oxygen depth penetration in the CANON granules was theoretically determined taking into account the internal mass transfer and the chemical bio reaction rate of ammonia oxidation (zero order) within the volume of the granules, assuming them as spherical particles (Figure 6.5) in steady state conditions and with no external mass transport resistance.

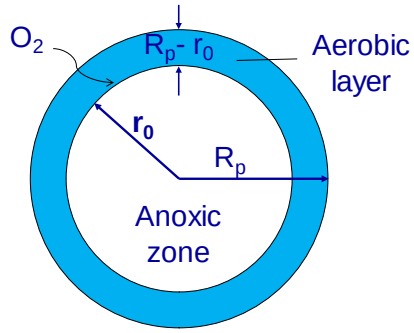

Figure 6.5. Representation of the oxygen depth penetration in the granules.

The Thiele module (M_{T0}), the ratio between the reaction and mass transfer rates, was determined with the equation (6.13). Then the anoxic zone radius (r_0 in m) can be calculated using the equation (6.14).

$$M_{T0} = \frac{R_p}{3\sqrt{2}}\sqrt{\frac{r_{max}\cdot\rho_{granule}}{D_{O_2}\cdot C_{O_{2,S}}}} \quad (6.13)$$

$$r_0 = R_p\cdot\left[\frac{1}{2}+\text{sen}\left[\frac{1}{3}\text{arctg}\left(\frac{3M_{T0}^2-2}{2\sqrt{3M_{T0}^2-1}}\right)\right]\right] \quad (6.14)$$

Where R_p is the radius of the granule (m), D_{O_2} is the oxygen diffusivity in water ($7.57\cdot10^{-6}$ m²/h at 20 ºC and $6.55\cdot10^{-6}$ m²/h at 15 ºC) (Denny, 1993), $C_{O_{2,s}}$ is the dissolved oxygen concentration in the surface of the granule (mg O_2/L), $\rho_{granule}$ the density of the granules (g VSS/$L_{granule}$) and r_{max} the maximal biological rate of the biomass (mg O_2/g VSS·h). The r_{max} was calculated using the value of the overall specific activity of the biomass determined by Vázquez-Padín *et al.* (2010a) at the beginning of their experimental period. Then the iteration strategy suggested by Vázquez-Padín et al. (2010c) was applied to this data by dividing the initial value by the fraction of active biomass calculated from equation (6.15) in order to obtain the r_{max}. Temperature adaptation of this value was performed with Arrhenius equation using the E_a=70 kJ/mol (Strous *et al.*, 1999).

$$\text{Active biomass fraction} = \frac{4/3\,\pi\,R_p^{\,3} - 4/3\,\pi\,r_0^{\,3}}{4/3\,\pi\,R_p^{\,3}} \quad (6.15)$$

6.2.6.4 Ammonia oxidation rate ratio

For given values of the particles radius, dissolved oxygen concentration, granule density and/or temperature change, the ratio between the AOR rate corresponding to the initial operational conditions and that corresponding to the final conditions is given by

equation (6.16). As the biomass density of the granules ($\rho_{granule}$ in g VSS/$L_{granule}$) varied in some of the operational stages, this parameter was also taken into account for the prediction of the AOR modifying the equation obtained by Vázquez-Padín *et al.* (2011):

$$\frac{(-r_{NH_4^+})_1}{(-r_{NH_4^+})_2} = \frac{R_{p_2}}{R_{p_1}} \cdot \sqrt{\frac{C_{O_2 1} \cdot \rho_{granule_2}}{C_{O_2 2} \cdot \rho_{granule_1}} \cdot e^{\frac{-E_a}{R}\left(\frac{1}{T_1}-\frac{1}{T_2}\right)}} \qquad (6.16)$$

Where ($-r_{NH_4^+}$) is the specific consumption rate of ammonia (g NH_4^+-N/g VSS·d), E_a is the activation energy (70 kJ/mol); R is the ideal gas constant ($8.314 \cdot 10^{-3}$ kJ/mol·K); and T is the temperature (K).

As in some stages, the average DO concentration applied was below twice the half saturation constant for the oxygen, K_M = 0.74 mg O_2/L (Guisasola *et al.*, 2005), a first order kinetic should be included for these conditions. The inclusion of first and zero order kinetics lead to the equation (6.17).

$$\frac{(-r_{NH_4^+})_1}{(-r_{NH_4^+})_2} = \frac{R_{p_2}}{R_{p_1}} \cdot \sqrt{2 \cdot K_M \frac{C_{O_2,1} \cdot \rho_{granule_2}}{C_{O_2,2} \cdot \rho_{granule_1}} \cdot e^{\frac{-E_a}{R}\left(\frac{1}{T_1}-\frac{1}{T_2}\right)}} \qquad (6.17)$$

6.3 RESULTS

During the operation of SBR 1 (A-I to A-VI) and SBR 2 (B-I) the temperature and dissolved oxygen concentration varied as shown in Figure 6.6. SBR 1 reactor was started at 20 ºC (stage A-I) and when stable operational conditions were achieved, the temperature was decreased to 15 ºC (stage A-II). As the biomass was lost, and the system stability was not maintained during this period, the temperature was returned to 20 ºC (stage A-III) in order to test the recovering potential of the system. Once the stable conditions were recovered, the operation at low temperature was tested again with different values of DO concentration (stage A-IV).

In stage A-V, the operation at low temperature and low nitrogen loading rate was tested. Due to the observed mixing problems of the biomass during this stage, a mechanical stirring was installed inside the reactor during stage A-VI, and finally, a different reactor configuration was tested in stage B-I, again operated at low temperature and nitrogen concentration.

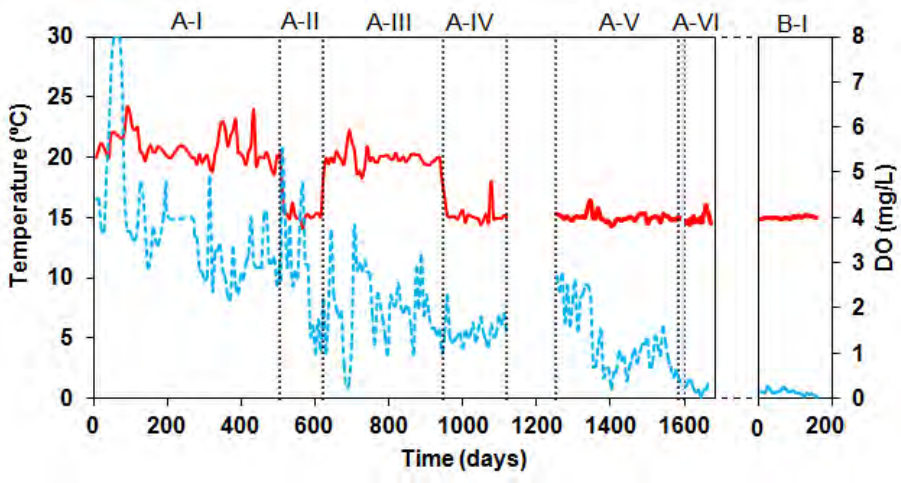

Figure 6.6. Temperature in ºC (—) and DO concentration in mg/L (---) in the CANON reactor SBR 1 in stages A-I to A-VI and in the SBR 2 in stage B-I.

6.3.1 Operation at 20 ºC and moderate nitrogen loading rates

The reactor was operated at 20 ºC in two different stages, A-I (1-506 d) and A-III (624-945 d), and in both stages the accumulation of granular biomass in the reactor was observed.

In the first stage, the reactor was started up with a concentration of 6.7 g VSS/L. After around 250 days, a concentration of about 12.0 g VSS/L was achieved (Figure 6.7 A). The biomass concentration was difficult to measure with precision at these high concentration levels, due to the high amount of granular biomass present in the reactor, which complicated the sampling method and caused the high variability observed in the measured values. However, the average biomass concentration value remained almost stable during the rest of the stage (250 days more).

Stage A-III was used to recover the biomass concentration and activity after the operation at 15 ºC in stage A-II (which will be described in section 6.3.2.1), where the biomass in the reactor dropped to 4.9 g VSS/L. Once the temperature of the reactor was elevated to 20 ºC, biomass concentration progressively rose and values of around 13.6 g VSS/L were reached at the end of this re-stabilization period, after around 300 days. The resulted biomass concentration in the reactor was quite similar to that observed during stage A-I, reaching a similar stable value, which ranged around 12-14 g VSS/L (Figure 6.7 B).

The average solid retention time (SRT) during stage A-I was of 103 ± 54 days while this value was 107 ± 61 days during stage A-III. These values indicate the good biomass

retention capacity achieved by the granular biomass system when the system was operated at 20 ºC. No difference was observed between the stage where the reactor was started up (A-I) and the recovery stage (A-III) in terms of biomass concentration inside the reactor and in the effluent. In stage A-I the solids concentration in the effluent varied between 5 and 60 mg VSS/L, with an average of 30 ± 13 mg VSS/L (Figure 6.7 A). In the recovering stage A-III, the average solids concentration in the effluent was around 33 ± 21 mg VSS/L (Figure 6.7 B).

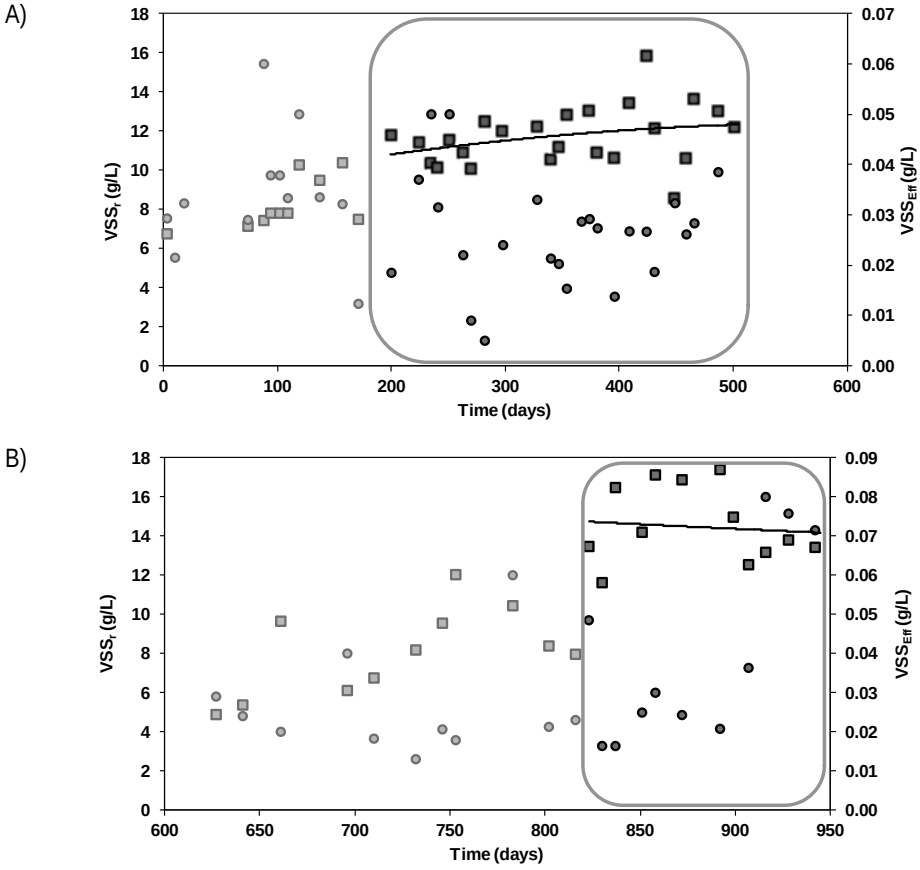

Figure 6.7. Solids concentration in the reactor (■) and in the effluent (●) in g VSS/L. A) In stage A-I. B) In stage A-III. Discussed steady state conditions are inside the square.

Regarding the nitrogen removal rates achieved, during stage A-I, ANR and AOR were 0.50 and 0.43 g N/L·d, respectively (Figure 6.8 A). Calculations were performed in order to evaluate the conversions corresponding to each of the three involved processes. The AOR/ANR ratio was 0.87, while the average value of the (AOR-NOR)/ANR ratio, calculated for the periods selected in Figure 6.7, was of 0.63. In fact, there was almost no accumulation of nitrite in the reactor, as the Anammox bacteria and NOB were using this

compound simultaneously to its production by the ammonia oxidizers, which was the limiting step of the process (Figure 6.9 A). On the other hand, the NOR values were around 0.1 g N/L·d due to the oxygen limitation. The average AR and NR efficiencies during this period were about 78% and 56% respectively, while the maximum values were obtained in the period from day 350 to day 450 which were of 85% and 68%, respectively.

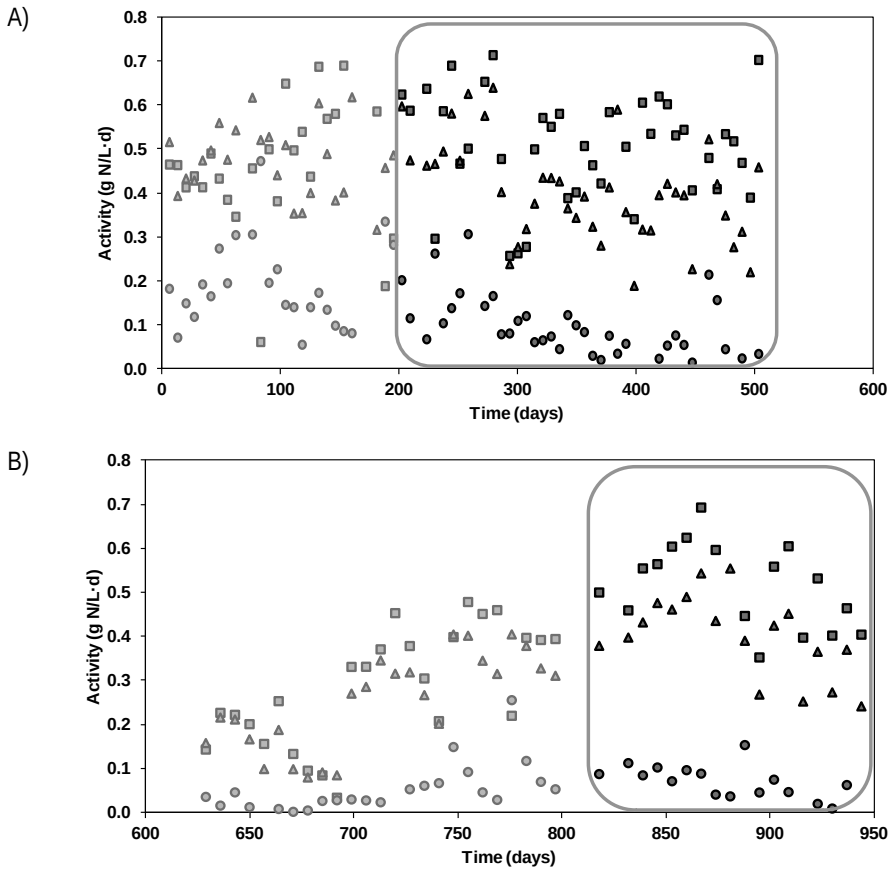

Figure 6.8. ANR (□), NOR (○) and AOR (△) in g N/L·d during A) stage A-I (20 ºC) and B) stage A-III (20 ºC). Discussed steady state conditions are inside the square.

In stage A-III changes in the reactor performance were applied to provide a better mixing of the biomass without increasing the oxygen transfer to the bulk liquid when low DO concentrations were tested. In this way a new gas diffuser was installed producing larger bubbles than the previous one. At the same time, the N_2/air ratio was increased. DO concentration was fixed at 2.0 mg O_2/L which involved obtained ANR and AOR (Figure 6.8 B), of 0.40 and 0.33 g N/L·d respectively. Average AR and NR efficiencies of 71% and 47%, respectively, were achieved, with no nitrite accumulation during the operation (Figure 6.9 B). The average NOR value was lower than 0.06 g N/L·d.

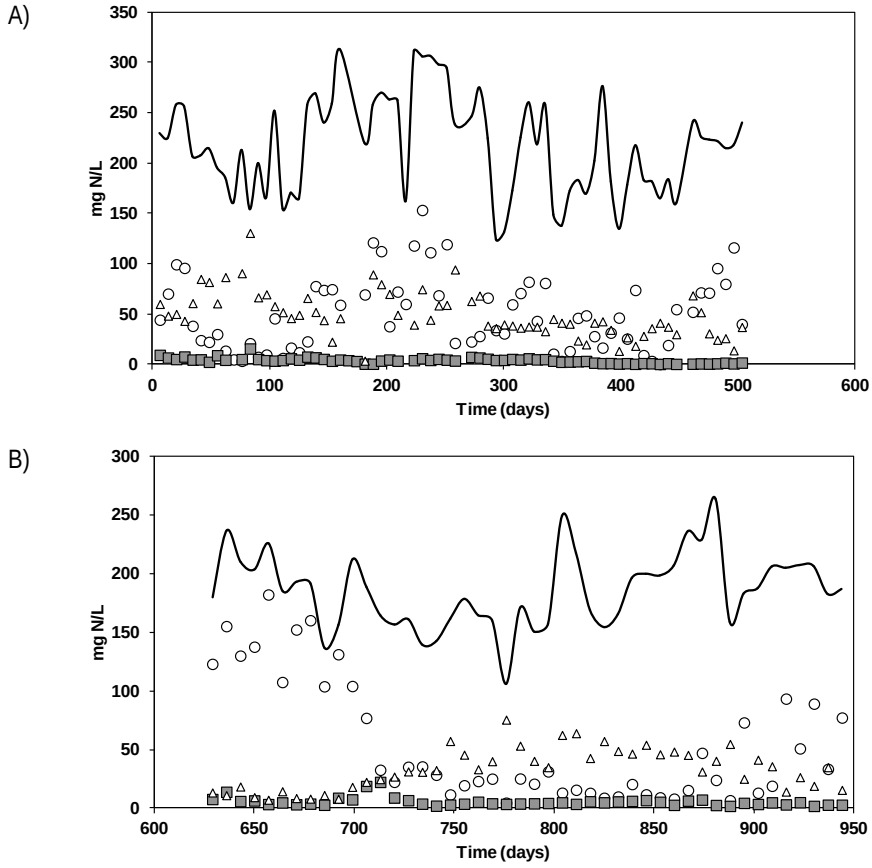

Figure 6.9. CANON operation in terms of NH_4^+-N concentration in the feeding (—) and NH_4^+-N (○), NO_2^--N (■) and NO_3^--N (△) concentrations in the effluent during A) stage A-I and B) stage A-III.

In spite of during stage A-III the reactor was recovering from previous operation at 15 ºC, the results obtained for the biomass retention in stage A-III were quite similar to those obtained in stage A-I. In addition, in the last days of stage A-III, when the biomass concentration reached its maximum (around day 800), values of ANR and AOR achieved in stage A-III were comparable to those reached in stage A-I: around 0.54 g N/L·d for Anammox activity and 0.41 g N/L·d for ammonia oxidation activity. The maximum AR and NR efficiencies were 86% and 64%, respectively in stage A-III. In addition, the ratios AOR/ANR and (AOR-NOR)/ANR were 0.75 and 0.64, indicating a suitable proportion of the different biomass activities.

The average diameter of the granules (Figure 6.10) measured during stage A-I and stage A-III were around 3.6 ± 0.2 mm and 3.4 ± 0.3 mm, respectively.

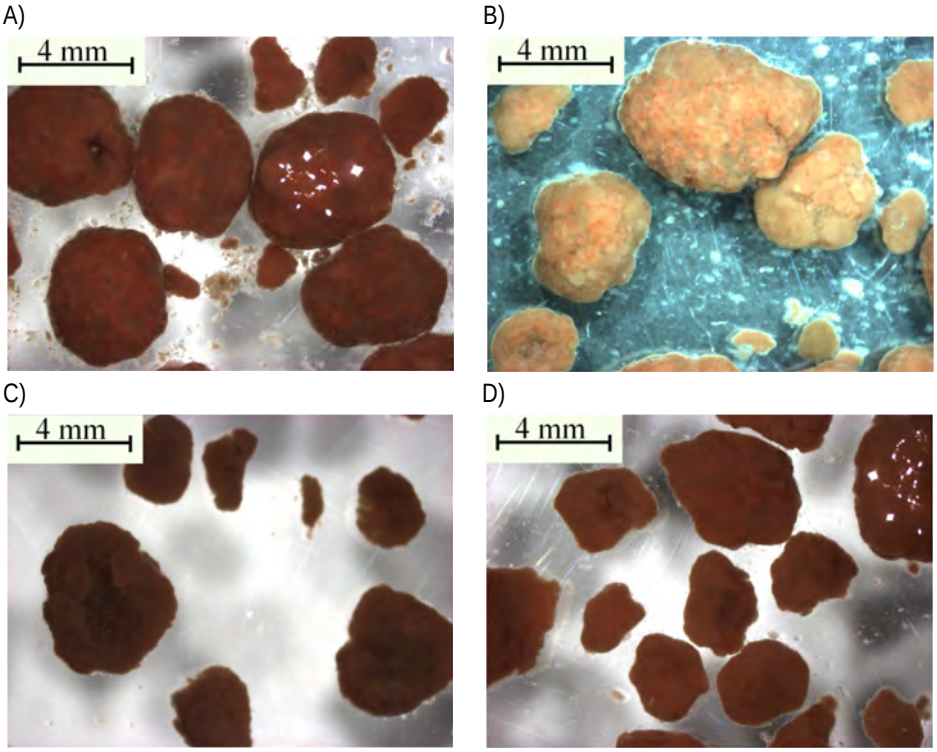

Figure 6.10. Images of the granules (zoom: 6.5x) during stage A-I: A) on day 242, B) on 263, and during stage A-III: C) on day 750 and D) on day 858.

To summarize, when the SBR 1 CANON reactor was operated at 20 ºC and fed with a moderate ammonia concentration, a high biomass retention in the form of granules was successfully achieved, exceeding the 10 g VSS/L, and nitrogen removal rate values higher than 0.5 g N/L·d were obtained. The control of the DO concentration in the bulk liquid limited the ammonia oxidation rate and regulated the balance with the Anammox bacteria; consequently, nitrite accumulation was not observed and NOB activity was limited. Similar average granular size, biomass concentration and removal capacity were achieved in both stages, independently from the initial conditions of the system. Reproducibility of the performed experiments is confirmed from obtained results.

6.3.2 Operation at 15 ºC and moderate nitrogen loads

The reactor SBR 1 was operated at 15 ºC and fed with moderate nitrogen loads in two different stages, A-II (507-623 d) and A-IV (946-1120), with the biomass obtained in the previous stages at 20 ºC. In order to maintain the oxygen penetration depth constant in the granules and therefore, the aerobic/anoxic volume ratio, the fixed DO concentration in the bulk liquid was reduced: from an average of 3.8 mg O_2/L in stage A-I to 2.8 mg O_2/L in stage A-II and from 2.0 mg O_2/L in stage A-III to 1.5 mg O_2/L in stage A-IV.

6.3.2.1 Stage A-II

The average SRT during stage A-II (507-623 d) was of 85 ± 15 days, and the biomass wash-out rate was slightly higher than during stage A-I (solids in the effluent of 35 ± 24 mg VSS/L). However, after fixing the temperature to 15 ºC, the biomass concentration progressively decreased from 12 to 6 g VSS/L (Figure 6.11 A). This effect was observed after a few weeks of operation at this temperature and could be attributed to the decrease of the biomass growth rate. This is corroborated by performed biomass balances which indicated that in this stage around 17.2 g VSS were removed with the effluent while the theoretically produced biomass due to the bacterial activity was around 8.0 g VSS (only 53% of the lost biomass) (see section 6.4). In this stage the biomass production did not compensate for the loss of biomass in the effluent.

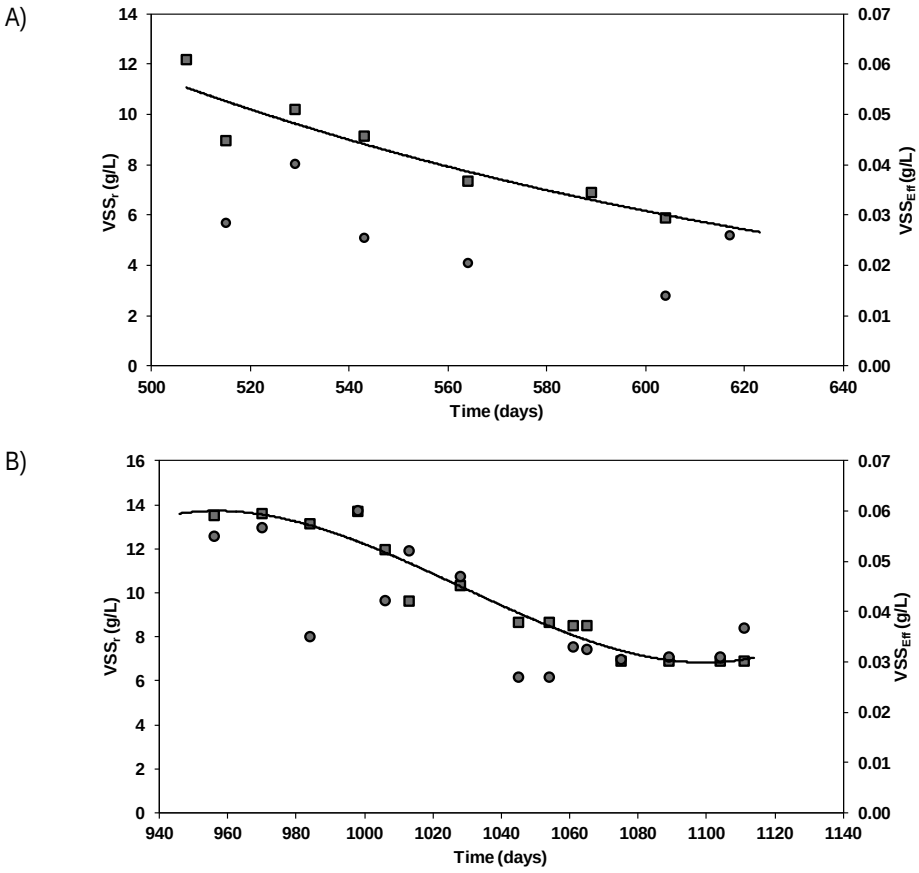

Figure 6.11. Solids concentration in the reactor (■) and in the effluent (●) in g VSS/L. A) in stage A-II and B) in stage A-IV.

The temperature decrease and the biomass loss caused the decrease of the ANR from around 0.5 to 0.1 g N/L·d and of the AOR from 0.4 to 0.2 g N/L·d (Figure 6.12 A). The average ammonia and nitrogen removal percentages reached values of 36% and 15%, respectively. In addition, during most part of the period, the ANR+NOR value was lower than that of AOR, which caused the accumulation of nitrite inside the reactor. In this way, in the last month of operation, the AOR reached values of 0.18 g N/L·d, while ANR remained below 0.1 g N/L·d. The AOR/ANR ratio varied from 0.82 to 3.62, and also the (AOR-NOR)/ANR ranged from 0.63 to 2.74. Consequently, the nitrite concentration measured in the last thirty days of operation was of 37 mg NO_2^--N/L (Figure 6.13 A). The average NOR was below 0.1 g N/L·d during this stage but reached a value of 0.2 g N/L·d between days 519 and 538 (Figure 6.12 A), which coincided with the higher levels of nitrate measured in the reactor, around 45 mg NO_3^--N/L (Figure 6.13 A).

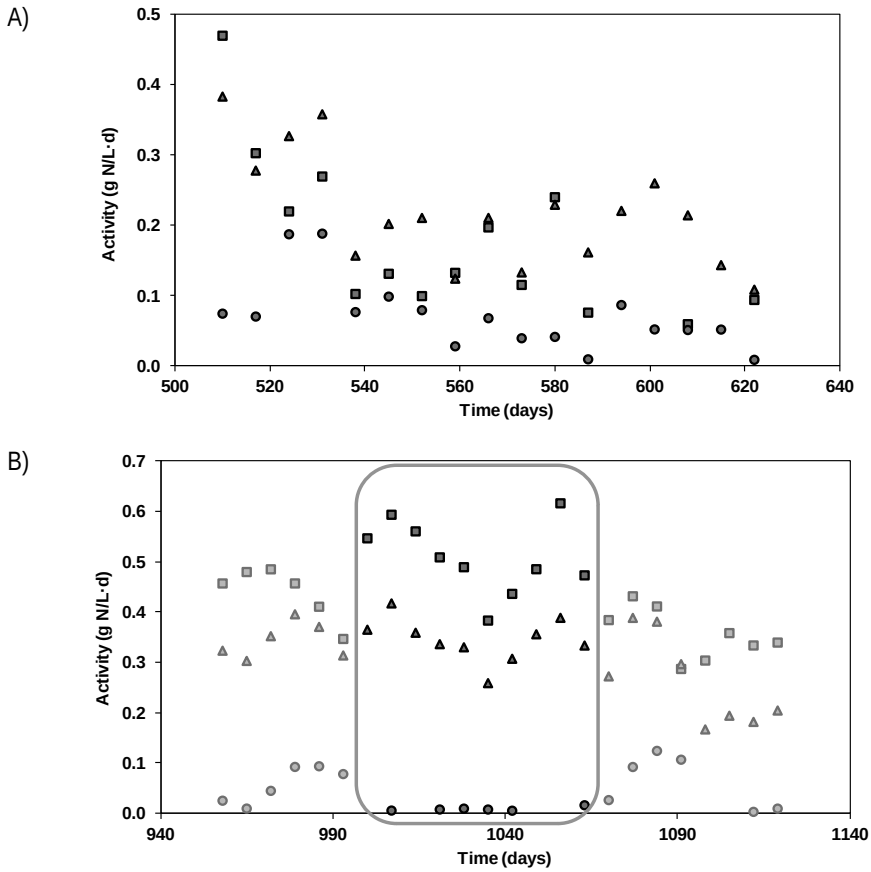

Figure 6.12. ANR (■), NOR (●) and AOR (▲) in g N/L·d. A) During stage A-II (15 ºC) and B) during stage A-IV (15 ºC). Discussed steady state conditions are inside the square.

The average diameter of the granules decreased from 3.8 mm at the end of stage A-I, to 3.2 mm at the end of stage A-II. The fraction of small granules increased, suggesting the disaggregation of bigger granules formed in the previous stage (Figure 6.14 A and B).

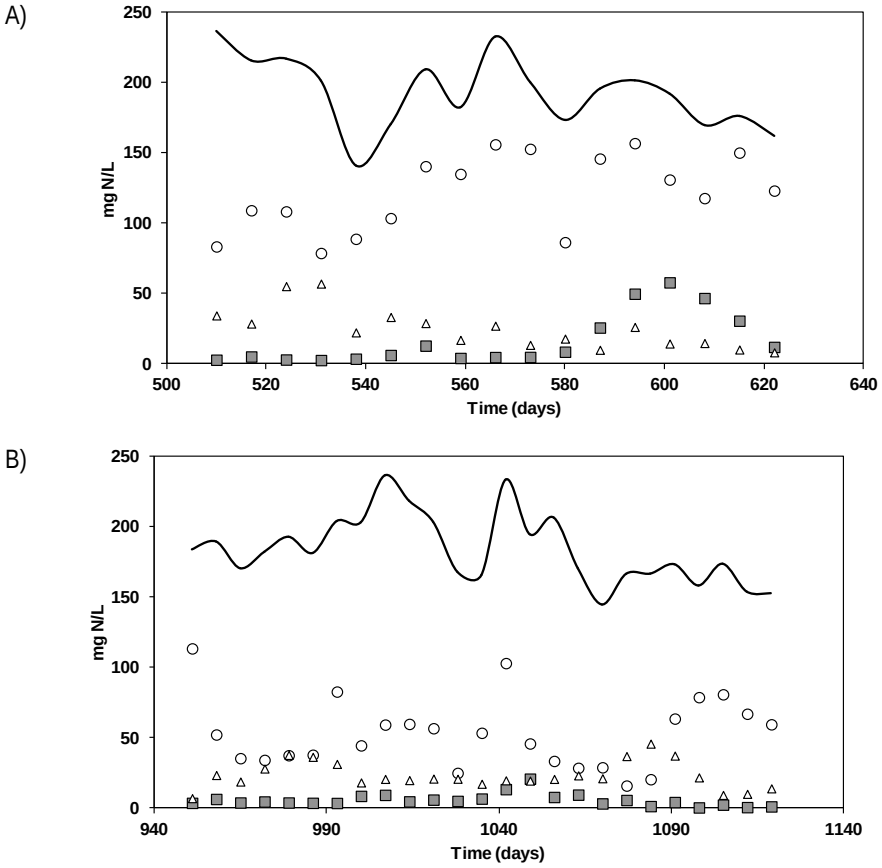

Figure 6.13. CANON operation in terms of NH_4^+-N concentration in the feeding (—) and NH_4^+-N (○), NO_2^--N (■) and NO_3^--N (△) concentrations in the effluent. A) During stage A-II and B) during stage A-IV.

In order to maintain a suitable DO level during this stage, the airflow applied to the system was reduced. This caused a negative effect in the mixing of the biomass. The aeration system was not able to completely mix the fast settling biomass, decreasing in this way the nitrogen removal capacity of the system. Thus, a recovery strategy was applied in stage A-III at 20 ºC as described before. Then a new experiment at 15 ºC was performed in stage A-IV, including the use of a different gas diffuser in the reactor which allowed obtaining a better mixing of the biomass without increasing the oxygen transfer and providing a better DO concentration control in the bulk liquid.

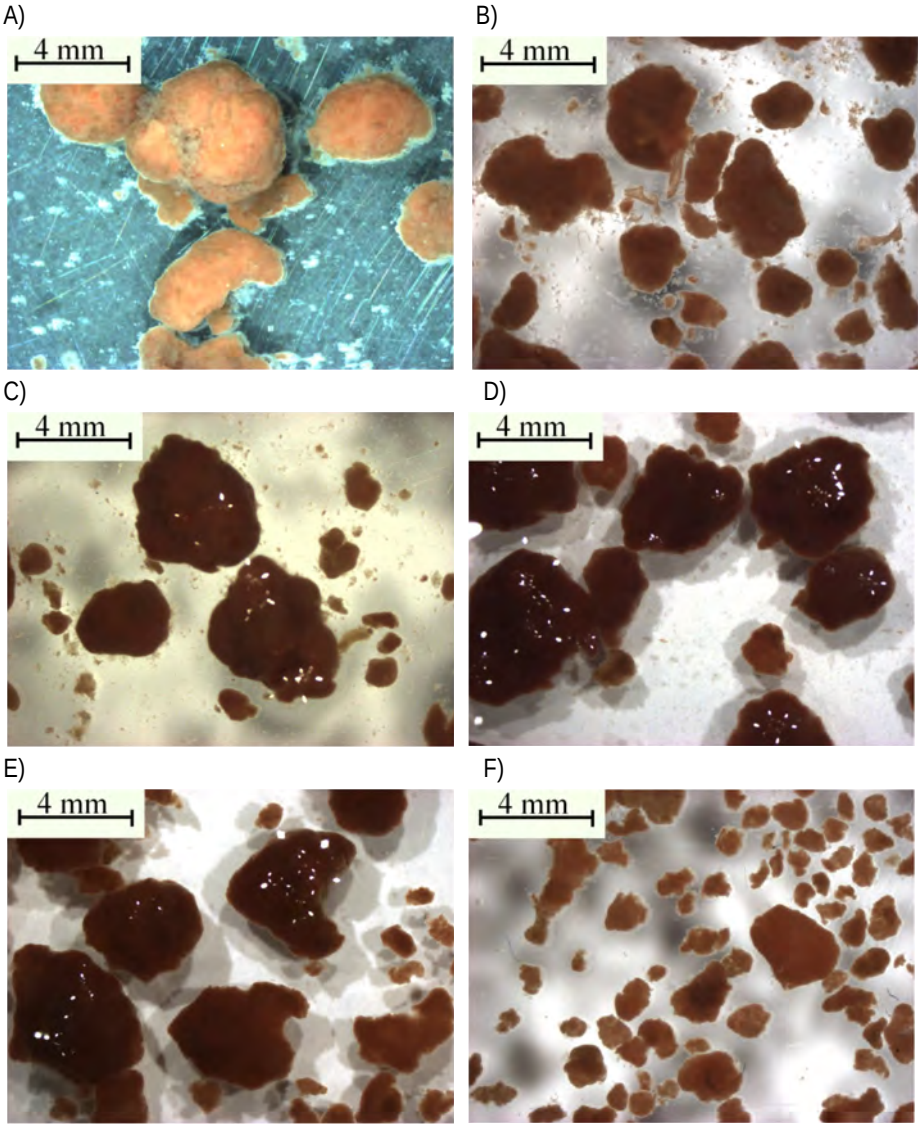

Figure 6.14. Images of the granules (zoom: 6.5x). Stage A-II: A) on day 537 and B) on day 621. Stage A-IV on days: C) on day 956, D) on day 1004, E) on day 1027, and F) on day 1094.

6.3.2.2 Stage A-IV

The CANON system was operated at 15 ºC in stage A-IV (946-1120 d), once the biomass and operation stability were recovered in stage A-III. The biomass concentration at the beginning of this stage was around 13.5 g VSS/L. Similar to what happened during stage A-II, the biomass concentration decreased progressively to 6.9 g VSS/L (Figure 6.11 B), however, at a smaller rate than that observed in the stage A-II. Furthermore, the

average SRT decreased, in this case to 62 ± 13 days. The measured concentration of solids in the effluent had an average value of 40 ± 11 mg VSS/L (Figure 6.11 B). This resulted in the loss of 42.0 g VSS during the stage, while the biomass production only accounted for 15.0 g VSS (see section 6.4). In consequence, the system was not able to maintain the biomass concentration stable when it was operated at 15 ºC.

However, unlike stage A-II, neither the ANR nor the AOR values dropped significantly. In addition, AOR and ANR had average values of 0.31 and 0.44 g N/L·d, respectively (Figure 6.12 B). An average AR and NR efficiencies of around 73 and 58%, respectively, were obtained in Stage A-IV.

A stable period from day 1000 to day 1070 d was observed, where ANR and AOR reached values of 0.50 and 0.34 g N/L·d respectively, while the NOR remained below 0.01 g N/L·d. The AOR/ANR and (AOR-NOR)/ANR ratios during these days were around 0.68. This resulted in AR and NR efficiencies of 76 and 62%, respectively, with a small nitrite accumulation. The average DO concentration measured during stage A-IV was 1.5 mg/L (in stage A-II it was 2.8 mg/L). The AOB limitation achieved in this period allowed obtaining a controlled nitrite concentration in the system (Figure 6.13 B).

Results indicated that a state of stable operation could be achieved, contrary to what happened in stage A-II when a continuously decreasing evolution of biomass concentration and activities was detected.

Granules appearance remained rather stable during the first weeks of operation with the conditions of stage A-IV (Figure 6.14 C, D and E). Granules average diameter at the beginning of this period was 2.8 mm. This parameter remained almost stable during the first months of operation. After around day 1060 it decreased progressively and dropped to 1.4 mm at the end of the stage, when a larger fraction of small granules was observed in the reactor (Figure 6.14 F). This observation suggested the disaggregation of bigger granules. This effect coincided with the progressive decrease of the Anammox and AOB activities and with the loss of biomass observed in stage A-IV.

6.3.3 Operation at 15 ºC and 70 mg NH_4^+-N/L

The inlet ammonia concentration in stage A-V (1250-1585 d) was diminished to around 70 mg NH_4^+-N/L which supposed a decrease of the applied nitrogen loading rate (NLR) from 0.76 to 0.34 g N/L·d. With the results from previous stages, the necessity of controlling the DO concentration in the bulk liquid and a possible reduction in the biomass concentration were expected. Since the NLR applied to the system was reduced, the inlet gas flow was also decreased in order to maintain a suitable DO concentration. However, the experimental setup was not able to manage the low gas flow required. It caused that the DO concentration measured in the bulk liquid until day 1346 varied between 1.5 and 2.7 mg

O_2/L. The relatively high DO concentration in the bulk liquid and the consequent increase in the oxygen depth penetration inside the granules caused that the Anammox activity was severely affected. The average ANR measured in that period was around 0.02 g N/L·d, while ammonia and nitrite oxidation was favored with values of the AOR and NOR around 0.11 g N/L·d (Figure 6.15). The AOR/ANR ratio was quite high, around 5.00, while the (AOR-NOR)/ANR ratio was around 0.64. Consequently, during these days, ammonia was mainly converted to nitrate (Figure 6.16) with an AR efficiency that reached an average of 60% but almost without overall nitrogen removal.

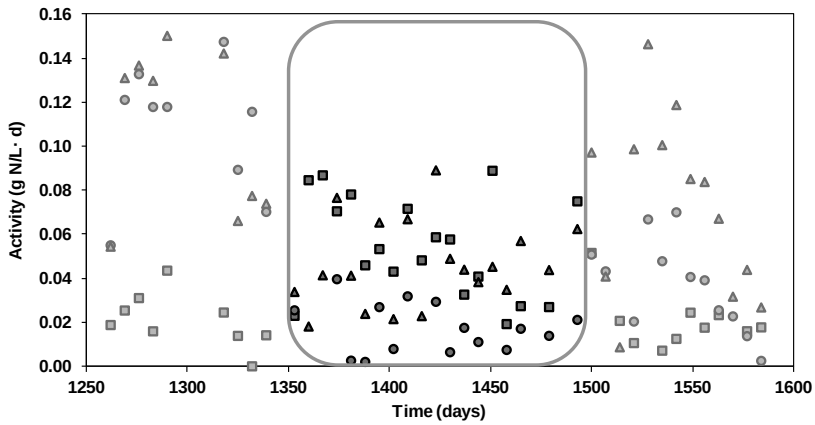

Figure 6.15. ANR (■), NOR (●) and AOR (▲) in g N/L·d, during stage A-V (15 ºC). Discussed steady state conditions are inside the square.

After day 1350, the experimental setup was adapted to manage the required gas flow and the DO level was controlled at 0.9 mg O_2/L. In spite of some mixing problems, the ANR slightly increased to 0.06 g N/L·d, while the AOR and NOR decreased to 0.05 and 0.01 g N/L·d, respectively. In this way, the AOR/ANR ratio decreased to 1.04. These values showed the straight relation between the low applied NLR the low DO level needed to maintain the balance between AOR and ANR and to avoid nitrite oxidation. However, the low gas flow used, provoked biomass homogenization problems, and the measurement of the biomass concentration was difficult, obtaining an average value of 2.3 g VSS/L (Figure 6.17). Average NR efficiency between days 1350 and 1500 reached the 16%.

In the last period of this stage, from day 1500 on, when a biomass re-inoculation was performed, the ANR fell under the AOR and NOR, with values of 0.02, 0.07 and 0.04 g N/L·d, respectively (Figure 6.15). The production of nitrite by AOB was higher than its consumption by both Anammox and NOB, which produced an accumulation of nitrite in some periods. Posterior increase of NOB activity produced also the accumulation of nitrate in the system (Figure 6.16). In consequence, the average nitrogen removal during the period did not exceed 10%.

Similarly, to what happened in the previous stages at 15 ºC (A-II and A-IV), the biomass concentration decreased progressively as the biomass growth did not compensate for the biomass loss in the effluent. In this way, the reactor was inoculated with fresh granules from a 200 L CANON reactor (Vázquez-Padín *et al.*, 2012) at the beginning of the stage, and had an average biomass concentration of 4.3 g VSS/L. This value dropped to 1-2 g VSS/L rapidly. A re-inoculation was performed around day 1500, but biomass dropped again (Figure 6.17) and average SRT was around 56 ± 30 days.

The appearance of the granules changed slightly during the operation (Figure 6.18). The presence of some light brown granules was detected in the last days of operation (Figure 6.18 C and D). In addition, inoculated granules had a density of 49.7 g VSS/$L_{biomass}$, while this value diminished to 31.8 g VSS/$L_{biomass}$ after 164 days of operation and the average diameter of the granules gradually decreased from 3.4 to 2.9 mm.

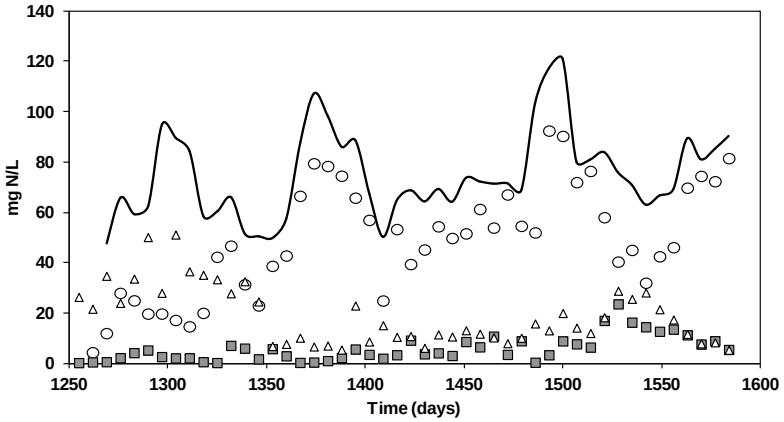

Figure 6.16. CANON operation in terms of NH_4^+-N concentration in the feeding (—) and NH_4^+-N (○), NO_2^--N (■) and NO_3^--N (△) concentrations in the effluent during stage A-V.

6.3.4 Operation at 15 ºC and 50 mg NH_4^+-N/L with mechanical stirring

In stage A-VI (1600-1676 d) low air flow was applied to the reactor in order to achieve a lower DO concentration in the bulk liquid than that maintained during the stage A-V, to avoid the development of NOB and of the total ammonia oxidation to nitrate. As it was shown in the previous stage, the air flow was not enough to provide an adequate mix of the fast settling granular biomass. So, a mechanical stirring system was implemented in the 1.5 L SBR reactor in order to complement the agitation provided by the aeration. The reactor was inoculated with granules from a 200 L CANON (Vázquez-Padín *et al.*, 2013).

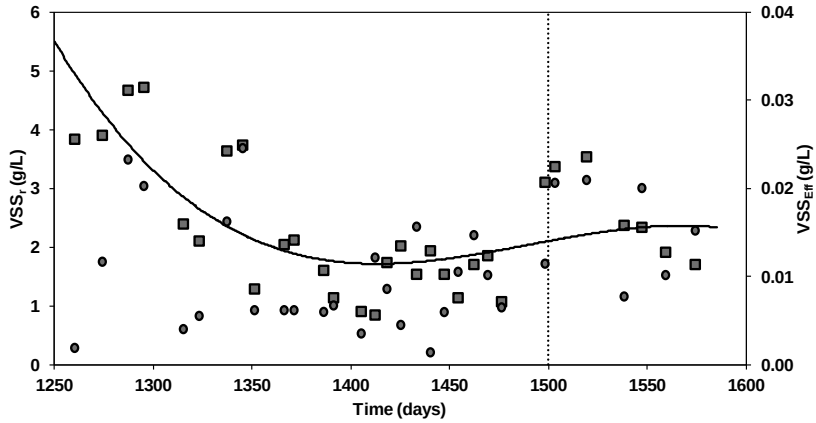

Figure 6.17. Stage A-V. A) Solids concentration in the reactor (■) and in the effluent (●) in g VSS/L. (Dotted line indicated the re-inoculation).

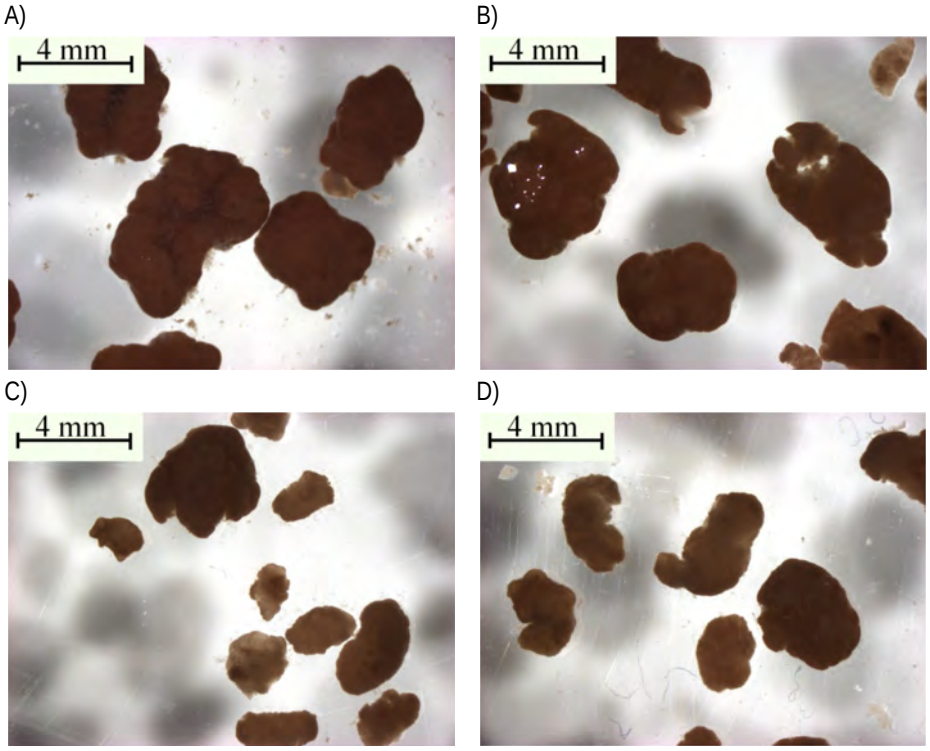

Figure 6.18. Images of the granules (zoom: 6.5x) on stage A-V on days: A) 1253 B) 1412 C) 1503 and D) 1540.

As in stages A-II, A-IV and A-V, the biomass concentration inside the reactor dropped continuously during this stage (Figure 6.19). A biomass concentration of around 4.0 g VSS/L was measured in first weeks of operation after the initial re-inoculation, when a settling time of 1 minute was applied. Then, even if the settling time was increased to 10 minutes, and finally to 30 minutes, the biomass concentration rapidly got lower as the biomass loss in the effluent did not decrease. Finally, a biomass concentration of around 1.0 g VSS/L was measured in the reactor.

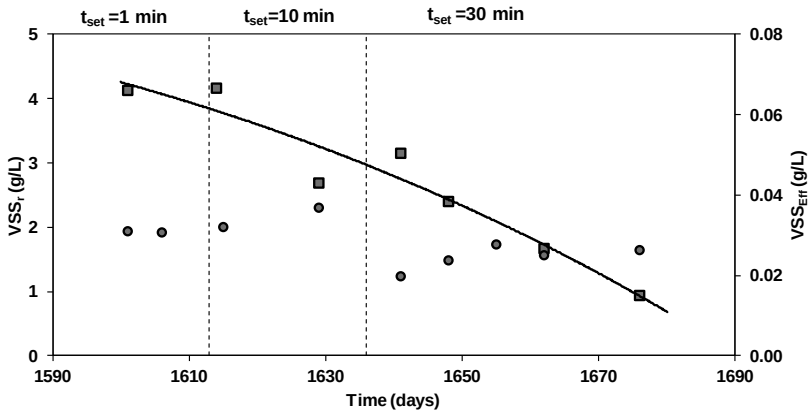

Figure 6.19. Solids concentration in the reactor (■) and in the effluent (●) in g VSS/L. t_{set} is the settling time applied during stage A-VI.

The NR efficiency, which had an average value of around 40% during first two weeks, dropped dramatically as the biomass was washed out from the reactor, and was almost negligible in the second half of the stage, after day 1650. The AOR was higher than the ANR, which dropped continuously during the operation (Figure 6.20). This conducted, firstly, to an accumulation of nitrite in the reactor, then, to the development of NOB and finally, to the excessive nitrate production (Figure 6.21). The breakage of the granules caused the complete loss of ANR, as Anammox bacteria were exposed to the dissolved oxygen present in the bulk liquid.

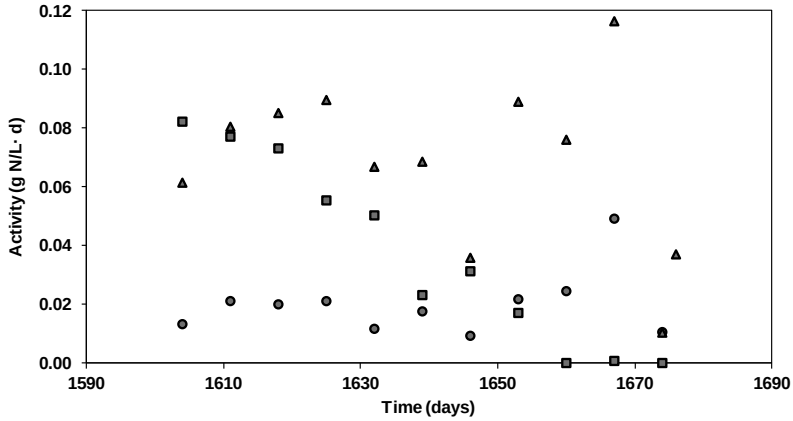

Figure 6.20. ANR (■), NOR (●) and AOR (▲) in g N/L·d, during stage A-VI (15 ºC).

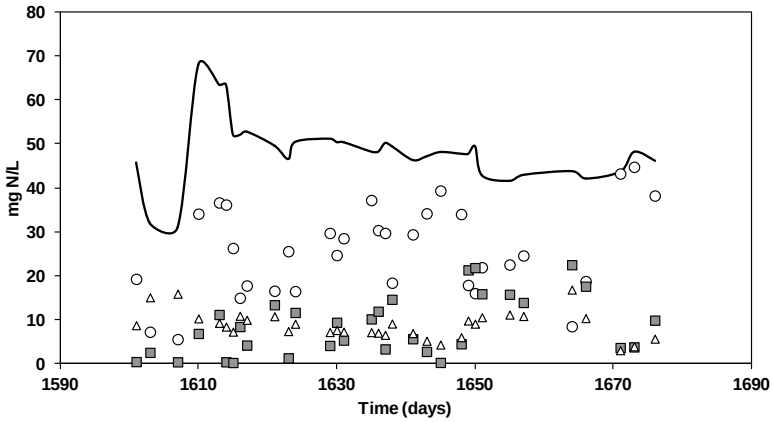

Figure 6.21. CANON operation in terms of NH_4^+-N concentration in the feeding (—) and NH_4^+-N (○), NO_2^--N (■) and NO_3^--N (△) concentrations in the effluent during stage A-VI.

In this case, the important event of granules breakage (Figure 6.22) was attributed to the mechanical stirring. The average diameter of the granules diminished rapidly in the reactor. Inoculated granules (Figure 6.22 A) had an average diameter of about 2.3 mm. Only after 10 days that value dropped to 1.6 mm (day 1610) and fragments of granules were observed in the reactor (Figure 6.22 B). After 27 days, average diameter of the particles dropped to 0.7 mm on day 1627 (Figure 6.22 C). Finally, almost no complete granules could be detected in the biomass at the end of the stage (Figure 6.22 D). Consequence of the progressive reduction of biomass concentration in the reactor, also the SRT diminished, with values of only 9 days at the end of the stage.

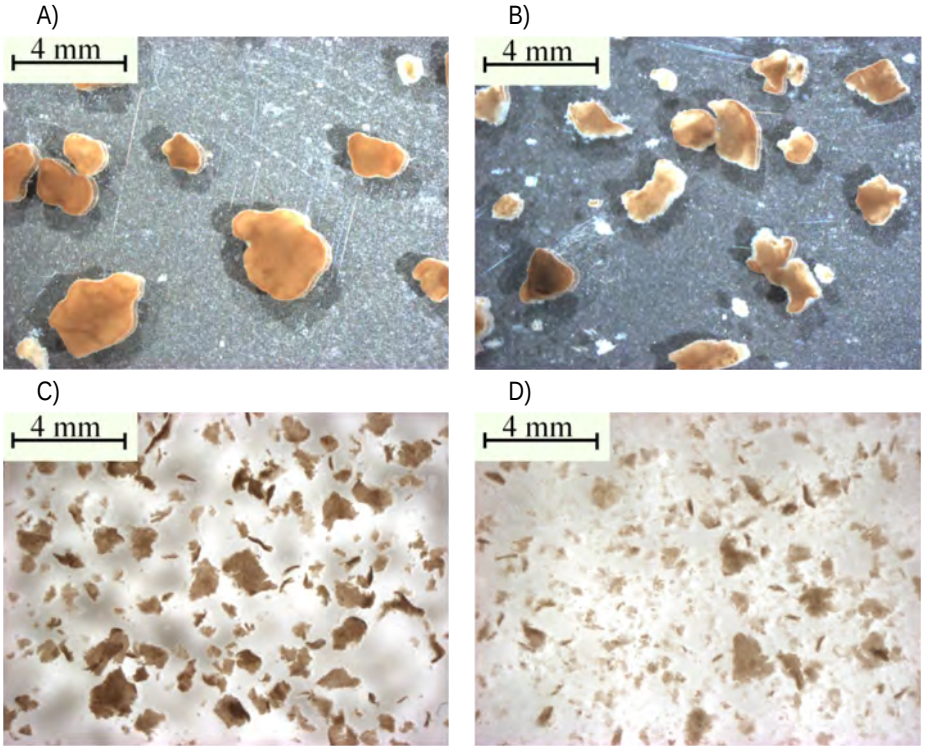

Figure 6.22. Images of the granules (zoom: 6.5x) on stage A-VI on days: A) 1600 (inoculums), B) 1610, C) 1627 and D) 1650.

6.3.5 SBR 2 operation

The biomass retention in the SBR 1 reactor when the mechanical stirring system was in operation during stage A-VI was not satisfactory, mainly due to the breakage of the granules. To avoid this problem, a bigger reactor, with an H/D ratio of around 1, was used in order to diminish the shear stress and the disintegration of the granules and to achieve better biomass retention (Stage B-I). In addition, the HRT of the system was increased to 0.5 d in order to achieve a lower biomass wash-out and, therefore, better nitrogen removal efficiency at 15 ºC.

Biomass concentration in the reactor remained rather stable during the stage B-I, with an average value of 10.4 ± 1.8 g VSS/L (Figure 6.23), in contraposition to the results obtained in all the previous stages when the CANON system was operated at 15 ºC (stages A-II, A-IV, A-V and A-VI). The system presented excellent biomass retention, with a SRT that exceeded 290 d. The average solids concentration measured in the effluent of the reactor was 23 ± 20 mg VSS/L, similar value to the solids concentration measured in the influent during this stage, 20 ± 14 mg VSS/L. Consequently, even if the biomass growth was low at this temperature, it can compensate for the biomass loss in the effluent.

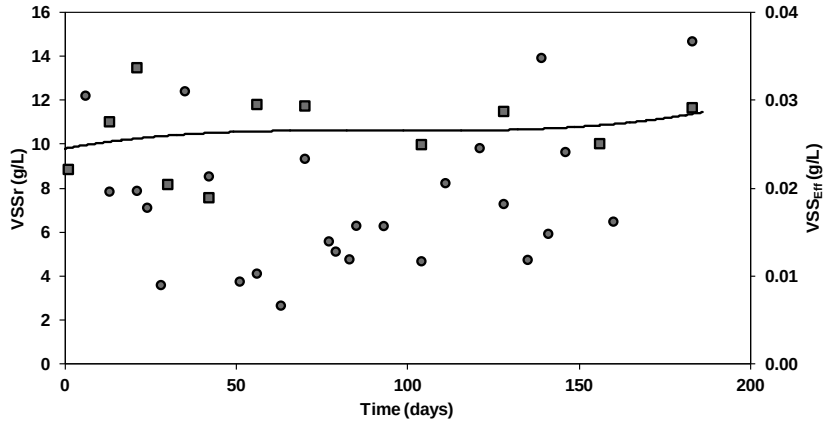

Figure 6.23. Stage B-I. Solids concentration in the reactor (■) and in the effluent (●) in g VSS/L.

During the first half of the experimental period until approximately day 80, the Anammox activity clearly overcame the AOB activity (Figure 6.24). Average ANR and AOR values were 0.08 and 0.06 g N/L·d, respectively. The AOR/ANR ratio was 0.77, while the (AOR-NOR)/ANR ratio was 0.64. Until day 77, the average AR and NR efficiency reached the 88% and 67%, respectively. These values were clearly higher than those obtained in stages A-V and A-VI, and similar to the obtained during stage A-IV when the ammonia in the influent was around 200 mg NH_4^+-N/L.

However, after approximately day 100, the situation turned over. The ANR value dropped progressively, while the AOR remained rather stable. The gradual increase of the NOB activity, with NOR values that reached 0.04 g N/L·d caused the nitrite consumption and nitrate production (Figure 6.25). The AOR/ANR ratio overcame 1.40, while the (AOR-NOR)/ANR remained close to the limit value of 0.64. NOB competed with the Anammox bacteria for the nitrite, which was completely removed from the reactor by both microorganism groups, in contrast with that observed in previous stages, where nitrite also accumulated in the reactor. That effect coincided with the observation of increasing amounts of floccular biomass in the reactor.

Average diameter of the granules remained rather stable, with a value of 2.4 ± 0.2 mm. Contrary to what happened in stage A-VI, granules maintained their size after 90 days (Figure 6.26 A, B, C and D). However, a deeper analysis of the pictures taken to the granules, revealed a slight transformation in their outer appearance. Even if, in general, the size did not change, the color and opacity of the granules diminished, especially after day 100 (Figure 6.26 D and E), in a similar or more accused way to what happened in stage A-V (Figure 6.18).

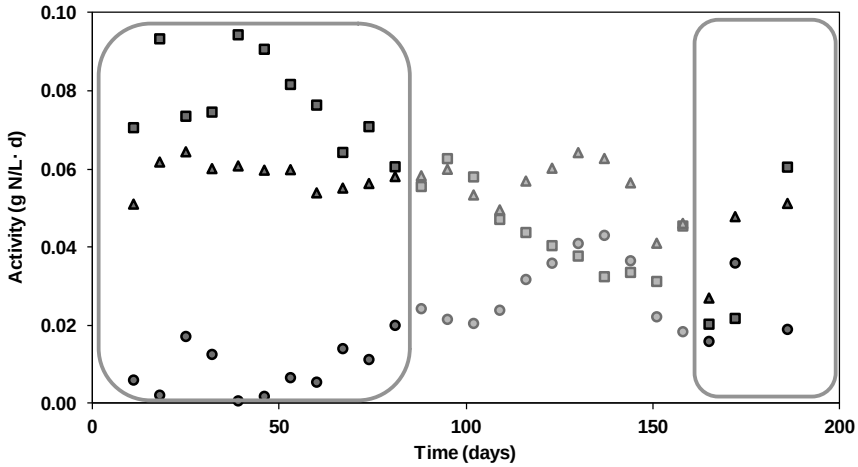

Figure 6.24. ANR (■), NOR (●) and AOR (▲) in g N/L·d, during stage B-I (15 ºC). Discussed steady state conditions are inside the square.

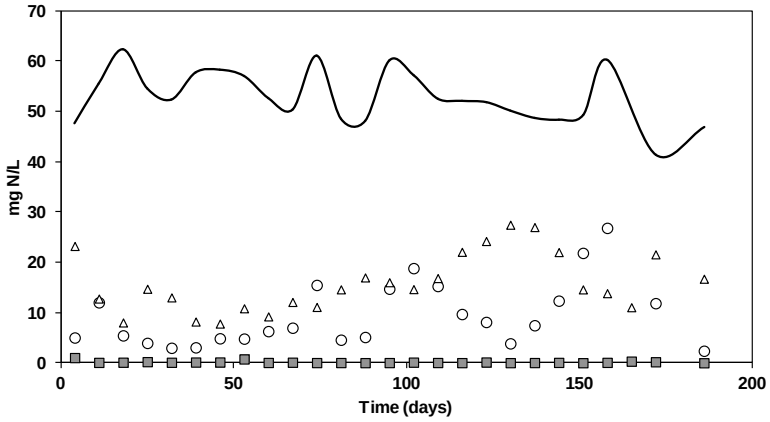

Figure 6.25. CANON operation in terms of NH_4^+-N concentration in the feeding (—) and NH_4^+-N (○), NO_2^--N (■) and NO_3^--N (△) concentrations in the effluent during stage B-I.

Winkler *et al.* (2011) observed the accumulation of white and lighter granules in their CANON reactor operated at room temperature (17-22 ºC) and fed with 230 mg N/L in the influent. Moreover, Liu *et al.* (2012) observed that the microorganisms were relatively scattered in their CANON reactors when the nitrogen concentration in the influent was low (100 mg N/L), in contrast with the more densely packed aggregates obtained when the concentration was higher (300-400 mg N/L).

In addition to the structural changes in the granules, a flocculent fraction of biomass was retained in the reactor. This biomass consisted mainly of NOB. This floccular biomass

fraction was not measured in the diameter of the granules determination due to its small size. This fraction, with lower settling velocity than the granules, accumulated over the granules during the settling period and was not observed in previous stages. In stage B-I, the granules remained unaltered and were not destroyed, almost until day 90 (Figure 6.26 D). Finally, in the last days of stage B-I, an accumulation of granules fragments was observed. The mechanical stirring was discarded as the cause of the accumulation of this biomass as it happened in stage A-VI. In stage A-VI, the breakage of the granules happened just after few days of operation with the mechanical stirring.

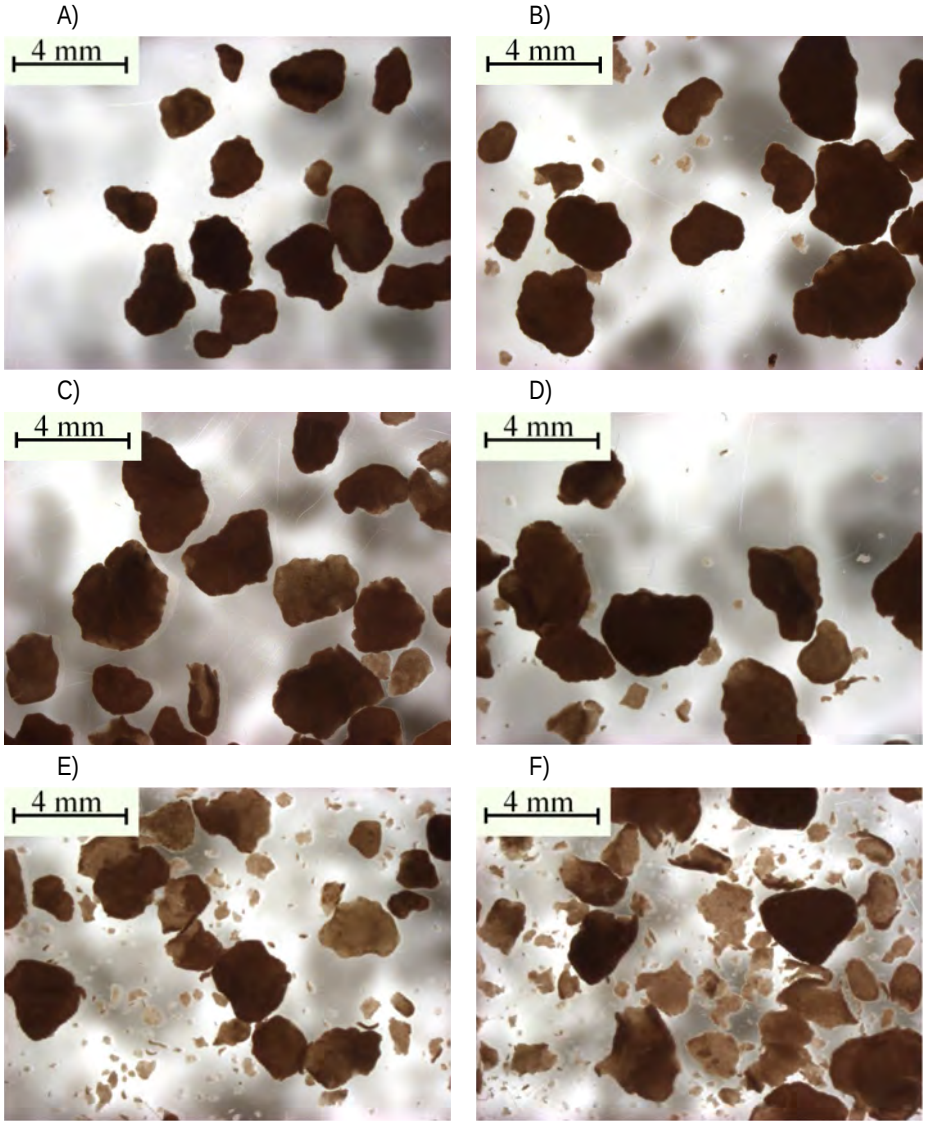

Figure 6.26. Images of the granules (zoom: 6.5x) on stage B-I on days: A) 1 (inoculums), B) 21, C) 63, D) 90, E) 105 and F) 120.

On day 126 (stage B-I), 0.6 g VSS/L of floccular biomass were substituted by 0.9 g VSS/L of granules in order to remove part of the NOB biomass associated with the floccular biomass. In addition, after day 169 the settling time was reduced to 10 minutes, in order to favor some wash out of floccular biomass from the reactor. After these actions, the NOR was reduced progressively, while both AOB and ANR increased their values (Figure 6.24). Winkler *et al.* (2011) also observed an accumulation of NOB in their reactor. These authors manually removed the granular biomass from the top of their CANON reactor, where biomass enriched in NOB accumulated and obtained a considerable increase in the nitrogen removal rate.

During this stage, the solids retention of the reactor was almost complete; the SRT reached values as high as 600 d, with an average of 280 d; in consequence, there was nearly no biomass washout from the reactor, which benefited the accumulation of the flocculent biomass and fragments of granules in the reactor.

6.4 DISCUSSION

Mean values of nitrogen removal rates and effluent characteristics obtained during the operation of both reactors are summarized in Table 6.3. Values obtained of these parameters during stable sub-periods are also summarized in Table 6.3 while characteristics of the granular biomass and biomass balances are summarized in Table 6.4.

From the results obtained in the different experimental periods, three main factors should be pointed out as crucial to control the performance of the AOB and Anammox bacteria in a CANON system operated at low temperature and nitrogen load: 1) to achieve a high biomass retention; 2) to achieve an equilibrium between the AOB and Anammox activities and 3) to avoid the NOB development in the biomass.

Table 6.3. Nitrogen removal efficiencies, nitrogen loading rate and nitrogen removal rates of the different biomass groups during the stages of the experimental period. Average and standard deviation for the overall period and average value in stable conditions in a sub-period (*values in italics*).

	A-I (20 ºC)	A-II (15 ºC)	A-III (20 ºC)	A-IV (15 ºC)	A-V (15 ºC)	A-VI (15 ºC)	B-I (15 ºC)
Period (d)	1-506	507-623	624-945	946-1120	1250-1585	1600-1676	1-186
Stable Sub-period	*(350-450)*	*(507-545)*	*(800-937)*	*(1000-1070)*	*(1350-1493)*	*(1604-1618)*	*(0-81)*
NLR (g N/L·d)	0.90 ± 0.19	0.79 ± 0.10	0.78 ± 0.12	0.76 ± 0.10	0.34 ± 0.08	0.21 ± 0.02	0.11 ± 0.01
AR (%)	78.1 ± 13.8 *(84.6)*	36.3 ± 14.1 *(49.9)*	70.7 ± 26.4 *(85.9)*	71.5 ± 13.5 *(75.1)*	36.2 ± 21.8 *(26.6)*	47.0 ± 20.3 (60.0)	80.5 ± 19.4 *(88.4)*
NR (%)	55.8 ± 14.3 *(67.9)*	16.4 ± 16.3 *(29.4)*	47.4 ± 26.0 *(63.9)*	57.1 ± 9.8 *(61.6)*	9.9 ± 11.5 *(16.2)*	17.5 ± 6.0 (34.0)	53.6 ± 19.5 *(67.0)*
AOR (g N/L·d)	0.43 ± 0.11 *(0.36)*	0.22 ± 0.8 *(0.30)*	0.33 ± 0.13 *(0.41)*	0.31 ± 0.08 *(0.34)*	0.07 ± 0.04 *(0.05)*	0.07 ± 0.03 (0.018)	0.05 ± 0.01 *(0.058)*
NOR (g N/L·d)	0.12 ± 0.10 *(0.03)*	0.07 ± 0.07 *(0.12)*	0.06 ± 0.06 *(0.07)*	0.03 ± 0.04 *(0.01)*	0.04 ± 0.05 *(0.08)*	0.02 ± 0.01 (0.004)	0.02 ± 0.01 *(0.009)*
ANR (g N/L·d)	0.50 ± 0.14 *(0.53)*	0.19 ± 0.11 *(0.27)*	0.40 ± 0.18 *(0.54)*	0.44 ± 0.09 *(0.50)*	0.04 ± 0.02 *(0.05)*	0.04 ± 0.03 (0.019)	0.06 ± 0.02 *(0.077)*

Table 6.4. Main characteristics of the obtained CANON biomass and production/loss of biomass balance. (Average and standard deviation).

	Units	A-I (20 ºC)	A-II (15 ºC)	A-III (20 ºC)	A-IV (15 ºC)	A-V (15 ºC)	A-VI (15 ºC)	B-I (15 ºC)
VSS_r	(g/L)	10.7 ± 2.2	8.7 ± 2.1	11.5 ± 3.9	9.9 ± 2.6	2.3 ± 1.1	2.7 ± 1.2	10.5 ± 1.8
Diameter	(mm)	3.64 ± 0.17	3.50 ± 0.47	3.44 ± 0.30	2.14 ± 0.59	3.36 ± 0.29	1.34 ± 0.74	2.46 ± 0.19
Density	($g/L_{biomass}$)	48 ± 5	48 ± 5	70 ± 22	57 ± 21	36 ± 6	--	45 ± 30
SRT	(d)	103 ± 54	85 ± 15	107 ± 61	62 ± 13	58 ± 29	25 ± 11	283 ± 165
AOB produced	(g VSS/d)	0.020 ± 0.008	0.010 ± 0.004	0.013 ± 0.004	0.011 ± 0.002	0.004 ± 0.002	0.005 ± 0.001	0.005 ± 0.001
NOB produced	(g VSS/d)	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001	0.0004 ± 0.0003	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001
Anammox biomass produced	(g VSS/d)	0.070 ± 0.010	0.024 ± 0.018	0.055 ± 0.019	0.066 ± 0.009	0.005 ± 0.003	0.005 ± 0.005	0.017 ± 0.006
Heterotrophic biomass produced	(g VSS/d)	0.027 ± 0.047	0.034 ± 0.040	0.010 ± 0.008	0.008 ± 0.009	0.005 ± 0.011	0.001 ± 0.003	0.006 ± 0.006
Total biomass produced	(g VSS/d)	0.120 ± 0.050	0.069 ± 0.055	0.075 ± 0.024	0.086 ± 0.011	0.015 ± 0.011	0.011 ± 0.003	0.028 ± 0.010
Solids* washed out	(g VSS/d)	0.174 ± 0.060	0.155 ± 0.040	0.188± 0.103	0.242 ± 0.056	0.065 ± 0.028	0.194 ± 0.053	0.181 ± 0.157

* "Solids washed out" includes the biomass loss in the effluent and the solids that came from the influent.

6.4.1 Biomass retention

As it was mentioned, to achieve an appropriate biomass retention in the reactor is crucial in order to obtain a good nitrogen removal performance, especially when the temperature of the system is 15 ºC. In this way, the settling time has to be high enough to maximize biomass retention. The SBR reactor operated at 20 ºC with only 1 minute of settling time in stages A-I and A-III, and it produced sufficient biomass to compensate for the biomass washed out. Overall biomass production was achieved during these stages at 20 ºC. During stage A-I, biomass balances performed taking into account the VSS measured in the effluent, the biomass accumulation inside the reactor and the calculated biomass production, indicated that around 0.174 g VSS/d were loss with the effluent; 0.011 g VSS/d were accumulated in the reactor, and 0.120 g VSS/d were produced. In stage A-III these values were 0.188, 0.029 and 0.075 g VSS/d, respectively.

Even if the solids in the influent were not systematically measured during these stages, the balance between production, accumulation and loss of biomass permitted to estimate that around 10-20 mg/L of solids in the influent (a reasonable value of the wastewater) would close the biomass balance.

The reduction in the activity of the biomass due to the use of relatively low temperatures is not necessary a problem, provided biomass retention is sufficient as suggested by Hendrickx *et al.* (2012). This observation is corroborated with results from this research in stages A-I and A-III when the reactor was operated at 20 ºC.

When the reactor was operated at 15 ºC, this temperature caused a further decrease in the slow biomass growth of the autotrophic microorganisms involved in the CANON system. Consequently, the biomass production alone did not compensate for the biomass loss in stages A-II and A-IV. Biomass production dropped from 0.120 to 0.069 g VSS/d. This decrease is even higher in the stage A-V and subsequent ones, when the temperature effect was combined with the reduction of the substrate concentration fed to the reactor.

The relaxation in the settling velocities imposed to the biomass applied in stage B-I (from 0.3 m/h at the end of stage A-VI to 0.1 m/h during first weeks of stage B-I) produced an improvement in the biomass retention, by the reduction of the biomass loss in the effluent. In stage B-I, the calculated daily biomass growth was around 0.028 g VSS/d, higher value than the amount of biomass 0.013 g VSS/d loss in the effluent, once subtracted the solids in the influent, which accounted for around 0.168 g VSS/d. Consequently, during the stage B-I the biomass concentration remained stable. However, a harmful effect showed up when this biomass retention is complete: the accumulation of floccular biomass, and the NOB growth mainly associated with this kind of biomass.

6.4.2 Balance between AOB and Anammox activities

In order to maintain the stability of the CANON system, the limiting step is the ammonia oxidation by the AOB. Then, some of the factors that affect the activity of these organisms are key factors for the control of the stability of the autotrophic nitrogen removal with the CANON granules.

6.4.2.1 Importance of oxygen depth penetration in the process stability

To maintain a constant oxygen depth penetration is quite important in order to protect the Anammox activity in the inner layers of the granules. This is of special importance when granules developed in CANON reactors under high nitrogen load and high temperature conditions are used to inoculate CANON reactors operated at notably lower temperature and the nitrogen loads. This is the case when a CANON reactor used to treat the main stream of a WWTP is inoculated with sludge from another reactor treating the reject water from the sludge line. Large CANON granules are grown at a high DO concentration, in order to develop a large AOB layer. When these granules are moved to the CANON reactor operated at a low temperature, this wide layer of AOB can protect the Anammox bacteria by consuming the oxygen.

Equations (6.13) and (6.14) can be used to determine both the oxygen depth penetration of the granules used as inocula in the initial conditions, and the DO concentration set point required in the new reactor, which is operated at different temperature. This DO concentration set point will allow maintaining the same aerobic/anoxic biomass ratio. In this way, the oxygen depth penetration calculated for the granules inoculated during stage A-V was 35.8 μm (for the average diameter) when they were subjected to the conditions of the pilot-scale plant: 29 ºC and 1.8 mg O_2/L. During first days of operation in stage A-V, the DO concentration was even higher than the used in the pilot-scale reactor: 2.3 mg O_2/L at 15 ºC. With these conditions, the calculated oxygen depth penetration was around 74.2 μm, which was twice the previous value. Consequently, the Anammox activity in the granules, 0.006 g N/g VSS·d, was not lower only due to the temperature decrease, but also due to the increase in the aerobic fraction of the granules. Later, DO concentration was reduced. The calculated oxygen depth penetration in the new conditions was limited to 42.5 μm. Then, Anammox activity increased to 0.04 g N/g VSS·d.

Similar situation happened when the temperature was reduced from 20 ºC to 15 ºC in the transition from Stages A-I to A-II. In the final days of operation of Stage A-I, the oxygen depth penetration was around 56 μm, when the DO concentration was 3.0 mg O_2/L. The ANR and AOR were 0.032 and 0.043 g N/g VSS·d, respectively. When the temperature was reduced, in first days of Stage A-II, the DO concentration raised up to 3.6 mg O_2/L. This high value of DO concentration produced an increase in the oxygen depth penetration, which reached 74 μm. The AOR increased slightly to 0.034 g N/g VSS·d, instead of

decreasing due to the temperature reduction. On the contrary, the ANR dropped to 0.032 g N/g VSS·d, probably as this larger oxygen depth penetration inhibited some of the Anammox bacteria. Consequently, the AOR/ANR activity ratio varied from 0.75 to 1.06. Later the loss of biomass and destabilization of the system caused the further drop of the ANR to 0.017 g N/g VSS·d.

In stage A-III, the DO concentration was lower than in Stage A-I, both stages operated at 20 ºC. Then, oxygen depth penetration was also lower, 40 µm when the DO concentration was 2.0 mg O_2/L (days 624 to 712), while the penetration in Stage A-I was 56 µm. The AOR was around 0.029 g N/g VSS·d and the Anammox bacteria activity recovered values around 0.032 g N/g VSS·d. Then, AOR and ANR reached averages of 0.030 and 0.037 g N/g VSS·d. Probably, the Anammox bacteria, which were inhibited and displaced deeper in the granule during Stage A-II, slowly occupied the new anoxic layer formed by the lower oxygen depth penetration during Stage A-III.

The oxygen depth penetration was estimated using the average diameter of the granules. This approach is adequate provided that the size distribution of the granules did not vary, and consequently, the initial aerobic/anoxic ratio within the granule was not expected to change. However, when the size distribution of the granules varies during the operation, the DO concentration should be modified in order to maintain the aerobic/anoxic ratio constant. The same should be applied when the biomass density of the biomass varies. The effect of these characteristics of the granules will be studied in the following sections.

Temperature and DO concentration are operational parameters. However, the average size, the size distribution and the biomass are characteristics of the granules which cannot be controlled. These characteristics can vary during the operation as a response to the temperature or DO concentration changes.

6.4.2.2 Effect of granules size on the aerobic fraction of biomass

According to the results obtained after the application of the equation (6.14), there were no significant differences between the oxygen penetration depth achieved in granules of different sizes, except for the smaller ones or floccular biomass (Figure 6.27 A). However, the main effect is over the relation between the biomass subjected to aerobic conditions and those subjected to anoxic conditions.

The oxygen depth penetration for stage A-I (20 ºC), for an average DO concentration of 3.8 mg O_2/L, varied from 66.8 µm for the larger granules (diameter 6.5-7.0 mm) to 69.7 µm for the granules with diameters of 0.5-1.0 mm, and to 74.1 µm for the smallest ones (diameter <0.5 mm). The difference resides in the fraction of biomass subjected to aerobic conditions (Figure 6.27). It means that more than 65% of the volume of small particles was penetrated by oxygen, but only a 6% for larger granules was (Figure 6.27 A). Taking into

account the granules size distribution, 92% of the overall volume of biomass was subjected to anoxic conditions, and 8% to aerobic conditions. This effect is schematized in Figure 6.28 where the aerobic and anoxic fractions of granules with two different sizes are represented.

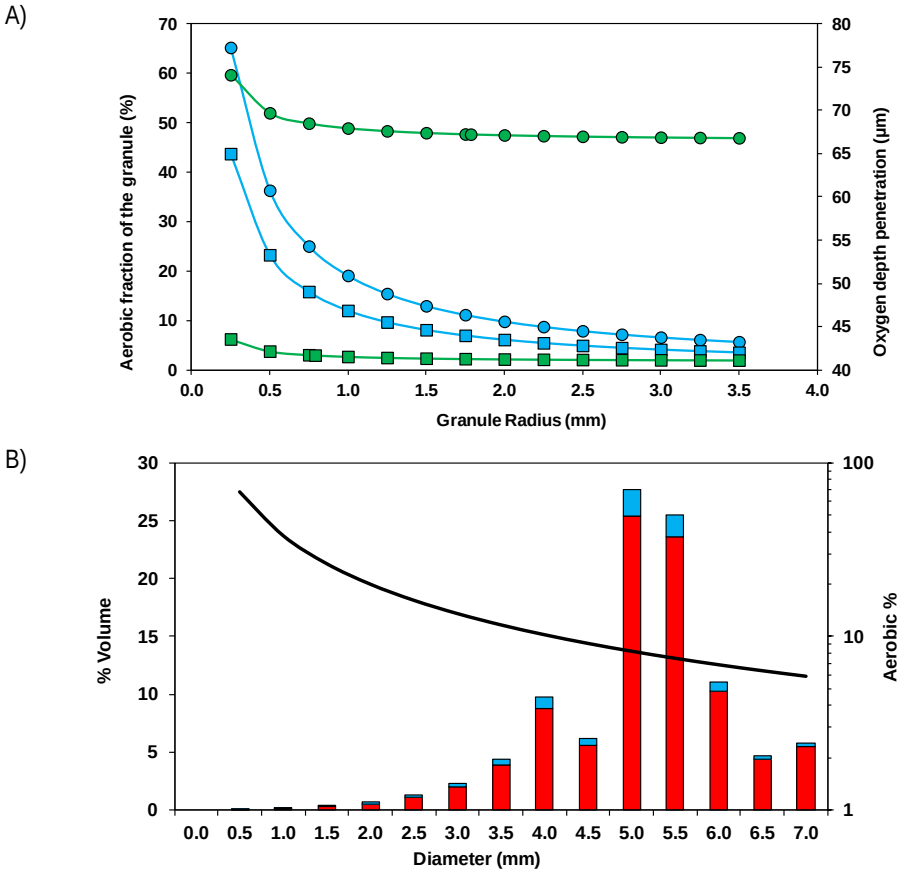

Figure 6.27. A) Aerobic fraction of the granule (blue) in % and oxygen depth penetration (green) in µm, in function of the granule radius (mm) for a DO concentration of 3.8 mg/L and 20 ºC (○), and for a DO concentration of 1.35 mg/L and 15 ºC (□).
B) Size distribution of the granules during stage A-I in percentage of volume of the total distribution when the DO concentration was 4.6 mg O_2/L (day 1 to 242). In each column, (■) represents the anoxic volume and (■) the aerobic volume. The line (—) represents the fraction of the granule penetrated by oxygen (subjected to aerobic conditions).

Even if the DO concentration applied in the different stages was selected in order to maintain the oxygen depth penetration and consequently, the aerobic fraction constant, the variations in the size distribution, conducted to variations in the different experimental periods. I.e., in stage A-IV the penetration depth varied in the range 41-45 µm (Figure 6.27A). However, the size distribution variation during the stage (Figure 6.29) produced a progressive variation in the aerobic fraction of the biomass, from 6% at the beginning of the

period (Figure 6.29 A) to 14% at the end (Figure 6.29 B), when the granules size distribution varied significantly, as bigger granules, in this case larger than 4.5 mm in diameter, were not observed.

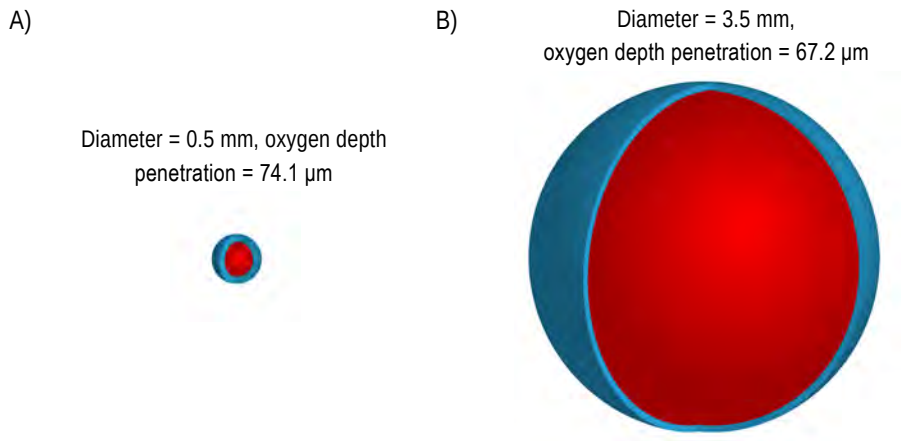

Figure 6.28. Schematic representation of the aerobic (■) and anoxic (■) fraction produced by the oxygen depth penetration, for granules with diameter 0.5 mm and 3.5 mm, in the conditions of stage A-I (20 ºC, DO = 3.8 mg O_2/L).

Thus, even if the oxygen penetration depth remained stable, the biomass fraction subjected to aerobic conditions increased due to the presence of smaller granules. In this way, in period 946-972 d at the beginning of the Stage A-IV, the AOR was 0.28 g N/L·d while the ANR was 0.42 g N/L·d, being the AOR/ANR ratio around 0.67 and the (AOR-NOR)/ANR ratio 0.60. At the end of the stage, in period 1075-1092 d, the AOR increased to 0.36 g N/L·d while ANR was around 0.38 g N/L·d. Therefore, the AOR/ANR increased to 0.95, while the (AOR-NOR)/ANR ratio was 0.66. The reduction of the Anammox activity can correspond with the loss of bigger granules, which have a larger anoxic fraction, and with the increment of percentage of small granules percentage, which have a larger aerobic fraction. The increase of the NOB activity, which reached 0.11 g N/L·d also reduced the overall nitrogen removal efficiency during this period, and avoid the nitrite accumulation in the system. In section 6.4.3, the effect of NOB development is discussed. This effect is more remarkable in small size granules.

The effect of oxygen penetration depth related to the size distribution agreed with results from other works. Nielsen *et al.* (2005) found that aggregates smaller than 0.5 mm were specialized in aerobic ammonium oxidation, and those larger than 0.5 mm were specialized in anoxic ammonium oxidation. Vlaeminck *et al.* (2009) obtained aggregates with a size between 0.1 and 1.0 mm (rather floccular) specialized in aerobic ammonium oxidation and granules with 1.8 ± 0.3 mm specialized in anoxic ammonium oxidation. These authors corroborated their findings through FISH analyses, which showed that Anammox bacteria were more abundant in the bigger particles while AOB dominated the flocs.

Anammox bacteria grow in the more external layers of the anoxic volume of the granules, where NH_4^+ and NO_2^- are available, but protected from oxygen. Vázquez-Padín et al. (2010b) observed that Anammox bacteria were located inside the granules in the range of depths between 400 and 1000 μm, when applied DO concentration was of 6.6 mg O_2/L (which would imply a wider aerobic layer than the obtained in the granules of the present study). These authors also observed that AOB and Anammox coexisted in the range 400-600 μm, as AOB were still present at 600 μm, but their share decreased when the depth increased.

6.4.2.3 Biomass density effect on oxygen depth penetration

An important factor in the determination of the oxygen penetration using the equation (6.14) is the biomass density of the granules. For a granule with an average diameter of 3.64 mm (the average diameter in stage A-I), the oxygen depth penetration and anoxic ratio vary as shown in Figure 6.30 as a function of the biomass density in g VSS/$L_{granule}$. Inoculated granules in stage A-I had an average biomass density of around 45 g VSS/$L_{granule}$ and this value ranged from 45 to 90 g VSS/$L_{granule}$ in the stages at 20 ºC (A-I and A-III). These important variations in the biomass density had a limited impact on the variation of oxygen depth penetration. On the contrary, granules inoculated in stage B-I had a biomass density value of 66 g VSS/$L_{granule}$, and after 170 days of operation under the conditions of stage B-I (DO=0.2 mg O_2/L, and 50 mg NH_4^+-N/L) the density dropped to 23 g VSS/$L_{granule}$. At this low biomass density, the effect over the oxygen depth penetration is more noticeable. Thus, in addition to the increase of the oxygen depth penetration due to the biomass activity decrease caused by the temperature reduction, the diminished in the biomass density also contributed to the increase in the oxygen depth penetration. This effect can be the responsible of the drop of Anammox activity in stage B-I.

Granules inoculated in stage B-I were cultivated previously at 24-30 ºC, fed with a solution containing an ammonia concentration of 940 mg NH_4^+-N/L and operated at a DO concentration of 1.8 mg O_2/L. Low substrate concentration can promote the formation of looser microcolonies, with less areal cell density (the number of cells per unit area), which facilitates substrate penetration into the biofilm (Okabe *et al*., 2004). In the present case both substrates for AOB, ammonia and oxygen, had always low concentrations.

6.4.2.4 Ammonia oxidizing bacteria activity prediction

To predict the ammonia oxidizing activity under different operational conditions is fundamental to control the processes involved in the autotrophic nitrogen removal.

Since both, temperature and dissolved oxygen concentration regulate ammonia oxidizing bacteria activity, once the temperature of the reactor was decreased from 20 ºC to 15 ºC, also the DO concentration had to be decreased to avoid the deeper oxygen

penetration inside the CANON granules. This deeper oxygen penetration within the granules can cause the inhibition of Anammox bacteria. To predict the AOB activity values at the different periods of operation is possible using equations (6.16) and (6.17) taking into account that the ammonia oxidation rate was limited by the internal mass transfer rate and oxygen was the limiting substrate of the process. The values measured during stage A-I were used as reference values for the stages A-II to A-IV (Table 6.5). As new granules from a pilot-scale reactor were inoculated in stages A-V, A-VI and B-I (Vázquez-Padín *et al.*, 2012, Vázquez-Padín *et al.*, 2013), the data from that reactor were used as reference for the aforesaid stages, taking into account its diameter, density and biomass activity values. These data were obtained from Vázquez-Padín (2013).

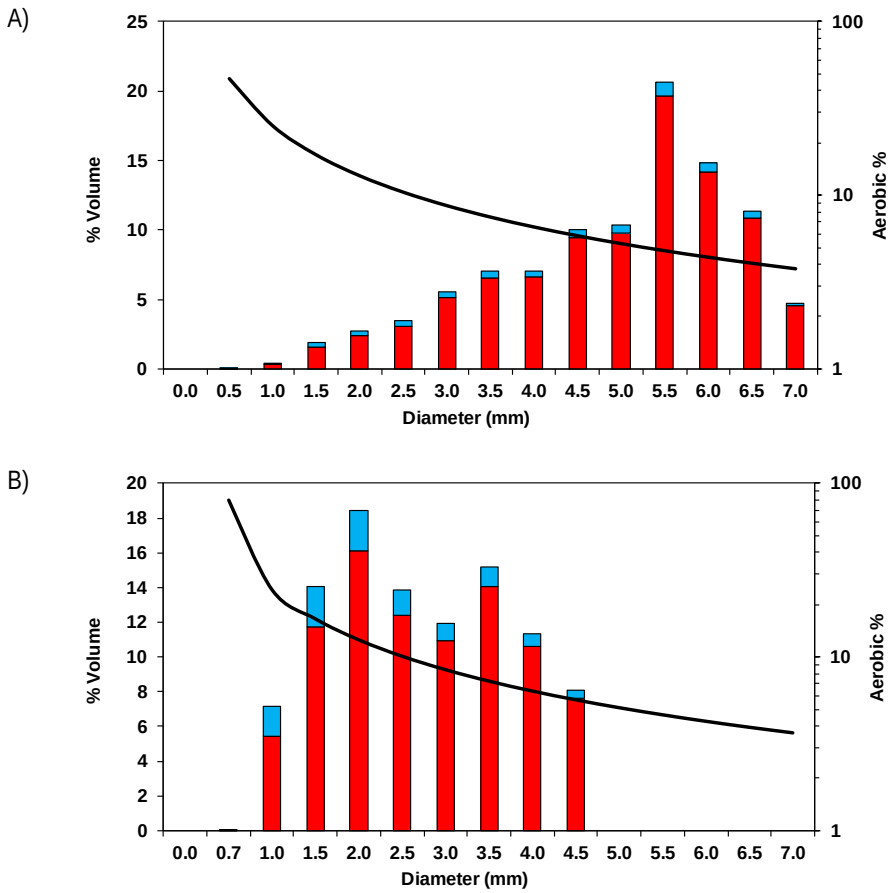

Figure 6.29. Columns represent the size distribution of the granules in percentage of volume of the total distribution. In each column, the red section (■) represents the anoxic volume fraction and the blue section (■) the aerobic volume fraction. The line (—) represents the fraction of the granule penetrated by oxygen (subjected to aerobic conditions). A) At the beginning of stage A-IV (days 946-970, DO=1.6 mg O_2/L). B) At the end of stage A-IV (1075-1092, DO=1.5 mg O_2/L)

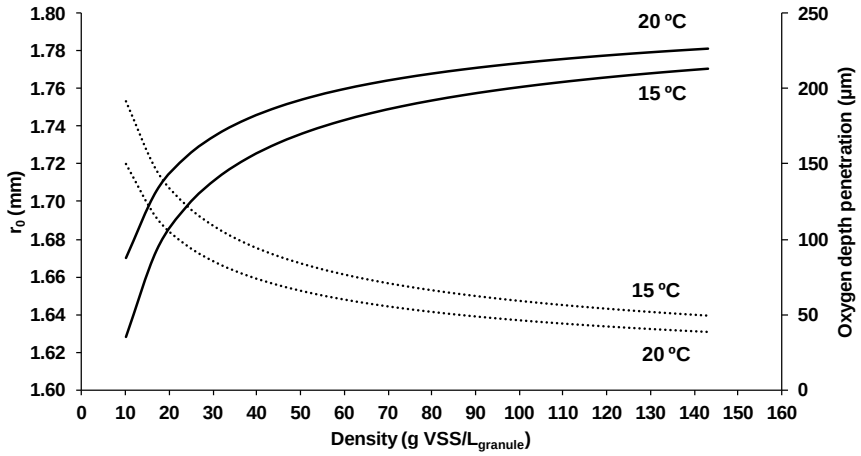

Figure 6.30. Anoxic radius (—) in mm and oxygen depth penetration (···) in µm as a function of the biomass density of the granule (g VSS/$L_{granule}$), calculated for a granule with a diameter of 3.64 mm in the conditions of stage A-I (20 ºC) and A-II (15 ºC).

The predicted values in the different stages were similar to the average AOR values observed in the reactor as it is shown in Table 6.5. The same procedure was applied for sub-periods in the stages at 15 ºC, where the activity had more variations (induced by DO concentration or variations in the average size of the granules). The predicted values in these sub-periods were even more similar to the observed ones.

Even when the DO concentration was controlled in order to maintain a constant aerobic/anoxic ratio, the performance of the reactor was altered by the occurrence of the nitrite oxidizing process in the equilibrium. The effect of the NOB development on the performance of the CANON system and alternatives to repress its development are discussed in the next section.

6.4.3 Nitrite Oxidizing Bacteria

6.4.3.1 NOB development

Especially in stage B-I, a continuous increment in the NOB activity was detected (Figure 6.24), which contributed to the reduction in the overall nitrogen removal in the system, as these bacteria compete with Anammox bacteria for the nitrite and with AOB for the oxygen. In order to evaluate the influence of the different biomass fractions, respirometric AOB and NOB activity tests, and specific Anammox activity tests were performed to samples of the biomass from the reactor. Respirometric tests were performed to separated fractions of the granules and the floccular biomass on stage B-I, when an excessive nitrate production was detected.

Table 6.5. AOB activities predicted and observed.

Stage	Period (days)	T (ºC)	DO (mg/L)	Diameter (mm)	Density (g VSS/ $L_{granule}$)	$AOR_{observed}$ (g N/ g VSS·d)	$AOR_{predicted}$ (g N/ g VSS·d)
A-I (reference)		20	3.8	3.6	54	0.041	--
A-II	Complete stage	15	2.8	3.5	54	0.028 ± 0.008	0.029
	(507-537)		3.8	3.5		0.034 ± 0.002	0.033
	(552-623)		2.4	3.5		0.027 ± 0.009	0.027
A-III		20	1.9	3.4	70	0.031 ± 0.009	0.028
A-IV	Complete stage	15	1.5	2.1	57	0.032 ± 0.011	0.034
	(946-1000)		1.4	2.7		0.024 ± 0.007	0.026
	(1007-1070)		1.4	1.9		0.035 ± 0.006	0.036
(Vázquez-Padín, 2013) Reference		29	2.1	3.6	41	0.066 ± 0.018	--
A-V	Complete stage	15	1.3	3.4	36	0.029 ± 0.015	0.029
	(1270-1340)		2.1	3.6	38	0.034 ± 0.012	0.034
	(1367-1493)		1.0	3.0	32	0.028 ± 0.015	0.031
(Vázquez-Padín, 2013) Reference		26	0.9	2.3	41	0.035	--
A-VI	Complete stage	15	0.3	1.1	41	0.026 ± 0.018	0.026
(Vázquez-Padín, 2013) Reference		23	2.0	2.5	66	0.044	--
B-I	Complete stage	15	0.17	2.5	44	0.005 ± 0.001	0.007
	(11-137)		0.15	2.4	50	0.006 ± 0.001	0.007
	(140-185)		0.05	2.5	23	0.005 ± 0.001	0.006

The measured specific Anammox activity of the overall biomass remained without significant variations during the stage (Figure 6.31). A small reduction in the Anammox activity can be inferred from the floccular sample (Figure 6.31), due to its lower anoxic fraction compared to the granular biomass. However, significant differences were found in the NOB activity tests. The NOB activity measured in the flocculent fraction was remarkably higher than the measured in the granules (Figure 6.32).

The NOB activity in the floccular sludge at 15 ºC was around 0.12 g N/g VSS·d, while in the granules it was around 0.02 g N/g VSS·d. This can indicate that NOB are preferably in the flocculent fraction of the biomass. Winkler *et al.* (2011) determined that a mix of AOB, NOB and Anammox formed the white and the smaller granules observed in their reactor, while larger granules were dominated by AOB and Anammox. Activity tests performed by these authors to biomass of their reactor showed similar ammonium oxidizing capacity for both biomass fractions. On the contrary, higher nitrite oxidizing capacity was measured for biomass which had a larger fraction of smaller white granules.

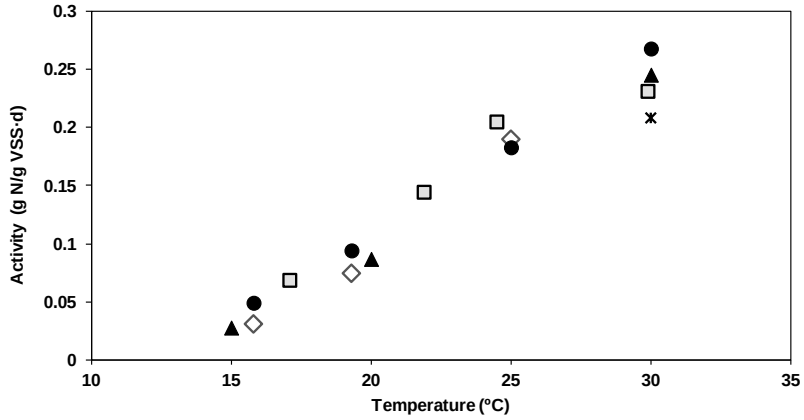

Figure 6.31. Anammox activity of the overall biomass on days: 1 (●), 69 (■), 91 (◇) and 167 (▲), and of the flocculent biomass on day 120 (✱), in g N/g VSS·d at different temperatures in stage B-I.

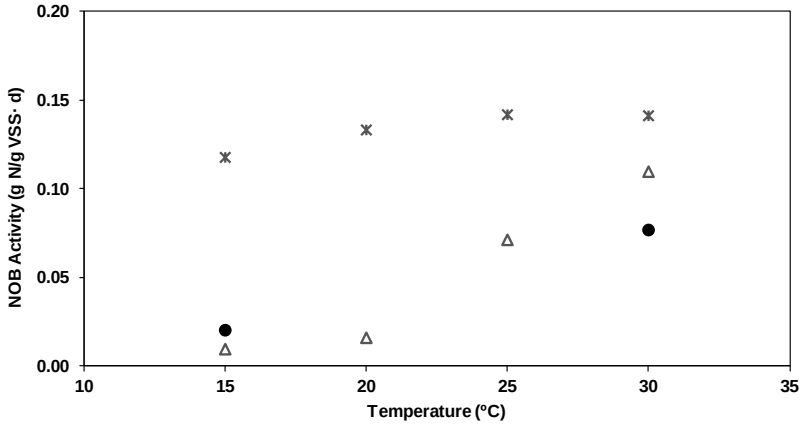

Figure 6.32. NOB activity of the overall biomass (△) on day 105, of the granules (●) and flocculent biomass (✱) on day 120 in g N/g VSS·d, at different temperatures during stage B-I.

NOB would be preferentially accumulated in smaller granules due to the higher availability of oxygen compared with larger granules. Gilbert *et al.* (2013) observed that the NOB activity decreased with increasing aggregate size for floccular biomass. Third *et al.* (2001) observed that NOB bacteria were present in their CANON biomass, even if the NOB activity test failed in their detection. While high temperature and ammonia concentration in excess were applied to their reactor, the nitrate production was near the stoichiometric of the Anammox process. However, a gradual increase in the NOB activity of the biomass was detected by these authors when the reactor was operated under ammonia concentration limitation. Volcke *et al.* (2012) determined that, in a single granular sludge reactor, NOB are present in the smallest granules (and for the same reason in the floccular fraction), while they are outcompeted by Anammox bacteria in larger granules. Gilbert *et al.* (2013) concluded that NOB can be inhibited in the system, but no extincted, even if the NOB activity is negligible. Furthermore, the FISH technique shows the presence of these microorganisms.

6.4.3.2 NOB repression

The continuous repression of the NOB bacteria appears as a key factor in order to achieve high nitrogen removal efficiency in CANON systems operated at low temperatures and ammonia loads. NOB activity in stage B-I was partially reduced after the increasing of the effluent withdrawal (increase of the settling velocity from 0.1 to 0.3 m/h), which mainly removed the floccular biomass from the system, and with the substitution of floccular biomass by re-inoculated granules. However, this strategy needs the availability of CANON granules developed in another reactor treating high loaded nitrogen streams.

Bin *et al.* (2011) observed in nitrifying granules that oxygen penetration was not restricted in the granules of less than 0.6 mm particle diameter. However, in the larger granules (d > 0.9 mm), a smaller aerobic volume fraction and inhibition of NOB growth can be achieved. According to these authors, in small granules, the location of the boundaries between AOB and NOB biomass species were distinct due to the limited existing space provided by granules for the growth of both microorganisms. The research conducted by Volcke *et al.* (2010) suggested that NOBs are favored to grow in small granules because of their high aerobic fraction. According to these authors, completely autotrophic nitrogen removal by the granular sludge can be achieved while NOB are outcompeted by Anammox bacteria, by controlling the DO concentration in the bulk liquid in a determined range. This optimal range of bulk DO concentration is broader for larger particles and for increasing influent ammonium concentrations. Consequently, systems with big granules are easier to control and more robust towards disturbances in bulk oxygen concentration than those with small ones. However, the granules size also has an effect over AOB, as activity and abundance of these bacteria decrease with increasing the aggregate size (Vlaeminck *et al.*, 2010).

Different strategies have been proposed by different authors with the objective of out-selecting the NOB and/or favor the AOB and Anammox selection. At high temperatures and high ammonia loads this objective has been successfully achieved by the use of these mechanisms: the AOB growth rate is larger than the NOB one at high temperatures; the inhibition by FA and/or FNA is larger for NOB than for AOB (Anthonisen *et al.*, 1976); DO affinity is larger for AOB than NOB, at least at high temperatures (Blackburne *et al.*, 2008). Nevertheless, batch oxygen half saturation (K_{O_2}) test performed more recently by Akintayo (2012) suggested that NOB had a higher affinity for oxygen at lower DO than AOB at 15 ºC. Wett *et al.* (2013) observed that the K_{O_2} of NOB decreased during the operation of their plant when the DO concentration was between 0.06 and 0.3 mg/L, while that value for the AOB remained stable. Then, when operating a reactor at low temperatures and nitrogen concentrations other selection mechanisms should be explored.

In this way, Winkler *et al.* (2013) concluded that for CANON granular sludge, mainly the diameter of the granular biomass and not the density differences must be used for the segregation of the biomass. Winkler *et al.* (2011) proposed the feeding of nitrite and ammonium during a CANON start up process at mesophilic temperatures in order to promote the growth of bigger granules, which are dominated by Anammox, and to favor the biomass segregation. Biomass selection based only on the density of the cells of the microorganisms is not viable, as the density of the NOB is higher than that of the Anammox bacteria (Winkler *et al.*, 2013). These authors stated that larger granules can achieve greater settling velocities, and consequently, smaller granules can accumulate in the top of the reactor and will be easily removed. These smaller granules are enriched in NOB.

A strategy based on the larger density of the granules was followed by Wett *et al.* (2013) in the Strass WWTP, where Anammox granules produced from sludge liquor treatment were seeded to the mainstream. Then, a hydrocyclone classifier selected for the high-density sludge fraction in order to retain these Anammox enriched granules. These authors also observed an excessive NOB activity in an Anammox process applied to the water line that conducted to an accumulation of nitrate and to a reduction in the overall nitrogen removal. They did not achieve a NOB repression as long a low DO concentration was applied (around 0.1 mg O_2/L). These authors suggested the application of rapid transitions from high to low DO levels in order to avoid NOB development. This strategy is based on the lag-time of the NOB behind the AOB when the system alternates between anoxic/anaerobic and aerobic conditions (Yoo *et al.*, 1999). This lag time was suggested by Turk and Mavinic (1986), however, these authors talked about several hours for the duration of such lag time.

Yao *et al.* (2013) proposed the addition of hydrazine (N_2H_4), a fundamental intermediate in the biochemical pathway of Anammox bacteria (Kartal *et al.*, 2011), to inhibit the NOB activity and enhance the performance of a CANON SBR reactor operated at 31 ºC. The hydrazine addition reduced at the same time the NO_3^- production below the

stoichiometric ratio. According to these authors, the electrons released from the oxidation of additional N_2H_4, which substituted the electrons from NO_2^- oxidation to NO_3^-, replenished the consumption of Anammox anabolism. The N-N bond in hydrazine is catalyzed in the Anammox metabolism by the hydrazine synthase, which low activity possibly explains the slow growth rates and long doubling times of the Anammox bacteria (Kartal *et al.*, 2012). However, the applicability of these alternative is complicated, as in the work of Yao *et al.* (2013), hydrazine in a concentration of around 7.5% of the ammonia influent concentration (in g N/L) was added to the reactor. In addition, hydrazine is a highly toxic and dangerously unstable compound (Carlsen *et al.*, 2009).

Liu *et al.* (2008) suggested the application of salinity, as the addition of 10 g NaCl per liter, to stand out AOB and Anammox activity and selectively suppress NOB activity under oxygen limited conditions in a non-woven rotating biological contactor (NRBC) CANON reactor operated at 35 ºC. The strategy is based on the fact that nitrite oxidation is more sensitive to salinity than ammonium oxidation. The Anammox process exhibits resistance to the presence of concentrations of NaCl up to 15 g/L, in addition salinity favors the aggregation of Anammox biomass in granules (Dapena-Mora *et al.*, 2010).

Other options to inhibit or to select AOB versus NOB in CANON granules may be studied in the future, as the heat-shock temperature selection proposed by Isaka *et al.* (2008) and developed to avoid NOB growth in gel carriers in a partial nitrification process. These authors found that AOB survive to heat-shock treatments at 90 °C for 1 h, while NOB were dead at 60 ºC when polyethylene glycol gel carriers were used to grow nitritation populations. These nitritation gel cubes were combined with Anammox gel cubes to perform the complete autotrophic denitrification in a single reactor at 30 ºC (Isaka *et al.*, 2013).

The inorganic carbon source limitations affect in larger grade to the AOB than to the NOB. Then, to guarantee the presence of HCO_3^- in excess in the system is fundamental. Higher growth rates for AOB than for NOB were achieved in an airlift-fluidized bed reactor packed with sponge cubes at 30 ºC when $NaHCO_3$ was used as the alkalinity source (Tokutomi *et al.*, 2010), while nitrate accumulated in the system, indicating the NOB development, when NaOH was used instead.

To keep appropriated conditions for the balanced performance of the AOB and Anammox bacteria is crucial for the application of the CANON process. Its operation at low temperature and nitrogen concentrations requires of further study to avoid NOB development and stable long-term operational conditions.

6.5 CONCLUSIONS

Three main factors should be pointed out as crucial to control the performance of the AOB and Anammox bacteria in a CANON system operated at low temperature and nitrogen load: 1) to achieve a high biomass retention; 2) to achieve an equilibrium between the AOB and Anammox activities and 3) to avoid the NOB development in the biomass.

The operation of the CANON system at 20 ºC treating moderate ammonia loads is feasible. In these conditions biomass growth compensated for the biomass loss in the effluent and the biomass concentration in the reactor reached a constant value of around 12 g VSS/L. Even more, the system was able to recover the biomass concentration loss observed at 15 ºC when the temperature was increased to 20ºC. AR and NR efficiencies of around 80 and 60% respectively, were achieved at 20 ºC, with an average ANR of 0.5 g N/L·d. In addition, no excessive NOB development was detected.

On the other hand, a progressive biomass loss was observed when the reactor was operated at 15 ºC. The low biomass growth observed at this temperature did not compensate for the biomass loss in the effluent, except in stage B-I. In this stage, a different reactor configuration with long settling times was used, and biomass retention was almost complete. Nevertheless, once this high biomass retention was achieved, the accumulation of flocculent and small particles was observed. This kind of biomass was enriched in NOB, which compete with the Anammox bacteria for the nitrite and with the AOB for the oxygen. Once this NOB enriched biomass fraction was removed, the Anammox activity was recovered, and the nitrogen removal increased.

The AR and NR efficiencies reached the 72 and 57%, respectively, when moderate ammonia concentrations were treated at 15 ºC. The ANR in this case was around 0.44 g N/L·d. When the ammonia concentration of the treated wastewater was low, the average ANR reached was 0.06 g N/L·d, and AR and NR efficiencies around 80 and 54% were reached, respectively. NOB competition becomes more important in these conditions.

The DO concentration in the bulk liquid should be fitted according to the operational temperature, the size distribution and biomass density of the granules, in order to avoid an excessive oxygen depth penetration in the granules which facilitate the development of NOB and the inhibition of Anammox. This dissolved oxygen concentration must be controlled in order to maintain constant the ratio between the aerobic and anoxic volumes within the granules, and then, the ratio between the AOB and Anammox activities.

NOB activity measured was larger for small particles and floccular biomass than for large granules. This difference can be exploited to wash out these microorganisms.

6.6 REFERENCES

Akintayo, O. (2012). Evaluating the Impact of Low Dissolved Oxygen in the Deammonification Process. 1529448 M.S. Thesis, Howard University, Washington D.C., U.S.A.

Anthonisen, A. C., Loehr, R. C., Prakasam, T. B. S. and Srinath, E. G. (1976). Inhibition of Nitrification by Ammonia and Nitrous-Acid. *Journal Water Pollution Control Federation* 48(5): 835-852.

APHA (1998). *Standard methods for the examination of water and wastewater*, American Public Health Association/American Water Works Association/Water Environment Federation. Washington DC, USA

Beun, J. J., van Loosdrecht, M. C. M. and Heijnen, J. J. (2002). Aerobic granulation in a sequencing batch airlift reactor. *Water Research* 36(3): 702-712.

Bin, Z., Zhe, C., Zhigang, Q., Min, J., Zhiqiang, C., Zhaoli, C., Junwen, L., Xuan, W. and Jingfeng, W. (2011). Dynamic and distribution of ammonia-oxidizing bacteria communities during sludge granulation in an anaerobic–aerobic sequencing batch reactor. *Water Research* 45(18): 6207-6216.

Blackburne, R., Yuan, Z. and Keller, J. (2008). Partial nitrification to nitrite using low dissolved oxygen concentration as the main selection factor. *Biodegradation* 19(2): 303-312.

Broda, E. (1977). Two kinds of lithotrophs missing in nature. *Zeitschrift für allgemeine Mikrobiologie* 17(6): 491-493.

Carlsen, L., Kenessov, B. N. and Batyrbekova, S. Y. (2009). A QSAR/QSTR study on the human health impact of the rocket fuel 1,1-dimethyl hydrazine and its transformation products: Multicriteria hazard ranking based on partial order methodologies. *Environmental Toxicology and Pharmacology* 27(3): 415-423.

Chang, X., Li, D., Liang, Y., Yang, Z., Cui, S., Liu, T., Zeng, H. and Zhang, J. (2013). Performance of a completely autotrophic nitrogen removal over nitrite process for treating wastewater with different substrates at ambient temperature. *Journal of Environmental Sciences* 25(4): 688-697.

Dapena-Mora, A., Fernandez, I., Campos, J. L., Mosquera-Corral, A., Mendez, R. and Jetten, M. S. M. (2007). Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. *Enzyme and Microbial Technology* 40(4): 859-865.

Dapena-Mora, A., Vázquez-Padín, J. R., Campos, J. L., Mosquera-Corral, A., Jetten, M. S. M. and Méndez, R. (2010). Monitoring the stability of an Anammox reactor under high salinity conditions. *Biochemical Engineering Journal* 51(3): 167-171.

Daverey, A., Su, S.-H., Huang, Y.-T., Chen, S.-S., Sung, S. and Lin, J.-G. (2013). Partial nitrification and anammox process: A method for high strength optoelectronic industrial wastewater treatment. *Water Research* 47(9): 2929-2937.

De Clippeleir, H., Yan, X., Verstraete, W. and Vlaeminck, S. (2011). OLAND is feasible to treat sewage-like nitrogen concentrations at low hydraulic residence times. *Applied Microbiology and Biotechnology* 90(4): 1537-1545.

De Clippeleir, H., Vlaeminck, S., Wilde, F., Daeninck, K., Mosquera, M., Boeckx, P., Verstraete, W. and Boon, N. (2013). One-stage partial nitritation/anammox at 15 °C on pretreated sewage: feasibility demonstration at lab-scale. *Applied Microbiology and Biotechnology*: 1-12.

Denny, M. (1993). *Air and Water: The Biology Adn Physics of Life's Media*, Precenton University Press.

Dosta, J., Fernandez, I., Vazquez-Padin, J. R., Mosquera-Corral, A., Campos, J. L., Mata-Alvarez, J. and Mendez, R. (2008). Short- and long-term effects of temperature on the Anammox process. *Journal of Hazardous Materials* 154(1-3): 688-693.

Frijns, J., Hofman, J. and Nederlof, M. (2013). The potential of (waste)water as energy carrier. *Energy Conversion and Management* 65(0): 357-363.

Garrido, J. M., Fdz-Polanco, M. and Fdz-Polanco, F. (2013). Working with energy and mass balances: a conceptual framework to understand the limits of municipal wastewater treatment. *Water science and technology* 67(10): 2294-2301.

Gilbert, E., Müller, E., Horn, H. and Lackner, S. (2013). Microbial activity of suspended biomass from a nitritation–anammox SBR in dependence of operational condition and size fraction. *Applied Microbiology and Biotechnology* 97(19): 8795-8804.

Guisasola, A., Jubany, I., Baeza, J. A., Carrera, J. and Lafuente, J. (2005). Respirometric estimation of the oxygen affinity constants for biological ammonium and nitrite oxidation. *Journal of Chemical Technology and Biotechnology* 80(4): 388-396.

Hao, X. D., Heijnen, J. J. and van Loosdrecht, M. C. M. (2002a). Model-based evaluation of temperature and inflow variations on a partial nitrification-ANAMMOX biofilm process. *Water Research* 36(19): 4839-4849.

Hao, X. D., Heijnen, J. J. and van Loosdrecht, M. C. M. (2002b). Sensitivity analysis of a biofilm model describing a one-stage completely autotrophic nitrogen removal (CANON) process. *Biotechnology and Bioengineering* 77(3): 266-277.

Hendrickx, T. L. G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H. and Buisman, C. J. N. (2012). Autotrophic nitrogen removal from low strength waste water at low temperature. *Water Research* 46(7): 2187-2193.

Hu, Z., Lotti, T., de Kreuk, M., Kleerebezem, R., van Loosdrecht, M., Kruit, J., Jetten, M. S. and Kartal, B. (2013). Nitrogen removal by a nitritation- anammox bioreactor at low temperature. *Applied and Environmental Microbiology*.

Isaka, K., Sumino, T. and Tsuneda, S. (2008). Novel nitritation process using heat-shocked nitrifying bacteria entrapped in gel carriers. *Process Biochemistry* 43(3): 265-270.

Isaka, K., Kimura, Y., Yamamoto, T., Osaka, T. and Tsuneda, S. (2013). Complete autotrophic denitrification in a single reactor using nitritation and anammox gel carriers. *Bioresource Technology* 147(0): 96-101.

Kartal, B., Kuenen, J. G. and van Loosdrecht, M. C. M. (2010). Sewage Treatment with Anammox. *Science* 328(5979): 702-703.

Kartal, B., Maalcke, W. J., de Almeida, N. M., Cirpus, I., Gloerich, J., Geerts, W., Op den Camp, H. J. M., Harhangi, H. R., Janssen-Megens, E. M., Francoijs, K.-J., Stunnenberg, H. G., Keltjens, J. T., Jetten, M. S. M. and Strous, M. (2011). Molecular mechanism of anaerobic ammonium oxidation. *Nature* 479(7371): 127-130.

Kartal, B., van Niftrik, L., Keltjens, J. T., Op den Camp, H. J. M. and Jetten, M. S. M. (2012). Chapter 3 - Anammox—Growth Physiology, Cell Biology, and Metabolism. In: *Advances in Microbial Physiology*. Robert, K. P. (ed), Academic Press. Volume 60: 211-262.

Liu, S. T., Yang, F. L., Gong, Z. and Su, Z. C. (2008). Assessment of the positive effect of salinity on the nitrogen removal performance and microbial composition during the start-up of CANON process. *Applied Microbiology and Biotechnology* 80(2): 339-348.

Liu, T., Li, D., Zeng, H., Li, X., Liang, Y., Chang, X. and Zhang, J. (2012). Distribution and genetic diversity of functional microorganisms in different CANON reactors. *Bioresource Technology* 123(0): 574-580.

Mara, D. and Horan, N. J. (2003). *Handbook of Water and Wastewater Microbiology*, Elsevier Science.

Morales, N., Val del Río, A., Vázquez-Padín, J. R., Méndez, R., Mosquera-Corral, A. and Campos, J. L. (2012). Application of the Anammox process to the main stream of WWTPs. *9th Edition Int. Symposium of Sanitary and Environmental Engineering (Sidisa2012)*. Milano (Italy).

Mulder, A., Vandegraaf, A. A., Robertson, L. A. and Kuenen, J. G. (1995). Anaerobic Ammonium Oxidation Discovered in a Denitrifying Fluidized-Bed Reactor. *Fems Microbiology Ecology* 16(3): 177-183.

Nielsen, M., Bollmann, A., Sliekers, O., Jetten, M., Schmid, M., Strous, M., Schmidt, I., Larsen, L. H., Nielsen, L. P. and Revsbech, N. P. (2005). Kinetics, diffusional limitation and microscale distribution of chemistry and organisms in a CANON reactor. *Fems Microbiology Ecology* 51(2): 247-256.

Qiao, S., Matsumoto, N., Shinohara, T., Nishiyama, T., Fujii, T., Bhatti, Z. and Furukawa, K. (2010). High-rate partial nitrification performance of high ammonium containing wastewater under low temperatures. *Bioresource Technology* 101(1): 111-117.

Rysgaard, S., Glud, R. N., Risgaard-Petersen, N. and Dalsgaard, T. (2004). Denitrification and anammox activity in Arctic marine sediments. *Limnology and Oceanography* 49(5): 1493-1502.

Siegrist, H., Salzgeber, D., Eugster, J. and Joss, A. (2008). Anammox brings WWTP closer to energy autarky due to increased biogas production and reduced aeration energy for N-removal. *Water Science and Technology* 57(3): 383-388.

Strous, M., Van Gerven, E., Kuenen, J. G. and Jetten, M. S. M. (1997a). Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (anammox) sludge. *Applied and Environmental Microbiology* 63(6): 2446-2448.

Strous, M., Van Gerven, E., Zheng, P., Kuenen, J. G. and Jetten, M. S. M. (1997b). Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (Anammox) process in different reactor configurations. *Water Research* 31(8): 1955-1962.

Strous, M., Kuenen, J. G. and Jetten, M. S. M. (1999). Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology* 65(7): 3248-3250.

Szatkowska, B., Cema, G., Plaza, E., Trela, J. and Hultman, B. (2007). A one-stage system with partial nitritation and anammox processes in the moving-bed biofilm reactor. *Water Science and Technology* 55(8-9): 19-26.

Third, K. A., Sliekers, A. O., Kuenen, J. G. and Jetten, M. S. M. (2001). The CANON system (completely autotrophic nitrogen-removal over nitrite) under ammonium limitation: Interaction and competition between three groups of bacteria. *Systematic and Applied Microbiology* 24(4): 588-596.

Tokutomi, T., Shibayama, C., Soda, S. and Ike, M. (2010). A novel control method for nitritation: The domination of ammonia-oxidizing bacteria by high concentrations of inorganic carbon in an airlift-fluidized bed reactor. *Water Research* 44(14): 4195-4203.

Turk, O. and Mavinic, D. S. (1986). Preliminary assessment of a shortcut in nitrogen removal from wastewater. *Canadian Journal of Civil Engineering* 13(6): 600-605.

van der Star, W. R. L., Abma, W. R., Blommers, D., Mulder, J.-W., Tokutomi, T., Strous, M., Picioreanu, C. and van Loosdrecht, M. C. M. (2007). Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. *Water Research* 41(18): 4149-4163.

van Dongen, U., Jetten, M. S. M. and van Loosdrecht, M. C. M. (2001). The SHARON-Anammox process for treatment of ammonium rich wastewater. *Water Science and Technology* 44(1): 153-160.

Vázquez-Padín, J. R., Pozo, M. J., Jarpa, M., Figueroa, M., Franco, A., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2009). Treatment of anaerobic sludge digester effluents by the CANON process in an air pulsing SBR. *Journal of Hazardous Materials* 166(1): 336-341.

Vázquez-Padín, J. R., Figueroa, M., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2010a). Nitrifying granular systems: A suitable technology to obtain stable partial nitrification at room temperature. *Separation and Purification Technology* 74(2): 178-186.

Vázquez-Padín, J. R., Mosquera-Corral, A., Campos, J. L., Méndez, R. and Revsbech, N. P. (2010b). Microbial community distribution and activity dynamics of granular biomass in a CANON reactor. *Water Research* 44(15): 4359-4370.

Vázquez-Padín, J. R., Mosquera-Corral, A., Campos, J. L., Méndez, R., Carrera, J. and Pérez, J. (2010c). Modelling aerobic granular SBR at variable COD/N ratios including accurate description of total solids concentration. *Biochemical Engineering Journal* 49(2): 173-184.

Vázquez-Padín, J. R., Fernández, I., Morales, N., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Autotrophic nitrogen removal at low temperature. *Water science and technology* 63(6): 1282-1288.

Vázquez-Padín, J. R., Fernández, R., Rogalla, F., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2012). *Fast start-up of a CANON pilot scale on reject water –influence of DO and HRT control.* Proceeding of IWA Nutrient Removal and Recovery 2012: Trends in NRR., Harbin, China.

Vázquez-Padín, J. R. (2013). Personal Communication.

Vázquez-Padín, J. R., Morales, N., Gutiérrez, R., Fernández, R., Rogalla, F., Barrio, J. P., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2014). Implications of full scale implementation of an anammox based process as post-treatment of a municipal anaerobic sludge digester operated with co-digestion. *Water science and technology* 69(6):1151-8

Vlaeminck, S. E., Cloetens, L. F. F., Carballa, M., Boon, N. and Verstraete, W. (2009). Granular biomass capable of partial nitritation and anammox. *Water Science and Technology* 59(3).

Vlaeminck, S. E., Terada, A., Smets, B. F., De Clippeleir, H., Schaubroeck, T., Bolca, S., Demeestere, L., Mast, J., Boon, N., Carballa, M. and Verstraete, W. (2010). Aggregate size and architecture determine microbial activity balance for one-stage partial nitritation and anammox. *Applied and Environmental Microbiology* 76(3): 900-909.

Volcke, E. I. P., Picioreanu, C., De Baets, B. and van Loosdrecht, M. C. M. (2010). Effect of granule size on autotrophic nitrogen removal in a granular sludge reactor. *Environmental Technology* 31(11): 1271-1280.

Volcke, E. I. P., Picioreanu, C., De Baets, B. and van Loosdrecht, M. C. M. (2012). The granule size distribution in an anammox-based granular sludge reactor affects the conversion—Implications for modeling. *Biotechnology and Bioengineering* 109(7): 1629-1636.

von Sperling, M. (2007). *Activated Sludge and Aerobic Biofilm Reactors*, IWA Publishing.

Wett, B. (2007). Development and implementation of a robust deammonification process. *Water Science and Technology* 56(7): 81-88.

Wett, B., Omari, A., Podmirseg, S., Han, M., Akintayo, O., Gómez-Brandón, M., Murthy, S., Bott, C., Hell, M. and Takács, I. (2013). Going for mainstream deammonification from bench to full scale for maximized resource efficiency. *Water science and technology* 68(2): 283-289.

Winkler, M. K. H., Kleerebezem, R., Kuenen, J. G., Yang, J. and van Loosdrecht, M. C. M. (2011). Segregation of Biomass in Cyclic Anaerobic/Aerobic Granular Sludge Allows the Enrichment of Anaerobic Ammonium Oxidizing Bacteria at Low Temperatures. *Environmental Science and Technology* 45(17): 7330-7337.

Winkler, M. K. H., R. Kleerebezem, and M. C. M. van Loosdrecht. (2012) Integration of Anammox into the Aerobic Granular Sludge Process for Main Stream Wastewater Treatment at Ambient Temperatures. *Water Research* 46(1): 136-44.

Winkler, M. K. H., Kleerebezem, R., Strous, M., Chandran, K. and Loosdrecht, M. C. M. (2013). Factors influencing the density of aerobic granular sludge. *Applied Microbiology and Biotechnology* 97(16): 7459-7468.

Yang, J., Trela, J. and Płaza, E. (2011). *Influence of aeration strategy on behavior of different microorganisms in deammonification process*. Int. Conf. Environmental (Bio)Technologies & EU-FP 7 Environment Brokerage, Gdansk, Poland.

Yao, Z.-B., Cai, Q., Zhang, D.-J., Xiao, P.-Y. and Lu, P.-L. (2013). The enhancement of completely autotrophic nitrogen removal over nitrite (CANON) by N_2H_4 addition. *Bioresource Technology* 146(0): 591-596.

Yoo, H., Ahn, K.-H., Lee, H.-J., Lee, K.-H., Kwak, Y.-J. and Song, K.-G. (1999). Nitrogen removal from synthetic wastewater by simultaneous nitrification and denitrification (SND) via nitrite in an intermittently-aerated reactor. *Water Research* 33(1): 145-154.

GENERAL CONCLUSIONS

The present thesis was focused on some of the new technologies under development in recent years in the field of wastewater treatment. Nowadays, new concepts are being taken into account in order to face up to new environmental, economic and social limitations, coping with the population growth, the global climate change, water scarcity, etc. The new designs should be done with sustainability in mind, as the energy-intensive and chemical-dependent systems in current use are completely unsustainable. The application of the technologies studied through this thesis is expected to lead to more efficient and sustainable wastewater treatment systems.

In this way, the space requirements of the Wastewater Treatment Plants (WWTPs) can be reduced by the use of aerobic granulation technology in discontinuous or continuous systems; the recovery of nutrients from the wastewater can be achieved by means of the separated urine treatment and ammonia stripping; and the energy efficiency of the WWTPs can be improved by means of the application of Anammox processes to the main stream.

The main conclusions of each experimental chapter of this thesis are detailed below:

Chapter 3. Operation of an aerobic granular pilot scale SBR plant to treat swine slurry

The aerobic granular technology was tested for its implementation as nitrogen and organic matter removal system for small swine farms in a pilot scale sequencing batch reactor (SBR). The reactor had a high height to diameter ratio and a high biomass retention capacity, which allowed reducing the area needed for its implantation.

Aerobic granules were formed after 9 days of operation and could withstand the variations in the feeding characteristics. In this way, the average granule diameter stabilized around 3 mm, with a Sludge Volume Index (SVI_{10}) lower than 50 mL/g TSS. Even though the reactor had a good biomass selection capacity, it was not able to retain the solids present in the pig slurry. Therefore, a pre- or post- treatment of these solids should be integrated in the overall treatment in order to achieve the required values for the effluent discharge.

The solids concentration in the reactor ranged between 5 and 12 g VSS/L. This biomass concentration is higher than the conventional value obtained in the activated sludge reactors. To obtain a high biomass concentration in the biological reactor allows treating a high loading rate, which permit to reduce the total volume of the reactor.

Aerobic granules could withstand the variations in the Organic Loading Rate (OLR), Nitrogen Loading Rate (NLR), and Chemical Oxygen Demand (COD) to Nitrogen ratio that can be usually observed in industrial effluents. Organic matter removal efficiencies were not affected by OLRs fluctuations, but by the non-biodegradable fraction of the swine slurry. Ammonia load was mainly oxidized to nitrite; the ammonia removal efficiency was around 76%. However, denitrification was practically not observed during the experiment as it was revealed by the cycle analysis.

The microbial population was followed through Fluorescent in situ Hybridization (FISH) analysis. The detected bacterial populations indicated an evolution from the inoculated sludge to those composing the aerobic granules. Filamentous organisms were present mainly in the inoculums while they were washed out from the system as granules developed. The nitrifying microbial population was mainly composed by members of *Nitrosomonas* spp. as ammonia oxidizing bacteria, and a fewer amount of nitrite oxidizing bacteria belonging to phylum *Nitrospirae*, in correspondence with the nitrite accumulation observed in the reactor.

Chapter 4. Aerobic granular-type biomass development in a continuous stirred tank reactor

A continuous stirred tank reactor (CSTR) was operated in order to define the appropriate operational conditions to obtain aerobic biomass grown in the form of granules. Microbial aggregates were formed in a reactor with similar geometry to the activated sludge reactors used in WWTPs, which usually have a height to diameter ratio of around 1. Aerobic granules are conventionally obtained in SBRs, where the selection pressure is achieved thanks to the cycle operation. However, in the continuous reactor operated in this chapter, the selection pressure was obtained in the effluent discharge tube, where the upflow velocity was controlled.

With this design, particles with a settling velocity smaller than the fixed upflow velocity were washed out from the reactor, while biomass with good settling properties was retained in the system. The formation of aerobic granules in the CSTR was achieved when a Hydraulic Retention Time (HRT) of 1 hour and an upflow velocity of 10 m/h in the effluent discharge tube were applied.

The diameter and settling velocities of the obtained granules were similar to those obtained in SBRs. In this way, the average diameter of the granules was 6.8 mm and the settling velocity varied around 36-48 m/h. However, the SVI value, which reached 127 mL/g

TSS, was worse than that of the granules grown in SBRs, but better than the value obtained in conventional activated sludge.

When the HRT was fixed at 6 and 3 hours filamentous shape bacteria appeared in large amounts and no granules were formed. On the other hand, when the HRT applied was 1 hour, the bacterial shape was mainly bacillus and filamentous shape bacteria were absent.

Transforming a continuous system into a SBR suitable to obtain aerobic granules is difficult. Then, the feasibility of the production of aerobic granules in a continuous reactor will open a new perspective to the application of the aerobic granular technology for the improvement of already existing WWTPs.

Chapter 5. Recovery of N and P from urine by struvite precipitation followed by combined stripping with digester sludge liquid at full scale.

In this chapter, a full-scale ammonia stripping reactor equipped with a CO_2 pre-stripper was used to recover ammonia from a nitrogen rich stream, and to produce the fertilizer ammonia sulphate. In the same study, a pilot-scale system was used to recover phosphorus from separated collected urine. Magnesium oxide was added to the urine tank in order to remove more than 95% of the phosphorus in the urine, through struvite precipitation. Struvite crystals with an average size of 42 to 80 μm were formed in the reactor.

Nevertheless, the solids recovery system during urine pre-treatment needs to be improved in order to retrieve a higher percentage of nutrients. In addition, the separated urine collecting system should be connected to the stripping reactor in order to combine both processes and optimize the nutrients recovery system.

Preliminary results obtained in the full scale ammonia stripping reactor, treating the supernatant of an anaerobic digester together with separately collected urine, showed the viability of this co-treatment for the recovery of nitrogen and phosphorus from wastewater. The addition of 10% volume of treated urine to the sludge liquid fed to the stripping treatment system produced an increase of 40% of the ammonia concentration. Even if the efficiency values obtained in this unit were lower than those obtained during the simulations, an increase in the ammonia sulphate production rate of 36% and 56% was measured during the two full-scale experiments performed in an urban WWTP. In addition, operational problems due to the treated urine addition were not reported by the WWTP operators; however, the length of the experiments was too short to discard this possibility.

The treatment of high concentrated nitrogen load streams, as separately collected urine and supernatant of an anaerobic digester, reduces the requirements of the biological nitrification/denitrification processes in the activated sludge reactor. In this way, the energy needed for aeration is saved, and the waste of organic matter for the heterotrophic denitrification process avoided.

Chapter 6. Dynamics of CANON process treating low nitrogen load at low temperature.

A Completely Autotrophic Nitrogen Removal Over Nitrite (CANON) reactor was operated at 15 and 20 ºC and fed with moderated and low concentrations of ammonia in order to test the viability of the application of the ANaerobic AMMonium OXidation (Anammox) based processes to the main line of urban WWTPs. The application of such processes opens the possibility of removing the nitrogenous compounds with less energy and chemical requirements. Furthermore, larger amounts of organic matter would be available for methane production, as this organic matter is not necessary for the heterotrophic denitrification process.

The CANON system operated under stable conditions at 20 ºC treating moderate ammonia loads. Under these operational conditions there was a net growth of the biomass and its concentration in the reactor increased up to 12 g VSS/L. Even more, the system was able to recover the biomass concentration loss observed at 15 ºC when the temperature was increased to 20ºC. Ammonia and nitrogen removal efficiencies of around 80 and 60% respectively, were achieved.

A progressive biomass loss was observed when the reactor was operated at 15 ºC, especially when the ammonia concentration simulated that of the water line in a WWTP and a consequent loss in the nitrogen removal activity and reduction of the removal efficiency were detected. This biomass loss forced to use a high settling time in the SBR, in order to achieve higher biomass retention. With these conditions, the development of nitrite oxidizing bacteria (NOB), the accumulation of flocculent particles and the loss of biomass density in the granules were observed.

Three main factors should be pointed out as crucial to control the performance of the AOB and Anammox bacteria in a CANON system operated at low temperature and nitrogen load: 1) to achieve a high biomass retention; 2) to achieve an equilibrium between the AOB and Anammox activities and 3) to avoid the NOB development in the biomass.

Low Dissolved Oxygen (DO) concentrations, adjusted to the granule size distribution and density of the granules variation, were needed to maintain when the temperature decrease. These low DO concentrations avoid an excessive oxygen depth penetration in the granules, which facilitate the development of NOB and inhibition of Anammox.

NOB activity measured was larger for small particles than for large granules. The use of periodical re inoculations and the control of the floccular biomass fraction are suggested to avoid the excessive development of NOB and maintain the stability of the CANON system.

LIST OF ACRONYMS AND SYMBOLS

Abs	Absorbance	
AD	Anaerobic Digester	
Anammox	Anaerobic Ammonium Oxidation	
ANR	Nitrogen Removal Rate by Anammox bacteria	g N/L·d, kg N/m^3·d
AOB	Ammonia Oxidizing Bacteria	
AOR	Ammonium Oxidation Rate	g N/L·d, kg N/m^3·d
AR	Ammonia Removal efficiency	%
ATES	Aquifer Thermal Energy Storage	
AUFB	Aerobic Upflow Fluidized Bed reactor	
AUSB	Aerobic Upflow Sludge Blanket reactor	
b	Endogenous respiration rate	d^{-1}
BAF	Biological Aerated Filters	
BAS	Biofilm Airlift Suspension Reactor	
C	Carbon	
CANON	Completely Autotrophic Nitrogen Removal Over Nitrite	
cap	per capita	
CAS	Conventional Activated Sludge	
ce	cattle equivalent	
CGSFDMBR	Continuous-flow bioreactor with aerobic granular sludge and self-forming dynamic membrane	
CHF	Swiss Franc	
COD	Chemical Oxygen Demand	mg/L, g/L
COD/N	Chemical Oxygen Demand to Nitrogen ratio	
C_s	Concentration of substance S	g/L, mg/L
CSTR	Continuous Stirring Tank Reactor	

CULTAN	Controlled Uptake Long Term Ammonium Nutrition fertilization	
CW	Constructed Wetlands	
Cy3	Cyanine 3	
Cy5	Cyanine 5	
D	Diameter	mm, cm
DAPI	4',6-DiAmidino-2-Phenylindole	
DEMON	Deammonification	
DO	Dissolved Oxygen concentration	mg O_2/L
D_s	Diffusivity coefficient of substance S	m^2/d
EAWAG	Swiss Federal Institute of Aquatic Science and Technology	
EBPR	Enhanced Biological Phosphorus Removal	
EEA	European Environment Agency	
EEC	European Community	
EGSB	Expanded Granular Sludge Blanket reactor	
EPA	United States Environmental Protection Agency	
EPS	Extracellular Polymeric Substances (exopolysaccharides)	
EU	European Union	
F	Formamide	
f_b	Biodegradable fraction of the biomass	
F/M	Food to Microorganism ratio	
FA	Free Ammonia	g N/L
FAS	Ferrous Ammonium Sulphate	
FISH	Fluorescent in situ hybridization	
FITC	Fluorescein IsoThioCyanate	
FNA	Free Nitrous Acid	g N/L
FOG	Fats, Oil and Grease	
GC	Gas Chromatography	
GHG	Greenhouse Gas	
GSBR	Granular Sequencing Batch Reactor	
H/D	Height to Diameter ratio	
H_c	Henry's law constant for compound c	
HR	Heterotrophic denitrification Rate	g COD/L·d

HRT	Hydraulic Retention Time	d, h
IC	Internal Circulating reactor	
IC	Inorganic Carbon	g/L
IFAS	Integrated Fixed Film Activated Sludge	
IN	Inorganic Nitrogen	
IWA	International Water Association	
K_s	Half saturation constant of substance S	g/L
MAP	Magnesium Ammonium Phosphate	
MBBR	Moving Bed Biofilm Reactor	
MBR	Membrane Biological Reactor	
MDG	Millennium Development Goal	
MFC	Microbial Fuel Cells	
N	Nitrogen	
NASA	Nitrifying Activated Sludge Airlift reactor	
ND	Not detected	
NLR	Nitrogen Loading Rate	g N/L·d, kg N/m^3·d
NOB	Nitrite Oxidizing Bacteria	
NOR	Nitrite Oxidation Rate	g N/L·d, kg N/m^3·d
NR	Nitrogen Removal	
NRR	Nitrogen Removal Rate	g N/L·d, kg N/m^3·d
OLAND	Oxygen-Limited Autotrophic Nitrification-Denitrification	
OLR	Organic Loading Rate	g COD/L·d, kg N/m^3·d
P	Phosphorus	
p.e.	Population equivalent	
PBS	Phosphate Buffer Solution	
PH2MV	Poly-Hydroxy-2-MethylValerate	
PHA	Poly-Hydroxy-Alkanoates	
PHB	Poly-Hydroxy-Butyrate	
PHV	Poly-Hydroxy-Valerate	
PLC	Programmable Logic Controller	
Q	Flow rate	L/d, Nm^3/h
R	Ideal gas coefficient	
RBC	Rotating Biofilm Contactor reactor	

rNH_4^+	Consumption rate for ammonia	mg N/L·h
$rNO_{x\ f}$	Consumption rate for nitrogen oxides in the feast phase	mg N/L·h
$rNO_{X\ h}$	Production rate of nitrogen oxides during famine phase	mg N/L·h
rRNA	Ribosomal Ribonucleic Acid	
S	Stripping factor	
SAA	Specific Anammox Activity	g N/g VSS·d
SBR	Sequencing Batch Reactor	
SCADA	Supervisory Control And Data Acquisition	
SEM	Scanning Electron Microscope	
SHARON	Single reactor system for High-activity Ammonia Removal over Nitrite	
SRT	Solids Retention Time	d
SVI_n	Sludge Volume Index, after n minutes of settling	mL/g TSS
T	Temperature	ºC, K
t	Time	
TC	Total Carbon	g/L
TF	Trickling or Percolating Filter	
TKN	Total Kjeldahl Nitrogen	g N/L
TN	Total Nitrogen	g N/L
TOC	Total Organic Carbon	g/L
Tris	Tris(hydroxymethyl)aminomethane	
TSS	Total Suspended Solids	g/L
U.N.	United Nations	
UASB	Upflow Anaerobic Sludge Blanket reactor	
UCBR	Ultra-Compact Biofilm Reactor	
USA / U.S.	United States of America	
UV	Ultraviolet	
V	Volume	L
VER	Volumetric Exchange Ratio	%
VFD	Variable Frequency Drives	
VOC	Volatile Organic Compounds	
$V_{s\ min}$	Minimum settling velocity for the biomass to be retained in the reactor	m/h

v_{set}	Settling velocity	m/h
VSS	Volatile Suspended Solids	g/L
v_{up}	Upflow velocity	m/h
W	Weight	
WFD	Water Framework Directive	
WWTP	Wastewater Treatment Plant	
X_{Eff} (or SSV_{Eff})	Biomass washed out in the effluent	
Y	Yield coefficient	$\frac{g\ VSS}{g\ Substrate_{removed}}$
ΔN	Nitrogen balance	
Δt	Length of period	d
ΔW_P	Amount of produced biomass in a certain period	g VSS
ΔX_r	The change of biomass concentration during a certain period	g VSS/L
μ	Biomass growth rate	d^{-1}
ρ	Density	g/L

Sub index:

air	Air
assimilated	Assimilated
biomass	biomass
denitrified	Denitrified
Eff	Effluent
G	Gas Phase
granule	Granule
Inf	Influent
L	Liquid Phase
obs	Observed
r	Reactor
recovered	Recovered
removed	Removed
s	Soluble
t	Total

LIST OF PUBLICATIONS

International journal publications:

Jungles, M. K., Figueroa, M., Morales, N., Val del Río, A., da Costa, R. H. R., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Start up of a pilot scale aerobic granular reactor for organic matter and nitrogen removal. *Journal of Chemical Technology and Biotechnology* 86(5): 763-768.

Val del Río, A., Morales, N., Isanta, E., Mosquera-Corral, A., Campos, J. L., Steyer, J. P. and Carrère, H. (2011). Thermal pre-treatment of aerobic granular sludge: Impact on anaerobic biodegradability. *Water Research* 45(18): 6011-6020.

Vázquez-Padín, J. R., Fernández, I., Morales, N., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Autotrophic nitrogen removal at low temperature. *Water science and technology* 63(6): 1282-1288.

Isanta, E., Suárez-Ojeda, M. E., Val del Río, Á., Morales, N., Pérez, J. and Carrera, J. (2012). Long term operation of a granular sequencing batch reactor at pilot scale treating a low-strength wastewater. *Chemical Engineering Journal (Lausanne)* 198–199(0): 163-170.

Val del Río, Á., Morales, N., Figueroa, M., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2012). Effect of coagulant-flocculant reagents on aerobic granular biomass. *Journal of Chemical Technology & Biotechnology* 87(7): 908-913.

Morales, N., Figueroa, M., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2012). Aerobic granular-type biomass development in a continuous stirred tank reactor. *Separation and Purification Technology* 89(0): 199-205.

Val del Río, A., Morales, N., Figueroa, M., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2013). Effects of the cycle distribution on the performance of SBRs with aerobic granular biomass. *Environmental Technology* 34(11): 1463-1472.

Morales, N., Boehler, M., Buettner, S., Liebi, C. and Siegrist, H. (2013). Recovery of N and P from Urine by Struvite Precipitation Followed by Combined Stripping with Digester Sludge Liquid at Full Scale. *Water* 5(3): 1262-1278.

Morales, N., Figueroa, M., Fra-Vázquez, A., Val del Río, A., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2013). Operation of an aerobic granular pilot scale SBR plant to treat swine slurry. *Process Biochemistry* 48(8): 1216-1221.

Vázquez-Padín, J. R., Morales, N., Gutiérrez, R., Fernández, R., Rogalla, F., Barrio, J. P., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2014). Implications of full scale implementation of an anammox based process as post-treatment of a municipal anaerobic sludge digester operated with co-digestion. *Water science and Technology* 69(6): 1151–1158.

Spanish journal publications:

Figueroa, M., Morales, N., Val del Río, A., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2011). Tratamiento de la fracción líquida de purín de cerdo en reactores granulares aerobios SBR. *InfoEnviro* 66: 108-111.

Figueroa, M., Val del Río, A., Morales, N., Mosquera-Corral, A., Méndez, R. and Campos, J. L. (2011). Anammox: Hacia una eliminación sostenible del amonio en los efluentes de digestores anaerobios de purines. *Revista Técnica de Medio Ambiente* 24(151): 84-90.

Vázquez-Padín, J.R., Morales, N., Gutiérrez, R., Fernández, R., Rogalla, F., Mosquera-Corral, A., Campos, J.L., and Méndez, R. (2013). Bacterias implicadas en la eliminación de nitrógeno en retornos de fango de EDAR — Desarrollo y escalado del sistema ELAN® de eliminación autótrofa de nitrógeno. IX Jornadas de Transferencia de Tecnología sobre Microbiología del Fango Activo. *Editorial GBS.*

Book Chapters:

Figueroa, M., Val del Río, A., Morales, N., Campos, J. L., Mosquera-Corral, A. and R., M. (2009). Nitrogen removal in aerobic granular systems. In: *Environmental Technologies to Treat Nitrogen Pollution, Principles and Engineering*. Cervantes, F. J. (ed), IWA Publishing. London: 373-401.

Mosquera-Corral, A., Figueroa, M., Morales, N., Val del Río, A., Campos, J.L. y Méndez, R. (2009). Tecnologías basadas en biomasa granular aerobia. En: *Tecnologías Avanzadas para el Tratamiento de Aguas Residuales*. 48-68. Editorial Lápices 4. Santiago de Compostela.

Mosquera-Corral, A., Figueroa, M., Morales, N., Val del Río, A., Campos, J. L. and Méndez, R. (2010). Chapter 2: Aerobic granulation technology. In: *Innovative Technologies for Urban Wastewater Treatment Plants*. Omil, F. *et al.*. (ed), Editorial Lápices 4. Santiago de Compostela.

Mosquera-Corral, A., Figueroa, M., Morales, N., Val del Río, A., Campos, J. L. and Méndez, R. (2012). Chapter 2: Aerobic granulation technology. In: *Innovative Technologies for Urban Wastewater Treatment Plants (2nd Edition)*. Omil, F. *et al.*. (ed), Editorial Lápices 4. Santiago de Compostela.

Campos, J. L., Figueroa, M., Morales, N., Fajardo, C., Vázquez-Padín, J. R., Mosquera-Corral, A. and Méndez, R. (2012). Advanced Systems for Nitrogen Removal from Effluents Produced in the Fish Canning Industry. In: *Agricultural Research Updates. Volume 2*. Hendriks, B. P. (ed), Nova Science Publishers 395-414

Conference Proceedings:

Morales, N., Figueroa, M., Val del Río, A., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2008). Start up and operation of a pilot scale aerobic granular SBR. *The Third International Meeting on Environmental Biotechnology and Engineering (3IMEBE)*. Palma de Mallorca (Spain).

Morales, N., Figueroa, M., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2010). Aerobic Granular Biomass in a Continuous Stirred Tank Reactor. *Sustainable Solutions For Small Water and Wastewater Treatment Systems S2Small 2010*. Girona (Spain).

Jungles, M. K., Figueroa, M., Morales, N., Vázquez-Padín, J.R., da Costa, R. H. R., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2010). Start up of a pilot scale aerobic granular reactor. *Sustainable Solutions For Small Water and Wastewater Treatment Systems S2Small 2010*. Girona (Spain).

Vázquez-Padín, J.R., Figueroa, M., Morales, N., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2010). Autotrophic nitrogen removal in granular reactors. *Conferencia Nacional de Jóvenes Profesionales del Agua de España (Spain National Young Water Professionals Conference, Spain NYWPC)*, Barcelona (Spain).

Vázquez-Padín, J.R., Fernández, I., Morales, N., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2010). Autotrophic nitrogen removal at low temperature. *IWA World Water Congress and Exhibition*. Montreal (Canada).

Val del Río, A., Morales, N., Figueroa, M., Palmeiro, T., Mosquera-Corral, A., Campos, J. L. Suárez-Ojeda, M.E., Isanta, E. and Méndez, R. (2011). Potential anaerobic biodegradability of aerobic granular sludge. *Water and Industry 2011. IWA Specialist Conference Chemical Industries*. Valladolid (Spain).

Val del Río, A., Morales, N., Figueroa, M., Mosquera-Corral, A., Campos, J. L. and Méndez, R. (2011). Effect of the Length of the Famine Period on Mature Aerobic Granular Biomass. *8th IWA Leading-Edge Conference on Water and Wastewater Technologies (LET 2011)*. Amsterdam (The Netherlands).

Morales, N., Figueroa, M., Val del Río, A., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2011). Treatment of Swine Slurry in an Aerobic Granular Pilot-Scale Reactor. *8th IWA Leading-Edge Conference on Water and Wastewater Technologies (LET 2011)*, Amsterdam (The Netherlands).

De Florio, L., Morales, N., Díez, R., Esteban, L., Rodriguez, L. and Tejero, I. (2011). Principles and basic technical devices for an Ecological Sanitation. In proceeding of: *Industrial Ecology, ECOMONDO 2011*, Rimini (Italy).

Böhler, M., Büttner, S., Morales, N., Liebi, C., Schachtler, M. and Siegrist, H. (2012). Recovery of nutrients from ammonia rich sludge liquids and urine for the production of fertilizer by full scale air stripping or membrane stripping. Proceedings of the *IWA World Congress on Water, Climate and Energy*, Dublin, (Ireland).

Morales, N., Figueroa, M., Val del Río, A., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2012). *Application of aerobic granular pilot scale SBR plant to treat swine slurry wastewaters*. *EcoSTP: EcoTechnologies for Wastewater Treatment*, Santiago de Compostela (Spain).

Morales, N., Val del Río, A., Vázquez-Padín, J. R., Méndez, R., Mosquera-Corral, A. and Campos, J. L. (2012). Application of the Anammox process to the main stream of WWTPs. *9th Edition Int. Symposium of Sanitary and Environmental Engineering (Sidisa2012)*, Milano (Italy).

Morales, N., Val del Río, A., Campos, J. L., Mosquera-Corral, A. and Méndez, R. (2013). *CANON process applied to the main stream of WWTPs*. *13th World Congress on Anaerobic Digestion,* Santiago de Compostela (Spain).

Vázquez-Padín, J.R., Morales, N., Gutiérrez, R., Fernández, R., Rogalla, F., Barrio, J.P., Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2013). Implications of full scale implementation of an Anammox based process as post-treatment of a municipal anaerobic sludge digester operated with co-digestion. *13th World Congress on Anaerobic Digestion,* Santiago de Compostela (Spain).

Vázquez-Padín, J.R., Morales, N., Gutiérrez, R., Fernández, R., Rogalla, F., Mosquera-Corral, A., Campos, J.L., and Méndez, R. (2013). Bacterias implicadas en la eliminación de nitrógeno en retornos de fango de EDAR ― Desarrollo y escalado del sistema ELAN® de eliminación autótrofa de nitrógeno. *IX Jornadas de Transferencia de Tecnología sobre Microbiología del Fango Activo,* Sevilla (Spain).

Novel technologies for WWTP optimization in footprint, nutrients valorization, and energy consumption.

The present thesis has been focused on the study of some alternatives to improve the Wastewater Treatment Plants (WWTPs). These alternatives are expected to lead to more efficient and sustainable wastewater treatment systems:

• The aerobic granulation technology allows reducing the excess sludge production in the WWTPs and the requirements of area needed for its implantation by the formation of granular biomass with good settling properties. This technology was tested in sequential and continuous reactors.

• The combination of urine separate treatment, production of struvite, and ammonia stripping can increase the nutrients reclamation from wastewater through the production of fertilizers.

• The use of Anammox based processes to autotrophically remove the nitrogen from the water line of the WWTPs can contribute to important energy savings in the treatment. These processes use less oxygen, produce less sludge and do not need organic matter compared to the conventional nitrification/denitrification processes.

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Tesis Doctoral 2014

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